UNIVERSIDAD DE MURCIA

FACULTAD DE MEDICINA

Comparative Study of the Radioprotective Effect of an Extract of *Pycnanthus angolensis* Against Damage Induced by X-rays

Estudio Comparativo del Efecto Radioprotector de un Extracto de *Pycnanthus angolensis* Frente al Daño Inducido por Rayos X

D. Daniel Gyingiri Achel

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UNIVERSIDAD DE MURCIA

FACULTAD DE MEDICINA Y ODONTOLOGÍA

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Instituto Universitario de Investigación en Envejecimiento de la Universidad de Murcia

Doctoral Thesis

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Tesis Doctoral

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April 2013

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D. Vicente Vicente Ortega, Catedrático de Universidad del Área de Anatomía Patológica y **Presidente Comisión Académica programa doctorado** * en Envejecimiento, INFORMA:

Que una vez evaluado, de conformidad con el procedimiento establecido en el artículo 21 del Reglamento de doctorado de la Universidad de Murcia, el expediente completo de la tesis doctoral titulada "Comparative study of the radioprotective effect of an extract of Pycnanthus angolensis against damage induced by X-rays"("Estudio comparativo del efecto radioprotectorde un extracto de Pycnanthus angolensis frente al daño inducido por rayos X)", realizada por D. Daniel Gyingiri Achel, bajo la inmediata dirección y supervisión de D. Miguel Alcaraz Baños, esta Comisión Académica, en sesión celebrada en fecha 19 de febrero de 2013, ha dado su autorización para su presentación ante la Comisión General de Doctorado.

Murcia, a 19 de febrero de 2013

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AUTORIZA:

La presentación de la Tesis de Doctoral titulada **"Comparative study of the radioprotective effect of an extract of *Pycnanthus angolensis* against damage induced by X-rays"** ("**Estudio comparativo del efecto radioprotector del extracto de *Pycnanthus angolensis* frente al daño inducido por rayos X**")**",** presentada por **D. DANIEL GYINGIRI ACHEL**, se ha realizado bajo mi inmediata dirección y supervisión, en el Departamento de DERMATOLOGIA, ESTOMATOLOGIA Y RADIOLOGIA Y MEDICINA FISICA**,** y que se presenta para la obtención del grado de Doctor por la **Universidad de Murcia**.

En Murcia, a 11 de abril de 2013.

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To my wife, Vivienne and daughter, Mamata,
for the endurance you had for me during the times spent to accomplish this assignment.

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Resumen

Resumen

Introducción.

El Magnonliflorae (Myristicaceae) superorden a la que pertenecen *angolensis P*. comprende 19 géneros de árboles y arbustos y 380 especies de árboles del bosque húmedo de tierras bajas distribuidas en Asia (cuatro géneros), África y Madagascar (nueve géneros) y América (seis géneros). Morfológicamente, el Myristicaceae es fácilmente reconocible en el campo por sus troncos rectos, típicos exudados de savia rojiza, pocas hojas y frutos parecidos a la nuez moscada que se consideran uno de los más primitivos de las angiospermas. Tienen estípulas simples alternas, de color verde oscuro y hojas habitualmente con pequeñas flores masculinas o femeninas en árboles distintos. Las flores masculinas tienen de 2 a 20 estambres unidos mientras que las flores femeninas tienen un solo ovario con un óvulo (semilla de potencial). Los frutos son de color rojo amarillento, ovoide, con semillas redondeadas u ovoideas, globulosas. Un árbol de hoja perenne, nuestro ***P. angolensis*** puede alcanzar una altura de 40 metros y una circunferencia de 150 cm. Florece en octubre y noviembre. Los frutos permanecen en el árbol hasta aproximadamente febrero. La dehiscencia se produce en el árbol, pero muchos de los racimos de la fruta caen sin abrir. El ***P. angolensis*** es originario de las zonas de bosque tropical de África, con una distribución geográfica que se extiende por África occidental de Guinea a Camerún, incluidos los países de Sierra Leona, Liberia, Costa de Marfil, Ghana, Togo, Nigeria, Guinea Ecuatorial, Angola y Uganda.

La importancia médica de la planta ya ha sido reconocida. En la práctica común su utilización terapéutica realizada por etnobotánicos y por la medicina tribal tradicional le han atribuido numerosos beneficios para la salud. La literatura está repleta de beneficios para la salud relacionados con el uso de diferentes partes del *P. angolensis* como agente antimicrobiano, analgésico, antihelmíntico, antihemorrágico, antiinflamatorio antihiperglucémico, así como para aumentar la fertilidad femenina. Por lo menos existe evidencia sustancial anecdótica importante que apoya los beneficios de salud de las diversas preparaciones de la planta, ya sea sola o en combinación con otros agentes. De hecho numerosos estudios científicos parecen indicar que algunas de estas afirmaciones son ciertas.

Objetivos.

Los objetivos de esta Memoria de Tesis doctoral son los siguientes:

1º. Obtener un extracto de ***Pycnanthus angolensis*** (PASE) con bioactividad adecuada para realizar el estudio comparativo mediante los ensayos celulares de radioprotección.

2º. Determinar la capacidad radioprotectora del PASE frente al daño genotóxico y citotóxico inducido por la radiación ionizante.

3º. Evaluar la capacidad antimutagénica y citoprotectora del PASE comparándolas con otras sustancias radioprotectoras utilizadas en oncología clínica y/o estudios de radiobiología.

Material y métodos

Sustancias químicas ensayadas

El extracto de *P. angolensis* (PASE) se extrajo a través de un procedimiento original. RPMI 1640, Ham-F10, Phytoheamaglutina A (PHA), cytochalasina B, estreptomicina, penicilina, tampón fosfato (PBS), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2h-tetrazolium bromide (MTT), Vitamin E (δ-tocopherol) (E), suero de albumina bovina (BSA fractionV), Eriodictiol (E), Coenzyma Q4 and Vitamin C (Ascorbic Acid) (C) se obtuvieron de Sigma-Aldrich chemicals SA (Madrid, España). El suero bovino fetal (FBS) se obtuvo de Gibco (USA); el acido acético glacial y el etanol se obtuvieron de Scharlab SL (Madrid, España). El metanol y el cloruro potásico se obtuvieron de Pancreac (Madrid, España); 5 % heparina sódica se obtuvo de Rovi Pharmaceutical Laboratories (Madrid, España). El ácido rosmarinico (ROS), Diosmina (D), Rutina (R) y Quercetina (Q) se obtuvieron de Extrasynthese (Extrasynthèse S.A., Genay, France). El dimethylsulfoxido (DMSO) se obtuvo de Laboratorios MercK (Merck, Darmstadt, Alemania). La amifostina (AMIF) (WR-2721) o Ethyol® se obtuvo de laboratorios Schering-Plough S.A. (Madrid, España). El acido zoledronico (Z) (Zometa®) se obtuvo de Novartis pharmaceuticals (Barcelona, España). El extracto de té (Té), Carnosol (COL), Carnosic Acid (CAR), extracto cítrico soluble (CE), Apigenina (API) y extracto de Procianidinas (P90) se obtuvieron de Nutrafur S.A (Alcantarilla, Murcia).

Análisis del efecto genotóxico:

Frecuencia de micronúcleos en cultivos de linfocitos humanos irradiados con bloqueo citocinético con citochalasina B *(*CBMN).

Muestras de sangre y procedimiento de irradiación

Las muestras de sangre humana se obtuvieron de dos jóvenes mujeres supuestamente sanas, no fumadoras que se conservaron en tubos heparinizados. Para la administración de todas las sustancias se añadieron 20 µl de las soluciones preparadas en 2 ml de la sangre humana heparinizada para obtener una concentración final de sustancia de 25 µM que se administraron inmediatamente antes de la irradiación. Para los administración post-irradiación se añadieron 20 µl de estas soluciones a 2 ml de sangre humana irradiada inmediatamente después de la exposición a rayos X.

Cultivo Celular

Tras la irradiación se realizó la técnica de micronúcleos (MN) en linfocitos humanos irradiados con bloqueo citocinético (CBMN) descrito por Fenech y Morley (1985) y adaptado por la Agencia Internacional de Energía Atómica (2011). Brevemente: las muestras de sangre (0,5 ml) se cultivaron a 37 °C durante 72 horas en 4,5 ml de medio F-10 conteniendo 15% de suero fetal bovino, 1,6 mg/ml de fitohemaglutinina, 1% de penicilina/estreptomicina y 1 mg/ml de glutamina. Cuarenta y cuatro horas después de la iniciación de los cultivos de linfocitos, 150 µl de citochalasina B se añadió para obtener una concentración de 6µg/ml. A las 72 horas los linfocitos se trataron con una solución hipotónica (KCl, 0,075 M) durante 3 min y se fijaron usando metanol:ácido acético (3:1). Se obtuvieron frotis que, secados al aire, se tiñeron con May-Grünwald Giemsa 24 horas más tarde. Cada experimento se repitió, al menos, en tres ocasiones.

Frecuencia de micronúcleos en eritrocitos policromatófilos de medula ósea de ratón (PCEs)

Animales

Se han utilizado ratones machos adultos Swiss de entre 25 y 30 g de peso y con edades comprendidas entre 9-12 semanas de edad al comienzo del estudio. Todos los ratones fueron aclimatados durante al menos 2 semanas antes de su empleo. Se mantuvieron en condiciones ambientales controladas de temperatura y humedad) con un

ciclo de 12 h de luz/12 h de oscuridad; fueron alimentados con granulado normal (Dieta de roedores Toxicología, B & K Universal de Beekay RSS, Evreux, Francia) y agua potable *"ad libitum"*. Cada grupo constaba de 6 ratones. Los animales fueron mantenidos y tratados de acuerdo con las pautas establecidas por la Unión Europea para la protección de los animales utilizados en los experimentos (86/609/CEE). Todas las soluciones se prepararon diariamente y las sustancias antioxidantes se disolvieron a 0,2% en el agua de bebida. Todos los procedimientos de manipulación de los ratones fueron aprobados por el Cuidado y uso de Animales de la Comisión de la Universidad de Murcia y fueron diseñados y llevados a cabo en el Servicio de Laboratorio Animal (SAI) de la Universidad de Murcia (Murcia, España, REGAES300305440012).

Además del control y los grupos de control irradiados, otros 18 grupos de animales se trataron con las sustancias ensayadas: 6 grupos no se irradiaron con rayos X con el fin de mostrar el efecto de las sustancias solas (RA, CA, API, D, Te y DMSO); a 6 grupos se les dio las sustancias diluidas a 0,2% en el agua de bebida durante 7 días (grupo pre-irradiación) y los otros grupos 6 comenzaron recibir las sustancias inmediatamente después de la exposición a rayos X (grupo post-irradiación).

Ensayo de Micronúcleos

El ensayo de micronúcleos se realizó sobre eritroblastos policromatófilos (PCEs) de la médula ósea de los animales, como se ha descrito por Schmid (1975). Brevemente: los animales se sacrificaron por dislocación cervical 24 h después de la irradiación, y las muestras de médula ósea se obtuvieron de ambos fémures de cada ratón. Los extremos proximal y distal de los fémures se cortaron, y las células de médula ósea se lavaron suavemente con suero bovino fetal. Las células de médula ósea se dispersaron mediante pipeteo repetido suave y se concentraron tras centrifugación a 1.000 rpm durante 5 min a 4 °C. Los sedimentos celulares se resuspendieron en un pequeño volumen de suero bovino fetal, y posteriormente se realizaron los frotis de médula ósea (tres preparaciones microscópicas por ratón). Las preparaciones fueron codificadas para evitar el sesgo atribuible al observador. Tras de 24 horas de secado al aire, las extensiones se tiñeron con May-Grünwald-Giemsa. Con este método, los eritroblastos policromáticos (PCEs), aparecen con citoplasma de color rojizo-azulado y los normocromáticos se tiñen de color naranja, mientras que el material nuclear presenta color púrpura oscuro.

Irradiación

Se ha utilizado un equipo productor de rayos X 200E Andrex SMART (YXLON International, Hamburgo, Alemania) a 4,5 mA, 36 cm FOD y a temperatura ambiente. Las dosis de radiación fueron controladas con un dosímetro UNIDOS ® Universal con PTW Farme ® TW con cámaras de ionización 30010 (PTW-Freiburg, Freiburg, Alemania) introducido en el interior de la cabina de irradiación. Posteriormente, las dosis de rayos X fueron confirmadas por medio de dosímetros termoluminiscencia (TLD) (GR -200A, Conqueror Electronics Technology Co. Ltd, China) suministrados y leídos por el CIEMAT (Ministerio de Industria y Energía, España). En el ensayo de micronúcleos con bloqueo citocinético (CBMN) se administraron 2 Gy de rayos X; mientras que se utilizaron diferentes dosis en la obtención de las curvas de supervivencia celular y/o cuantificación de la viabilidad celular (ensayo de MTT) y que fueron: 4, 6, 8, 10 y 0 Gy como control. La irradiación de los animales con rayos X (en exposición corporal total) se realizó de manera similar, aunque la distancia de la fuente animal fue de 74,5 cm, con una tasa de dosis de 102 mGy / min y una dosis total administrada para cada animal irradiado de 50 cGy.

Recuento de Micronúcleos.

El número de micronúcleos (MN) se determinó en cada caso con un recuento de al menos 3.000 células bloqueadas citocinéticamente (células CB) que fueron estudiadas por dos especialistas que analizaron las preparaciones de todos los grupos en un estudio doble ciego. Estas preparaciones se examinaron usando un microscopio de luz Ziess (Oberkochem, Alemania) con aumento de 400X de forma habitual y 1000 aumentos para confirmar la presencia de MN en las células. El número de micronúcleos en los eritrocitos policromáticos (MNPCEs) se expresaron por 500 PCEs. Se determinó también el número de eritrocitos normocromáticos (ECN) y la suma total de eritrocitos en la médula ósea (NCR + PCEs = E). Estos determinaciones nos permitió establecer la relación PECs/NCE (P / N) y la relación PCEs/eritrocitos totales (P / E) cuya alteración podría detectar un posible efecto citotóxico de las sustancias antioxidantes utilizadas.

Curvas de supervivencia celular y cuantificación de la viabilidad celular: ensayo del MTT

Líneas celulares y condiciones de cultivo celular.

La línea celular PNT2 utilizada en este estudio se obtuvo de la Colección Europea de Cultivos Celulares (ECACC), Agencia de Protección de la Salud Culture Collection (Catálogo no 95012613, HPACC, UK). La línea de células B16F10 (establecido desde una línea primaria de melanoma cutáneo de ratón C57BL/6) fue proporcionado amablemente por el Dr. Ambrose del Instituto Nacional del Cáncer (Bethesda, Maryland, EE.UU.). Se realizaron las pruebas durante todo el estudio para descartar la presencia de Mycoplasma spp. Las células PNT2 fueron cultivadas en RPMI 1640 suplementado con 10% de suero fetal bovino (FBS), glutamina (2 mM) y estreptomicina más penicilina (100µg/mL y 100 UI/ml, respectivamente). Las células B16F10 se cultivaron en medio esencial mínimo de Eagle (EMEM). Todos los procesos se llevaron a cabo en un equipo Cultair ASB I cámara de flujo laminar vertical. Los cultivos se mantuvieron a 37 °C y 95% de humedad relativa, en atmósfera de 5% de CO2, en un incubador Cytoperm. El medio de cultivo se cambió cada 2 días o cuando la acidificación fue indicada por el indicador de pH (rojo fenol).

MTT test.

Para analizar los efectos de las sustancias estudiadas sobre la viabilidad celular y la supervivencia celular PNT2, se utilizó la 3 - (4,5 - dimetiltiazol-2-il) -2,5-difenil tetrazolio (MTT) para los períodos estudiados (24 o 48 horas). Brevemente, los cultivos celulares se incubaron en medio de crecimiento de 200 µl y se dejaron adherir durante 24 horas. Después del tratamiento con las dosis de incubación antes mencionados se adicionó medio de cultivo suplementado y se añadió 50 µl de MTT (5 mg / ml) a cada pocillo en placas de 96 pocillos y las microplacas se incubaron adicionalmente a 37 °C durante 4 horas en una atmósfera de 5% CO2. Después, las microplacas se centrifugaron a 90 rpm durante 8 minutos para eliminar cuidadosamente el medio y el MTT no metabolizado, y se añadió 100 µl de DMSO a cada pocillo para solubilizar el MTT absorbido por las células vivas. Después de agitar durante 30 min a temperatura ambiente, las microplacas se leyeron con un espectrofotómetro Multiskan MCC/340P usando 570 nm y 690 nm. Los pocillos de control negativo se utilizaron para la línea de base cero. Cada experimento se repitió en tres ocasiones.

Análisis estadístico

En el estudio de genotoxicidad, el grado de dependencia y la correlación entre variables se evaluaron mediante análisis de la varianza complementado por un contraste

de medias considerándose los valores inferiores a 0,05 ($p<0,05$). Los resultados cuantitativos se compararon por análisis de regresión y correlación lineal. Además, se utilizó la fórmula descrita por Sarma y Kesavan (1993) para evaluar el Factor de protección:

Factor de Protección (%)=((Fcontrol irradiado-Ftratados irradiado) / Fcontrol irradiados) x 100.

Donde Fcontrol irradiado = frecuencia de MN en linfocitos de sangre no tratadas pero irradiado y la frecuencia Ftratados = irradiada de MN en linfocitos de sangre tratadas con sustancias y irradiados (administrada antes y después de irradiación).

El factor de reducción de la dosis (DRF) se ha calculado como la relación de la dosis de radiación requerida para producir el mismo efecto biológico en presencia y en ausencia del radioprotector. Se utilizó la fórmula descrita por Hall (1974): DRF = dosis de reducción en la presencia de radioprotector / dosis de radiación en ausencia del radioprotector, para producir un nivel dado de MN

En los ensayos de citotoxicidad, se utilizó un análisis de varianza (ANOVA) de medidas repetidas para comparar los porcentajes de células supervivientes en los cultivos con diferentes concentraciones de las sustancias estudiadas. Esto se complementó con la menor diferencia significativa para contraste de pares y medias. Los análisis se llevaron a cabo por logarítmicamente transformar los datos para cumplir con las condiciones de ANOVA.

Para determinar el factor de protección (FP) en los estudios de supervivencia celular se ha utilizado una modificación de la fórmula anterior que se ha aplicado a los resultados obtenidos en la inhibición del crecimiento a 10 Gy:

FP (%) = ((MT-MC) / MC) x 100.

Donde MT = mortalidad celular en las muestras irradiadas y tratadas con las sustancias estudiadas; MC = mortalidad celular en las células de control irradiados.

Resultados

El extracto obtenido

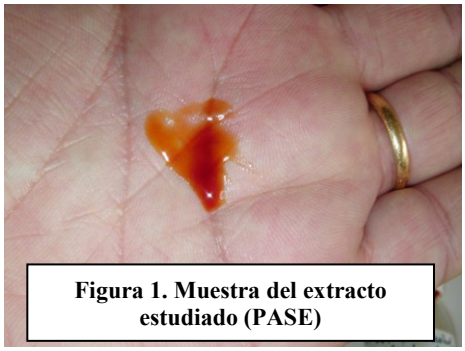


Figura 1. Muestra del extracto estudiado (PASE)

Se ha obtenido un extracto mediante un procedimiento propio. En base a los resultados obtenidos es consistente considerar que la estructura molecular del principio activo principal presente en el extracto obtenido (PASE) es un derivado del ácido sargahydroquinoico.

Resultados Morfológicos

Cultivos de linfocitos humanos irradiados con bloqueo citocinético con citochalasina B (CBMN)

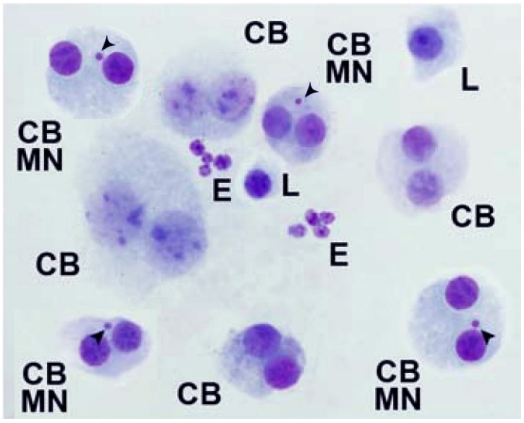


Figura 2: Fotomicrografía de un cultivo de linfocitos humanos con bloqueo citocinético teñido con la técnica de May-Grünwald-Giemsa (400X)(L: linfocito; CB: células binucleadas con bloqueo citocinético; CBMN: células binucleadas con bloqueo citocinético con micronúcleo en su citoplasma; E: restos de eritrocitos hemolizados.

Eritrocitos policromatófilos de medula ósea de ratón (PCEs)

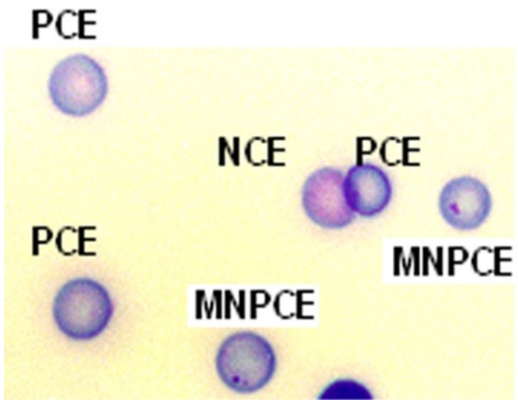


Figura 3: Fotomicrografía de médula ósea de ratón teñida con la técnica de May-Grünwald-Giemsa (1000X)(PCE: eritroblasto policromatófilo; MNPCE: eritroblasto policromatófilo con micronúcleo; NCE: eritroblasto normocromátófilo).

Curvas de supervivencia celular y cuantificación de la viabilidad celular: ensayo del MTT: Líneas celulares y condiciones de cultivo celular.

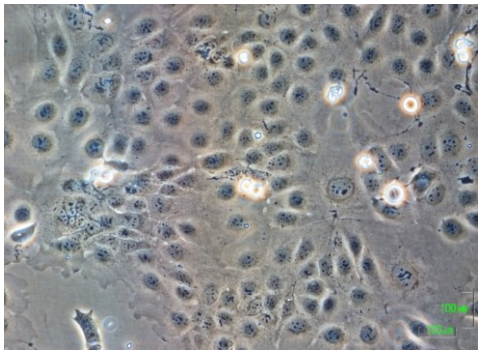


Figura 4.Microfotografía de células epiteliales normales de próstata humana (cultivo control, 24 horas de incubación, 200X).)

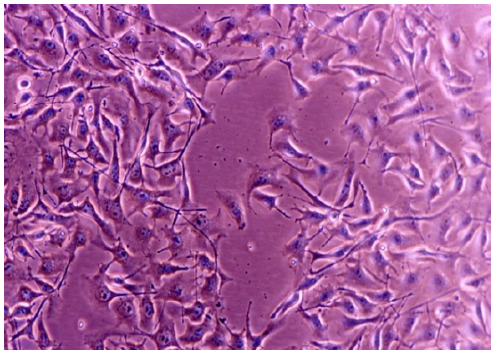


Figura 5. Microfotografía de células de melanoma B16F10 (cultivo control, 48 horas de incubación, 200X

Resultados actividad genoprotectora: actividad antimutagénica

Cultivos de linfocitos humanos irradiados con bloqueo citocinético con citochalasina B (CBMN)

Nuestros resultados ponen de manifiesto una diferente capacidad genoprotectora de las sustancias analizadas dependiendo de su presencia en el medio biológico durante la exposición a la radiación ionizante o si estas sustancias se administran inmediatamente después de la exposición a la radiación ionizante.

Si la administración de las sustancias se realiza **antes de la exposición** a la radiación se observa que su capacidad genoprotectora podría expresarse de la siguiente forma ordenadas de mayor a menor capacidad protectora:

Ácido rosmarínico (ROS)> Procianidinas (P90) > ácido Carnósico (CAR) = Apigenina (API) = Vitamina E > Carnosol (COL) = Extracto Cítrico (CE) > Vitamina C (C) = Amifostina (AMIF) > Extracto P. Angolensis (PASE) > Extracto de Té (Te) = Rutina (RUT) = Dimetilsulfosido (DMSO) ($p<0.001$)

Sin embargo, si la administración de las sustancias se realiza inmediatamente **después de la exposición** a la radiación ionizante se observa que su capacidad genoprotectora es menor y diferente. Podría expresarse de la siguiente forma ordenadas de mayor a menor capacidad protectora determinada:

Carnosol (COL) > ácido Carnósico (CAR) > Procianidinas (P90) = Extracto P. Angolensis (PASE) = Apigenina (API) = Vitamina E > = Extracto Cítrico (CE) > Vitamina C (C) > Extracto de Té (Te) = Rutina (RUT) = ácido rosmarínico (ROS) = Dimetilsulfosido (DMSO) (p<0.001)

En ambos casos, el extracto PASE estudiado presenta una significativa capacidad genoprotectora que se mantiene en ambas formas de administración y cuyos resultados comparativos pueden observarse en las Figuras 6 y 7) y en donde se ha podido determinar un Factor de Protección entre el 27% y el 35%, dependiendo del momento en el que se administre (pre o post-irradiación).

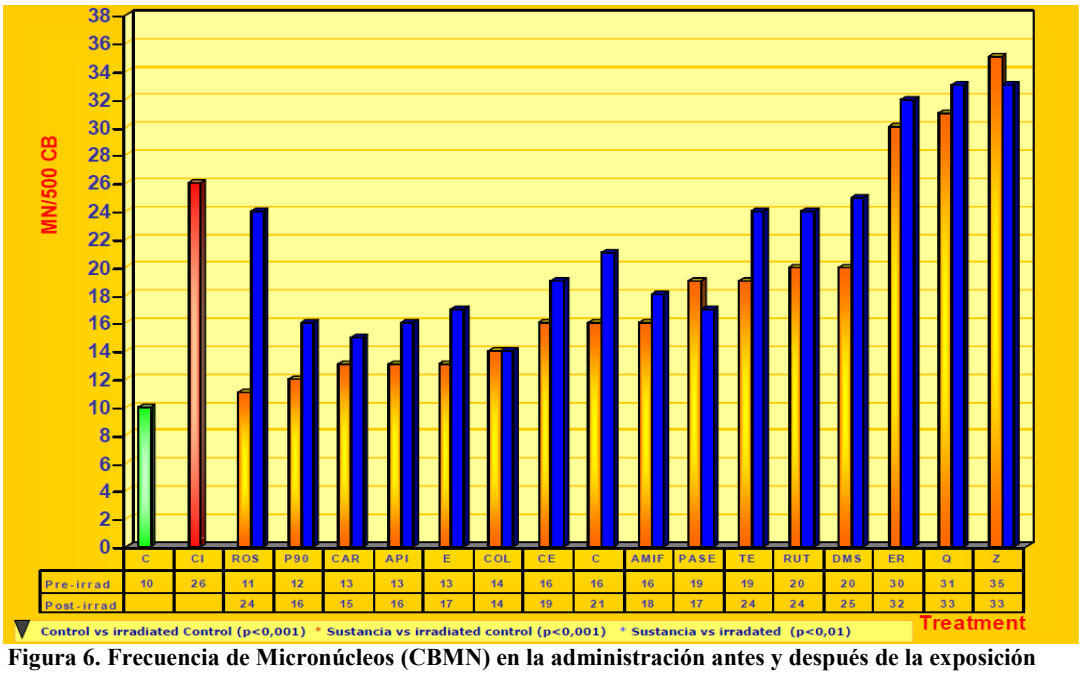


Figura 6. Frecuencia de Micronúcleos (CBMN) en la administración antes y después de la exposición a rayos X.

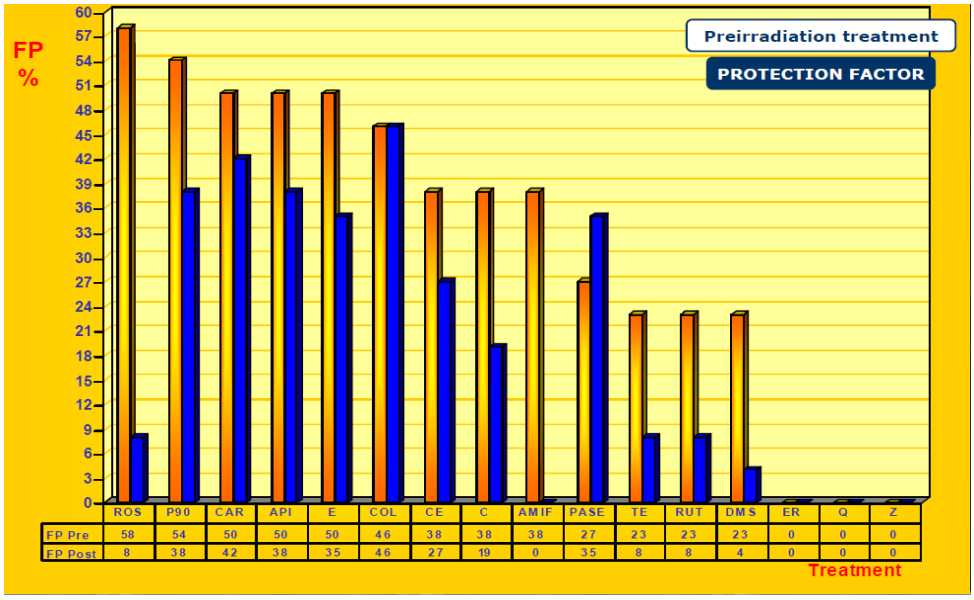


Figura 7. Magnitud de Protección (Factor de Protección obtenidos tras la administración antes y después de la exposición a la radiación (tratamientos pre- y post-irradiación)

Eritrocitos policromatófilos de medula ósea de ratón (PCEs)

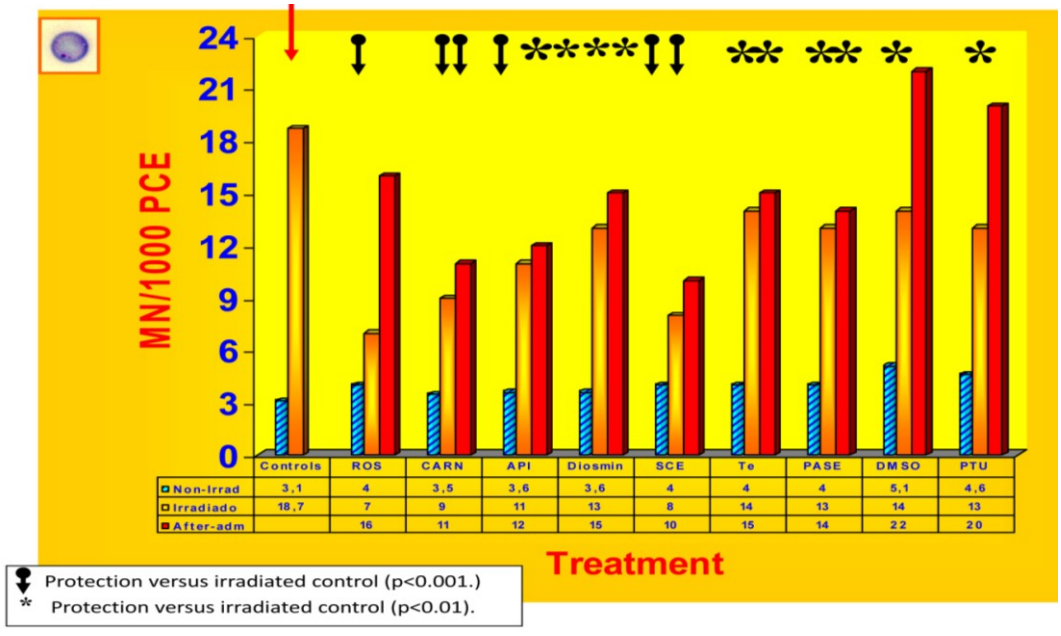
Nuestros resultados ponen de manifiesto una diferente capacidad genoprotectora de las sustancias analizadas dependiendo de su presencia en el animal de experimentación antes de la exposición a la radiación ionizante o si estas sustancias se administran al animal de experimentación inmediatamente después de la exposición a la radiación.

Si la administración de las sustancias se realiza **antes de la exposición** a la radiación se observa que su capacidad genoprotectora podría expresarse de la siguiente forma ordenadas de mayor a menor capacidad protectora:

Acido rosmarínico (ROS) > ácido Carnósico (CAR) = Extracto Cítrico (SCE) > Apigenina (API) > Diosmina (D) = Extracto P. Angolensis (PASE) = Extracto de Té (Te) = Dimetilsulfosido (DMSO) (p<0.001)

Sin embargo, si la administración de las sustancias se realiza inmediatamente **después de la exposición** a la radiación ionizante se observa que su capacidad genoprotectora podría expresarse de la siguiente forma ordenadas de mayor a menor capacidad protectora:

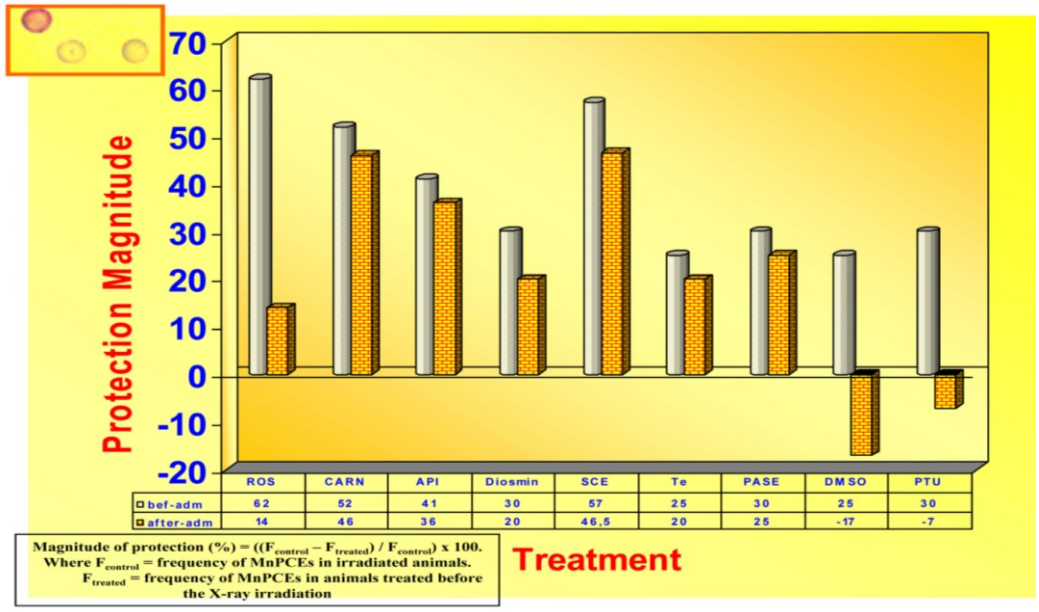
Acido Carnósico (CAR) = Extracto Cítrico (CE) > Extracto P. Angolensis (PASE) = Apigenina (API) = Diosmina (D) > ácido rosmarínico (ROS) = Extracto de Té (Te) (p<0.001)



	Controls	ROS	CARN	API	Diosmin	SCE	Te	PASE	DMSO	PTU
Non-Irrad	3,1	4	3,5	3,6	3,6	4	4	4	5,1	4,6
Irradiado	18,7	7	9	11	13	8	14	13	14	13
After-adm		16	11	12	15	10	15	14	22	20

Figura 8. Frecuencia de Micronúcleos (PCEs) en la administración antes y después de la exposición a rayos X (↓: (p<0.001) versus grupo control irradiado; (*): p<0.01 versus grupo control irradiado)

En ambos casos el extracto estudiado PASE presenta una significativa capacidad genoprotectora que se mantiene en ambas formas de administración, en donde se ha determinado un factor de Protección entre el 25% y el 30%, en función del momento en el que se administre (períodos pre y post-irradiación); y cuyos resultados comparativos pueden observarse en las Figuras 8 y 9).



e

Protección (Factor de Protección) obtenidos tras la administración antes y después de la exposición a la radiación (tratamientos pre- y post-irradiación)

Resultados actividad radioprotectora

Curvas de Supervivencia celular en la línea celular PNT2 (línea de células epiteliales normales de próstata humana).

A las 24 horas de evolución del cultivo celular, el porcentaje de supervivencia de las células tratadas con PASE es un 23,3% mayor que el de las células controles en los cultivos irradiados con 10 Gy. A las de 48 horas de incubación, la supervivencia es de un 23,8% mayor en las células tratadas con PASE que en las células controles irradiadas, poniendo de manifiesto un Factor de protección del 86% en las células tratadas con PASE y mostrando un incremento significativo de la supervivencia celular en los cultivos tratados con PASE y que muestra su capacidad radioprotectora ($p<0,001$) (Figura 9).

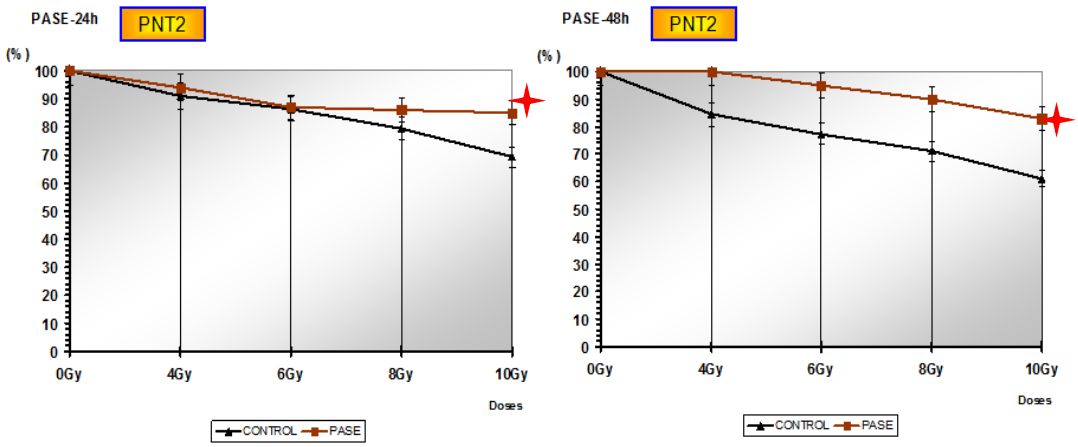


Figura 9. Curva de supervivencia celular de células epiteliales normales de próstata humana tratadas con PASE y expuestas a diferentes dosis de rayos X evaluadas tras 24h y 48h de cultivo respectivamente (*): (p<0.001) versus cultivos controles irradiados).

En las células tratadas con una mezcla de PASE + CAR, a las 24 horas de evolución del cultivo celular el porcentaje de supervivencia de las células tratadas con PASE es un 27,3% mayor que en las células controles irradiadas con 10 Gy. A las de 48 horas de incubación, la supervivencia es de un 12% mayor que las células irradiadas, poniendo de manifiesto un Factor de protección que ha variado desde el 88% en los cultivos de 24 h, hasta un 30% en los cultivos mantenidos durante 48 h. En ambos casos se ha determinado un incremento significativo de la supervivencia celular en los cultivos tratados con PASE + CAR y que muestra su capacidad radioprotectora (p<0,001) (Figura 10).

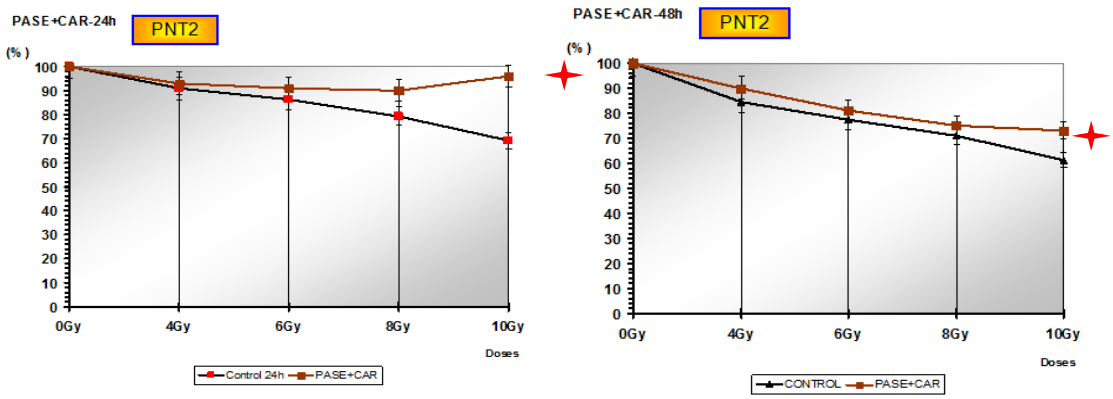


Figura 10. Curva de supervivencia celular de células epiteliales normales de próstata humana tratadas con (PASE + CAR) y expuestas a diferentes dosis de rayos X evaluadas tras 24h y 48h de cultivo respectivamente (*): (p<0.001) versus cultivos controles irradiados).

En las células tratadas con una mezcla de PASE + ROS, a las 24 horas de evolución del cultivo celular el porcentaje de supervivencia de las células tratadas con PASE es un 30,8% mayor que en las células controles irradiadas con 10 Gy. A las de 48 horas de incubación, la supervivencia es de un 38,8% mayor en las células tratadas que en las células controles irradiadas, poniendo de manifiesto un Factor de protección del 100% en los cultivos de 24h y de 48h, respectivamente. En ambos casos se ha determinado un incremento significativo de la supervivencia celular en las células tratadas con PASE + ROS y que muestra su capacidad radioprotectora ($p<0,001$) (Figura 11).

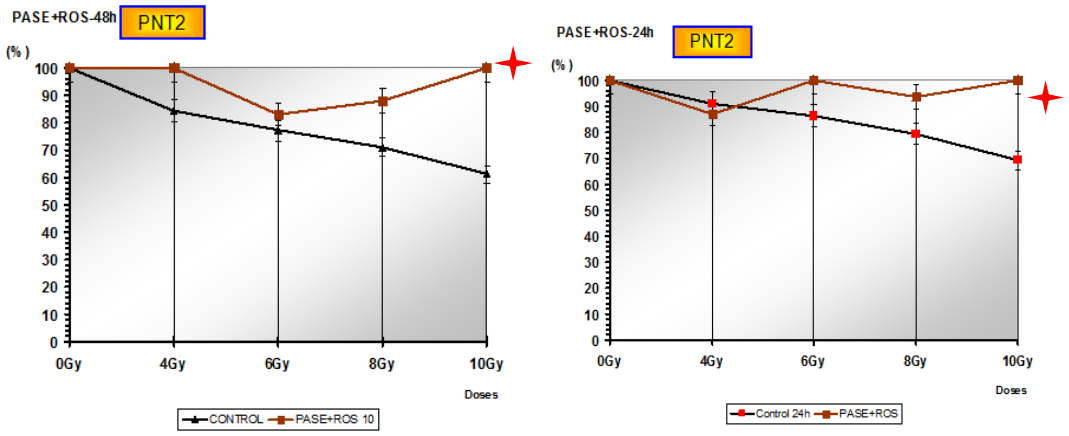


Figura 11. Curva de supervivencia celular de células epiteliales normales de próstata humana tratadas con (PASE + ROS) y expuestas a diferentes dosis de rayos X evaluadas tras 24h y 48h de cultivo respectivamente (*): (p<0.001) versus cultivos controles irradiados).

Curvas de Supervivencia celular en la línea celular B16F10 (línea metastásica de melanoma murino).

A las 24 horas de evolución del cultivo celular, el porcentaje de supervivencia de las células tratadas con PASE es un 18,8% mayor en las células tratadas con PASE que en las células controles irradiadas con 10 Gy. A las de 48 horas de incubación, la supervivencia es de un 24% mayor en las células tratadas con PASE que en las células irradiadas, poniendo de manifiesto un Factor de Protección del 28% a las 24h y del 41,2% en los cultivos de 48h; mostrando un incremento significativo de la supervivencia celular como muestra de su capacidad radioprotectora ($p <0,001$) (Figura 12).

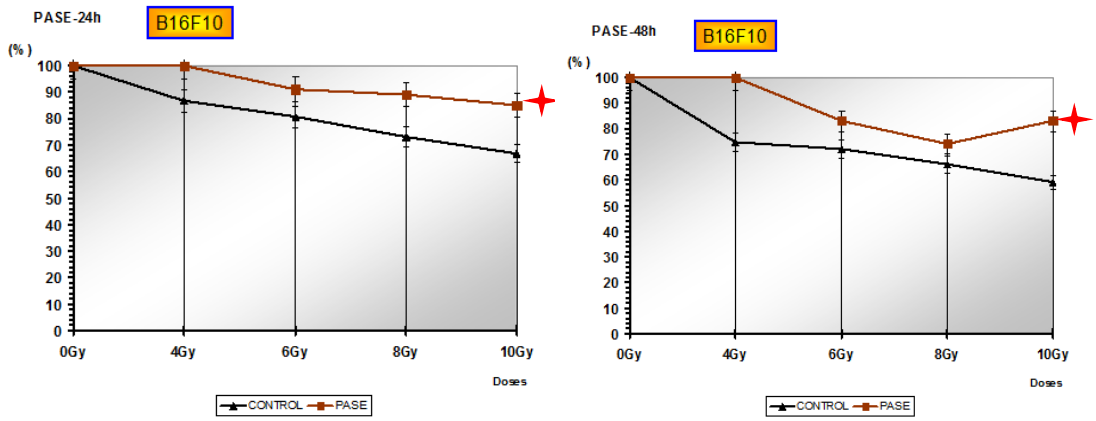


Figura 12. Curva de supervivencia celular de células metastásicas de melanoma murino B16F10 tratadas con PASE y expuestas a diferentes dosis de rayos X evaluadas tras 24h y 48h de cultivo celular respectivamente ((*): (p<0.001) versus cultivos controles irradiados).

En las células tratadas con una mezcla de (PASE + CAR), a las 24 horas de evolución del cultivo celular el porcentaje de supervivencia de las células tratadas con PASE + CAR es un 46,9% menor que en las células controles irradiadas con 10 Gy. A las de 48 horas de incubación, la supervivencia es de un 8,3% menor que en las células irradiadas, poniendo de manifiesto una ausencia de cualquier tipo de Factor de Protección durante ambos períodos estudiados. Además, se ha determinado un descenso significativo de la supervivencia celular a las 48h de incubación celular, poniendo de manifiesto una significativa capacidad radiosensibilizante de la mezcla de sustancias utilizada (p<0,001) (Figura 12).

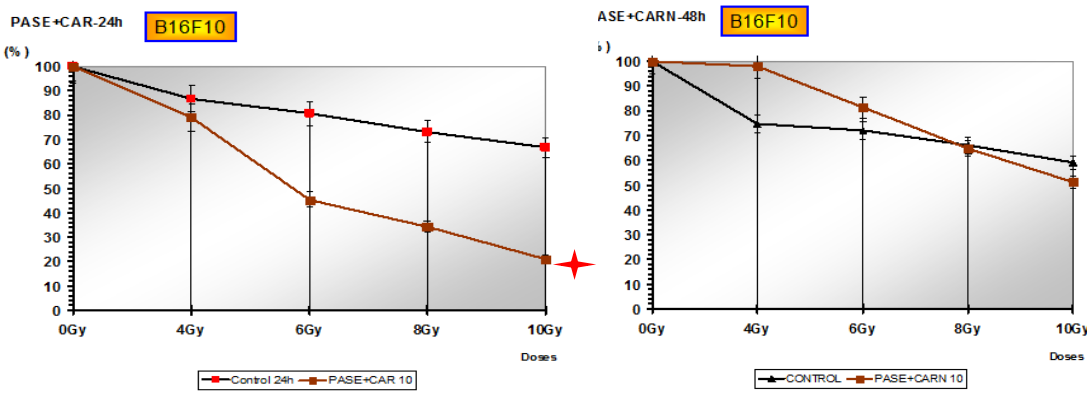


Figura 13. Curva de supervivencia celular de células metastásicas de melanoma murino B16F10 tratadas con (PASE + CAR) y expuestas a diferentes dosis de rayos X evaluadas tras 24h y 48h de cultivo celular respectivamente ((*): (p<0.001) versus cultivos controles irradiados).

En las células tratadas con una mezcla de (PASE + ROS), a las 24 horas de evolución del cultivo celular el porcentaje de supervivencia de las células tratadas con PASE + ROS es un 9,1% mayor que en las células controles irradiadas con 10 Gy. A las 48 horas de incubación, la supervivencia es de un 22,6% menor que las células controles irradiadas, poniendo de manifiesto una ausencia de cualquier tipo de Factor de Protección durante ambos períodos estudiados. Además, se ha determinado un descenso significativo de la supervivencia celular a las 48h de incubación celular poniendo de manifiesto una significativa capacidad radiosensibilizante de la mezcla de sustancias utilizada (p <0,001) (Figura 13).

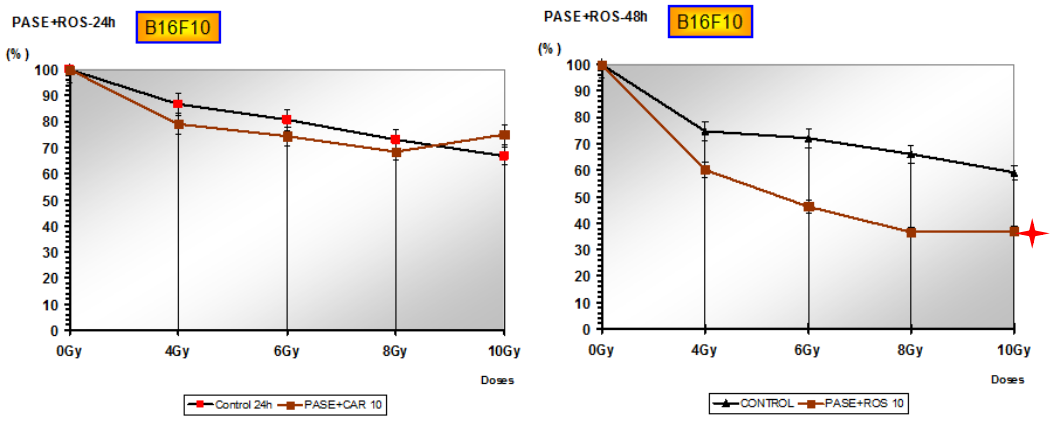


Figura 14. Curva de supervivencia celular de células metastásicas de melanoma murino B16F10 tratadas con (PASE + ROS) y expuestas a diferentes dosis de rayos X evaluadas tras 24h y 48h de cultivo celular respectivamente ((*): (p<0.001) versus cultivos controles irradiados).

Discusión

Actualmente, la capacidad de una sustancia para eliminar o disminuir las lesiones genotóxicas y citotóxicas inducidas por la radiación ionizante se mide en términos de la disminución de producción de estos radicales libres (ROS) y de sus efectos biológicos indeseables (ALCARAZ et al., 2009; 2010; 2013). Anteriormente hemos utilizado el test de MN para evaluar la actividad genoprotectora de diferentes compuestos. Así, hemos descrito cómo algunos flavonoides puros (por ejemplo, diosmina y apigenina) y algunos extractos polifenólicos muestran una capacidad genoprotectora mayor que los radioprotectores tradicionales como, por ejemplo, compuestos azufrados que presentan en su estructura química puentes sulfhidrilos (DMSO y PTU), tanto contra los efectos de los rayos X "in vivo" (CASTILLO et al, 2000; BENAVENTE-García et al, 2002;. 2005) como para la radiación γ "in vitro" (BENAVENTE-GARCÍA et al, 2005). También hemos descrito cómo estas capacidades

de protección dependían del grado de polimerización y de la solubilidad de las sustancias ensayadas, ya que consiguen modificar su biodisponibilidad celular (CASTILLO et al., 2001, 2002). En concordancia con lo descrito por otros autores (ABRAHAM et al, 1993; PRASSAD et al, 2004, 2005), hemos descrito que los extractos antioxidantes polifenólicos obtenidos a partir de diferentes plantas tales como hoja de olivo (Olea europaea) (BENAVENTE et al, 2002; CASTILLO et al, 2010) y cítricos (citroflavonoides) (BENAVENTE-GARCÍA et al, 2005; CASTILLO et al, 2000, 2002) muestran un mayor capacidad de protección cuando se administra solos que cuando se realiza su administración tras la confección de diferentes mezclas de sustancias.

En este estudio, hemos intentado extraer y cuantificar la capacidad radioprotectora del PASE administrado antes e inmediatamente después de la exposición a la radiación ionizante, en línea con resultados obtenidos en estudios anteriores. En estos estudios, las diferentes sustancias ensayadas proporcionaron una significativa capacidad radioprotectora frente al daño provocado por la radiación ionizante que pretendemos comparar, ahora, con la obtenida por el extracto PASE obtenido. Para lograr este objetivo y poder establecer comparaciones eficaces, hemos utilizado los mismos protocolos experimentales que utilizamos en nuestros estudios anteriores, aunque con un nuevo agente ionizante, los rayos X.

Los resultados obtenidos con respecto a la magnitud de la protección ofrecida por los diferentes tratamientos administrados antes y después de la irradiación con rayos X sugieren la probable existencia de diferentes mecanismos de radioprotección. En los pre-tratamientos, los efectos radioprotectores (actividad anti-mutagénica) de estas sustancias se basan, teóricamente, en la capacidad de de eliminación del anión superóxido (O2•) y especialmente del radical hidroxilo (OH•), que es masivamente generado durante la irradiación. De hecho, de acuerdo con las consideraciones estructurales, la actividad mutagénica de los compuestos ensayados es consistente con sus propiedades antioxidantes y demás actividades específicas como captadores de radicales libres.

Cuando las sustancias ensayadas se incorporan después de la irradiación, el único radical presente, de acuerdo con la vida media de anión superóxido y radicales

hidroxilo, son los radicales lipoperoxy (R-OO•), que son los responsables de la continuidad del daño cromosómico a lo largo de un periodo mayor. Además, la radiación ionizante podría aumentar la producción de la enzima lisosomal y la liberación de ácido araquidónico en las membranas a través de las lipooxigenasa, de la ciclooxigenasa y de las actividades fosfolipasa, durante el incremento de la respuesta inflamatoria inducida por la radiación ionizante. Bajo estas condiciones de estrés oxidativo, más complejas, es muy difícil determinar los elementos estructurales responsables de los datos experimentales obtenidos para la actividad antimutagénica de las diferentes sustancias administradas post-irradiación (ALCARAZ 2009; 2010).

Nuestros resultados sugieren que no hay una única estructura química relacionada con la existencia de una capacidad genoprotectora. Nuestros resultados sugieren que el efecto antimutagénico descrito es proporcional a la capacidad antioxidante de cada sustancia, aunque también es dependiente de sus características de biodisponibilidad en el medio biológico. En consecuencia, nosotros hemos determinado que la estructura flavan-3-ole ha mostrado la mayor capacidad de protección de todos los polifenoles (CASTILLO et al, 2000, 2001.); mientras que otros flavonoides con una mayor capacidad antiproliferativa y antineoplásica han mostrado una menor capacidad antimutagénica (BENAVENTE-GARCÍA et al, 2005; MARTÍNEZ-CONESA et al, 2005; YAÑEZ et al, 2004). Continuando con la búsqueda de compuestos con capacidad antioxidante mayor, se han descrito otras sustancias que presentan una mayor capacidad genoprotectora con una estructura química diferente como son ROS y CAR (DEL BAÑO et al., 2006). Sin embargo, el presente estudio muestra que las sustancias ensayadas en el tratamiento post-irradiación no siguen los criterios exclusivamente mencionados anteriormente. Así, se demuestra que la capacidad de protección es menor cuando se realiza una administración de la sustancia inmediatamente después de la irradiación, lo que subraya la importancia que subyace en seleccionar el momento de administración en relación con la exposición a radiación ionizante.

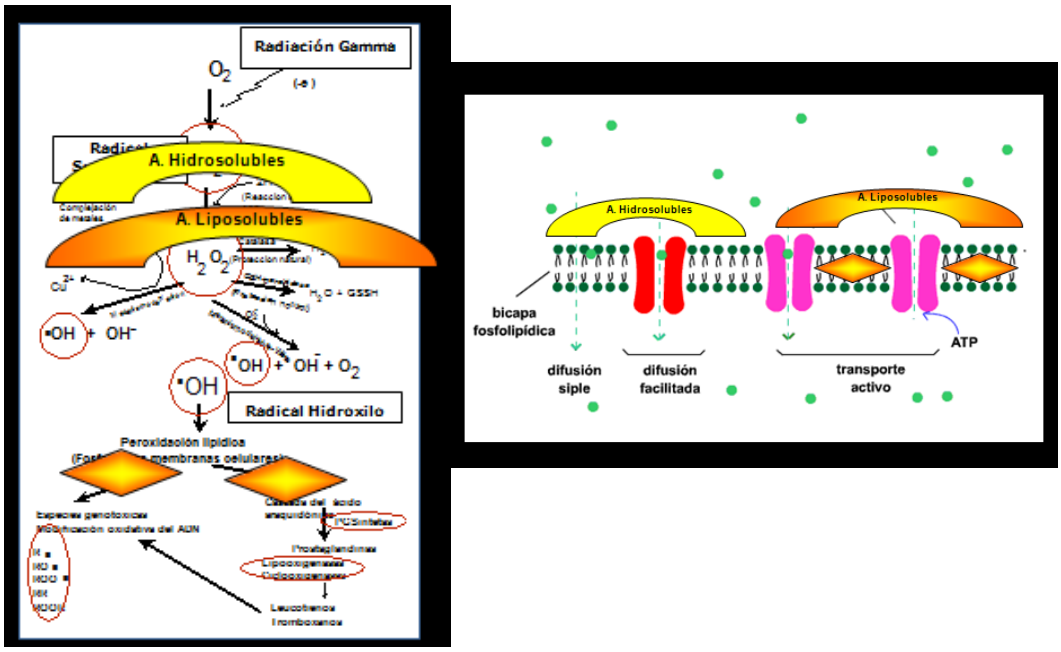
A partir de nuestros resultados, se ha puesto de manifiesto que el PASE también tiene una capacidad radioprotectora significativa, aunque con una estructura química diferente a las sustancias anteriores con las que se le compara. Por ello, hemos querido determinar su estructura química y relacionarla con la actividad radioprotectora frente a la radiación ionizante. Se puede inferir que en mayor o menor medida, la eficacia

radioprotectora del extracto no puede ser debido exclusivamente a una estructura química específica, sino a una combinación de elementos estructurales presentes en el extracto.

Parece que, cuando los agentes protectores se administran antes de la irradiación, la capacidad radioprotectora parece estar relacionada con su capacidad antioxidante, independientemente de los parámetros estructurales que confieran esta propiedad para el compuesto estudiado. Sin embargo, cuando la administración se realiza inmediatamente después de la irradiación, la eficacia parece estar relacionada con la capacidad liofilica del compuesto, además, por supuesto, de sus propiedades antioxidantes.

Cuando la administración de sustancias se lleva a cabo después de la exposición a la radiación ionizante, los radicales hidroxilo y superóxido habría desaparecido del entorno biológico y provocado la secuencia posterior de una cascada de reacciones que inducen las lesiones por radiación más tardías. Por lo tanto, algunas de las características adicionales que debemos buscar en las sustancias de radioprotectoras es sean sustancias lipídicas capaces de atrapar los radicales peroxi- inducidos en los fosfolípidos de las membranas celulares por la radiación y conseguir con ellos un mayor grado de protección contra el daño inducido por la radiación ionizante. En este caso, estas sustancias pueden funcionar mecánicamente contra la peroxidación lipídica y evitar así los efectos nocivos causados por estos productos secundarios (ALCARAZ et al, 2013).

La peroxidación lípidica se puede prevenir o eliminar eliminando los radicales libres del oxígeno mediante inactivadores o inhibidores. Se ha demostrado en el ensayo TBARS que la adición de CAR conduce a una disminución en compuestos oxidantes durante la auto-oxidación de ácido linoleico. También los resultados obtenidos en la determinación de la capacidad de CAR para secuestrar los ABTS•+ confirman la capacidad antioxidante del extracto concentrado de CAR. La presencia de dos anillos catecol, conjugado con un grupo de ácido carboxílico es, probablemente, el elemento estructural más importante de la actividad antioxidante de este compuesto (DEL BAÑO et al., 2003).



Esquema 1. Representación esquemática de los mecanismos de acción y localización del efecto de las sustancias antioxidantes contra el daño causado por la radiación ionizante.

En nuestro estudio hemos querido complementar el análisis de este efecto genoprotector de estas sustancias con el análisis radioprotector de las mismas sobre la viabilidad celular utilizando el ensayo del MTT. En este estudio de citotoxicidad, la amifostina que hemos utilizado como radioprotector ha presentado reacciones cruzadas en la técnica de MTT y no ha permitido su valoración en estos ensayos de supervivencia celular. En su lugar, DMSO, un eliminador de radicales libres conocido, lo hemos utilizado para su comparación con el resto de sustancias ensayadas (MURRAY et al, 1988; WEISS et al, 1990).

Evidentemente en nuestro estudio, utilizando las células epiteliales de próstata normal (PNT2), la administración del PASE, RA, CA y DMSO, así como de todas las demás diferentes mezclas ensayadas, han actuado como antioxidantes que pueden eliminar los radicales libres inducidos por el exceso de radiación ionizante, sumándose al sistema redox intracelular defensivo, y demostrando también una significativa capacidad radioprotectora (ALCARAZ et al., 2013).

De igual forma, en las células tumorales de melanoma metastásico B16F10, el PASE administrado sólo, también ha mostrado su capacidad radioprotectora, aunque en menor grado de protección. Esta respuesta se considera adecuada ya que son células muy radiorresistentes, tradicionalmente refractarias a cualquier tratamiento radiológico y, por ello, confirman la capacidad radioprotectora de nuestro extracto PASE. Sin embargo, esta capacidad radioprotectora desapareció completamente cuando se administraba el PASE mezclado con CAR y/o ROS. Este extraño resultado nos ha obligado a analizar las posibles causas de este efecto contradictorio y paradójico en su capacidad radioprotectora y evaluar, con mayor profundidad las características de esa sustancia que acompañaban al PASE durante los ensayos. Estas características se atribuyen a aspectos específicos de las sustancias y se discuten en la memoria presentada (ALCARAZ *et al*, 2013). Así, sorprendentemente, hemos determinado una falta de capacidad radioprotectora en las células tumorales de melanoma metastásico B16F10, que incluso ha llegado a ser todo un efecto radiosensibilizante de estas sustancias y mezclas.

En resumen, no hay una estructura química única relacionada la capacidad de ciertos compuestos para prevenir los efectos mutagénicos ni la toxicidad celular inducidos por la radiación ionizante. La capacidad antioxidante parece determinar el grado de protección de los compuestos administrados antes de la radiación. Sin embargo, parece que la liposolubilidad proporciona una mayor protección, si la sustancia se administra inmediatamente después de la exposición a radiación ionizante. El PASE que hemos ensayado en este estudio se puede considerar como una buena sustancia radioprotectora si se encuentra antes de la exposición a la radiación ionizante en el medio biológico, pero también si se administra inmediatamente después de haber ocurrido una exposición a la misma.

De acuerdo con Prasad [2003], sería un buen momento para realizar un ensayo clínico que permitiría evaluar la capacidad radioprotectora de estas sustancias antioxidantes en pacientes que reciben radiación durante las exploraciones médicas de radiodiagnóstico, evaluando las consecuencias que suponen dichas exploraciones sobre el estrés oxidativo y el daño cromosómico, y que permitiera incorporarlas como protección a los pacientes frente al daño secundario inducido por la radiación ionizante. Sin embargo, parece poco probable, ya que suelen ser sustancias naturales, componentes

habituales de la dieta humana, carentes de toxicidad, pero poco rentables comercialmente, ya que no suelen ser subsidiarias de protección comercial bajo patentes.

Conclusiones

Las conclusiones obtenidas en este estudio son las siguientes:

1ª. Se ha obtenido un extracto de semillas de ***Pycnanthus angolensis*** **(PASE)** adecuado para los ensayos de radioprotección celular en las concentraciones similares al resto de sustancias ensayadas y cuyo componente mayoritario es el ácido sargahydroquinoico.

2ª. Se ha determinado **un descenso significativo** del daño cromosómico y de la citotoxicidad inducida por la radiación ionizante tras la administración *"in vitro*" e *"in vivo"* del PASE, lo que muestra la capacidad radioprotectora del PASE frente al daño inducido por la radiación ionizante.

3ª. El extracto de semillas de ***Pycnanthus angolensis*** **(PASE**) presenta una capacidad protectora mayor que algunas sustancias radioprotectoras utilizadas actualmente en Oncología radioterápica, aunque menor que otros antioxidantes ensayados, al menos en las concentraciones del extracto utilizado en este estudio.

I. Justification.

I. Justification.

The dawn of twentieth century has witnessed an increase in nuclear technologies particularly in the areas of industrial radiology (mining and oil refinery), military, medical radiology, engineering, scientific research and electrical power/energy management. Radiation/nuclear accidents or unplanned radiation exposures may occur not withstanding strict regulations and safety measures that may be put in place. In parallel to the increased health concerns regarding occupational and medical exposures to radiation is the need for new radioprotectors that are effective, harmless and inexpensive, and for compounds that are able to raise the efficacy of radiotherapy when treating oncological diseases.

In the light of these arguments, it imperative for Ghana to be actively involved in biodosimetry and the development of radioprotectors, given that Ghana aims at including nuclear power in its energy mix in the not too distant future and more so, the GAEC is a member of the International Nuclear Library Network.

As part of the International Atomic Energy Agency`s (IAEA) strategy to preserve and transfer Nuclear Science Education and Training in the African Region, the IAEA and the Ghana Atomic Energy Commission (GAEC) signed agreements to support education, training and outreach in the nuclear field, including e-learning capabilities, and to collaborate in promoting nuclear knowledge management in IAEA Member States in the African Region. In consonance with this agreement, the GAEC will serve as a regional hub for the African region to install and operate an e-learning platform. In this regard, the GAEC receives support from the IAEA to foster capacity building initiatives through knowledge transfer between IAEA Member States and to develop materials relevant to nuclear education and training. Thus this fellow was supported by the IAEA as part of its initiative to preserve and transfer nuclear knowledge and transfer same to the younger generation (i.e. training of trainers), in order to secure a qualified workforce for safe and sustainable nuclear development.

As part of its quota towards achieving the overall vision of the Ghana Atomic Energy Commission, the Applied Radiation Biology Centre (ARBC) of the Radiological and Medical Sciences Research Institute (RAMSRI) of the GAEC of which this fellow is a member, has been working towards establishing competence in biological dosimetry and the need to development of non-toxic and effective radioprotective compounds from plant origin.

Through a research proposal submitted to the IAEA by this fellow for sponsorship geared towards the development of **non-toxic plant based radioprotectors and radiation biodosimetry** to fulfil his desires of obtaining a terminal degree in science, the IAEA aptly identified researchers in the Laboratory of Experimental and Clinical Radiology of the Department of Radiology and Physical Medicine at the Faculty of Medicine-Dentistry, Espinardo Campus, Murcia, Spain as the right place to conduct this research. This research group under leadership of Professor Miguel Alcaraz Baños has a vast research knowledge and experience in biological dosimetry and development of radioprotectors and their work is internationally acclaimed.

Indeed this request from the IAEA to the University of Murcia to have this fellow trained for his doctorate degree fitted in well with their current programme sponsored by National Spanish R&D Programme CENIT (Acronym:SENIFOOD) Nutrafur-Furfural Español S.A., Camino Viejo de Pliego s/n Murcia, Spain. For years, this research group have been contracted and supported by grants from SENIFOOD to assist their Research and Development department to evaluate the toxicological implications of food additives using tools of biological dosimetry as well as develop novel non-toxic radioprtotetive substances from plant origin. The SENIFOOD graciously agreed to co-sponsor this research work.

This trigonal collaboration (i.e. GAEC, IAEA, UM) which started in February 2011 colluminated in this fellow being admitted to the “Doctor Europeus” programme in the University of Murcia (UM), Spain for Accreditation of the European Doctorate at the end of his study period. This gesture, it is hoped, will forge a stronger triangle and strengthen ties among IAEA, GAEC and UM which would contribute to a shared goal and facilitate the nurturing and growth radiation biology in Ghana.

With this exposure, the trainee has been armed with the basic impetus in radiobiology in general but biological dosimetry and the development of radioprotectors in particular. This puts him in the right frame of mind to lead research back in his laboratory in Ghana and to provide mentorship to graduate students of the Graduate School of Nuclear and Allied Sciences (SNAS) which was jointly established by Ghana Atomic Energy Commission and University of Ghana in 2006 in collaboration with the International Atomic Energy. With this background, this fellow was chosen to lead a team to develop a curriculum in Radiation and Cancer Biology (RACB) which has since been presented to SNAS and is currently being considered to be mounted as a post graduate (Master of Philosophy) programme, needless to add that he will play a central role in mentorship when the curriculum is finally accepted and the programme mounted.

II. Introduction.

II. Introduction.

1. Ionizing radiation (IR) in everyday life

The dawn of twentieth century has witnessed an increase in nuclear technologies particularly in the areas of industrial radiology (mining and oil refinery), military, medical radiology, engineering, scientific research and electrical power/energy management. Notwithstanding strict regulations and safety measures that may be put in place, terrorist mediated activities, latent proliferation of nuclear weapons and illegal distribution of radioactive materials posse a serious threat to nuclear security issues. The risk and likelihood of radiation exposure in contemporary times far surpasses what pertained during the era of cold war, thus putting the military personnel, first responders and civilians at high risk. Further to these exposure scenarios other possible sources of radiation occur on a daily basis and these include planned exposures like diagnostic and therapeutic sources that include X-rays and radiation therapy. The clinical utility of ionizing radiation was first appreciated after the discovery of X-rays in 1895 by Wilhelm Conrad Röntgen and has since formed the basis for radiotherapy. Substantive progress in this field continues to be boosted by advances in imaging techniques enabling clinicians to detect and monitor many types of cancers as well as other diseases, and to assess the extent of injuries thus improving the prognosis and quality of life of cancer patients (BRODSKY and KATHREN, 1989). Radiotherapy, an adjuvant mode of malignant tumour treatment, is frequently used, either alone or in combination with chemotherapy or surgery, to achieve local or regional control of tumour growth. (KINSELLA, 2011; BASKAR *et al.*, 2012). A prospective survey on the practice of radiotherapy affirm that approximately 50% of all cancer patients will receive radiation therapy during their course of treatment (CITRIN *et al.*, 2010; BASKAR *et al.*, 2012) underscoring the importance of this treatment modality.

Treatment with IR invariably exposes some neighbouring normal non-cancer cells (tissues) to doses of radiation inflicting damage to their genetic apparatus, resulting in self-limiting acute toxicities, mild chronic symptoms and even severe organ

dysfunction or the development of secondary tumours. Despite many practical uses of radiation, its deleterious effects on human health cannot remain an insignificant issue.

Need for research into biodosimetry and radiation response modifiers (RSM)

The effective medical management of a suspected acute-radiation overexposure incident necessitates recording dynamic medical data, measuring absorbed doses by appropriate bioassays and estimating dose from dosimeters and radioactivity assessments in order to provide diagnostic doses to the treating physician and a dose assessment records for radiation protection personnel (BLAKELEY, *et al*., 2005). In the face of increasing risk of exposure to ionizing radiation, it is important to build competencies in biological dosimetry in order to provide much needed service of estimating the right doses of radiation received by subjects in the event of a mass-casualty radiological emergency. Amongst others, such a scenario requires that local, national and international resources be integrated to provide suitable dose assessment and continuing clinical triage and diagnosis. This calls for the creation of sustainable networks in biological dosimetry involving a number of experienced laboratories to significantly improve the accident and emergency response capabilities in case of a large-scale radiological emergency .

Biodosimetry is of little practical value unless there are effective therapies thus, in parallel with the health concern regarding occupational and medical exposures to radiation such as accidental exposures (e.g. industrial nuclear accident), and therapeutic applications (e.g. radiotherapy and nuclear medicine) there is a critical need to identify the hazards caused by ionizing radiation and develop counter-measures to tackle radiation-induced damage.

In this regard, the need for the development of new non-toxic, effective and inexpensive radiation response modifiers (RRM) for medical use is currently in vogue. RRMs in use today have dose-limiting toxicities. Since 1946, considerable efforts have been made towards the development of RRMs that would be effective on pre- or post-irradiation application albeit with limited success. Indeed since its inception over sixty years ago, many RRMs including synthetic compounds and natural products, have been

investigated and evaluated. Amiphostine (WR-2721), the synthetic thiol compound developed by the Walter Reed Army Research Institute after screening over 4,000 compounds was found to be and is still the most effective and only radioprotective compound approved by the FDA in clinical practice today (WEISS, 1997). Even so, its dose-limiting toxicity coupled with nausea, vomiting, hypotension, allergic reactions restricts its use. Crrently there does not appear to be any ideal synthetic or natural radioprotector in clinical use today that meets all the ideal requirements for a good RRM.

Therapies for acute injuries will have very few long-term benefits unless there are therapies for late effects that will occur in people who receive high doses and for whom hematological toxicity cannot be prevented. Conversely, therapy for chronic radiation injuries will be of little use without development of better biodosimetry tools, and better methods for decreasing acute hematological toxicity.

Natural radioprotectors from vegetable origin

Taking the above enumerated downsides into consideration, several plants have been screened for potential radioprotection given their non-toxicity, suitability for oral application, proven therapeutic benefits and ease of regulatory approval since they have been utilized during ancient times to cure various ailments. The World Health Organization estimates that 80% of the people in developing countries of the world rely on traditional medicine for their primary health care needs, and about 85% of traditional medicine involves the use of plant extracts. Plants produce many chemicals that are biologically active and provide medicinal benefits to humans and several of them have been reported to be beneficial for ameliorating free radical-mediated disease conditions in humans such as arthritis, atherosclerosis, cancer, Alzheimer's disease, Parkinson's disease, aging and inflammatory disorders. It is therefore, logical to expect that plants may contain groups of compounds that can protect against radiation-induced reactive oxygen species (ROS) and reactive nitrogen species (RNS) mediated damage. ROS and RNX have been identified as the likely cause of cell injury in many diseases. Exposure to ionizing radiation initiates the process of water radiolysis leading to the generation of ROS in the cellular milieu contributing to oxidative stress. For example, it has been estimated that about two thirds of X-ray and γ-ray damage is caused by free radicals

especially hydroxyl (•OH) radicals generated from the radiolysis of water (COIA and MOYLAND, 1998). Naturally, cells manifest potent antioxidant defences against ROS, using detoxifying enzymes and exogenous free radical scavengers (vitamins). The major enzymes that convert ROS to less reactive molecules are superoxide dismutase (SOD), catalases, and glutathione peroxidases (GPX). Antioxidants have been touted as free radical sinks and when present during the generation of free radicals could be very useful in that regard.

Some plants are rich sources of antioxidant polyphenolic compounds that scavenge ROS thereby complementing endogenous antioxidants enumerated above. By virtue of their free radical scavenging abilities, some of these plant based antioxidants might be endowed with radioprotective abilities.

In this context, this work focused on *Pycnanthus angolensis* Warb (*P. kombo*) which has substantial evidence of antioxidant potential. A review of the medicinal properties and applications of *P. angolensis* (welw) warb has been discussed elsewhere (ACHEL *et al.,* 2012). In particular its free radical scavenging, antioxidant, anticancer, immunomodulatory, anti-inflammatory and antimicrobial properties strategically positions it as a possible repository of radiomodulators, however, there is a dearth of sound scientific evidence to confirm this hypothesis. Studies are needed to fill this gap in order for full scale realization to unlock some hidden wealth in the plant in this regard.

2 REVIEW OF THE LITERATURE

Botany and distribution of *Pycnanthus angolensis* Warb *(P. kombo)*.

The Magnonliflorae (Myristicaceae) super-order to which *P. angolensis* belong comprises 19 genera of trees and shrubs and 380 species of lowland rainforest trees distributed in Asia (four genera), Africa and Madagascar (nine genera) and America (six genera). Morphologically, the Myristicaceae which are easily recognizable on the fields by their straight trunks, characteristic blood-like sap exudates, few leaves and nutmeg-like fruits are considered as one of the most primitive of the Angiosperms. They have simple alternate stipules, habitually dark green and leathery leaves with tiny male or female petalless flowers on different trees [WIART, 2006a]. Male flowers have 2 to 20 united stamens while female flowers have a single ovary with one ovule (potential seed). A fleshy covering, known as an aril, surrounds the fluted seed. The fruits are yellowish-red, ovoid, drupes, with laciniate seed almost obovoid-globose to base embedded in a fleshy aril (see images of *P. angolensis* in figure 1). An evergreen tree, *P. angolensis* may attain a height of 40 m and girth of 150 cm. It flowers in October and November, at the same time as the previous year's fruits are ripening. The fruits remain on the tree until about February. Dehiscence takes place on the tree, but many o the fruit clusters fall unopened [ORWA *et al.,* 2009; ABBIW, 1990]. *P. angolensis* is native to the forest zones of tropical Africa with a geographical distribution stretching across western Africa from Guinea to Cameroon, including Sierra Leone, Liberia, Cote D'Ivoire, Ghana, Togo, Benin, Nigeria, Equatorial Guinea, Angola and Uganda.

a

b

c

d

d

e

Figure 1: Images of *P. angolensis* (welw) Warb. a) Fruit flowers and leaves; b) Bole; c). Inflorescence; d) Braches with fruits; d) Young plant; e) Opened fruits. Photos courtesy: http://database.prota.org/dbtw-wpd/protabase/Photfile Images/Pycnanthus angolensis

Folkloric uses

The medical importance of the plant has long been recognized and at present scientific research is rife on its therapeutic utility because traditional healers and ethnobotanists have ascribed numerous health benefits to the use of the plant.

Literature is replete with numerous health benefits linked to the use of different parts of *P. angolensis* including but not limited its role in fighting hyperglycaemia, sterility in women, as an antimicrobial agent, analgesic, anthelmintic, antidote for poisoning, anti-bleeding agent, anti-inflammatory and pain soothing agent. At least there exists substantial anecdotal evidence supporting the health benefits of the various preparations of the plant either alone or in combination with other agents some of which have been summarized in table 1. Indeed numerous scientific studies seem to suggest that some of these claims may be accurate.

Table 1. Documented ethnomedical information *for P. angolensis* warb (*P. kombo*) (adapted from ACHEL *et al.,* 2012).

Part Used	Place	Documented Use	Type of Extract	Method	Ref.
Leaves	Ghana	Haemorrhoids, stomach ulcer, chronic wounds	Decoction		(AGYARE *et al*., 2009)
		Dropsy	Decoction	enema	(ORWA et al., 2009)
	Nigeria, Ghana	Toothache, chronic fungal infection			(ORWA *et al*., 2009), (OMOBUWAJO *et al*., 1996)
	Ghana	enema to treat ascites	Decoction		(ABBIW, 1990, MAPONGMETSEM, 2007)
		Oral thrush in children	Leaf-juice		(ABBIW, 1990)
Stem-bark	Ghana	Haemorrhoids, stomach ulcer, chronic wounds	Decoction		(AGYARE *et al*., 2009, FORT *et al*., 2000)
	Ghana, Cote d'Ivoire, São Tomé, Cameron	Malaria, anaemia, prevent abortion, scabies	Decoction /infusion		(ANCOLIO *et al*., 2002, ASASE and OPPONG-MENSAH, 2009, DO CÉU *et al*., 2002, IRVINE 1961, JIOFACK *et al*., 2009, MAPONGMETSEM, 2007, ODUGBEMI *et al*., 2006, ZIRIHI *et al*., 2005)
	Cameroon	Intestinal helminthic infection, constipation, vomiting, vaginal toilet	Not described		(BETTI 2002, FORT *et al*., 2000)
		Jaundice	Macerate	Not described	(BETTI and LEJOLY, 2009)
	Guinea, Côte d'Ivoire. Guinea-Bissau	Purgative, leprosy, female sterility, purify breast milk	Hot water decoction,	Oral	(FORT *et al*., 2000, LOK *et al*., 1983, MAPONGMETSEM, 2007, NONO *et al*. 2010, ONOCHA *et al*., 2008, TSAASSI *et al*., 2010)
Bark (root/ stem)	Nigeria	Tuberculosis, cancer, coated tongue	Maceration, poultice, Mix with other plants	Oral	(ASHIDI *et al*., 2010, NONO, *et al*.,1994 , OGBOLE and AJAIYEOBA,, OLOWOKUDEJO,*et al*., 2008)
	Ghana, Nigeria	Analgesic (headache, body aches, chest pain)	Made into paste with *Piper guineense* and water	Topical application	(FORT *et al*., 2000, IRVINE, 1961, MAPONGMETSEM, 2007, ONOCHA and OTUNLA, 2010, TSAASSI *et al*., 2010)
	Congo	Gynaecological problems, infertility, gonorrhoea	Not described		(MAPONGMETSEM, 2007)
	Guinea, Ghana	Emeto-purgative, antidote for poisoning, leprosy, stomach-ache, anthelminthic	Decoction		(ABBIW, 1990, GBOLADE and ADEYEMI, 2008, OMOBUWAJO *et al*., 1996, ONOCHA and OTUNLA, 2010, ORWA *et al*., 2009, TSAASSI *et al*., 2010)
	Ghana	Oral thrush in children	Bark exudate		(FORT *et al*., 2000, TSAASSI *et al*., 2010, UBILLAS *et al*., 1997)
	Ghana, Nigeria	Appetizer and for the treatment of stomach-ache vesiculopustular skin lesions caused by Onchocerca toothache, poison antidote	Pounded bark, bark infusion or latex		(ABBIW, 1990, ONOCHA and OTUNLA, 2010, UBILLAS *et al*., 1997)
	Cameroon	Toothache, lactation failure	Decoction	Brush teeth; oral	(BETTI, 2004)
Root- Bark	Ghana, Nigeria	Anthelminthic	Hot water infusion	Oral	(DIEHL *et al*., 2004, GBOLADE and ADEYEMI, 2008, ORWA *et al*., 2009)
	Cote d'Ivoire	Schistosomiasis	macerate		(MAPONGMETSEM, 2007)
Twigs/wood	Nigeria, other parts of West Africa	Toothache, skin disease, stomachache, oral thrush, shingles	Pulverize		(FORT *et al*., 2000, KAYODE *et al*., 2009, OMOBUWAJO *et al*., 1996, TSAASSI *et al*., 2010)
Sap/Latex	Ghana, Nigeria	Arrest bleeding (syptic)	Neat Sap	Apply topically	(ABBIW, 1990, GBOLADE and ADEYEMI, 2008, ONOCHA and. OTUNLA, 2010 , ORWA *et al*., 2009, UBILLAS *et al*., 1997)
		Eye troubles, e.g. cataract, filarial in eye, schistosomiasis	Latex	Not described	(ONOCHA and OTUNLA, 2010)
Seeds/fruits	Cameroon, Ghana	cure thrush, skin and fungal infections (antimicrobial)		Fat as mouthwash and topical application	(FORT *et al*., 2000, ORWA *et al*., 2009, TCHINDA *et al*., 2008, TSAASSI *et al*., 2010, WABO *et al*., 2007)
	Nigeria	Anti-inflammatory, pneumonia infections, craw-craw, analgesic			(GBOLADE and ADEYEMI, 2008, ONOCHA and OTUNLA, 2010)
		Rhinitis, cough and sore throat			(AKENDENGUÉ, 1992, FORT *et al*., 2000, NONO, *et al*. 2010)
		Rheumatism and haemorrhoids			(AKENDENGUÉ, 1992, FORT *et al*., 2000, TSAASSI *et al*., 2010)
shoot, bark, leaves	Cameroon	Dysentery, anaemia	Not described	Not described	(JIOFACK *et al*., 2009)
nnamed part	Ghana	Blood tonic, constipation, menstrual pains, unstable pregnancy, stomach ulcer	Not described	Not described	(GOVINDASAMY *et al*., 2007)

Biological activities of crude extracts from *P. angolensis*

Most investigations regarding the bioactivities of medicinal plants are limited to examination of crude aqueous or organic solvent extracts and in most cases, the investigators have sought to validate the traditional medicinal use of the plant. Various workers using different types of extracts looking for answers to the scientific rationale for the use of different parts of *P. angolensis* in folklore have demonstrated different important biological activities of its extracts thereby adding confidence to the prescriptions of local herbalist as well as paving the path for the effective screening for therapeutically relevant lead molecules. It is noteworthy to stress that crude plant extracts could be biologically active against a test model(s); however, the observed activity may be significantly reduced or virtually abolished when the compounds are isolated as pure substances as experienced by Abrantes *et al.,* (2008). In this light it seems logical to suggest that it may not always be essential to seek pure compounds to develop a therapeutic agent that requires a synergistic action of some of the natural components of the plant. As a matter of course, the biologically active extracts need to be evaluated for general toxicity, since there may be unacceptably low distinctions between toxicity towards the test model and that shown towards the host. Table 2 highlights some biological activities of *P. angolensis* extracts reported in literature which are discussed in more details in the following sections.

Antimicrobial activity

The antimicrobial properties of different crude solvent extracts of *P. angolensis* have been investigated and reported by a number of researchers. Anti-microbial, antifungal and anti-mycobacterial activities have been reported on these phyto-preparations (crude or fractionated extracts and sometimes individual compounds) under laboratory conditions. The antimicrobial activity of crude ethanol leaf extracts of *P. angolensis* on clinical strains of bacteria and fungi were investigated by Onocha and Otunla, (2010) using the disc diffusion method. The leaf extracts inhibited the growth of two bacterial and three fungal strains. The mean zones of inhibition produced by the crude ethanol extracts of the leaf at 75 mg/ml was found to be around 15 mm for the bacterial species and 10-20 mm for the fungi while the reference drugs, ampicillin and tioconazole at a concentration of 12.5 µg/ml yielded zones of inhibition of 19 mm and 12-16 mm respectively. Similarly Atindehou *et al*., 2002 demonstrated the bactericidal properties of ethanol extracts of the roots of *P. angolensis* against some gram positive

strains of bacteria. Again, Oladimeji *et al*., (2006) showed that leaf extracts of *P. angolensis* abrogated the *in vitro* survival of *Bacillus subtilis*, and *Staphylococcus aureus,* were inactive against *Escherichia coli, Salmonella typhi* and *Klebsiella pneumonia*; and exhibited activity against the fungal isolates *Aspergilus niger* and *Candida albicans* although the potency in these assays may not have been clinically significant. Purified essential oils from the stem-bark and leaves have also proven to have some antimicrobial activities against various fungal and bacterial strains [SIMIC *et al*., 2006; OGUNWANDE *et al*., 2007]. The fact that most crude extracts of the plant demonstrated antimicrobial activity probably supports its use by traditional healers for the treatment of microbial infestations.

Antiprotozoal

Several vector-borne tropical diseases are protozoal infections, caused by protozoan parasites. Some of these diseases, including leishmaniasis, trypansosomias, and malaria, persist without effective treatment. Although many drugs are available, some have become ineffective due to the development drug resistant strains. By far the most important vector-borne protozoal infection is malaria caused by Plasmodium parasites which are transmitted through the bite of some species of Anopheles mosquitoes.

Traditional remedies are continually being investigated as plant derived antimalarial drugs become more sought after. The anti-plasmodial activities of different extracts of *P. angolensis* have been investigated with promising outcomes. The chloroform and ethanol extracts of its stem-back showed activity against chloroquine-sensitive *Plasmodium falciparum* strain D6 and the chloroquine-resistant strain W2 with both extracts giving an IC_{50} of less than 25 µg/ml [ANCOLIO, *et al*., 2002]. Similarly, Abrantes *et al*., 2008 showed that the dichloromethane, methanol and aqueous ethanol extracts of the stem bark afforded *in vitro* anti-plasmodial activity against 3D7 *P. falciparum* strain. While crude CH_2Cl_2 extracts produced remarkable activity with an IC_{50} of 1.6 µg/ml, the purified fractions lacked anti-plasmodial activity. Zirihi *et al.* 2005 confirmed that the ethanol extracts of the stem back of this plant possess moderate activity against chloroquine-resistant FcB1/Colombia strain of *P. falciparum*. In a related study, Do Céu de Madureira *et al*., (2002) previously demonstrated that crude ethanol extracts of the stem bark of *P. angolensis* cleared female balb/C mice of

parasitemia due to *P. berghei* ANKA infection thereby validating the traditional use of pyto-preparations from *P. angolensis* against malaria fever.

P. angolensis extracts have shown promising anthelmintic properties. For instance, the crude chloroformic and methanolic extracts of the leaves and methanolic extracts of the stems exhibited *in vitro* anthelmintic activities against *Fasciola gigantica*, *Taenia solium* and *Pheritima pasthuma* consistent with the folkloric use of the plant as an anthelminthic [ONOCHA and OTUNLA, 2010]. Moreover, success was chalked when ethanolic extracts of the roots were evaluated for anthelminthic activities against the larvae of *Haemonchus contortus* [DIEHL *et al*., 2004]. Similarly methanolic and chloroformic extracts of stem bark exhibited anthelmintic activity when tested *in vitro* against *Eudrilus eugeniae* with the methanolic extract being more effective [GBOLADE and ADEYEMI, 2008]. *In vitro* antileishmanial assay using methanolic extracts of the stem against promastigotes of *L. major* exhibited a leishmanicidal action with an IC_{50} of 70.59 µg/ml. On the other hand, the methanolic extracts of the root was not leishmanicidal but cytotoxic while same solvent extracts of the leaves showed neither leishmanicidal nor cytotoxic activity [ONOCHA *et al.,* 2008]. When the nematicidially active crude fractions of *P. angolensis* (against *C. elegans*) were further fractionated, the lignan dihydroguaiaretic acid, with an LD_{50} of 10 µg/ml was obtained [NJOKU *et al.,* 1997].

Plant Part	Activity Tested	Type Extract	Test Model	Results	Ref
leaves, roots and stem	Antimicrobial, Antibacterial, antifungal, Anthelmintic	Chloroform and methanol	*S.typhii, ,P. aeruginosa, A. niger, Candida albicans, Dermatophyte. sp,Fasciola gigantica,T. solium, Pheritima pasthuma.*	Methanol extract of leave active against 2 bacterial test models and 3 fungal models used. Methanol extracts of leaf and stem possesses anthelmintic activity	(ONOCHA and OTUNLA, 2010)
	Antileishmaniasi s,cytotoxicity	Methanol	*Lemna. major, Artemia salina* (brine shrimps)	Stem extract was leishmanicidal with IC_{50} of 70.59µg/ ml but wasn't cytotoxic. Root extract cytotoxic with an D_{50} 727.70 µg/ ml but was not leishmanicidal; Leave extract was inactive.	(ONOCHA *et al.*, 2008)
Leaves and Bark	Antimicrobial	Steam (fractionated into pure products)	*K. pneumoniae, E. coli, S. enteritidis, . S. aureus, Aspergillus niger,Candida albicans, Ps aeruginosa*	Leave oil more potent on *P. aeruginosa, E. coli S. enteritidis and S. aureus* than bark oil. But bark oil more potent on *C. albicans*, *A. niger* and *K. pneumonia* than leave extracts	(OGUNWANDE *et al.*, 2007, SIMIC *et al.*, 2006)
Stem bark	Antiplasmodial	Chloroform and methanol	chloroquine-sensitive strain *Plasm falciparum* strain D6 and chloroquine-resistant (strain W2)	IC_{50} = < 25µg/ml: IC_{50} chloroquine = 0.055µg/ml	(ANCOLIO *et al.*, 2002)
	Antiplasmodial	dichloromethane, methanol and aqueous ethanol	3D7 *Plasmfalciparum* strain Dd2 P. falciparum strains	Crude CH_2Cl_2 extract was most active with an IC_{50} of 1.6 µg/mL; pure isolates gave weaker anti-plasmodial activity	(ABRANTES *et al.*, 2008)
	Anti-cancer	Methanol	MCF-7, COR-L23 and SVK-14 cell lines	IC50 < 50µg/ml	(ASHIDI *et al.*,2010)
Stem bark	Anti-cancer	Ethyl acetate fractionated to pure compounds	HuH-7 cell lines	Nine flavonoid compounds isolated. Testing at 20 µM for each compound genetisin gave highest cytotoxicity of 50% compared with the control. For apoptosis induction compounds exhibited higher potency than well-known control compounds	(MANSOOR *et al.*, 2011)
	anti-diabetic (antihyperglycemic)	Ethanol (fractionation to pycnanthuquinone A and B)	db/db mouse model for Type 2 diabetes	Significant blood glucose lowering effects observed for pycnanthuquinones A and B when administrated orally at 100 and 25(mg/kg. Effects observed for pycnanthuquinone A on days 1 and 2, and for pycnanthuquinone B on day 2, at 100 and 250 mg/kg dose, respectively.	(FORT, *et al.*, 2000)
	Anthelminthic	Methanolic and chloroform	*Eudrilus eugeniae*	Methanol extracts more active than chloroform extract significant effects shown at tested concentrations (10–20mg/ml).	(GBOLADE and ADEYEMI, 2008)
	Anticancer/ cytotoxicity	methanol	MiaPaCa-2, CCRF-CEM, CEM/ADR5000 (MDR sub-line) and (HUVEC normal cell lines).	Active against CCRF-CEM at 20µg/ml, but no activity noted for MiaPaCa-2 and CEM/ADR5000 at same concentration.	(KUETE *et al.*, 2011)
Stem bark	Antimalarial	Ethanol	Chloroquine-resistant FcB1/Colombia strain of *Plasmodium falciparum*	Active against test model IC_{50} = 18.2±2.7 µg/ml	(ZIRIHI *et al.*, 2005)
Roots	Anti-microbial	CH_2Cl_2-CH_3OH (1:1) further fractionated to pure compounds	Drug-resistant microorganisms; *S. aureus, E.Coli*, *Shigella dysenteriae, K. pneumonia, P. aeruginosa, S. typhi* and *Citrobacter freundii* and two pathogenic fungi used were *Candida albicans an*d *Microsporum audouinii.*	Significant antimicrobial activities. Ten compounds isolated, pycnanthulignenes A and C assessed foe antimicrobial activity. Significant activity against all organisms (S. aureus most sensitive). MIC for A varied from 28.7-230.9 µM. C exhibited inhibition against eight of the nine tested microorganisms. The lowest MIC value was 63.8 µM. Both compounds less active than the reference antibiotics	(NONO *et al.* 2010
Roots	Anti-bacterial and antifungal	Ethanol	*E. coli, P. aeruginosa, S. aureus, E. faecalis C. albicans and C. cucumerinum*	Bactericidal against gram positive strains but no report on antifungal activity	(ATINDEHOU *et al.*, 2002)
	anthelminthic activities	Ethanol	Haemonchus contortus	Active MIC 1.7mg/ml	(DIEHL *et al.*, 2004)

Stem and root barks	Memory enhancement	Methanol	In vitro: acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE)	Inhibited AchE and BuChE activities L: C 43.96±3.04%; B= 43.59 ± 1.77% Rb: C= 15.51 ±0.64%; B = 0% Sb: C = 66.52 ±5.02%; B = 86.05±8.32%	(ELUFIOYE *et al.,* 2010)
Seed/fruits	Anti-diabetic	CH_2Cl_2-MeOH	α-glycosidase	Demonstrated α-glycosidase inhibitory activity IC_{50} = 522.0 µM. Deoxynojirimycin IC_{50} = 425 ± 8.14 (a potent α-glycosidase inhibitor) Acarbose IC_{50} = 780 ± 28 µM clinically used drug for type-2 diabetes	(TCHINDA *et al.,* 2008)
Seed	anticancer cholesterol lowering	Alcohol or supercritical CO_2	Rat liver microsome, Human low density lipoproteins, tumour bearing mice, MDAMB-435 cells, MCF-7 estrogen receptor-positive human breast cancer cells	Extracts showed stronger antioxidant activity than vitamin E, inhibited proliferation of MDAMB- 435 and MCF-7 cancer cells synergistically with tamoxifen. IC_{50} comparable with the tocotrienols but gave lower IC_{50} in combination with tamoxifen than either of the compounds alone. Reduced elevated plasma levels of total and LDL cholesterol as well as plasma apolipoprotein B concentrations in humans.	(LEONARD, 2004)
Seed	Osteoporosis and osteoarthritis rheumatoid arthritis musculoskeletal conditions	Cetyl myristoleate (a cetyl alcohol ester of myristoleic acid acid).	Horse , human subjects	Restored swollen and stiff knees, arthritis and pain in horses. 92% of human subjects showed marked reduction of pain and inflammation when treated with cetyl myristoleate.	(BRILL *ET AL.*, 2004, LEONARD and SIMO 2010)
	Cognitive disorders,cancer, cell proliferation inhibition	Aq NaOH followed by acidification and purification	Rat liver microsomes, Human low density lipoproteins, cancer cells e.g. human breast cancer cells MCF-7, clooxygenase-2	Effective at inhibiting cell proliferation. Inhibits cyclooxygenase 2 activity	(LEONARD, 2004)

Table 2. Documented biological activities for extracts of *Pycnanthus angolensis* (adapted from ACHEL *et al.,* 2012).

Pain and Anti-inflammatory Activity

Excessive production of some prostaglandins (PG) is known to provoke pain and inflammation and enhance blood clotting action. The cyclooxygenases have been implicated in the regulation of prostaglandin synthesis and two isoforms of this enzyme (COX-1 and COX-2) have been reported. COX-I is a constitutive isoform that exists in most tissues and is responsible for the production of prostanoids involved in homeostasis whilst the inducible isoform (COX-2) is responsible for the production of prostanoids involved in inflammation [DANNHARDT and KIEFER, 2001]. Enhanced levels of COX-2 have been found in humans during the course of numerous inflammatory conditions including rheumatoid arthritis, osteoarthritis and acute or chronic inflammatory disease.

Conventional Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) non-selectively inhibit both COX isoforms. Due to its influence on the on the production of PG at the site of inflammation, COX-2 represents a possible therapeutic target. Currently researchers are keenly interested in finding NSAIDs which selectively inhibit COX-2 with little or no interference to COX-1. Compounds that selectively inhibit COX-2 are highly promising new agents for the treatment of pain and inflammation, and for the prevention of cancer [DANNHARDT and KIEFER, 2001; MARNETT and KALGUTKAR, 1998].

Herbal therapies present an attractive approach for the treatment of various inflammatory disorders. The important value of extracts and compounds from *P. angolensis* as anti-inflammatory remedies and treatment of adverse health conditions associated with inflammation and/or the presence of free radicals, joint diseases, arthritis, and allergies have been documented. Products from the seed of *P. angolensis* are believed to be useful for the prevention and treatment of conditions linked to inflammation. For instance, Simon *et al.,* (2010) determined that agents in the fruits of *P. angolensis* possess anti-inflammatory activities based on *in vitro* experiments utilizing lipopolysachharide induced murine (RAW 264.7) macrophage cells. They showed that the extracts of this plant suppressed inflammation-mediated conditions in the experimental model used and these agents from *P. angolensis* have since been adjudged to possess beneficial inhibitory activities on the inflammatory response in cells, both *in vitro* and *in vivo* (PEREZ-CASTORENA *et al*., 2002). Further exposition

on the anti-inflammatory potentials of extracts and compounds of *P. angolensis* is elaborated elsewhere [BARCLAY *et al.,* 2010; SIMON *et al.,* 2010].

Anti-atherosclerotic effect (Cholesterol lowering)

Hypercholesterolemia remains to be one of the major risk factors of coronary heart disease, the leading cause of death in the world [MANNU et *al*., 2012]. Atherosclerosis is a complex multi-cellular process involving oxidation of cholesterol and the intracellular accumulation of oxidized cholesterol. Currently available hypolipidemic drugs like gemfibrozil, bezafibrate, lovastatin, and nicotinic acid are not totally safe particularly when used for prolonged periods [MUKHERJEE, 2003].

Several plant species are known to possess antihypercholesterolemic action and may afford a suitable alternative to current day cholesterol lowering medicaments. Extractives of *P. angolensis* have been shown to be efficient at reducing elevated plasma levels of total and LDL cholesterol as well as plasma apolipoprotein B (ApoB) concentrations in humans. ApoB is the dominant protein constituent of low-density lipoprotein (LDL) thought to stabilize lipid emulsions, serve as a cofactor and modulator of enzymatic reactions, manage export of lipids out of cells and direct lipids to target organs. ApoB levels are positively correlated with the risk of coronary disease. Extracts from *P. angolensis* have been demonstrated to cause a dose-dependent reduction of medium ApoB in cultured HepG2 cells (which have the capacity to secrete and catabolize lipoproteins similar to LDL) proving to be useful cholesterol-lowering agents [LEONARD, 2004].

Wound healing

Over the years, different herbal products have been used in the management and treatment of wounds particularly in folklore. In Nigerian traditional herbal practice, a mixture with ratio 1:1:2 of exudates of *Anchomones difformis, Cyrtosperma senegalense* and *P. angolensis* respectively, is prescribed for treatment of corneal ulcers. Similarly there are reports that decoctions of the stem bark and leaves of *P. angolensis* are employed to stem haemorrhoids, stomach ulcer and chronic wounds in Ghana [ASASE AND OPPONG-MENSAH, 2009]. Following these folkloric leads Agyare *et al*., 2009 have confirmed the *in vitro* wound healing capability of ethanol and aqueous extracts of the stem bark of *P. angolensis* in human keratinocytes and dermal fibroblasts.

Furthermore, Onwukaeme *et al*., 2007 showed that the exudates of *P. angolensis* exhibited antibacterial activity, and healed *in vivo* corneal ulcers induced in rabbits even in the absence of prescribed exudates of *A. differmis* and *C. senegalensis*. Even though further confirmations and clinical studies are warranted, these initial findings points to bioactive principles present in both the exudates and stem bark of *P. angolensis* that are capable of stimulating wound healing and may thus justify its traditional use to heal wounds. Besides, the plant is known to elaborate other known antimicrobial agents which together might have some level of wound healing activity [BEUTH *et al., 2006;* MARAGAKIS *et al., 1995;* WILLITAL and HEINE*, 1994*]. Therefore, an interesting development from these reports would be a systematic investigation of compounds responsible for these wound healing properties of *P. angolensis*.

Cognitive disorders

Current records point to a progressive rise in the number of individuals afflicted with neurological disorders including Alzheimer's disease (AD), senile dementia, ataxia and myasthenia gravis for which special research attention is required to develop better treatment regimen for victims. While butycholinesterase (BuChE) activity is thought to increase progressively in patients with AD, acetylcholinesterase (AChE) activity remains unchanged or declines. AChE and BuChE inhibitors are considered promising therapeutic agents for the treatment of AD to help maintain or elevate the levels of acetylcholine in the brain [SRAMEK *et al.,* 2000*;* SRAMEK *et al.,* 2002; GREIG, 2002]. Only few synthetic medicines which are known to poses significant adverse effects exist for treatment of cognitive dysfunction and memory loss associated with these diseases. Hence, developing potential AChE inhibitors from botanicals could be helpful. Quiet recently, the Amaryllidaceae alkaloid, galanthamine was approved in a number of European countries for the treatment of Alzheimer's disease [WILKINSON and MURRAY, 2001]. Following leads from folklore, Elufioye *et al*, 2010 screened methanolic extracts of *P. angolensis* (leaves, root bark and stem bark) for both AChE and BuChE inhibitory activities. They demonstrated that the crude stem-bark extracts exhibited the highest *in vitro* AChE and BuChE inhibitory of 66.52 ± 5.02% and 86.05±8.32% respectively while the leaf extracts gave nearly equal inhibitory activity of 43.96 ±3.04 and 43.59± 1.77% in that order. The root bark showed 15.51% inhibition towards AChE but could not inhibit the activity of BuChE. Barclay *et al*, (2010) recognised the pro-cognitive properties of extracts from the seeds of *P. angolensis* and

traced it to the presence of hydroquinones specifically sargahydroquinoic acid. The authors suggest the use of these compounds in Alzheimer disease to enhance cognitive function, myasthenia gravis, glaucoma, learning and memory. Earlier studies by Choi *et al*., 2007, demonstrated the effectiveness of sargaquinoic acid and sargachromenol derived from a marine plant towards the inhibition of BuChE and AChE activities which is in consonance with the findings of Barclay and others [BARCLAY *et al*., 2010] and thus confirming one of the folkloric uses of *P. angolensis*.

Anticancer/antitumor activity

Cancer is a disease recognised by seven hallmarks: unlimited growth of abnormal cells, self sufficiency in growth signals, insensitivity to growth inhibitors, evasion of apotosis, sustained angiogenesis, inflammatory microenvironment and eventually tissue invasion and metastasis [MANTOVANI, 2009; GALLO and SARACHINE, 2009]. Extracts of *P. angolensis* have shown antitumor activities in several cancer cell lines. *In vitro* assay for anticancer activity of methanol extracts from the mature stem bark *of P. angolensis* have exhibited cytotoxicity and anti-proliferative action on various cancer cell lines [ASHIDI *et al.,* 2010; KUETE *et al.,* 2011]. When nine flavonoids isolated from the ethyl acetate extracts of the plant were screened for apoptosis induction in human hepatoma HCH-7 cancer cell lines, the compounds tested showed higher apoptosis induction profiles compared with the control. The apoptosis inducing activity of these flavonoids was confirmed by caspase activity assays [MANSOOR *et al.,* 2011]. Pure compounds other than flavonoids and belonging to the hydroquinones such as kombic acid, sargahydroquinoic acid, sargachromeol, and hydroquinoic acid isolated from the seeds of *P. angolensis* and related compounds have been shown to be efficient at preventing and/or retarding the development of cancer [LEONARD , 2004; 2004*;* BARCLAY *et al.,* 2010*;* SIMON *et al.,* 2010]. Results of these studies may be a first step in the verification of the anecdotal claims of the folkloric use of *P. angolensis* as an anticancer agent.

Antioxidant Action

Phytochemical antioxidants are touted as free radical sinks, effectively neutralizing free radicals, active atoms or molecules that can damage DNA and corrode cell membranes. Free radicals play a key role in the development of a number of

adverse health conditions, including cancer, cardiovascular disease, and cataracts, and have also been implicated in both initiation and acceleration of the aging process. Due to the adverse health conditions associated with free radicals and other inflammatory factors, antioxidant and anti-inflammatory agent with capacity to ameliorate health conditions like cancer, cardiovascular disease, cataracts, rheumatic diseases, fibromyalgia, Alzheimer's disease and other neurodegenerative conditions are currently being perused in this regard; phyto-preparations could come in handy.

Extracts of *P. angolensis* possess important liposoluble antioxidant activities comparable to or even better than well-known antioxidants like Vitamin E [LEONARD, 2002*; IBID,* 2004; BARCLAY *et al.,* 2010]. Simon *et al*., (2010) have demonstrated the strong antioxidant properties of some extracts from the seeds of *P. angolensis* using standard free radical scavenging assay protocols and trolox as standard. A DPPH assay yielded IC_{50} values of 17.3 and 9 µg/ml respectively for purified sargachromenol and sargahydroquinoic acid and served as ample proof that the compounds are good free radical scavengers. Also, kombo butter acid extract, with an IC_{50} of 25.2 µg/ml, was found to be a much better DPPH free radical scavenger than kombo butter. Results of the ABTS free radical scavenging assays corroborated the outcomes of the DPPH assay.

Furthermore during a search for anti-inflammatory properties in some extracts derived from the seeds of *P. angolensis,* these extracts were found to suppress NO production, iNOS mRNA and protein expression as well as COX-2 mRNA and protein expression in murine macrophages and LPS-induced RAW 264.7 mouse macrophage cells [LEONARD, 2002]. In consonance with these reports, Tchnida *et al*., 2008 demonstrated in a nitric oxide scavenging assay that crude CH_2Cl_2-MeOH extracts of the fruits of *P. angolensis* possess 99.0 % compared with 90.3 % radical scavenging activity elicited by n-propyl gallate standard (TCHINIDA et al., 2008). Despite its beneficial role in host defence mechanisms, excessive nitric oxide (NO) production by activated macrophages has been implicated in several inflammatory diseases. Perez Cantona *et al*., 2002 also showcased the anti-inflammatory and antioxidant properties of sargahydroquinoic acid and sargachromenol isolated from *Roldana barba-johannis (*but *present in P. angolensis)*, alongside their mixtures and methyl esters.

The antioxidant components have also been proposed for use in different cosmetic and herbal formulations for topical applications.

Major chemical constituents

In order to evaluate the potential of *P. angolensis as* a source of additional bioactive compounds, various parts of the plant have been investigated and important compounds with high therapeutic potentials reported some of which have been discussed below and summarised in table 2.

Flavonoids

P. angolensis elaborates a number of flavonoid compounds including the flavonones genkwainin [KUETE *et al*., 2011; NONO *et al* 2010], 8-hydroxykanzakiflavone-2 [NONO *et al* 2010], liguiritigentin [MANSOOR *et al.,* 2011] (–)-epicatechin and (+)-catechin [YAZAKI *et al.,* 1985]. Isoflavonoids present in *P. angolensis* are biocchanin A [KUETE *et al.,* 2011; NONO *et al* 2010; TSAASSI *et al.,* 2010; WABO *et al*., 2007], formononetin [NONO *et al* 2010 : MANSOOR *et al.,* 2011; TSAASSI *et al.,* 2010; MAPONGMETSEM, 2007; OMOBUWAJO *et al.,* 1996; ADESANYA, 2005], 7, 4'-dimethoxy-2'-hydroxyisoflavone [MANSOOR *et al.,* 2011; OMOBUWAJO *et al.,* 1996], prunetin [KUETE *et al*., 2011; MANSOOR *et al.,* 2011], calycosin [KUETE *et al*., 2011], irilone [MANSOOR *et al.,* 2011; WABO *et al*., 2007], tectorigenine, genistein, and 2'-hydroxybiochanin A [MANSOOR *et al.,* 2011]. A study on cytotoxic effects of some nine isoflavonoid compounds isolated from *P. angolensis* against hepatoma HuH-7 cells (at a concentration 20μM) revealed that genetisin had the highest cytotoxicity of 50% when compared with the control compound used. Furthermore, when screened against a panel of human hepatoma HuH-7 cells for apoptosis induction, most of these isoflavonoids exhibited higher potency than acclaimed control compounds used [MANSOOR *et al.,* 2011]. Other useful bioactivities of some of these flavonoids and isoflavonoids have been reported elsewhere [WABO et *al*., 2007].

Flavonoids have long been known to exhibit several pharmacological properties; acting as anti-allergenic, anti-inflammatory, antiviral, antiproliferative, anti-cancer, antibacterial, vasodilatory, and antioxidative properties [YAO *et al*., 2004; SANDHAR *et al.,* 2011]. Flavonoids have also been proven to be good photo-protective shields against UV and ionizing radiations [SANCHEZ-CAMPILLO *et al*., 2009]. Circumstantial evidence exist supporting the relationships between the intake of flavonoids and reduced risk of coronary heart disease [KURIYAMA *et al.,* 2006],

neurodegenerative disorders [CHECKOWAY *et al.,* 2002] lung cancer [CUI *et al.,* 2008; NEUHOUSER, 2004], stomach and other forms of cancer [NEUHOUSER, 2004]. In particular, isoflavonoids have been identified as chemopreventive against various types of cancer and cardiovascular diseases [WATANABE *et al*., 2002; WIDYARINI *et al.,* 2005]. We have previously demonstrated that the structure of flavonoids extracted from citrus and other plants is related to their antioxidant, anti-proliferative and anti-metastatic properties [YANEZ *et al.,* 2004; RODRIGUEZ *et al.,* 2002; BENAVENTE-GARCÍA *et al.,* 2007]. Furthermore, our team have shown the antiproliferative action of several phenolic and ployhydroxylated flavonoids on metastatic melanoma B16F10 and melanocyte cell lines [MARTINEZ *et al.,* 2005a; 2005b; 2003]

Terpenes and Sesquiterepene

Terpenes represent a large group of compounds responsible for the fragrance of plants and comprise the essential oil fraction. They differ structurally from fatty acids in that they are branched and cyclized. When additional elements, such as oxygen, are added they are called terpenoids. Terpenes particularly triterpenes are known to display a wide spectrum of biological activities including anitumour, antiviral, bactericidal, fungicidal, analgesic, anti-inflammatory spermicidal and cytotoxic activities [PATOÈKA, 2003]. Simic *et al*, (2006) analysed the volatile oils from the stem bark of *P. angolensis* and isolated a number of compounds with the major constituents being; α-bergamotene (25.1%), terpinen-4-ol (16.6%), α-terpineol (15.6%), trans β- bergamotene (12.9%), α--curcumene (6%), piperitone (4.5%) and β-farnesene (4%). Other constituents include borneol (2.8%), bornyl acetate (1.6%), β- santalene (0.6%), acoradene (0.3%), β-bisabolene (3%), δ-cadinene (2.3%), germacrene B (1.0%), cembrene A (1.0%) and farnesyl acetone (2.0%). During screening of the leaf oils, only two compounds, spathulenol (82%) a sesquiterepene alcohol and caryophyllene oxide (14%) a sesquiterepene were detected. The authors further demonstrated the antimicrobial effects of these volatile oils against a panel of bacterial and fungal species. Even though the MICs were not reported, the authors found that the leaf essential oils were in general more potent on the bacterial strains than on the fungal strains (see Table 2).

On the other hand, the fungal species seemed to succumb more to the essential oils obtained from the plant bark. Further purification of the dicholoromethane extracts

of the stem bark afforded the labdane-type diterpene ozic acid ($C_{20}H_{30}O_2$) which demonstrated moderate antiplasmodial activity against D*d2*-chloroquine resistant *P. falciparum* strain [ABRANTES *et al.,* 2008].

Terpene-type quinones: the Pycnathuquinones

Quinones constitute a structurally diverse class of phenolic compounds with a wide range of pharmacological properties. Terepinoid quinones have frequently been isolated from microorganisms and plants to exploit their antimicrobial, antiviral and anticancer properties. Recently attention has been drawn to their anti-mycobacterial properties. *P. angolensis* is known to elaborate a series of unusual 6, 6, 5-tricyclic geranyltoluquinone terpene-like quinones called pycnanthuquinones (fig 2). A search for physiologically active compounds against diabetes using *in vitro* bioassay-guided fractionations of alcohol extracts of the root and leaves of *P. angolensis* (Warb.) against ob/ob and db/db mice models for type 2 diabetes led to the discovery of two terepene-type quinones, pycnanthuquinones A and B [FORT *et al.,* 2000; UBILLAS *et al,* 1997; LUO *et al., 1999*]. Further screening of the stem bark afforded another novel compound; pycnanthuquinones C [WABO, 2007] with antifungal properties against *Trichophyton soudanense*. Recently too, Liard and Co-workers reported the presence of pycnanthuquinone C in the Western Australian brown alga *Cystophora harveyi* [LAIRD *et al*., 2007] however, in both cases the authors have been silent about the antihyperglycemic property of the pycnanthuquinone C. Total synthesis of pycnanthuquinone C has since been achieved in the laboratory via the Diels-Alder type synthesis [LÖBERMANN *et al.,* 2010] however, the absolute configuration of the pycnanthuquinones still remain to be established. During the past decade and a half, Fort *et al*., (2000) have convinced the scientific community that the pycnanthuquinones represent a new class of anti-diabetic compounds which is structurally distinct from the currently available oral type 2 diabetes remedies namely sulfonylureas, biguanides, disaccharidase inhibitors, and thiazolidinediones.

Short of clinical data, this could well be the heralding of a new class of drug for type -2 antidiabetic therapies and a classic case of a modern drug with an origin from ethnopharmacology and traditional medicine; undeniably, numerous drugs have entered the international pharmacopoeia via the study of ethnopharmacology and traditional medicine [FABRICANT and FARNSWORTH, 2001]. Remarkable advances into the

study and discovery of this novel class of antidiabetic principles from *P. angolensis* has been extensively expounded elsewhere [FORT *et al.,* 2000; UBILLAS *et al.,* 1997; LUO *et al.,* 1999].

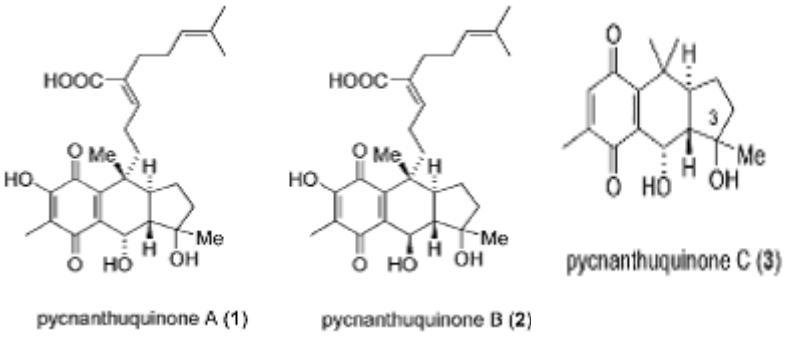


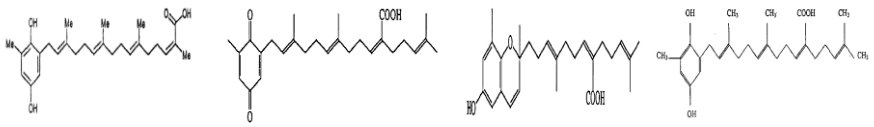
Figure 2. The Pycnathuquinones

Plastoquinones and Ubiquinones

Fractionation of the crude ethanol extracts of the seeds of *P. angolensis* yielded a series of medicinally relevant plastoquinones namely kombic acid, sargahydroquinoic and sargaquinoic acids and sargachromenol (an ubiquinone, Fig. 3) [MAPONGMETSEM, 2009; LOK *et al.,* 1976]. The antiinflammatory, antiproliferative, anti-aging, UV protecting and anti-oxidative activities of these compounds have been established [LEONARD*,* 2002; 2004a; 2004b; BARCLAY *et al., 2010*]. Further *in vitro* and *in vivo* studies have revealed other bioactivities inherent in them including cholesterol lowering effects, reduction of elevated plasma levels of Low Density Lipoproteins (LDL) cholesterol, inhibition of inducible nitric oxide synthase (iNOS) and Cyclooxygenase-2 (COX-2) protein expression, inhibition of LPS-induced iNOS and COX-2 mRNA expression.

Park *et al*., (2008) extracted sargahydroquinoic acid from *Sargassum micracanthum* obtained from the East Coast of the Korean sea which induced vasodilatation in the basilar and carotid arteries of rabbits in a dose-dependent manner. The EC_{50} values for the basilar and carotid arteries were 11.8 ± 0.28 and 140 ± 0.6μM respectively. It selectively dilated the basilar artery more than 10-fold over the carotid artery without

lowering systemic blood pressure. This implies sargahydroquinoic acid may accelerate cerebral blood flow through dilatation of the basilar artery without influencing systemic blood pressure. The authors suggests that compounds with similar core structure as sargahydroquinoic acid, such as plastquinones and hydroquinones may be provide novel leads to compounds with selective pharmacological activity for the human vascular system [PARK *et al*., 2008].



A **B** **C** **D**

Figure 3. Kombic acid (A), Sargaquinoic acid (B), sargachromenol (C)Sargahydroquinoic acid (D)

The acetylcholinesterase (AChE) and butylcholinesterase (BuChE) inhibitory activities of plastoquinones have been amply demonstrated [CHOI *et al.*, 2007] both sargaquinoic acid and sargachromenol isolated from *Sargassum sagamianum* showed AChE inhibitory activity at micromolar concentrations with IC_{50} 23.2 and 32.7 μM respectively. However, sargaquinoic acid demonstrated higher inhibitory activity on BuChE than on AChE; the observed IC_{50} of 26 nM was 1000-fold greater than the corresponding IC_{50} value for AChE inhibition. BuChE is a new drug target for the treatment of Alzheimer's disease. The authors remark that sargaquinoic acid represents an effective and selective inhibitor of BuChE with potency similar to or greater than the anticholinesterases in current clinical use, making it an interesting potential drug candidate for AD [CHOI *et al.*, 2007]. These results corroborate the findings of Elufioye *et al*., (2010) following their attempt to find a scientific rationale for the use of extracts of *P. angolensis* for the treatment of cognitive problems in Nigerian folklore.

Only recently, Tchinda *et al*., (2008) demonstrated the promising anti-diabetic potentials of sargaquinoic acid and sargachromenol sourced from the seeds of *P. angolensis*. While these compounds furnished IC_{50} values of 3 ± 0.123 and 4.6 ± 0.123μM respectively reputed α-glucosidase inhibitors deoxynojirimycin and acarbose had IC_{50} values of 425 ± 8.14μM and 780 ± 28μM.

Fatty acids

Some fatty acids are common plant and animal constituents and usually exist *in vivo* as esters. Some of these fatty acids are known to have diverse and potent biological activities. The fatty acid constituents of *P. angolensis* have been studied in detail. In addition to medically important myristoleic acid (9-tetradecenoic acid) and other FAs such as lauric, oleic, palmitic acids and myristic acid (tetradecanoic acid) have been characterized from the seeds of *P. angolensis* [MAPONGMETSEM, 2007; LEONARD and SIMONTON, 2010]. Leonard and Simonton, 2010 demonstrated the effectiveness of cetyl myristoleate (CMO), a derivative of myristeoleic acid, in the treatment of osteoarthritis, joint inflammatory diseases of musculoskeletal system and other stress related traumas in animals preferably in equines. In a randomised controlled double-blind study, myristeoleic acid formulated to CMO was compared with phenylbutazone in subjects with rheumatoid arthritis. CMO was found to significantly improve morning stiffness, walking time and joint swelling, however, its effects were less than that elicited by phenylbutazone. A substantial drop in knee pain and disability due to osteoarthritis was observed during a randomised, double-blind placebo, controlled crossover study of 42 patients [LEONARD and SIMONTON, 2010; BRILL *et al*., 2004].

Myristoleic acid, identified as a cytotoxic component in *Serenoa repens* extracts has been found to induce apoptosis and necrosis in human prostate cancer LNCaP cells. Treating LNCaP cells with 130 mg/ml of *S. repens* extract or 100 mg/ml myristoleic acid for 24 hr produced a proportion of 16.5 and 8.8%, apoptotic cells and 46.8 and 81.8% necrotic cells respectively. Thus, the extract from *S. repens* and myristoleic acid seem to induce both apoptosis and necrosis in LNCaP cells at the same time. When another prostatic cell line (PC-3 cells) was used in place of LNCaP cells, apoptotic/necrotic cell death was also observed after treatment with the extract [IGUCHI *et al, 2001;* VACHEROT *et al., 2000*]. Furthermore, myristeoleic acid a by-product of cheese has been shown to be one of three by products effective at inhibiting *in vivo Candida albicans* germination with a minimum inhibitory concentration (MIC) of 9 µM [CLÉMENT *et al.,* 2007]. Moreover, a massive body of overriding evidence has been presented to show CMO can reduce disability related to migraine headaches,

psoriasis broad spectrum anti-inflammatory and analgesic applications [BRILL *et al*., 2004].

P. angolensis presents a very important vegetable source of myristoleic acid which was formally largely obtained from ovine and porcine sources. The presence of myristoleic acid in commercially relevant quantities in the seed fats of *P. angolensis* is significant in the sense that it satisfies all classes of users; animal sources raises doubt in the minds of some users as to whether the product meets kosher and/or halal requirements. Besides, fatty acids sourced from land animal or marine animal origins are non-vegetarian and when sourced from beef tallow run the risk, although slight, of inducing bovine spongiform encephalitis (mad-cow disease) [LEONARD and SIMONTON, 2010].

Steroids

Chemical investigation into the stem, and roots resulted in the isolation of stigmast-4-en-6β-ol-3- one, stigmasterol [ABRANTES *et al.,* 2008], β-sitosterol [TSAASSI *et al.,* 2010; ABRANTES *et al.,* 2008] and other known non-steroidal compounds. These steroids showed partial *in vitro* suppression of the growth of *P. falciparum* parasites upon evaluation of their antimalarial activity [ABRANTES *et al.,* 2008]. Stigmast-4-en-6β-ol-3-one has been found elsewhere to possess potent antitumor-promoting activity [HABSAH *et al*., 2005], while ß-sitosterol has also been implicated in the mitigation of cardiovascular diseases through cholesterol reduction [MUKHERJEE, 2003].

Cerebrosides (Pycnangloside)

A biologically uncharacterised novel molecule, pycnangloside (Fig. 4) ($C_{44}H_{85}NO_{10}$), has been structurally elucidated from the bark of *P. angolensis* using comprehensive analyses of its 1D and 2D NMR spectroscopic, and ESI mass spectrometric data [TSAASSI *et al*., 2010]. The fact that its biological activities remain relatively unknown is ostensibly because it has only recently been reported.

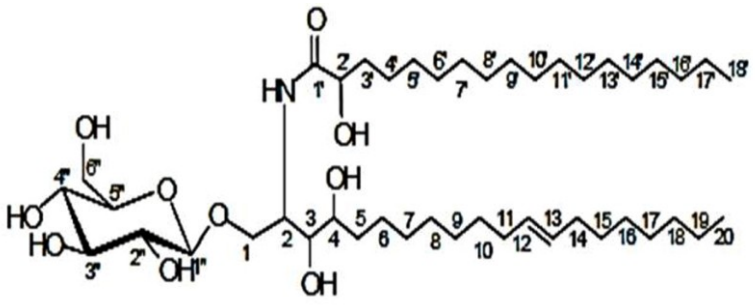


Figure 4. Structure of pycnangloside from *P. angolensis* *(Adapted from Tsaassi et al., 2010).*

Cerebrosides are a family of pharmacologically active and important glycosphingolipids, present in a wide variety of tissues and organs in biological systems. The cerebrosides are currently receiving attention because of their therapeutic potential including antitumor/cytotoxic and anti-HIV-1 moderating potentials amongst others [TAN and CHEN, 2003]. Cerebrosides are known to be involved in a very wide range of biological activities such as cell agglutination, intracellular communication, cellular development, and antitumor/cytotoxic effects.

Allantoin

Allantoin ($C_4H_6N_4O_3$) a diuredide of glyoxylic acid has been reported to be present in the bark of *P. angolensis* [OMOBUWAJO *et al.,* 1996, FORT *et al.,* 2000; ONOCHA *et al.,* 2008]. Allantoin is well known for its healing, moisturizing, soothing and anti-irritating, keratolytic and non-toxic effect in dermatological, cosmetic and veterinary preparations. In cosmetology, allantoin is used for sunscreen and skin-care products. It has shown clinical potentials for the treatment of skin ulcers, wounds, scalds, (sun) burns, carbuncles, acne, skin eruptions, fissures, impetigo, eczema and psoriasis. It has also been used in various oral hygiene preparations such as toothpaste and mouthwash as well as in eye drops to treat watering eyes and in ear drops to clean the ear canal. It is effective at quite low concentrations, 0.1% up to 2%.

Lignans

P. angolensis is known to produce lignans in its stems, roots leaves and seed including 3,4- dimethoxy-3',4'-methylenedioxy-7,7'-epoxylignan [KUETE *et al*., 2011] 4,5-dimethoxy-3',4'-methylenedioxy-2,7'-cyloligna-7,7'-diene, 4'-methoxy-4,5-methylenedioxy-2,7-cyclolign-7-ene-4,5-dimethoxy- 2,7'-cyclolign-7-en-4'-ol (pycnanthulignene A), [KUETE *et al*., 2011; NONO *et al.,* 2010], 2,7-dimethoxy-3,6 dimethylnaphthalene, 4,5- dimethoxy-3',4'-methylenedioxy-2,7'-cyclolign-7-ene (pycnanthulignene B) 3,6-dimethoxy-4,5- methylenedioxy-2,7'-cycloligna-7,7'-dien-4'-ol (pycnanthulignene D), 4'-methoxy-4,5- methylenedioxy-2,7'-cyclolign-7-ene [NONO *et al.,* 2010], Calopiptin, (12 S, 13 S)-12, 13-dihydroxylabda-8, 14-dien-18-oic acid, (12 R,13 S)-12,13-dihydroxylabda-8 (CUI *et al.,* 2008),14-dien- 18-oic acid, [WABO *et al*., 2007], threo-4,4'-dihydroxy-3- methoxylignan (pycnantolol) [ABRANTES *et al.,* 2008],

(−)- dihydroguaiaretic acid, heliobuphthalmin, talaumidin and hinokinin [MAPONGMETSEM, 2007; ABRANTES *et al.,* 2008; NJOKU *et al.,* 1997].

Dihydroguaiaretic acid showed non-selective cytotoxicity to a panel of cancer cell lines with an ED_{50} of 1.63 – 3.10 µg/ml [NJOKU *et al.,* 1997] and prevented harmful effects of UV [MOON et al., 2005] which facilitates skin aging. Furthermore, dihydroguaiaretic acid is important for the treatment of shock, neoplasms [DAVIS *et al*., 2009], sepsis, melanoma, and lymphoma [ANONYMOUS, 2003] and is a promising candidate for the treatment of Alzheimer's disease [SANDSON and FELICIAN, 1998; RÍOS *et al.,* 2002]. Working on a different plant Moon *et al,* (2008) isolated meso-dihydroguaiaretic acid from *Saururus chinensis* and showed that it inhibited the cyclooxygenase-2 (COX-2)-dependent phase of prostaglandin D generation in bone marrow-derived mast cells with an IC_{50} of 9.8 µM. However, it did not inhibit COX-2 protein expression in these cells at concentrations up to 30µM, suggesting that the lignan directly inhibits COX-2 activity. In addition, meso-dihydroguaiaretic steadily abrogated the production of leukotriene with an IC_{50} of 1.3 µM demonstrating that it inhibits both COX-2 and 5- lipoxygenase. Furthermore meso-dihydroguaiaretic was found to strongly inhibit the degranulation reaction in bone marrow-derived mast cells with an IC_{50} of 11.4µM providing strong basis for novel anti-inflammatory drug development [MOON *et al*., 2008]. Again mesodihydroguaiaretic acid from the stem bark of *Machilus thunbergii* showed significant matrix metalloproteinase MMP-9 inhibition in human keratinocyte cells caused by ultraviolet irradiation.

Li *et al,* demonstrated that the *in vitro* enzymatic activity of topoisomerase I and II was inhibited by 93.6 and 82.1% respectively at a concentration of 100 mM [LI *et al*., 2004; WIRAT, 2006b]. Wiart (2006a) notes that the Myristicaceae abounds in lignans with biological activities against topoisomerases and advocates the search for lignans with potent topoisomerase inhibitory activities from amongst the Laurales–Magnoliales plant group. Nono *et al,* 2010, have shown that pycnanthulignenes A and C possess antibacterial activity against a panel of drug-resistant bacterial and pathogenic fungal strains using gentamicin and nystatin as references for antibacterial and antifungal tests respectively (see Table 2.). The MIC values for pycnanthulignenes A varied from 28.7 µM (against *S. aureus*) to 230.9 µM (against *K. pneumoniae* and *P. aeruginosa*) while

lowest MIC values of 63.8 μM observed with pycnanthulignenes A were against *S. aureus, E. coli,* and *C. albicans.*

Glyceryl-1, 3-ditetradecanoate

Tcinida *et al*., (2008) examined in detail the fruits o*f P. angolensis* and reported for the first time, the presence of a diglyceride; glyceryl-1, 3- ditetradecanoate together with two known plastoquinones. The authors further demonstrated the α- glucosidase inhibitory tendencies of these compounds with glycerol-1, 3-ditetradecanoate registering an IC_{50} of 522.0 μM. This IC_{50} value compared favourably with deoxynojirimycin a potent α- glucosidase inhibitor which had an IC_{50} of 425 ± 8.14 μM, and acarbose a popular drug used for type- 2 diabetes therapy which had an IC_{50} of 780 ± 28 μM.

As indicated above, the plastoquinones proved to be better glycosidase inhibitors compared with the glycerol derivative. These findings led the authors to propose the use of extracts from the fruits of *P. angolensis* for the management of type-2 diabetes and related diseases. In our literature search, the mention of glycerol-1, 3-ditetradecanoate as a potential α-glycosidase (protease) inhibitor was rare and this could well be one of the pioneering works involving this compound in the moderation type-2 diabetes. Apha-glucosidases are key enzymes catalyzing the final step in the digestive process of carbohydrates. Therefore, α-glucosidase inhibitors can retard the liberation of D-glucose from dietary complex carbohydrates and delay glucose absorption, resulting in reduced postprandial plasma glucose levels and suppression of postprandial hyperglycemia. An effective way to manage non-insulin dependent diabetes mellitus (NIDDM) then is by inhibiting or reducing the activity of these enzymes (i.e. α-glucosidase and α-amylase) in the digestive organs there by retarding absorption of glucose and decreasing postprandial hyperglycemia.

Presently, there is renewed interest in plant based medicines and functional foods modulating physiological effects in the prevention and cure of diabetes and obesity. The plant kingdom is a wide field to search for natural effective oral hypoglycaemic agents that have slight or no side effects. Therefore, natural α-

glucosidase and α-amylase inhibitors from plant sources offer an attractive strategy for the control of hyperglycaemia [TUNDIS *et al.,* 2010; SUBRAMANIAN *et al*., 2008].

3. Current & future perspectives

From the above review, *P. angolensis* appears to be a rich source of phytochemicals that can be used to design and develop potentially useful therapeutic agents. Some extracts and compounds isolated from *P. angolensis* have been found to have antitumor (cytotoxic), cholesterol lowering, possess significant antioxidant and anti-inflammatory, iNO inhibitory, anti-ulcerogenic, antimicrobial and anti-hyperglycaemic activities. Indeed, some of these promising results are worthy of further investigation. For example, mesodihydroguaiaretic acid has been ascertained to inhibit the enzymatic activity of DNA topoisomerase I and II. The topoisomerases have recently been identified as molecular targets of a variety of pharmaceutical agents. Some of the drugs that target the topoisomerases are anticancer drugs.

Thus further investigation into the structure-activity relationship could lead to a better understanding of the mechanism of action of these anticancer principles. The AchE and BuChE inhibitory activities observed could be the action of different bioactives expressed in different parts of the plants as crude extracts of the stem bark and pure sargaquinoic acid (from the seed) both exhibited inhibitory activities. Moreover, mention of a new class of antidiabetic compounds in pycnanthuquinones A and B elaborated by *P. angolensis* is crucial to say the least. It will be particularly encouraging to see results of the clinical trials on the pycnanthuquinones as well as studies on their safety and efficacies either alone or in combination with conventional therapies. This will go a long way to help control and reduce the overall burden of diabetes as *P. angolensis* also expresses the α-glycosidase inhibitors, glyceryl-1, 3-ditetradecanoate, sargaquinoic acid and sargachromenol. It seems likely that more than one antidiabetic therapy could be derived from the plant. As noted earlier the butter extracted from the seeds of *P. angolensis* could become the much needed vegetable substitute for animal-fat based cetyl myristoleate employed in arthritis management.

The current paradigm is to evaluate antioxidants from flora (for obvious reasons) for their radioprotective potential in order to exploit them for the development of countermeasure agents for radiation exposure [ALCARAZ *et al.,* 2009]. The *in vitro*

and in vivo antioxidant potential of *P. angolensis* has been amply demonstrated. In particular the plastoquinones/ubiquinones kombic acid, sargahydroquinoic acid, sargachromenol and sargahydroquinone and the flavonoid compounds if evaluated could lead to promising radioprotective compounds.

Literature search for the radioprotective evaluation of these compounds or other phytochemicals elaborated by *P. angolensis* did not yield results probably due to the fact that there is no place in folklore for radioprotection to offer research leads in this direction. It will be interesting to investigate this property of the plant extracts and if confirmed, this might increase the economic potential and prospects for the plant.

Further work on this topic should be encouraged, and one can reasonably expect interesting results to radioprotective leads. Again, through the literature search, it has been noted that the potential genotoxic effects that may follow usage of the preparations from *P. angolensis* have not been investigated even though there is extensive coverage on cytotoxicity. In our view this constitutes a very vital aspect of the genetic safety evaluation of herbal extracts in a bid to uncover lead molecules with therapeutic utility. Previous pharmacological screening and toxicology assays on plants used medically showed that several plants used for medicinal purposes cause damage to the genetic material and, therefore, should be used with caution [FENNELL *et al.,* 2004].

4. Principles of Radiation Therapy (Radiotherapy)

Radiation therapy is one of the most important treatment modalities in the management of human cancer. In this modality, calculated doses of ionizing radiation (IR) is targeted directly at the cancerous area to safely and effectively purge off or reduce the growth of cancer cells. It has also found application in other aspects of curative therapy, to prevent tumour recurrence after surgery to remove primary malignant tumours. Oftentimes it is used in palliative care only to reduce the pain caused by growing tumours and to improve quality of life of the patient.

Ionizing radiation (IR) and it effects

Ionizing radiation, a physical agent, consists of high energy stream of either particles (such as neutrons or alpha particles) or photons (such as x- or γ-rays) with sufficient energy to ionize atoms or molecules in the process creating ion-pairs (ionizing) without raising the temperature of the bulk material.

The quality of IR may be differentiated on the basis of their linear energy transfer (LET); a physical parameter that describes average energy deposited along the track of a particle per unit length. The density of the ionization separates low- from high-LET. Particle radiations cause dense ionization along their track and are accordingly referred to as high-linear-energy-transfer (high-LET) radiation. Biophysical studies of track structure have revealed that while low-LET radiations are more inclined to produce localized clusters of ionizations within a single electron track, high-LET radiation induce relatively more ionizations that are close in spatial extent (clustered DNA damage). Localized DNA damage caused by dense ionizations is more difficult to repair than the diffuse DNA damage. This makes high-LET radiations more lethal to biological material than low-LET radiations at same doses. Indeed, the biological effectiveness of radiation is dependent on the LET but also on the total dose, fractionation rate and radiosensitivity of the targeted cells or tissues.

Mechanisms of biological damage by Ionizing Radiation

For years the biological effects of radiation has been thought to arise solely from damage induced in the DNA molecules of “hit” cells. However, the discovery of non-targeted responses to radiation (the so-called bystander response), has called this paradigm into question. Ionizing radiation-induced bystander responses are effects

occurring in cells that did not directly receive radiation doses but are thought to be induced by signals from nearby or neighboring irradiated cells. The bystander effect has been described in *in vitro* cell culture systems (TERZOUDI *et al.*, 2010), however, overriding scientific evidence exits supporting the occurrence of this phenomenon in subcutaneous tumours (AZZAM *et al.*, 2001). Moreover, as far as epigenetic damage or modifications of other cell constituents are concerned, cytoplasmic changes and mitochondrial or membrane damage also play a role in certain types of radiation effects.

Damage to DNA (supported by the target theory) is one of the single most important factors leading to cell death in radiation biology (HAN and YU, 2010). Even so, it is believed that the entire DNA is not the target *per se,* but only alteration/s to specific "hot spots" located on the DNA molecule constitutes the target for cellular inactivation. A variety of DNA lesions, such as single strand breaks (SSBs), double-strand breaks (DSBs), AP sites (either apyrimidinic or apurinic), clustered/complex/multiple DNA damage (MSBs), multiple base and sugar modifications, DNA-DNA and DNA-protein cross-links are induced by IR (WARD, 1998). These lesions may result from direct ionization of DNA (direct effect) or through the interaction of free radicals (produced through the radiolysis of water) with DNA (indirect effect). Even though cells have in-built DNA repair mechanisms to repair these lesions with high fidelity, DSB and MDS have been found to be extremely difficult to salvage (AZZAM *et al.*, 2001; HADA and GEORGAKILAS, 2008; HAN and YU, 2010).

Indirect Mechanisms on DNA damage

During the radiolysis of water reactive oxygen species (ROS) are released and water constitutes 70-85% by weight of the cell. ROS include hydroxyl radical ($^{\bullet}OH$), hydrogen radical ($^{\bullet}H$), hydronium ion (H_3O^+), hydrated electron ($^{\bullet}e_{aq}^-$) and hydrogen peroxide (H_2O_2) (Fig. 5). Normal cell metabolism is a source of some of these ROS; however, their excessive generation leads to oxidative stress, a highly injurious condition to cells. Consequently, under normal healthy conditions, a balance is maintained between oxidative stress and antioxidant requirements. By virtue of the fact that IR generates these ROS *in vivo*, it is considered as an exogenous source of ROS and exposure to IR initiates a burst of these unsafe entities in cells leading to a loss of balance of ROS in the system. Highly penetrating low-LET radiations mediate most of

their harmful effects via the indirect mechanism (Fig.6). Generally lifetimes of simple free radicals ($^{\bullet}$H or $^{\bullet}$OH) are very short, of the order of 10^{-10} sec and react with nearby molecules in a very short time, resulting in breakage of chemical bonds or oxidation of the affected molecules (ARENA, 1971).

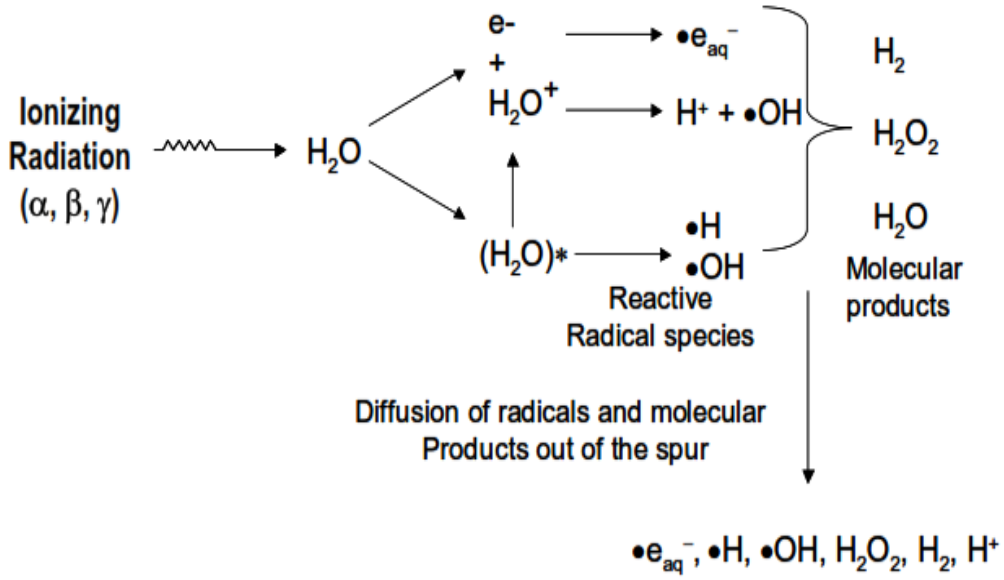


Figure 5. Steady state radiolysis (Formation and ROS)

Direct action on DNA

Two basic signature tunes of complex DNA damage are double strand breaks and non-DSB oxidative clustered DNA lesions (OCDL). One of the current theories invoked to explain the higher RBE observed in favour of high-LET over low LET radiations is the formers superior capacity to generate clustered DNA damage (PAINTER, 1980; WARD, 1998.; BASKAR *et al.*, 2012). Theoretical analysis and experimental data provide evidence of increased complexity and severity in complex DNA damage with increasing LET (HADA and GEORGAKILAS, 2008).

For a long time, it was difficult to relate the effect of clustering of DNA damage with biological effects, as ionizing radiation induces various kinds and numbers of damage in DNA at random positions. Recently, Shikazono and co-workers 2006, proved through the cluster damage experimental model system that the two base lesions

clustered DNA damage comprising 8-oxo-7,8-dihydroguanine (8-oxoG) and dihydrothymine (DHT) are more mutagenic than isolated base lesions in close proximity (SHIKAZONO *et al.*, 2006). Moreover, lesions within these complex DNA damage sites induced by ionizing radiation are less readily repaired than when present as isolated lesions (NIKJOO *et al.*, 2001; GULSTON *et al.*, 2004). These findings place the radiobiological properties and applications of high-LET in perspective.

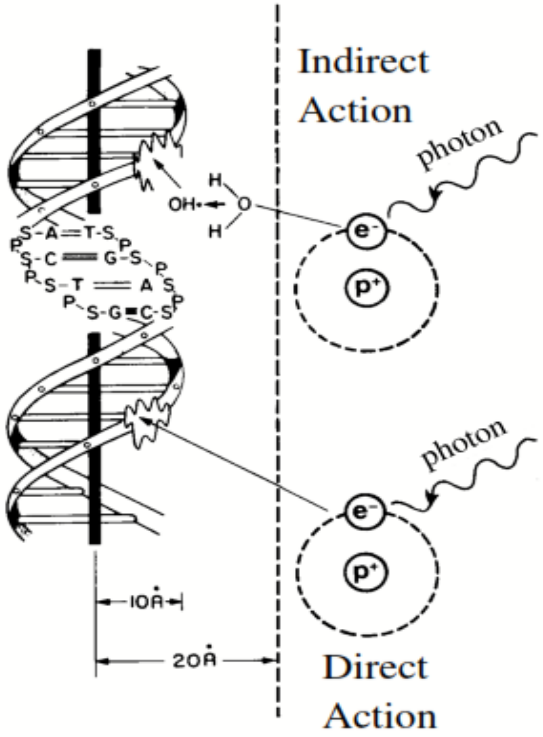


Figure 6: Direct and indirect radiation effects on DNA)

Radiation Induced biological membrane damage

The structural integrity of the plasma membrane is paramount to the maintenance of cellular viability given its capacity to ensure cellular compartmentalization, transfer extracellular signals to the cytosol through cascades and control of metabolic pathways. Recent findings have demonstrated that many important radiobiological effects are due to compromised integrity of biological membranes and overt doses to IR has been acknowledged as a key culprit in plasma membrane damage.

Essentially the cell membrane is made up of a lipid bilayer of polyunsaturated fatty acids (PUFA) embedded in proteins. Unsaturated lipids are highly prone to free radical attack resulting in lipid peroxidation and consequently adverse alterations (Fig

7). Oxidative damage to the active sites of proteins by ROS generated through the radiolysis of water has been implicated in the enhancement of enzyme degradation which leads to altered enzyme activity, receptor deformations, and defective membrane transport. Membrane-protein structural genomics and membrane-lipid structural studies have shown that alterations in the integrity of integral membrane proteins and PUFAs do seriously alter membrane permeability to various molecules and thus affect their specific functions (Le MAIRE *et al.*, 1990; BERROUD *et al.*, 1996).

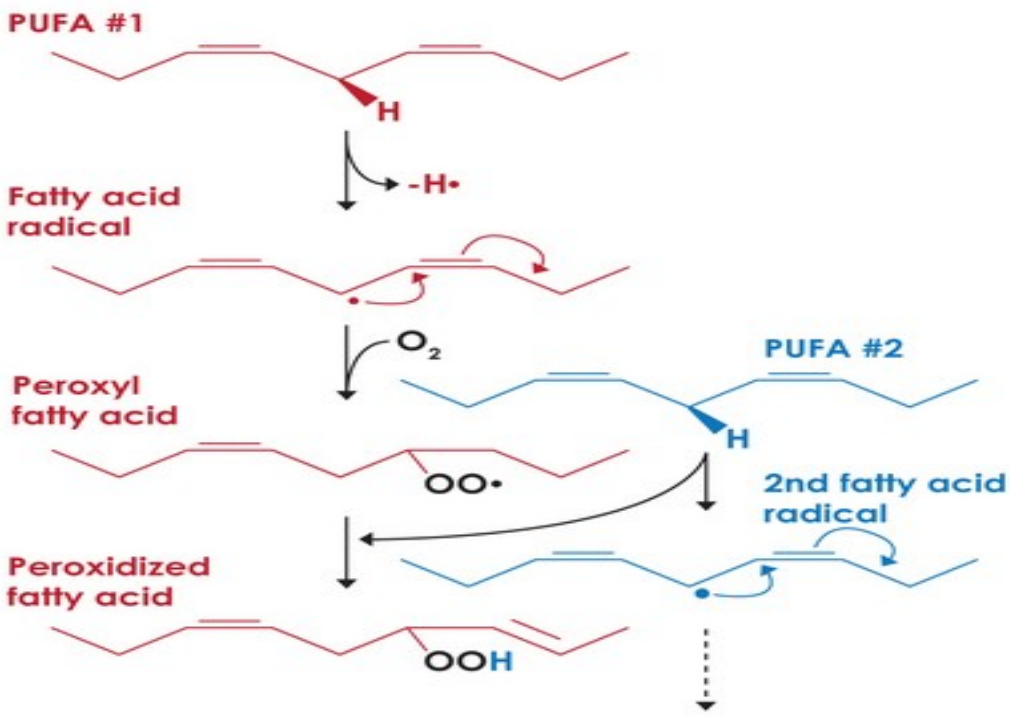


Figure 7. Lipid peroxidation in lipid membrane bilayer (chain reaction)

Radiation-induced bystander effect (RIBE)

The phenomenon of RIBE is now a well-established consequence of exposure to IR whereby biologic responses occur in cells that are not traversed by an ionizing radiation track and thus, are not subject to energy deposition events, i.e., the response(s) take place in un-irradiated cells. Some authors argue that bystander effects are somehow communicated from an irradiated cell to an un-irradiated cell, via cell-to-cell gap junction communication and/or by the secretion or shedding of soluble factors whose precise nature is unknown, although reactive oxygen and nitrogen species and various cytokines have been suspected to play a role (AZZAM *et al.*, 2001; SOKOLOV *et al.*, 2005; SEDELNIKOVA *et al.*, 2007; PRISE and O'SULLIVAN, 2009; TERZOUDI *et*

al., 2010; LOBACHEVSKY *et al.*, 2011). The levels of these molecular signals have been found to be elevated in growth medium conditioned with irradiated cells (PRISE AND O'SULLIVAN, 2009; IVANOV *et al.*, 2010; HEI *et al.*, 2011).

Radiation as a cytotoxic and genotoxic agent

IR is both a genotoxic and cytotoxic agent due to its ability to induce the formation of lethal DNA damage, initiate cell membrane lipid peroxidation and protein denaturation and potentiate the bystander effect leading to cell lethality at high doses. Reliable *in vitro* and *in vivo* genotoxicity and cytotoxicity assays are essential in order to assess the responses of cells to therapeutic agents but also for the identification and pre-clinical evaluation of new drugs. Furthermore, safety concerns when absence of physical, chemical, or biochemical dose meters or in scenarios where absorbed doses of radiation cannot be accurately established such as mass exposure of victims to radiation in the case radiation accidents, terrorism mediated nuclear attacks or in the military context mandate the use of genotoxic and cytotoxic assays.

Genotoxicity assays

Genotoxicity is a broad term denoting the ability of a chemical or agent to interact directly with DNA and/or the cellular apparatus that regulates genome fidelity and which can potentially harm the genome. Genotoxicity tests are designed to detect drugs or agents that can induce genetic damage either directly or indirectly and may be assessed through *in vitro* and *in vivo* tests. This usually covers two main end points of concern (gene mutations and chromosome aberrations); for reliable insights of the genotoxic properties of chemicals and agents, both genotoxic endpoints are essential. Over the years, several *in vitro* and *in vivo* methods have been developed to identify genotoxic chemicals and agents.

***In vitro* chromosomal aberration assays.**

In vitro assays offer significant advantages in that they are relatively inexpensive and easy to conduct, and do not directly involve the use of animals but require nutrient, enzyme and vitamin supplementation to mimic mammalian systems. By their *in vitro* nature their inherent disadvantage is that they do not take into account mammalian metabolism, pharmacodynamics and DNA-repair mechanisms. This notwithstanding, *in*

vitro assays are typically used to provide an initial indication of the genotoxicity of a chemical or agent, and the results often serve as a guide to subsequent *in vivo* studies. Ionizing radiation-induced DNA damage has been associated with increased frequency of chromosomal aberrations and induction of cell death (PATEL *et al.*, 2011).

The importance of chromosomal aberrations as a bioindicator for radiation exposure and genotoxicity has long been recognized (IAEA, 2011), and used extensively to investigate radiation induced damage mechanisms. Depending on the phase of the cell cycle, chromosome mutations will manifest as chromosome-type aberrations (when they occur during the G1- or S-phase), or as chromatid-type aberrations when the mutations occur during the G2-phase. Ionizing radiation induces chromosome-type aberrations in the G0 or G1 stage of the cell cycle, while chromatid-type aberrations are produced during the S or G2 stage. Chromosome aberrations induced by ionizing radiations are generally formed by errors of DNA repair, whereas those produced by non-radiomimetic chemicals are generally formed by errors of DNA replication on a damaged DNA template (PRESTON and HOFFMANN, 2001). Various methods for measuring DNA damage/genotoxicity exist some of which have been catalogued below.

Dicentric (DC) and Centric rings chromosome assay

The dicentric chromosome assay is widely appreciated as an excellent genotoxic index for dose estimation in individuals. A sensitive assay system, DC and Centric ring assays are capable of detecting exposures to doses as low as 100 mGy whole-body exposure and may probably even detect lower doses with extra care and effort (IAEA, 2011). The dicentric assay using blood lymphocytes has been in use for several years and is currently considered the “gold standard” for dose detection, an ultimate measure for radiation genotoxicity and remains the most frequently used biodosimetric technique (IAEA, 2011). Basically, dicentric chromosomes arise as a result of an exchange between the centromeric pieces of two broken chromosomes which in its complete form are accompanied by an acentric fragment composed of the acentric pieces of affected chromosomes. During exposure to high dose radiation and/or chemical toxins, additional multicentric variants such as tricentrics and quadricentrics may also be evident.

Genotoxicity may also be manifested as centric rings in human lymphocytes. In this type of aberration the centric rings are formed as a result of an exchange between

two breaks on separate arms of the same chromosome and are accompanied by an acentric fragment (IAEA, 2011).

Chromosome painting (Fluorescence *in-situ* hybridization [FISH]) assay

The fluorescent *in-situ* hybridization (FISH) technique is a powerful chromosome painting analytical tool for estimating genotoxicity. It has the power to localize specific DNA sequences within interphase chromatin and metaphase chromosomes and is capable of identifying both structural and numerical chromosome changes (LEVSKY and SINGER, 2003). Here, nucleic acid probes are hybridized to complementary sequences in chromosomal DNA. Probes are labeled with a fluorescent dye so that the chromosomal location to which it binds is visible by fluorescence microscopy (NATH and JOHNSON, 2000). The ushering-in of the FISH technology as a biological end-point for genome damage has revolutionized the area of genetic toxicology increasing both the sensitivity and ease of detection of chromosomal aberrations, especially stable chromosomal aberrations. Developed in the 1980s, FISH offers advantage over traditional chromosome banding methods in the sense that with solid giemsa stain, translocations are not reliably observed, however by the FISH method, these are visualized as bicoloured monocentric chromosomes. FISH protocols including a wide variety of current applications of FISH technology have been extensively reviewed elsewhere (NATH and JOHNSON, 2000; LEVSKY and SINGER, 2003; BISHOP, 2010).

Sister-Chromatid Exchange (SCE) assay

SCE is an inexpensive analytical tool widely used to assess genotoxicity of chemicals or agents. The SCE is mostly conducted as an *in vitro* test and the endpoints are presumed to be reciprocal exchanges of DNA segments between sister-chromatids of chromosomes produced during the S phase of cell growth. Molecular mechanisms of these exchanges remain obscure, however, it is postulated to proceed via chromosome breakage, exchange of DNA at homologous loci and repair (PAINTER, 1980). Detection of SCEs is achieved through differential labeling of sister chromatids accomplished through the incorporation of bromodeoxyuridine (BrdU) into chromosomal DNA for two cell cycles. Briefly, mammalian cells are exposed to the test chemical with and without an exogenous mammalian metabolic activation system and cultured for two rounds of replication in BrdU containing medium. After treatment with

a spindle inhibitor (e.g. colchicine) to accumulate cells in a metaphase-like stage of mitosis (c-metaphase), cells are harvested and chromosome preparations are made and visualized (WOLFF, 1991).

Studies have confirmed the ability of low-LET radiation to induce sister chromatid exchanges in rodent cells (GEARD, 1993) and human lymphocytes (GRUNDY *et al.*, 1984). Low- LET ionizing radiation and radiomimetic chemicals are, however, not very effective at inducing sister chromatid exchanges when compared with S-dependent agents such as UV light (WOLFF *et al.*, 1974), alkylating agents (YAGER *et al.*, 1990), and cross-linking agents (SAHAR *et al.*, 1981). High-LET radiation, however, induce sister chromatid exchanges in normal human peripheral lymphocytes at G_0 confirming its higher RBE for the induction of sister chromatid exchange compared with low-LET exposures (ADAMS, 1992). The induction of sister chromatid exchange as a function of charged-particle LET in Chinese hamster cells has been established (GEARD, 1993). At each LET examined there was a dose-dependent increase in the frequency of sister chromatid exchanges. In contrast to the majority of biological end points, however, where RBEs increases as LET increases up to a maximum and then declines, it was found that sister chromatid exchange induction already declined as LET changed from 10 to 120 keV mm (GEARD, 1993). These observations can be explained on the basis of repair differences for DNA damage induced by radiations of different LET, i.e. the faster the repair, the less likelihood there will be unrepaired DNA damage at the time of replication when sister chromatid exchanges are formed.

Unscheduled DNA synthesis (UDS) in mammalian cells

The UDS assay measures DNA synthesis induced for the purposes of repairing an excised segment of DNA containing a region damaged by chemical and physical agents. The induction of repair mechanisms is presumed to have been preceded by DNA damage. Measuring the extent to which DNA synthesis has occurred thus offer indirect evidence of the DNA damaging ability of a test chemical or an agent.

Technically, the method involves measuring radiolabelling of the cell nucleus in order to quantify how much DNA synthesis has taken place to repair the damage. Higher amount of labeling is observed when damage is high and *vice versa* (CASCIANO, 2000). Basically, DNA synthesis is measured by detecting tritium-

labeled thymidine (^{3}H-TdR) incorporation into DNA of mammalian cells which are not in the S phase of the cell cycle. The uptake of ^{3}H-TdR may be determined by autoradiography or by liquid scintillation counting (LSC) of DNA from the treated cells (WILLIAMS, 1976;1977). The detailed recommended methodology is described in OECD Test Guideline 487.

The Premature Chromosome Condensation (PCC) assay

During mitosis, the chromatin of cycling cells condenses into the usual shaped chromosomes, however, when the "cell system" is coerced to condense its chromatin when it is not naturally prepared for this process, "premature chromosome condensation" is said to occur. PCC may be achieved by fusing interphase cells with mitotic Chinese Hamster Ovary (CHO) or HeLa cells using Sendai virus or polyethylene glycol as fusing agent (PANTELIAS and MAILLIE, 1983; PANTELIAS and MAILLIE, 1984). Chemical methods of inducing PCC, using inhibitors of DNA phosphorylation, such as okadaic acid or calyculin A, have also been developed. Most of these methods require the cells to be cycling in culture. Peripheral blood lymphocytes may be fused with mitotic CHO cells and the resulting PCCs analysed by means of a light microscopy. The number of chromatid fragments can be counted in each cell (2n=46) and excess of single chromatid chromosomes above 46 represents chromosome damage in the peripheral mononuclear blood cells (PANTELİAS and MAİLLİE, 1983; IAEA, 2011). This technique enables direct observation of radiation-induced chromosome damage in non-stimulated interphase human peripheral blood lymphocytes.

The PCC method in combination with a conventional technique such as C-banding and molecular cytogenetic assays like FISH with chromosome specific DNA libraries and/or a pan-centromeric probe, provide further refinemets to the PCC method (IAEA, 2011). With these combinations it is possible to detect breaks, dicentrics, rings and translocations immediately after irradiation.

Gamma-H2AX as a biomarker of DNA damage

Histone γ-H2AX is a member of the histone H2A family, which is part of the nucleosome complex. γ-H2AX not only enhances the efficient packaging DNA but also play an important role in the maintenance of genomic stability (CELESTE *et al.*,

2002.). Histone γ-H2AX gets rapidly phosphorylated at serine 139 following exposure to IR. Phosphorylated γ-H2AX is essential for efficient recognition and/or repair of DNA DSBs, and many molecules of γ-H2AX become rapidly phosphorylated at the site of each nascent DSB. Using immunofluorescence techniques, it has been demonstrated that these phosphorylated histone γ-H2AX form discrete nuclear foci at sites of DNA DSBs. These γ -H2AX foci can be visualized within 3 min after exposure to IR. The number of γ-H2AX foci increases gradually during the first 10–30 min after exposure to IR before reaching a plateau which correlates directly to the dose of IR and the predicted number of slowly repaired DSBs (STASZEWSKI *et al.*, 2008)

Currently techniques are being refined to apply quantum dot technology to detect γ -H2AX in cells recovered from blood with the view to incorporating it into clinical protocols. This assay has potential to impact on monitoring of radiation exposure and chemotherapy with regard to both toxicity and efficacy in the clinic (WATTERS *et al.*, 2009; SHEIKH *et al.*, 2012).

***In vitro* gene mutation assays**

The Ames bacterial reverse mutation test (gene mutation test in bacteria), the mouse lymphoma assay (gene mutation test in mammalian cells using the tk locus) and the hprt assay (gene mutation test in mammals using the hypoxanthine-guanine phosphoribosyl transferase (*Hprt*) locus) are popular *in vitro* tests commonly employed to access the genotoxicity of chemicals and mutagenic agents.

The Ames bacterial reverse mutation assay, detects chemicals and agents that cause point mutations or frame shift mutations in histidine auxotrophic strains of *Salmonella typhimurium* (e.g. strains TA100, TA98, TA102), and a reverse mutation assay using a tryptophan auxotrophic strain of *Escherichia coli* (e.g., WP2*uvr*A) (AMES *et al.*, 1975;GATEHOUSE *et al.*, 1994;OECD, 2004)]. In this type of assay, revertant cells grow on minimal agar containing trace amounts of histidine or tryptophan, while wild type cells rapidly deplete the limiting amino acid and stop growing. A significant increase in the number of revertant colonies (usually a two-fold or greater increase) compared with the results of the concurrent negative control is considered to be mutagenic and the chemical or agent used to create the revertant cell is concluded to be mutagenic.

In the mammalian forward mutation gene assays, such as the thymidine kinase (Tk) assay or the Hprt assay, mutations at the heterozygous Tk or hemizygous Hprt gene are evaluated (JOHNSON, 2012). Cells such as L5178Y mouse lymphoma cells (Tk locus), Chinese hamster cell lines (Hprt locus), and human lymphoblastoid cells (Tk locus) are readily used to realize the objectives of these assays. Mutations are selected by incubation of the cell cultures with the selective agents; trifluorothymidine (Tk assay) or 6-thioguanine (Hprt assay). Cells having forward mutations at the Tk or Hprt genes survive in the presence of the selective agent, while wild-type cells accumulate a toxic metabolite and do not proliferate (JOHNSON, 2012). The mutant frequency, calculated by the ratio between the selective and the non-selective plates is an indication for the mutagenicity of test chemical or agent.

The comet assay

The comet assay is a rapid and very sensitive fluorescent microscopy-based method for measuring DNA damage, protection and repair at the level of individual cells (COLLINS *et al.*, 2008). The comet assay has diverse applications in fundamental research for DNA damage and repair, in genotoxicity testing, human biomonitoring and molecular epidemiology and ecotoxicology (WONG *et al.*, 2005; SPIVAK *et al.*, 2009). In this assay, cells are embedded in agarose, lysed and then electrophoresed. Negatively charged broken DNA strands exit from the lysed cell under the electric field and form a comet with “head” and “tail.” The amount of DNA in the tail, relative to the head, is proportional to the amount of strand breaks. The sensitivity limit of the comet assay is approximately 50 strand breaks per diploid mammalian cell (TICE *et al.*, 2000;SPEIT and HARTMANN, 2006; SPIVAK *et al.*, 2009). It reveals mainly early, yet repairable, moderate DNA damage and can be used in virtually any eukaryotic cell. In order to achieve various objectives, different modifications of the comet assay have been developed. In its alkaline version, which is mainly used, DNA SSBs, DNA DSBs, alkali-labile sites, and SSBs associated with incomplete excision repair sites provoke increased DNA migration (KLAUDE *et al.*, 1996). In the neutral variant, DNA molecules are preserved in their native double stranded structure enabling the detection of double stranded DNA breaks (KLAUDE *et al.*, 1996). Cross linkage of DNA-DNA/DNA-protein leading to decreased DNA migration can be identified by the failure to detect single-strand breaks that were known to be present. Oxidized purines and

pyrimidines, may be revealed by incubating damage lysed cells with base specific endonucleases before electrophoresis (COLLINS *et al.*, 2008).

The micronucleus (MN) Test

The *in vitro* and *in vivo* MN assays are simple short-term screening tests generally accepted as valid alternatives to the chromosome aberration assay. In the *in vitro* method, chromosome aberrations are detected indirectly via chromatin loss from the nucleus leading to the formation of MN in the cytoplasm of the cell (FENECH, 1997; 2000). During the metaphase/anaphase transition of mitosis, chromosome fragments (acentric fragments) and/or whole lagging chromosomes may not be dragged to the nuclear poles either because there is no centromere to bind microtubules or due to a damaged spindle fibre. As a result of this, these chromosome fragments lag behind at the equatorial plane and cannot integrate into the daughter nuclei. During telophase, a nuclear envelope forms around the lagging chromosomes and fragments to form a small nucleus (micronucleus). Analysis of the induction of micronuclei in human lymphocytes cultures has revealed that the most convenient stage to score micronuclei in this cell system is the binucleated interphase stage (FENECH and MORLEY, 1985a;1985b). Such cells have completed one cell division after chemical or agent treatment and are therefore capable of expressing micronuclei. When cells in culture are treated with cytochalasin B (an inhibitor of actin polymerisation) the resultant effect is the "trapping" of cells at the binucleate stage where they can be easily identified (FENECH and MORLEY, 1985a; 1985b).

The *in vivo* micronucleus assay involves microscopic examination of cytological preparations of polychromatic erythrocytes obtained from the bone marrow of animals. Unlike the *in vitro* assay, the *in vivo* assay takes into account whole animal processes, like absorption, tissue distribution, metabolism and excretion of a foreign chemical and its metabolites, and repair of lesions. In this assay, young erythrocytes formed by expulsion of the nuclei from erythroblasts present a bluish colour when stained with Geimsa because they still contain ribosomal RNA in the cytoplasm, and they are called polychromatic erythrocytes (PCEs). Mature erythrocytes present an orange colour when stained with Geimsa and are called normochromatic erythrocytes (NCEs). The distinction between PCEs and NCEs is of fundamental importance to confirm whether the increased incidence of MN is actually due to exposure to a given chemical or agent,

since there is a constant migration of normochromatic cells from the peripheral circulation to the bone marrow. The *in vivo* bone marrow micronucleus test plays a key role in current testing strategies for genotoxicity.

Micronucleus (MN) formation is an established signature tune of genetic toxicity and is accordingly used extensively as a bioindicator of genotoxicity both *in vitro* and *in vivo* and has probably over taken all other chromosomal aberration assays in this endeavour. The *in vitro* micronucleus assay is part of the recommended regulatory test battery for genotoxicity. It is an alternative to the *in vitro* chromosomal aberration assay. Multiple published studies show a high level of concordance (80-90%) between the *in vitro* micronucleus assay and *in vitro* chromosomal aberration assay. The accuracy of the MN test (scores more cell thus has more statistical power), coupled with its simplicity of scoring, rapidity, wide applicability in different cell types and amenability to automation makes it particularly robust and a more appropriate tool for this purpose. Currently it finds applications as an attractive, effective and reliable tool for mutagenic evaluation of chemicals and physical agents (MATTER and SCHMID, 1971; Von LEDEBUR and SCHMID, 1973; MILLER, 1973; SCHMID, 1975) and is perhaps one of the most commonly used methods in genotoxicity testing and biomonitoring populations at risk (IAEA, 2011).

Emerging genotoxic assays

The 'omic' technologies

The introduction of the so-called "omic" techniques which encompass transcriptomics, proteomics and metabolomics is gradually revolutionizing methodologies and approaches for genotoxic and carcinogenic substances. These methods provide the opportunity to gain insights into genotoxic mechanisms of carcinogenicity beyond the initial DNA-damaging events, provide new *in vitro* and *in vivo* markers and may possibly increase the accuracy of discriminating carcinogens from non-carcinogens. When fully developed and appropriately standardized it will probably provide useful information on emerging and newly-identified health risks. However, at present technological difficulties remain as anticipated in the development of all new technologies probably backed by the complexity of responses to genotoxic stresses, cumbersome management of large volumes of raw data generated, database challenges, and high costs. Comprehensive reviews concerning these new technologies

and their possible roles in genotoxicology are available elsewhere (CABA and AUBRECHT, 2006).

Cell proliferation and viability assay methods

Testing the effects of compounds and agents on the viability of cells grown in culture is widely used as a predictor of potential toxic effects of these chemicals or agents in whole animals. Cell viability assays may also be used to assess the biocompatibility of a material or test substances and provide an excellent way to screen materials prior to *in vivo* tests.

Conventionally the release of radio-isotopic labels (^{3}H or ^{51}Cr) was measured to assess cell viability, based on the rate of DNA synthesis in cell populations but for a variety of reasons, including requirement for specialized skills, these techniques are currently being less patronized by scientists. The trypan blue dye exclusion method has also previously been extensively used for this purpose, however, the low throughput of the technique coupled with its tedium has restricted its use. Most current assays for measuring cytotoxicity are based on alterations of plasma membrane permeability and the consequent release (leakage) of components into the supernatant or the uptake of dyes, normally excluded by viable cells. A serious disadvantage of such permeability assays is that the initial sites of damage of most cytotoxic agents are intracellular and therefore, cells may be irreversibly damaged and committed to die with the plasma membrane still intact, thus these assays tend to underestimate cellular damage when compared to other methods.

Another family of assays that generally measure the metabolic activity in cell populations is colorimetric in nature and focus on biological functions inside intact cells as an alternative to permeability testing. Viable cells reduce various tetrazolium salts (MTT, XTT, or WST-1) to coloured formazan compounds, mainly using extra-mitochondrial dehydrogenases and permitting the use of the colorimetric assays to measure cell survival.

These enzymes based colorimetric assay methods using tetrazolium salts are very useful for quantitating factor-induced cytotoxicity within 24 to 96 h of cell culture and can accurately quantify as few as 950 cells. Technically, these colorimetric methods

(e.g. the MTT formazan assay) measure the reduction of yellow 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide by mitochondrial succinate dehydrogenase. Because the cellular reduction is only catalyzed by living cells, it is possible to quantify the percentage of living cells in a solution. Practically, these tests can be used to evaluate the cytotoxicity of medical devices, toxic compounds, toxins and environmental pollutants, potential anti-cancer drugs, medicinal plant extracts and antibodies to examine their growth inhibiting potentials. The main strengths of the MTT assay reside in their quantitative abilities, un-subjective interpretation of results and high throughput (on 96-well microplates) allowing for fast screening of multiple samples. These assays are however limited by their inability to discriminate specific cellular death mechanisms e.g., apoptosis versus induced cell death and may underestimate cellular damage. Besides they only detect death at the last stages of the cellular dying process.

For regulatory purposes, the prescribed maximum concentration of compounds is 5000 μg/ml or 10 mM. The maximum recommended test concentration of substances may also be guided by the limit of its solubility in the solvent or culture medium, or the highest level allowed as adjudged by a cytotoxicity assay. It is instructive to note that testing excessively toxic concentrations can lead to problems with data interpretation or may lead to artefactual positive results (KIRKLAND, 1992; GALLOWAY, 2000; FELLOWS and O'DONOVAN, 2007)

Radioprotectors in radiotherapy

Radiation has been embraced as an inevitable therapeutic and diagnostic tool in medicine and industry even so its useful benefits are restricted due to radiation-induced damage to normal tissues. This considerably lowers the therapeutic doses that can be delivered to tumours thereby lowering the therapeutic gain. Thus use of radiation in medicine has always been rationalized on the basis of risk versus gain.

In everyday life, radiation exposures can occur in several settings including, radiography, nuclear medicine, radiotherapy, radiological imaging, radionuclide production, biomedical research, military, nuclear reactors, air and space travel.

Since the use of IR has become inextricably woven with life supporting human activities, the best possible option available is to develop acceptable radiation countermeasures to reduce the harm it may bring along with its use. The three cardinal principles of radiation protection are: (a) shielding (usually by lead) of unexposed areas, especially radiosensitive organs such as bone marrow, gonads and thyroid; (b) increased distance between the radiation source and radiation workers or patients; and (c) reduction of exposure time. Lately, the ALARA strategy adopted for people working directly with radiation was introduced to address the growing concerns of radiation-induced somatic and heritable mutations (PRASAD *et al.*, 2004;ALCARAZ *et al.*, 2011).

Even though these physical radiation protection measures enumerated has served and continue to serve useful purposes, a plethora of limitations still remain. Firstly, lead shielding and other physical measures are cumbersome and impractical in some situations, for example during fluoroscopy, it may not be possible to protect the gastrointestinal tract (one of the most radiosensitive organs) against radiation damage by lead shielding. Secondly, increasing the distance between the radiation source and exposed individuals may not be practical for many radiation workers, patients or astronauts. Finally, reducing exposure time may also not be relevant to all populations, for instance during nuclear accidents and nuclear terror attacks (PRASAD *et al.*, 2004; ALCARAZ *et al.*, 2011).

In order to find an effective radiological countermeasure to protect normal tissues from potential radiation damage in most scenarios listed above, pharmacological interventions known as chemical radioprotectors have been proposed (ALLALUNIS-TURNER *et al.*, 1989;WEISS and LANDAUER, 2000; JAGETIA, 2007). This represents an obvious strategy to improve the therapeutic index in radiotherapy, help extend the concept of ALARA by protecting patients against radiation damage during diagnostic procedures, and for use in case of a radiological event. Moreover, military personnel and emergency responders urgently need non-toxic countermeasures to ionizing radiation. All these scenarios have deepened scientific motivation to research for chemical radioprotectors.

Medical methods to cope with radiation catastrophe

Three accepted treatment modalities categorised base on their mechanisms of action are recognised as far as pharmacological radiation countermeasures is concerned. These include radioprotectors, mitigators and treatment and are well depicted in fig. 8 (STONE *et al*., 2004, CITRIN *et al.,* 2010)

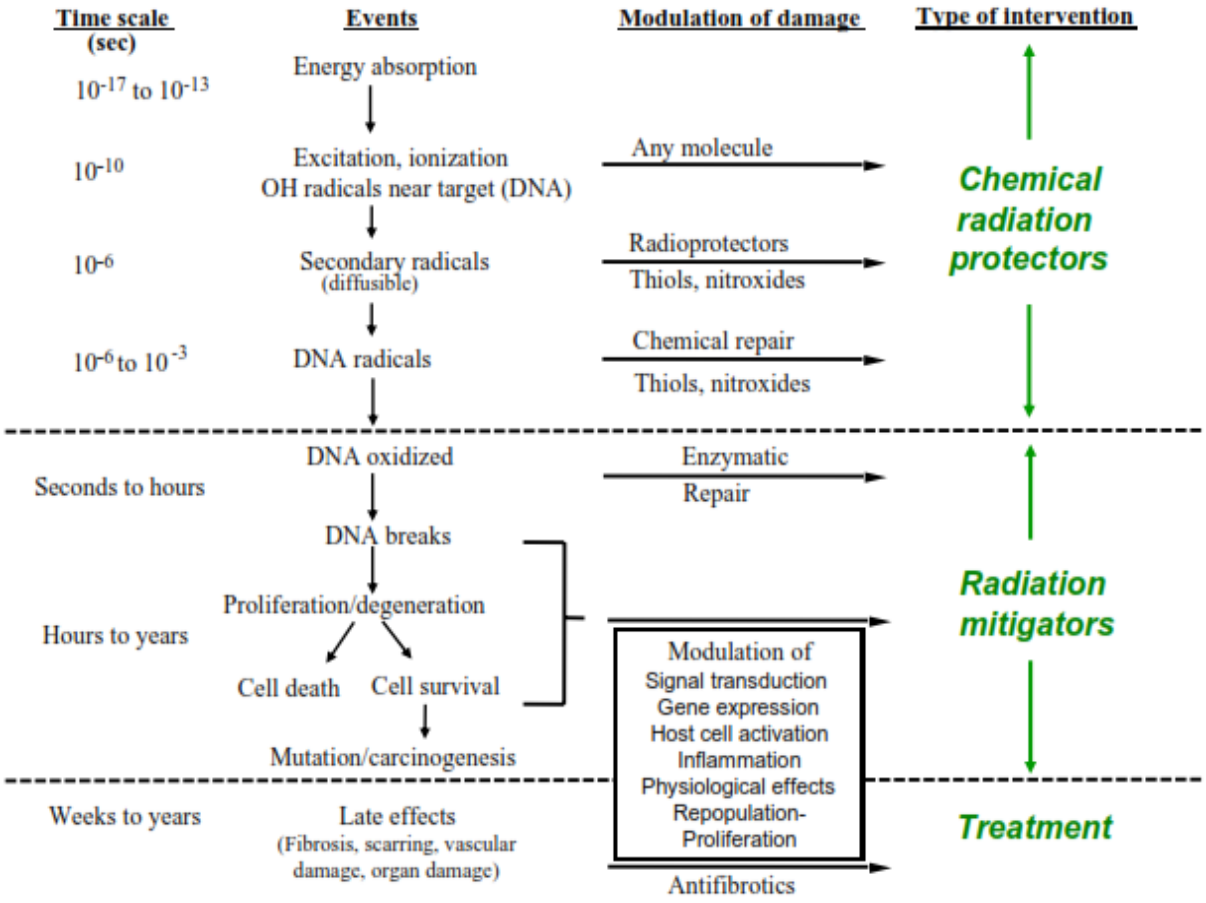


Figure 8. Sequence of events following radiation exposure. The chart is divided into three parts by dashed lines suggesting events and reactions that might be modified by radiation protectors (top), radiation mitigators, and treatment (adapted from (CITRIN *et al.*, 2010)

Radiation mitigaors

This largely comprise of agents that stimulate the proliferation and function of hematopoietic and immunopoietic stem cells. When administered during or after radiation exposure but prior to the onset of the symptoms of radiation of injury. These have the capacity to speed up the recovery or repair of depleted stem-cell populations after radiation injury by stimulating the proliferation of surviving stem and progenitor

cells (GIAMBARRESI and WALKER, 1991). Radiation mitigators may be grouped into under two main categories namely immunomodulators and cytokines.

The immunomodulators considered as inducer molecules are generally nonspecific immunostimulants that function as external stimuli for a broad range of cell types in the hematopoietic system. In regards to ameliorating radiation injury, the most effective compounds appear to be those that act primarily on the macrophages. Glucan, a beta-1, 3-linked polysaccharide isolated from the cell wall of the yeast *Saccharomyces cerevisiae* is a potent immune modulator capable of enhancing a variety of immunopoietic and hematopoietic responses.

Cytokines considered as effector molecules, are hormone-like peptides that function as molecular signals between cells. They are synthesized and released primarily by macrophages and lymphocytes that have been stimulated by inflammatory agents or immunomodulators. Included in this class of compounds are the interleukins-1 through -6, tumour necrosis factor (TNF), a variety of hematopoietic growth factors (such as granulocyte-macrophage colony-stimulating factor, granulocyte colony-stimulating factor, and erythropoietin), and the alpha-, beta-, and gamma-interferons. All of these act in a variety of ways to stimulate proliferation, differentiation, or function of cells in the hematopoietic system. Several cytokines have been examined for their ability to mitigate radiation injury, and of these, IL-1 and TNF have been found to be the most effective.

Therapeutic agents (Treatment)

Treatments or therapeutic agents refer to those agents that are administered after the development of radiation symptoms. They are capable of repairing the molecular damage caused by radiation. These class of radioprotective agents are normally administered after the exposure to the radiation such as after completing cancer therapy when symptoms develop and may be used to modulate the progression of ionizing radiation-induced fibrosis or late vascular damage (BEGG *et aL.*, 2011;PATEL *et al.*, 2011). Therapeutic agents are administered after radiation exposure to treat or facilitate recovery from various aspects of the acute radiation syndrome (ARS) and delayed effects of radiation. Treatment agents include some of the drugs that are effective as mitigators, but also include agents such as pentoxifylline to treat radiation fibrosis and

growth factors to facilitate recovery from hematological injury (Stone *et al*., 2007; CITRIN *at al*., 2010)

Radioprotectors

Radioprotectors have been used in radiobiology to refer to prophylactic agents that must be given before radiation exposure. Since the idea of probing chemicals for radioprotective agents was kindled in the 1940s, several chemical compounds and their analogues have been screened for radioprotection. Despite these efforts the development of suitable radioprotectors for clinical practice has been elusive and daunting due to reasons of severe toxicity and other unbearable side effects. Pioneering studies by Patt (PATT, 1953) and Bacq *et al.*(BACQ *et al.*, 1953**)** demonstrated the radioprotective effect of aminothiols against radiation lethality in a large number of animal species, proving for the first time that these compounds could be used in humans. However, their clinical utility is offset by their high toxicities at physiologically relevant concentrations, inability to differentiate between tumour and normal cells coupled and their failure to provide post-irradiation protection (JAGETIA, 2007).

Herbal radioprotectors

Issues of toxicity of the aminothiols have necessitated the search for alternative non-toxic and highly effective pharmacological radiation countermeasures (ARORA *et al.* 2008; UPADHYAY, *et al,* 2005; JAGETIA, *et al*, 2003a; 2003b; GIAMBARRESI and WALKER 1991).

Herbal radioprotectors are defined as extracts, fractionated extracts, isolated bioactive constituents, or bioactivity modulating molecules derived from natural (including all natural flora both marine and land) or genetically modified plant sources or their artificially generated combinations, that render protection against the deleterious effects of IR when administered before irradiation (GOEL *et al.*, 2004; PRASAD *et al.*, 2004; ARORA *et al.*, 2005; 2008).

Many phytochemicals have the advantage of low toxicity, and are protective when administered at pharmacological doses. Moreover, natural agents have clear practical advantages with regard to availability, suitability for oral application and regulatory approval amongst others.

A rational approach of selecting possible plant candidates for radioprotective evaluation is to first audit the existing authentic properties of the various parts or extracts of the plant. Critical characteristics that may lead to the election of a plant for radioprotective explorations include immunemodulatory, anti-inflammatory, antioxidant, antimicrobial, free radical scavenging and anti-stress properties. These properties may offer leads to radioprotective or radiosensitizing agents (CASTILLO *et al.*, 2000; WEISS and LANDAUER, 2000; CASTILLO *et al.*, 2001; BENAVENTE-GARCIA *et al.*, 2002; DEL BANO *et al.*, 2006; BENAVENTE-GARCIA *et al.*, 2007; THOMAS *et al.*, 2009; ALCARAZ *et al.*, 2009; SANCHEZ-CAMPILLO *et al.*, 2009).

Indeed, several of such natural compounds with radioprotective promise have been isolated and characterized from diverse flora (ARORA *et al*., 2005, ALCARAZ, *et al*., 2009). Table 3, below report on some of the major important findings on medicinal plants with promising radioprotective properties.

Table 3: Radioprotective effects of some herbs and plants (adapted from ACHEL *et al.,* 2012).

Plant	Family	Test system	Route	observation	References
Acanthopanax senticosus	Araliaceae	Mice	i.p	Survival, recovery of spleen colony-forming units (CFU-S), protective effect on endogenous CFU-S. and effect on self-renewal of CFU-S	(Miyanomae and E., 1988)
Acorus calamus		Mice	oral	damage in the genomic DNA of peripheral blood leucocytes, bone marrow cells and splenocytes	(Sandeep and Nair, 2010)
Aegle marmelos	Rutaceae	HPBLs, Mice	i.p, oral	radiation sickness, survival, micronuclei, free radical scavenging	(Jagetia and Venkatesh, 2005;Jagetia, 2007)
Ageratum conyzoides	Asteraceae	Mice	i.p	Radiation sickness, GI and BM deaths, free radical scavenging	(Jagetia *et al.*, 2003)
Allium cepa	Alliaceae	Cell lines		DNA damage	(Chang *et al.*, 2012)
Allium sativum	Alliaceae	Cell lines		DNA damage	(Chang *et al.*, 2012)
Aloe arborescens	Liliaceae	Mice		Free radical scavenging, and reducing alterations in enzyme activity (SOD and glutathione peroxidase activity)	(Arora *et al.*, 2005)
Aloe vera	Liliaceae	Mice	i. p	Survival, testicular damage	(Pande *et al.*, 1998)
Ambrosia maritima		Rats	p.o	Neurotransmitter disturbances	(Abdel-Hamid and Tarabanko, 2004)
Amaranthus paniculatus	Amaranthaceae	Mice	p.o, oral	Survival, CFU, spleen weight, lipid peroxidation, GSH	(Yadav, 2004,)
Angelica sinensis	Apiaceae	Mice	oral	peripheral leucocyte and lymphocyte counts, micronuclei frequency of PCE in bone marrow, transformation rates of peripheral lymphocyte, SOD and MDA of liver cells	(Sun *et al.*, 2007)
Aphanamixis polystachya	Meliaceae	Mice	i.p	Chromosomal aberrations, aberrant cells	(Jagetia and Venkatesha,

					2006;Jagetia, 2007)
Aparagus racemosus	Liliaceae	rat liver/brain mitochondria		lipid peroxidation, protein oxidation and depletion of thiols	(Kamat and Venkatachalam, 2004)
Archangelica oficinalis	Umbelliferae	mice		survival rate	(Narimanov *et al.*, 1991)
Centella asiatica	Umbelliferae	Rats, Mice	p.o oral	Survival , weight loss	(Sharma and Sharma, 2002)
Citrus aurantium	Rutaceae	Mice	i.p	Clastogenic effects, MnPCEs, MnNCEs	(Hosseinimehr *et al.*, 2003;Benavente-Garcia *et al.*, 2007)
Crataegus microphylla	Rosaceae	Mice, HPBLs	i.p	Micronuclei, MnPCEs	(Yamini and Gopal, 2010)
Curcuma longa	Zingiberaceae	Rats	oral	8-hydroxy-2'-deoxyguanosine (8-OHdG) in urine. The lipid profile and antioxidant status, as well as levels of transaminases, interleukin-6 (IL-6), and tumour necrosis factor α (TNFα)	(Inano and Onoda, 2002;Nada *et al.*, 2012)
Emblica officinalis	Euphorbiaceae	Mice	p.o	Survival, weight loss	(Singh *et al.*, 2005)
Ginkgo biloba	Ginkgoaceae	Rat	i.v	Eye lens total (enzymatic plus non-enzymatic) superoxide scavenger activity (TSSA), non-enzymatic superoxide scavenger activity (NSSA), glutathione reductase (GRD), and glutathione-S-transferase (GST)	(Okumus *et al.*, 2011)
Glycyrrhiza glabra	Fabaceae	plasmid pBR322 DNA, HPBLs,	i.p	Chromosomal aberrations in in plasmid DNA, lymphocytes and BM, Radical scavenging	(Gandhi *et al.*, 2004;Arora *et al.*, 2005)

		Mice			
Hippophae rhamnoides	Elaegnaceae	Rats, mice	i.p	Survival, ACTH, CFU, Micronuclei, spleen Colony forming units (CFU) and haematological parameters	(Goel *et al.*, 2002)
Lycium chinense	Solanaceae	Mice	i.p	bone marrow death, CFUs	(Hsu *et al.*, 1999)
Mentha arvensis	Lamiaceae	Mice	i.p	Radiation sickness, GI and BM deaths	(Baliga and Rao, 2010)
Mentha piperita		Mice	p.o	Radiosensitive organs; testis, gastrointestinal and hemopoetic system	(Baliga and Rao, 2010)
Moringa olifera	Moringaceae	Mice	i.p	Bone marrow protection by scoring aberrations in metaphase chromosomes and micronucleus induction in polychromatic erythrocytes and normochromatic erythrocytes	(Rao *et al.*, 2001)
Myristica fragrans	myristicaceaea	Mice	p.o	Survival hepatic GSH and testicular LPO	(Sharma and Kumar, 2007)
Osmium sanctum	Lamiaceae	Mice	i.p	Survival, CFU, lipid peroxidation, GSH, chromosomal damage	(Devi and Ganasoundari, 1995)
Olea europaea	Oleaceae	Mice	i.p	micronucleus test for anticlastogenic activity and Micronucleated polychromatic erythrocytes (MnPCEs) in bone marrow of mouse	(Castillo *et al.*, 2001;Benavente-Garcia *et al.*, 2002)
Panax ginseng	Araliaceae	Mice	i.p	Jejunal crypt survival, endogenous spleen colony formation and apoptosis in Jejunal crypt cells	(Lee *et al.*, 2006)
Phyllanthus amarus	Euphorbiaceae	rat liver mitochondria and pBR322 plasmid DNA	i.p	lipid peroxidation and protein oxidation in mitochondria and DNA strand breaks in pBR322 plasmid DNA	(Londhe *et al.*, 2009)

Podophyllum hexandrum	Berberidaceae	Mice		Survival	(Goel *et al.*, 1998)
Spinacia oleracea	Amaranthaceae	Mice	oral	lipid peroxidation (LPO) product and tissue levels of glutathione	(Bhatia and Jain, 2004)
Syzigium cumini	Myrtaceae	Mice, HPBLs	i.p	Micronuclei, radiation sickness, GI and BM deaths	(Jagetia *et al.*, 2003)
Rosemarinus officialis	Labiatae	Mice, HPBLs	oral	micronucleus test, cutaneous alterations caused	(Sanchez-Campillo *et al.*, 2009)
Rubia cordifolia	Rubiaceae	Mice	i.p	Survival, hemopoietic cell protection and micronucleus assay, lipid peroxidation measured by the inhibition of TBARS.	(Tripathi and Singh, 2007)
Terminalia chebula	Combretaceae	pBR322 plasmid DNA, Mice, HPBLs	i.p	Chromosome/DNA aberration, peroxidation of membrane lipids	(Gandhi and Nair, 2005)
Tinospora cordifolia	Menispermaceae	Mice	i.p	whole body survival, spleen colony forming units (CFU), hematological parameters, cell cycle progression, and micronuclei induction	(Goel *et al.*, 2004)
Vitis vinifera	Vitaceae	HPBLs	*In vitro*	Micronucleus test for anticlastogenic activity with the cytokinesis-blocked.	(Castillo *et al.*, 2001)
Zingiber officinalis	Zingiberaceae	Mice	i.p	Radiation sickness, mortality, GSH, LPO	(Jagetia *et al.*, 2003)

GSH: glutathione; GST: glutathione-s-transferase; SOD: superoxide dismutase; GI: gastro intestinal; BM: bone marrow; Human peripheral blood lymphocytes; DPPH: diphenyl picryl hydrazyl radical; LPO: lipid peroxidation; MnPCEs: micronucleated polychromatic erythrocytes; MnNCEs: micronucleated normochromatic erythrocytes; i.p: interperitoneal; BM: bone marrow; CFU colony formig units; TBARS: thiobarbituric acid reactive substance

The fractionation and characterization of specific natural products for their roles in radiation protection is an important strategy in radioprotective drug development industry.

Pycnanthus angolensis is a tropical African plant widely used in traditional medicine. Its medicinal properties and uses has been reviewed elsewhere (ACHEL *et al.*, 2012). In particular its free radical scavenging, antioxidant, anticancer, immunemodulatory, anti-inflammatory and antimicrobial properties strategically positions it as a possible repository of radiomodulators, however, there is a dearth of sound scientific evidence to confirm this hypothesis. Studies are needed to fill this gap in order for full scale realization to unlock some hidden wealth in the plant in this context.

Activity guided fractionation is an important strategy in new drug screening regimen aimed at focusing researchers on the fractions of interest while sparing scarce resources. The evaluation of crude herbal extracts for bioactivity is as relevant as is the evaluation of pure isolated compounds for bioactivity in the sense that the desired activity could be elicited in either form. In the case of the crude extract, the interaction of the different component present may be essential for functional activity. Compounds or fractions with the potential desired activity need to be evaluated for biocompatibility and this include such exploratory studies as *in vitro* and *in vivo* cytotoxicity, genotoxicity and cytoprotective studies. The present study was conducted to assess the *in vitro* and *in vivo* radioprotective potential of an extract obtained from *P. angolensis* in comparison with other established herbal radioprotectors using X-rays as a mutagenic agent.

III. Objectives.

III. Objectives.

The main purpose of this doctoral studies was to achieve the following **objectives:**

1º. Obtain a minimally toxic **extracts** from ***Pycnanthus angolensis*** (PASE) with suitable bioactivity to support analysis in cellular radioprotection assays for for human application.

2º. Determine the radioprotective capacity of **PASE** against cytotoxic and genotoxic damage induced by ionizing radiation.

3º. Evatuate the cytoprotective and antimutagenic capacities of **PASE** comparing these with other radioprotective substances used in radiation oncology and /or radiobiology studies.

IV. Materials and methods.

IV. Materials and methods.

Blood samples

Whole human blood samples were kindly donated by healthy volunteers. The blood samples were collected into sterile conical VENOSAFE ™ (Terumo Europe NV, Leuven, Belgium) blood collection vacuum tubes containing heparin (Heparin sodium 5%; Rovi Lab, Madrid).

Donors were non-smokers, with no known medical illness and no record of exposure to clasterogenic agents, therefore they were presumed to be healthy individuals. In all cases express consent was sort from each donor for the use of their blood samples in the present investigation.

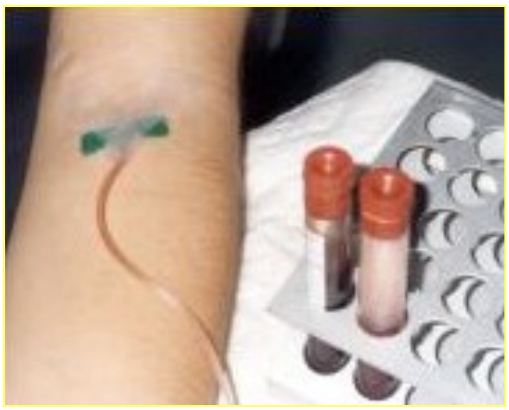


Figure 9. Bblood being drawn from doner into vacutainers.

The blood donors comprised two females aged 18 and 20 years. The blood samples were drawn on the mornings of the day of experimentaion and used immediately for the cytokinesis-blocked (CBMN) micronucleus assay.

Animals

This experiment used 9-11 weeks old male Swiss mice weighing approximately 20-25g. The animals were maintained under identical environmental conditions of temperature, light and moisture. They were offered food and water *ad libitum*. All animals were from the animal unit of the Experimental Sciences Support Service (SACE), University of Murcia (Official License No 30030-2A). The international ethical standards for the use and handling of laboratory animals for biomedical research were strictly adhered to (Guidelines on the Use of Living Animals in Scientific Investigations. London, UK).

Figure 10. Experimental animals being irradiated

Cell lines

PNT2 cells

PNT 2 cell lines (European Collection of Cell Cultures (Salisbury, United Kingdom).used corresponds with non-tumour adherent cells obtained from the prostate epithelial tissue of a 33 year old man at post-mortem and transfected with a plasmid containing a simian virus 40 (SV40) genome with a defective origin of replication. The cells were cultivated in RPMI 1640 medium (Table MM4), at neutral pH 7.2-7.4, supplemented with 10% FBS, 2 mM glutamine, penicillin (100 UI/ml) and streptomycin (100 µg/ml). The cells were grown in 25-cm^2 tissue culture flask containing 5ml tissue culture medium with phenol red pH indicator, in an incubator maintained at 37ºC with a humidified atmosphere (90-95% relative humidity) and 5% CO_2.

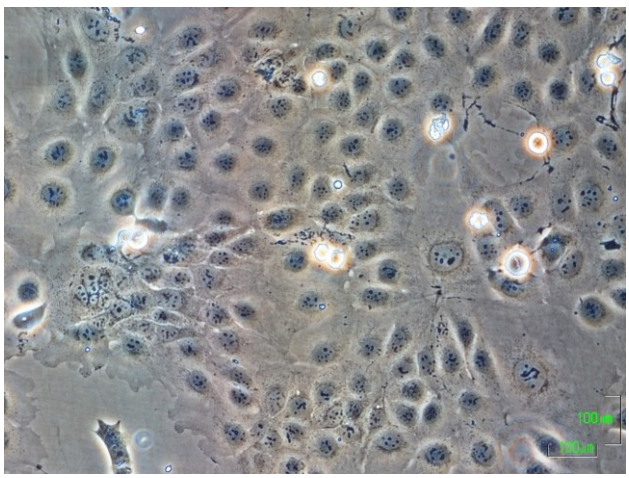


Figure 11. Morphological appearance of PNT2 cells cultivated for 72 hours (200X)

B16F10 mouse melanoma cells

The metastatic B16F10 mouse melanoma cell line used is a variant of the original metastatic melanoma cell line established in 1954 from a spontaneous melanoma at the base of the ear of a C57BL/6 mouse in the laboratories of the National Institutes of Health (NIH) U.S. and provided by Dr. V. Hearning (NIH, Bethesda MD). Currently, the commercial source of this cell line is the Naval Biosciences Laboratory, School of Public Health, University of California (Berkeley, California, USA). The cells were cultured in Dulbecco's Modified Eagle´s Medium (DMEM, Table MM5), (Sigma-Aldrich St. Louis: USA) buffered to pH 7.2 – 7. 4, containing L-glutamine and 4.5 g / l glucose but without sodium bicarbonate (Table 6). It was supplemented with 10% FBS (Sigma-Aldrich, St. Louis: U.S.), 4 mM L-glutamine, penicillin (100 IU / ml) and streptomycin (100 µg / ml). The cultures were maintained at 37 ° C with 90-95% relative humidity and an atmosphere of 7.5% CO_2. Thses cells frequently adopt distinctive spindle shape morphology but changes to polygonal shape with increasing cell density. The cells were passaged in 25 cm^2 flasks, in growth medium containing pH colour indicator (phenol red)

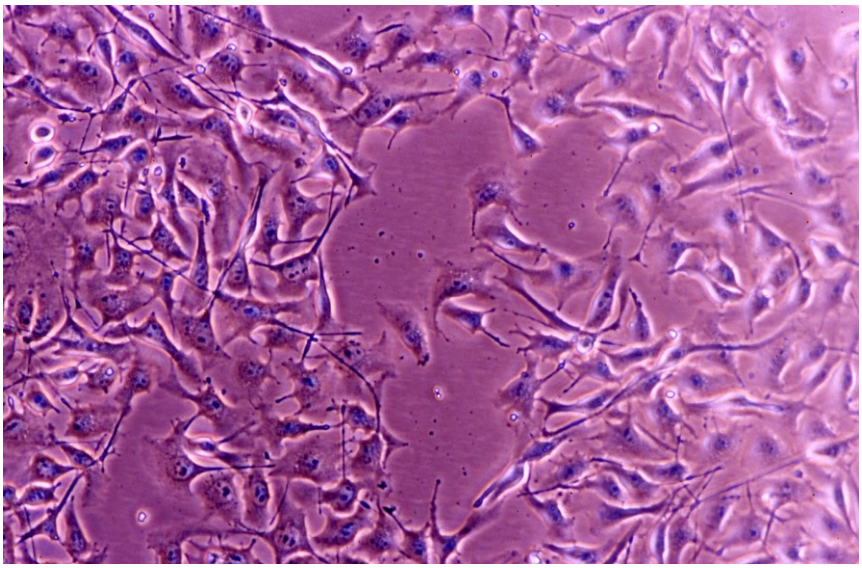


Figure 12: Morphological appearance of B16F10 cells cultivated for 72 hours (200X)

Equipment used

Positive Pressure Laminar Flow Hood (Chamber)

To provide a sterile workstation, a Cultiar ASB type II positive pressure vertical laminar flow hood (Fig. 13) was used at all times when sterility was paramount. The laminar flow hood used incorporate 99.99% efficient HEPA modules that remove airborne particulates to ensure a uniform, contamination free flow of air to the bench surface.

Figure 13: Laminar flow hood used for the mainpulation and cultivation of peripheral blood lymphocytes

X-Ray beam Irradiator.

A Smart Laboratory-ray irradiator, Model 583 E, equipped with a 200 E, X-ray tube (200 kV and 4.5 mA), YXLON International AS Copenhagen, Denmark located in the Radiation Protection and Waste Management Department of S.A.C.E (Service Research Support), University of Murcia (Figure 14) was used as radiation source.

Figure 14. YXLON X-ray Irradiator

Electrometer.

A UNIDOSE E model electrometer equipped with a TW 30010-1 (PTW Freiburg, Germany) probe/ionization camera, was used to measure the doses of X-rays administered to blood and cell samples under culture. This ultra-high sensitive probe enabled the measurement of doses administered to our experimental models inside the X-ray irradiator (Figure 15).

Figure 15. Electrometer with probe

HPLC System

For quantification and authentication of the various substances used, HPLC analysis were performed using a Hewlett-Packard (HP), HPLC 1100 series System (Agilent technologies, Sta. Clara, USA, Figure 16) equipped with diode-array detectors. A C_{18} LiChrospher 100 (250 x 4 mm id) column with an average particle size of 5 microns was employed as stationary phase. A column temperature of 30 ºC, flow rate of 1ml/min and a wavelength of 280 nm were also maintained.

Figure 16. High Performance Liquid chromatography (HPLC) equipment

Microplate reader.

A Multiskan Model MCC/340 microplate reader equipped with 340, 380, 405, 520, 550, 560 and 690 nm filters and located in the Tissue Culture Service Centre of SAI, University of Murcia (Figure 17) was used to determine absorbance in 96 well plates.

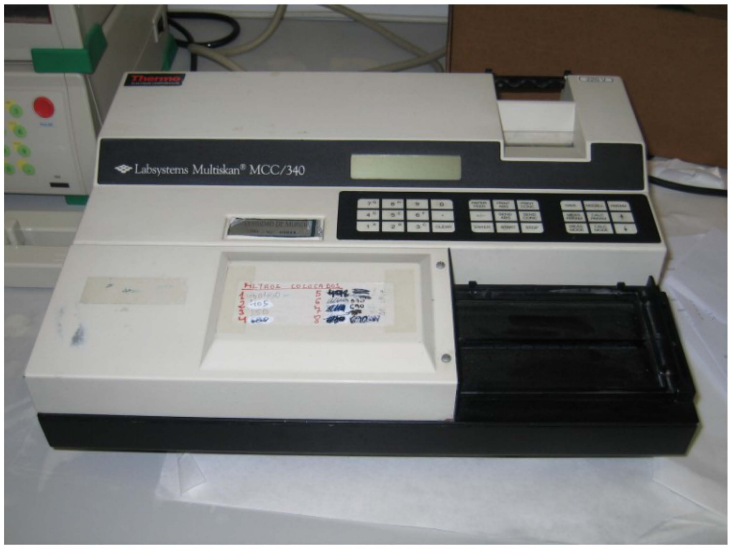


Figure 17. Multiskan MCC/340 Microplate Reader

Mass spectrometry (HPLC-MS) HPL200

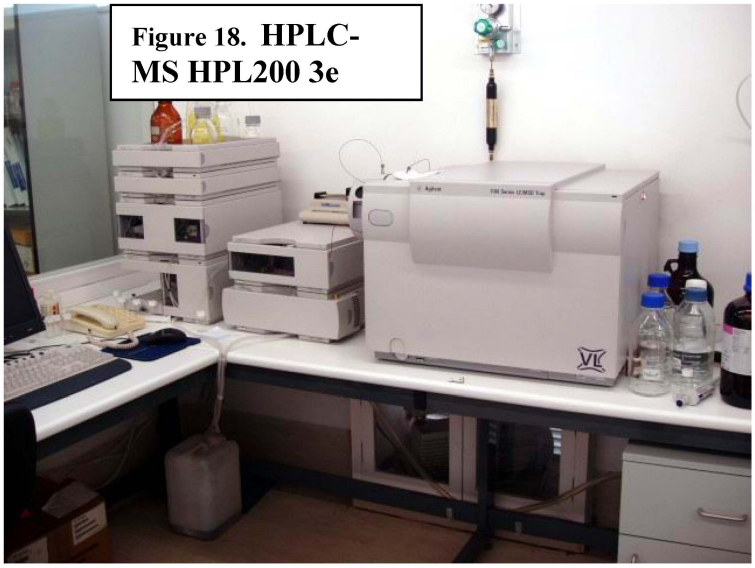


Figure 18. HPLC-MS HPL200 3e

An Agilent HPLC 1100 series coupled with ion trap mass spectrometer (HPLC/MSD, Figure 18) was employed to assist in the identification of some of the compounds studied. It is an extemely versatile instrumental tool whose roots lie in the application of more traditional liquid chromatography to theories and instrumentation that were originally developed for gas chromatography (GC).The primary advantage HPLC/MS has over GC/MS is that it is capable of analysing a much wider range of components. Compounds that are thermally labile, exhibit high polarity or have a high molecular mass may all be analysed using HPLC/MS..

Optical Microscopes

For chromosome aberration studies, three different optical microscopes were used i.e., Laborlux 12 (Leitz, Germany), Hund V200 (Wetzlar, Germany) and Olympus BX40 (Barcelona, Spain) (Figure 19). A magnification of 400 was used to examine the slides, however, in ambiguous cases, a magnification of 1000 was used for confirmation.

Figure 19. OLYMPUS BX40 Microscope (lateral and front views)

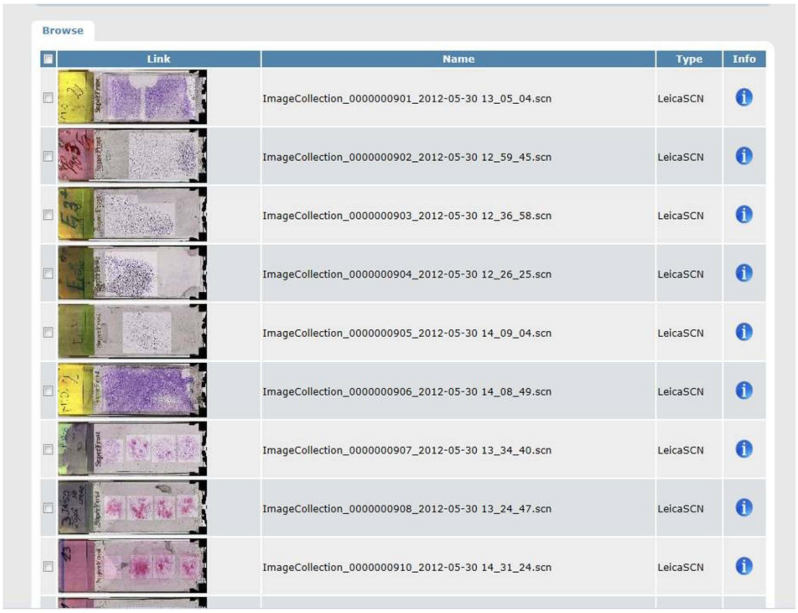


Figure 20. Digital Virtual Microscope Slides for genotoxicity studies with on-line database

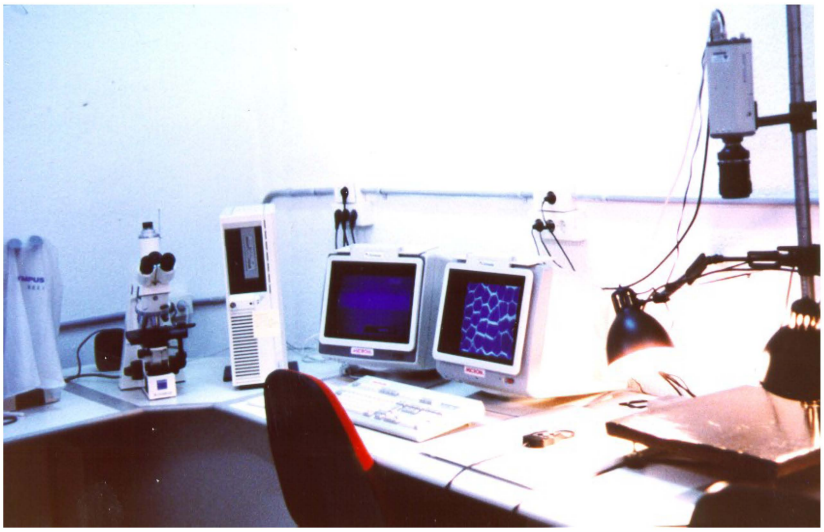


Figure 21. Morphometric analysis using image analysis for the identification ofbinucleated (BN) and micronucleated binucleated (MNBN) cells.

For high-resolution and preserving the quality image of the slides, bright field scanning and fluorescent imaging was used. This was a forded by a Leica SCN400 F slide scanner which combines both fluorescence and bright field imaging on a single platform was used**.** This technique permitted an ¨on-line¨ double-blind study to evaluate biological endpoints (Micronuclei frequency) through the Digital Image Hub Management Web Service System of the university of Murcia which was installed to support Research and development in the university.

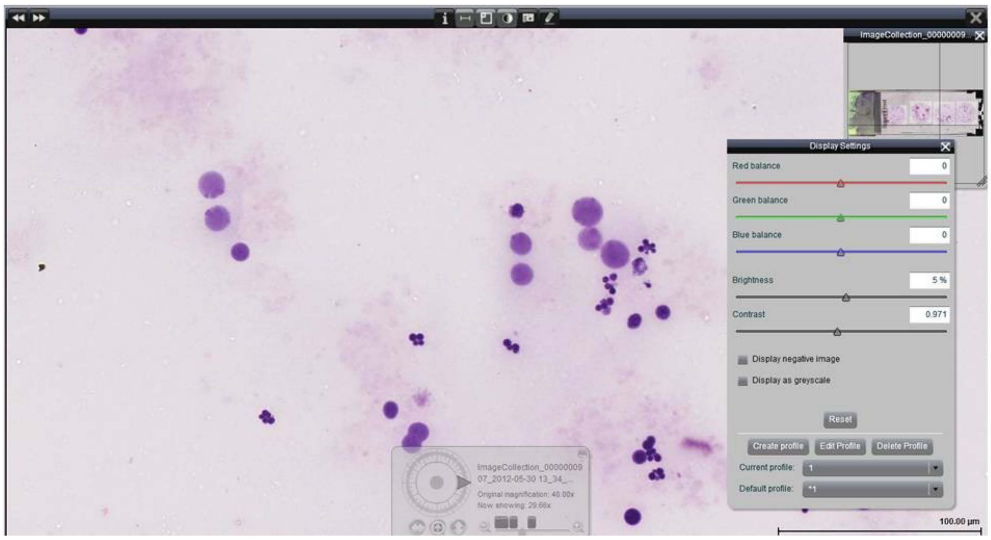


Figure 22. A closer view the Digital Virtual Microscope Slides for Genotoxicity studies

Thermoluminiscent dosimeters (TLDs)

The evaluation of X-radiation doses received by the samples of blood under investigation is very a fundamental step to establishing the biological effects sought in this study and further permits the construction of dose-response curves for the study. Thermoluminiscent dosimeters (TLD) were used to ascertain the average absorbed doses delivered to the experimental models. TLDs have been widely used for several decades and are widely accepted as an appropriate technique for dose measurements. TLDs with radiation sensitive lithium fluoride crystals doped with Mg, Cu, P (GR-200r, Conqueror Electronics Technology Co. Ltd, China) were placed in pairs in sealed transparent plastic bags measuring 8x16 mm and protected thermally from the exterior. The bags were coded and labeled with indelible ink. The small size of the dosimeters facilitated their positioning next to blood samples in the irradiation chamber. The TLDs consisted of rounded screens of 4-5 mm diameter and 1 mm of thickness (Figures 23). A total of 50 dosimeters were placed in pairs in 24 bags; however, one of these bags contained 4 dosimeters that were not exposed to ionizing radiation and served as negative control dosimeters providing background radiation values. The radiation doses read from these badges were considered as the background from natural emissions covering the period before and after the experimental work and were subtracted from the final absorbed doses measured in the samples. For any lot of dosimeters this correction factor was deemed valid for an approximate period of 2 to 3 months.

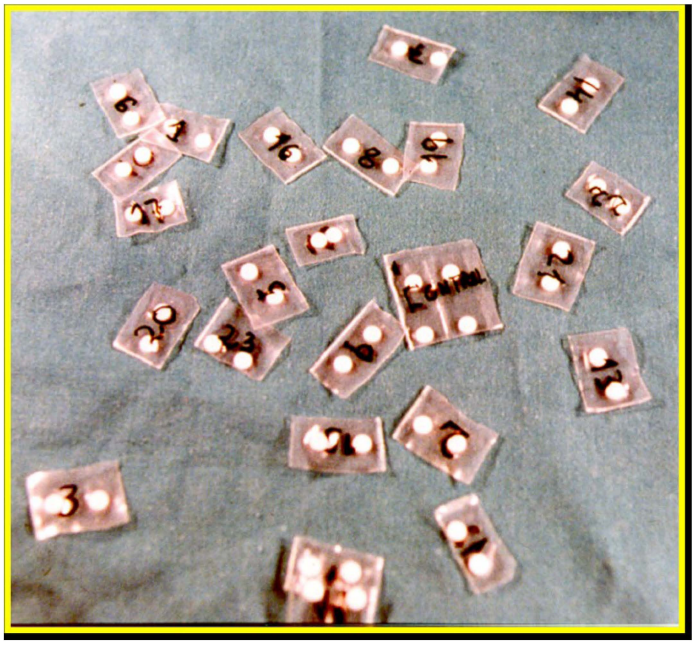


Figure 23. Thermoluminescent dosimeters (TLDs).

The dosimeters were processed in a Harshaw 5500 TDL automatic reader with a programmable heating system. The heating was based on the injection of hot nitrogen, through a time-temperature programming profile adapted for different thermo luminescent materials. Based on the experience at CIEMAT, for the (LiF: Mg, Cu, P) sintered pellets, a reading cycle that subjects the detector to a linear heating rate of 15 ºC/s from 50 ºC up to 275 ºC was defined. The heating was maintained at this temperature for 12.7 second for a total reading period of 26.7 seconds. This cycle assures the complete reading of the detector up to doses as high as 1 Gy ensuring its immediate re-use. The thermoluminiscent dosimeters were provided by the Centre for Energy, Environment and Technology (CIEMAT), Ministry of Industry and Energy who also monitored the readings on the TLDs to determine the actual radiation doses used in this work. The evaluation of the doses is based on the simplified analysis of the emission curves using the computer programs developed by CIEMAT. A dose of 2 Gy was established to be appropriate for the experimental procedures.

Chemicals, reagents and extracts used for assays

Chemicals and Reagents

P. angolensis seed extract (PASE) was extracted from the seeds as described below. Zoledronic acid (Z) (Zometa®) was obtained from Novartis pharmaceuticals (Barcelona, Spain) and was administered in concentrations, 5 and 100 %, in different volumes 80 (5–50µl). RPMI 1640, Ham´s F10, Phytoheamagglutinin A (PHA), cytochalasin B, streptomycin, penicillin, phosphate-buffered saline (PBS), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2h-tetrazolium bromide (MTT), Vitamin E (δ-tocopherol), Bovine serum albumen (BSA fraction V), Eriodictiol, Coenzyme Q4,6-n-propyl-2-thiouracil (PTU) and Vitamin C (Ascorbic Acid) were from Sigma-Aldrich chemicals SA (Madrid, Spain). Foetal bovine serum (FBS) was obtained from Gibco (USA); glacial acetic acid and ethanol were obtained from Scharlao SL (Madrid, Spain). Methanol and potassium chloride were obtained from Panreac (Madrid, Spain); 5 % sodium heparin was obtained from Rovi Pharmaceutical Laboratories (Madrid, Spain). Rosmarinic acid (95% ROS), Diosmin, Rutin and Quercetin were obtained from

Extrasynthese (Extrasynthèse S.A., Genay, France). Dimethyl sulfoxide (DMSO) (Merck, Darmstadt, Germany). Amifostine (WR-2721) or Ethyol® (Schering-Plough S.A., Madrid Green Tea Extract (TE), Carnosol (COL), Carnosic Acid (CARN), Soluble Citrus Extracts (CE) Apigenin, P90 were from Furfural Español S.A., Alcantarilla, Murcia.

Overall process of obtaining and identifying the active ingredients of interest *Pycnanthus angolensis* Warb. *(P. Kombo).*

Quantitative analysis of the active principles of interest present in the plant material (Selection of plant material with high amount and yield of active principle)

The main aim and objective of this study is to obtain an extract rich in some bioactive principles of *P. angolensis* warb (*P. kombo*) known for their potent antioxidant activity but also to analyze the different parts of the plant in order to establish a correct selection of plant material to be used. The extraction procedure was performed with the technical supervision of Nutrafur SA (Alcantarillal, Spain), who modified the patented process to adapt to the new material provided, reducing the potential toxicity of the original method

Leaves, roots, stem-bark and seeds of *P. angolensis* Warb were collected from plants growing wild around cocoa farms in Nkawkaw in the eastern region of Ghana. Voucher specimen were prepared and the plant authenticated by Mr. Amponsah, an established Curator at the Ghana Herbarium, located in the Department of Botany of the University of Ghana, Legon, Accra. The material was dried under vacuum at room temperature in order to maintain the integrity of the different active principles potentially present in the said plant materials. The final moisture content of the plant materials under investigation was measured according to the criteria and methodology described in the European Pharmacopoeia version 7.0 and was in all cases less than 5%.

The direct quantification of the active ingredients of interest in the plant extract was used as end point for this determination. To directly establish the identity of active principle present in extracts obtained from these materials, the plant materials were all milled to powder using a laboratory attrition mill. The plant materials obtained from the

various parts were extracted independently with different solvents, filtered and analyze using the high-performance liquid chromatography (HPLC) technology. Working with the seeds proved very tough due to high oil content and hard cuticle. After repeated extractions and analysis using various solvent compositions the most suitable solvent which gave optimal yield of the active principle of interest in all the plant material used was methanol/water (80/20 v/v) (data not shown). The seeds afforded the highest yield of the active principle of interest.

The chromatographic technique has widely been used to separate mixtures of compounds in analytical chemistry and biochemistry with the purpose of identifying, quantifying and/or purifying the individual components of the mixtures. The HPLC method of measurement is sensitive, accurate, unbiased and above all non-subjective. This chromatographic technique, including sample preparation has been described in detail below in this report.

Figure 24. Various parts of plant used for the study. a) root-bark; b) milled seeds; c) leaves; d) stem-bark

Preparation of seeds for extraction

After selecting the most suitable plant material (i.e. the seeds) from which to obtain reasonable quantities of the active ingredients based on quantitative chromatographic analysis, the main extraction process proceeded.

The seeds of *P. angolensis* are high-oil content seeds with dense and/or relatively hard outer core or seed coat that is very difficult to crush. Because of the very high oil content and the hardness of the outer cuticle, obtaining a finely divided powdered product with the desired texture for extraction posed a serious challenge. With the various laboratory analytical mills available, uneven particle size distribution was formed and irregular microparticles were produced which ultimately would affect the extraction process. Accordingly, there was the need for a milling process that will provide a meal/flour with a particle size that is suitable for a correct and effective extraction operation.

To obviate the problems enumerated the seeds were first crushed into small bits and mixed with carriers to facilitate a further grinding operation that will yield the desired product quality. Tests were made with different materials (excepients) including maltodextrin, gum arabic and diatomaceous earth to select the best exceipient for the process. The best results were obtained when 50% seed + 45% diatomaceous earth + 5% silicon dioxide (SiO_2) (w/w) was used. With this combination a caking problems ("gumming") encountered previously during milling were circumvented, furthermore, a hygroscopic property of the milled material that occurred probably due to high proportion of polysaccharides was also eliminated. The above combination made it possible to obtain a stable seed powder with a uniform particle size of less than 1 mm in diameter.

Following this feat, 100 g of crushed seeds were mixed with 10 grams of SiO_2 and 90 grams of diatomaceous earth and ground in a laboratory mill to a particle size 1μm. The milled material was first extracted in 99% methanol. Amongst various solute/solvent ratios assessed, the optimal extraction yield was obtained with 10 % (w/v) ratios of extraction material (milled material) and solvent (99% methanol) at room temperature. Thus a solid-liquid ratio 1:10 (i.e. 200 grams of ground material;

equivalent to 100g of seed were extracted in 2000 ml of 99% methanol). The extraction was done in a 3000 ml Erlenmeyer flask on a Heidolph mechanical stirrer model RZR 2020 at room temperature of 22-24°C and for 1 hour.

Upon completion of the solid-liquid extraction process the alcoholic extract was separated from the marc by filtration using a Büchner funnel attached to a Kitasato flask fitted with a polypropylene filter cloth which is more suitable for the filtration of plant materials than the normal filter paper. A vacuum pump was attached to the filtration unit to facilitate the filtration process.

After filtration, the damp residual solid plant material (marc) was pressed to recover most of the solvent into the extraction flask. The marc was washed on the funnel with fresh 99% methanol (200 ml) affording a total volume of 1924 ml of recovered extracting solvent (total volume of extracting solvent used was 2,200 ml).

The methanolic extract containing the active ingredients was concentrated under reduced pressure at 40-50 °C in a Heidolph Rotary Evaporator (Laborota 4000). The evaporation was continued until the extract was reduced to one-twelfth its original volume (i.e. 150-160 ml) yielding a semi-viscous syrupy liquid.

The concentrate contained an undesirable gummy material co-extracted from the seeds. Addition of 30 ml of deionised water to the concentrated alcoholic extract yielded a methanol: water ratio of 5:1 and led to flocculation of the gummy materials, which were accordingly removed through a specific clarification operation. This filtration system employed a Büchner-Kitasato filter system fitted with a 1 micron cellulose/silica AF100 filter membrane (Ref 2036-Filtrox, Switzerland) afforded a 188 ml clarified extract. The gummy solid residue, obtained in the filtration was washed thoroughly on the same filter with 25 ml of methanol/water (5:1) and dried.

The clarified extract was further evaporated under vacuum at 40-45 °C using a Heidolph Rotary Evaporator, (Laborota 4000). The initial volume was reduced by 5-6 times yielding a final volume of 36 ml of viscous "syrup". The syrupy extract was subjected to liquid-liquid extraction, using ethyl ether as extractant as follows: the ratio of the syrupy extract to ethyl ether was 1:7 (i.e. 36 ml of syrup to 250 ml of ethyl ether) at room temperature (22-24 °C) for 3 hours in a 500 ml Erlenmeyer flask stirred on a Heidolph

mechanical stirrer (model RZR 2020). One hour was allowed for liquid-liquid partitioning in a 500 ml separatory funnel with PTFE valve and threaded screw cap.

The liquid-liquid extraction process was repeated three times, ensuring that the organic phase showed no taint of yellow-orange colour after decantation. A final volume of 737 ml of organic extract was obtained. This solution was evaporated to dryness at 30-35 ° C in a Heidolph Rotary Evaporator, (Laborota 4000). The process afforded 16.34 grams of a dark brownish oily and viscous semisolid as final product. Previous studies have reported the presence of sargahydroquinoic acid, sargaquinoic acid and sargachromenol (SIMON *et al*., 2008; LEONARD, 2004a; 2004b) in the seeds of *P. angolensis*. The scheme in the results section summarizes the detailed process of obtaining an extract from the seeds of *P. angolensis*.

Preparation of plant extract for Chromatographic analysis

65-70 mg of the dark brown viscous seed extract were weighed accurately (Sartorius SACP124S) into a 20 ml volumetric flask. Some HPLC grade methanol (Panreac, Spain) was added with continuous stirring using a magnetic stirrer, and the volume made up to the mark with methanol. The content was stirred for 30 min till complete dissolution and the solution was filtered through a 0.45 μM nylon filter membrane and then passed through a guard column attached to the HPLC system before passing through the main HPLC system for analysis. The most suitable mobile phase for this HPLC-MS analysis proved to be a methanol-water mixture (80: 20 v/v).

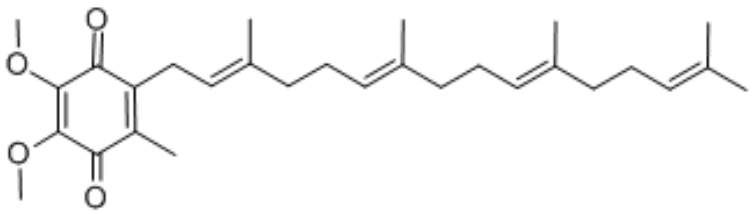
Preparation of reference sample for chromatographic analysis:

In general chromatographic methods rely heavily on reference standards to provide accurate data. However, it is significant to note that reference materials for the chemical compounds in the plant material under investigation which is the main focus of this study was not readily available from commercial suppliers thus making it impossible to perform a strict content quantification of these compounds in the plant extracts material obtained.

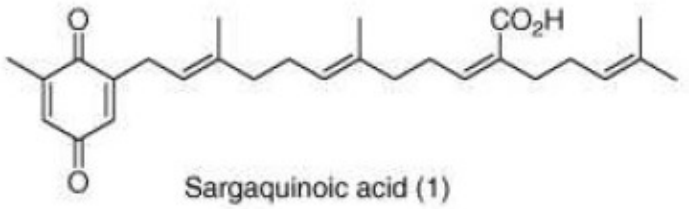
Under these circumstances, the usual technique is to identify a compound that can easily be obtained from commercial suppliers specialized in the distribution of standard chromatographic reference substances with a similar molecular structure as the

compound to be quantified and use same as the reference substance for the assay being performed. In this case, the choice was Co-enzyme Q4; $C_{29}H_{42}O_4$, 454.64 g/mol (Sigma-Aldrich Reference C2470) because of its similar chemical and structural properties with the compounds of interest (e.g., it shares a quinine chemical group with the plastoquinones).

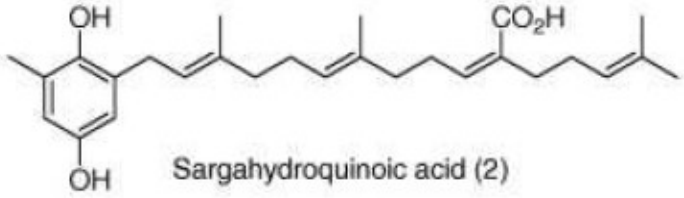
The quantification of the active ingredient content of the extract was thus performed, as in the case of plant material used, using the chromatographic elution profile of Co-enzyme Q4 as reference material.



Co-enzyme Q4



Sargaquinoic acid (1)



Sargahydroquinoic acid (2)

Figure 25. Structures of HPLC reference compound (co-enzyme Q4) and two active principles of interest in PASE (Sargaquinoic and sargahydroquinoic acids)

A standard solution of the reference sample was prepared at a concentration of 1 mg/ml in HPLC grade methanol. Briefly 100 mg of the coenzyme Q4 standard was weighed precisely and transferred into a 100 ml volumetric flask. After dissolving in a few millilitres of the solvent, the volume was made by adding more of the solvent. The solution was filtered on a 0.45µM Millipore filter disc and used for injection into the HPLC instrument.

High-performance liquid chromatography coupled to a diode array detector (HPLC-DAD)

HPLC was performed according to the general criteria for good manufacturing practices (GMPs). Triplicate analyses were conducted on each sample while solvent blanks were intermittently injected into columns (column washing) to eliminate peak splitting or tailing during the analysis.

The chromatographic profile of different extracts obtained from *P. angolensis* was monitored by a UV-Vis diode array detector at a wavelength of 250nm. Analyses were performed in an HP 1100 liquid chromatographic system (Hewlett- Packard, Waldbronn, Germany) series and an LC-6A double pump (Shimadzu Corporation, Kyoto, Japan) using a C_{18} column (Shimadzu Shim-Pack CLC(M) 250 x 4 mm id 5 μm, 100 Å) maintained at 30 °C. The chromatograms were recorded at 250 nm using a gradient between mobile phase A (1% acetic acid) and phase B (Methanol). Chromatographic separation was carried out at a flow rate of 1ml/min, as follows: 0–5 min, 50% of A and B; 5–25 min, 50% A and B; 25–35 min, 100% B. The column was finally re-equilibrated after 40 min with 50% A and 50% B.

Chemical composition of seed extract (SE)

The composition of the extracts in the chromatograms was assessed by comparing their relative retention times and UV spectra which are a function of their molecular structure and subsequently confirmed by mass spectrometry (HPLC-MS).

High-performance liquid chromatography coupled to mass spectrometer

Further analysis was performed on an Agilent 1100 series HPLC (Agilent Technologies, Germany) coupled to an ion trap VL mass spectrometer (Agilent Technologies, Germany).

The separation was executed on a Waters SunFire® C_{18} column (5 μm particle size and column dimensions of 120 μ, 4.6 mm × 150 mm). The mobile phase was composed of 20/80 water/methanol and 1% acetic acid with the isocratic elution system at a flow rate of 0.8 ml/min and a total run time of 20 minutes. The sample volume

injected through the rheodyne sample loop was 20 µl and the column temperature was set at 30 °C.

Two signals were acquired, one with a diode array detectors (Agilent Technologies, Germany) at a wavelength of 280 nm, and a range from 190-380 nm. The mass spectrometer was operated in a scan mode with the electrospray (ESI) source in the positive ion mode (ESI +), and a mass detection range of 100-800 amu and a target mass of 400 m/z. The optimized conditions were nebulizer pressure 60 psi, a drying gas flow rate of 9 ml / min and a gas temperature of 350 ° C.

Identification of the active principles:

The active principles were identified from signals produced. In this study a compound was eluted at 27.4 minutes with a molecular weight of 448. The signals observed were 449 (M + H) + and 471 (M + Na).

Quantification of compounds

Quantification of the compounds was carried out from the peak area, in comparison with the peak area of the reference standard used (CoQ4). To find the percentage by mass (%M) of each active principle the following relation was used:

$$\% M = (ArPb/ArQ4Std) \times (Wt\ Q4Std / Wt\ Pb) \times 100$$

Where: ArPb: is the area of active principle in unknown sample

ArQ4Std: is the area of CoQ4 in the reference sample ("standard").

Wt Pb: Weight of sample in mg and;

WtQ4Std: weight of the reference sample ("standard") in mg

Preparation of stock and working solutions of *P. angolensis* seed extracts for cell culture assays

The brown oily and viscous seed extract was insoluble in aqueous medium but dissolved in DMSO thus the samples were first solubilised in DMSO, which was then diluted in PBS prior to the experimentation. A stock concentration of 90µM plant extract was prepared in 4 % DMSO solution by weighing out exactly 0.4mg of the oily extract on an analytical balance and transferred into a 100 ml volumetric flask. The solute was dissolved slowly in a few millilitres 50% DMSO. After dissolving in a few millilitres of the solvent, the volume was made up by adding more of the solvent. The solution was then filter-sterilized by passing through a 0.22 micron membrane filter.

The resulting 90 µM stock solution was then serially diluted to the required concentrations and used for cell assays.

Optimization for non-toxic concentration of extract for cell analysis

Cells were exposed to the different concentrations of PASE prepared serially for 24 or 48 hrs to evaluate viability and proliferation. Different volumes (ranging from 10 µl to 50 µl) of 90, 75, 50 and 25 µM of the stock solution were delivered into wells containing 200 µl suspensions of normal prostate epithelial PNT2 and mouse metastatic B16F10 melanoma cells and evaluated after 24 and 48 hrs of incubation to assess cell viability and proliferation to establish non-cytotoxic concentrations of the extract.

Handling, storage and preparation of reagents for use in assays

MTT

MTT (MW 414.3) was stored at 0-5º C. A stock solution of 8 mg/ml was prepared in phosphate buffered saline (PBS) and stored at 4º in the dark until needed. The maximum storage period was 1 month.

Dimethyl sulfoxide.

DMSO (MW 78 and 99.5% purity) with specific gravity of 1.1 at 20ºC was maintained under anhydrous conditions at ambient temperature (never below 4º C) until required.

Bovine Serum Albumen.

Lyophilized bovine serum albumen, fraction V, with a minimum purity of 96% was stored at 0-5º C. Stock solutions of 20 mg/ml in PBS were prepared and filter-sterilized.

Carnosol and Carnosic acid

Carnosol ($C_{20}H_{26}O_4$, 98% purity) a white crystalline solid in its pure form, is a phenolic antioxidant present in the rosemary plant *Rosmarinus officinalis*. Together with carnosic acid, this duo (Fig. 26) constitutes two very important components of *R. officinalis* and are purported to be part of the main diterpenes of the plant. The two compounds are probably responsible for about 90% of the antioxidant properties of rosemary extracts and have strong abilities to inhibit lipid peroxidation in microsomal and liposomal systems (ALCARAZ et al., 2009). They are also thought to be good peroxide, superoxide anion and radical scavengers. In addition to their antioxidant

properties, they are known to possess anti-carcinogenic properties. Both carnosol and carnosic acid are insoluble in water, partially soluble in alcohol but completely miscible in organic solvents and oils, in this regard, stock solutions (1mg/ml) of the two were prepared in DMSO and filter sterilized.

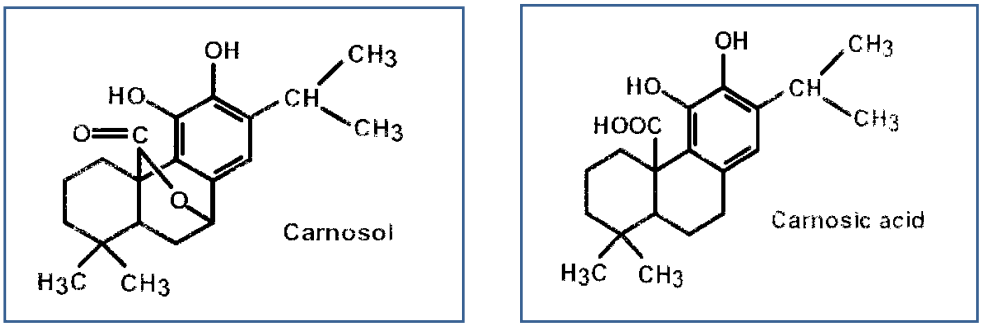


Figure 26. Chemical structures of Carnosol and Carnosic Acid

Preparation of carnosic acid for addition to cell cultures:

A stock solution of 1 mM was prepared by dissolving 0.01 g of carnosic acid in 2.5 ml of DMSO. 22.5 ml of this solution was added to 247.5 ml of supplemented phenol red-free growth medium, previously incubated at ambient temperature. The stock solution was filter-sterilized by passage through a 0.22 micron membrane filter.

A 1 in 50 dilution of this stock solution was prepared by introducing 26.46 ml carnosic acid of supplemented phenol red-free growth medium into sterile flask and adding 540 µl of the stock solution (1 mM) to obtain a 20 µM solution of carnosic acid.

The 20 µM solution was diluted two fold with supplemented phenol red-free growth medium under aseptic conditions to obtain 10 µM carnosic acid. Thus two working solutions of carnosic acid; 10 and 20 µM were obtained. The volume of each dilution was prepared in relation to the number microwells to be used for the experiment.

For animal studies, CARN solubilized in DMSO before further diluting in the drinking water to a concentration of 0.2% and administered to the animals for 5 days prior to irradiation in the pre-irradiation treated animal group. This implies an approximate intake of 25 mg/animal. After 5 days of commencing treatment with CARN the animals in this group were exposed to 500 mGy of X-rays and sacrificed by cervical dislocation 24 hrs after exposure to radiation.

The post-irradiation treated groups of animals were exposed to a total of 500 mGy of X-rays before they were administered with CARN 6 hours after receiving the radiation. 180 mg of the substance was administered by a single dose injection of 0.6 ml of a 300mg/ml of CARN solution directly into the gastric lumen of each animal 6 h after exposure to X-irradiation with the animal lightly anesthetized with ethyl ether.

Rosmarinic Acid (ROS)

Rosmarinic acid ($C_{18}H_{16}O_8$, molecular weight: 360.31 g/mol, Fig 27) is an ester of caffeic acid and 3, 4-dihydroxyphenyl lactic acid. It is present in different species of the family Boraginaceae, especially in *R. officinalis* L. It is a polyphenol with known antioxidant and anti-inflammatory activities with the propensity to neutralize superoxide-anions and inhibit lipid peroxidase activity. Its antioxidant activity on the inhibition of the oxidation of certain substances like linoleic acid suggest that it is liposoluble and has been found to be superior to ascorbic acid, a frequently used water soluble antioxidant (ALCARAZ et al., 2009). In this study, rosmarinic acid was dissolved in DMSO to a concentration of 1 mg/ml and used for all the relevant assays.

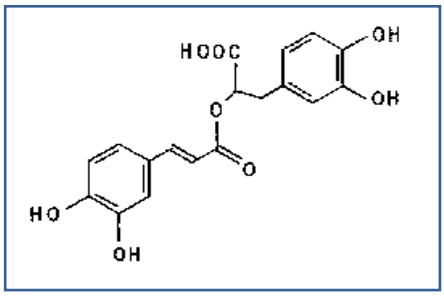


Figure 27. Rosmarinic acid

Preparation of Rosmarinic acid for addition to cells cultures:

A stock solution of 2 mM ROS was prepared by dissolving 0.01 g of the acid in 13.88 ml of pre-warmed supplemented phenol red-free growth medium. The resulting stock solution was filter sterilized through a 0.22 micron membrane filter.

27 ml of phenol red-free supplemented sterile growth medium was introduced in a flask and 540µl of the medium replaced with the same volume of the 2 mM ROS stock solution to obtain a 40 µM solution (1/50 dilution). A twofold dilution of this of this solution was prepared aseptically by mixing equal volumes of phenol red-free supplemented growth medium and 40 µM solution to produce a 20 µM solution of ROS.

The volume of each dilution was prepared in relation to the total volume required to execute the entire experiment.

For *in vitro* anti-mutagenic activity, ROS was also dissolved in physiological saline to obtain a final concentration of 25 µM in 2 ml of whole human blood. The control experiment only received the 25µM solution of ROS and the cells were incubated. For the pre-X-ray irradiation treatments, 20 µl (25µM) of the solution was added to 2 ml of human blood and the samples were homogenized just before X-irradiation. In the case of the post-X-irradiation treatments, 20 µl (25µM) of ROS solution was added to 2 ml of irradiated human blood and homogenized for 5 min after X-ray irradiation.

Apigenin

Apigenin (4′, 5, 7-trihydroxyflavone) is a flavonoid found in diverse vegetables such as celery, parsley artichoke, basil, in the flowers of the chamomile herb (*Matricaria chamomilla*) (McKAY and BLUMBERG, 2006: SHULKA AND GUPTA, 2010) and in aromatic plants such as thyme (*Thymus vulgaris*) or oregano (*Origanum vulgare* L. spp. hirtum) (KULISIC *et al*., 2007) and plant-derived beverages (e.g., tea and wine). It is a radioprotective antioxidant compound with anti-mutagenic and anti-inflammatory properties (ALCARAZ et al., 2009). It has been shown to have growth inhibitory properties in several cancer cell lines, including breast, colon, skin, thyroid and leukemia cells and to inhibit pancreatic cancer cell proliferation. Extracts of the compound can be obtained from grape or either of these sources motioned. Apigenin was dissolved in DMSO to a concentration of 0.5 mg/ml due to its poor solubility in water

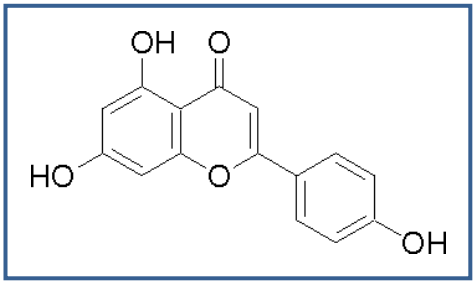


Figure 28. Apigenin

Preparation of apigenin for addition to cells cultures

A stock solution of 2 mM apigenin was prepared by dissolving 0.01 g of apigenin in 13.88 ml of pre-warmed phenol red-free supplemented growth medium and passed through filter membranes to sterilize. Due to pore clogging issues, two filters were employed; first it was passed through a 0.45 micron membrane filter and subsequently through a 0.22 micron filter. Filtering directly with the 0.022 μm filter was not feasible.

A stock solution of 2 mM apigenin was prepared by dissolving 0.01 g of apigenin in 13.88 ml of pre-warmed phenol red-free supplemented growth medium. The stock solution was filter sterilized using membrane filters. Due to pore clogging issues, two filters were employed; first it was passed through a 0.45 micron membrane filter and subsequently through a 0.22 micron filter. Filtering directly with the 0.022 μm filter was not feasible.

A 27 ml of phenol red-free supplemented growth medium was introduced into a sterile flask under aseptic conditions, 540 μl, of this medium was replaced with an equal volume of the 2 mM stock solution, producing a 40 μM solution of apigenin. A 1/2 dilution of this 40 μM solution was prepared by mixing 9 ml with 9ml of phenol red-free supplemented growth medium in a sterile flask producing 18 ml of a 20 μM solution of apigenin. Thus 20 and 40 μM working solutions of apigenin were prepared.

For animal studies, the API was administered dissolved in the drinking water at a concentration of 0.2% for 5 days prior to irradiation in the pre-irradiation treated animal group. This implies an approximate intake of 25 mg/animal. After 5 days of commencing treatment with API , the animals in this group were exposed to 500 mGy of X-rays.

The post-irradiation treated groups of animals were exposed to a total of 500 mGy of X-rays before they were administered with API 6 hours after receiving the radiation. 180 mg of the substance was administered by a single dose injection of 0.6 ml of a 300mg/ml of API solution directly into the gastric lumen of each animal 6 h after exposure to X-irradiation with the animal lightly anesthetized with ethyl ether

Green Tea Extract (TE)

Green tea extract contain catechins, a class of low molecular weight polyphenols that consist mainly of flavan-3-ol monomers and known to for their physiological and

pharmacological properties. Catechins are present mainly as catechin, epigallocatechin gallate, epicatechin gallate and gallocatechin gallate. The extract used for this study comprised 80% gallocatechins, with the main active substance being epigallocatechin-3-O-gallate whose chemical structure is $C_{22}H_{18}O_{11}$ (fig. 29) the extract also contains 0.3% caffeine among other substances. For use in cell culture, the extract was dissolved in water to 0.5mg/ml of water and filter sterilized through a 0.45µM nylon membrane.

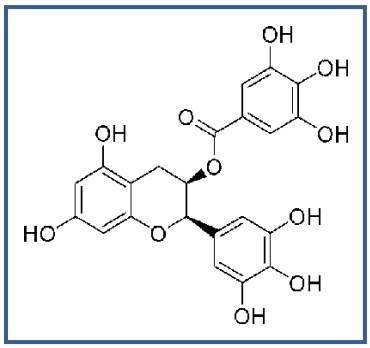


Figure 29. Green tea extract

For animal studies, TE was administered dissolved in the drinking water at a concentration of 0.2% for 5 days prior to irradiation in the pre-irradiation treated animal group. This implies an approximate intake of 25 mg/animal. After 5 days of commencing treatment with TE, the animals in this group were exposed to 500 mGy of X-rays.

The post-irradiation treated groups of animals were exposed to a total of 500 mGy of X-rays before they were administered with TE 6 hours after receiving the radiation. 180 mg of the substance was administered by a single dose injection of 0.6 ml of a 300mg/ml of TE solution directly into the gastric lumen of each animal 6 h after exposure to X-irradiation with the animal lightly anesthetized with ethyl ether.

Dimethyl sulfoxide (DMSO)

Dimethyl sulfoxide (C_2H_6OS is a colorless aprotic organosulphur compound that dissolves both polar and non-polar compounds and is miscible in a wide range of organic solvents. It has widely been used in *in vitro* cell culture and *in vivo* studies. Amongst others, it a known for its antioxidant properties and is generally believed to offer protection against deleterious effects of ionizing radiation. In this study, DMSO (Fig. 30) was generally used as a solvent to dissolve most of the test substances investigated.

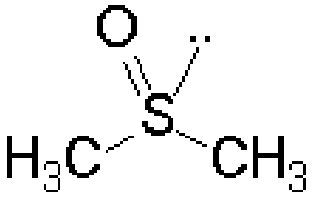


Figure 30. Dimethyl sulfoxide

Preparation of the DMSO for use in cell culture studies

0.2% and 0.1% solutions of DMSO were prepared in phenol red-free supplemented growth medium. To obtain a concentration of 0.2% of DMSO from the stock, 30 ml of growth medium were introduced into a sterile flask from which 60µl were pipetted out and replaced with the same volume of pure DMSO (i.e., 1/500 dilution). The resulting solution was then sterilized by passage through a 0.22 micron filter membrane. For the 0.1% solution of DMSO equal volumes of the 0.2% DMSO solution and phenol red-free supplemented growth medium were aseptically mixed (i.e., a 1/2 dilution). This process gave 0.1% and 0.2% working solutions of DMSO.

Optimization of DMSO concentration to be used as solvent for cell analysis

To establish the maximum tolerable dose of DMSO to our experimental models (cells), serial dilutions of DMSO were prepared in PBS and exposed to the cells for 24 or 48 hrs for viability and proliferative evaluation.

For cell culture studies, cells were seeded into microwells in 200µl cell suspensions in growth medium. Various volumes of the serial dilutions DMSO stated above were delivered into microwells containing the same number of cells and maintained over 24 or 48-hour incubation periods whereupon cell viability and proliferation was evaluated for each concentration of DMSO to be used, PBS was used as control.

The DMSO was administered to the animal by subcutaneous injection using a 25G needle. 100µl of 0.1% and 0.2% DMSO solutions were delivered subcutaneously to different cohorts of the experimental animals. Control animals received only the DMSO treatment but were not exposed to radiation while the pre-irradiation treatment group received 500 mGy of X-rays after the DMSO. These sets of animals were sacrificed after 24 hours by cervical dislocation.

The post-irradiation treated groups of animals were exposed to a total of 500 mGy of X-rays before they were treated 6 hours after receiving the radiation. The DMSO was administered at the same rate as with the pre-irradiation and test substance control animals

Ethyol® (Amifostine)

Amifostine, also known as WR-2721is an organic thiophosphate cytoprotective agent for its role against ionizing radiation when administered prior to radiotherapy in the treatment of various forms of cancers. An analogue of β-mercaptoethylamine (MEA), amifostine is known chemically as S-2-[(3-aminopropyl) amino] ethanethiol dihydrogen phosphate (ester), amifostine is a white crystalline powder which is freely soluble in water. It has an empirical formula of $C_5H_{15}N_2O_3PS$, a molecular weight of 214.22 and its structural formula is depicted in fig 31. The compound was used according to the manufacturer's recommendations i.e., 375 mg was dissolved in 7.3 ml 0.9% NaCl. For cell culture purposes, the resulting solution was sterilized by passing through a 0.45 micron nylon membrane. For use in blood culture protocols, 40µl of the sterilized solution were added of 2 ml of cultured human blood to obtain a final concentration of 1 mg/ml of the compound in culture medium. The control blood samples did not receive irradiation. Another cohort of blood samples that received the treatment were exposed to 2 Gy of X-rays and constituted the pre-irradiation treatment group. Finally another batch of blood samples were exposed to the 2 Gys of X-rays and the sample of amifostine added within 15 after exposure and mixed thoroughly before incubation. This cohort stood acted as the post-irradiation treatment group.

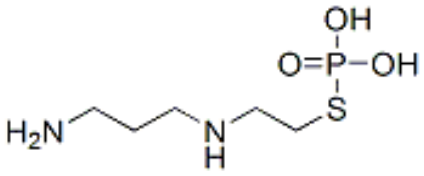


Figure 31. Amifostine

Zoledronic acid (Z)

Zoledronic acid (Z) Zoledronic acid is a white crystalline powder with a molecular formula of a $C_5H_{10}N_2O_7P_2 \bullet H_2O$, molar mass of 290.1g/mol and structural formula:

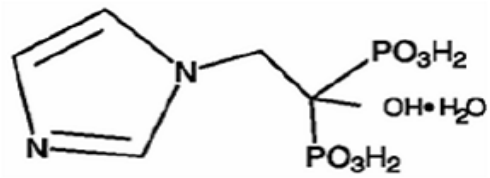


Figure 32. Structure of Zoledronic acid

Known chemically as (1-Hydroxy-2-imidazol-1-yl-phosphonoethyl) phosphonic acid monohydrate, it was administered at 100 % (Z100). Z was further diluted in sterile PBS to obtain 5 %(Z5), following the indications of the manufacturer for therapeutic use. To assess the genotoxic capacities of this substance, 20 µl and 40 µl each of these solutions (i.e., Z100 and Z5) were added to 2 ml of human blood and the samples were homogenized before cell culture. For the genoprotective capacities, 2 ml of human blood was exposed to 20 µl and 40 µl each of these solutions and the samples were homogenized just before X-irradiation. For the post-X-irradiation treatments, 20 µl and 40 µl each of these solutions was also added to 2 ml of irradiated human blood and homogenized for 5 min after X-ray irradiation. The substance control group of blood samples only received the same treatment with Z as above but was not exposed to radiation.

Propylthiouracil

6n-2n-propylthiouracil (PTU) (purity > 97%, Sigma, Madrid) was dissolved in 0.15 N NaOH to a concentration of 20 mg/ ml and the pH adjusted to 8.5 with 1N HCl. 25 ml of the resulting solution was diluted in 225 ml of 1% sucrose solution and was prepared daily when needed. The PTU was been administered dissolved in the drinking water at a concentration of 0.2% for 5 days prior to irradiation in the pre-irradiation treated animal group. This implies an approximate intake of 25 mg /animal. After 5 days of commencing treatment with PTU, the animals in this group were exposed to 500 mGy of X-rays.

The post-irradiation treated groups of animals were exposed to a total of 500 mGy of X-rays irradiation before they were fed with PTU 6 hours after receiving the radiation. 180 mg of the substance was administered by a single dose injected at a rate 0.6 ml/animal directly into the gastric lumen 6 h after exposure to X-irradiation with the animal lightly anesthetized with ethyl ether.

Procyanidins (P90)

P90 (Furfural Spanish SA, Sewer, Murcia) is a grape seed extract rich in procyanidins (polymers of flavan-3-ols). It (P90) is mixture of low molecular weight polyphenolic compounds and was obtained by passing the spent aqueous solution after the extraction of GSE-PS or P120 fraction from mercerated grape seed through an Amberlite XAD-2 column (to separate these compounds from other soluble polyphenolic components present in the extract). Methanol was used to elute the polyphenols from the amberlite column. The methanol was subsequetly eliminated by evaporation under vacuum in a rotary evaporator at a temperature of 40 °C, in the process, a dry solid dark orange crystalline material known as P90 was obtained.

P90, was dissolved in methanol to a concentration of 3 mg/ml and filtered through a 0.45 micron nylon membrane. The resulting solution was anlalysed by HPLC using a linear gradient of the following mobile phases: (A) water, (B) acetic acid: water (10:90) and (C) methanol: acetonitrile.

The P90 extract has a high concentration of polymers ≥ C4 (90.92%) while the monomers including, (+)-catechin (2.11%) and (-) -epicatechin (1.06%) are present in low proportions. The distribution of the remaining procyanidins was: B4 dimer (0.96%), B3 (0.71%), B1 (0.52%), B2 (0.48%) and esterified gallic acid derivatives in position 3 B1 dimer (0.52%) (Table 7, Figure 5). The activities of P90 was evaluated by dissolving it in DMSO to a concentration of 0.15 mg ml. Twenty microliters (20 µl) of the test substance were added to a cohort of unirradiated human periperal blood samples (non-irradiated P90 treated samples). Another 20 µl of the test substance was administered to another cohort of human peripheral blood samples before they were exposed to X-ray irradiation (pre-irradiation P90 treated samples).

The effect of administered P90 immediately after exposure to 2 Gy of X-rays was evaluated in a third set of blood samples to which 20 µl of the solution was administered after having exposed the samples to X-ray (post-irradiation treated samples).

Table MM1. Relative distribution of the major flavan-3-ols present in P90 by HPLC

Peak	Flavan-3-ol	Rt	ative amount of P90 present (%)
G	Gallic acid	9.7	0.83
1	B3 (dimer)	34.3	0.71
2	(+)-catechin	37.6	2.11
3	B1 (dimer)	40.6	0.52
4	T2 (trimer)	45.4	0.11
5	B4 (dimer)	49.9	0.96
6	B2 (dimer)	54.0	0.48
7	B2-3'-O-gallate	59.1	0.16
8	(-)-epicatechin	62.9	1.06
9	B1-3-O-gallate	64.4	0.52
10	C1 (trimer)	67.4	0.20
11	Polymers ≥ C_4 units	74.4	90.92
Others	Others dimer and trimer flavan-3-ols		

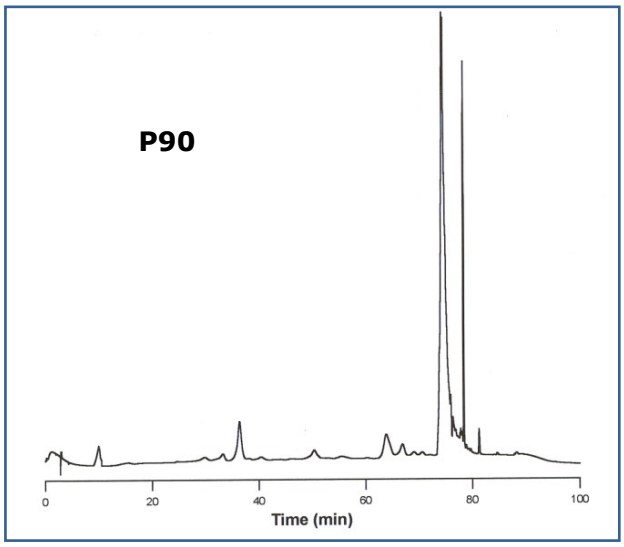


Figure 33. HPLC profile of the of P90 extract

Rutin

The rutin (purity > 90%, Extrasynthèse SA, Genay, France) a flavonoid with chemical formula $C_{27}H_{30}O_{16}$ and structure below (see figure 34) was dissolved in DMSO to a concentraytion of 1.0 mg/ml and filter sterilised. For use in the assays, 20 µl of this solution was dispensed into 2 ml of non-irradiated blood samples to evaluate its genotoxicity (non-irradiated rutin treated samples). Then 20 µl of rutin was also added

to peripheral blood samples before the samples were exposed to 2 Gys of X-rays to evaluate the possible protective effect (pre-irradiation rutin treated samples). Lastly, 20 µl of the rutin solution were added 2 ml sample of blood previously exposed to 2 Gy of X-rays. These samples represented the post-irradiation rutin treated samples. The rutin was administered to the blood samples within 5 minutes after exposure to irradiation.

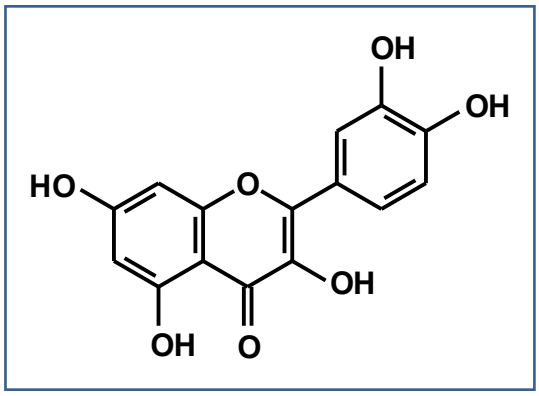


Figure 34. Rutin

Quercetin

Quercetin (Extrasynthèse SA, Genay, France) is a plant-based phytochemical called (2 - (3.4-dihydroxyphenyl) -3, 5, 7-trihydroxy-4H-[1-benzopyran]-4-one or 3 ', 4', 3, 5, 7-pentahydroxy-flavone). It is a flavonoid characterized by the possession of a hydroxyl group in the 3 position of the flavonoid skeleton. It lacks an aglycone moiety in its structure and can be obtained by acid hydrolysis of rutin, a rhamnoglucoside of quercetin in 1N HCl.

Quercetin was dissolved in DMSO (5% in sterile distilled water) to a concentration of 1 mg /ml for use in the preceding assays. 20 µl of this solution was dispensed into 2 ml of non-irradiated blood samples to evaluate its genotoxicity (non-irradiated quercetin treated samples). Then 20 µl of quercetin was also added to peripheral blood samples before the samples were exposed to 2 Gys of X-rays to evaluate the possible protective effect (pre-irradiation quercetin treated samples). Lastly, 20 µl of the quercetin solution were added 2 ml sample of blood previously exposed to 2 Gy of X-rays. These samples represented the post-irradiation quercetin treated samples. The quercetin was administered to the blood samples within 5 minutes after exposure to irradiation.

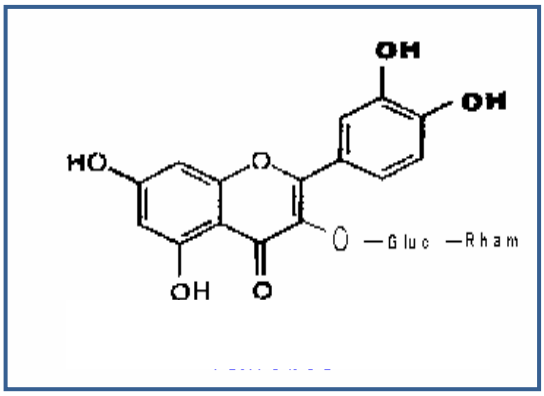


Figure 35. Structure of Quercetin

Eriodictyol

Eriodictyol (Sigma-Aldrich, Madrid) is a flavonoid known chemically as (S) 2-[3, 4-Dihydroxyphenyl] 2, 3-dihydro-5, 7-dihydroxy-4H-1benzopyran-4-one) **or** 3′, 4′, 5, 7-tetrahydroxyflavanone**)**. It is presents in several plant species, particularly in the form of glycosides (or rhamnoglucosides) and occurs as eriocitrin in lemon and neoeriocitrin in bitter orange. The compound may be obtained from quercetin following aqueous hydrolysis with 1N sulfuric acid.

For its genoprotective evaluation, eriodictyol was dissolved in DMSO to a concentration of 1 mg/ml. 20 µl of the resulting solution were dispensed into 2 ml of human peripheral blood samples (non-irradiated eriodictyol treated samples), another 20 µl were added to another lot of blood samples and mixed thoroughly before exposed to 2 Gy of X-radiation (pre-irradiation treatment group) and a finally, 20 µl eriodictyol was added to a last group blood samples that had previously been irradiated with 2 Gy of X-radiation. These samples represented the post-irradiation eriodictyol treated group. The sample of eriodictyol was added administered within 5 minutes after the blood samples were exposed to the radiation.

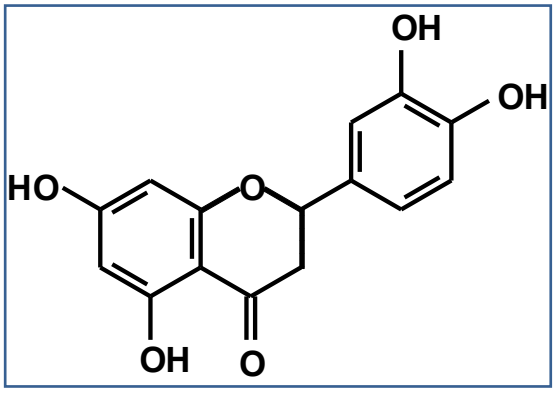


Figure 36. Eriodictyol

Soluble Citrus Extracts (CE)

Soluble Citrus Extract or CE (Spanish Furfural SA, Sewer, Murcia) is characterized by a the prescence of a high concentration of flavanones and flavones. The CE used in this study was obtained from immature fruits harvestef from three citrus species growing in the Autonomous Community of the Region of Murcia namely; *Citrus limonia* (lemon), *Citrus sinensis* (sweet orange) and *Citrus aurantium* (bitter orange)

To extract the flavone type compounds, the immature fruits were dried and milled in an atrition mill to a fine particle size. Extraction was conducted at ambient temperature for three hours using an extraction solvent of water -methanol (20:80 v/v) at a ratio of 5% w /v. The extract was then filtered and the polyphenol-rich hydroalcoholic extract concentrated under vacuum in a rotavapor at a maximum temperature of 55 °C. The flavanoids were subsequently crystalized out of solution by chilling and slowly agitaing the concentrate over a 24-hour period. The suspension was then filtered, washed with water and dried under vacuum at a maximum temperature of 55 °C to obtain a light brown solid powder which was as branded soluble Citrus Extract (CE), this was saved and used the various assays.

An HPLC analysis was performed on CE-50 by dissolving the the extract in DMSO to a concentration of 2 mg/ml and filtering the resulting solution through a 0.45 micron nylon membrane. A C_{18} LiChrospher 100 (250 x 4 mm id) column with a mean particle size of 5 µM ((Merck, Darmstadt, Germany) was employed as the stationary phase. The column temperature was maintained at 30 °C and a flow rate of 1 ml/min was used. The flavanones were visualised at a wavelength of 280 nm while the flavones were detected at 340 nm. Mobile phases of : (A) acetic acid: water (1:99), (B) methanol and (C) acetonitrile were used and an elution method composed of stages isocratic and linear gradients was employed.

The most abundant flavonoids in the citrus extract were the flavanones naringin, hesperidin and neohesperidin, however, other flavanones with very significant biological activity such as eriocitrin and neoeriocitrin and small concentrations of some other flavones were also present. Also present in the CE were luteolin glucoside**,** two

diosmetin flavonoids (diosmin and neodiosmin) along with rhoifolin, a neohesperidoside of apigenin.

The compposition of the the main flavonoids present in CE-50 used in this study is os displayed in table MM2 and in figure 38.

Table MM2. Retention times and absolute percentagea of the main flavonoids in Soluble Citrus Extract (SCE-50).

Peak	Poly phenols	R_t(min.)	%
1	Eriocitrin	8.24	1.72
2	Neoeriocitrin	10.12	1.25
3	Luteolin 7-O-rutinoside	11.56	0.60
4	Luteolin-7-O- luteolin 7-O-neohesperidoside	12.42	0.41
5	Isonaringin	15.23	1.10
6	Naringin	16.89	10.76
7	Hesperidin	19.96	6.68
8	Neohesperidin	22.35	7.65
9	Rhoifolin	25.31	1.62
10	Diosmin	30.21	0.87
11	Neodiosmin	36.52	0.56
12	Didimin	52.23	0.25

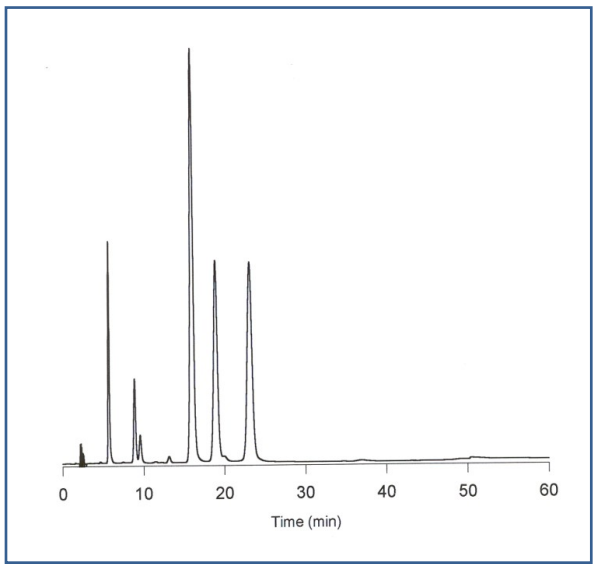


Figure 37. Chromatographic analysis of components soluble Citric Extract (CE-50)

For CE exploratory studies, the extract was dissolved in DMSO to a concentration of 1.0 mg/ml abd filtere sterilised. Twenty microiliters (20 µl) were administed diffrently to three different cohorts of blood samples. The first lot of 2 ml blood received 20 µl of the CE solution without exposure to irradiation and was dubbed the (non-irradiated CE treated samples). A second group of blood samples received 20 µl of CE solution after which they were exposed to 2 Gys of X-rays (pre-irradiation CE treated samples). Finally 20 µl of CE were added to a third cohort of blood which were previously exposed to X-rays (post-irradiation CE treated samples).

CE was administered dissolved in the drinking water at a concentration of 0.2% for 5 days prior to irradiation in the pre-irradiation treated animal group. This implies an approximate intake of 25 mg/animal. After 5 days of commencing treatment with CE, the animals in this group were exposed to 500 mGy of X-rays.

The post-irradiation treated groups of animals were exposed to a total of 500 mGy of X-rays before they were administered with CE 6 hours after receiving the radiation. 180 mg of the substance was administered by a single dose injection of 0.6 ml of a 300mg/ml of CE solution directly into the gastric lumen of each animal 6 h after exposure to X-irradiation with the animal lightly anesthetized with ethyl ether

Vitamin C (Ascorbic Acid)

Vitamin C or ascorbic acid (Sigma-Aldrich, Madrid) is a water soluble vitamin which is not stored in the body for long periods and is removed in small quantities through urine. Pure vitamin C is a white substance and stable in dry form but easily oxidized when in solution, especially when exposed to heat. Its chemical structure is $C_6H_8O_6$ and is represented graphically in figure 38.

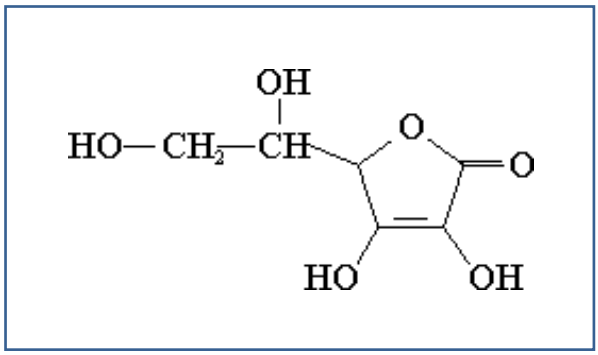


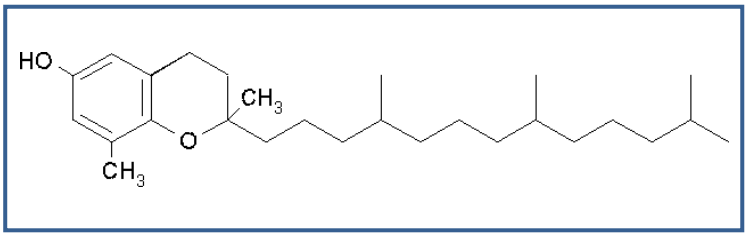
Figure 38. Vitamin C (Ascorbic acid)

To evaluate the possible protective effects of vitamin C in this study a 2.5 mg/ml solution was prepared in DMSO and filterer sterilized from which 20 µl was added to 2ml of non-irradiated blood samples (non-irradiated Vitamin C treated samples). For the pre-irradiation group, 20 µl of the vitamin E sample was added to the blood samples and mixed thoroughly before they were exposed to 2 Gy of X-rays. A third group of blood samples were treated with2 Gy of X-rays, after which 20 µl of the test vitamin C solution was added, and this constituted the post irradiation group.

Vitamin E (δ-tocopherol)

Vitamin E (δ-tocopherol) (Sigma-Aldrich, Madrid) is a fat soluble vitamin that is stored in the fatty tissue of the body and has an important antioxidant role. Eight different forms of vitamin E (figure 39) are known and each has its own biological activity which is measured by its potency or functional use in the body. δ-tocopherol ($C_{27}H_{46}O_2$), which has a very high antioxidant activity, is the form of Vitamin E that was used in this study. A solution of 2.5 mg/ml was prepared in a 50% (v/v) DMSO/ethanol solvent and filter sterilized.

Figure 39: Vitamin E (δ-tocopherol)



The effect of different concentrations of Vitamin E before exposure to radiation was also evaluated by adding 25 µl of the test solution to 2ml of non-irradiated blood before, a cohort of this blood samples were not exoposed to radiation and acted as the substance control group while the other cohort was exposed to 2 Gys of X-rays (pre-irradiation Vitamin E treated samples).

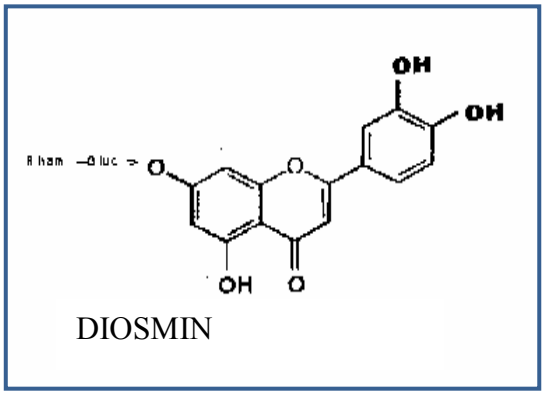
To third group of 2 ml blood samples previously irradiated with 2 Gy X-radiation (post-irradiation vitamin E treated samples), 25µl of the test vitamin E solution ws added within 15 mins post irradiation.

Diosmin

Diosmin (Extrasynthèse SA, Genay, France) is a pure flavonoid, specifically a flavone. The sample used in this research work had a minimum purity of 90% of the

flavonoid as adjudged by HPLC. Its chemical structure is $C_{27}H_{32}O_{15}$ and the structure is displayed in the figure below. For use dissolved in DMSO to a concentration of 1.0 mg /ml and filter sterilized. It may be obtained in the lab by, selective dehydrogenation of C2 = C3 bond present in the hesperidin flavonoid structure. It is one of the most abundant flavonoid in nature and occurs primarily in citrus (orange and lemon) plants.

Figure 40: Diosmin



DIOSMIN

The Diosmin was administered to the animals dissolved in the drinking water at a concentration of 0.2% for 5 days prior to irradiation in the pre-irradiation treated animal group. This implies an approximate intake of 25 mg/animal. After 5 days of commencing treatment with Diosmin, the animals in this group were exposed to 500 mGy of X-rays.

The post-irradiation treated groups of animals were exposed to a total of 500 mGy of X-rays before they were administered with Diosmin 6 hours after receiving the radiation. 180 mg of the substance was administered by a single dose injection of 0.6 ml of a 300mg/ml of Diosmin solution directly into the gastric lumen of each animal 6 h after exposure to X-irradiation with the animal lightly anesthetized with ethyl ether.

Preparation of supplements (additives) to culture medium

Preparation o f phytohemagglutinin (PHA)

Lyophilized phytohemagglutinin (PHA) were obtained either in 1 or 5 mg glass ampoules and stored at 2-8 ° C until required. When required a stock solution of 16 µg / ml of PHA was preared by dissolving 1,600 µg (1.6 mg) PHA in 100 ml of sterile Ham´s F10 culture medium (Table MM1) and aliquoted into two-mililiter vials for storage at -20 ° C. During cell cultures, stock PHA was added to a final concentration of 1.0µg/ml in the culture medium. The function of PHA is to artificially stimulate cell division or proliferation in normal, non-dividing lymphocytes.

Preparation of cytochalasin B (Cyt B)

Cyt B was used in this study to lock up dividing cells at cytokinesis by blocking first mitotic division in human peripheral blood lymphocytes. This substance was added to each of the cultures after 44 hours of initiation of cell culture in a sterile environment. Freeze-dried preparations Cyt B were obtained in lots of 1 and 5 mg in glass ampoules and stored at -20 ° C until required. For use, each bottle was reconstituted by initially dissolving in an appropriate volume of 100% dimethylsulfoxide (DMSO) in a sterile environment to obtain a stock solution of 2 mg/ml of Cyt B. Subsequently a 1: 9 dilution of this stock solution was prepared in PBS producing a 200 µg/ml of Cyt B which was aliquoted into two-mililiter vials and stored at -80 ° C and only thawed just in time of use. To obtain a final concentration of 3 µg/ml of Cyt B in each culture flask containing 10 ml of culture medium, 150 µl of the Cyt B solution corresponding to 30 µg of Cyt B was added.

Preparation of hypotonic solution of potassium chloride

A hypotonic solution of potassium chloride (0.075 M) was prepared by dissolving 5.59 g of KCl (Probos, Madrid) in a litre of distilled water. Suspending the cells in hypotonic saline causes an osmotic influx of water in the cells due to the concentration gradient caused by the less-than-physiological concentration outside of the cells, this result in the cells swelling up and allowing a better view of cells under a microscope.

Preparation of phosphate buffer

A solution of 10 mM phosphate buffer (pH 6.2) was obtained by preparing 10mM stock solutions of the diabasisc and monobasic phosphate salts of sodium separately in 1L volumetric flasks. The following weights of the sodium phosphate salts were weighed into separate 1L volumetric flask and dissolved in distilled water. The volumes were then made to the 1L mark with more distilled water.

$NaH_2PO_4.2H_2O$ ----------1.560 g

$Na_2HPO_4.7H_2O$ ----------2.681 g

Different volumes of the stock solutions were mixed until the desired pH was obtained.

Preparation of the fixing solution

The fixative solution used to fix lymphocytes cells was prepared by mixing 3 parts of methyl alcohol with 1 part of glacial acetic acid (v/v). With the addition of fixative, each cell is preserved in its swollen form. The fixative was always freshly prepared when needed.

Cell Culture media

Various growth mediums were used to maintain the differnt types of cells used in this study. Some of these mediums and their respective compositions have been provided in the following tables (Tables MM3 to MM5):

Table MM3. Composition of Ham's F-10 medium

Inorganic salts/ Concentration (mg/l)	
KCI	285
Na_2HPO_4	154
KH_2PO_4	83
$CaCI_2 \cdot 2H_2O$	44
$MgSO_4 \cdot 7H_2O$	153
$FeSO_4 \cdot 7H_2O$	0.834
$CuSO_4 \cdot 5H_2O$	0.00249
$ZnSO_4$ x $7H_2O$	0.0288
$NaHCO_3$	1200
Other components/ Concentration (mg/l)	
D-glucose	1100
Na-pyruvate	110
Phenol red	1.2*
Amino acids/Concentration (mg/l)	
L-alanine	9
L-arginine·HCI	211
L-asparagine	13
L-aspartic acid	13
L-cysteine	25
L-glutamine	146
L-glutamic acid	14.7
Glycine	7.51
L-histidine.HCI·H_2O	21
L-isoleucine	2.6
L-leucine	13
L-lysine·HCI	29
L-methionine	4.48
L-phenylalanine	5
L-proline	11.5
L-serine	10.5
L-threonine	3.57
L-tryptophan	0.6
L-tyrosine	1.81
L-valine	3.5
Vitamins/Concentration (mg/l)	
Biotin	0.024
D-Ca-pantothenate	0.715
Choline chloride	0.698
Folic acid	1.32
Myo-inositol	0.541
Nicotinamide	0.615
Pyridoxine·HCI	0.21
Riboflavin	0.376
Thiamine·HCI	1
Vitamin B12	1.36
Hypoxanthine	4.1
Thymidine	0.73
Lipoic acid	0.2

* Liquid media contain 10 mg/l of phenol red

Table MM4. Composition of RPMI 1640 medium

Inorganic salts/Concentration (mg/ml)	
$Ca(NO_3)_2.4H_2O$	100
KCl	400
$MgSO_4$ (unhydrous)	48.84
NaCl	6.000
Na_2HPO_4 (unhydrous)	800

Other components/Concentration (mg/ml)	
D-Glucose	2.000
Glutathione (reduced)	1.0
Phenol red	5.0

Amino acids/concentration (mg/ml)	
L-Arginine HCl	241.86
L-Asparagine (free base)	50.0
L-Aspartic Acid	20.0
L-Cysteine HCl	65.15
L-Glutamic Acid	20.0
L-Glutamine	300.0
Glycine	10.0
L-Histidine (free base)	15.0
L-Hydroxyproline	20.0
L-Isoleucine	50.0
L-Leucine	50.0
L-Lysine. HCl	40.0
L-Methionine	15.0
L-Phenylalanine	15.0
L-Proline	20.0
L-Serine	30.0
L-Threonine	20.0
L-Tryptophan	5.0
L-Tyrosine (disodium salt)	28.94
L-Valine	20.0

Vitamins/Concentration (mg/ml)	
D-Biotin	0.20
D-pantothenic acid	0.25
Choline chloride	3.0
Folic acid	1.0
myo-Inositol	35.0
Nicotinamide	1.0
P-aminobenzoic acid	1.0
Pyridoxine HCl	1.0
Riboflavin	0.20
Thiamine HCl	1.0
Vitamin B_{12}	0.005

Table MM5. Composition of Dulbecco's Modified Eagle´s Medium(DMEM)

Inorganic salts/Concentration (mg/l)	
$CaCl_2$ (unhydrous)	200
KCl	400
$MgSO_4$ (unhydrous)	97.67
NaCl	6.400
Na_2HPO_4.	109
$Fe(NO3)_3.9H_2O$.	0.1

Other components/Concentration (mg/l)	
D-Glucose	4.500
Phenol red.Na	5.9

Amino acids/ Concentration mg/l	
L-Arginine.HCl	84
L-Cysteine.2HCl	62.6
L-Histidine HCl. H_2O	42
L-Isoleucine	105
L-Leucine	105
L-Lysine.HCl	146
L-Methionine	30
L-Pheynylalanine	66
L-Threonine	95
L-Tryptophan	16
L-Tyrosine (dibasic salt)	103.79
L-Valine	94
L-Serine	42
L-Glutamine	584
Glycine	30

Vitamins/Concentration (mg/l)	
D-Pantothenate	4
Choline chloride	4
Folic acid	4
Myo-Inositol	7.2
Niacinamide (Vitamin B3)	4
Pyridoxal HCl	4
Riboflavin	4
Thiamine HCl	4

CYTOTOCICITY AND GENOTOXIC METHODOS

Cytoprotective studies

Maintenance and conservation of cell cultures

For cytoprotective studies normal human prostate PNT2 and mouse B10F16 melanoma cell lines were used. Both cell lines were grown and maintained from multiple passages and conserved by cryopreservation. They were maintained in a continuous logarithmic culture in appropriate growth medium in 25-cm^2 tissue culture flasks. All cell culture manipulations were carried out in appropriately sterilized vertical laminar flow hoods (Cultair ASB type II).

Cell growth was monitored daily by inspection using a phase contrast microscope. The medium was changed when the pH indicator signalled medium exhaustion. PNT2 cells were cultured in RPMI 1640 supplemented with FBS (10 %) and glutamine (2 mM) and streptomycin plus penicillin (100 µg/ml and 100 IU/ml, respectively). While B16F10 cells were cultured in DMEM supplimeted with essetial growth factors. All the processes were carried out in a Cultair ASB type II vertical laminar flow chamber. The PNT2 cell cultures were kept at 37 ºC and 95 % relative humidity, in 5 % CO2 atmosphere, in a Cytoperm Heather. The culture medium was changed every 2 days or when acidification was indicated by the pH indicator (phenol red). Cells were allowed to grow until confluent and subsequently trypsinized by aspirating the exhausted medium and adding 2 ml of trypsin (0.1 mg/ml), EDTA (0.2 mg/ml) to the cell monolayer and gently swirling the flask to ensure that the entire surface was covered with trypsin. The flask was returned to the incubator at 37ºC for 8 minutes and further examined under an inverted microscope to confirm that cells were loosened, when necessary, the flask was gently tapped to facilitate detachment of adhering cells from the base. Subsequently, 4 ml of cold medium supplemented with FBS was added and the contents transferred aseptically into 15ml centrifuge tubes, followed by low speed centrifugation (214 x g) for 10 minutes at 22 ºC to eliminate and/or inactivate the trypsin. The pelleted cells were uniformly dispersed in 2 ml of fresh medium. Assessment of cell viability was accomplished by the tryptan blue viability assay using a haemocytometer and the number of cells needed for seeding calculated.

Samples were regularly drawn from cell cultures and screened to confirm the absence of *Mycoplasma spp*. by direct fluorescence with DNA specific dye H33233 (Hoechst, Germany). For cryopreservation, cells were viewed using an inverted microscope to check confluence and to confirm absence of bacterial and fungal contaminants. The confluent cells were trypsinized as above and centrifuged to remove medium. The cells were dispersed in fresh medium supplemented with foetal bovine serum (FBS) at a concentration of 1 x 10^6 cells/ml and distributed into cryovials containing 10% (v/v) of DMSO, a conventional cryoprotectant.

For cell storage, cryopreservation was initially carried out in 100% isopropyl alcohol which is known to have a stepwise cooling rate of 1ºC/minute. The vials containing the cells were initially maintained under these conditions until it attained a temperature of -80ºC, subsequently, the vials were transferred into in a liquid nitrogen tank to reduce the temperature to -196ºC and for indefinite storage at this temperature.

To re-establish the cultures from frozen stocks, vials containing cryopreserved cells were fast thawed in a water bath at 37ºC. The vials were wiped with 70% alcohol and transferred into a laminar flow hood. In the laminar flow hood, the content of the vials were transferred into a centrifuge tube containing 10 ml of pre-warmed appropriate growth medium to dilute out the DMSO present in the freezing medium. The centrifuge tubes together with its contents were centrifuged at 214 g for 10 minutes and the supernatant discarded. The pelleted cells were re-suspended in 2 ml growth medium and counted in haemocytometer in the presence of the cell viability assessment dye tryptan blue. After counting, the appropriate number of cells were seeded in a cell culture flask containing 5ml of appropriate growth medium to restart the sub-culturing process, changing the growth medium 24 hours and supplementing with higher amounts of FBS in the case of worse recovery cells.

Optimization of cell numbers

Cell viability and proliferative assays were conducted at 24, and 48 hours after treatment with test substance with and without exposure to X-radiation. Cell cultures were performed in 300µl/well capacity Nunc ® MicroWell 96 well optical bottom plates. Positive controls were included for each treatment and cell line and subjected to the same conditions as the experimental cells. Cells in the exponential growth phase

were used for the assays and the culture conditions in the microwells were the same as pertained in the tissue culture flasks. The treatments were incubated for 24 hours after cell seeding in both types of assays to get cells adapted to the culture conditions and to adhere to the bottom of the wells. To determine the optimal cell seeding concentration the appropriate number of cells were seeded into each well to avoid cell overgrowth during the treatments out of which growth curves were constructed. After analysing the growth curve of for each cell line it turned out that the most convenient number to seed per well was 3200 cells for PNT2 cells and 2, 500 for BF16F10 melanoma cells.

Influence of *P. angolensis* seed extract (Q52) on cell viability with and without X-rays

Cell numbers were manipulated such that 200µl of medium containing 3,200 or 2,500 cells were introduced into each of the wells and different concentrations of the test substances to be assayed added into each well, (at least 6 wells per test substance). After 15 minutes, the plates were irradiated with different dose of X-rays (0, 4, 6, 8 and 10 Gy). The samples were then incubated for 24 or 48 hours and the MTT colorimetric assay performed to determine the cytoprotective effects of the test substances.

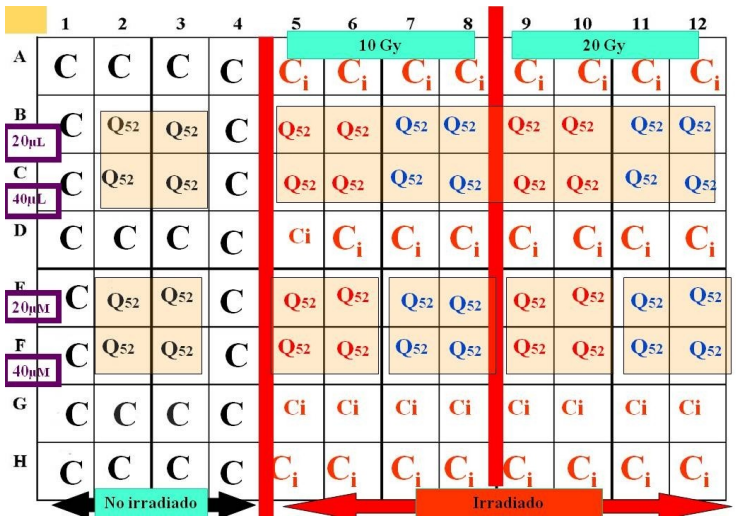


Figure 41. Scheme used to study cytoprotective activity of extracts from *P. angolensis* (Q52 =PASE)

Cell survival and viability quantification: MTT test

To analyze the effects of the substances studied on cell viability and survival for 24 and 48 hours, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) cell viability assay method was used. This method has been extensively used to measure cell proliferation and survival capacities. The quantity of the living cells is

proportional to the quantity of formazan produced. The methods of Carmichael *et al*. (1987a and 1987b) and Alley et al, (1988) were adapted to the culture conditions to quantify cell viability. Briefly, 200µl of growth medium containing 3,200 PNT2 (or 2,500 B16F10) cells were seeded into 96 well micropipettes and incubated for 24 hr for the cells to adhere. After treatment with the above stated concentrations of test substances studied, radiation doses and the stated incubation periods, the spent medium in each well was aspirated and replaced with 200µl of fresh supplemented growth medium enriched with PBS. Fifty micro litres (50 µl) of MTT (8 mg/ml) were added to each well in 96 well plates and the microplates were further incubated at 37°C for 4 hours in a 5% CO_2 atmosphere. Afterwards, the plates were centrifuged at 1000 rpm for 8min to carefully remove the medium and non-metabolized MTT, 100 µl of DMSO was added to each well to solubilize the MTT taken up by the living cells. After shaking for 30 min at room temperature, microplate absorbance was read on a Multiskan MCC/340P microplate reader with a test wavelength of 570 nm and a reference wavelength of 690. The negative control wells were used for the baseline zero absorbance. Each experiment was repeated on three occasions.

The protocol used to perform the MTT assay in this experiment is detailed as follows:

1. 3,200 PNT-2 (or 2, 500 B10F16) cells were seeded into 96-well plate and incubated overnight for cells to adhere to the substratum.
2. The spent growth medium in each microwell was aspirated from each well after 24 hrs.
3. The aspirated growth medium was replaced with 200µl of fresh growth medium enriched with FBS.
4. 50µl of MTT (8mg/ml) were added into each well and the multiwall plates incubated at 37º C for 4 hours in an incubator at 5% of CO_2 tension.
5. The cells were centrifuge at 1000 rpm for 8 minutes to eliminate the growth medium and non-metabolized MTT.
6. 100µ l of DMSO was added into each well to solubilize the MTT formazan taken up by the cells and the microplates agitated to mix at ambient temperature for 30 minutes.
7. Absorbance readings were made on a Multiskan MCC/340P microplate reader equipped with a double wavelength system (560 and 690 nm).

Relative cytoprotective activities of Q52 extracts and other herbal and non herbal substances on PNT2 and B10F16 melanoma cells via the MTT assay

Cell viability

The effect of various test substances on the viability of the PNT2 and B10F16 melanoma cells was determined using the MTT (3-4, 5-dimethylthiazol- Z-yl-2, 5-diphenyltetrazolium bromide) assay. PNT2 and B10F16 cells were seeded in a 96 well plate at a concentration of 3, 200 and 2, 500 cells/ml respectively. Twenty four hours after plating, cells were treated with two different concentrations of each of the following substances either pre- or post- irradiation; Carnoisic Acid, Apigenin, Rosmarinic Acid (ROS), and DMSO. Preparations of the solutions and their respective volumes and concentrations administered to the cells have been described above.

For the pre-irradiation treatments, after 15 minutes, the plates were irradiated with different dose of X-rays (0, 4, 6, 8 and 10 Gy). The samples were then incubated for 24 or 48 hours following which their cytoprotective abilities was assessed via the MTT viability assay. For the post-irradiation experiments, cultured cells were first exposed to radiation before addition of the relevant amounts of the test substances and subsequent incubation for 24 and 48 hours where upon the cytoprotective capacities of the test substances were evaluated.

The relative cell viability was determined by the amount of MTT converted to the insoluble formazan salt. Absorbance reductions of the samples viability was determined by absorbance reductions of the samples at various concentrations when compared with untreated control. Cell viability was defined relative to untreated control cell cultures as follows:

Cell viability = (absorbance of treated/absorbance of control cells culture) x 100

All data are presented as mean ± standard deviation (SD) for at least six replications for each prepared sample.

Cytoprotective effect of combined treatment of test substances: Two-drug combination Assay

Combination treatments of the test substances were also assessed for synergistic cytoprotective properties. In this regard, equimolar amounts of carnosic acid and apigenin were evaluated for cytoprotecion at two different concentrations i.e., equal

volumes (10μL) of (10 μM and 20 μM) of these test concentrations were pipetted into microwells containing cells, exposed to radiation and the treatments monitored over a period of 24 and 48 hours. Similarly, other treatment pairs included; Apigenin and Rosmarinic acid (API + ROS); PASE and Carnosic acid (PSAE + CAR); and PASE and Rosmarinic Acid (PASE + ROS). Preparation of the solutions and the respective amounts administered to the cell cultures are detailed in the sections above. As usual the cytoprotective activity of the above treatment was assessed by the cell viability and the PNT2 cell survival was analyzed by an MTT assay for 24, or 48 as previously described (CARMICHAEL *et al.,* 1987a; 1987b; ALLEY *et al.,* 1988; MOSSMAN, 1983).

Genoprotective Activity

In vitro cytokinensis-blocked Micronucleus(CBMN) Assay

Peripheral blood Lymphocyte sam ples

Whole human blood samples from healthy volunteers used in this study (see blood sample section). The blood samples were collected into sterile conical VENOSAFE ™ (Terumo Europe NV, Leuven, Belgium) blood collection vacuum tubes containing heparin (Heparin sodium 5%; Rovi Lab, Madrid). The blood was aliquoted in 2ml portions into cryovials. The blood aliquots from the donors were divided into two cohorts for the construction of a dose-response curve and for the evaluation of the anti-mutagenic activity of some of the test substances.

Construction of Dose response curves

Blood donors were divided into 2 groups;

Group 1:

Fourteen cryovials containig 2ml of donor blood were used for the construction of a dose-reponse curve. Of these, 2 vials conatining blood were not irradaited and served as control vials (baseline micronuclus frequency) while the remailing 12 blood samples were exposed to different doses of X-rays (2, 4, 6, 8 and 10 Gy).

***In vitro* Genoprotective activity of test substaces**

Group 2:

The second group consisted of the remaing 2ml aliquots of blood in cryovials, and these were used to evaluate the antimutagenic effect of different test substance used (pre- and post-irradiation). The test substances used comprised PASE, ROS,

P90, CARN, API in DMSO, Vit E, VIT C, COL, CE, AMFI TE, Rutin, DMSO, Eriodictyol, Quercetin, and Zoledronic Acid.

Two of these blood samples were used as experimental controls (baseline micronucleus frequency or or non-irradiated control), irradiated control. A sample of blood was reserved for each test substance used, and this served as test substance control. These samples only received the test substance but were not exposed to radiation. Some blood samples were treated with the test substances before exposure to irradiation and were designated the pre-irradiation group, the rest were irradiatated before the addtion of the test substances and were accordingly called the post-irradiation group.

***In vitro* genoprotective (antimutagenic) properties of test substances**

PASE was administered dissolved in physiological saline to obtain a final concentration of 25 µM in 2 ml of blood. For the pre-X-ray irradiation treatments, 20 µl of these solutions was added to 2 ml of human blood and the samples were homogenized just before X-irradiation. For the post-X-irradiation treatments, 20 µl of these solutions was added to 2 ml of irradiated human blood and homogenized for 5 min after X-ray irradiation.

The present study was also designed to examine if the test compounds could afford protection against the mutagenic effects of x-irradiation, using the micronucleus test for antimutagenic activity and evaluating the reduction in the frequency of micronuclei (MN) in cytokinesis-blocked cells of human lymphocytes before and after x-ray irradiation. Briefly 25µl PASE was added to 2ml aliquots samples of whole donor blood in heparinised. No substance was added to the control blood. Two controls were set up both containing no test substance; one was not exposed to radiation and served as control for zero dose (i.e. un-irradiated control). The other control tube received 2Gy of x-irradiation together with the other blood samples and served as irradiated control. Soon after irradiation, 25 µl of the test substance were added separately to the three irradiated tubes containing blood which previously did not receive test substance and these served as post irradiation addition.

Micronucleus assay

The assay used here was based on evaluating the MN frequency in cultured peripheral blood human lymphocytes blocked at cytokinesis during the first mitotic cell

division, as described by Fenech (1986, 1990, 2000). This method is currently considered as one of the most appropriate procedures for cytogenetic analysis of genotoxic damage induced by ionizing radiation in a systematic (IAEA, 2001).

To perform the micronucleus assay, the blood samples were mixed thoroughly and 500 µl of each (irradiated or non irradiated and treated or not treated) aliquoted into 25mm^2 tissue culture flasks containing 4.5ml F-10 medium supplemented with 15 % FBS, 1.6 % phytohaemagglutinin, 1 % penicillin/100 IU streptomycin and 1 µg/ml of glutamine. The cultures were maintained at 37°C, ad 5% CO_2 in a humidified atmosphere. Forty-four hours after initiation of the lymphocyte culture, 150 µl of cytochalasin B was added at a concentration of 3 µg/ml. After 48 h, the the contents of the culture flasks were transferred into 15 ml conical tubes and centrifuged (Heraeus, Minifuge T, Madrid) for 5 minutes at 1000 rpm to harvest the cells The supernatant was discarded and the pelleted cells re-suspended in 8 ml of hypotonic solution of KCl (0.075 M), votexed for 3 min and fixed in a methanol: acetic acid (3:1) fixative. Microscopic smear preparations were made by placing the cell suspension dropwisely on pre-chilled slides followed by drying over a stream of warm air and allowing for complete drying in the open air for at least 24 hours. Slides were then stained by immersing them completely in pure May-Grunwald (Braun, Melsungen, Germany) solution of for 3 minutes, then in 50% May-Grünwald solution (50% May-Grünwald and 50% distilled water for 2 minutes. The slides were then transferred into a pure solution of Giemsa (Merck, Darmstadt, Germany) stain for 2 minutes. Finally, the stained slides were dipped individually in 100 mM phosphate buffer pH 6.2 to washed off excess stain after which they were air dried and coverslips mounted on each microscopic slide for light microscopic studies (Figure 42).

Figure 42. Prepared slides after staining with May-Grünwald and Giemsa stains

Scoring of micronuclei

Binucleated and micronucleated binucleated cells were scored in each slide using a ligt microscopy and/or a Lecia SCN 400 scanner. The light microscopes and details of their usage have been described above (see section on optical microscopes). Micronuclei in the human lymphocyte preparations were counted in 500 binucleated cells (BC). Six preparations were obtained from the two replicate cultures performed on each blood sample (1 blood sample, 2 replicate cell cultures and 6 Microscope slide preparations). Thus even though figures have been presented as micronuclei per 500 cytokinesis-blocked cells (MN/500 BC), approximately 3,000 BC were evaluated for MN for each blood sample analyzed. Counting was performed in a double-blind fashion and the results averaged out to yield a single value with each point corresponding to the mean of at least six readings.

The adopted criteria for scoring micronuclei was the version described by Almassy, et al, 1987 and Fenech *et a*l., 2003 and enumerated below for the sake of brevity:

i. A micronucleus must have the same structure as the main nucleus.
ii. A micronucleus must be smaller than the main nucleus but must not exceed half the size of the core nucleus or twice the normal length of a chromosome.
iii. A micronuclei must be rounded or spherical in shape and be visibly separated from the main nucleus.
iv. Micronuclei are non-refractile and should therefore be readily distinguished from artifacts such as stained extraneous particles (Figures 43 and 44).
v. Micronuclei are counted only in cells that have retained the cytoplasm.
vi. Micronuclei usually have the same staining intensity as the main nuclei but occasionally staining may be more intense.

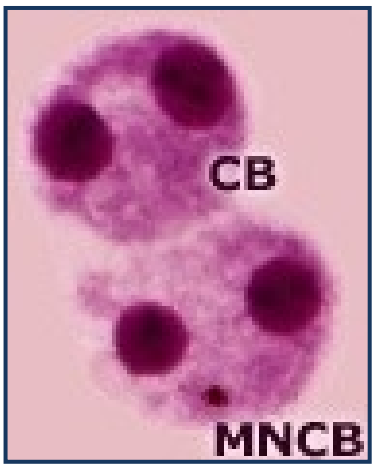


Figure 43. Image of two binucleated cells with preserved cytoplasm (BC) with one having a micronucleus in the cytoplasm (MNCB) (MG-G. 400x)

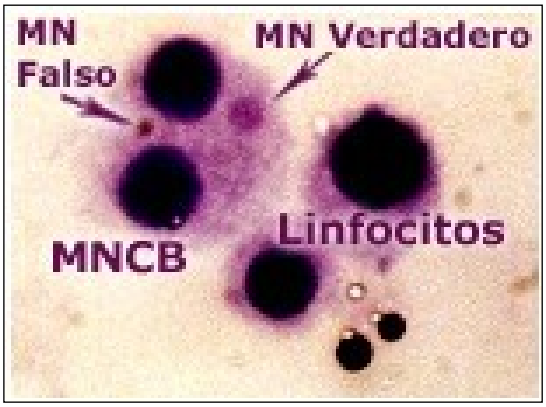


Figure 44. A binucleated lymphocyte cell (BC) with 2 micronuclei (MN) in the cytoplasm: one, of which is a refractive (false MN) micronucleus and the other, a true MN. Note the presence of two lymphocytes that have completed the process of cell division. (400x MG-G)

In vivo genoprotective activity of test substances

Animal study groups

9 to 11 -week-old male Swiss mice weighing approximately 20-25g were used for this assay. The animals were maintained under identical environmental conditions of temperature, light and moisture. They were offered food and water *ad libitum*. All animals were from the Animal unit of the Experimental Sciences Support Service (SACE), University of Murcia (Official License No 30030-2A). The international ethical standards for the use and handling of laboratory animals for biomedical research were strictly adhered to (*Guidelines on the Use of Living Animals in Scientific Investigations.* London, UK).

Whole body irradiation of animals with X-ray:

To induce bone marrow MN formation, mice treated with different test substances namely PASE, ROS, CAN, API, Diosmin, CE, TE, PTU and DMSO were exposed to different radiation doses of (0, 2, 4, 6 8 and 10Gy) (0.8 Gy/min, 200 kV, 4.5 mA) X-ray (total body irradiation), using an Andrex Smart 583 Röntgen source (YXLON International, Copenhagen, Denmark) located in the Radiation Protection and Waste Management Department of S.A.C.E (Service Research Support), University of Murcia (Figure 14).

The experimental animals were divided into three groups of eight as follows:

1. The first group which consisted of 2 mice comprised the control group. Both were not exposed to any of the test substance. Of these, one was exposed to 2Gy X-

rays, while the other did not receive radiation and acted as the non-irradiated control.

2. A second group consisting of 3 mice was exposed to the same dose (0.1 ml of 25µM of PASE. Of these two received 2 Gy of radiation while the other one did not receive any bout of radiation.
3. A third group consisting of 3 mice was exposed to the same dose (0.1 ml of 25µM of PASE). Of these two received 2 Gy of radiation while the other one did not receive any bout of radiation.
4. A fourth group consisting of 3 mice was exposed to the same dose (0.1 ml of 20mg/ml of 5% Zolendronic acid). Of these two received 2 Gy of radiation while the other one did not receive any bout of radiation.
5. The animals that received the test substances but without radiation served as the positive control group for each of the tested substances and served to directly determine the *in vivo* genotoxic effect of the test compound in the test animal in the absence of radiation

The *in vivo* mouse bone marrow micronucleus assay

In vivo experiments performed above were used to evaluate the genotoxic and/mutagenic effects of some of the test substances. This *in vivo* mouse bone marrow test was originally described by Schmid in 1975, and is a widely used technique for determining *in vivo* genotoxic and mutagenic effect of different chemicals and physical agents.

Bone marrow cells were extracted from the two femurs of each experimetal animal using the technique described by von Ledebur and Schmid (1973) and Schmid (1975) which is concisely detailed below:

1. The animals were sacrificed by cervical dislocation and immediately dissected to extract the two femurs, removing all traces of muscles and tendons.
2. The epiphysis of the femurs were cut off and using an insulin syringe (24G) a total of 2 ml foetal bovine serum (Sigma, Madrid) was run through the femoral shaft to flush out the bone marrow which were collected into 15 ml conical tubes.
3. Cells were disaggregated by gently pipetting up and down several times to obtain single cell suspensions.

4. Cells were washed in BSA by repeated centrifugation and re-suspension in BSA for at least three times. Centrifugation was at 1000rpm for 7 min. after the final wash, the BSA was completely aspirated from the cells.
5. Cells were re-suspended n appropriate amount of BSA.
6. The MN slides were prepared by dropping the single cell suspensions on to coded grease free slides and air-dried for at least 24 hours.
7. The slides were stained by first dipping them into pure May-Grünwald stain (Analemma, Vorquimica SL, Vigo) for 3 minutes, followed by 50% of May-Grünwald dye in distilled water for 2 minutes, and finally in Giemsa stain (Probus, Madrid) diluted 1:6 in 0.01M phosphate buffer, pH 6.2 for 2 minutes.

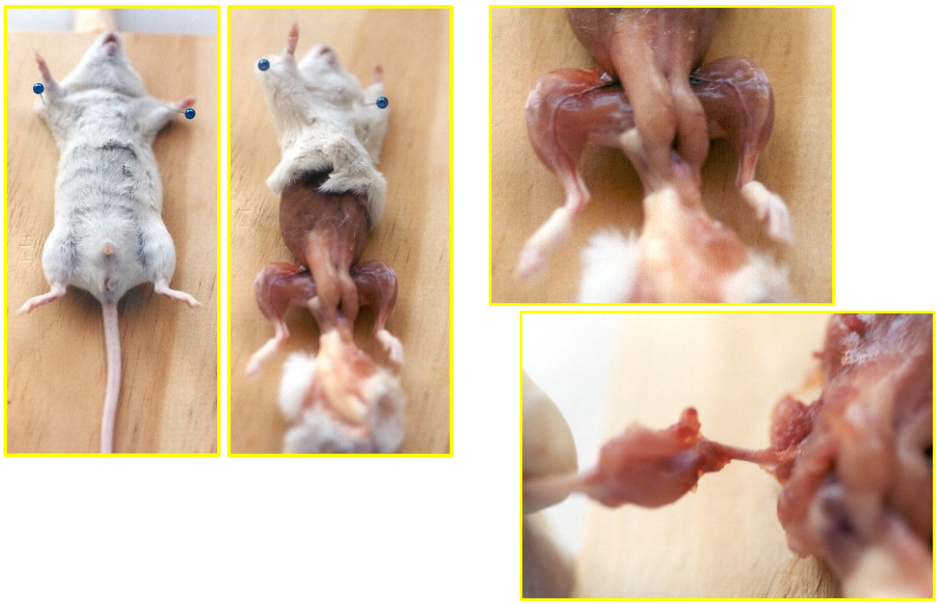


Figure 45. Dissection of mice to extract femurs.

Determination of micronuclei frequency

Bone marrow preparations obtained in the experiments above were analyzed using Laborlux 12 optical microscopes (Leitz, Germany), at the Electron Microscopy Service, Experimental Sciences Support Service (SACE), and a Hund Wetszlar V200 optical microscope located in the Department of Radiology and Physical Medicine, University of Murcia.

A 400X magnification was used to effect counting and analysis of micronuclei (MN). For confirmation of the results especially for ambiguous cases, an oil immersion with 1000X magnification was used

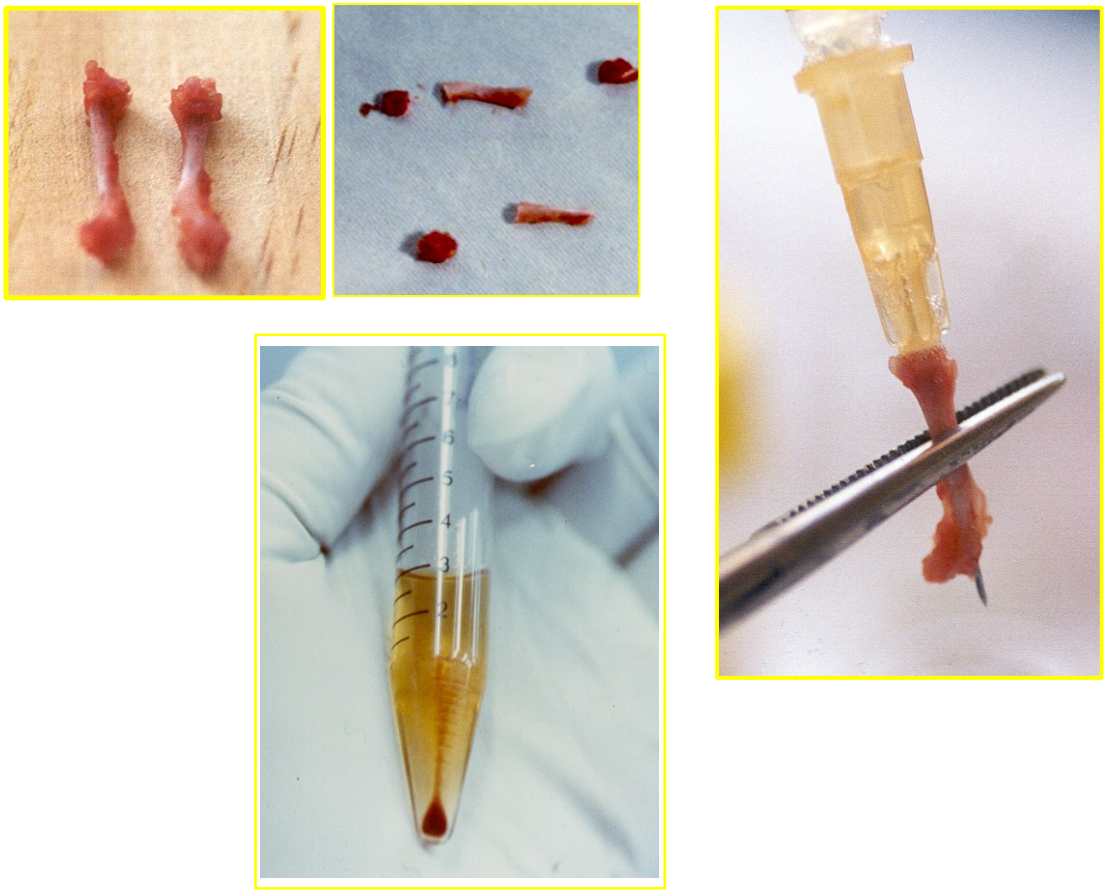


Figure 46. Bone marrow extraction from femurs

Three slide preparations per animal were made and 1000 polychromatophilic erythythrocytes (PCEs) were counted to determine the frequency of micronuclei in each animal. This value was expressed as MN/1000 PCEs for each of the animals. The slides were coded to avoid obsserver bias and three values were obtained for each data point and averaged out to obtain the mean experimetal value.

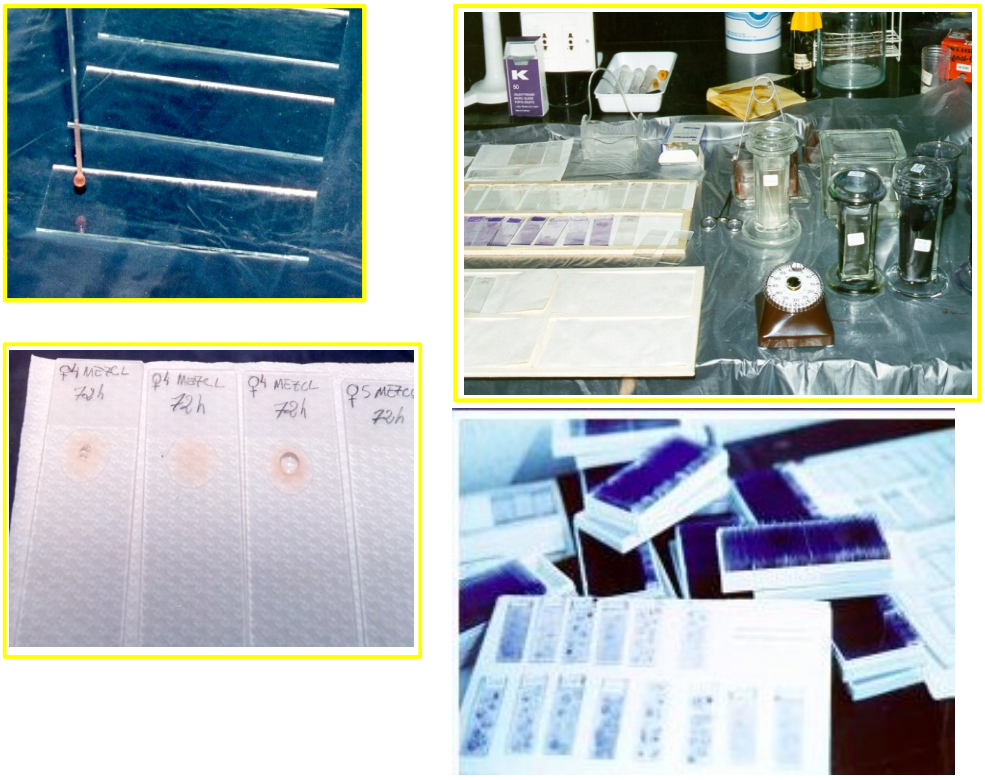


Figure 47.Representative images of polychromatic erythrocytes (PCEs), normochromatic erythrocytes (NCEs) and micronucleated polychromatic erythrocytes (MNPCEs) for in the mouse bone marrow cells analyzed.

Statistical methods

Each point on the graph of cell viability or survival corresponds with a concentration of the evaluated compound and represents the mean value from four to twelve replicate measurements while the bars on the graphs correspond to the standard errors of the means. In the cytotoxicity assay, an ANOVA of repeated means was carried out to compare the percentages of surviving cells in the cultures with different concentrations of the various compounds, complemented by least significant difference (LSD) analyses to contrast pairs and means. The analyses were carried out by logarithmically transforming the data to conform to the assumptions of ANOVA conditions.

In the genotoxicity study, the degree of dependence and correlation between variables were assessed using analysis of variance complemented by a contrast of means ($p<0.05$). Quantitative means were compared by regression and linear correlation analysis. In addition, the formula used to evaluate the protection factor

described by Sarma and Kesavan 1993, and which has on several occasions been used to evaluate the sensitization factor (FS) was adapted. For the MN analysis:

$$Fs = \frac{Ft - Fc}{Fc} \times 100$$

where Ft is the frequency of micronuclei in the irradiated samples and treated with test substance and FC is the frequency of micronuclei in the irradiated control samples. Findings were considered to be statistically significant when p values of less than 0.05 ($p < 0.05$) were obtained.

The LD_{50} values were extrapolated from the adjusted curves with regards to the averages of the absorbance quotients of the control experiments. Statistical analysis was performed in collaboration with the Department of Biostatistics, University of Murcia, using the BMDP Statistical Software package, Inc. (Version 1988, IBM PC / DOS).

Protection Factor (PF)

In addition, the Protection Factors of each test compound that registered radioprotective activity was evaluated. The formula described by Sarma and Kesavan, 1993 (see equation below1993) was adopted to evaluate the Protection Factor (PF) for each of the tested substances.

$$\mathbf{FP\ (\%) = (F_{control} - F_{treated} / F_{control}) \times 100}$$

Where: FP is the magnitude of protection expressed in %. $F_{control}$ is the frequency of micronuclei in irradiated control animals. $F_{treated}$ is the frequency of micronuclei in animals treated and irradiated.

Dose reduction factors (DRF)

The effectiveness of radioprotectors is expressed by a dose reduction factor. DRF is defined as the ratio between the dose of radiation needed to produce a given effect in the presence of a radioprotective compound and the dose of radiation needed to produce the same effect in the absence of the said compound (i.e. the ratio of radiation doses producing identical effects in the presence or absence of radioprotectors) and is expressed by the formula:

$$DRF = \frac{\text{Dose with RP to obtain X MN}}{\text{Dose without RP to obtain X MN}}$$

Where: DRF is the Dose reduction Factor; RP is the substance used, and X MN is the number of micronuclei produced (TRAVIS, 1979).

V. Results

IV.1. RESULTS OF EXTRACTION ASSAY

IV.1. RESULTS OF EXTRACTION ASSAY

Results of extraction studies.

The extraction process performed on seeds of *P. angolensis*, in yielded the product displayed in figure CH1. It shows the physical appearance and some characteristics features of the product:

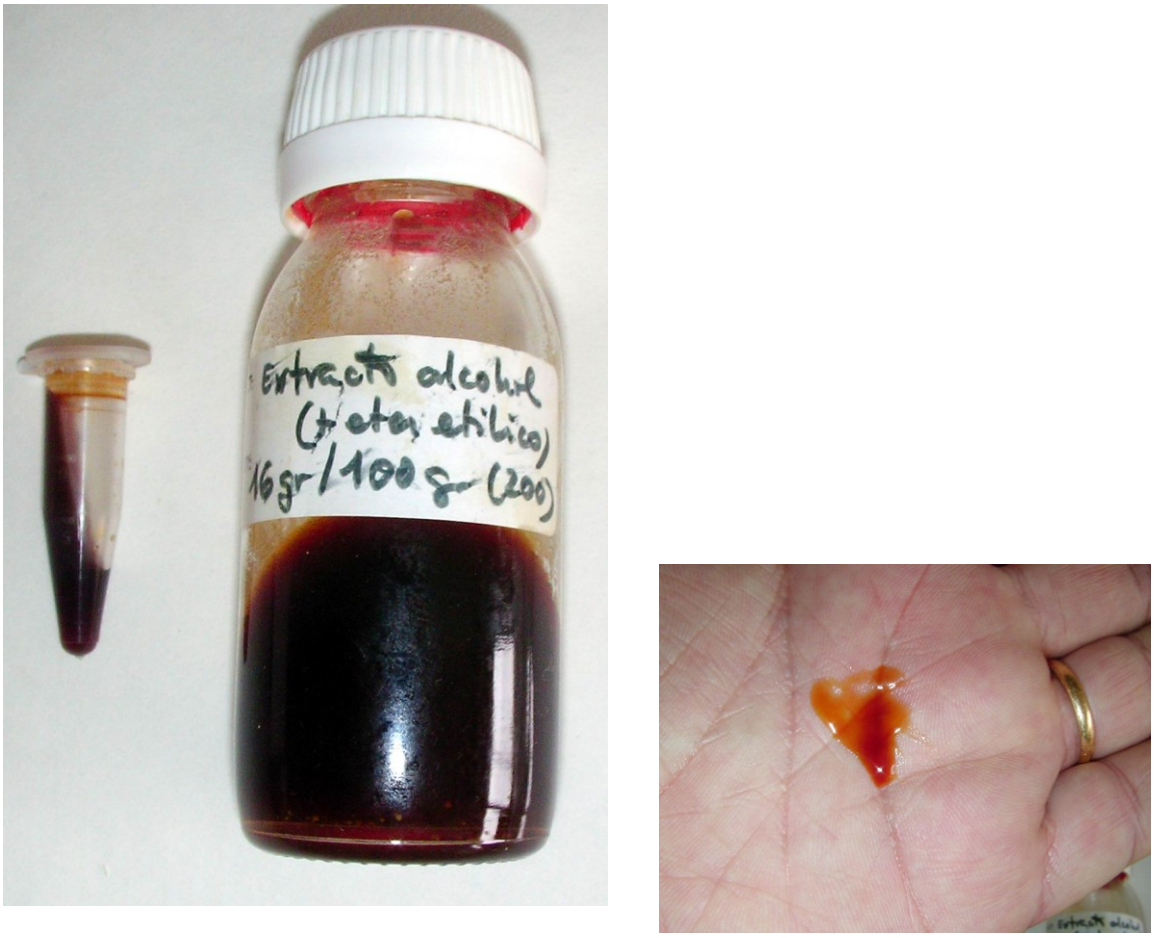


Figure CH1. ***Pycnanthus angolensis*** **seed extract (PASE) obtained after the extraction processing**

Figure CH2 shows the flow diagram which summarizes the process for extraction performed. After various tests on the leaves, bark (stem and root) and seeds, the latter was selected as the ideal material from which to obtain optimal quantities of the extract studied.

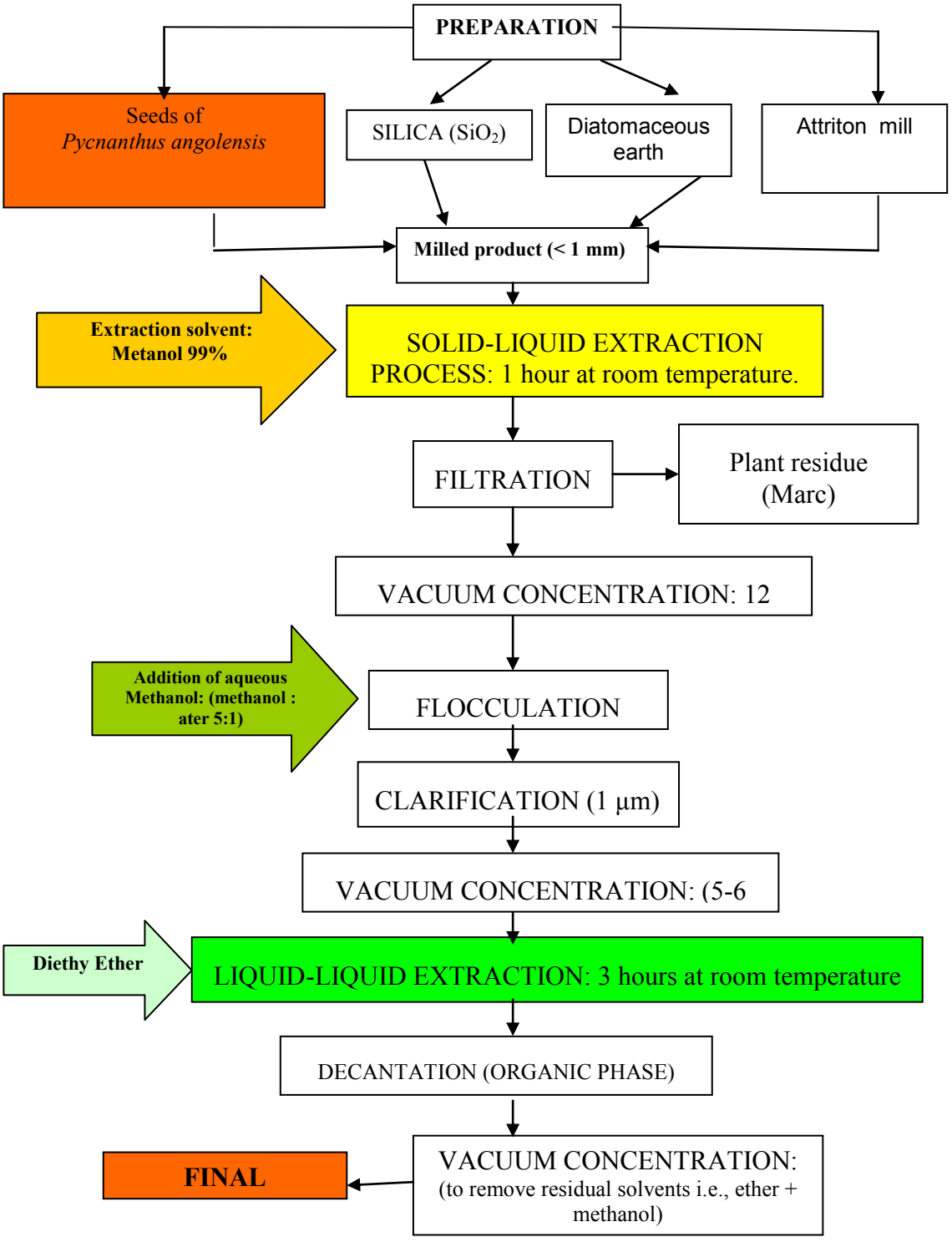


Figure CH2. Flow diagram of the extraction process.

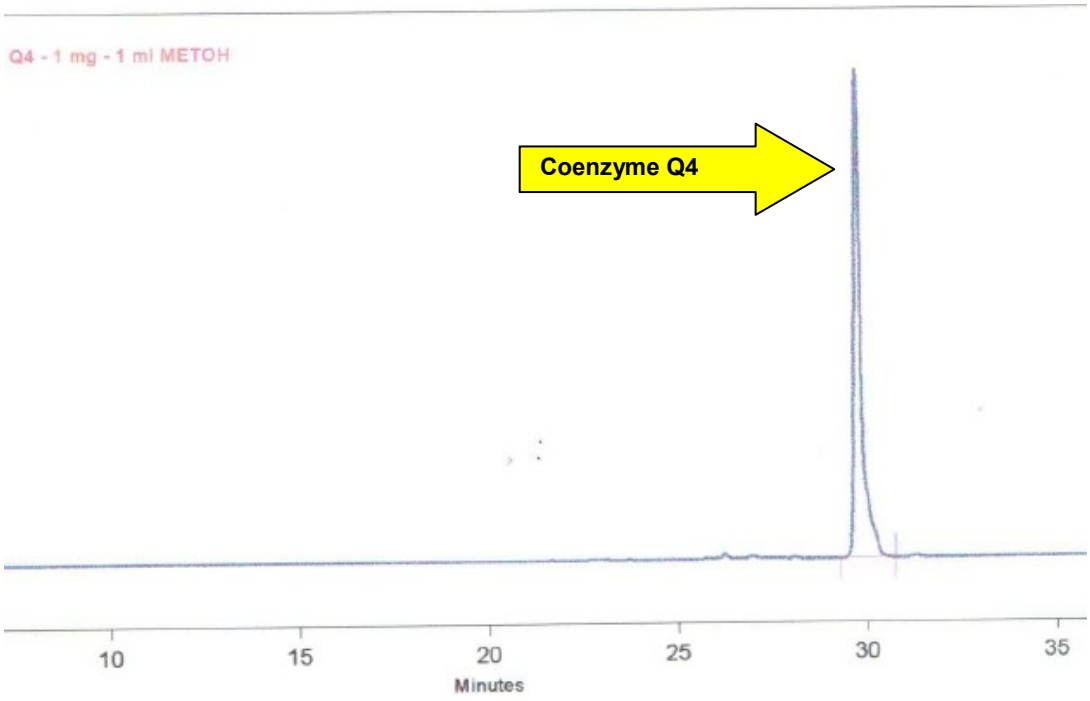


Figure CH3. HPLC chromatogram of co-enzyme Q4 reference standard

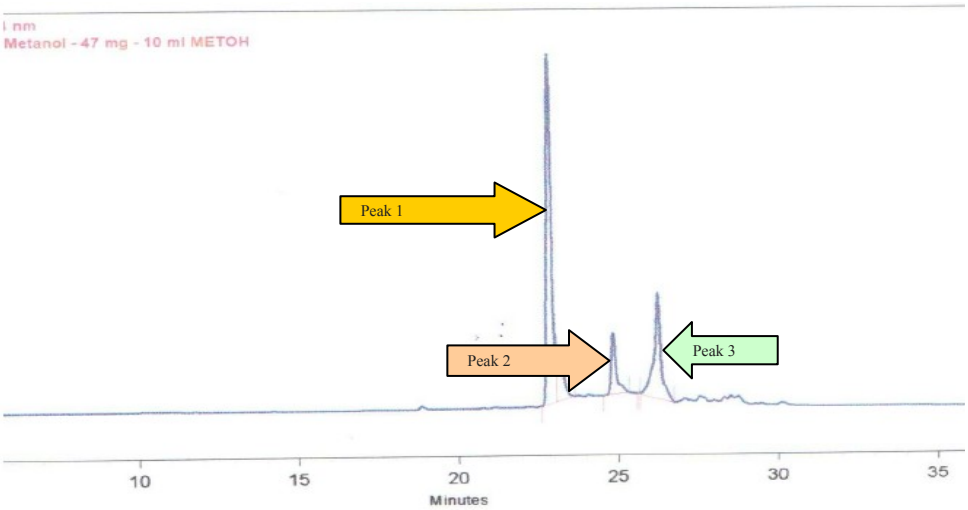


Figure CH4. High performance liquid chromatography quantitative analysis of the three active principles of interest present in the final extracts of the various parts of *P. angolensis*.Warb (*P. kombo*); seeds, root-bark, stem-bark and leaves: Sargahydroquinoic acid (peak 1), sargaquinoic acid (peak 2) and sargachromenol (peak 3).

Table CH1. Content of active principles found in the different parts of *P. angolensis* analyzed after similar extraction conditions: For extraction procedure see experimental.

Part of plant	% by weight of Compound A (Rt 22-23 min)	% by weight Compound (Rt 24-25 min)	% by weight of Compound (Rt 26-27 min)
Leaves	0.08%	0.24%	0.16%
Stems	0.11%	0.04%	0.04%
Stem bark	1.61%	0.14%	0.16%
Seeds	**7.48%**	**0.41%**	**1.42%**

Based on these results, it was decided that only the seeds of *Pycnanthus angolensis* Warb which contained the highest amount of the active principles be used for the extraction of active ingredients of the plant for this study. The figure below is an HPLC elution profile of the seed extract (PASE). It shows the relative distribution of active principles of interest in the seed extract. Clearly there seem to be a high specific extraction of compound A which was initially shown to be more abundant in all the different parts of the plant surveyed in the exploratory stages of the experiment. The other components are present, appearing in relatively much lower proportions (see chromatogram of the raw material used).

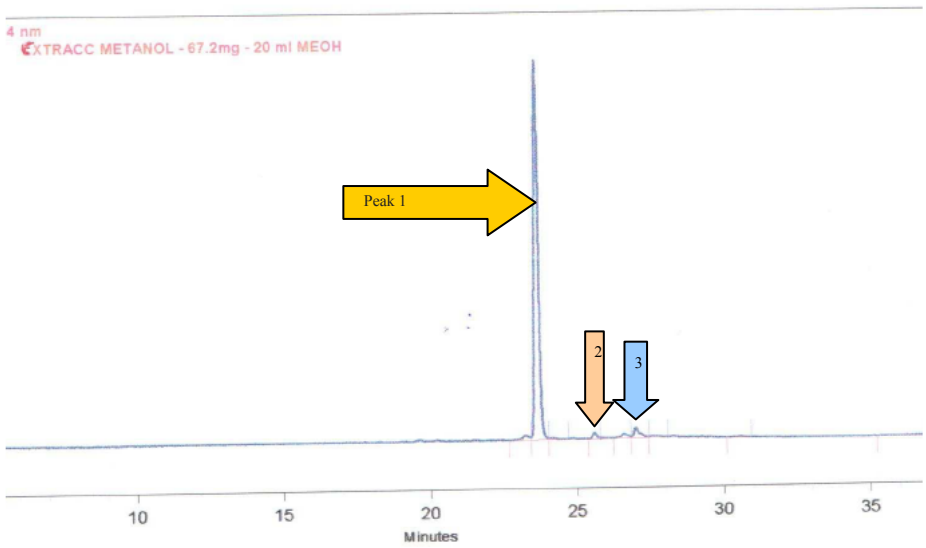


Figure CH5. HPLC elution profile of Pycnanthus angolensis Seed Extract (PASE) used for assays.

Table CH2. The following table shows the results obtained in the quantization characteristic of the 3 active principles present in the extract of seeds obtained.

Simple anlaysed	% by weight of compound A (Rt 22-23 min)	% by weight of compound B (Rt 24-25 min)	% by weight of compound C (Rt 26-27 min)
Seed extract	**40.14%**	0.35%	0.56%

The delay in the retention time of the reference substance, Co-enzyme Q4 could be attributed to two elements of it its molecular structure which both influence an overall decrease in polarity of the compound, directly affecting its interaction with the chromatographic column (i.e. the Silica-C_{18} stationary phase). Firstly, the absence of carboxyl group and secondly, the presence of two methoxy- groups (-OCH3) on the aromatic ring (i.e. the compounds on interest are more polar than the reference standard).

Calculating extraction yield

The gravimetric extraction yield was evaluated from the results obtained in order to establish the effectiveness of the extraction method used as follows:

100 g of seeds x 0.0748 (7.48%) = 7.48 g of Compound A
16.34 g of extract obtained x 0.4014 (40.14%) = 6.56 g of Compound A

Gravimetric yield of the major theoretical active principle, Compound A (sargahydroquinoic acid) is given by:
6.56 g / 7.48 g = 0.877; i.e., gravimetric yield obtained = **87.70 %**

From the viewpoint of the efficiency of an extraction process, these results may be regarded as encouraging even from an industrial viewpoint, where obtaining extraction yields of active ingredient higher than 80% is considered satisfactory.

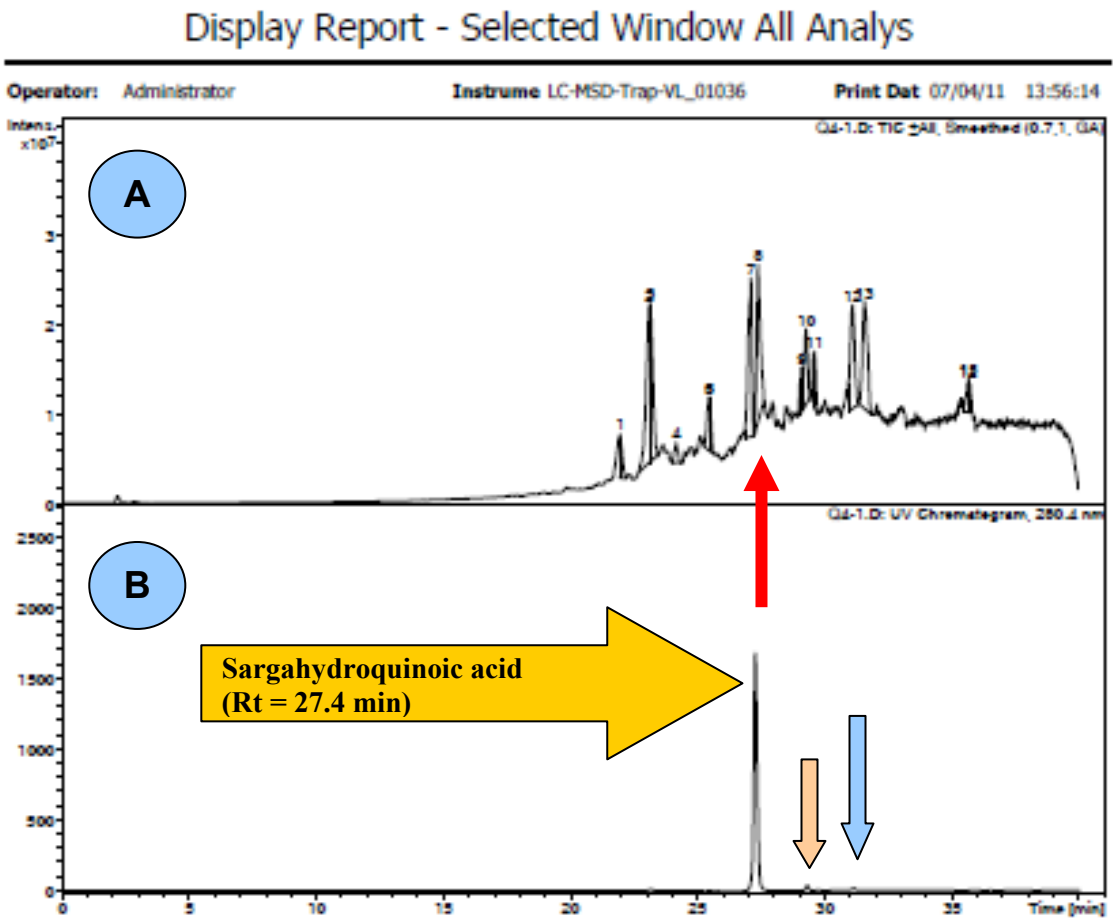


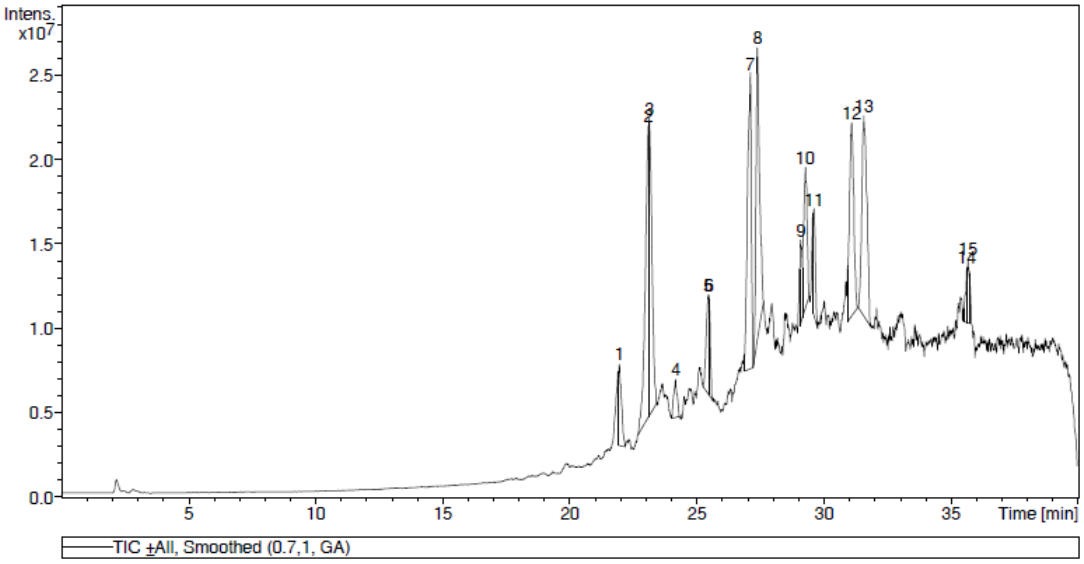
Figure CH6. Chromatographic separation of the components of PASE using LC-ESI-MS; (a) Total ion chromatogram b) extracted ion chromatogram

Compound Mass Spectrum List Report - MS

Analysis Name: Q4-1.D **Instrument:** LC-MSD-Trap-VL_01036 **Print Date:** 07/04/11 13:55:05
Method: Copy of TOCOFERO.M **Operator:** Administrator **Acq. Date:** 07/04/11 13:09:13
Sample Name: Q-4
Analysis Info:

Acquisition Parameter:

Mass Range Mode	Std/Normal	Trap Drive	33.8	Scan Begin	100 m/z
Ion Polarity	Positive	Skim 1	38.3 Volt	Scan End	800 m/z
Ion Source Type	ESI	Skim 2	6.0 Volt	Averages	5 Spectra
Dry Temp (Set)	350 °C	Octopole RF Amplitude	150.0 Vpp	Max. Accu Time	300000 µs
Nebulizer (Set)	60.00 psi	Capillary Exit	113.0 Volt	ICC Target	30000
Dry Gas (Set)	9.00 l/min	Cap Exit Offset	74.7 Volt	Charge Control	on



Compound List:

#	RT [min]	Range [min]	Height	Area	Area %	S/N
1	21.9	21.9 - 22.2	4794367	34991500	2.8	22.9
2	23.1	22.7 - 23.1	17269529	154801898	12.6	82.7
3	23.1	23.1 - 23.4	17582140	147800604	12.0	84.2
4	24.1	24.0 - 24.3	2237993	20637278	1.7	10.7
5	25.4	25.2 - 25.4	5835764	35292115	2.9	27.9
6	25.5	25.4 - 25.6	5916724	27305049	2.2	28.3
7	27.1	26.9 - 27.2	17411245	175867553	14.3	83.3
8	27.4	27.2 - 27.6	17275245	171372722	13.9	82.7
9	29.1	29.0 - 29.1	4911106	25827701	2.1	23.5
10	29.3	29.1 - 29.4	8270634	77663311	6.3	39.6
11	29.6	29.6 - 29.7	6292685	31589794	2.6	30.1
12	31.1	30.9 - 31.3	11421193	138428942	11.2	54.7
13	31.6	31.3 - 31.8	11813080	160781739	13.0	56.5
#	RT [min]	Range [min]	Height	Area	Area %	S/N
14	35.6	35.5 - 35.6	3255413	16708146	1.4	15.6
15	35.6	35.6 - 35.7	3768803	13885152	1.1	18.0

Figure CH7. High Performance liquid chromatography mass spectrometry (HPLC/MS) analysis of PASE

Table CH4. Experimental results versus theoretical structural data of proposed compounds present in PASE.

Parameter	Compound A	Compound B	Compound C
Proposed structure	Sargahydroquinoic acid	Sargaquinoic acid	Sargachromenol
Molecular weight of proposed compound	426	424	424
Molecular formula of proposed compound	$C_{27}H_{38}O_4$	$C_{27}H_{36}O_4$	$C_{27}H_{36}O_4$
Retention time in HPLC-MS analysis (R_t HPLC-MS)	27.4 min	29.3 min	31.6 min
$M + H^+$	449	447	447
M + Na	471	469	469
Molecular weight obtained from HPLC-MS	448	446	446
Theoretical and experimental mass difference	+ 22	+ 22	+ 22

Given the constant difference of 22 amu between the molecular weight of the proposed compounds and the experimental values obtained using the HPLC-MS analysis (i.e., the three most significant compounds mentioned throughout the extraction process and obtained from the material under study), under the limited isolation and identification work conducted on this plant material, it is postulated that the main principles of the studied extract would be an undefined derivative of sargahydroquinoic acid.

In an attempt to identify the possible molecular structure of the proposed derivative compounds with a high degree of precision, an exhaustive literature review of all previously isolated compounds with identified molecular structures and weights in extracts obtained from this plant was conducted (BARCLAY *et al*., 2010; FORT *et al*., 2000: LEONARD, and SIMONTON 2010: LEONARD, 2004a and 2004b; LEONARD, 2002; LOK *et al.*, 1983; LUO *et al*., 2011; MANSOOR *et al*., 2011; , NJOKU *et al*., 1997; NONO *et al*., 2010; SIMIC *et al*., 2006; SIMON *et al*., 2010; TCHINDA *et al.,* 2008; UBILLAS *et al*., 1997; WABO *et al.,* 2007).

The following figure shows the main compounds and their chemical structures previously isolated and identified from the plant under study.

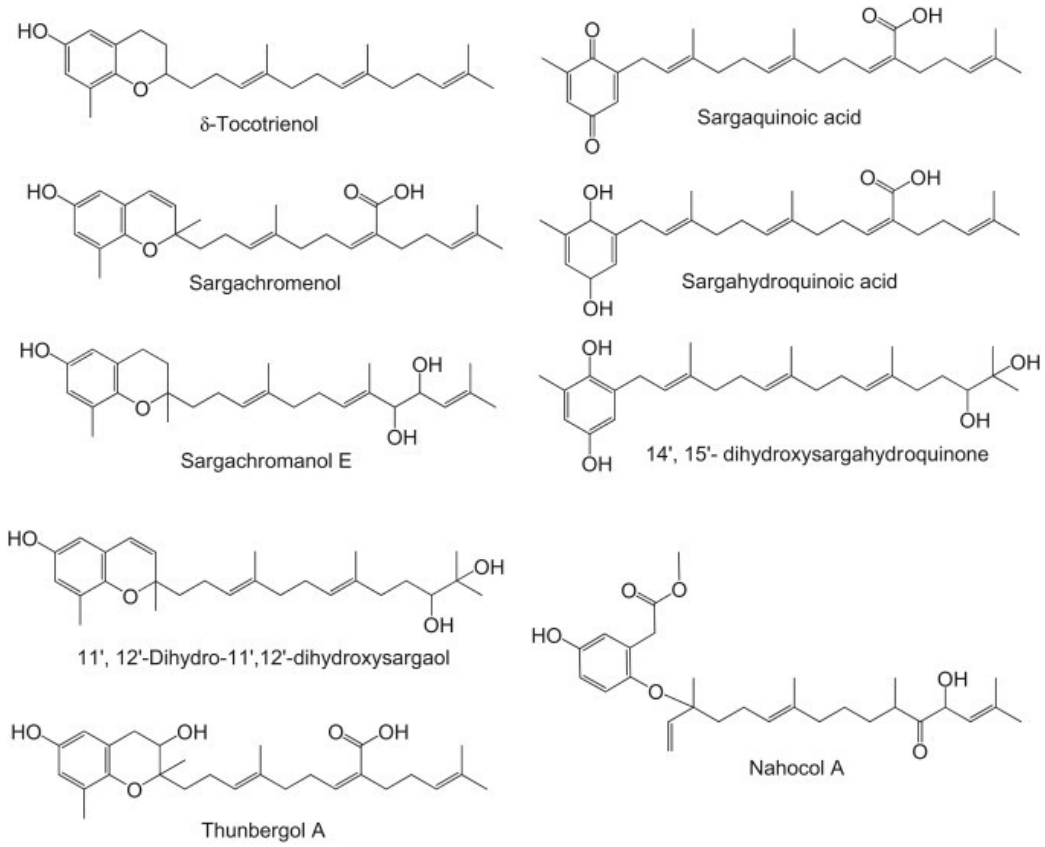


Figure CH 8. Structures of compounds previously isolated from P. angolensis

Table CH5: Molecular formulae and molar masses of compounds previously extracted from *P. angolensis.*

Compound	Molecular formula	Molecular mass
δ-Tocotrienol	$C_{26}H_{38}O_2$	382
Sargaquinoic acid	$C_{27}H_{36}O_4$	424
Sargachromenol	$C_{27}H_{36}O_4$	424
Sargahydroquinoic acid	$C_{27}H_{38}O_4$	426
Kombic acid	$C_{27}H_{38}O_4$	426
11',12'-Dihydro-11',12'-dihydroxysargaol	$C_{27}H_{38}O_4$	426
Sargachromanol E	$C_{27}H_{40}O_4$	428
14',15'-Dihydroxysargahydroquinone	$C_{27}H_{43}O_4$	431
Thumbergol A	$C_{27}H_{38}O_5$	442
Nahocol A	$C_{29}H_{40}O_6$	484

Different permutations were made starting from the parent compound to establish the possible true identity of the compound by combining it with different functional

groups and mechanistically reasonable (permissible) common neutral fragments. In doing this, assumption of fragment substitutions and additions made were consistent with normal metabolic processes occurring in eukaryotic plant cells e.g., hydroxylation processes (phenoloxidases), methylation (methyltransferases), decarboxylation (decarboxylases), acetylation (acetyltransferases) to enable us arrive at a plausible compound. None of these approaches either alone or in combination with each other yielded a candidate compound with known structure or desired mass of 448 mass units as the possible main active ingredient in the extract.

As a result of these encumbrances the molecular mass of the compounds studied was reassessed using the electrospray technique. (General Principles of electrospray mass spectrometry are provided in the figure below).

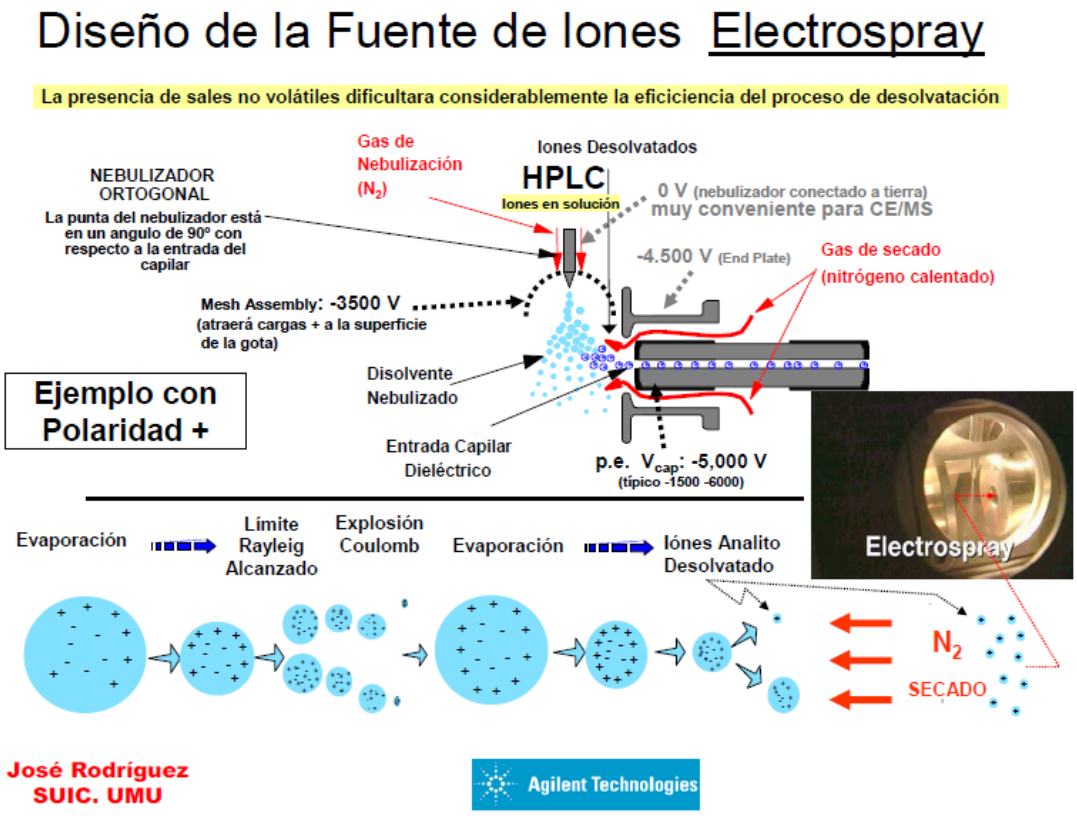


Figure CH9: A detailed schematic of the components and mechanism of an Electrospray Mass Spectrometry (UMU, 2013)

The mineral content of different plant extraction are generally known to be high especially aqueous extractions. It seems logical then to imagine the presence of significant amounts of monovalent ions, especially Na^+. It is well known in the in the art of electrospray technology that very frequently Na adducts are formed and most authors believe that it is derived from hygroscopic Na existing in water vapor in the atmosphere**.** In chemistry, an adduct AB may be formed by direct binding of two molecules, A and B without any structural changes arising in the topology of the A and B. It is also possible for other stoichiometries other than 1:1, for example 2:1 (A2B). Thus it is very likely that other different but common forms of Na + adducts could be generated in this analysis. These Na adducts would be related to the presence and number of sodium "binding" sites in the molecular structure of the test compound. In various analyses of plant materials, adducts such as $[M + Na]^+$, $[M +2 Na-H]^+$ and even $[M +3 Na-2H]^+$ were detected depending on the different locations and potential docking or binding site available for sodium in the compound to be analyzed.

Based on these results, we propose that the peaks obtained in HPLC-MS correspond to the potential generation of two adducts, one of which is probably due o Na binding $[M + Na]^+$ to the primary, the carboxyl group in the sargahydroquinoic acid molecule, and the other on the sterically less hindered hydroxyl group on the phenol ring of the type [M +2 Na-H] +, which have:

- 426 (theoretical molecular weight) + (1 x 23) + = 449 (signal shown).
- 426 (theoretical molecular weight) + (2 × 23) -1 H] + = 471 (signal appearing)

Based on the deductions made from these results supported by the literature search conducted, it may be reasonable and consistent to imagine that the molecular structure of the main active principle (Peak) present in the extract of study is compatible with sargahydroquinoic acid. The aim of the current research work is to evaluate the radioprotective and antimutagenic activity of the plant extract which is in concordance with the results obtained.

It is recommended that future work be done focusing more extensively on confirming the actual structure of the main active principle of this extract, by indulging in further purification and using more complex MS techniques including fragmentation studies.

IV.2. MORPHOLOGICAL RESULTS.

IV.2. MORPHOLOGICAL RESULTS

CYTOKINESIS-BLOCKED MICRONUCLEUS (CBMN) ASSAY/CBMN CYTOME ASSAY IN HUMAN LYMPHOCYTES AFTER *IN VITRO* IRRADIATION.

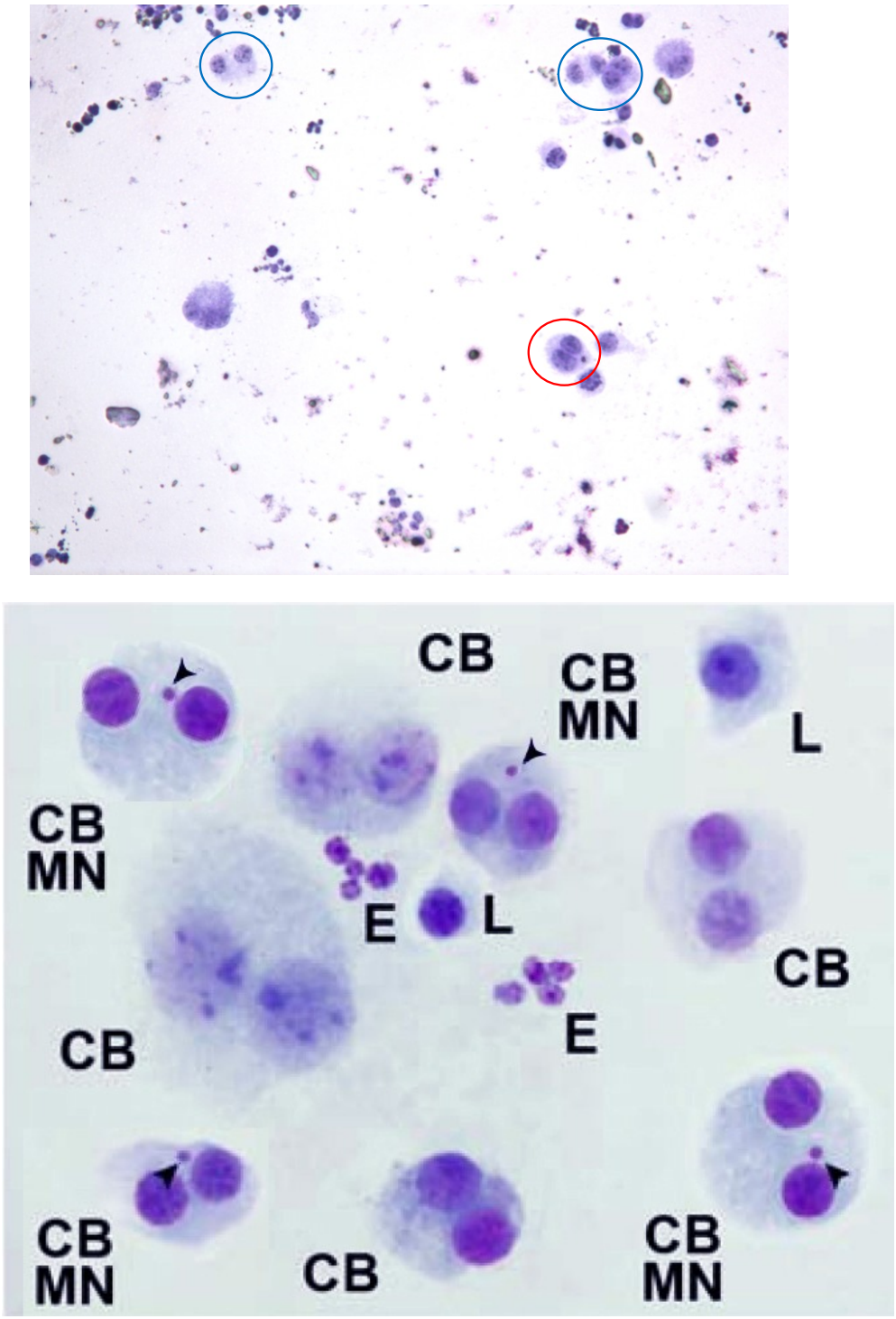


Figure MOR1: Photomicrograph of a human lymphocyte culture using the cytokinesis-block technique and stained with May-Grünwald-Giemsa (400X) (L: lymphocyte, CB: cytokinesis-blocked binucleated cells; CBMN: cytokinesis-blocked binucleated cells with micronucleus in cytoplasm, E: remains of hemolyzed erythrocytes.

MOUSE BONE MARROW MICRONUCLEUS ASSAY

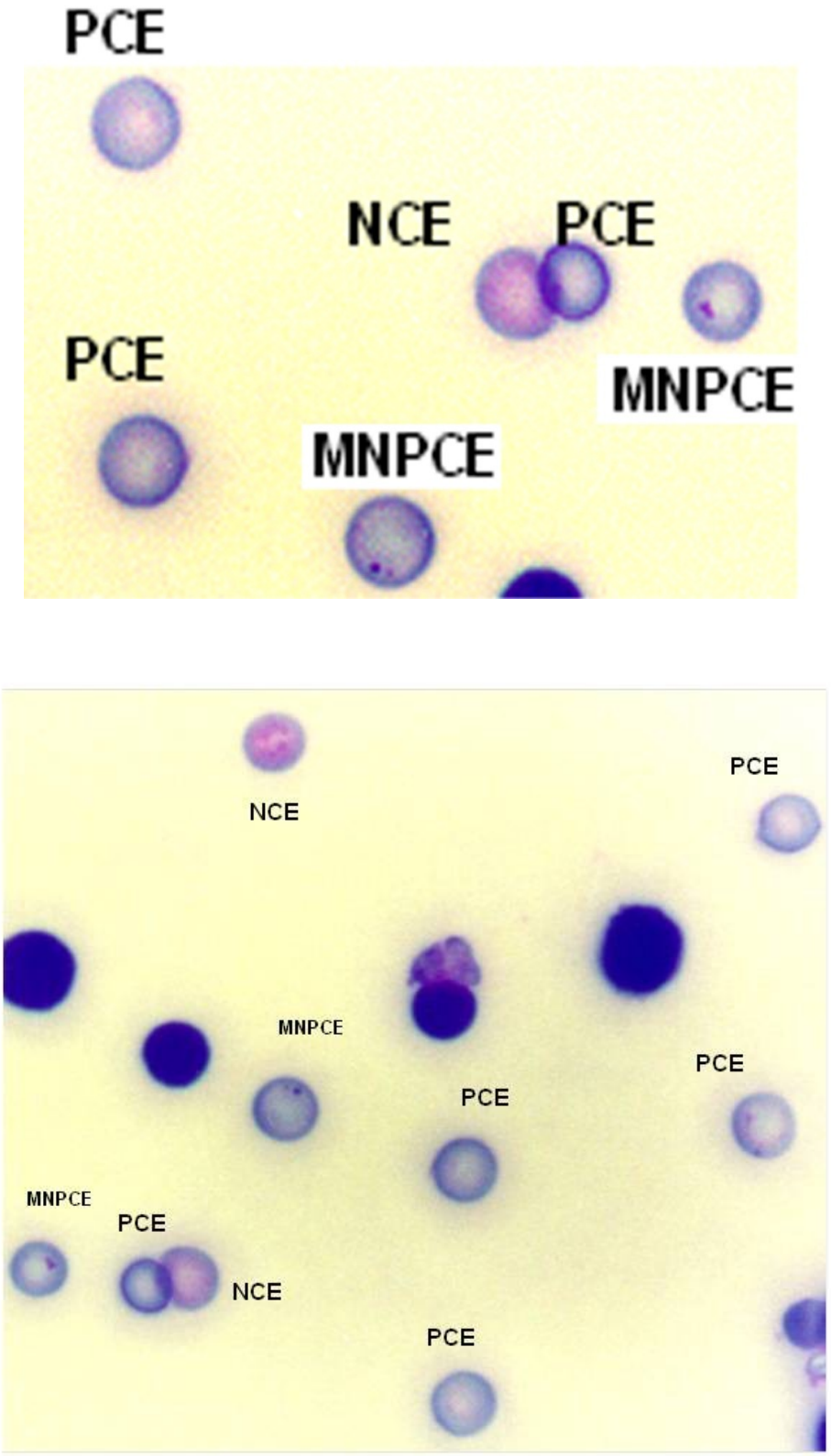


Figure MOR2: Photomicrograph of May-Grünwald-Giemsa stained mouse bone marrow cells (1000X) (PCE: polychromatic erythrocytes; MNPCE: polychromatic erythrocytes with micronucleus; NCE: normochromatic erythrocytes).

RESULTS OF CYTOPROTECTIVE ASSAYS (MTT ASSAY)

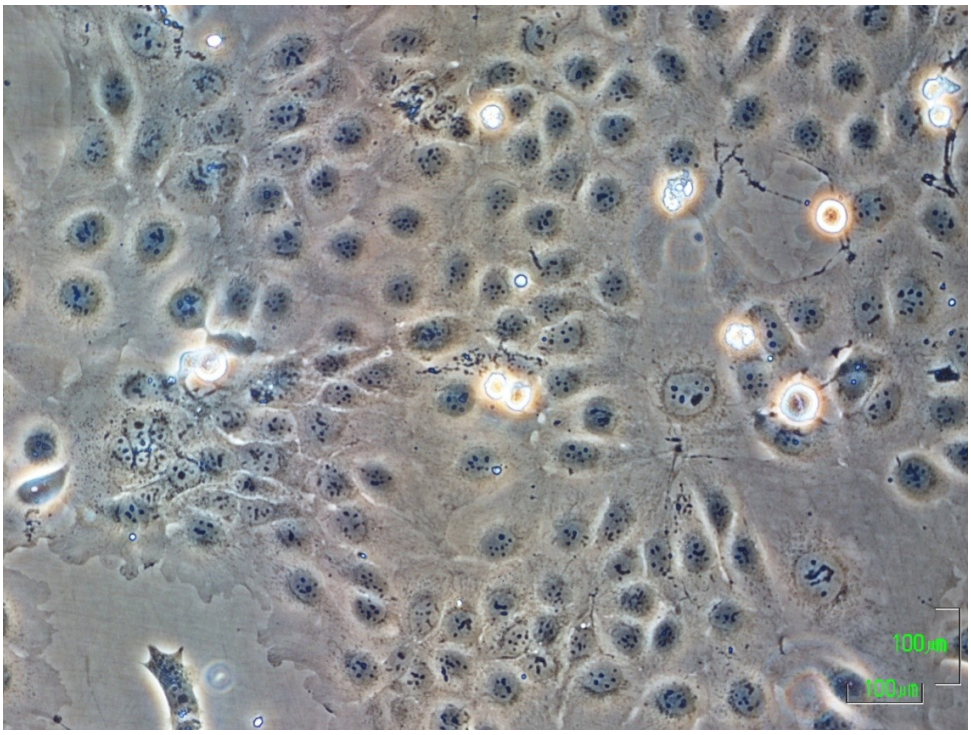


Figure MOR3. Morphological appearance of prostate epithelial PNT2 cells observed using a phase contrast microscope (control cell cultures: after 24 hours of cultivation 200X).

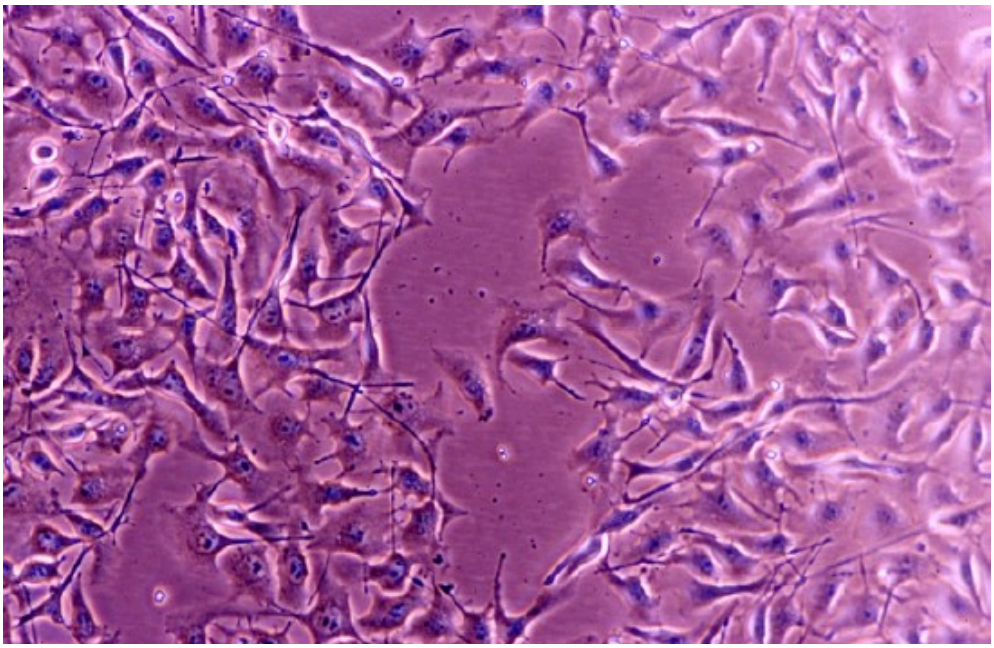


Figure MOR4. Morphological appearance of B16F10 melanoma cells observed using a phase contrast microscope (control cell cultures: after 48 hours of cultivation 200X).

V. 3. Results.

CYTOKINESIS-BLOCKED MICRONUCLEUS ASSAY (CBMN) IN IRRADIATED HUMAN LYMPHOCYTES

V. 3. Results

CYTOKINESIS-BLOCKED MICRONUCLEUS ASSAY (CBMN) IN IRRADIATED HUMAN LYMPHOCYTES

CONTROL SAMPLES

Micronucleus frequency was between 5 and 15 MN/500 BC with an average of 10 MN/500 BC. This value corresponded to the baseline or spontaneous micronuclei frequency in the samples (Table CB1; figure CB1).

An increased number of binucleated cells were observed to express micronuclei in their cytoplasm when the samples were exposed to 2 Gy of radiation (irradiated control CI, samples) when referenced with the frequency of MN in non-irradiated controls. Two hundred and fifty (250) binucleated cellswere observed to be positive for the presence of micronuclei out of 5000 binucleated cells (i.e., 250 MN/5000 BCs), of which 242 expressed single micronuclei, 6 expressed two micronuclei and 2 expressed three micronuclei. The frequencies of occurrence of MN were also observed to increase, and ranged from 11 to 32 MN/500 BC with an average of 26 MN/500 BC (**$p < 0.001$**) (Table CB1; figure CB1).

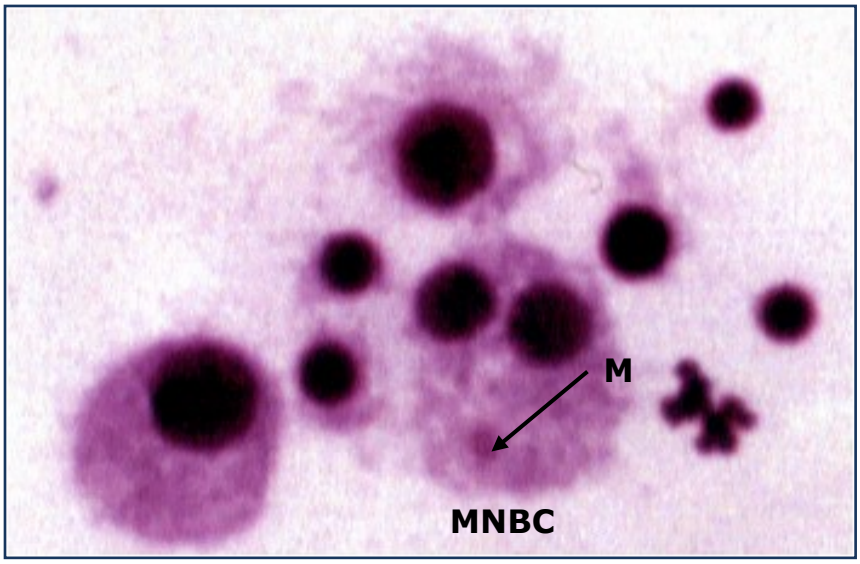


Figure CB1. An unblocked mononucleated lymphocyte cell and a cytokinesis-blocked lymphocyte with a micronucleus in its cytoplasm (MNBC) (400x)

Table CB1. Micronuclei distribution in non-irradiated control (C) and irradiated control (CI) samples (CI >C p> 0.001)

SAMPLE	POSITIVE CELLS	MNCB 1 MN	2 MN	3 MN	TOTAL MN	MEAN MN
C 1	11	11			11	10
C2	7	6	1		8	
C3	13	13			13	
C4	5	5			5	
C 5	9	9			9	
C6	15	15			15	
C7	12	12			12	
C8	10	10			10	
C9	11	11			11	
C 10	6	6			6	
CI 1	29	29			29	26(*)
CI 2	25	22	2	1	29	
CI 3	31	31			31	
CI 4	23	21	1	1	26	
CI 5	30	29	1		31	
CI 6	29	29			29	
CI 7	32	32			32	
CI 8	27	27			27	
CI 9	13	11	2		15	
CI 10	11	11			11	

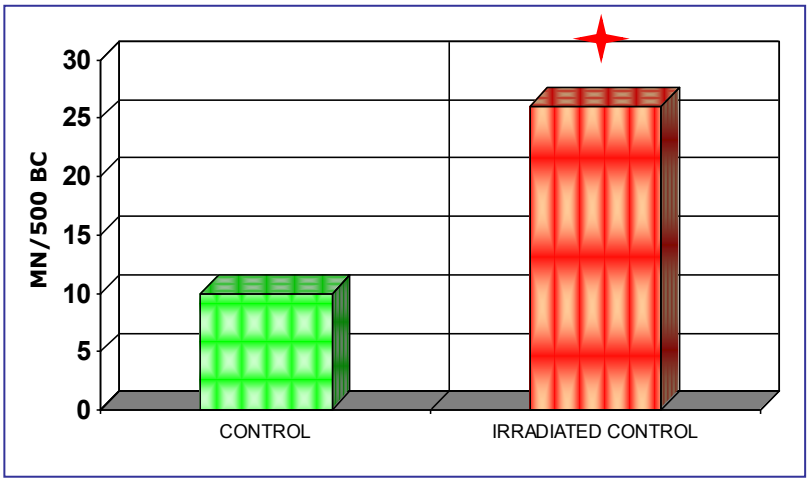


Figure CB2. Micronuclei frequency in non-irradiated (C) and irradiated control (CI) samples ((+): (p<0.001) versus irradiated contol cells)

A number of different substances were examined for their ability to protect against DNA damage caused by ionizing radiation in human peripheral blood lymphocytes when administered before and after exposure to radiation (pre-irradiation treatment and psot-irradiation treatment). The results of these assays are presented as follows:

SAMPLES TREATED WITH *P. ANGOLENSIS* SEED EXTRACTS (PASE)

1. Samples treated with **PASE** expressed a MN frequency that ranged from 8 to 10 MN/500 BC with an average of 9 MN/500 BC. Thus this can be said to show no significant difference with the non-irradiated control samples whose average frequency of occurrence of MN was 10 MN/500 BC.

2. Samples treated **PASE** before radiation exposure expressed an average MN frequency occurrence of 19 MN /500 BC. This suggestive of a significant increase (**$p<0.01$**) in the frequency of appearance of MN over the non-irradiated control samples treated with the same substance and whose average frequency of occurrence of MN was 9 MN/500 BC.

3. Samples treated with **PASE** after exposure to radiation expressed an average frequency of 17 MN /500 BC. This represents a significant increase (**$p <0.05$**) in the frequency of appearance of MN over the control non-irradiated samples treated with the same substance and whose average frequency of occurrence of MN was 9 MN/500 BC.

4. Samples treated with PASE before exposure to radiation expressed an average frequency occurrence of 19 MN micronuclei per 500 BC. Statistical analysis of the frequency of MN in irradiated samples pretreated with PASE shows a significant decrease (**$p < 0.05$**) in MN frequency in comparison with the irradiated control samples (26 MN/500 BC). This means that treatment with PASE before exposure to radiation decreases the expected frequency MN and therefore offer some measure of protection against radiation induced chromosome damage.

5. The samples treated with **PASE** after exposure to radiation produced an average frequency of occurrence of 17 MN /500 BC. Analysis conducted on the frequency of MN occurrence in samples that were irradiated before subsequent treatment with **PASE** showed a significant decrease (**$p < 0.001$**) in MN frequency in comparison with irradiated control samples (26 MN/500 BC). This observation implies that post-irradiation treatment with PASE decreases the expected frequency of MN and therefore offer protection against radiation induced DNA damage.

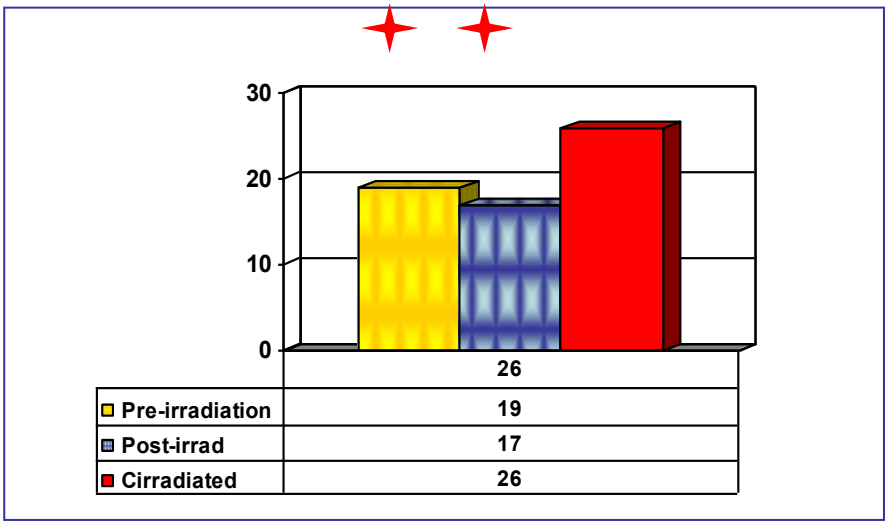


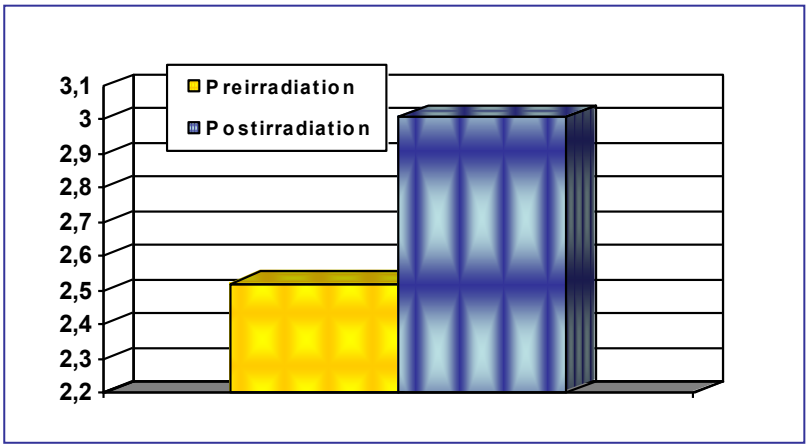
Figure CB3. Frequency of Micronuclei (CBMN) in human peripheral blood samples treated with PASE ((+): ($p<0.01$) versus irradiated contol cells)

6. The **Protection Factor (PF)** of PASE treated samples before irradiation was 26.92%, but samples treated with PASE after exposure to radiation showed a PF of 34.61%.

PF (%)	
Pre-irradiation	**Post-irradiation**
26.92	34.61

Table CB2. Protection Factor obtained fwhen human peripheral blood samples were treated with PASE

7. The pre-irradiation PASE treated samples expressed a **Dose Reduction Factor** (DRF) of 2.52 while a DRF of 3.009 was obtained with samples treated with PASE after exposure to irradiation.



DRF	
Pre-irradiation	Post-irradiation
2.52	3.009

Figure CB4; Table CB3. Dose Reduction Factors obtained obtainedwhen human peripheral blood samples were treated with PASE

- **ROSMARINIC ACID TREATMENT (ROS)**

1. In the samples treated with **Rosmarinic acid** a MN frequency ranging from 9 to 15 MN/500 BC with an average of 12 MN/500 BC was established. There was no significant difference between these results when compared with those obtained for the non-irradiated control samples whose average MN frequency of occurrence was 10 MN/500 BC.

2. In samples treated with **Rosmarinic acid** before exposure to radiation a MN frequency that ranged between 10 and 14 MN/500 BC with an average of 11 MN/500 BC cells was determined. This Shows a significant difference with respect to the irradiated control samples treated with the same substance whose average MN frequency of occurrence was 26 MN/500 BC. There was also difference in the pre-

irradiation frequency of MN occurrence when the volume of administered substance was doubled (i.e., from 12.5 to 25 µl).

3. In samples treated with **Rosmarinic acid** after exposure to radiation a MN frequency ranging between 20 and 29 MN/500 BC with an average frequency of appearance of 24 MN/500 BC was determined, and this represents a significant increase (**$p < 0.001$**) in the average frequency of MN occurrence with respect to the irradiated control samples treated with the same substance which had an average frequency of MN occurrence of 12 MN/500 BC. The administration of two different volumes of the substance gave a MN frequency of 25.5 MN/500 BC when a volume of 12.5 µl was added but this frequency decreased to 20 MN/500 BC when the volume was increased to 25 µl.

4. In samples treated with **Rosmarinic acid** before exposure to radiation, an average frequency of occurrence of micronuclei of 11 MN/500 BC was determined. Statistical analysis of the frequency of MN in samples pretreated with Rosmarinic acid before exposure to irradiation, compared to irradiated control samples (26 MN/500 BC) showed a significant difference (**$p < 0.001$**). This implies that when rosmarinic acid is administered to the samples before exposure to radiation, a decrease in the frequency of MN is expected and therefore it offers some protection against radiation induced damage.

5. In samples treated with **Rosmarinic acid** after exposure to radiation (post-irradiation treatment), an average MN frequency of occurrence of 24 MN/500 BC was established. Statistical analysis of the frequency of MN in the post-irradiation Rosmarinic acid treated samples, shows that there was a significant difference in the frequency of MN occurrence in the irradiated control samples (26 MN/500 BC) when compared with the post-irradiation Rosmarinic acid treated samples. This observation can be interpreted to mean that post-irradiation treatment of samples with Rosmarinic acid does not lead to a decrease in the expected frequency of MN and therefore offers no protection against damage induced by radiation.

6. **The Protection Factor (PF**) of sample treated with **Rosmarinic acid** before exposure to irradiation ranged from 46.15% to 61.54%. However, the PF of the samples treated after radiation exposure ranged from a minimum value of -11.54% to a

maximum value of 23.08%. When different volumes (12.5µ1 and 25 µl) of the same concentration of the substance were used, the average MN frequency values obtained for the pre- and post-irradiation was 11 and 24 MN/500 BC, respectively. Therefore, an average pre-irradiation PF of 57.69% was evaluated while the mean post-irradiation PF was 7.69% (Figure CB5; Table CB4).

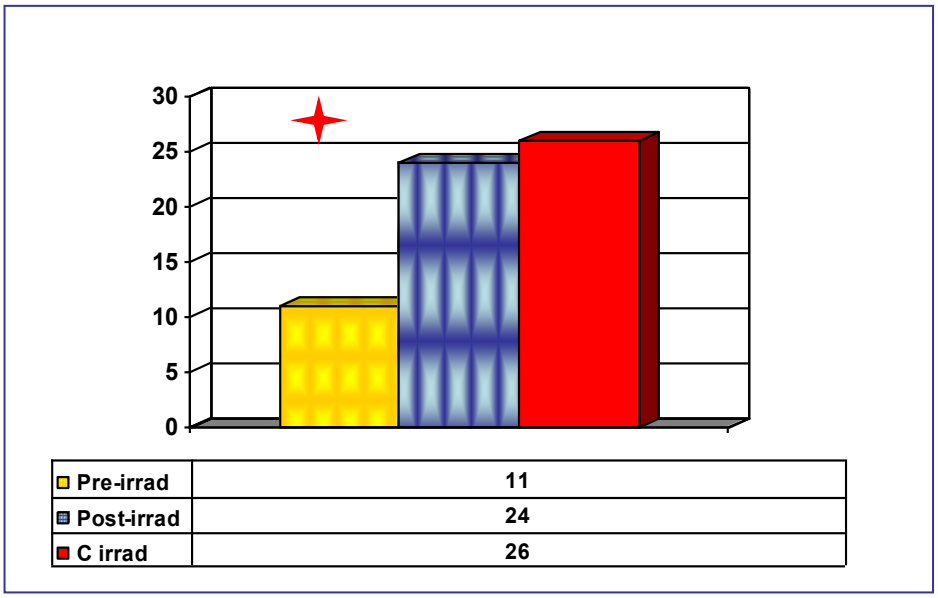
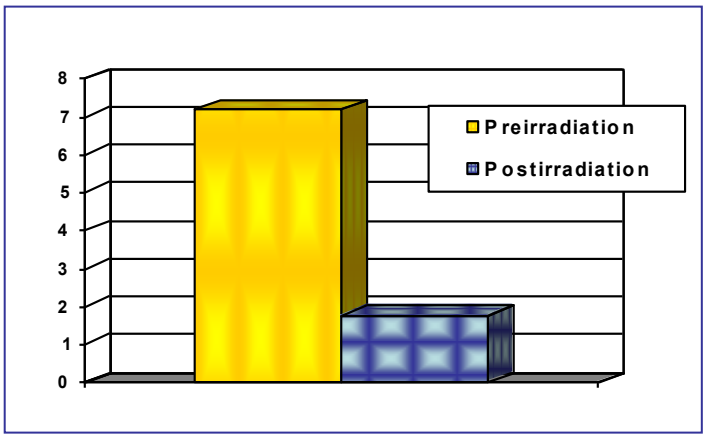


Figure CB5. Frequency of Micronuclei (CBMN) in human peripheral blood samples treated with ROS ((+): (p<0.001) versus irradiated contol cells)

PF (%)	
Pre-irradiation	**Post-irradiation**
57.69	7.69

TableCB4. Protection Factors obtained when human peripheral blood samples were treated with rosmarinic acid treatment.

7. **The Dose Reduction Factor (DRF)** of the **Rosmarinic acid** pre-irradiation treated samples was 7.18 while a **DRF** of 1.793 was obtained with the ROS post-irradiation treated samples (Figure CB6;Table CB5).



DRF	
Pre-irradiation	**Post-irradiation**
7.18	1.793

Figure CB6; Table CB5. Dose Reduction Factor obtained when human peripheral blood samples were treated with Rosmarinic acid

- **P90 TREATMENT (P90)**

1. In the samples treated with **P90,** a MN frequency of occurrence ranging from 6 to 12 MN/500 BC with an average of 9 MN/500 BC was determined. This can be said to show no significant difference with the non-irradiated control samples whose average frequency of MN occurrence was 10 MN/500 BC.

2. In samples treated with **P90** before exposure to radiation, a MN frequency ranging between 7 and 25 MN/500 BC with an average frequency of appearance of 12 MN/500 BC was determined. This shows that there are no significant differences between samples treated with this substance and the non-irradiated control samples treated with the same substance which had an average frequency of occurrence of MN of 9 MN/500 BC.

3. In the samples treated with **P90** after exposure to radiation, a MN frequency of occurrence ranging between 10 and 22 MN/500 BC and an average frequency of appearance of 16 MN/500 BC was computed. This observation can be interpreted to mean that there are no significant differences with respect MN frequency between these

samples and the non-irradiated control samples treated with the P90 whose average frequency of occurrence of MN was 9 MN/500 BC.

4. In samples treated with **P90** before exposure to radiation an average MN frequency of occurrence of 12 MN/500 BC was determined. Statistical analysis of the frequency of MN in irradiated samples previously treated with **P90** compared to irradiated control samples (26 MN/500 BC) and reflects a significant (**$p < 0.001$**) difference which means that treatment with P90 before exposure to radiation is expected decrease the frequency of MN and therefore offer some protection against radiation induced damage.

5. In the samples treated with **P90** after exposure to radiation, an average frequency of occurrence 16 MN/500 BC was determined. Statistical analysis of the frequency of MN in the samples irradiated before treatment with **P90** reflects a significant difference (**$p < 0.001$**) compared to irradiated control samples (26 MN/500 BC). These results means that post-irradiation treatment with P90 decreases the frequency of expression of MN in the cultured lymphocytes and therefore offer some level of protection to DNA against damage induced by radiation.

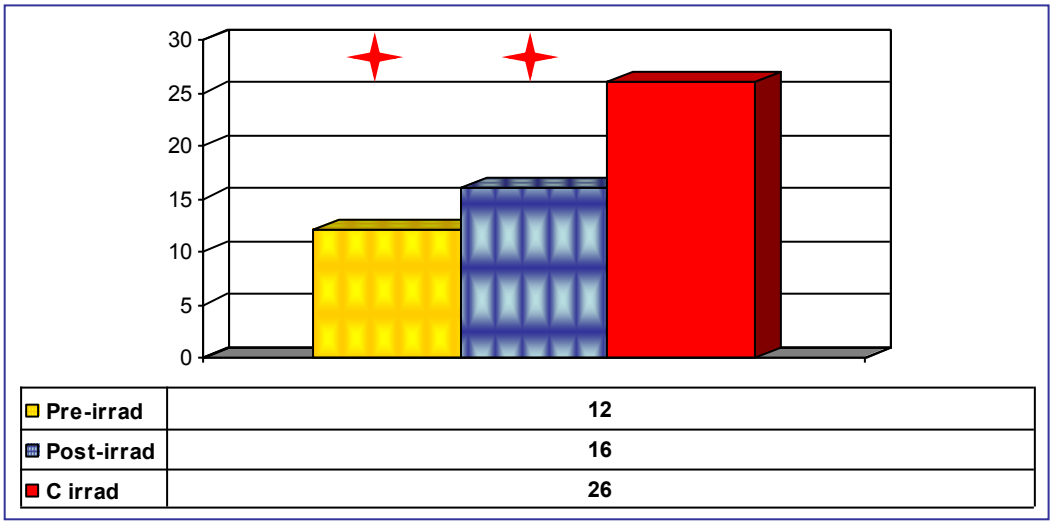


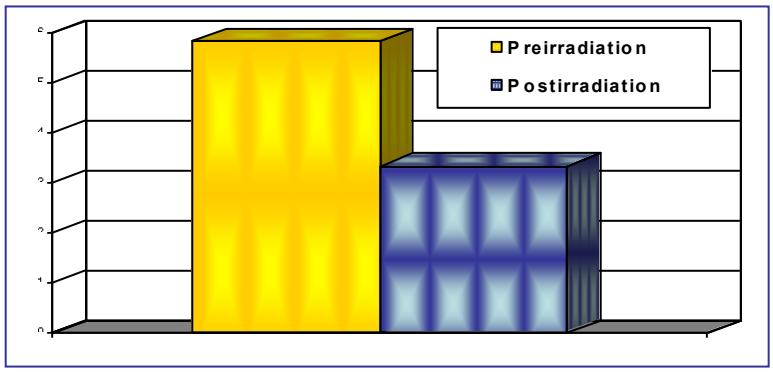
Figure CB7. Frequency of Micronuclei (CBMN) in human peripheral blood samples treated with P90. ((+): (p<0.001) versus irradiated contol cells)

6. The **Protection Factor (PF)** obtained for the **P90** pre-irradiation treated samples was 53.85%, but samples treated with P90 after exposure to radiation showed a PF of 38.46% (Figure CB7; Table CB6).

PF (%)	
Pre-irradiation	Post-irradiation
53.85	38.46

Table CB6. Factor of Protection obtained when human peripheral blood samples were treated with P90

7. The Dose Reduction Factor (DRF) for the pre-irradiation P90 treated samples was 5.833 while post irradiation P90 treated samples expressed a DRF of 3.331 (Figure CB8; Table CB7).



DRF	
Pre-irradiation	Post-irradiation
5.833	3.331

Figure CB8; Table CB7. Dose Reduction Factor obtained when human peripheral blood samples were treated with P90

- **CARNOSIC ACID TREATMENT (CARN)**

1. In the samples treated with **Carnosic acid** a MN frequency ranging between 9 and 11 MN/500 BC and an average of 10 MN/500 BC was established. This can be said to show no significant difference in terms of the frequency of occurrence of MN with respect to the non-irradiated control samples whose average frequency of occurrence of MN was 10 MN/500 BC.

2. In the samples treated with **Carnosic acid** before exposure to radiation a MN frequency ranging between 10 and 15 MN/500 BC and an average frequency of 13 MN per 500 binucleated cells was determined. This shows that there is no

significant difference with what pertains in the non-irradiated control samples treated with the same substance whose average frequency of occurrence of MN was 10 MN/500 BC. When two different volumes of the same concentration of the substance were administered, a MN frequency of 11 MN/500 BC was observed with the 12.5 µl volume, however, this frequency increased to 14.5 MN/500 BC on the administration of 25 µl of the sample.

3. In the samples treated with **Carnosic acid** after exposure to radiation, a MN frequency ranging between 12 and 17 MN/500 BC and an average frequency of 15 MN/500 BC was demonstrated. This presupposes that there is no significant difference in the MN frequency between post-irradiation carnosic acid treated samples and their non-irradiated control counterparts treated with the same substance whose average frequency of occurrence of MN was 10 MN/500 BC. On the administration of two different volumes of the same concentration of carnosic acid, an average MN frequency of 16 MN/500 BC was observed with the 12.5 µl volume treatment, however, the frequency decreased to 13 MN/500 BC when the volume of 25 µl was administered.

4. In the samples treated with **carnosic acid** before exposure to radiation the average frequency of occurrence of micronuclei was 13 MN/500 BC. Statistical analysis of the frequency of MN in the samples pre-treated with carnosic acid before irradiation shows a significant (**$p < 0.001$**) difference compared with irradiated controls samples (26 MN/500 BC). This means that the treatment with carnosic acid before exposure to radiation decreases the frequency of MN occurrence and therefore offer some degree of protection against radiation induced damage.

5. In the post-irradiation **Carnosic acid** treated samples an average MN frequency of occurrence of 15 MN/500 BC was established. Statistical analysis of the frequency of MN in the samples initially irradiated before subsequent treatment with carnosic acid reflects a significant difference (**$p < 0.001$**) with the irradiated control samples (26 MN/500 BC). This implies that the post-irradiation treatment with Carnosic acid decreases the frequency of MN and therefore offers protection against chromosome damage induced by radiation.

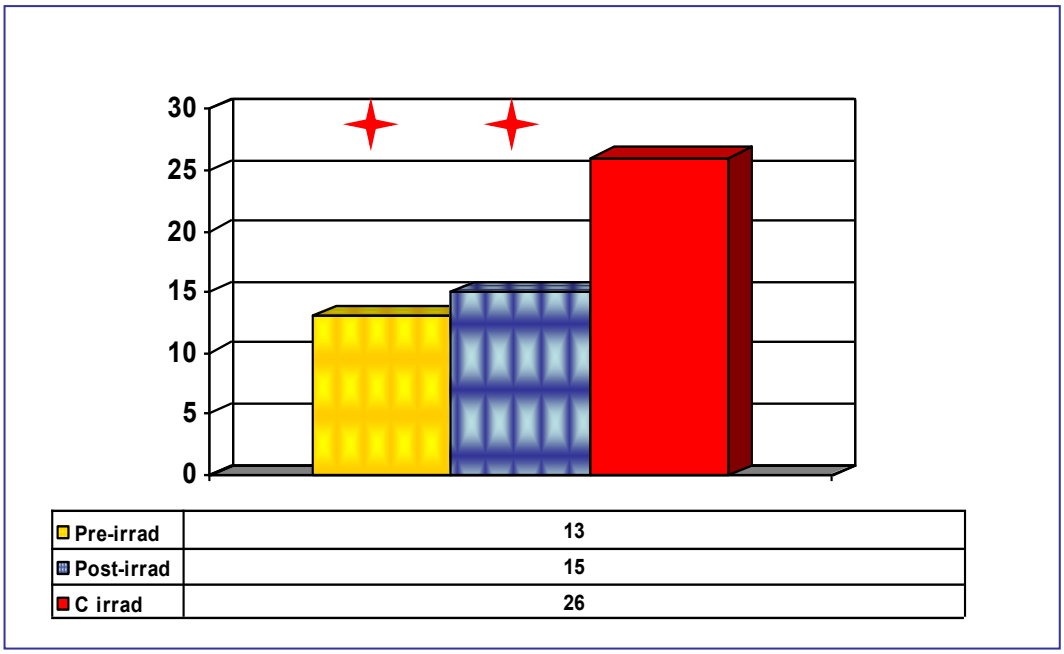


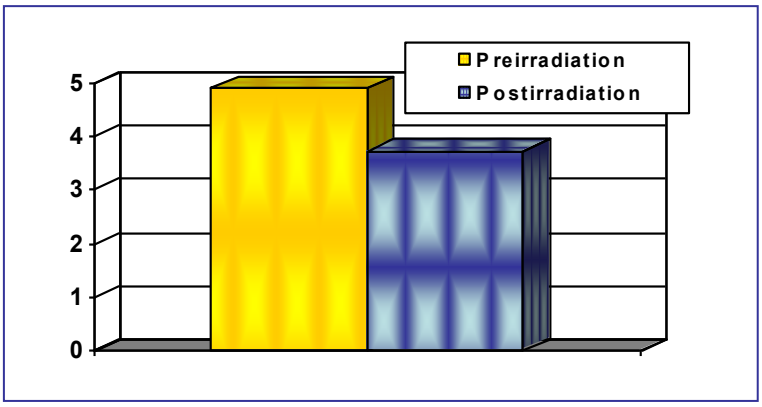
Figure CB9. Frequency of Micronuclei (CBMN) in human peripheral blood samples treated with Carnosic acid ((+): ($p<0.001$) versus irradiated control cells)

6. **The Protection Factor (PF)** of pre-irradiation **carnosic acid** treated samples ranged from 44.23% to 57.69%, however, the values of PFs for the samples treated with carnosic acid after exposure to radiation ranged between a minimum of 38.46% to a maximum of 50%. When different concentrations of carnosic acid were administered the average MN frequency value obtained for the pre- and post-irradiation treatments were 13 and 15 MN/500 BC respectively. Therefore, an average pre-irradiation PF of 50% and a mean post irradiation PF of 42.31% were determined (Figure CB9; Table CB8).

PF (%)	
Pre-irradiation	Post-irradiation
50	42.31

Table CB8. Factor of Protection obtained when human peripheral blood samples were treated with Carnosic acid

7. **The Dose Reduction Factor (DRF)** obtained for the pre-irradiation **carnosic acid** treated samples was 4.91 whereas the DFR for the post-irradiation treated samples was 3.731 (Figure CB10; Table CB9).



DRF	
Pre-irradiation	**Post-irradiation**
4.91	3.731

Figure CB10; Table CB9. Dose Reduction Factors obtained when human peripheral blood samples were treated with Carnosic acid

- **APIGENIN (API) DISSOLVED IN DMSO**

1. In the samples treated with **Apigenin dissolved in DMSO**, the frequency of MN detected ranged between 7 and 11 MN/500 BC and a mean of 9 MN/500 BC. This can be said to show no significant difference with that of the non-irradiated control samples whose average frequency of MN occurrence was 10 MN/500 BC.

2. In the samples pre-treated with **Apigenin dissolved in DMSO** prior to radiation exposure, an average frequency of occurrence of 13 MN/500 BC was established. This revelation means that there are no significant differences in MN frequency of occurrence with respect to the non-irradiated control samples treated with the same substance and whose average frequency of MN occurrence was 9 MN/500 BC.

3. In the samples treated with **Apigenin dissolved in DMSO** after exposure to radiation, an average frequency of occurrence of 16 MN/500 BC was determined. This find means that there are no significant differences in MN frequency of occurrence of this cohort with respect to the non-irradiated control samples treated with the same substance and whose average frequency of MN occurrence was 9 MN/500 BC.

4. In the samples pre-treated with **Apigenin dissolved in DMSO** before radiation exposure, an average frequency of occurrence of 13 micronuclei per 500 BC was determined. Statistical analysis of the frequency of MN in the samples pre-treated with Apigenin dissolved in DMSO before exposure to irradiation, shows a significant difference (**$p < 0.001$**) compared with irradiated control samples (26 MN/500 BC). This means that the treatment with Apigenin dissolved in DMSO prior to radiation exposure leads to a decrease in the expected frequency of MN and therefore offer some protection against radiation induced damage.

5. In the samples treated with **Apigenin dissolved in DMSO** after exposure to radiation, an average frequency of occurrence of 16 MN/500 BC was observed. Statistical analysis of the frequency of MN in the samples pre-irradiated before treatment with Apigenin dissolved in DMSO, showed a significant difference (**$p < 0.001$**) in MN frequency compared to irradiated control samples (26 MN/500 BC). This observation suggests that post-irradiation treatment with Apigenin dissolved in DMSO decreases the expected frequency of MN and thus protects against radiation induced damage.

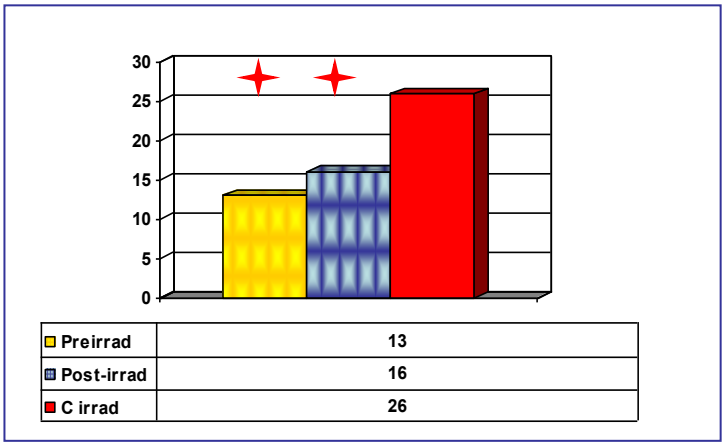


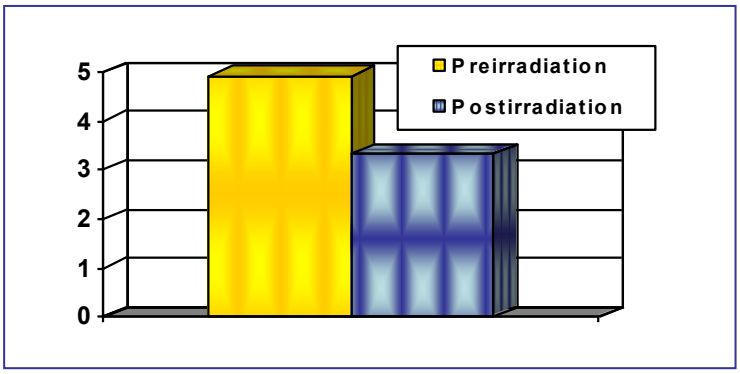
Figure CB11. Frequency of Micronuclei (CBMN) in human peripheral blood samples treated API dissolved in DMSO ((+): ($p<0.001$) versus irradiated contol cells)

6. **The** Protection **Factor (PF)** of samples treated with **Apigenin dissolved in DMSO** before irradiation was 50%, but samples treated with Apigenin dissolved in DMSO after exposure to radiation expressed a PF of 38.46 % (Figure CB11; Table CB10).

PF (%)	
Pre-irradiation	**Post-irradiation**
50	38.46

TableCB10. Protection Factors obtained when human peripheral blood samples were treated with Apigenin dissolved in DMSO

7. **The Dose Reduction Factor (DRF)** for the pre-irradiation samples treated with Apigenin dissolved in DMSO was 4.91 while the **DRF** obtained in the post-irradiation treated samples was 3.331 (Figure CB12; Table CB11).



DRF	
Pre-irradiation	**Post-irradiation**
4.91	3.331

Figure CB12 and Table CB11. Dose Reduction Factor obtained when human peripheral blood samples were treated with Apigenin dissolved in DMSO

- **VITAMIN E TREATMENT (E)**

1. In the samples treated with **Vitamin E,** the MN frequency of occurrence ranged between 7 and 13 MN/500 BC with an average of 10 MN/500 BC. This indicates that there is no significant difference in the MN frequency of occurrence between this cohort and the non-irradiated control samples whose average frequency of occurrence of MN was 10 MN/500 BC.

2. In the samples treated with **Vitamin E**, before exposure to radiation an average frequency of occurrence of 13 MN /500 BC was observed, which means a no significant difference in MN frequency of occurrence between this group and the non-

irradiated control samples treated with the same substance whose average frequency of occurrence of MN was 10 MN/500 BC.

3. In the samples treated with **Vitamin E** after exposure to radiation, an average MN frequency of occurrence of 17 MN /500 BC was observed, which represents a significant increase (**$p < 0.05$**) in the frequency of occurrence of MN in this group compared with the non-irradiated control samples treated with the same substance whose average frequency of occurrence of MN was 10 MN/500 BC.

4. In the samples treated with **Vitamin E** before exposure to radiation, an average frequency of occurrence of 13 micronuclei per 500 binucleated cells was determined. Statistical analysis of the frequency of MN in the samples pre-treated with Vitamin E before exposure to irradiation shows a significant difference (**$p < 0.001$**) when compared to irradiated control samples (26 MN/500 BC), which means that treatment with Vitamin E before exposure to radiation decreases the frequency of occurrence of MN and therefore offer some protection against radiation induced damage.

5. In the samples treated with **Vitamin E** after exposure to radiation, an average frequency of occurrence of 17 MN/500 binucleated cells was observed. Statistical analysis of the frequency of MN in the post-irradiation vitamin E treated samples, reflects a significant difference (**$p < 0.001$**) with respect to the irradiated control samples (26 MN/500 BC). This is suggestive that post-irradiation treatment with Vitamin E decreases the expected frequency of MN and therefore offers some level of protection against damage induced by radiation.

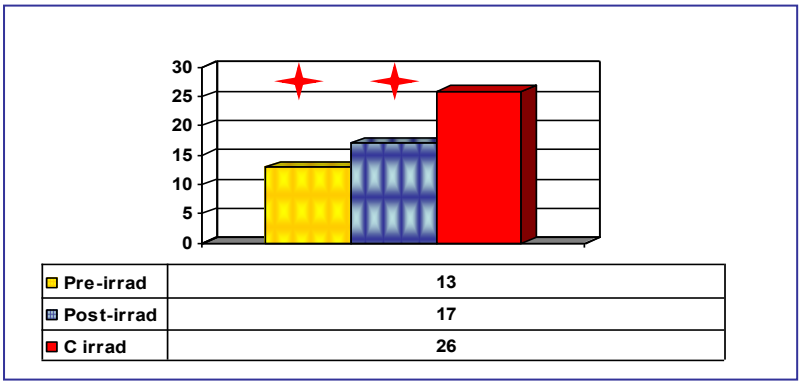


Figure CB13. Frequency of Micronuclei (CBMN) in human peripheral blood samples treated with Vitamin E ((+): (p<0.001) versus irradiated contol cells)

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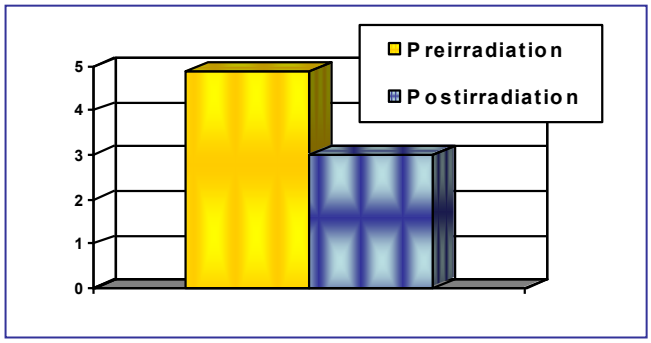
6. The **Protection Factor (PF)** of the samples treated with **Vitamin E** before irradiation was 50%, while samples treated with Vitamin E after exposure to radiation produced a PF of 34.61% (Figure CB13 and Table CB12).

PF (%)	
Pre-irradiation	**Post-irradiation**
50	34.61

Table CB12. Protection Factors obtained when human peripheral blood samples were treated with Vitamin E

7. The **Dose Reduction Factor (DRF)** for the pre-irradiated samples treated with **Vitamin E** was 4.91 while DRF obtained with post-irradiation vitamin E treated samples was 3.009 (Figure CB14; Table CB13).



DRF	
Pre-irradiation	**Post-irradiation**
4.91	3.009

Figure CB14; Table CB13. Dose Reduction Factor obtained when human peripheral blood samples were treated with Vitamin E.

- **CARNOSOL TREATMENT (COL)**

1. In the samples treated with **Carnosol,** the frequency of occurrence of MN ranged between 8 and 12 MN/500 BC and a mean of 10 MN/500 BC. This showed no significant difference with the non-irradiated control samples, whose average frequency of MN occurrence of was 10 MN/500 CB.

2. In samples treated with **Carnosol** before exposure to radiation the MN frequency ranged between 13 and 17 MN/500 binucleated cells and an average

frequency of appearance of 14 MN/500 BC was demonstrated. This shows no significant differences with respect to the non-irradiated control samples treated with the same substance but whose average frequency of occurrence of MN was 10 MN/500 BC. On the administration of two different volumes of the same concentration of Carnosol, a frequency of 15.5 MN was observed when 12.5 µl was administered, however, when a volume of 25 µl was administered the MN frequency dropped to 13 MN/500 binucleated cells.

3. In post-irradiation **Carnosol** treated samples, the frequency of occurrence of MN ranged between 13 and 16 MN/500 binucleated cells with an average frequency of appearance of 14 MN/500 BCs. This observation indicates that there is no significant differences in the MN frequency of occurrence between this group when compared with the non-irradiated control samples treated with the same substance and whose average MN frequency of occurrence was 10 MN/500 BC. Also, no difference in the frequency of occurrence of MN was observed when the volume of the substance administered was doubled (i.e., from 12.5 to 25 µl) and administered post-irradiation.

4. The **Carnosol** treated samples before exposure to radiation expressed an average MN frequency of occurrence of 14 micronuclei /500 BCs. Statistical analysis of the frequency of MN in the samples pre-treated with Carnosol before irradiation reflects a significant (**$p < 0.001$**) difference when compared to irradiated control samples (26 MN/500 BC). This indicates that treatment with Carnosol before exposure to radiation decreases the expected frequency of occurrence of MN and therefore offer some degree of protection against radiation induced damage.

5. In post-irradiation **Carnosol** treated samples, an average MN frequency of occurrence of 14 MN /500 BCs was determined. Statistical analysis conducted on the frequency of MN occurrence in the irradiated samples which were subsequently treated with Carnosol reflects a significant (**$p < 0.001$**) difference when compared to irradiated control samples (26 MN/500 CB). This observation means that post-irradiation treatment with Carnosol decreases the expected frequency of occurrence of MN and is therefore protecting against chromosome damage induced by radiation.

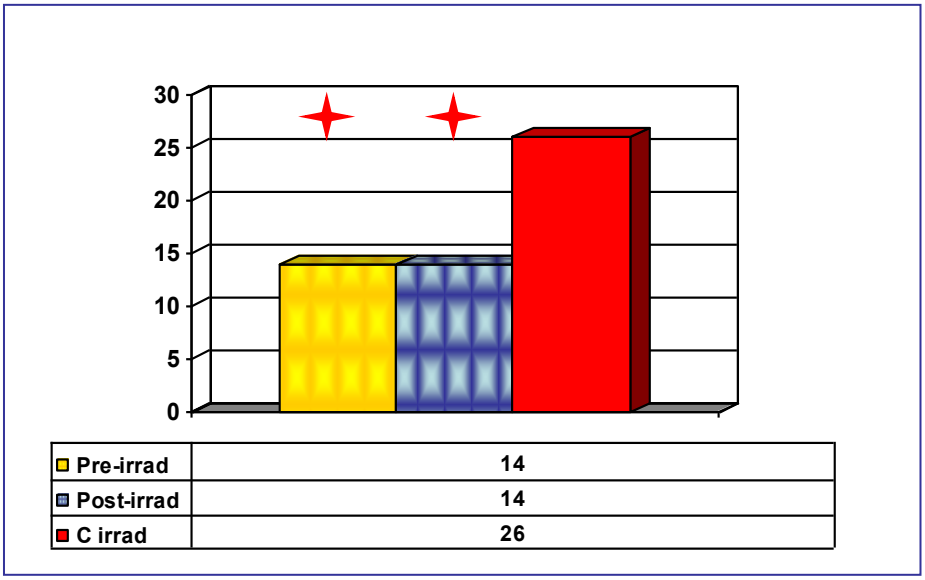


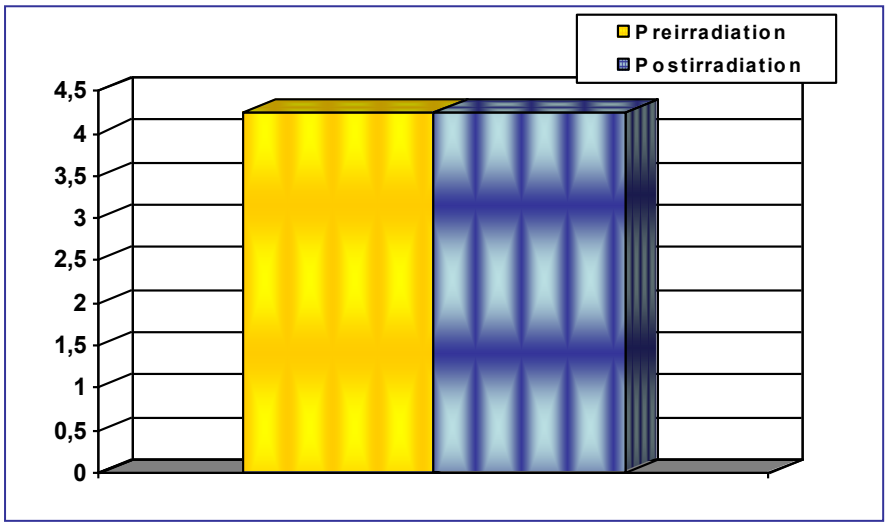
Figure CB15. Frequency of Micronuclei (CBMN) in human peripheral blood samples treated with COL ((+): (p<0.001) versus irradiated contol cells)

6. The **protection Factor (PF)** of the samples treated with **Carnosol** before exposure to irradiation ranged from 40.38% to 50.0%, however, the PF of the samples treated with Carnosol after exposure to radiation was 46.15%. The administration of different concentrations produced the same average pre- and post-irradiation MN frequency value of 14 MN/500 BC. Therefore, an average pre- and post-irradiation PF of 46.15% was established (Figure CB15; Table CB14).

PF (%)	
Pre-irradiation	Post-irradiation
46.15	46.15

Table CB14. Factor of Protection obtained when human peripheral blood samples were treated with of Carnosol

7. **The Dose Reduction Factor** (DRF) for the pre-irradiation Carnosol treated samples was 4.24 and proved to be equal when the substance was administered post irradiation (Figure CB16; Table CB15).



DRF	
Pre-irradiation	**Post-irradiation**
4.24	4.24

Figure CB16;Table CB15. Dose Reduction Factor obtainedwhen human peripheral blood samples were treated with Carnosol

- **SOLUBLE CITRUS EXTRACTS (CE) TREATMENT**

1. In the samples treated with **CE**, a MN frequency distribution ranging between 8 and 12 MN/500 BC with an average of 10 MN/500 BC was determined. This can be interpreted to mean that there were no significant differences in the occurrence of MN frequencies between this group and the non-irradiated control samples whose average MN frequency of occurrence was 10 MN/500 BC.

2. In the samples treated with **CE** before exposure to radiation, the frequency of MN determined ranged between 13 and 20 MN/500 BC with an average frequency of occurrence of 16 MN/500 BC. This represents a significant increase (**$p < 0.05$**) in the frequency of appearance of MN when compared with the non-irradiated control samples treated with the same substance and whose average frequency of occurrence of MN was 10 MN/500 BC. Also, the frequency of occurrence of micronuclei varied with the amount of CE added to the peripheral blood samples, from 14 MN/500 BC when 2.5 µl

of CE was administered through 16 MN (5 µl); 13 MN (10 µl) to 20MN per 500 BCs when 25 µl of CE was administered to the sample.

3. In the samples treated with **CE** after exposure to radiation the MN frequency ranged between 16 and 22 MN/500 BC with an average frequency of appearance of 19 MN /500 BC. This represents a significant increase (**$p < 0.01$**) in the frequency of appearance of MN compared with the non-irradiated control samples treated with the same substance but whose average frequency of occurrence of MN was 10 MN/500 BC. Regarding the amount of CE administered, an increasing trend in the number of MN was established with 19, 16, 21 and 22 MN/500 BC as the volume of substance added to the sample rose steadily from 2.5, 5, 10 to 25 µl respectively.

4. In the samples treated with **CE** before exposure to radiation, an average frequency of occurrence of 16 micronuclei /500 BC was determined. Statistical analysis of the frequency of MN in the samples pretreated with CE before exposure to irradiation showed a significant decrease (**$p < 0.001$**) compared to irradiated control samples (26 MN/500 CB). This is suggestive that treatment with CE before exposure to radiation decreases the expected frequency occurrence of MN and therefore offer some level of protection against radiation induced chromosome damage.

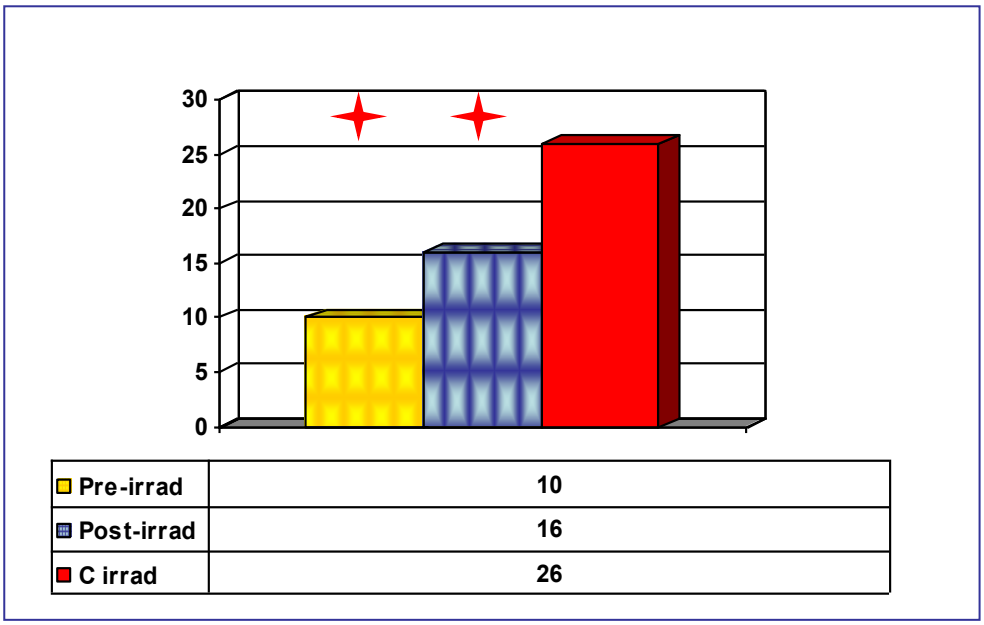


Figure CB16. Frequency of Micronuclei (CBMN) in human peripheral blood samples treated with CE ((+): ($p<0.00$) versus irradiated contol cells)

5. In the samples treated with **CE** after exposure to radiation, the average frequency of occurrence of MN was 19 MN/500 BC. Analysis of the frequency of MN in the irradiated samples which were subsequently treated with CE showed a significant decrease (**$p < 0.05$**) in MN frequency when compared with the irradiated control samples (26 MN/500 BC). This indicates that post-irradiation treatment with CE decreases the expected frequency of MN and therefore offer some protection against radiation induced chromosome damage.

6. The **Protection Factor (PF)** of the samples treated with **CE before irradiation** ranged from 23.08% to 50%, however, the PF for samples treated CE **after exposure to radiation** ranged from a minimum of 15.38% to a maximum of 38.46%. Upon treatment with different concentrations of the test substance, the average pre- and-post irradiation MN frequency values obtained were 16 and 19 MN/500 BC respectively. Therefore, an average pre-irradiation PF of 38.46% and a mean post-irradiation PF of 26.92% were obtained (Figure CB16; Table CB15).

PF (%)	
Pre-irradiation	Post-irradiation
38.46	26.92

Table CB15. Protection Factor obtained when human peripheral blood samples were treated with CE

7. The **Dose Reduction Factor (DRF)** for the samples treated with CE pre-irradiation was 3.331 while the DRF for the CE post-irradiation treated samples was 2.52 (Figure CB17; Table CB16).

DRF	
Pre-irradiation	Post-irradiation
3.331	2.52

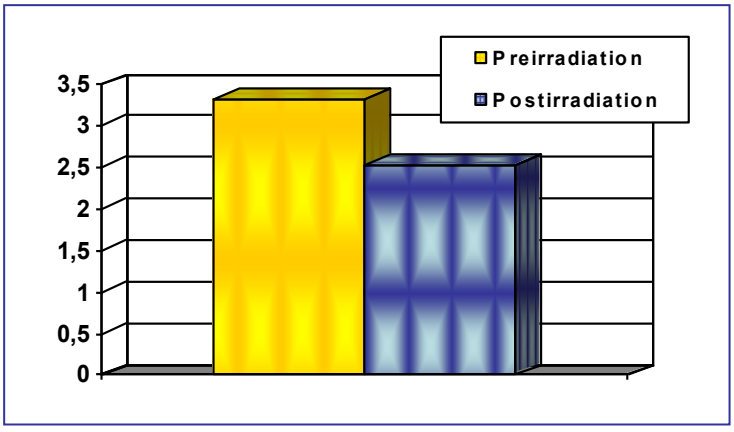


Figure CB17; Table CB16. Dose Reduction Factor obtained when human peripheral blood samples were treated with CE

- **VITAMIN C TREATMENT (C)**

1. In the samples treated with **Vitamin C,** a MN frequency ranging between 6 and 12 MN/500 BC with an average of 9 MN/500 BC was found. This can be said to show no significant difference with the non-irradiated control samples whose average frequency of occurrence of MN was 10 MN/500 BC.

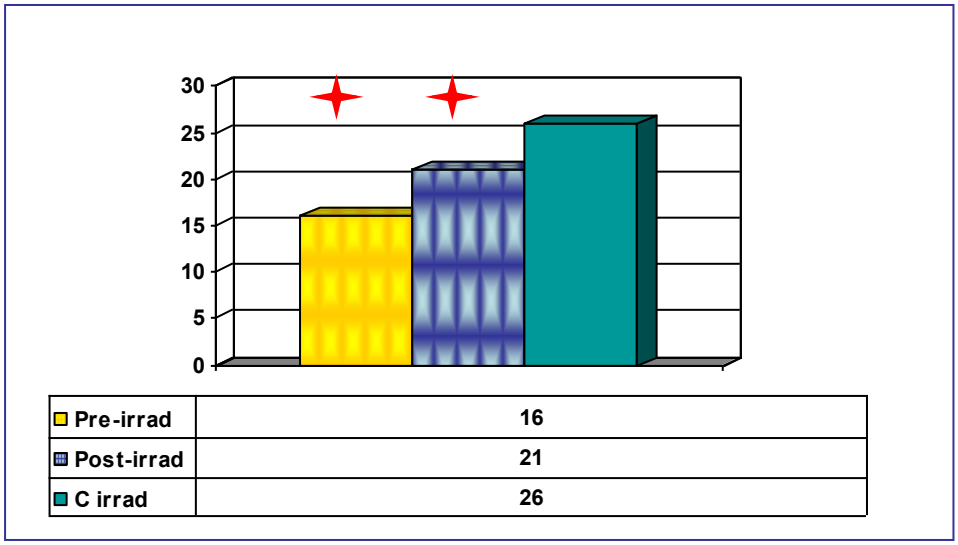
2. In the samples treated with **Vitamin C** before exposure to radiation, the MN frequency distribution ranged between 12 and 21 MN/500 BC with an average frequency of appearance of 16 MN/500 BC. This observation represents a significant increase (**p <0.05**) in the frequency of appearance of MN compared with the non-irradiated control samples treated with the same substance but whose average frequency of occurrence of MN was 9 MN/500 BC.

3. In the post-irradiation **Vitamin** C treated samples, a MN frequency ranging between 18 and 23 MN/500 BC with an average frequency of appearance of 21 MN/500 BC was determined. This represents a significant increase (**p <0.01**) in the frequency of appearance of MN over that of the non-irradiated control samples treated with the same substance but whose average frequency of occurrence of MN was 9 MN/500 BC.

4. In the samples treated with **Vitamin C** before exposure to radiation an average frequency of occurrence of 16 micronuclei /500 BC was determined. Statistical analysis of the frequency of MN in the samples pre-treated with vitamin C before exposure to irradiation shows a significant decrease (**p < 0.001**) compared with irradiated control samples (26 MN/500 BC). This suggests that treatment with Vitamin C before exposure to radiation decreases the expected frequency of MN and therefore protects somewhat against radiation induced chromosome damage.

5. In the **Vitamin C** post-irradiation treated samples, an average frequency of occurrence of 21 MN/500 BC was displayed. Analysis of the frequency of MN in the samples irradiated before treatment with Vitamin C reflects a significant decrease (**p < 0.05**) compared with irradiated control samples (26 MN/500 BC). This is to say that the post-irradiation treatment with Vitamin C decreases the expected frequency of

occurrence of MN and therefore offer some degree of protection against radiation induced chromosome damage.



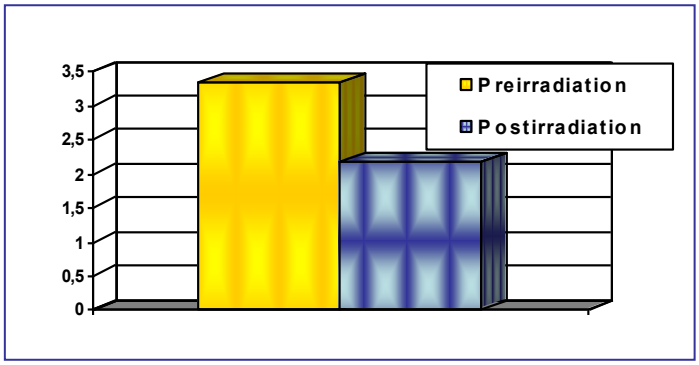
FigureCB18. Frequency of Micronuclei (CBMN) in human peripheral blood samples treatedt with Vitamin C ((+): (p<0.001) versus irradiated contol cells)

6. The **protection Factor (PF)** of the samples treated with **Vitamin C** before exposure to irradiation was 38.46%, but samples treated with Vitamin C after exposure to radiation gave a PF response of 19.23%. (Figure CB18; Table CB17)

PF (%)	
Pre-irradiation	Post-irradiation
38.46	19.23

Table CB17. Factor of Protection obtained when human peripheral blood samples were treated with Vitamin C

7. The **Dose Reduction Factor (DRF)** for the samples treated with Vitamin C pre-irradiation was 3.331 while the post-irradiation Vitamin C treated samples elaborated a DRF of 2.169 (Figure CB19; Table CB18).



DRF	
Pre-irradiation	**Post-irradiation**
3.331	2.169

Figure CB19; Table CB18. Dose Reduction Factor obtained when human peripheral blood samples were treated with Vitamin C

- **AMIFOSTINE (WR-2721 OR ETHYOL®) TREATMENT (AMIF)**

1. In the samples treated with **amifostine,** a MN frequency distribution ranging between 8 and 14 MN/500 BC with an average of 11 MN/500 BC was confirmed. This could be interpreted to mean that there is no significant difference between this and what pertains with the non-irradiated control samples whose average frequency of occurrence of MN was 10 MN/500 BC.

2. In the samples treated with **amifostine before exposure to radiation** the average MN frequency of occurrence established was 16 MN/500 BC. Thus no significant differences in MN frequency were inferred with respect to the non-irradiated control samples treated with the same substance but whose average frequency of occurrence of MN was 11 MN/500 BC.

3. In the samples treated with **amifostine after exposure to radiation** an average frequency of occurrence of 27 MN /500 BC was determined and this represents a significant increase (**p <0.001**) in the MN frequency over the non-irradiated control samples treated with the same substance but whose average frequency of MN occurrence of was 11 MN/500 BC.

4. In the samples treated with **amifostine before exposure to radiation** an average frequency of occurrence of 16 micronuclei /500 BC was determined. Statistical analysis of the frequency of MN in the samples pretreated with amifostine before exposure to irradiation reflects a significant decrease (**p < 0.001**) compared to the control irradiated samples (26 MN/500 BC). This infers that treatment with amifostine before the exposure to radiation decreases the expected frequency of MN and is therefore capable of offering some level of protection against radiation induced damage.

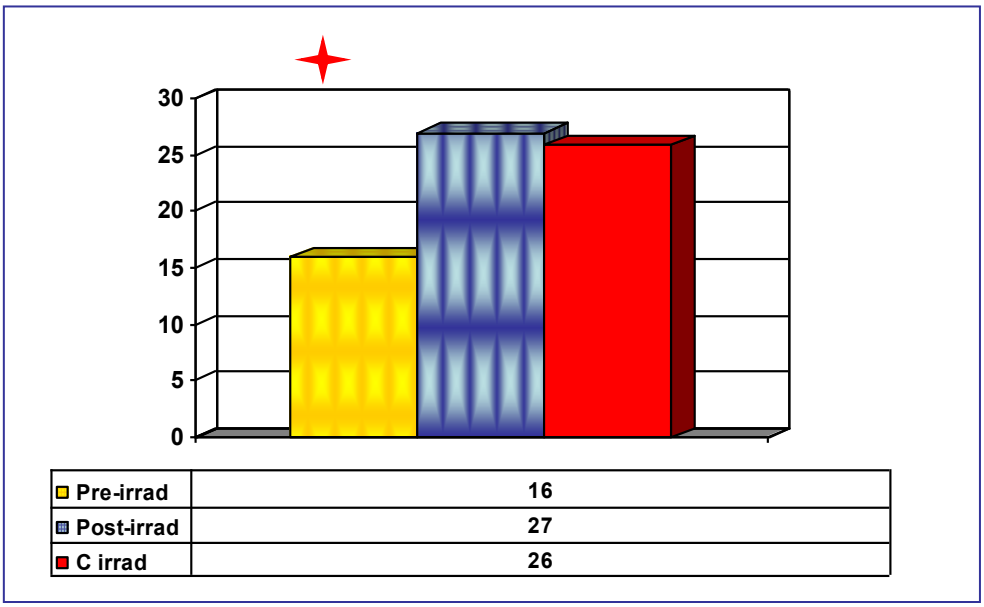


Figure CB20. Frequency of Micronuclei (CBMN) in human peripheral blood samples treated with Amifosstine ((+): (p<0.001) versus irradiated contol cells)

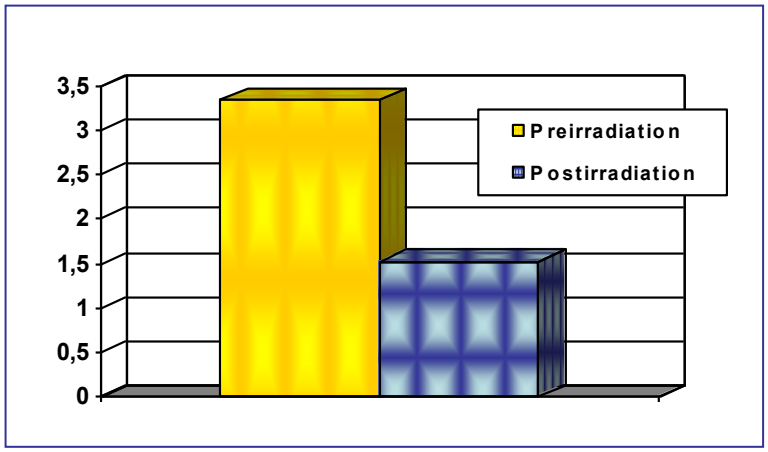
5. In the samples **treated with amifostine after exposure to radiation** an average frequency of occurrence of 27 MN/500 BC was determined. Statistical analysis of the frequency of MN in the samples irradiated before subsequent treatment with amifostine reflects no significant differences with respect to irradiated control samples (26 MN/500 CB). This observation suggests that post-irradiation treatment of samples with amifostine does not decrease the expected frequency of MN and therefore does not offer any level of protection against radiation induced damage.

6. The **Protection Factor (PF)** of the samples treated with amifostine before irradiation was 38.46%, but samples treated with amifostine after exposure to radiation showed a negative PF value of -3.85% (Figure CB20;Table CB19).

PF (%)	
Pre-irradiation	**Post-irradiation**
38.46	-3.85

Table CB19. Protection Factor obtained when human peripheral blood samples were treated with Amifostine

7. **The Dose Reduction Factor (DRF)** obtained for the blood samples treated with amifostine pre-irradiation was 3.331 while a post-irradiation amiphostine treated samples gave a DRF of 1.528 (Figure CB21; Table CB20).



DRF	
Pre-irradiation	**Post-irradiation**
3.331	1.528

Figure CB21; Table CB20. Dose Reduction Factor obtained in human peripheral blood samples treated with Amifostine

- **GREEN TEA EXTRACTS TREATMENT (TE)**

1. In the samples treated with **TE,** the frequency of MN distribution ranged between 9 and 11 MN/500 BC with an average of 10 MN/500 BC. This observation shows that there is no significant difference in the frequency of occurrence of MN in this cohort in comparison with the non-irradiated control samples whose average frequency of occurrence of MN was 10 MN/500 BC.

2. In samples treated with **TE before exposure to radiation**, the average MN frequency distribution established was 20 MN/500 BC, representing a significant increase (**p <0.01**) in the frequency of appearance of MN with respect to the non-irradiated control samples treated with the same substance but whose average frequency of occurrence of MN was 10 MN/500 BC.

3. In samples treated with **TE after exposure to radiation** the average MN frequency of occurrence determined was 24 MN/500 BC, representing a significant increase **(p <0.001)** in the MN frequency over the non-irradiated control samples treated with the same substance but whose average frequency of occurrence of MN was 10 MN/500 BC.

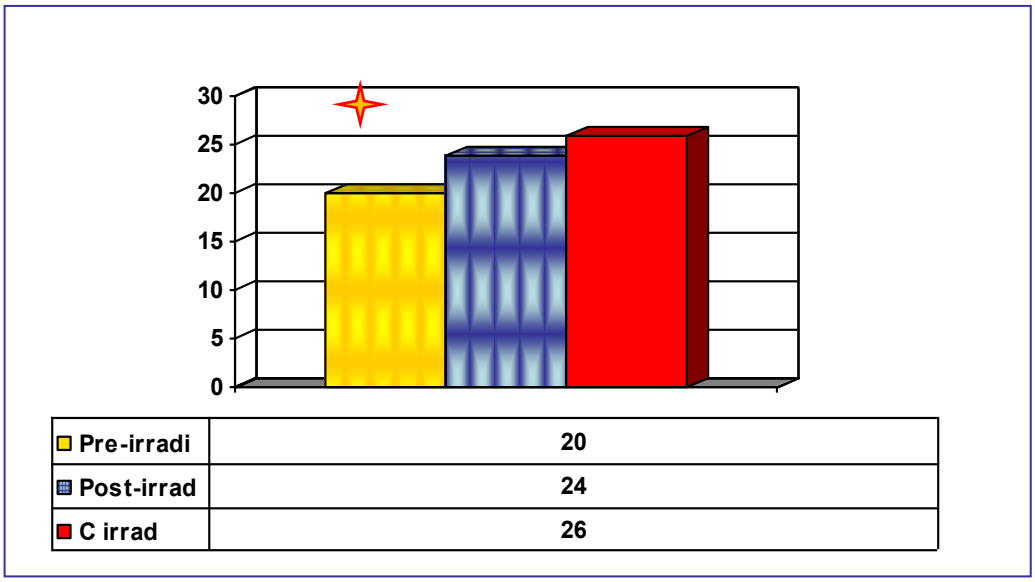


Figure CB22. Frequency of Micronuclei (CBMN) in human peripheral blood samples treated with TE (++: (p<0.01) versus irradiated contol cells)

4. In samples treated with **TE** before exposure to radiation an average frequency of occurrence of 20 micronuclei per 500 BC was established. Statistical analysis of the frequency of MN in irradiated samples pretreated with **TE** reflects a significant decrease in MN (**p < 0.05**) compared to irradiated control samples (26 MN/500 BC). This observation reveals that treatment with **TE** before exposure to radiation decreases the expected frequency of occurrence of MN and therefore offers some level of protection against radiation induced DNA damage.

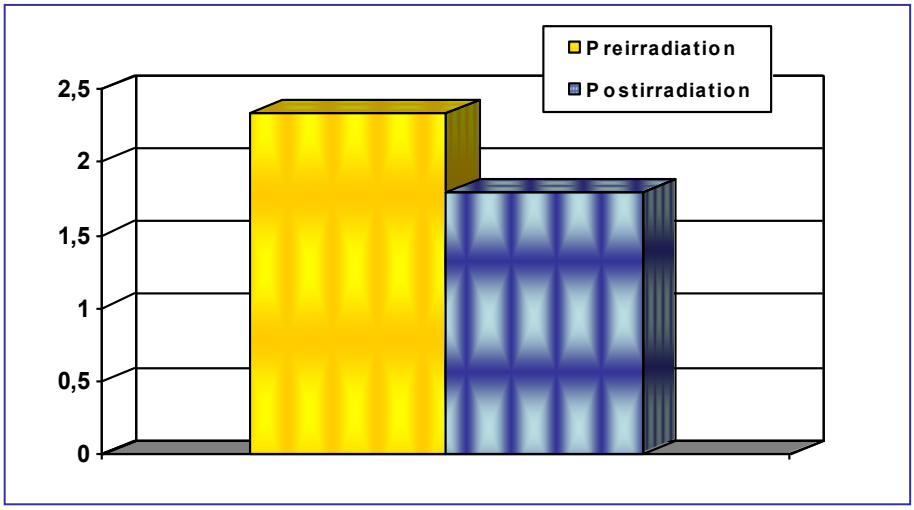
5. In samples treated with **TE after exposure to radiation,** an average MN frequency of 24 MN/500 BC was established. Analysis conducted on the frequency of MN in the samples irradiated before subsequent treatment with TE reflects no significant differences with the irradiated control samples (26 MN/500 BC). This suggests that post-irradiation treatment with TE does not decrease the expected frequency of MN occurrence and therefore does not offer protection against radiation induced DNA damage.

6. **The protection Factor (PF)** of the samples treated with TE before irradiation was 23.08%, but samples treated with TE after exposure to radiation had a PF of 7.69% Figure CB22; Table CB21).

PF (%)	
Pre-irradiation	**Post-irradiation**
23.08	7.69

Table CB21. Protection Factor obtained when human peripheral blood samples were treated with TE

7. The **Dose Reduction Factor (DRF)** obtained for the samples treated with TE before exposure to irradiation was 2.331 while the post-irradiation TE treated samples produced a DRF of 1.793 (Figure CB23; Table CB22).



DRF	
Pre-irradiation	**Post-irradiation**
2.331	1.793

Figure CB23; Table CB22. Dose Reduction factor obtained when human peripheral blood samples were treated with TE

- **RUTIN TREATMENT (R)**

1. In samples treated with **Rutin,** the frequency of MN distribution ranged between 8 and 10 MN/500 BC with an average of 9 MN/500 BC. This shows no significant difference with the non-irradiated control samples whose average frequency of occurrence of MN was 10 MN/500 BC.

2. In samples treated with **Rutin before exposure to radiation**, the frequency MN ranging between 15 and 29 MN/500 BC with an average frequency of appearance of 22 MN/500 BC, which shows a significant increase (**p <0.01**) in the frequency of appearance of MN over the non-irradiated control samples treated with the same substance but whose average frequency of occurrence of MN was 9 MN/500 BC.

3. Samples treated with Rutin after exposure to radiation, expressed a MN frequency ranging between 23 and 25 MN/500 BC with an average frequency of appearance of 24 MN/500 BC. This shows a significant increase (**p <0.001**) in the frequency of MN over the non-irradiated control samples treated with the same substance but whose average frequency of occurrence of MN was 9 MN/500 BC.

4. Samples treated with **Rutin before exposure to radiation,** expressed an average MN frequency of occurrence of 22 micronuclei per 500 BC. Statistical analysis of the frequency of MN in the irradiated samples pretreated with Rutin reflects a significant decrease (**p < 0.05**) compared to irradiated control samples (26 MN/500 BC), which means that treatment with rutin before exposure to radiation decreases the expected frequency of MN occurrence and is therefore offering some level of protection against radiation induced chromosome damage.

5. In samples treated with **Rutin after exposure to radiation** the average frequency of occurrence of MN was 24 MN/500 BC. Analysis of the frequency of MN in the samples irradiated before subsequent treated with Rutin revealed that there are no significant differences between this and the irradiated control samples (26 MN/500 BC). This is indicative that, post-irradiation treatment with Rutin does not lead to a decrease in the expected frequency of MN and so post-irradiation treatment with rutin does not offer protection against radiation induced DNA damage.

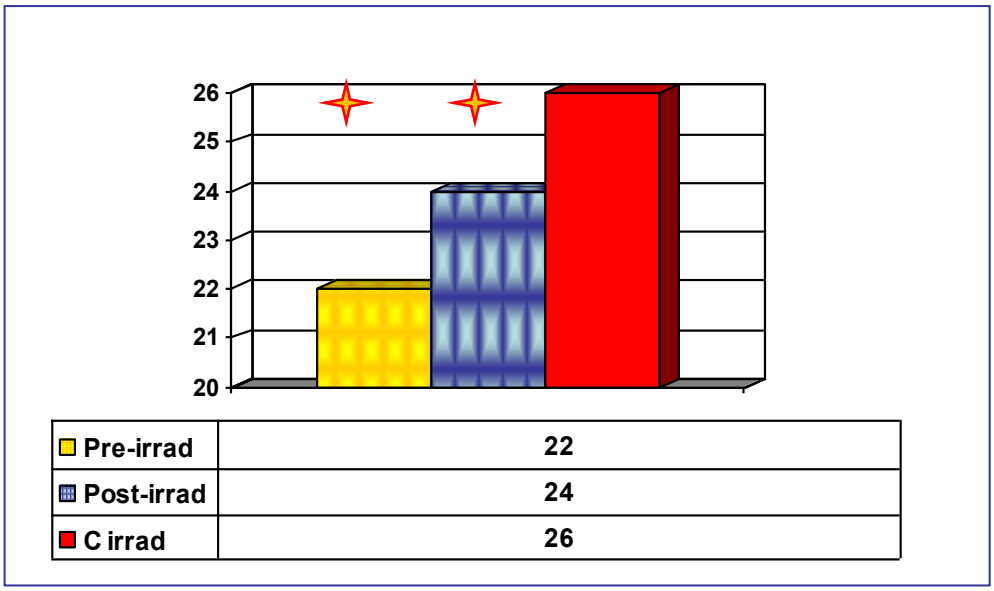


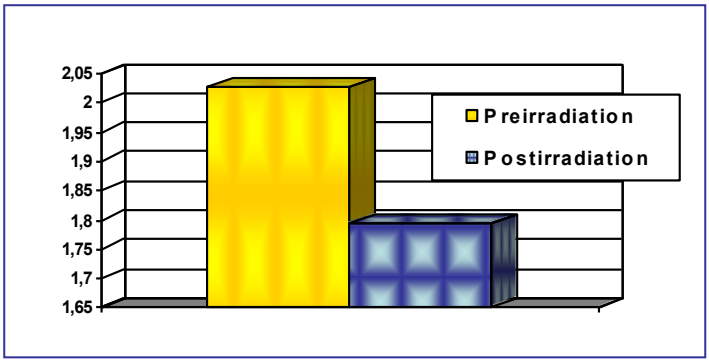
Figure CB24. Frequency of Micronuclei (CBMN) in human peripheral blood samples treated with Rutin (+: (p<0.01) versus irradiated contol cells)

6. The **Protection Factor (PF)** obtained for samples treated with Rutin **before irradiation** was 15.38%, but **post-irradiation** Rutin treated samples had a PF of 7.69% (Figure CB24; Table CB23).

PF (%)	
Pre-irradiation	**Post-irradiation**
15.38	7.69

Table CB23. Factor of Protection obtained when human peripheral blood samples were treated with Rutin

7. The **Dose Reduction Factor (DRF**) obtained for the samples treated with Rutin **before exposure to irradiation** was 2.027 while the **post-irradiation Rutin** treated samples expressed a DRF of 1.793 (Figure CB25; Table CB24).



DRF	
Pre- irradiation	**Post-irradiation**
2.027	1.793

Figure CB25; Table CB24. Dose Reduction Factor obtained when human peripheral blood samples were treated with Rutin

- **DIMETHYLSULFOXIDE TREATMENT (DMSO)**

1. **DMSO-**treated samples expressed a frequency of distribution of MN ranging between 10 and 12 MN/500 BC with an average of 11 MN/500 BC. This can be interpreted to mean that there is no significant difference between this and the non-irradiated control samples whose average frequency of occurrence of MN was 10 MN/500 BC.

2. In the samples treated with **DMSO prior to exposure to radiation,** a frequency of MN ranging between 12 and 34 MN/500 BC with an average frequency of appearance of 20 MN/500 BC was determined. This showed a significant increase (**$p < 0.001$**) in the frequency of MN over the non-irradiated control samples treated with the same substance but whose average frequency of occurrence of MN was 11 MN/500 BC.

3. In samples treated with **DMSO after exposure to radiation**, the frequency of distribution of MN ranged between 11 and 32 MN/500 BC with an average frequency of appearance of 25 MN/500 BC. This represents a significant increase (**$p < 0.001$**) in the frequency of MN over the non-irradiated control samples treated with the same substance but whose average frequency of occurrence of MN was 11 MN/500 BC.

4. In the samples treated with **DMSO prior to radiation exposure**, an average frequency of occurrence of 20 micronuclei per 500 binucleated cells was observed. Statistical analysis of the frequency of MN in the samples pretreated with DMSO before exposure to irradiation reflects no significant differences in the occurrence of MN in comparison with the irradiated controls samples (26 MN/500 BC). This means that treatment of the samples with DMSO before exposure to

irradiation does not decrease the expected frequency of occurrence of MN and therefore does not offer protection against radiation induced DNA damage.

5. In samples treated with **DMSO after exposure to radiation** an average frequency of occurrence of 25 MN/500 BC was determined. Statistical analysis conducted on the frequency of occurrence of MN in the samples irradiated before subsequent treatment with DMSO reflected no significant differences in comparison with the irradiated control samples (26 MN/500 BC). This revelation means that post-irradiation treatment with DMSO does not decreases the expected frequency of MN occurrence and thus, does not offer protection against radiation induced chromosome damage.

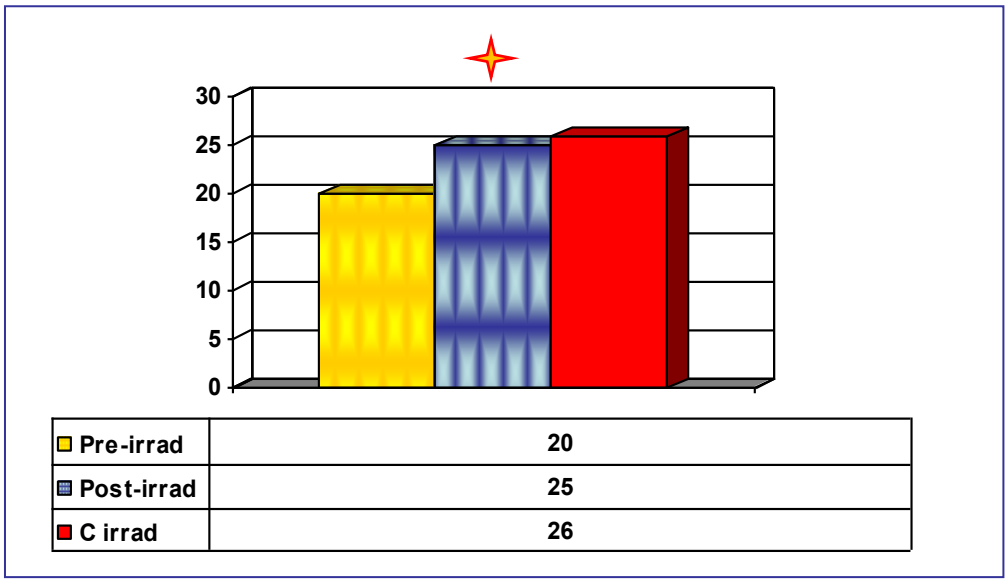


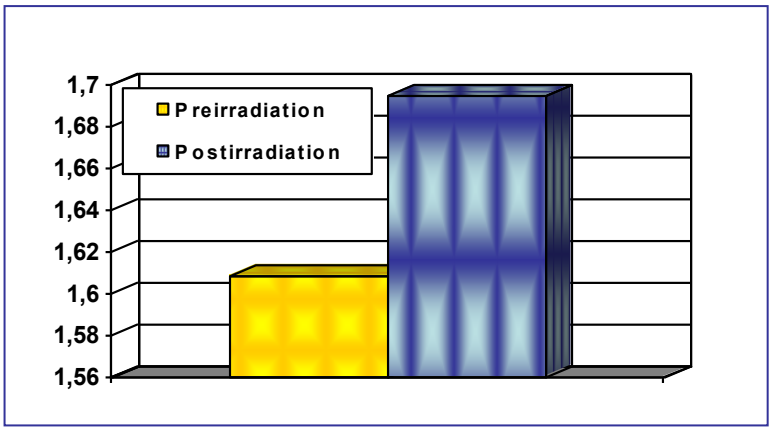
Figure CB 26. Frequency of Micronuclei (CBMN) in human peripheral blood samples treated with DMSO (++: (p<0.01) versus irradiated contol cells)

6 The **Protection Factor (PF)** of the samples treated with DMSO **before exposure to irradiation** ranged from a minimum of -17.69% to a maximum of 53.85%, however, the PF of the samples treated with DMSO **after radiation exposure** ranged between a minimum of -23.08% and a maximum of 57.69%. When different concentrations of DMSO were used, the average pre- and post-irradiation MN frequency values obtained were 26 and 25 MN/500 BC respectively. Therefore, an average pre-irradiation PF of 0% was obtained while the mean post-irradiation PF was 3.85% (Figure CB26; Table CB25).

PF (%)	
Pre-irradiation	**Post-irradiation**
23.1	**3.85**

Table CB25. Protection Factors obtained when human peripheral blood samples were treated with DMSO

7. The **Dose Reduction Factor (DRF)** obtained for the pre-irradiation DMSO treated samples was 1.608 while the post-irradiation DMSO treated samples gave a DRF value of 1.695 (Figure CB 27; Table CB26).



DRF	
Pre-irradiation	**Post-irradiation**
1.608	1.695

Figure CB27; Table CB26. Dose Reduction Factors obtained when human peripheral blood samples were treated with DMSO

- ERIODICTYOL TREATMENT (ER)

1. Samples treated with **eriodictyol** expressed a MN frequency ranging between 8 and 10 MN/500 BC with an average of 9 MN/500 BC. This shows that there is no significant difference between MN frequencies in this sample lot in comparison with the non-irradiated control samples whose average MN frequency of occurrence was 10 MN/500 BC.

2. Samples treated with **eriodictyol before exposure to radiation** expressed a MN frequency distribution ranging between 30 and 33 MN/500 BC with an average frequency of 31 MN/500 BC. This represents a significant increase (**p <0.001**) in the frequency of MN over the non-irradiated control samples treated with the same substance but whose average frequency of occurrence of MN was 9 MN/500 BC.

3. Samples treated with **eriodictyol after exposure to radiation** expressed a MN frequency ranging between 29 and 35 MN/500 BC with an average frequency of appearance of 32 MN/500 BC. This represents a significant increase (**p <0.001**) in the frequency of MN over the non-irradiated control samples treated with the same substance but whose average frequency of occurrence of MN was 9 MN/500 BC.

4. Samples treated with **eriodictyol before exposure to radiation** expressed an average frequency of occurrence of 31 micronuclei per 500 BC. Statistical analysis conducted on the frequency of occurrence of MN in samples pre-treated with eriodictyol before exposure to irradiation showed that there was no significant differences between these and the irradiated control samples (26 MN/500 CB). This suggests that pre-irradiation treatment with eriodictyol does not decrease the expected MN frequency, therefore does not offer protection against radiation induced DNA damage.

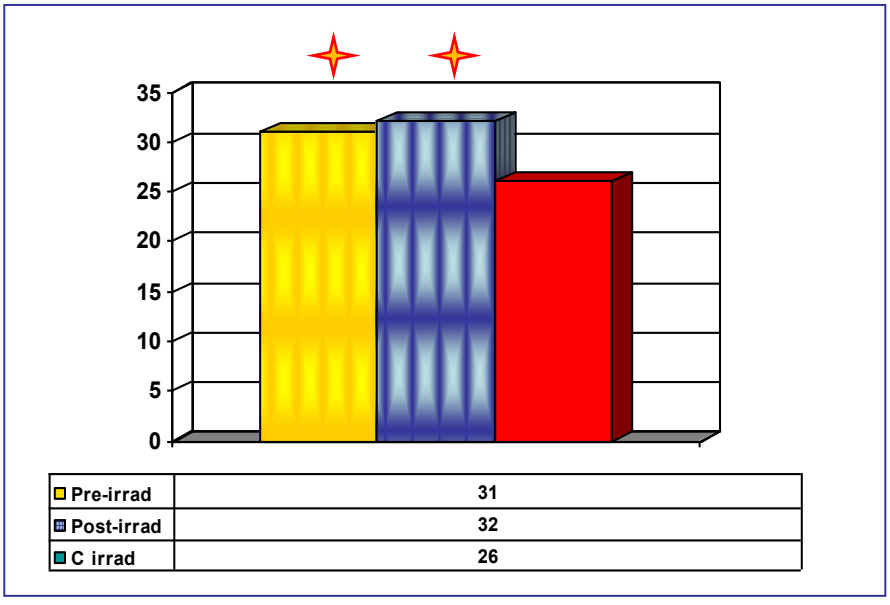


Figure CB28. Frequency of Micronuclei (CBMN) in human peripheral blood samples treated with Eriodictyol (+: (p<0.01) versus irradiated contol cells)

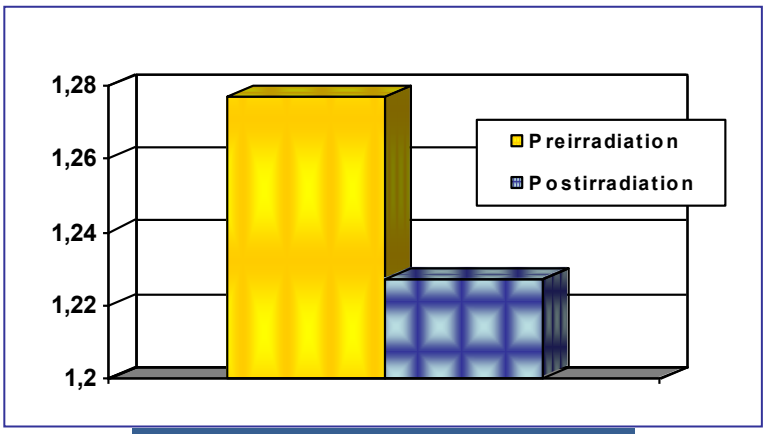
5. Samples treated with **eriodictyol after exposure to radiation**, expressed an average frequency of occurrence of 32MN per 500 BC. Analysis conducted on the frequency of MN occurrence in the samples irradiated before subsequent treatment with eriodictyol reflects no significant differences with respect to irradiated control samples (26MN/500 BC). This means that post-irradiation treatment with eriodictyol does not decrease the expected frequency of occurrence of MN and therefore does not offer protection against radiation induced chromosome damage.

6. The **Protection Factor (PF)** of the samples treated with eriodictyol before irradiation was -19.23%, however, when samples were treated with eriodictyol after exposure to radiation PF of -23.08% was realized (Figure CB28; Table CB27).

PF (%)	
Pre-irradiation	**Post-irradiation**
-19.23	-23.08

Table CB27. Protection Factors obtained when human peripheral blood samples were treated for Eriodictyol

7. The **Dose Reduction Factor (DRF)** of samples treated with eriodictyol before exposure to irradiation was 1.277 while post-irradiation DRF was 1.227 (Figure CB29; Table CB28).



DRF	
Pre-irradiation	**Post-irradiation**
1.277	1.227

Figure CB29; Table CB28. Dose Reduction Factor obtained when human peripheral blood samples were treated with Eriodictyol

- **QUERCETIN TREATMENT (Q)**

1. Samples treated with **Quercetin** expressed a MN frequency ranging between 9 and 11 MN/500 BC with an average of 10 MN/500 BC. This is an indication of a non-significant difference in the occurrence of MN between these samples and the non-irradiated control samples whose average frequency of occurrence of MN was 10 MN/500 BC.

2. Samples treated **Quercetin before exposure to radiation** expressed a MN frequency that ranged between 25 and 38 MN/500 BC with an average frequency of appearance of 35 MN/500 BC. This represented a significant increase (**p <0.001**) in the frequency of occurrence of MN over the non-irradiated control samples treated with the same substance but whose average frequency of occurrence of MN was 10 MN/500 BC.

3. Samples treated with **Quercetin after exposure to** radiation expressed a MN frequency of between 30 and 36 MN/500 BC with an average frequency of appearance of 33 MN/500 BC. This represents a significant (**p <0.001**) increase in the frequency of MN over the non-irradiated control samples treated with the same substance but whose average frequency of occurrence of MN was 10 MN/500 CB.

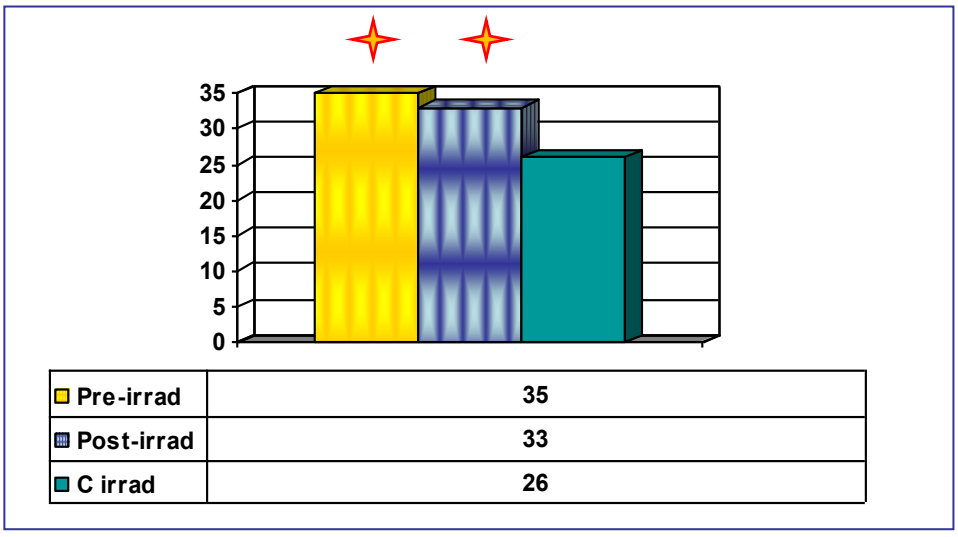


Figure CB30. Frequency of Micronuclei (CBMN) in human peripheral blood samples treated with Quercetin (+): (p<0.01) versus irradiated contol cells)

4. Samples treated with **Quercetin before exposure to radiation** expressed an average MN frequency of occurrence of 35 micronuclei per 500 BC. Statistical analysis of the frequency of MN in the samples pretreated with Quercetin before exposure to irradiation indicated that there is no significant difference between this and the irradiated control sample (26 MN/500 CB). This means that pre-irradiation treatment with Quercetin does not decrease the expected frequency of occurrence of MN and therefore offer no protection against radiation induced DNA damage.

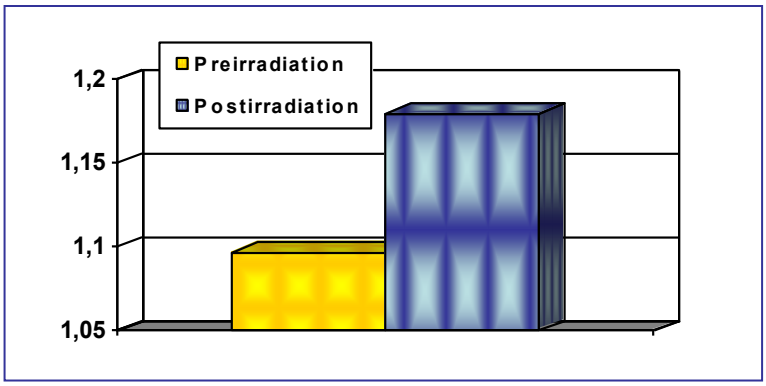
5. Samples treated with Quercetin **after exposure to radiation** expressed an average MN frequency of occurrence of 33 MN/500 BC. Statistical analysis of the frequency of MN in the samples irradiated before subsequent treatment with Quercetin demonstrate no significant differences with respect to irradiated control samples (26 MN/500CB). This suggests that post-irradiation treatment with Quercetin does not decrease the expected frequency of occurrence of MN in the samples and thus do not offer protection against radiation induced DNA damage.

6. The **Protection Factor (PF**) of the samples treated with Quercetin before irradiation was -34.61%, however, samples treated with Quercetin after exposure to radiation had a PF of -26.92% (Figure CB 30; Table CB29).

PF (%)	
Pre-irradiation	**Post-irradiation**
-34.61	-26.92

Table CB29. Protection Factors obtained when human peripheral blood samples were treated with Quercetin

7. The **Dose Reduction Factor (DRF)** of pre-irradiation Quercetin treated sample was 1.09 while post-irradiation Quercetin treated samples had a DRF of 1.18 (Figure CB31; Table CB30).



DRF	
Pre-irradiation	**Post-irradiation**
1.097	1.180

Figure CB31;Table CB30. Dose reduction Factors obtained when human peripheral blood samples were treated with Quercetin

- **ZOLEDRONIC ACID TREATMENT (Z).**

2. In the samples treated with Z expressed a MN frequency that ranged from 28 to 31 MN /500 BC with an average of 29 MN/500 CB. This observation shows that there is no significant difference in the MN frequency of occurrence in this group and that observed in the non-irradiated control samples whose average frequency of occurrence of MN was 10 MN/500 BC.

3. In the samples treated with **Z** before exposure to radiation, an average MN frequency of occurrence of 19 MN/500 CB was established. This represents a significant increase (**p <0.01**) in the frequency of appearance of MN over the non-irradiated control samples treated with the same substance and whose average frequency of occurrence of MN was 9 MN/500 CB.

4. Samples treated with **Z** after exposure to radiation expressed an average MN frequency of occurrence of 17 MN/500 BC. This represents a significant increase (**p <0.05)** in the frequency of appearance of MN over the non-irradiated control samples treated with the same substance and whose average frequency of occurrence of MN was 9 MN/500 BC.

5. The administration of pure Z (100%) and at the clinically recommended dose of 5% Z (Z5%) induced a significant increase in the frequency of MN/500 BC (**p<0.001**) when compared with the controls, which represents a genotoxic effect induced by Z. This chromosome damage compared favourably with chromosome damage caused by exposure of cells to 2 Gy of X-rays. Thus, given that the test quantifies the degree of chromosome damage induced by mutagenic agents, Z could be described as having a mutagenic capacity similar to that of 2 Gy of X-rays.

6. The administration of **Z** before exposure to X-rays showed no significant differences in the MN frequency of occurrence when compared with the samples treated with Z alone, showing that there is no additive or synergic effect in this situation. However, the administration of Z after irradiation led to a significant increase in the number of MN compared with the samples treated with Z alone or treated with Z prior to radiation (**p<0.01**). This reflects a greater genotoxic potential of Z when it is administered immediately after irradiation than when administered under the other treatment modalities described (Figure CB32).

7. The is an increase in sensitization (FS) produced during a combined treatment of IR + Z immediately after irradiation which manifest a value of about 35.5%

PROTECTION FACTORS (PFs)

1. Administration of substances before irradiation: Protection Factors

The highest protection factor (PF) values for the pre-irradiation treated samples showed the Rosmarinic acid treated group with the highest radioprotective capacity of 57.69% followed by the P90 treated group with a PF of 53.85%, then samples treated with carnosic acid, apigenin dissolved in DMSO and vitamin E which all presented with a PF of 50%.

Next in the series of pre-irradiation treated substances that offered relatively high PFs were samples treated with Carnosol which gave a PF of 46.15%. CE, Vitamin C and Amifostine all yielded a PF of 38.46% while PASE (Q52) produced a PF 26.92%, Green Tea Extract showed almost the same PF with a value of 23.08%, while Rutin portrayed a PF of 15.38%.

The Eriodictyol and Quercetin treated groups reordered negative protection factor values since treatment with these samples significantly increased the micronuclei frequency with respect to MN frequencies in the control groups (PFs of -19.23 and -34.61 respectively). A **significant increase in the frequency** of MN was recorded when the samples were treated with Zoledronic acid which showed a complete lack of protective capacity (Table CB30).

2 Administration of substances after irradaiation: Protection factors.

When samples were treated with the test-substances after exposure to irradiation, Carnosol showed the highest PF of 46.15% followed by carnosic acid with 42.31% and then Apigenin dissolved in DMSO and P90 both with a PF of 38.46%. Following closely behind these were samples treated with PASE and Vitamin E with a PF of 34.61% and CE with a PF of 26.92%. Continuing in descending order, was Vitamin C with 19.23%, then Rutin, Rosmarinic acid and TE all expressing a PF of 7.69%. DMSO showed a slight post-irradiation protective effect with a PF of 3.85%. Samples treated with Amifostine, eriodictyol and Quercetin showed negative PF values of -3.85, -23.08- and -26.92% respectively. Zoledronic acid gave the highest frequency of MN occurrence and showed a complete lack of protective capacity. (Table CB30).

Table CB30. Protection factors obtained when human peripheral blood samples were treated with the various test substance.

TEST-SUBSTANCE	PF (%)	
	Pre-irradiation	Post-irradiation
Rosmarinic acid	57.69	7.69
P90	53.85	38.46
Carnosic acid	50	42.31
Apigenin-DMSO	50	38.46
Vitamin E	50	34.61
Carnosol	46.15	46.15
CE	38.46	26.92
Vitamin C	38.46	19.23
Amifostine	38.46	-3.85
Green Tea Extr. (TE)	23.08	7.69
Rutin	15.38	7.69
PASE	26.92	36.61
DMSO	0	3.85
Eriodictyol	-19.23	-23.08
Quercetin	-34.61	-26.92
Zoledronic acid	0	- 35.7

DOSE REDUCTION FACTORS (DRFs)

1. **Administration of substances before irradiation: Dose Reduction Factors**

Highest pre-irradiation treatment values of Dose Reduction Factors (DRF) was shown in groups treated with **Rosmarinic Acid** which registered a DRF value of 7.18, the **P90** treatment group with a DRF of 5.833, followed by **carnosic acid, apigenin dissolved in DMSO and Vitamin E** all with a DRF of 4.91. Very close to these substances and with a DRF value of 4.24 was **Carnosol** which was also followed closely by samples treated with **CE, Vitamin C** and **Amifostine** with DRF of 3.331.

PASE, TE with a DRF value of 2.331 were next in sequence followed by **Rutin** with a DRF value of 2.027 and then DMSO came next with a DRF of 1.608. The worse pre-irradiation treatment dose reduction performers of this study were **eriodictyol** and **quercetin** which produced DRFs values of 1.277 and 1.097 respectively (Table CB31).

2. **Administration of substances after irradiation: Dose Reduction Factors**

Carnosol and Carnosic acid gave the highest post-irradiation treatment DRF values of 4.240 and 3.731 respectively while **P90** and **Apigenin dissolved in DMSO** both expressed a DRF value of 3.331 which was followed sequentially by **Vitamin E**, **CE and Vitamin C** with post-irradiation treatment DRF values of 3.009, 2.52 and 2.169 respectively. Continuing in descending order of DRF values which fell between 1.5 and 2 was most of the remaining substances, including: **Rutin, and rosmarinic acid**, Green **Tea Extract**, **DMSO** and **Amifostine** and **PASE**. Finally **eriodictyol and quercetin** produced post-irradiation DRF values of 1.227 and 1.180 respectively (Table CB31).

Table CB31. Dose Reduction Factors obtained when when human peripheral blood samples were treated with the various test substance.

TEST SUBSTANCE	DRF	
	Pre-irradiation	Post-irradiation
Rosmarinic acid	7.18	1.793
P90	5.833	3.331
Carnosic Acid	4.91	3.731
Apigenin-DMSO	4.91	3.331
Vitamin E	4.91	3.009
Carnosol	4.24	4.24
CE	3.331	2.52
Vitamin C	3.331	2.169
Amifostine	3.331	1.528
PASE	2.52	3.009
Green Tea Extr. (TE)	2.331	1.793
Rutin	2.027	1.793
DMSO	1.608	1.695
Eriodictyol	1.277	1.227
Quercetin	1.097	1.180
Zoledronic acid	-	-

RESULTS OF STATISTICAL ANALYSIS IN THE MNCB ASSAY

1. Control Substances

1. Statistical analysis has established a positive relationship between radiation dose and the frequency of micronuclei occurrence through the linear relationship ($y = \alpha + \beta D$) with a linear correlation coefficient $r^2 = 0.9974$.

2. Irradiated control blood samples showed a significant (**$p < 0.001$**) increase in micronuclei relative to non-irradiated controls samples. This means that the

administration of ionizing radiation significantly increases in the frequency of micronuclei.

3. Non-irradiated blood samples administered with **Rosmarinic Acid, P90, carnosic acid, apigenin dissolved in DMSO, Vitamin E, Carnosol, CE, Vitamin C, Amifostine, Green Tea Extract, Rutin, DMSO, eriodictyol and Quercetin** do not present significant differences in the frequency of occurrence of MN with respect to non-irradiated control samples, which means that the administration of various substances do not cause chromosomal damage with respect to the control samples.

2 Administration of substances before irradiation.

4. Blood samples treated with **Rosmarinic Acid, P90, carnosic acid, apigenin dissolved in DMSO, Vitamin E, P90, Carnosol, CE-50, vitamin C,** and **Amifostine**, before exposure to irradiation showed a significant decrease (**$p < 0.001$**) in the frequency of occurrence of micronuclei with respect to irradiated control samples. This means that the treatments carried out decreases the expected frequency of occurrence of micronuclei as a result of the irradiation and therefore present some level of protection against chromosomal damage induced by ionizing radiation (Figures CB32).

5. Blood samples treated with **PASE, Green Tea Extract and Rutin** before irradiation showed a significant decrease (**$p < 0.05$**) in the frequency of micronuclei compared with the irradiated control samples. This means that the treatments decrease the expected frequency of occurrence of micronuclei as a result of irradiation and therefore afford some protection against chromosomal damage induced by ionizing radiation.

6. Blood samples treated with **eriodictyol, Quercetin and zoledronic acid** before irradiation showed no significant decrease in the frequency of micronuclei with respect to irradiated control samples. This means that the treatment with the substances before exposure to radiation produces no significant changes in the appearance of micronuclei expected as a result of irradiation of the samples and therefore possesses no protection against chromosomal damage induced by radiation ionizing.

3 Administration of substances after irradiation.

7. Blood samples treated with **P90, carnosic acid, apigenin dissolved in DMSO, Vitamin E and Carnosol** after radiation exposure showed a significant decrease (**$p < 0.001$**) in the frequency of micronuclei with respect to the irradiated control samples. This means that the treatments meted out decreases the expected frequency of occurrence micronuclei as a result of irradiation and therefore present some degree of protection against chromosomal damage induced by ionizing radiation.

8. Blood samples treated with **CE, vitamin C**, and **PASE** after radiation exposure showed a significant decrease ($p < 0.05$) in the frequency of occurrence of micronuclei with respect to irradiated control samples. This means that the treatments delivered decreases the expected frequency of occurrence of micronuclei as a result of irradiation and therefore present some protection against chromosomal damage induced by ionizing radiation.

9. Blood samples treated with **Rosmarinic acid, amifostine, Green Tea Extract, Rutin, DMSO, eriodictyol, Quercetin** and **zoledronic acid** after radiation exposure showed no significant decrease in the frequency of occurrence of micronuclei with respect to the irradiated control samples. This means that treatment with substances 5 minutes after the irradiation of the samples did not produce significant changes in the expected frequencies of appearance of micronuclei as a result of irradiation and therefore possess no protection against damage induced chromosomal ionizing radiation.

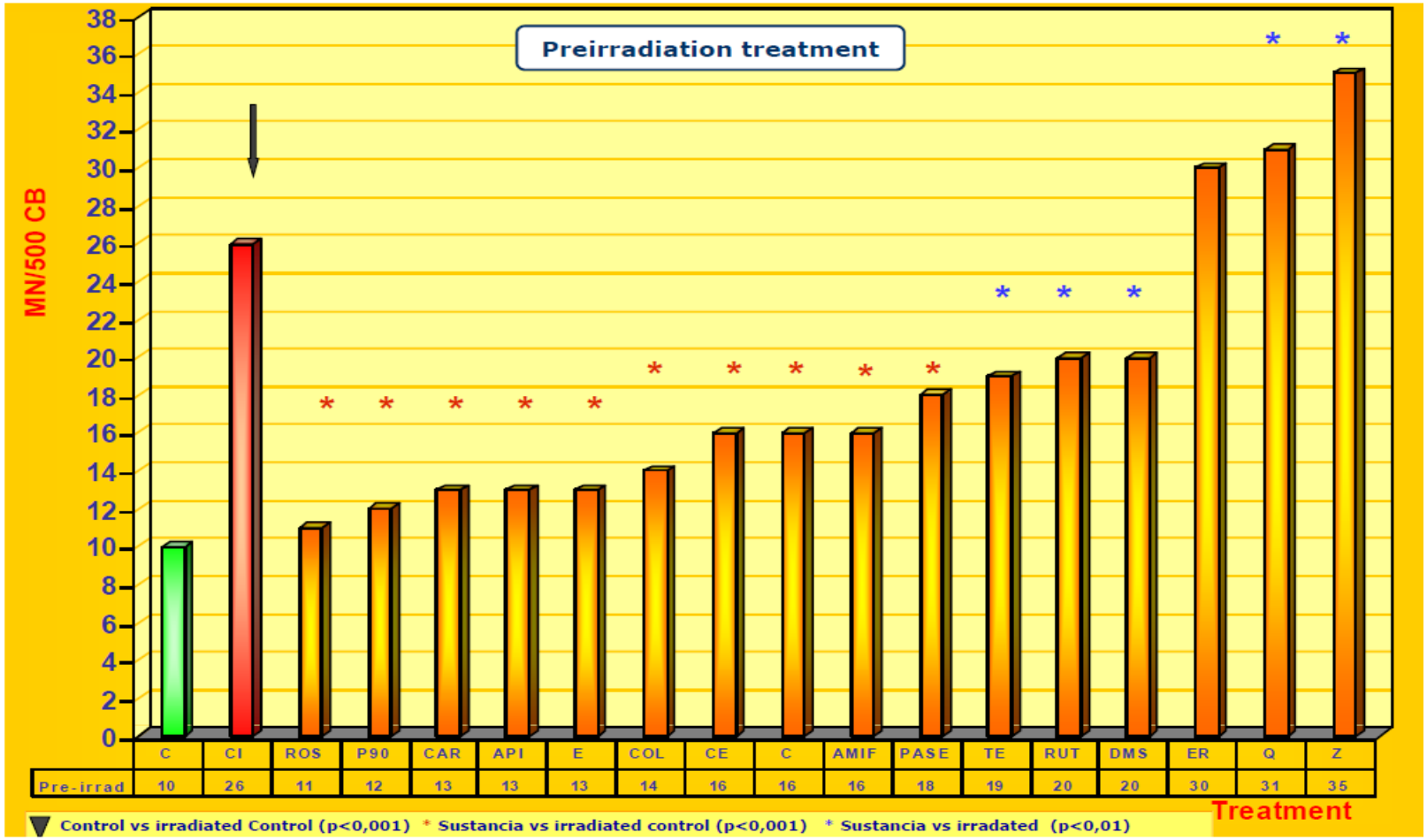


Figure CB32. Frequency of Micronuclei (CBMN) in humaan peripheral blood treated with the test substances prior to irradiation (preirradiation treatment) ((*): (p<0.001) versus irradiated contol cells; ((*): (p<0.01) versus irradiated contol cells).

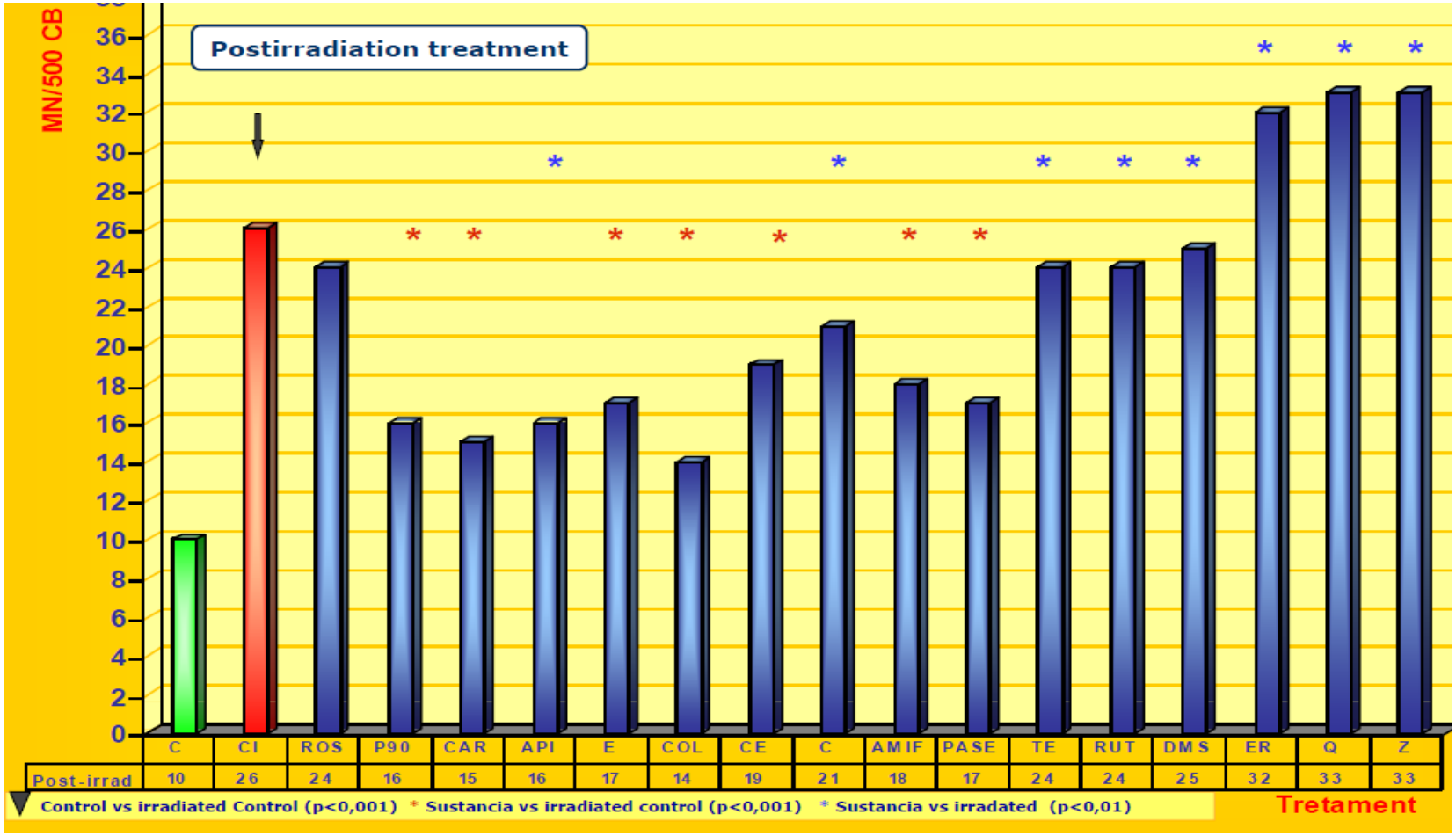


Figure CB33. Frequency of Micronuclei (CBMN) in human peripeheral blood samples treated with the test substances after irradiation (post-irradiation treatment) ((*): (p<0.001) versus irradiated contol cells; ((*): (p<0.01) versus irradiated contol cells).

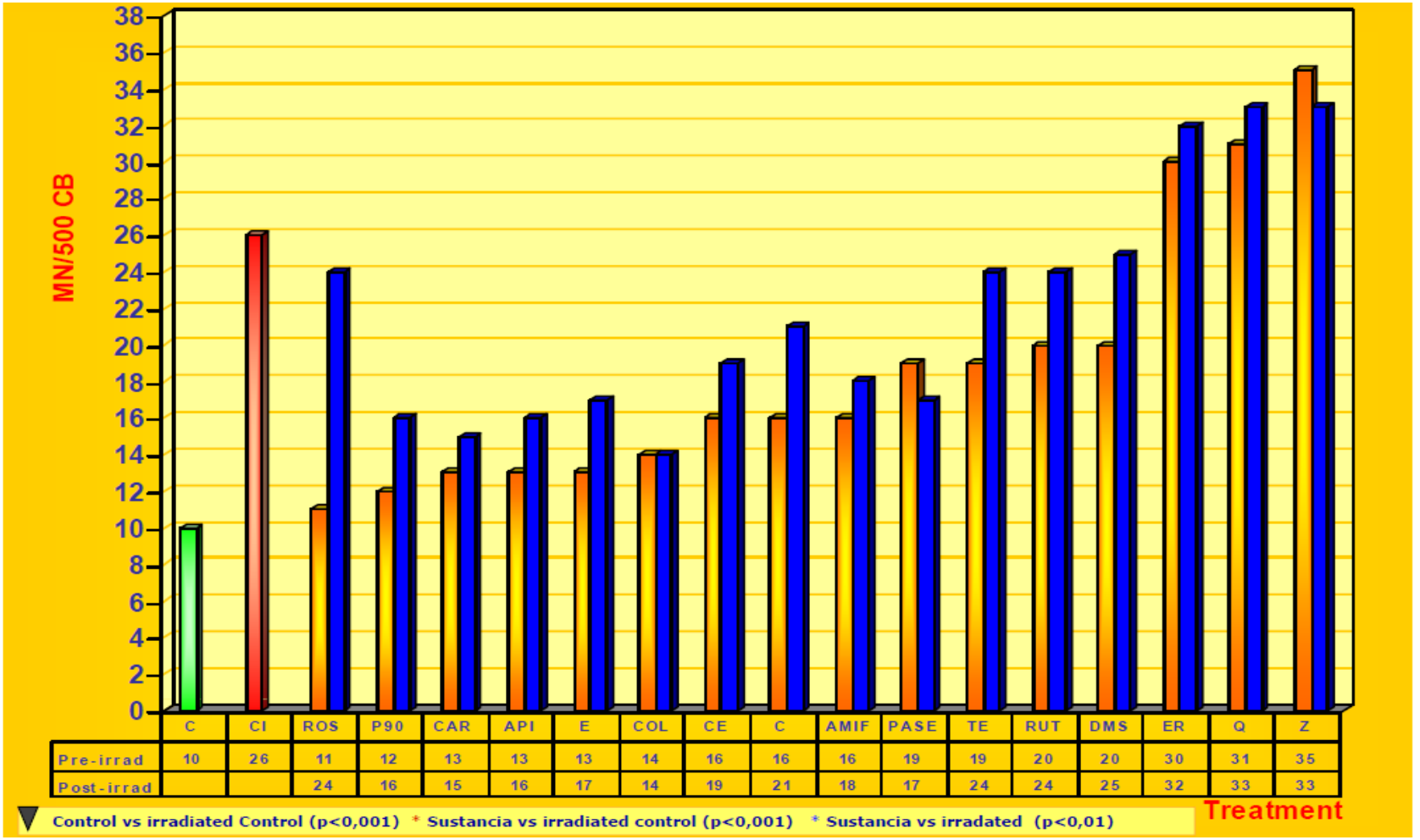


Figure CB34. Frequency of Micronuclei (CBMN) in human peripheral blood treted with the test substances before- and after irradiation

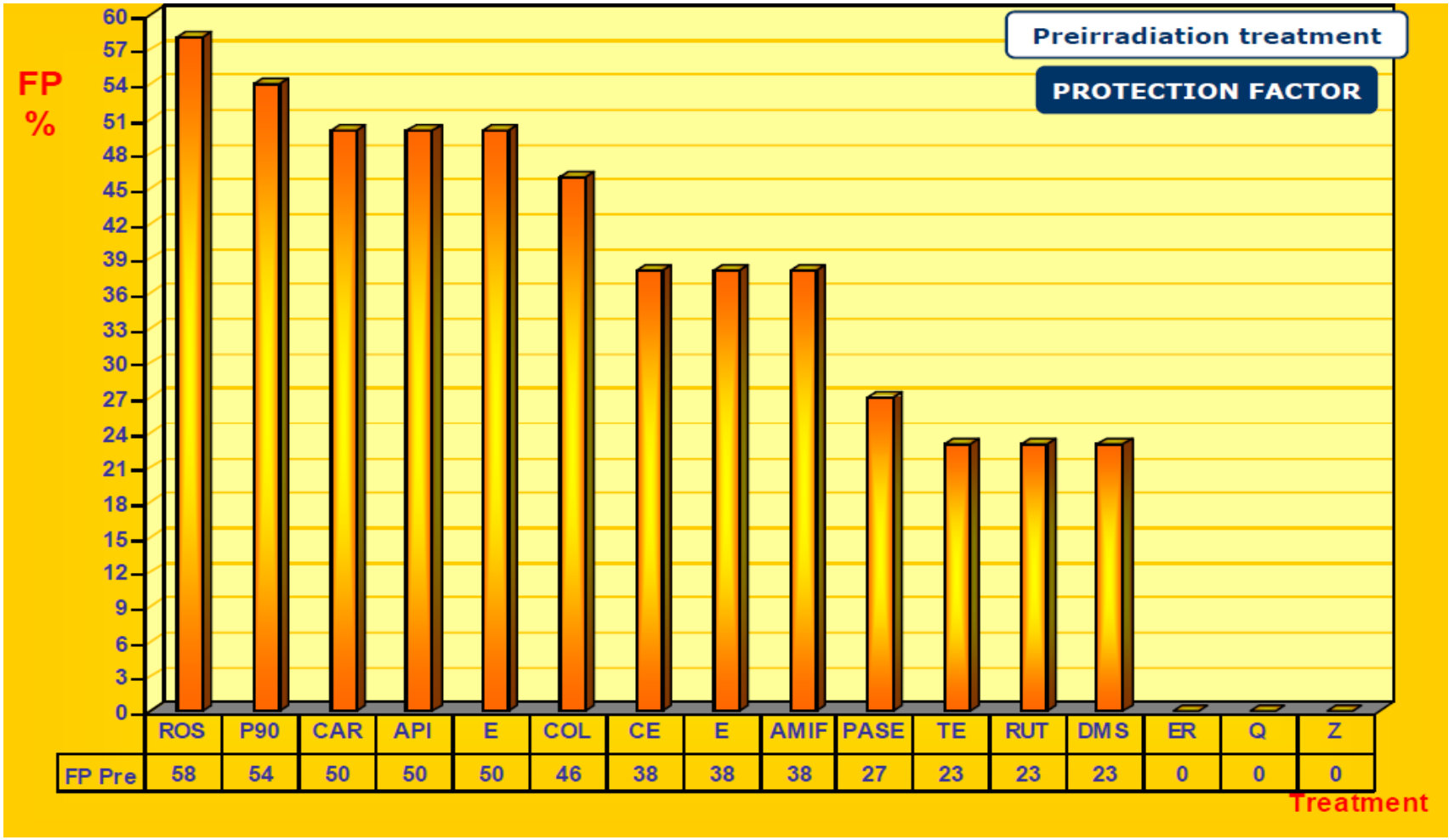


Figure CB35. Magnitude of Protection (Protection factor) measured in human peripeheral blood treted with the test substances before irradiation (pre-irradiation treatment)

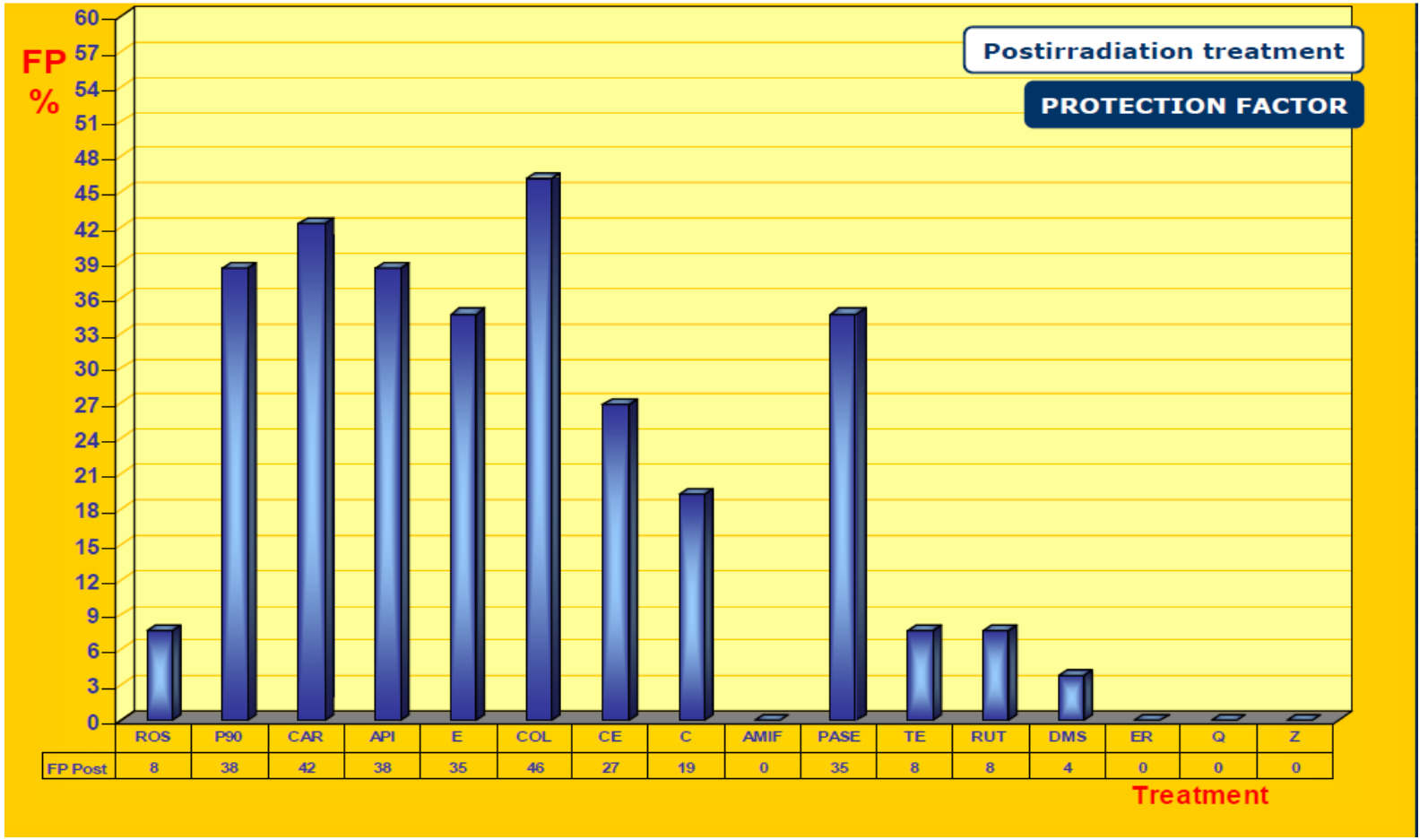


Figure CB36. Magnitude of Protection (Protection factor) measured in human peripheral blood treated with the test substances after irradiation (post-irradiation treatment)

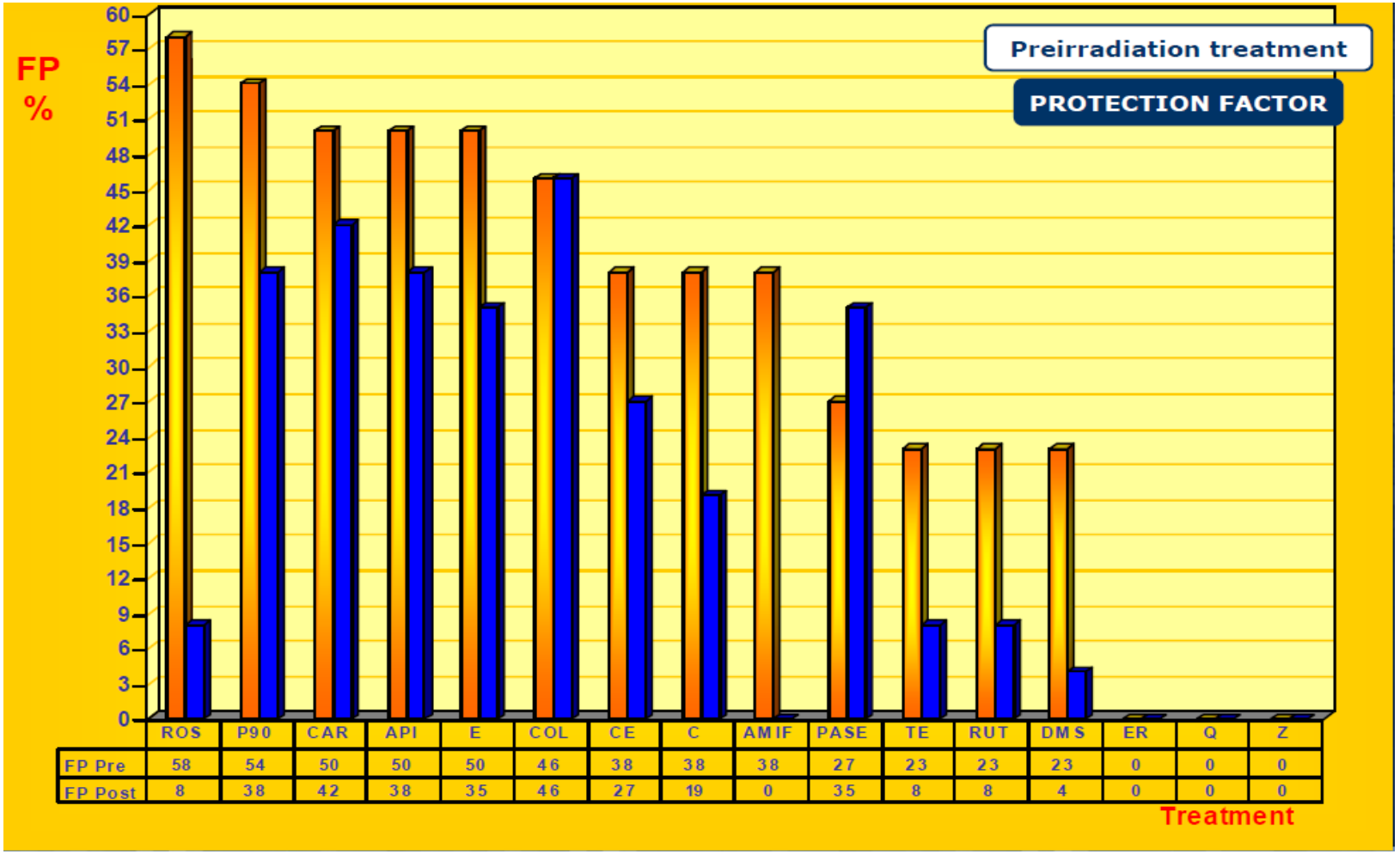


Figure CB37. Magnitude of Protection (Protection factor) measuerd in human peripehral blood treated with the test substances before and after irradiation (pre- and post-irradiation treatment)

V.3.Results.
MICRONUCLEUS ASSAY IN MOUSE BONE MARROW POLYCHROMATIC ERYTHROCYTES (MNPCEs)

V. 3. Results
MOUSE BONE MARROW MICRONUCLEUS ASSAY

The results of micronucleated polychromatic erythrocytes (MNPCEs) in bone marrow cells of animals treated with test substances before exposure to ionizing radiation are presented below.

CONTROL GROUPS

Animals in the non-irradiated control group exhibited a frequency of micronuclei ranging between a minimum of 1 and maximum of 5 MN/1000 PCEs with a mean of 3.1 MN/1000 PCEs. This value corresponds to the background frequency or frequency of spontaneous occurrence on MN in the bone marrows of animals under study (Figure PCE1).

The analysis of results obtained in batches of animals that were treated with the test substances but not exposed to radiation (non-irradiated) revealed no significant differences in the frequency of occurrence of MN/1000 PCEs with respect to the non-irradiated control animal batch, which can be seen as a non-genotoxic effect expressed by THE test substances administered (Figure PCE1).

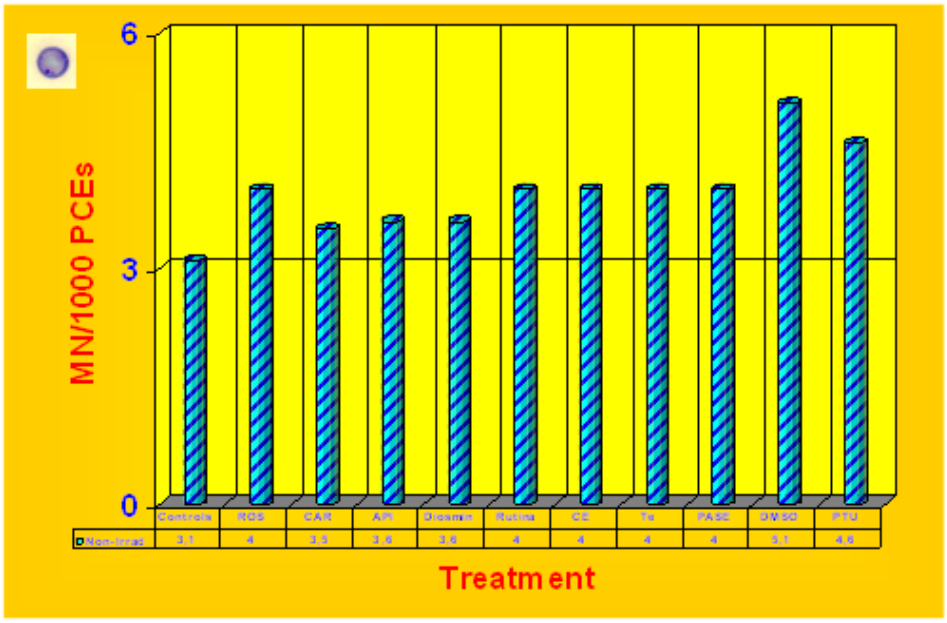


Figure PCE1. Frequency of MNPCEs in bone marrow cells of non-irradiated mice exposed to various test substances (test substance control)

Control animals irradiated with 500 mGy showed an increase in the occurrence of micronuclei frequency ranging between 17 and 24 MN/1000 PCEs with an average frequency of micronuclei occurrence of 18.7 MN/1000 PCEs (Figure PCE2).

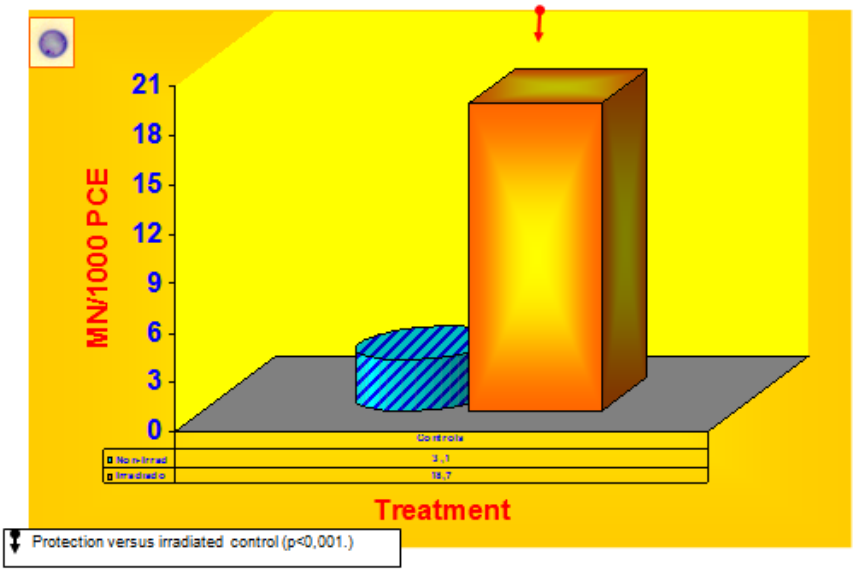


Figure PCE2. Baseline frequency of MNPCEs in irradiated and non- irradiated control animals (control groups) (↓ p< 0.001) versus irradiated control group)

Statistical analysis revealed significant differences in the frequency of MN occurrence between batches of irradiated and non-irradiated animals and show that exposure to ionizing radiation leads to significant increase in the production of micronucleated polychromatic erythrocytes of irradiated animals (**p<0.001**) (Figure PCE2).

Group treated with *Pynanthus angloensis* Seed Extract (PASE)

Non-irradiated animals treated with **PASE** expressed a micronucleus frequency ranging between 4 and 5 MN/1000 PCEs and an average frequency of 4 MN per 1000 PCEs observed (Figure PCE3).

The batch of animals treated with **PASE** before subsequent exposure to 500 mGy of X-ray irradiation expressed a MN frequency of between 12 and 15 per 1000 PCEs observed. The mean MN frequency in these set pre-**PASE** treated irradiated mice was 13 MN/1000 PCE (Figure PCE3).

The batch of animals irradiated with 500 mGy before receiving treatment with **PASE** expressed a MN a frequency that ranged between 13 and 15 MN per 1000 PCEs observed. The mean frequency of micronuclei in this cohort that was administered with **PASE** only after X-ray exposure was 14 MN/1000 PCEs (Figure PCE3).

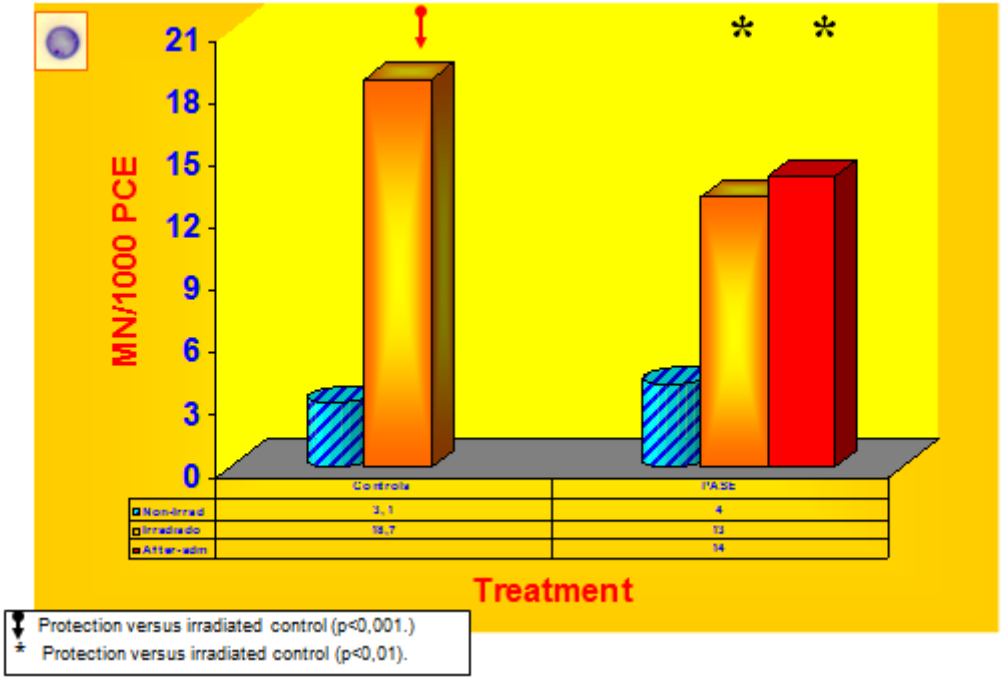


Figure PCE3. Frequency of MNPCEs in pre- and post-irradiation PASE treated mouse bone marrow cells ((*): p<0.01 versus irradiated control group).

Statistical analysis revealed significant differences in the frequency of MNPCEs occurrence between batches of animals treated with **PASE** and the irradiated control group of animals. This observation shows that when treated with **PASE** before or after exposure to ionizing radiation, a significant decrease in the appearance of micronuclei in polychromatic erythrocytes of irradiated animals is produced compared with irradiated control animals (**p<0.01**); suggesting that **PASE** shows genoprotective capacity whether administered prior or after exposure to radiation (Figure PCE3).

A protection magnitude of 30% was obtained when the **PASE** was administered before exposure to radiation and 25% when given immediately after exposure to X-rays (Figure PCE4).

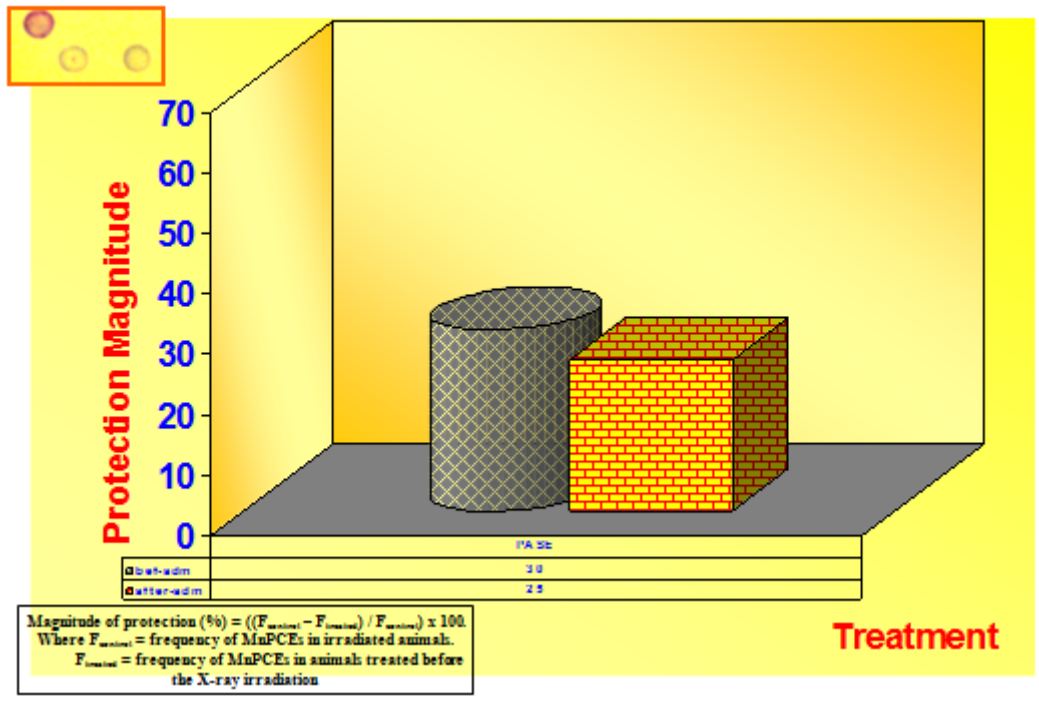


Figure PC4. Magnitude of protection of PASE treatment before and after exposure to 500mGy of X-rays.

Group treated with Rosmarinic acid *(*ROS)

The group of animals treated with ROS but not exposed to irradiation presented a micronucleus frequency ranging between 3 and 6 MN/1000 PCEs and an average frequency of 4 MN/1000 PCEs (Figure PCE 3). However, the batch of animals treated with ROS and subsequently exposed to a radiation dose of 500 mGy presented a frequency of between 6 and 10 MN /1000 PCEs. The mean frequency of MN in the ROS pre-treated irradiated group was 7 MN/1000 PCEs (figure PCE5).

The post-irradiation (500 mGy of X-rays) treated animal lot treated with ROS expressed a MN frequency that ranged between 14 and 18 MN /1000 PCEs. The mean frequency of micronuclei in this lot of animals that received ROS after X-ray exposure was 16 MN/1000 PCE (Figure PCE5).

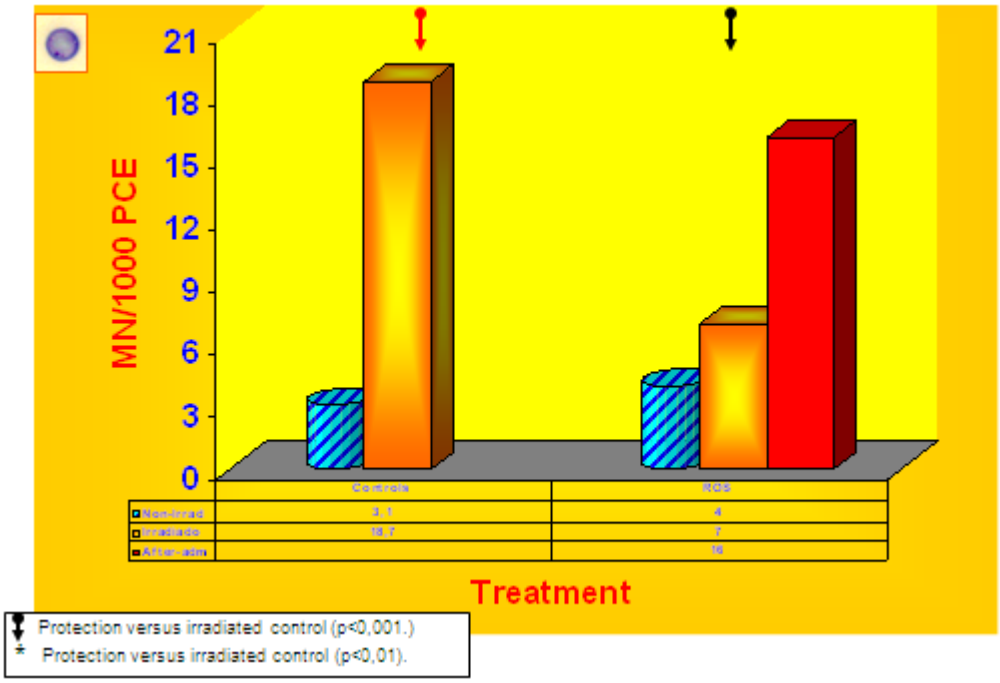


Figure PCE5. Frequency of MNPCEs in pre- and post-irradiation Rosmarinic acid treated mouse bone marrow cells (↓: (p<0.001) versus irradiated control group).

Statistical analysis revealed **significant differences** in the frequency of MNPCEs occurrence between batches of animals treated with ROS before and after irradiation exposure. This observation shows that when treated with ROS before exposure to ionizing radiation, a **significant decrease** in the appearance of micronuclei in polychromatic erythrocytes of irradiated animals is produced compared with irradiated control animals and animals treated with ROS post-irradiation (**p<0.001**); suggesting that RA shows genoprotective capacity only when administered prior to exposure to radiation (Fig PCE5).

A radiation **protection factor** of 62% was obtained when ROS was administered before exposure to radiation and 14% when given immediately after exposure to X-rays (Figure PCE6).

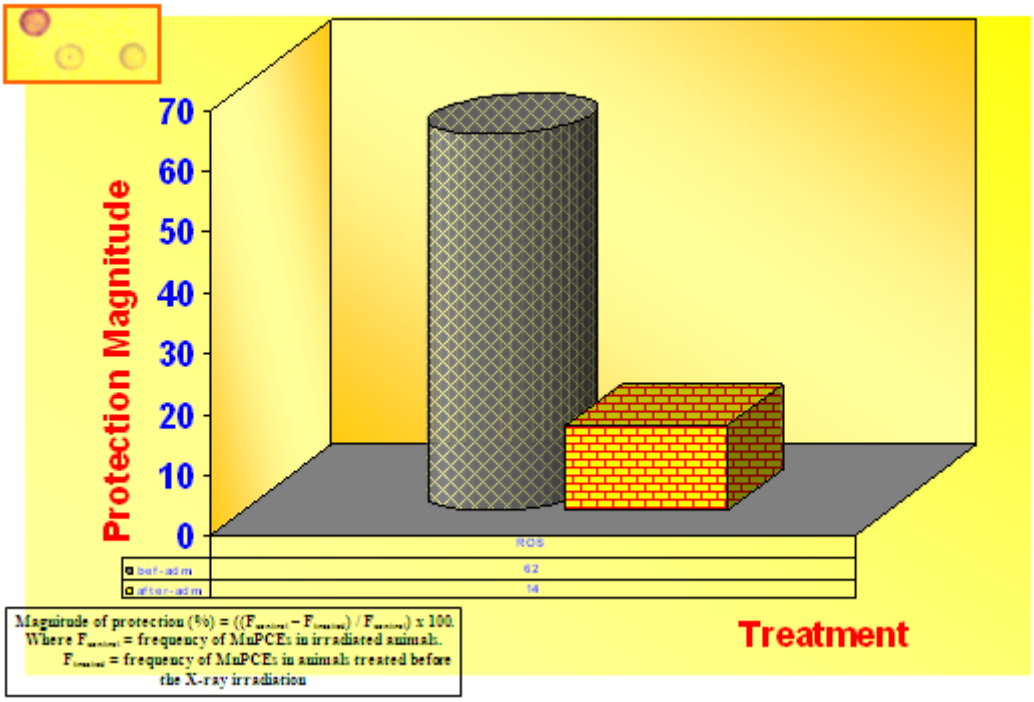


Figure PCE6. Magnitude of protection of Rosmarinic acid (ROS) in animals treated before and after exposure to low dose of X-rays (500mGy).

Group treated with Carnosic Acid (CARN)

Non-irradiated batch of animals treated with CARN expressed a micronucleus frequency ranging between 3 and 5 MN/1000 PCEs and an average frequency of 3.5 MN/1000 PCEs (Figure PCE7).

The pre- CARN treated batch of irradiated (500 mGy) animals expressed MN frequencies ranging between 7 and 10 per 1000 PCEs counted. The mean frequency of micronuclei in this set of irradiated pre-CARN treated animals was 9 MN/1000 PCE (figure PCE7).

The batch of animals exposed to 500 mGy of irradiation before receiving CARN treatment presented a MN frequency of between 9 and 13 per 1000 PCS observed. The mean frequency of micronuclei in this lot of animals that were administered with CARN after X-ray exposure was 11 MN/1000 PCEs (figure PCE7).

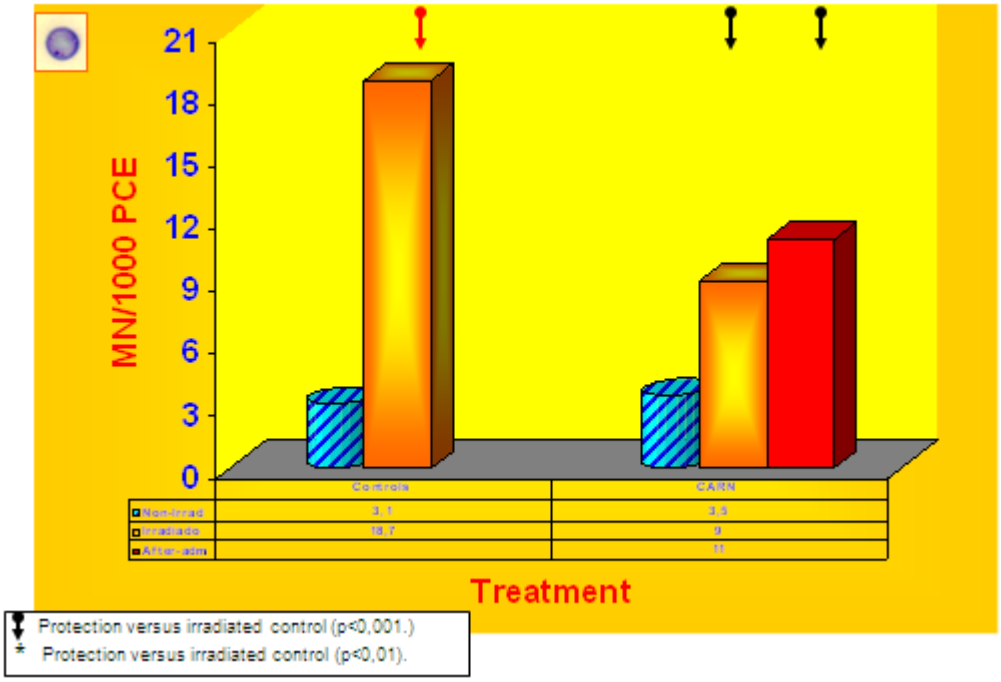


Figure PCE7. Frequency of MNPCEs in pre- and post-irradiation CARN treated mouse bone marrow cells (↓: (p<0.001) versus irradiated control group).

Statistical analysis revealed **significant differences** in the frequency of MNPCEs occurrence between batches of animals treated with CARN and the irradiated control group of animals. This observation shows that when treated with CARN before or after exposure to ionizing radiation, a significant decrease in the appearance of micronuclei in polychromatic erythrocytes of irradiated animals is produced compared with irradiated control animals (**p <0.001**); suggesting that CARN shows genoprotective capacity whether administered prior to or after exposure to radiation (Figure PCE7).

A protection magnitude of 52% is obtained when the CARN is administered before exposure to radiation and 46% when given immediately after exposure to X-rays (Figure PCE8)

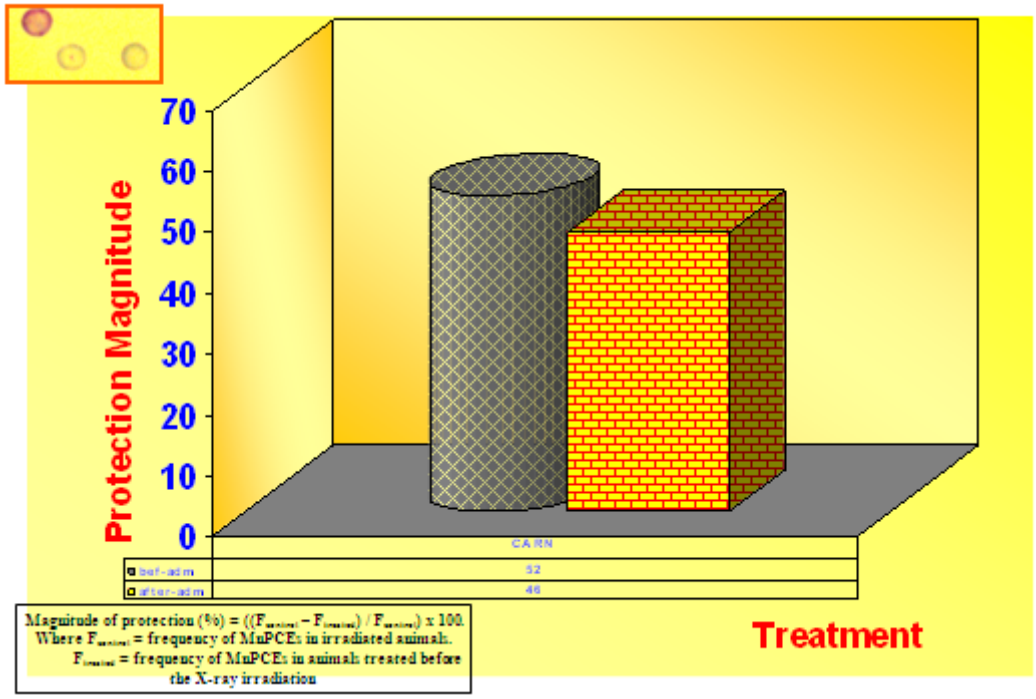


Figure PCE8. Magnitude of protection of CARN in animals treated before and after exposure to 500mGy of X-rays.

Group treated with Apigenin (API)

Non-irradiated animals treated with **API** expressed a micronucleus frequency ranging between 3 and 5 MN/1000 PCEs and an average frequency of 3.6 MN per 1000 PCEs observed (Figure PCE9).

The batch of animals treated with API before subsequent exposure to 500 mGy of X-ray irradiation expressed a MN frequency of between 9 and 13 per 1000 PCEs observed. The mean MN frequency in these set of pre-**API** treated irradiated mice was 11 MN/1000 PCE (Figure PCE9).

The batch of animals irradiated with 500 mGy before being administered with **API** expressed a MN a frequency that ranged between 10 and 14 MN per 1000 PCEs observed. The mean frequency of micronuclei in this cohort that was administered with **API** only after X-ray exposure was 12 MN/1000 PCE (Figure PCE9).

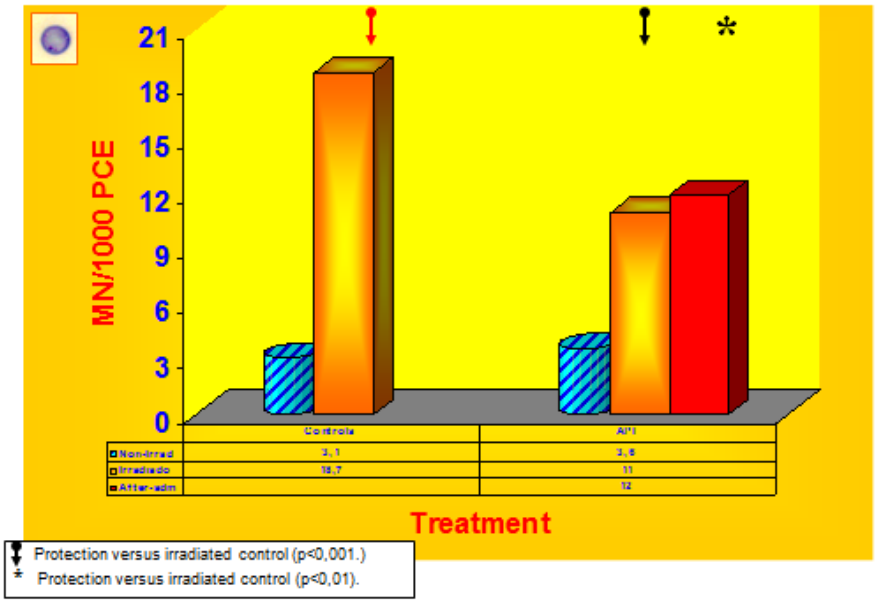


Figure PCE9. Frequency of MNPCEs in pre- and post-irradiation API treated mouse bone marrow cells (↓: (p<0.001) versus irradiated control group; (*) (p<0.01) versus irradiated control group)

Statistical analysis revealed significant differences in the frequency of MNPCEs occurrence between batches of animals treated with **API** and the irradiated control group of animals. This observation shows that when treated with **API** before or after exposure to ionizing radiation, a significant decrease in the appearance of micronuclei in polychromatic erythrocytes of irradiated animals is produced compared with irradiated control animals (**p<0.01**); suggesting that **API** shows genoprotective capacity whether administered prior or after exposure to radiation (Figure PCE9).

A protection magnitude of 41% was obtained when the API is administered before exposure to radiation and 36% when given immediately after exposure to X-rays (Figure PCE10).

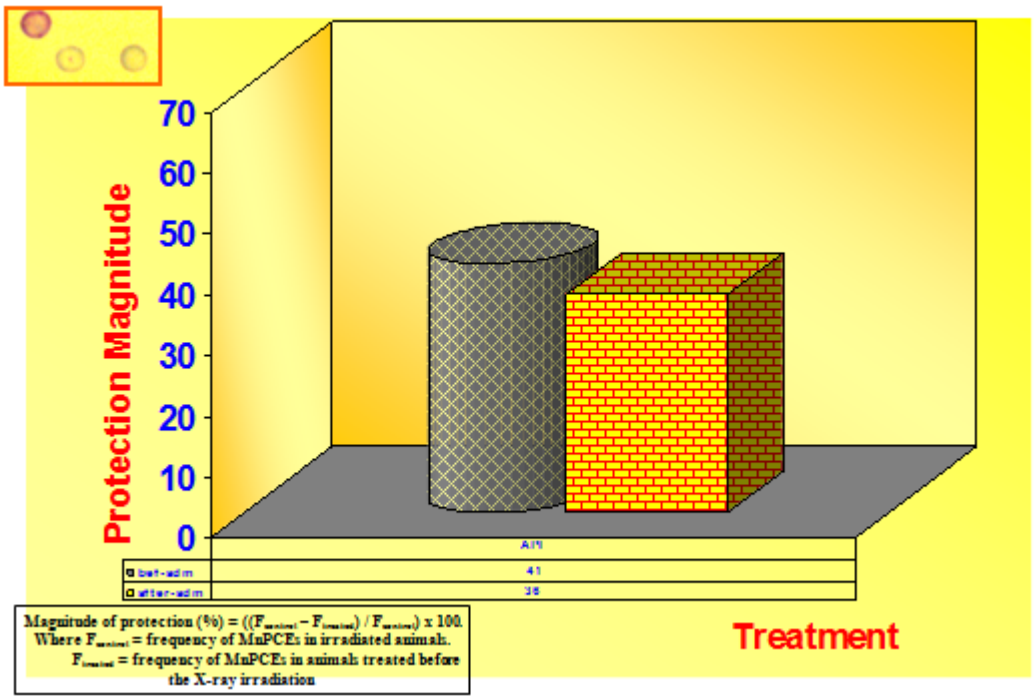


Figure PCE10. Magnitude of protection of Apigenin in animals treated before and after exposure to 500mGy of X-rays.

Group treated with Diosmin

Non-irradiated animals treated with **Diosmin** expressed a micronucleus frequency ranging between 3 and 5 MN/1000 PCEs and an average frequency of 3.6 MN per 1000 PCEs observed (Figure PCE11).

The batch of animals treated with **Diosmin** before subsequent exposure to 500 mGy of X-ray irradiation expressed a MN frequency of between 10 and 15 per 1000 PCEs observed. The mean MN frequency in these set of pre-API treated irradiated mice was 13 MN/1000 PCE (Figure PCE11).

The batch of animals irradiated with 500 mGy before receiving Diosmin expressed a MN frequency that ranged between 11 and 14 MN per 1000 PCEs observed. The mean frequency of micronuclei in this cohort administered with Diosmin after X-ray exposure was 13 MN/1000 PCE (Figure PCE11).

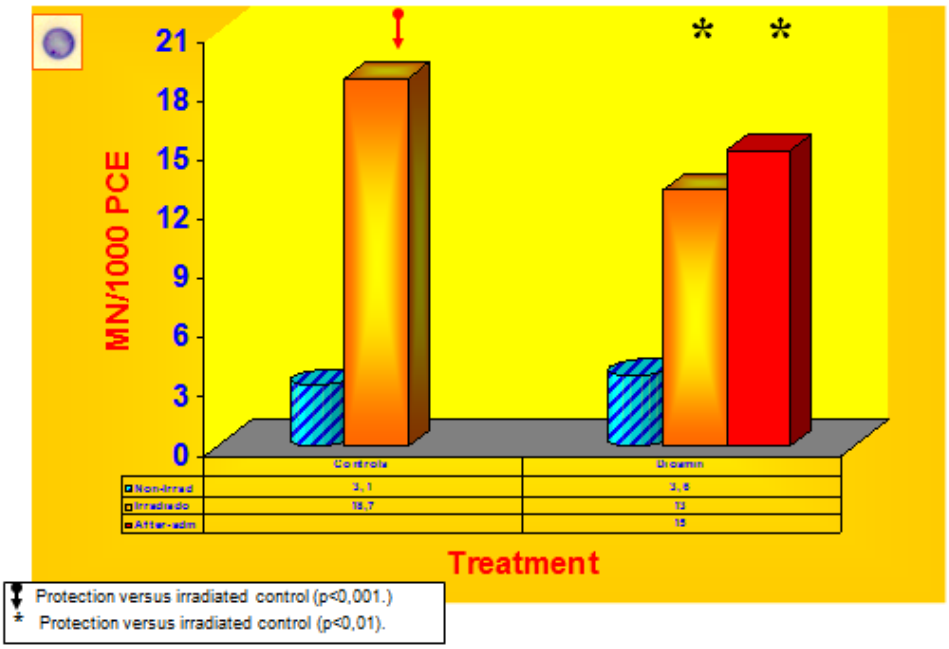


Figure PCE11. Frequency of MNPCEs in pre- and post-irradiation Diosmin treated mouse bone marrow cells ((*): (p<0.01) versus irradiated control group).

Statistical analysis revealed significant differences in the frequency of MNPCEs occurrence between batches of animals treated with Diosmin and the irradiated control group of animals. This observation shows that when treated with diosmin before or after exposure to ionizing radiation, a significant decrease in the appearance of micronuclei in polychromatic erythrocytes of irradiated animals is produced compared with irradiated control animals (**p<0.01**); suggesting that Diosmin shows genoprotective capacity whether administered prior or after exposure to radiation (Figure PCE11).

A protection magnitude of 30% is obtained when the Diosmin is administered before exposure to radiation and 20% when given immediately after exposure to X-rays (Figure PCE12).

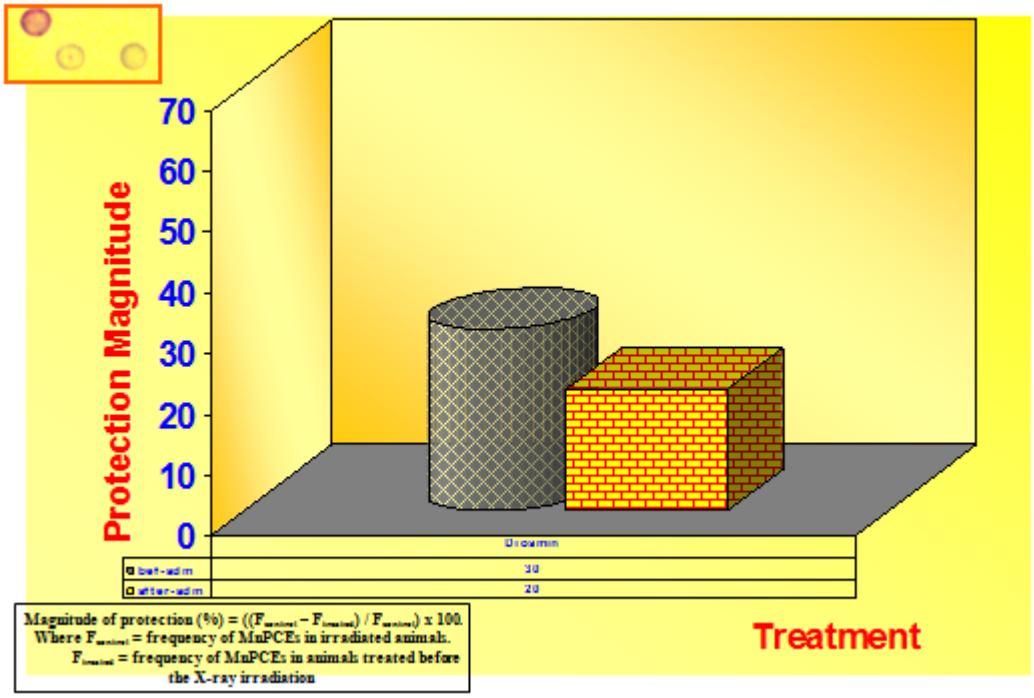


Figure PCE12. Magnitude of protection of Diosmin in animals treated before and after exposure to 500mGy of X-rays.

Group treated with soluble Citrus Extracts (CE)

Non-irradiated animals treated with CE expressed a micronucleus frequency ranging between 3 and 5 MN/1000 PCEs and an average frequency of 4 MN per 1000 PCEs observed (Figure PCE13).

The batch of animals treated with CE before subsequent exposure to 500 mGy of X-ray irradiation expressed a MN frequency of between 7 and 10 per 1000 PCEs observed. The mean MN frequency in this set of pre-CE treated irradiated mice was 8 MN/1000 PCE (Figure PCE13).

The batch of animals irradiated with 500 mGy before being administered with CE expressed a MN a frequency that ranged between 8 and 12 MN per 1000 PCEs observed. The mean frequency of micronuclei in this cohort that was administered with CE only after X-ray exposure was 10 MN/1000 PCE (Figure PCE13).

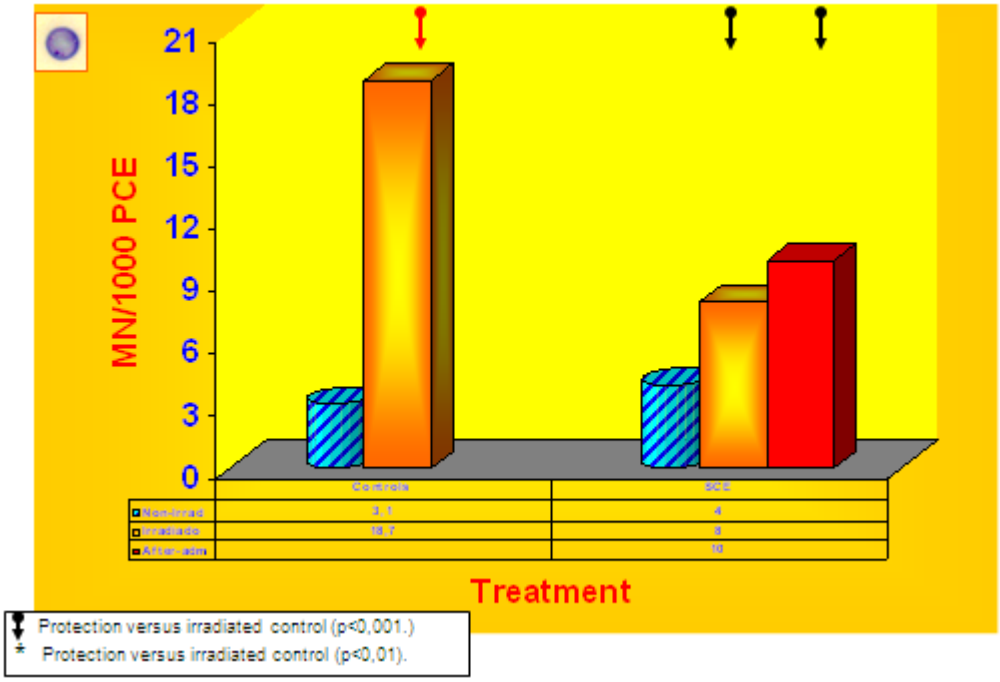


Figure PCE13. Frequency of MNPCEs in pre- and post-irradiation Soluble Citrus Extract treated mouse bone marrow cells ((↓): (p<0.001) versus irradiated control group).

Statistical analysis revealed **significant differences** in the frequency of MNPCEs occurrence between batches of animals treated with CE and the irradiated control group of animals. This observation shows that when treated with CE before or after exposure to ionizing radiation, a significant decrease in the appearance of micronuclei in polychromatic erythrocytes of irradiated animals is produced compared with irradiated control animals (**p<0.001**); suggesting that CE shows genoprotective capacity whether administered prior to or after exposure to radiation (Figure PCE13).

A protection magnitude of 57% is obtained when the CE is administered before exposure to radiation and 46.5% when given immediately after exposure to X-rays (Figure PCE14).

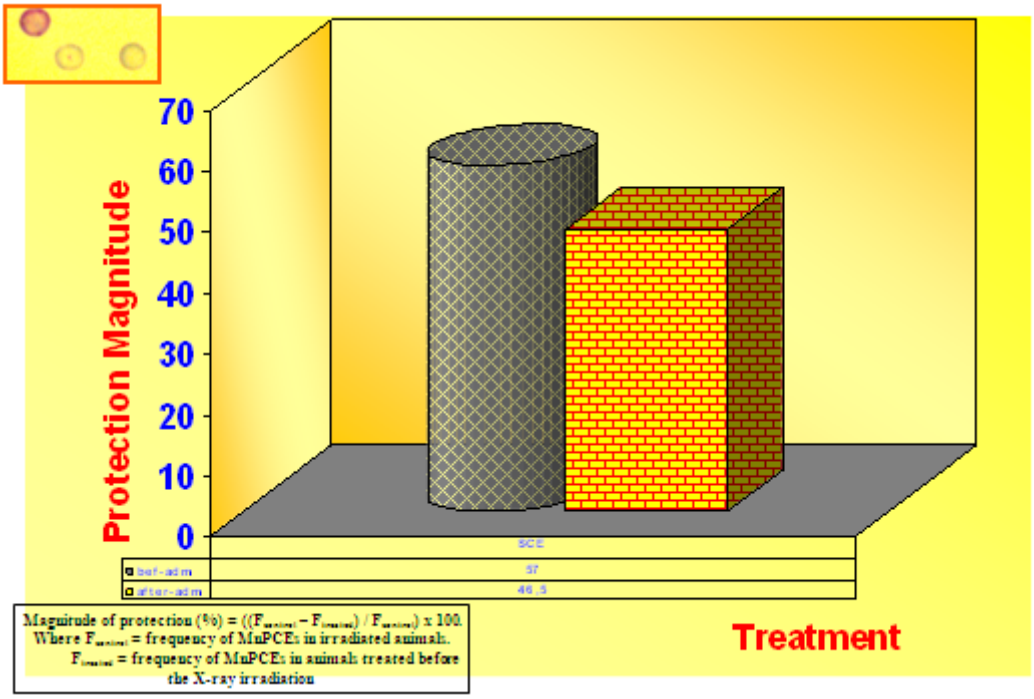


Figure PCE14. Magnitude of protection of Soluble Citrus Extract in animals treated before and after exposure to 500mGy of X-rays.

Group treated with Green Tea Extracts (TE)

Non-irradiated animals treated with **TE** expressed a micronucleus frequency ranging between 3 and 5 MN/1000 PCEs and an average frequency of 4 MN per 1000 PCEs observed (Figure PCE15).

The batch of animals treated with **TE** before subsequent exposure to 500 mGy of X-ray irradiation expressed a MN frequency of between 12 and 15 per 1000 PCEs observed. The mean MN frequency in these set pre-TE treated irradiated mice was 14 MN/1000 PCE (Figure PCE15).

The batch of animals irradiated with 500 mGy before being administered with **Te** expressed a MN a frequency that ranged between 12 and 16 MN per 1000 PCEs observed. The mean frequency of micronuclei in this cohort that was administered with **TE** only after X-ray exposure was 15 MN/1000 PCE (Figure PCE15).

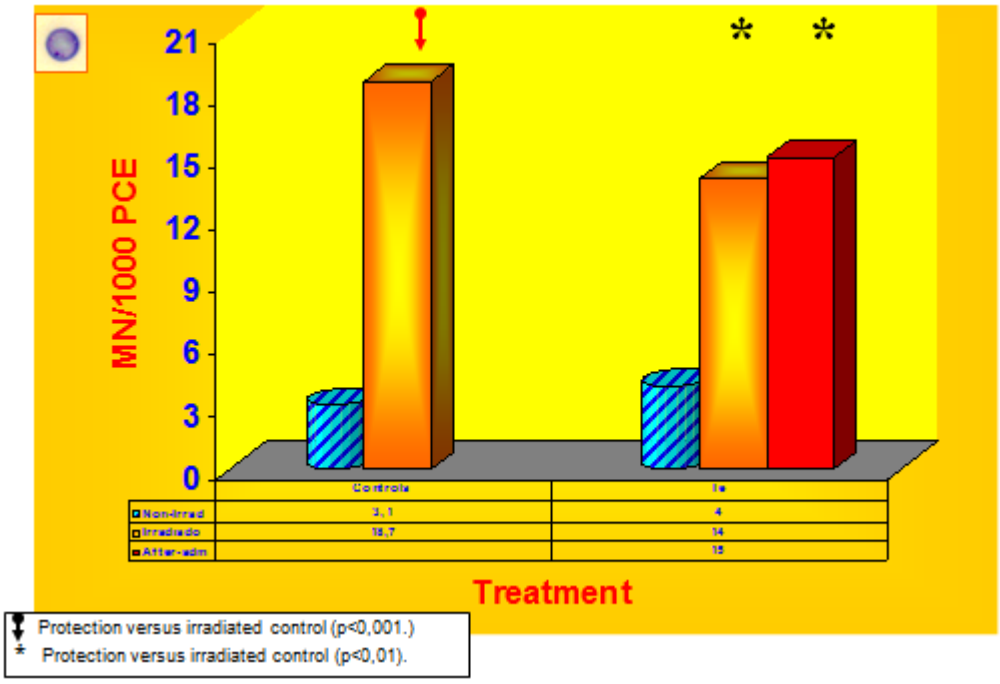


Figure PCE15. Frequency of MNPCEs in pre- and post-irradiation Green Tea Extract (Te) treated mouse bone marrow cells ((*): (p<0.01) versus irradiated control group.

Statistical analysis revealed **significant differences** in the frequency of MNPCEs occurrence between batches of animals treated with TE and the irradiated control group of animals. This observation shows that when treated with TE before or after exposure to ionizing radiation, a significant decrease in the appearance of micronuclei in polychromatic erythrocytes of irradiated animals is produced compared with irradiated control animals (**p<0.01**); suggesting that TE shows genoprotective capacity whether administered prior to or after exposure to radiation (Figure PCE15).

A protection magnitude of 25% is obtained when the TE is administered before exposure to radiation and 20% when given immediately after exposure to X-rays (Figure PCE16).

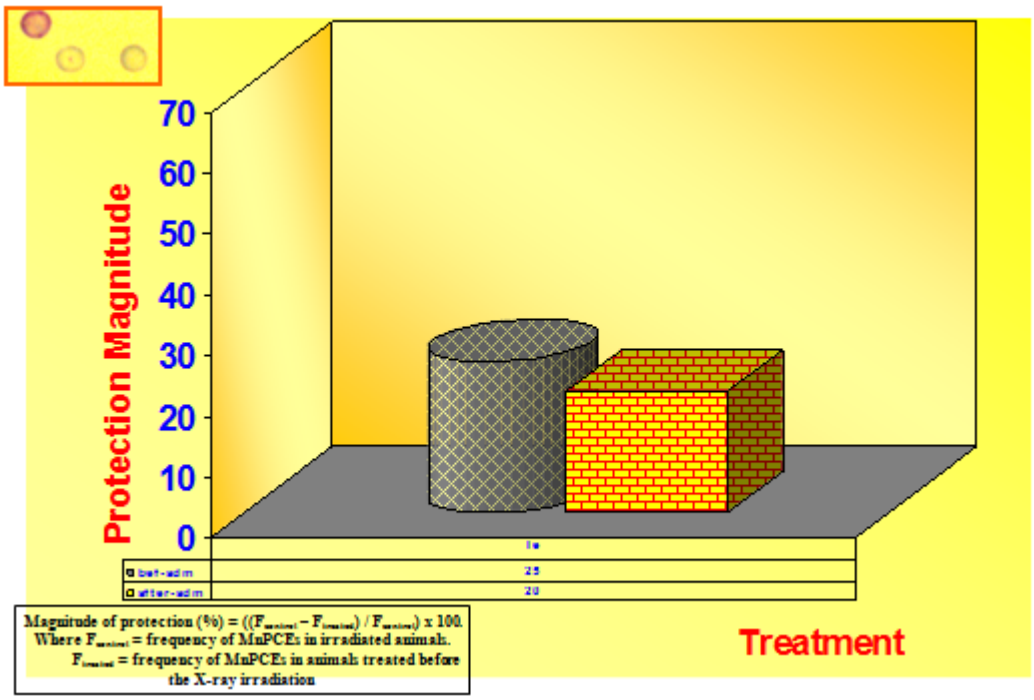


Figure PCE16. Magnitude of protection of Green Tea Extracts (TE) in animals treated before and after exposure to 500mGy of X-rays.

Group treated with Propylthiouracyl (PTU).

Non-irradiated animals treated with **PTU** expressed a micronucleus frequency ranging between 3 and 5 MN/1000 PCEs and an average frequency of 4.6 MN per 1000 PCEs observed (Figure PCE17).

The batch of animals treated with **PTU** before subsequent exposure to 500 mGy of X-ray irradiation expressed a MN frequency of between 12 and 15 per 1000 PCEs observed. The mean MN frequency in this set of pre-PTU treated irradiated mice was 13 MN/1000 PCE (Figure PCE17).

The batch of animals irradiated with 500 mGy before administered with **PTU** expressed a MN a frequency that ranged between 18 and 22 MN per 1000 PCEs observed. The mean frequency of micronuclei in this cohort that was administered with **PTU** only after X-ray exposure was 20 MN/1000 PCE (Figure PCE17).

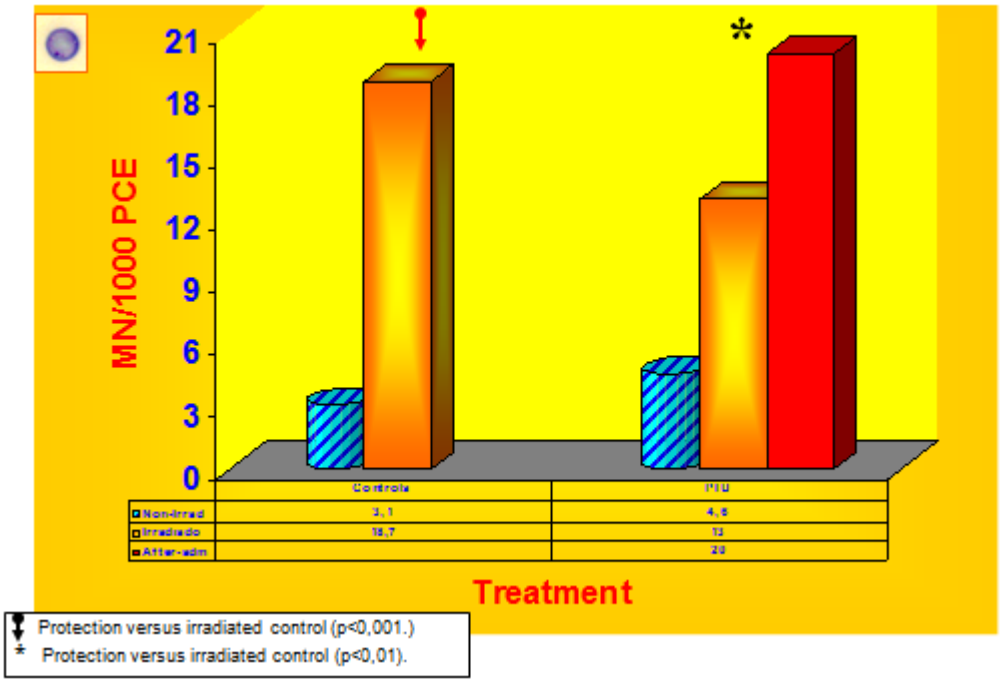


Figure PCE17. Frequency of MNPCEs in pre- and post-irradiation PTU treated mouse bone marrow cells ((*): (p<0.01) versus irradiated control group).

Statistical analysis revealed **significant differences** in the frequency of MNPCEs occurrence between batches of animals treated with **PTU** before and after irradiation exposure when referenced with the irradiated control cohort. The analysis revealed that when treated with **PTU** before exposure to ionizing radiation, a significant drop in the appearance of micronuclei in polychromatic erythrocytes of irradiated animals is observed compared with irradiated control animals and the animals treated with PTU post-irradiation (**p<0.01**); suggesting that PTU shows genoprotective capacity only when administered prior to exposure to radiation (Figure PCE17).

A magnitude of protection or protection factor of 30% was obtained when PTU was administered before exposure to the radiation, but it lacked any genoprotective capacity when administered immediately after exposure to X-rays (Figure PCE18).

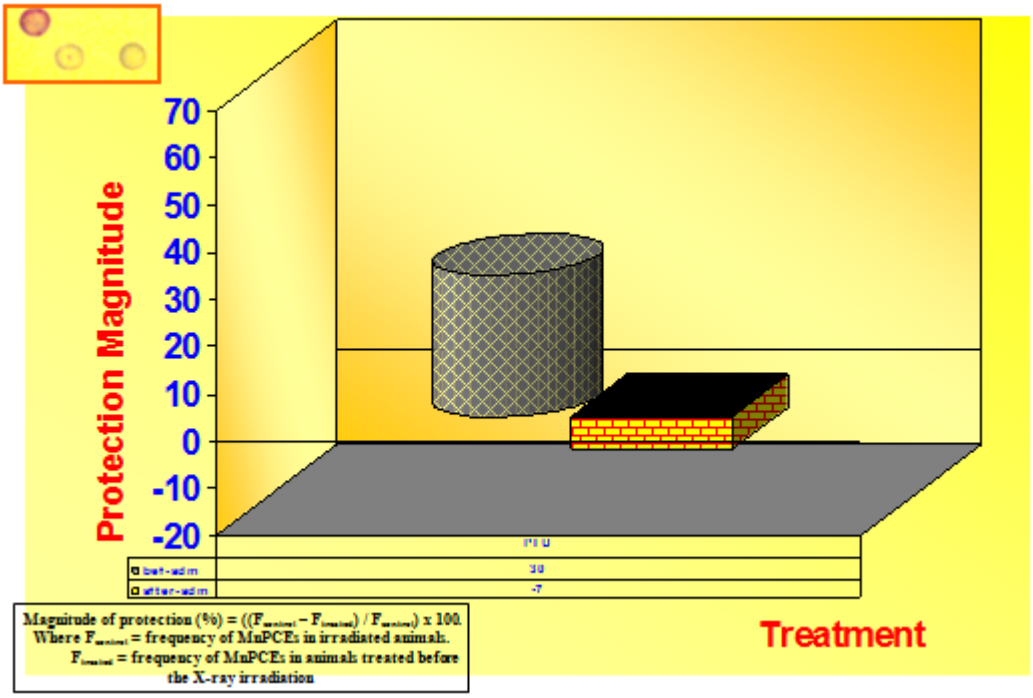


Figure PCE18. Magnitude of protection of PTU in animals treated before and after exposure to 500mGy of X-rays.

Group treated with Dimethylsulphoxide (DMSO).

Non-irradiated animals treated with **DMSO** expressed a micronucleus frequency ranging between 4 and 6 MN/1000 PCEs and an average frequency of 5.1 MN per 1000 PCEs observed (Figure PCE19).

The batch of animals treated with **DMSO** before subsequent exposure to 500 mGy of X-ray irradiation expressed a MN frequency of between 12 and 15 per 1000 PCEs observed. The mean MN frequency in these set pre-**DMSO** treated irradiated mice was 14 MN/1000 PCE (Figure PCE19).

The batch of animals irradiated with 500 mGy before **DMSO** treatment expressed a MN a frequency that ranged between 19 and 22 MN per 1000 PCEs observed. The mean frequency of micronuclei in this cohort that was administered with **DMSO** only after X-ray exposure was 22 MN/1000 PCE (Figure PCE19).

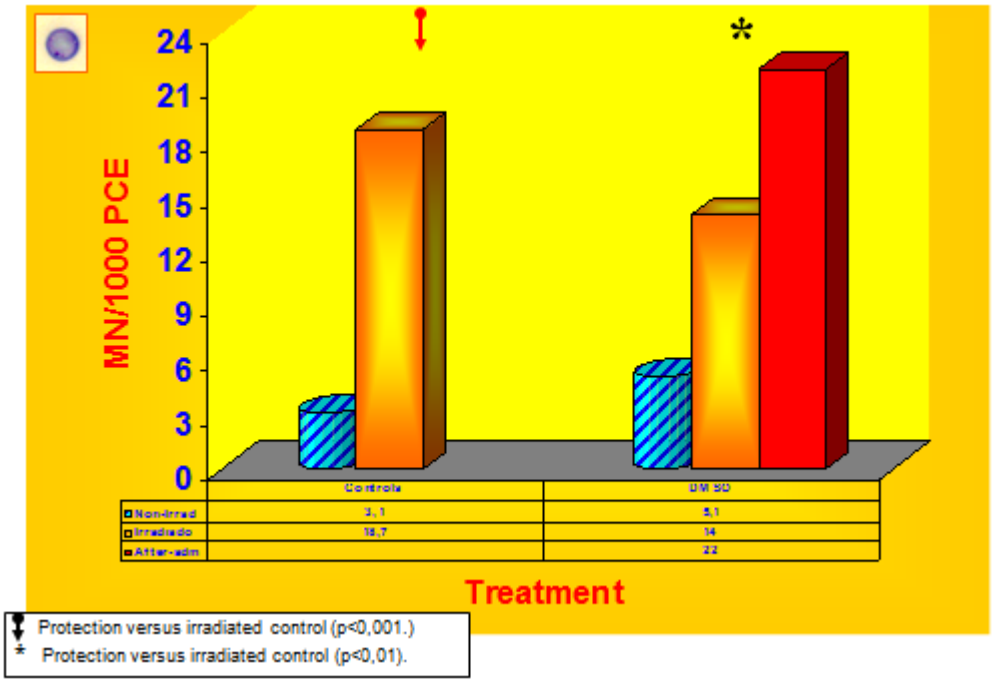


Figure PCE19. Frequency of MNPCEs in pre- and post-irradiation DMSO treated mouse bone marrow cells ((*): (p<0.01) versus irradiated control group)

Statistical analysis revealed significant differences in the frequency of MNPCEs occurrence between batches of animals treated with **DMSO** before and after irradiation exposure when referenced with the irradiated control cohort. The analysis revealed that when treated with **DMSO** before exposure to ionizing radiation, a significant drop in the appearance of micronuclei in polychromatic erythrocytes of irradiated animals is observed compared with irradiated control animals and the animals treated with DMSO post-irradiation (**p<0.01**); suggesting that **DMSO** shows genoprotective capacity only when administered prior to exposure to radiation (Figure PCE19).

A magnitude of protection or protection factor of 25% was obtained when **DMSO** was administered to the animals before exposure to irradiation, but it lacked genoprotective capacity when administered after irradiation exposure (Figure PCE20).

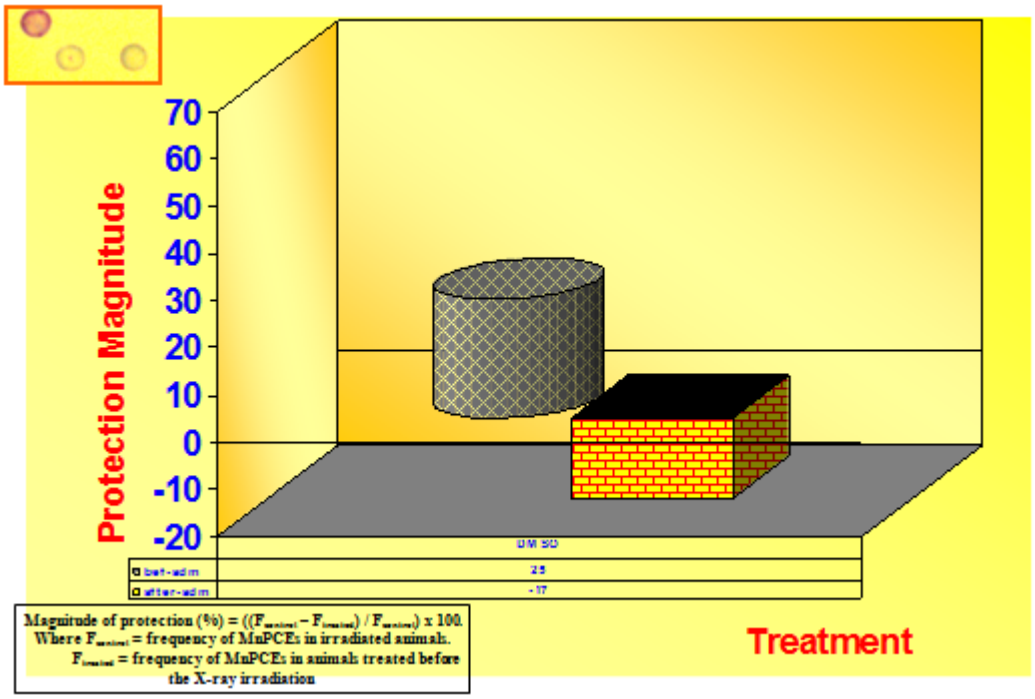


Figure PCE20. Magnitude of protection of DMSO treatment given to animals before and after exposure to 500mGy of X-rays.

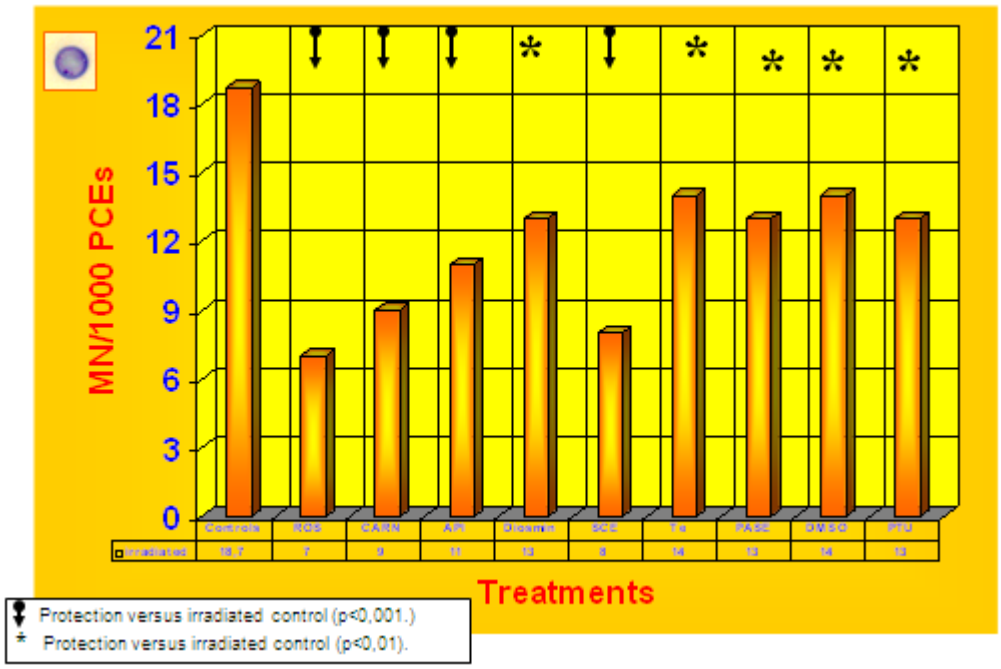
Results of Statistical Analysis on Micronucleated Polychromatic Erythrocytes.

1. The irradiated control animals showed a **significant increase** of MN over the non-irradiated control animals (**p<0.001**). This means that exposure to ionizing leads to a significant increase in the frequency of MN (Figures PCE3 and PCE21).

2. When non-irradiated animals were treated with only the test substances, there were **no significant differences** observed in the frequency of occurrence of MNPCEs of the animals with respect to non-irradiated control animals. Thus the different substance preparations did not manifest any chromosome damage to the animals (Figures PCE2 and PCE21).

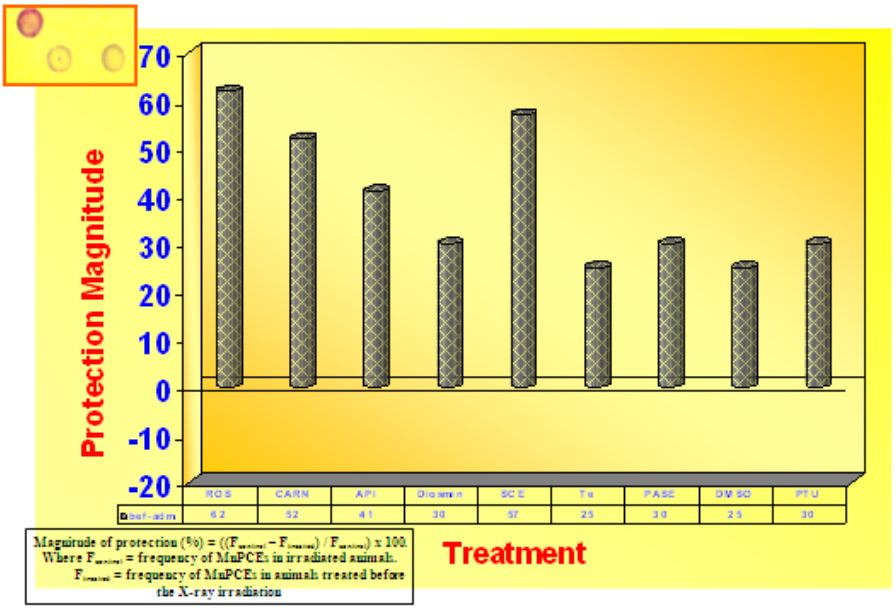
3. The batches of animals treated with ROS, CAR, API and CE before irradiation showed a **significant decrease in the frequency** of micronuclei in polychromatic erythrocytes with respect to the irradiated control batch (**p<0.001).** The batches of animals treated with diosmin, TE, PASE, DMSO and PTU before irradiation,

showed a **significant decrease** in the frequency of micronuclei batch respect irradiated control (**p<0.01).** This means that the treatments meted out led to decreases in the expected frequency of MN occurrence in the PCEs of irradiated animals as a result of exposure to irradiation and therefore represent a protection against chromosomal damage induced by radiation (Figure PCE21; PCE22).

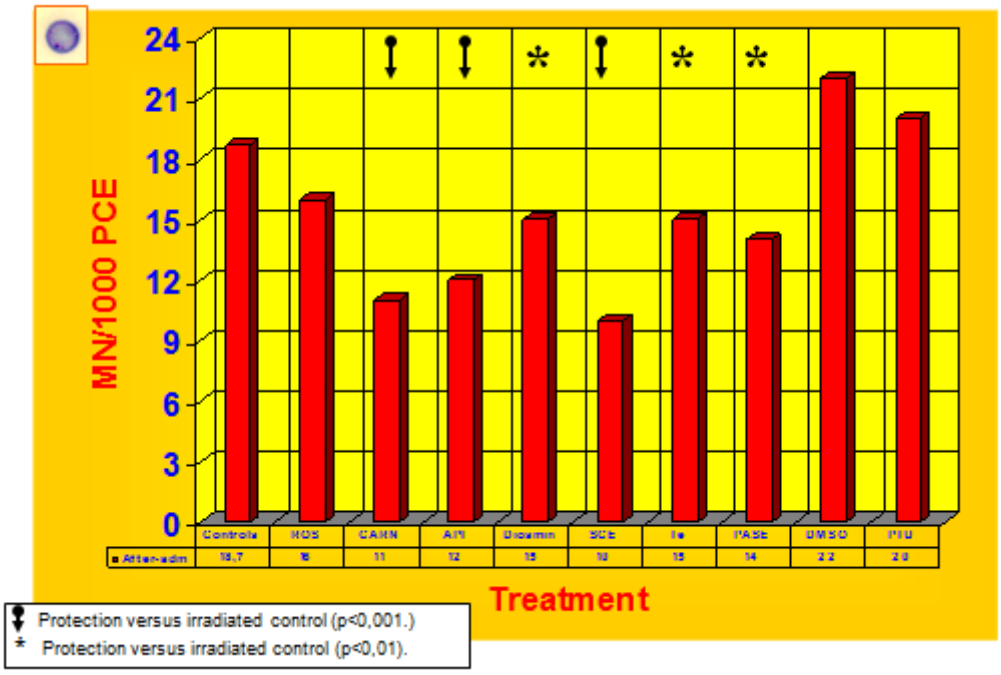


PCE21. Frequency of micronucleated polychromatic erythrocytes (MnPCE) in mouse bone marrow cells of mice pre-treated with test substances before exposure to low dose X-rays (↓: (p<0.001) versus irradiated control group;((*)(p<0.01) versus irradiated control group)

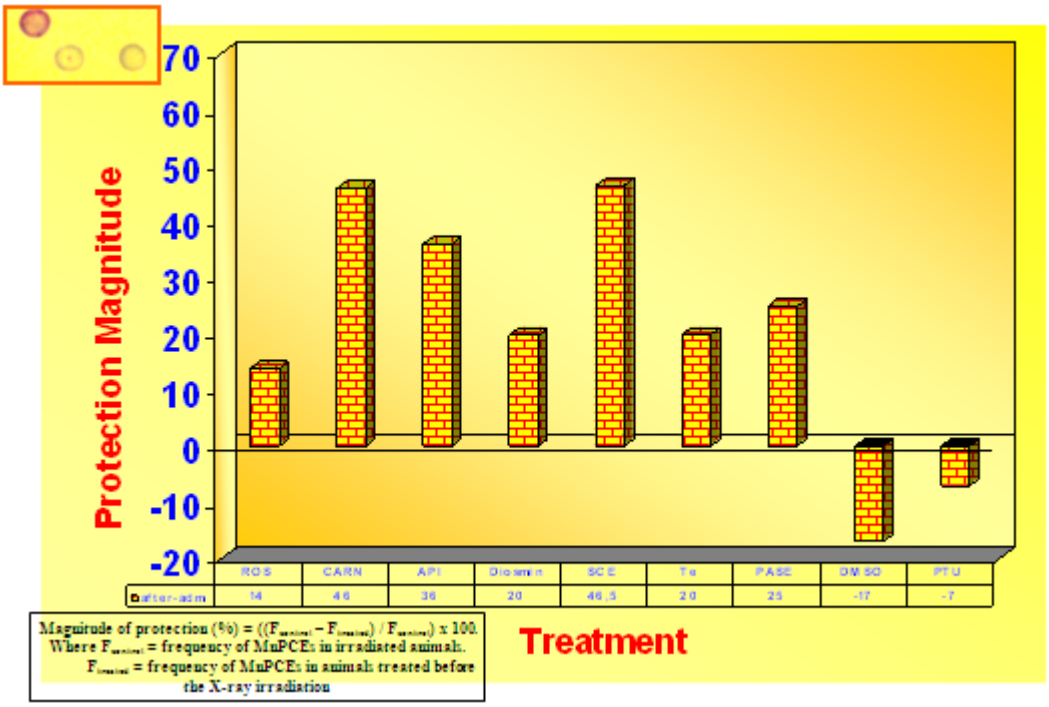
4. Lots of animals that received post-irradiation treatment with CARN, API and CE showed a **significant decrease** in the frequency of micronuclei in comparison with the irradiated control lot (p **<0.001**), while the batches of animals that received post-irradiated treatment with Diosmin, TE and PASE showed a **significant decrease in the frequency** of micronuclei with respect to irradiated control (**p <0.01**). This means that the treatments given significantly reduced the expected frequency of occurrence of MN as a result of radiation exposure are therefore offering protection against chromosomal damage induced by ionizing radiation.



PCE22. Magnitude of protection obtained when mice were administered with different test substance before exposure to low dose X-irradiation



PCE23. Frequency of micronucleated polychromatic erythrocytes (MnPCE) in mouse bone marrow cells of mice pre-exposed to irradiation before test substance administration (↓): (p<0.001) versus irradiated control group;((*): (p<0.01 versus irradiated control group).



PCE24. Magnitude of protection obtained when mice were exposed to low dose X-irradiation before treated with different test substances.

The overall results obtained with the micronucleus test conducted on mouse bone marrow polychromatic erythrocytes cells are shown in Figures PEC25 and PCE26.

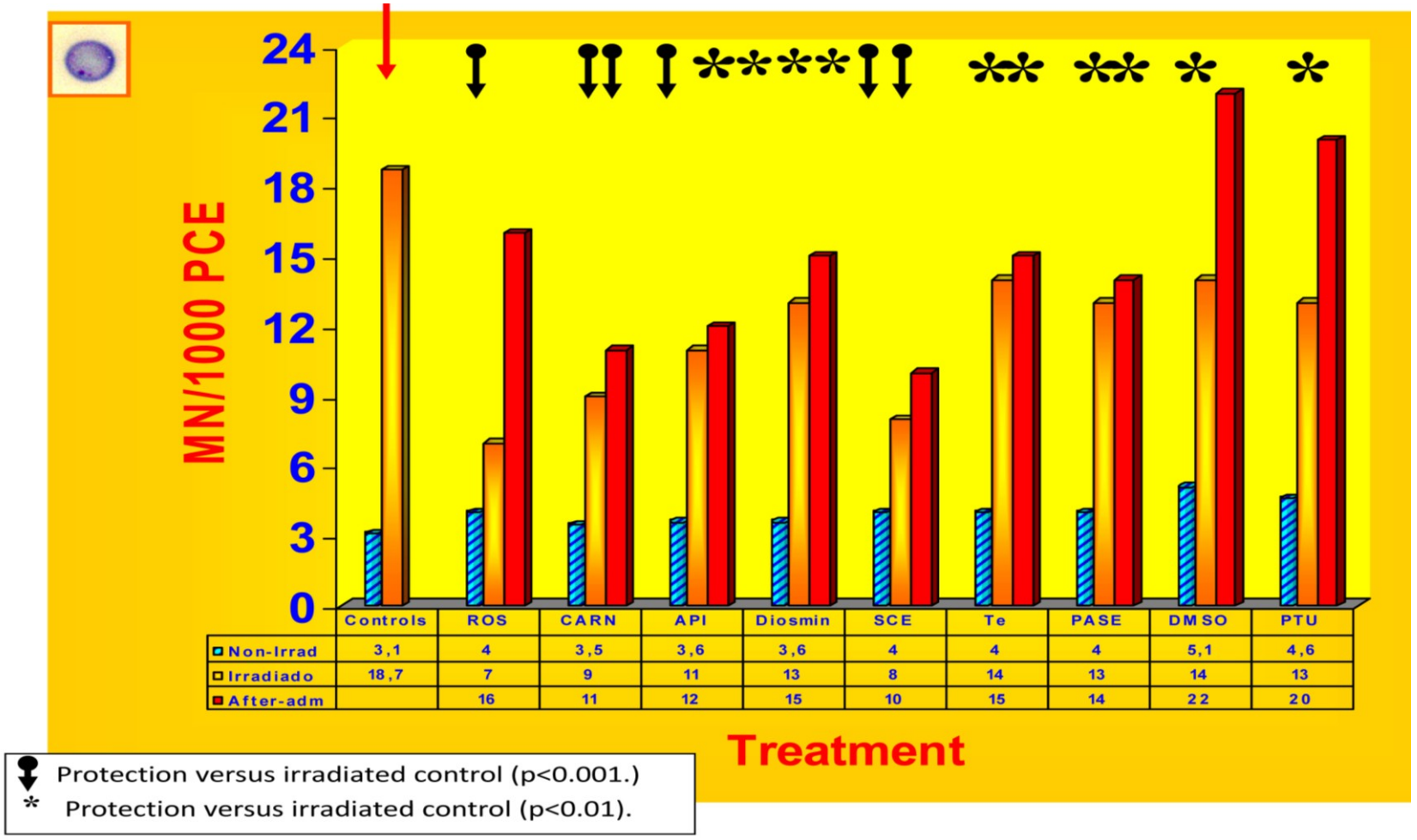
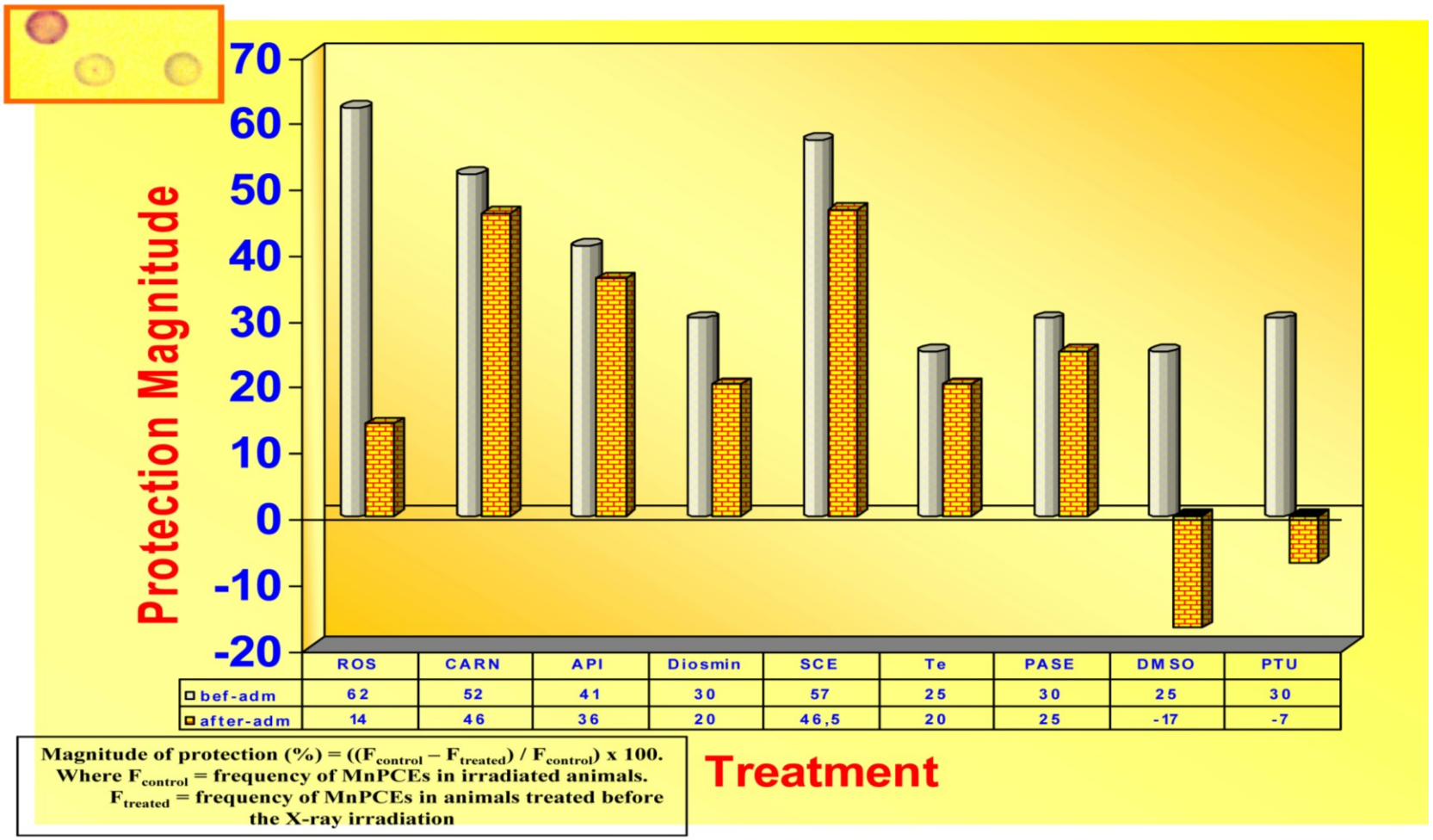


Figure PCE 25: Influence of pre- and post-irradiation treatments of different substances on the frequencies of MnPCEs in mouse bone marrow cells (↓: (p<0.001) versus irradiated control group; (*): p<0.01 versus irradiated control group).



	ROS	CARN	API	Diosmin	SCE	Te	PASE	DMSO	PTU
bef-adm	62	52	41	30	57	25	30	25	30
after-adm	14	46	36	20	46,5	20	25	-17	-7

Figure PCE26. Pre- and post-irradiation treatment magnitudes of protection obtained with adult male Swiss mice treated with different test substances and 500 mGy of X-rays.

V. Results.

RESULTS OF CYTOPROTECTION ASSAYS
PNT2 CELLS (HUMAN PROSTRATE EPITHELIAL CELLS)

RESULTS OF CYTOPROTECTION ASSAYS

CELL SURVIVAL CURVES WITH NORMAL HUMAN PROSTATE EPITHELIAL PNT2 CELLS.

- **Percentage cell survival in control cultures after exposure to x-rays**

The results of analysis conducted on PNT2 cells that were not treated with any substance but exposed to X-ray doses of 0 Gy, 4 Gy, 6 Gy, 8 Gy and 10 Gy are displayed in the table below (Table M1). A progressive dose-related decrease in the number of live cells which varies inversely with the administered radiation dose but directly with the post-irradiation assay period is portrayed i.e., the higher the radiation dose and the longer the post-irradiation incubation period, the lower cell survival. After exposure to 10 Gy of irradiation, the proportion of surviving cells reduced by 30.8% and 38.8% when examined 24 and 48 hours post irradiation respectively (**p<0.001**) (Table M1; Fig. M1).

Table M1. Percentage cell survival (%) of normal human prostrate epithelial PNT2 control cell cultures

Time/Radiation dose	0 Gy	4 Gy	6 Gy	8 Gy	10Gy
24 hours	100.0 ± 5.8	91.0 ± 7.1	86.2 ± 7.2	79.4±5.4	69.2±5.1*
48 hours	100.0 ± 4.5	84.5 ± 8.2	77.3 ± 7.4	71.1±5.6	61.2±5.2*

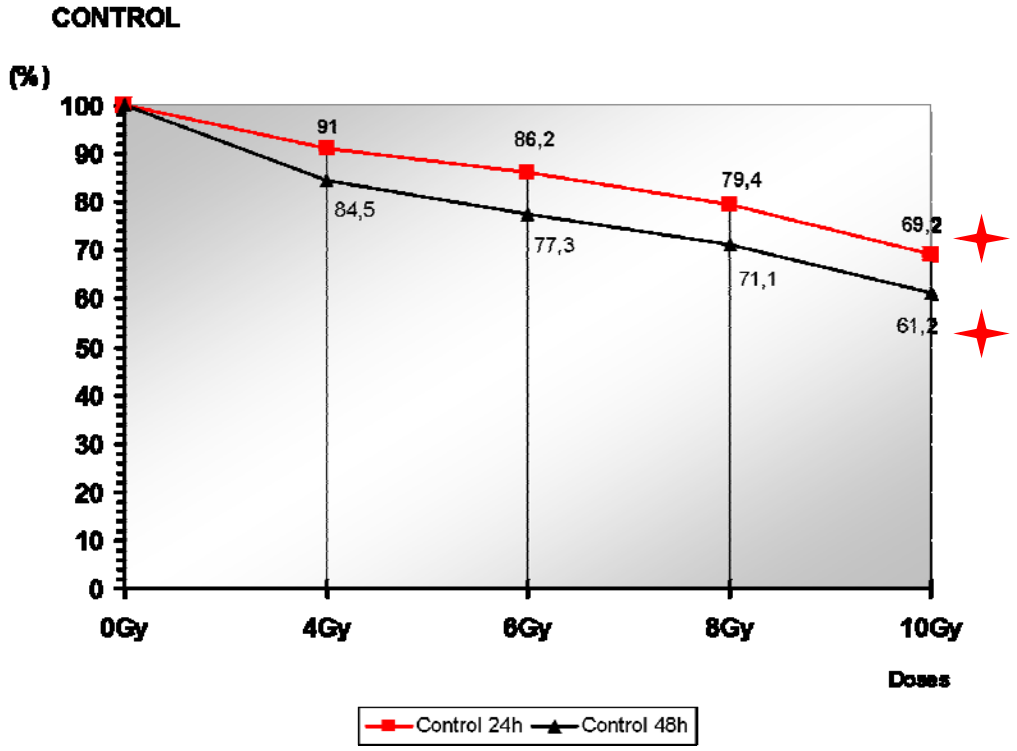


Figure M1. Cell survival curves of normal human prostrate epithelial PNT2 control cell cultures ((*): (p<0.001) versus irradiated control cells.

- **SUBSTANCES ASSAYED**

Figure M2 shows the radioprotective effect after the administration of different substances and mixtures to normal human prostrate epithelial PNT2 cells. With the exception of the effects elicited by *Pycnanthus angolensis* seed extract (PASE) which is shown alone and in different scenarios in Figure M22. Different substance and mixture concentrations (20µM and 40 µM) were used in this quest for cellular radioprotection by different substances. The results obtained after treating normal human prostrate epithelial PNT2 cell cultures with these test substances and incubating the cell cultures for 24h have been summarized in Figure M2.

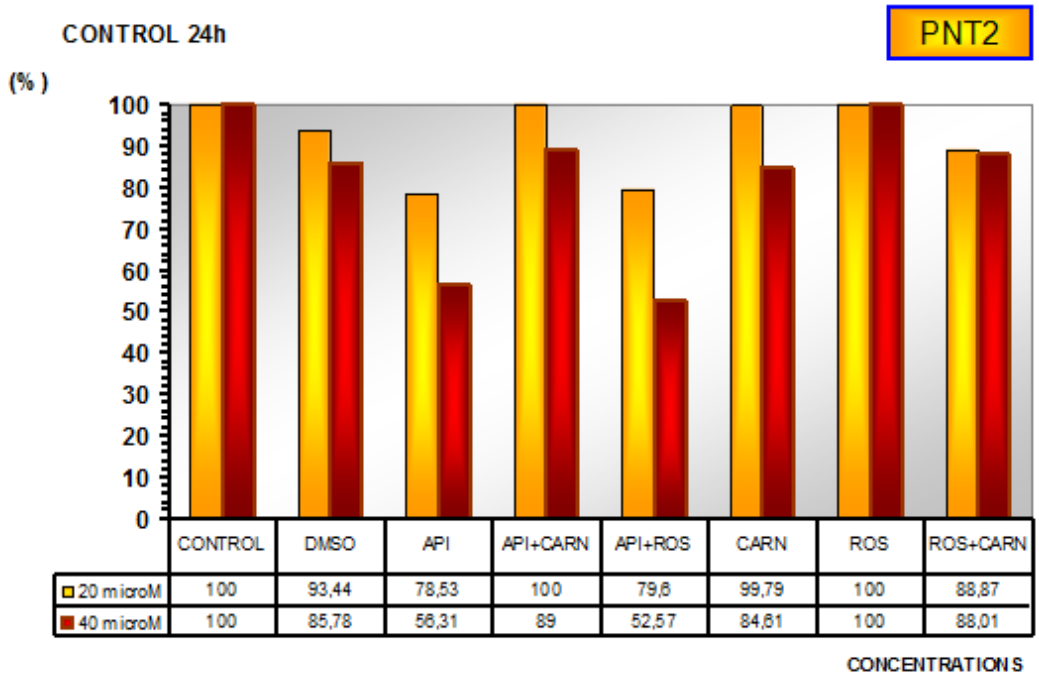


Figure M2. Percentage cell survival of control cell cultures after the administration of the diffrent substances studied on PNT2 cells evaluated after a 24-hour of incubation period (DMSO: Dimethysulphoxide; API: Apigenin; CARN: Carnosic acid; ROS: Rosmarinic Acid)

Figure M2 shows the radioprotective effect of the administration of different substances and mixtures on normal human prostate epithelial PNT2 cells. With the exception of the effects elicited by *Pycnanthus angolensis* seed extract (PASE) which is shown in Figure M2. Different substance and mixture concentrations (20µM and 40 µM) were used in this quest to evaluate their cellular radioprotection. The results obtained after treating normal human prostate epithelial PNT2 cell cultures with these test substances and incubating the cell cultures for 48h have been summarized in Figure M3.

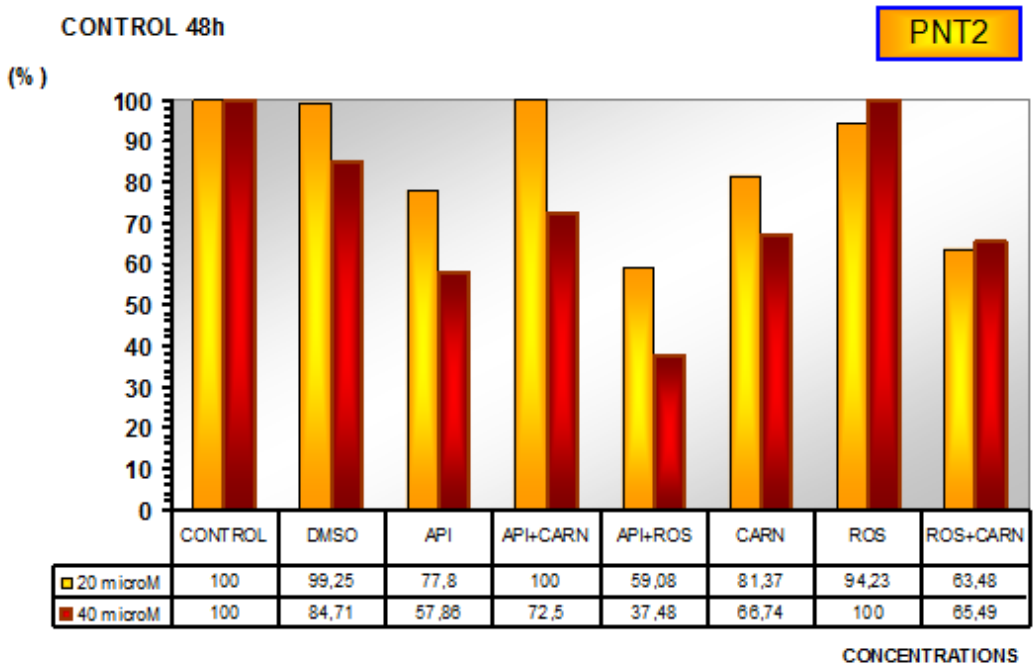


Figure M3. Percentage cell survival of control cell cultures after the administration of the diffrent substances studied on normal human prostate epithelial PNT2 cells evaluated after 48 hours of incubation (DMSO: Dimethysulphoxide; API: Apigenin; CARN: Carnosic Acid; ROS: Rosmarinic acid)

- APIGENIN

To evaluate the cytotoprotective capacity of apigenin, cells were treated with two different concentrations of apigenin i.e., 20µl each of 20 µM and 40 µM solutions of apigenin for 30 min before exposed to 2 Gy of X-irradiation. Cytotoxicity was evaluated after 24 h of incubating the cell cultures; control cell cultures were treated with the two concentrations of apigenin but were not irradiated.

In the non-irradiated cell cultures, apigenin administration at 20 µM and 40 µM to normal human prostate epithelial PNT2 cell cultures led to a 21.47% and 43.69% respective decreases in the proportion of surviving cells when compared with the control cultures following 24 and 48 h cell incubation periods (figure M4).

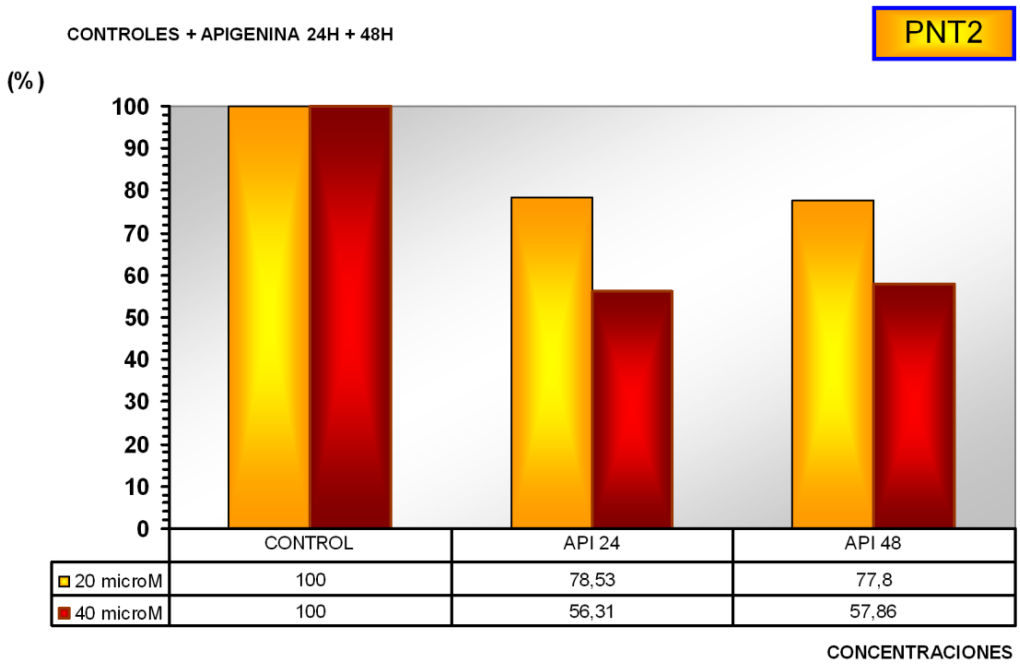


Figure M4. Effect of the administration of API on the PNT2 cell cultures at different concentrations i.e., 20 µM and 40 µM evaluated after 24h and 48h incubation periods.

After 24 hours following treatment of PNT2 cells with 20µM and 40 µM solutions of apigenin and exposure to different doses of irradiation, a higher cell survival was demonstrated when compared with the irradiated cell control group. At 10 Gy, the irradiated control group recorded the highest percentage reduction of 30.8% in surviving cells. At both concentrations of apigenin studied, a significantly increased PNT2 cell survival was observed compared with irradiated control cell cultures (**p<0.001**) (Table M2; Figure M5).

Table M2. Percentage cell survival (%) of normal human prostate epithelial PNT2 cell cultures, treated with 20 µM and 40 µM of Apigenin and exposed to different doses of X-rays evaluated after 24 hours of incubation.

Radiation dose	0 Gy	4 Gy	6 Gy	8 Gy	10Gy
Control	100.0 ± 5.8	91.0 ± 7.1	86.2 ± 7.2	79.4 ± 5.4	69.2 ± 5.1
Apigenin 20 µM	100.0 ± 6.7	95.4 ± 7.1	86.3 ± 7.9	86.3 ± 7.6	90.8 ± 8.1*
Apigenin 40 µM	100.0 ± 7.3	95.4 ± 8.0	100.0 ± 5.5	100.0 ± 7.2	100.0 ± 7.8*

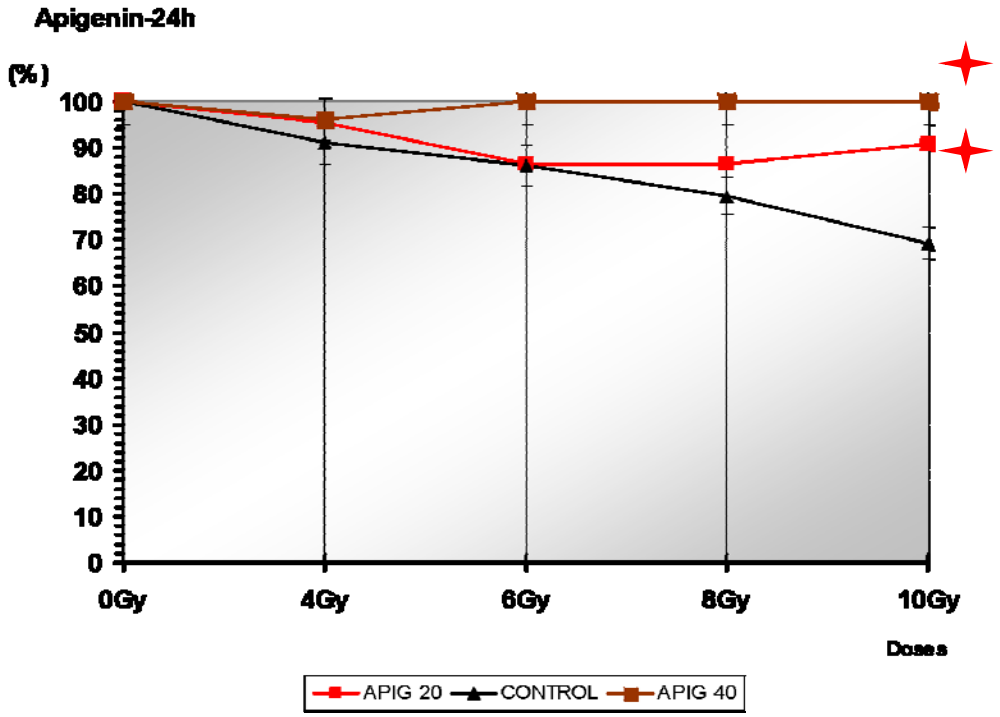


Figure M5. Cell survival curves of normal human prostate epithelial PNT2 cell cultures treated with 20 µM and 40 µM of apigenin and exposed to different doses of X-rays evaluated after 24 hours of incubation(*): (p<0.001) versus irradiated control).

Protection Factors (**PF**) were also was evaluated for the 48 h apigenin treated cultures. At the highest radiation dose administered (i.e., 10Gy), the PF obtained from PNT2 cell cultures treated with 20 µM apigenin was 70% while cultures treated with 40 µM apigenin expressed a PF of 100%.

Whereas it was not practically possible to directly derive a **Dose Reduction Factor** (DRF) from the 40 µM apigenin treated PNT2 cell cultures at 10 Gy after 48 hr of incubation, a DRF of 2.0 was determined from the cultures treated with 20 µM apigenin at this dose after 48 hours of cell incubation.

After 48 hours of incubation of PNT2 cells treated with the two concentrations of apigenin (20 and 40 µM) the results showed higher cell survivals than the irradiated control cultures portraying an increment of nearly 22.8% cell survival for the two concentrations at the highest radiation dose used over the control cultures. At these concentrations, a **significant increase** in the cellular survival was observed compared with irradiated control cells (**p<0.001**) (Table M3; Figure M6)

Table M3. : Percentage cell survival (%) of normal human prostate epithelial PNT2 cell cultures treated with 20 µM and 40 µM of Apigenin and exposed to different doses of X-rays evaluated after 24 hours of incubation.

Radiation Dose	0 Gy	4 Gy	6 Gy	8 Gy	10Gy
Control	100.0 ± 4.5	84.5 ± 8.2	77.3 ± 7.4	71.1 ± 5.6	61.2 ± 5.2
Apigenin 20 µM	100.0 ± 6.5	100.0 ± 7.5	100.0 ± 8.9	100.0 ± 7.5	85.0 ± 5.6*
Apigenin 40 µM	100.0 ± 6.6	100.0 ± 7.3	87.0 ± 8.4	87.6 ± 7.2	84.0 ± 6.3*

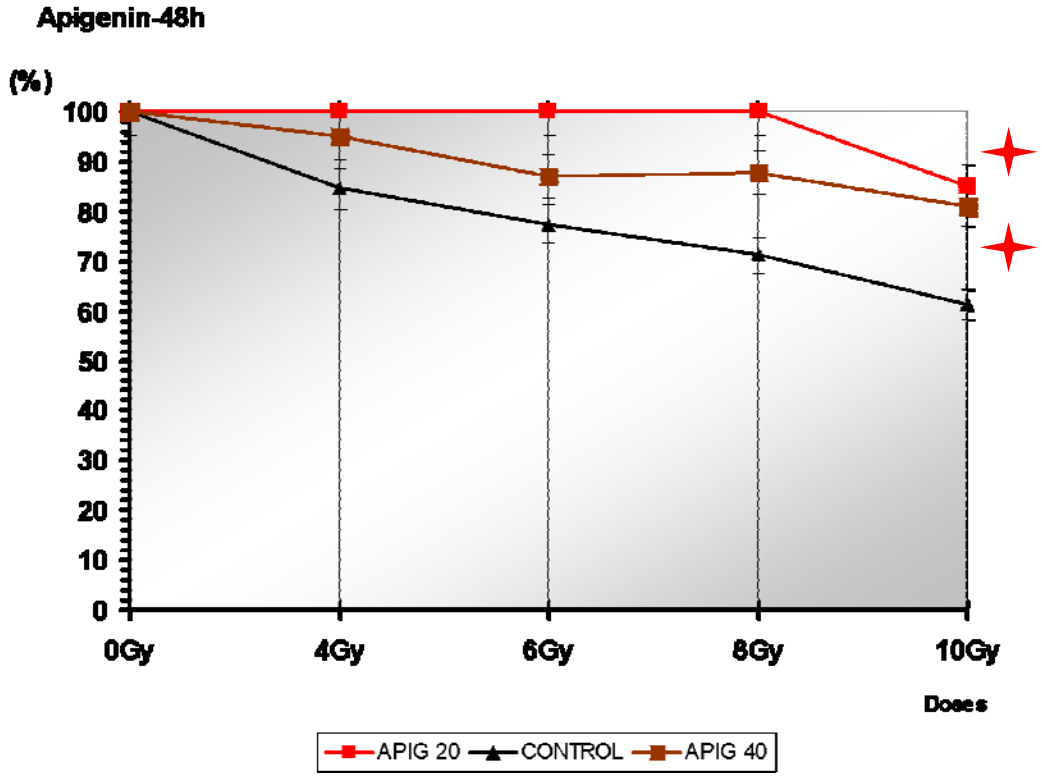


Figure M6. Cell survival curves of normal human prostate epithelial PNT2 cell cultures treated with 20 µM and 40 µM of Apigenin and exposed to different doses of X-rays evaluated after 48 hours of incubation((*): (p<0.001) versus irradiated control).

The **Protection Factor** (**PF**) obtained when PNT2 cells were treated with 20 µM of apigenin and exposed to a radiation dose of 10 Gy following a 48 hour incubation period was 58.7% while a corresponding value of 61.3% was observed when 40 µM of apigenin was used.

A **Dose Reduction Factor** (**DRF**) of 2.5 was obtained when cells were treated with 20 µM apigenin and exposed to the highest dose (10 Gy) used in this study while a DRF of 1.8 was obtained when a 40 µM of apigenin was used.

- **DIMETHYLSULFOXIDE (DMSO)**

The addition of 0.1% and 0.2% DMSO to the PNT2 cell cultures produced 6.56% and 14.22% decrease in cell survival respectively after 24 hour incubation when compared with cell survival results obtained with the control cell cultures; this produced the minimum growth inhibitory effect of DMSO on PNT2 cell survival (Figure M7).

After 48 hours of incubation and maintaining the DMSO concentrations (i.e., 0.1% and 0.2%) a respective decrease of 0.75% and 15.29% in cell proliferation was observed when compared with the control cultures of the same cell line (Figure M7).

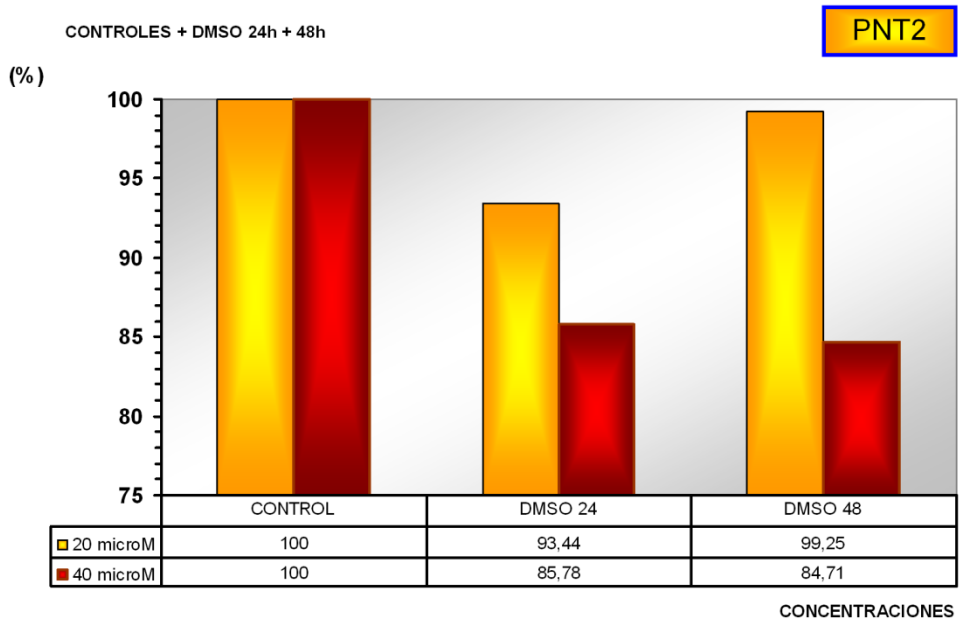


Figure M7. Effect of administration of 0.1% and 0.2% DMSO solutions on PNT2 cells evaluated after 24h and 48h incubation periods.

After 24 hours of incubation the results obtained from PNT2 cell cultures with the two concentrations of DMSO (0.1% and 0.2%) studied showed higher cell survival with respect to the irradiated control cells. While a 30.8% reduction in cell proliferation was observed for the irradiated control cultures at the highest dose employed, a 22.3% reduction in cell proliferation was obtained when cells were exposed to 0.2% DMSO. When cells were exposed to 0.1% DMSO and irradiated at 10 Gy, no reduction in cell proliferation was noticed. At both concentrations of DMSO studied, an increase in cell survival was observed compared with irradiated control cell cultures (**$p<0.001$**) (Table M4; Figure M8).

Table M4. Percentage cell survival (%) of normal human prostate epithelial PNT2 cell cultures treated with 0.1% and 0.2% of DMSO and exposed to different doses of X-rays evaluated after 24 hours of incubation.

Radiation dose	0 Gy	4 Gy	6 Gy	8 Gy	10Gy
Control	100.0 ± 5.8	91.0 ±7.1	86.2 ± 7.2	79.4 ± 5.4	69.2 ± 5.1
DMSO 0.1%	100.0 ± 7.0	100.0 ± 6.3	100.0 ± 5.4	100.0 ± 5.9	100.0 ± 3.4*
DMSO 0.2%	100.0 ± 6.5	100.0 ± 7.0	100.0 ± 6.2	95.1 ± 5.4	83.7 ± 6.3*

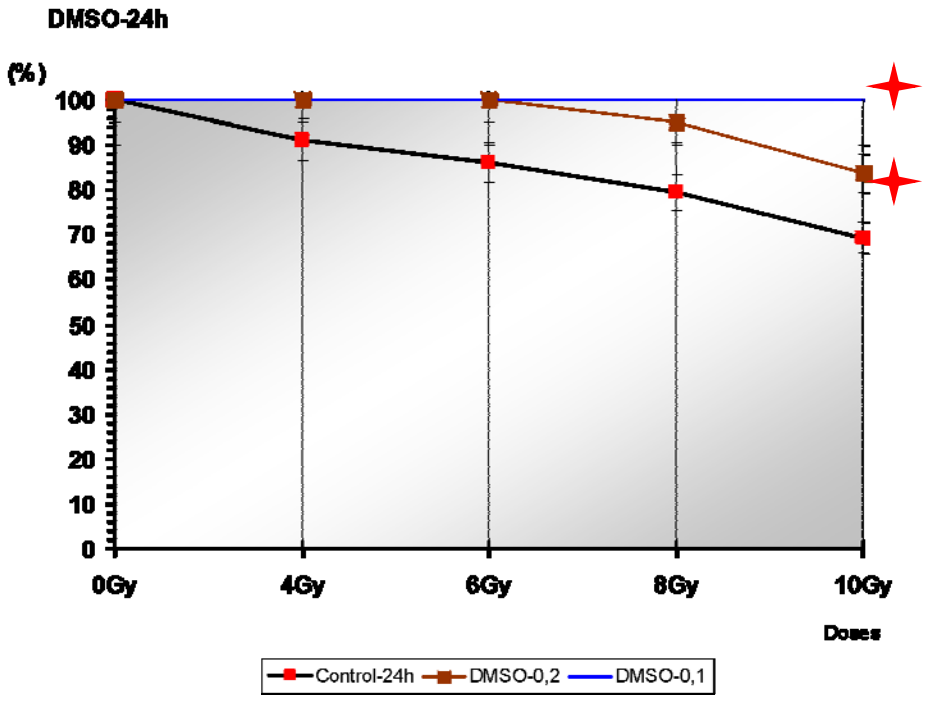


Figure M8. Cell survival curves of normal human prostate epithelial PNT2 cell cultures treated with 0.1% and 0.2% of DMSO and exposed to different doses of X-rays evaluated after 24 hours of incubation ((*): (p<0.001) versus irradiated control cells.

The **Protection Factor** (**PF**) obtained for cultures treated with 0.1% DMSO following 24- hour incubation and exposure to the highest irradiation dose (10 Gy) was 100% and 47% when 0.2% DMSO solution was used at the same radiation dose.

From the data obtained, it was not possible to directly obtain the DRF from the cultures treated with 0.1% DMSO and exposed to the highest dose (10 Gy) following 24-hour cultures, however, at the higher concentration of DMSO (0.2%) used, a DRF 1.4 was determined.

After 48 hours of incubation, the cell survival results obtained for the PNT2 cell cultures treated with the two levels of DMSO (0.1% and 0.2%) were equal but higher than that in the irradiated control group. Both concentrations produced the same effect of 38.8% increase in cell survival numbers compared with the irradiated controls. At both concentrations of DMSO employed, an increase in cellular survival was shown compared with irradiated control cells (**p<0.001**) (Table M5; Figure M9).

Table M5. Percentage cell survival (%) of normal human prostate epithelial PNT2 cell cultures treated with 0.1% and 0.2% of DMSO and exposed to different doses of X-rays evaluated after 48 hours of incubation.

Radiation dose	0 Gy	4 Gy	6 Gy	8 Gy	10Gy
Control	100.0 ± 4.5	84.5 ± 8.2	77.3 ± 7.4	71.1 ± 5.6	61.2 ± 5.2
0.1% DMSO	100.0 ± 7.0	100.0 ± 5.4	100.0 ± 7.4	100.0 ± 5.7	100.0 ± 6.2*
0.2% DMSO	100.0 ± 6.1	100.0 ± 7.2	100.0 ± 6.2	100.0 ± 8.2	100.0 ± 9.4*

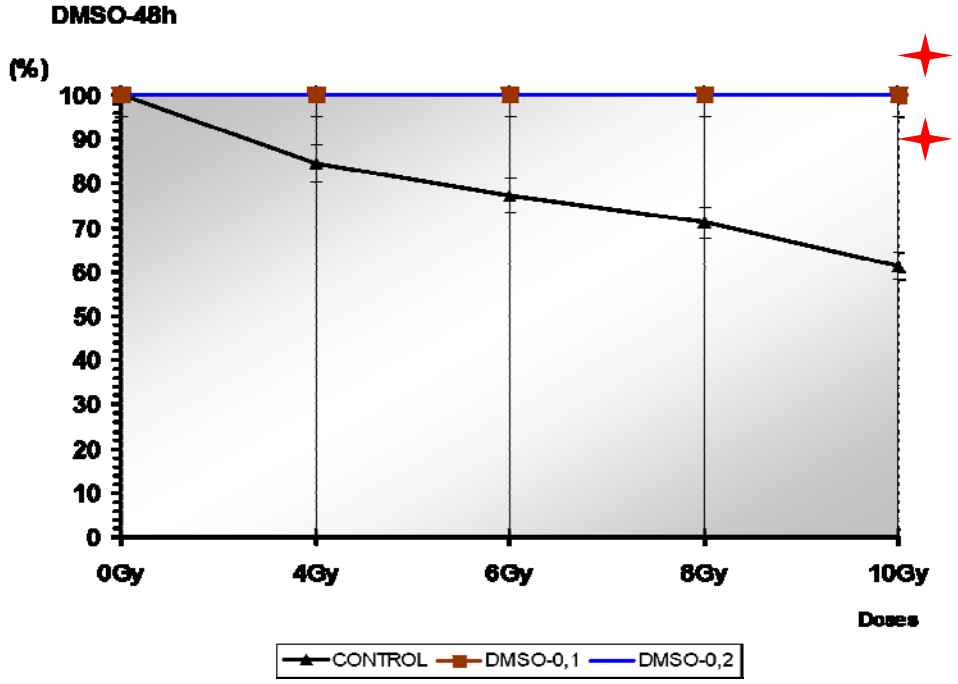


Figure M9. Cell survival curves of normal human prostate epithelial PNT2 cell cultures treated with 0.1% and 0.2% of DMSO and exposed to different doses of X-rays evaluated after 48 hours of incubation (*): (p<0.001) versus irradiated control cells.

The PF obtained with cell cultures treated with the two concentrations of DMSO for 48 hours after exposure to the highest experimental radiation of dose (10 Gy) was 100% for both 0.1% and 0.2% DMSO.

The DRF could not directly be evaluated from the data obtained with the cell culture experiments treated with DMSO at the highest experimental doses (10 Gy) for either of the DMSO concentrations (0.1% and 0.2%) employed in the study.

- **CARNOSIC ACID**

The administration of 25µl of 10 µM and 20 µM carnosic acid after 24 hours PNT2 cell incubation led to a 0.21% and 15.39% decrease in PNT2 cell viability respectively using data obtained from control cell cultures as a point of reference. After incubating for 48 hours and maintaining the same test concentrations i.e., 10 µM and 20 µM, the decrease in cell viabilities were 18.63% and 33.26% respectively (Figure M10).

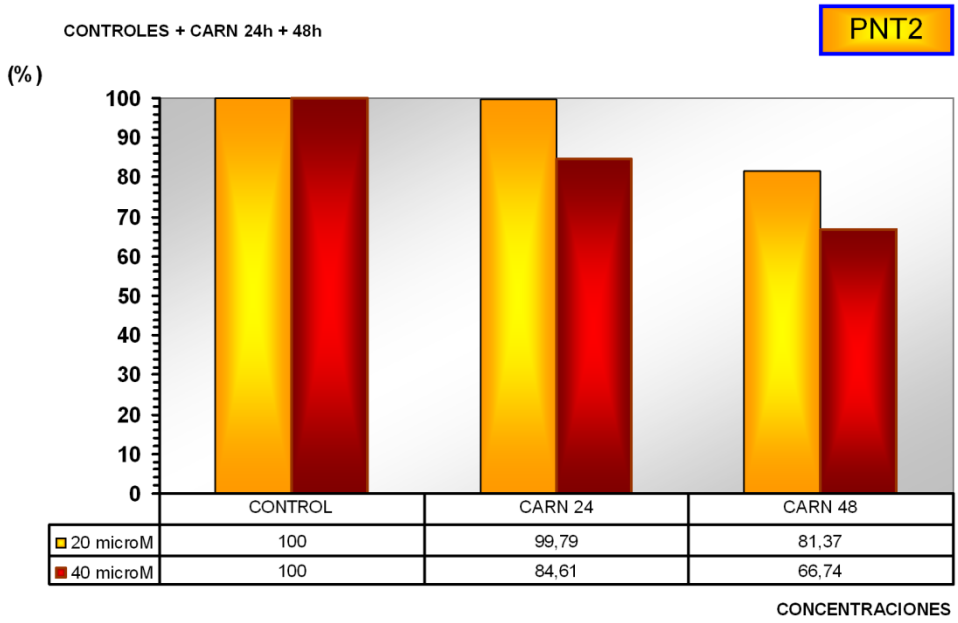


Figure M10. Effect of administration of 10 µM and 20 µM solutions of CARN on PNT2 cell survival evaluated after 24h and 48h incubation periods.

In the CARN-treated PNT2 cell cultures that were exposed to irradiation and evaluated after 48 hours of incubation, the results obtained from the cell cultures treated with 10µM and 20 µM of carnosic acid portrayed a significantly better cell survival response compared with the irradiated control cell cultures. At the highest radiation dose of 10 Gy used in this study, the proportion of surviving cells in the irradiated control cultures dropped by 30.8% when compared with cell populations in cultures that were sensitized with the two test doses of carnosic acid used. At both concentrations of CARN studied, a significant increase in cell survival was shown compared with irradiated control cell cultures ($p<0.001$) (table M6; Figure M11).

Table M6. Percentage cell survival (%) of normal human prostate epithelial PNT2 cell cultures treated with CARN (20µM and 40 µM) and exposed to different doses of X-rays evaluated after 24 hours of incubation

Radiation dose	0 Gy	4 Gy	6 Gy	8 Gy	10 Gy
Control	100.0 ± 5.8	91.0 ± 7.1	86.2 ± 7.2	79.4 ± 5.4	69.2 ± 5.1
10µM Carnosic acid	100.0 ± 8.2	100.0 ± 7.6	100.0 ± 6.9	100.0 ± 7.5	100.0 ± 9.2*
20µM Carnosic acid	100.0 ± 7.9	100.0 ± 8.3	100.0 ± 7.5	100.0 ± 9.0	100.0 ±10.2*

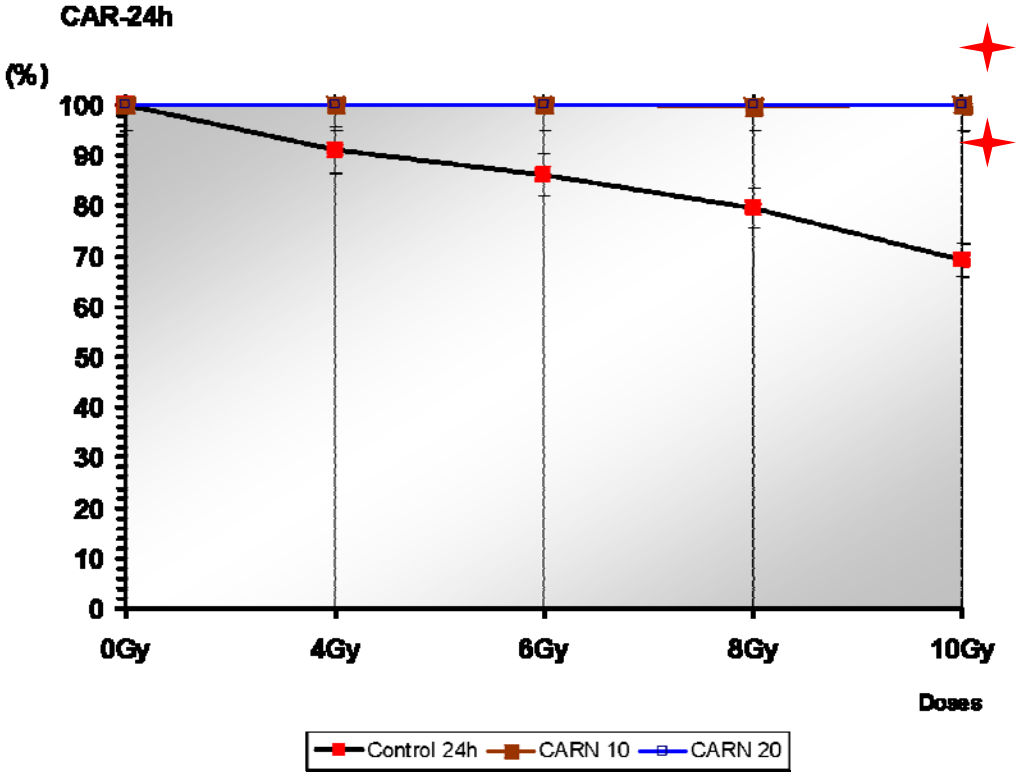


Figure M11. Cell survival curves of normal human prostate epithelial PNT2 cell cultures treated with CARN (20µM and 40 µM) and exposed to different doses of X-rays evaluated after 24 hours of incubation. ((*): (p<0.001) versus irradiated control cells).

The **Protection Factor** (**PF**) obtained from the cultures sensitized with carnosic acid and exposed to the highest experimental radiation dose of 10 Gy following 24 hours incubation was 100% for both 10 µM and 20µM of carnosic acid solutions used.

After 24hr of incubation, it was not possible to directly obtain the **dose Reduction Factor** (**DRF**) from experimental results obtained from the cell cultures sensitized with both concentrations of Carnosic acid (10 µM and 20 µM) and exposed to highest radiation dose (10 Gy).

After 48 hours following incubation, the results obtained with PNT2 cell cultures treated with 10 and 20 µM of carnosic acid and exposed to the highest experimental radiation dose of 10 Gy showed a higher rate of surviving cells when compared with the irradiated control cell cultures. At the end of the two incubation periods (24 h and 48 h), cellular survival was equal, standing at 38.8% compared to irradiated controls at this radiation dose. At both concentrations used, a significant increase in PNT2 cell survival was demonstrated compared with irradiated control cell cultures ($p<0.001$) (Table M7; Figure M12).

Table M7. Percentage cell survival (%) of normal human prostate epithelial PNT2 cell cultures treated with CARN (20µM and 40 µM) and exposed to different doses of X-rays evaluated after 48 hours of incubation.

Radiation dose	0 Gy	4 Gy	6 Gy	8 Gy	10 Gy
Control	100.0 ± 4.5	84.5 ± 8.2	77.3 ± 7.4	71.1 ± 5.6	61.2 ± 5.2
10 µM CARN	100.0 ± 7.5	100.0 ± 7.4	100.0 ± 9.7	100.0 ± 5.7	100.0 ± 4.9*
20µM CARN	100.0 ± 5.7	100.0 ± 9.1	100.0 ± 6.4	100.0 ± 5.7	100.0 ± 5.3*

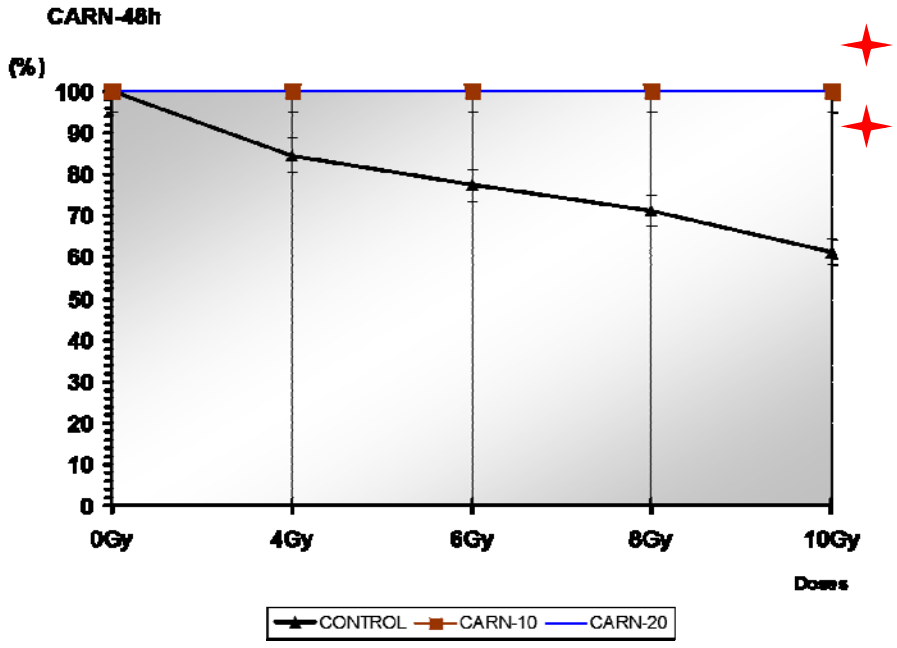


Figure M12. Cell survival curves of normal human prostate epithelial PNT2 cell cultures treated with CARN (20µM and 40 µM) and exposed to different doses of X-rays evaluated after 48 hours of incubation. ((*): (p<0.001) versus irradiated control cells).

The **Protection Factor** (**PF**) obtained of the cultures treated with 10 µM and 20µM of carnosic acid following 48 hours and exposure to the highest radiation dose of 10 Gy was 100% for both concentrations of carnosic acid.

Upon exposure to the highest radiation dose of 10 Gy, and incubating for 48 h, it was not possible to directly obtain the DRF from the cell cultures exposed to both 10 µM, and 20 µM of carnosic acid.

- **Rosmarinic acid (ROS)**

The administration of 25 µl of 20 µM and 40 µM solutions of ROS to the PNT2 cell cultures and incubating for 24 hours did not induce a decrease in percentage cell proliferation with respect to data obtained from control cultures. However, when cells were cultured for 48 hours, at the same test concentrations of 20 µM and 40 µM ROS solutions, a 5.77% decrease in percentage of cell proliferation was observed in the cultures sensitized with 20 µM test solution, while the 40 µM test solution did not show any decrease in proliferating cells with respect to the irradiated control culture values (Figure M13).

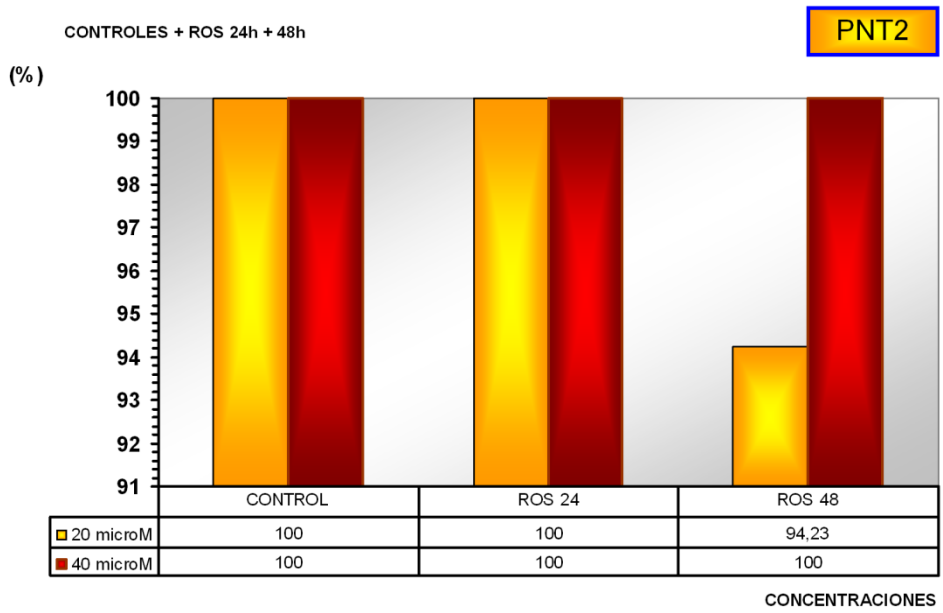


Figure M13. Effect of administration of 20 µM and 40 µM solutions of ROS on PNT2 cell survival evaluated after 24h and 48h incubation periods.

After irradiation and 24 hours of incubation, the results obtained from the PNT2 cell cultures conditioned with the two concentrations of ROS solutions (20 µM and 40 µM) showed a higher cell survival with respect to irradiated control cell cultures. At the highest experimental radiation dose (10 Gy) and test substance concentration (40 µM) used in this study, the cell survival was 21.7% superior to the irradiated control cells. At

both concentrations studied, a significant increase in cell survival was determined compared with irradiated control cell cultures ($p<0.001$) (Table M8; Figure M140).

Table M8. Percentage cell survival (%) of normal human prostate epithelial PNT2 cell cultures treated with ROS (20µM and 40 µM) and exposed to different doses of X-rays evaluated after 24 hours of incubation.

Radiation dose	0 Gy	4 Gy	6 Gy	8 Gy	10 Gy
Control	100.0 ± 5.8	91.0 ± 7.1	86.2 ± 7.2	79.4 ± 5.4	69.2 ± 5.1
Rosmarinic acid 20 µM	100.0 ± 6.0	95.4 ± 6.3	86.0 ± 5.0	88.8 ± 7.1	87.5 ± 6*
Rosmarinic acid 40 µM	100.0 ± 9.2	91.2 ± 7.1	84.5 ± 4.7	93.5 ± 9.4	90.9 ± 6*

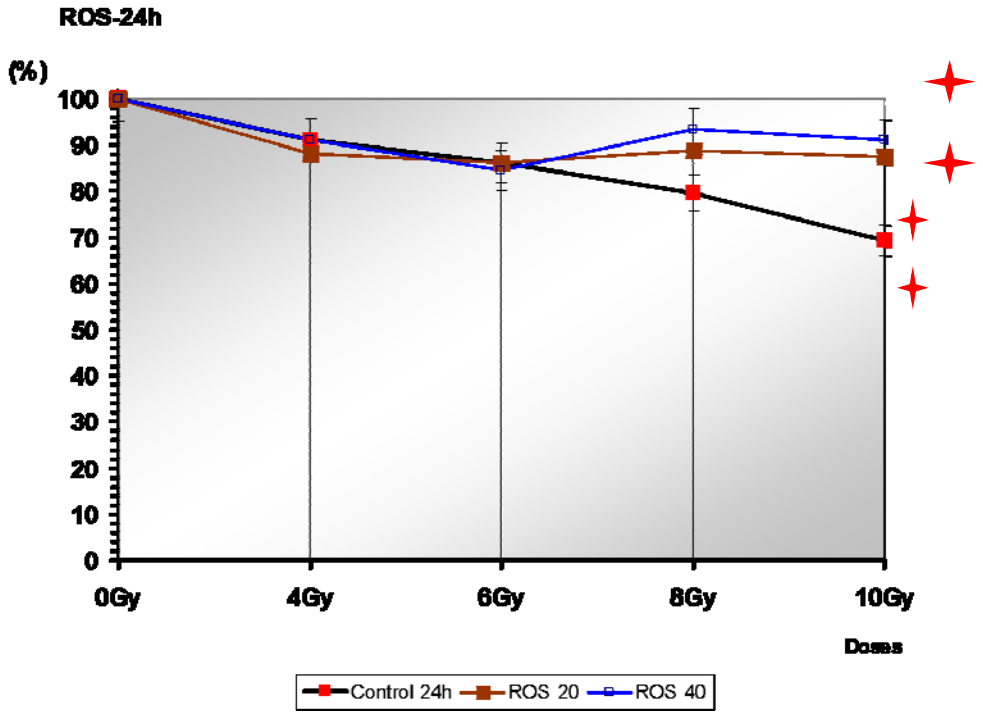


Figure M14. Cell survival curves of normal human prostate epithelial PNT2 cell cultures treated with ROS (20µM and 40 µM) and exposed to different dosed of X-rays evaluated after 24 hours of incubation ((*): ($p<0.001$) versus irradiated control cells).

The **Protection Factor** (**PF**) obtained in the samples conditioned with rosmarinic acid for 48 hours and exposed to the highest radiation dose of 10 Gy was 59.4% for cells sensitized with the 20 µM rosmarinic acid and 30.5% when the 40 µM rosmarinic acid solution was used.

The **Dose Reduction Factor** (**DRF**) obtained with the cultures conditioned with rosmarinic acid at the highest experimental radiation dose of 10 Gy employed in this study was 1.7 for the 20 µM solution and of 2.3 for the 40 µM rosmarinic acid solution.

After 48 hours of incubation, the results obtained for the PNT2 cell cultures conditioned with the two concentrations of ROS (20 and 40 µM) showed a higher cell survival with respect to the irradiated control cell cultures. At the highest experimental radiation dose and test substance concentration used in this study, the cell survival was 29.1% superior to the irradiated control cell cultures. At both concentrations, a significant increase of cellular survival was shown compared with irradiated control cells ($p<0.001$) (Table M9; Figure M115).

Table M9. Percentage cell survival (%) of normal human prostate epithelial PNT2 cell cultures treated with ROS (20µM and 40 µM) and exposed to different dosed of X-rays evaluated after 48 hours of incubation.

Radiation dose	0 Gy	4 Gy	6 Gy	8 Gy	10 Gy
Control	100.0 ± 4.5	84.5 ± 8.2	77.3 ± 7.4	71.1 ± 5.6	61.2 ± 5.2
20 µM Rosmarinic acid	100.0 ± 8.4	100.0 ± 9.1	95.7 ± 7.4	88.1 ± 9.4	89.7 ± 7*
40 µM Rosmarinic acid	100.0 ± 9.3	91.6 ± 8.7	87.4 ± 8.3	89.0 ± 7.3	90.3 ± 6*

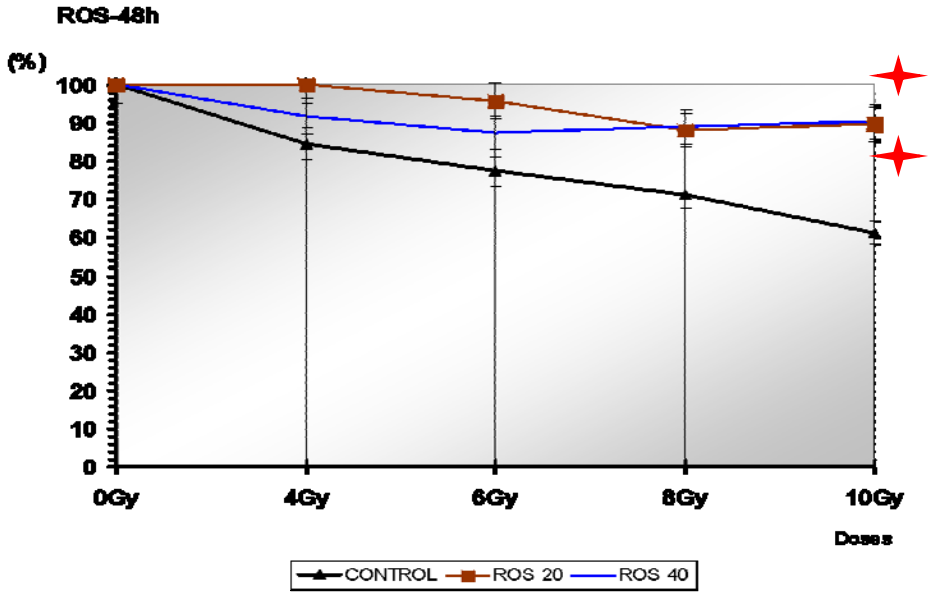


Figure M15. Cell survival curves of normal human prostate epithelial PNT2 cell cultures treated with ROS (20µM and 40 µM) and exposed to different dosed of X-rays evaluated after 48 hours of incubation ((*): ($p<0.001$) versus irradiated control cells).

When cells were irradiated with the highest dose of 10 Gy and incubated for 48 hours, PFs of 73.4% and 75% were obtained when cells were treated with 20 µM and 40 µM rosmarinic acid solutions respectively.

The **Dose Reduction Factor** (**DRF**) obtained in the samples treated with Rosmarinic acid at the highest radiation dose employed in this study (10 Gy) was 3.7 when 20 µM, and of 4.2 when 40 µM rosmarinic acid solutions were employed respectively.

- **MIXTURES APIGENIN AND CARNOSIC ACID (API +CARN)**

The simultaneous administration of equimolar amounts of Apigenin and CARN i.e., 25 µl of 10 µM (10µM API + 10µM CARN) and 20 µM (µ20 µM API + 20µM CARN) was also evaluated on cell proliferation. After 24 hour incubation, the 10 µM equimolar mixture did not lead to a decrease in the percentage of cell proliferation; however, the 20 µM equimolar mixture elicited an 11% reduction in percentage of surviving cells compared with cell viability values obtained in control cell cultures. Following 48 hours of incubation, there was no decrease in cell survival values at the lower concentration (10µM), but a decrease of 27.5% in percentage cell survival was observed with the 20 µM mixture compared with control cell cultures (Figure M16).

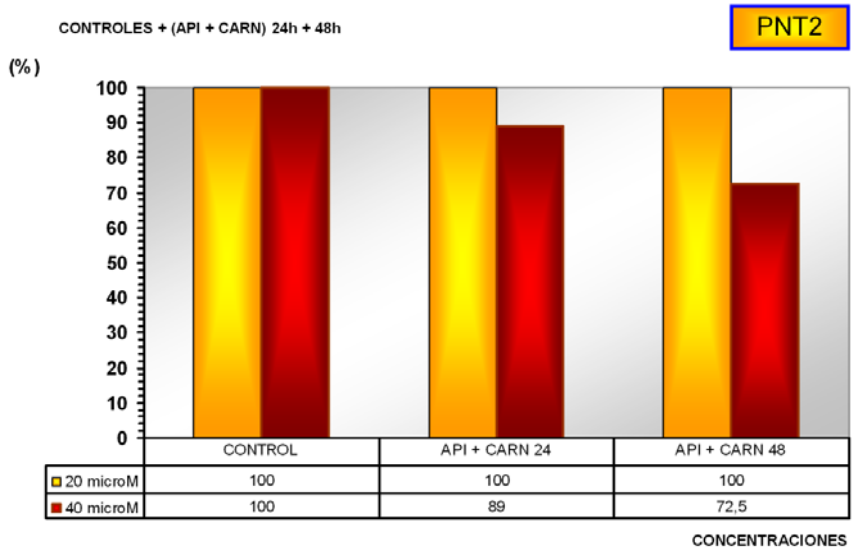


Figure M16. Effect of administration of different concentrations (10µM + 10 µM) and (20µM +20 µM) of (API + CARN) mixtures on PNT2 cell survival evaluated after 24h and 48h incubation periods).

After exposure to the highest experimental radiation dose of 10 Gy and incubating cells for 48 hours, the results obtained with the PNT2 cell cultures co-exposed to the two test substances at both concentrations showed a superior cell survival of 27.3% in comparison with the irradiated control cells. In both concentrations, a significant increase in cell survival was demonstrated compared with control cell cultures ($p<0.001$) (TableM10; Figure M17)

Table M10. Percentage cell survival (%) of normal human prostate epithelial PNT2 cell cultures treated with 10 µM and 20 µM mixtures of (API + CARN) and exposed to different doses of X-rays evaluated after 24 hours of incubation.

Radiation dose	0 Gy	4 Gy	6 Gy	8 Gy	10 Gy
Control	100.0 ± 5.8	91.0 ± 7.1	86.2 ± 7.2	79.4 ± 5.4	69.2 ± 5.1
10 µM (API + CARN)	100.0 ± 7.8	90.0 ± 9.4	90.3 ± 9.8	90.3 ± 7.5	96.5 ± 7.6*
20 µM (API + CARN)	100.0 ± 8.1	96.0 ± 7.5	86.2 ± 5.4	97.3 ± 6.9	94.4 ± 8.2*

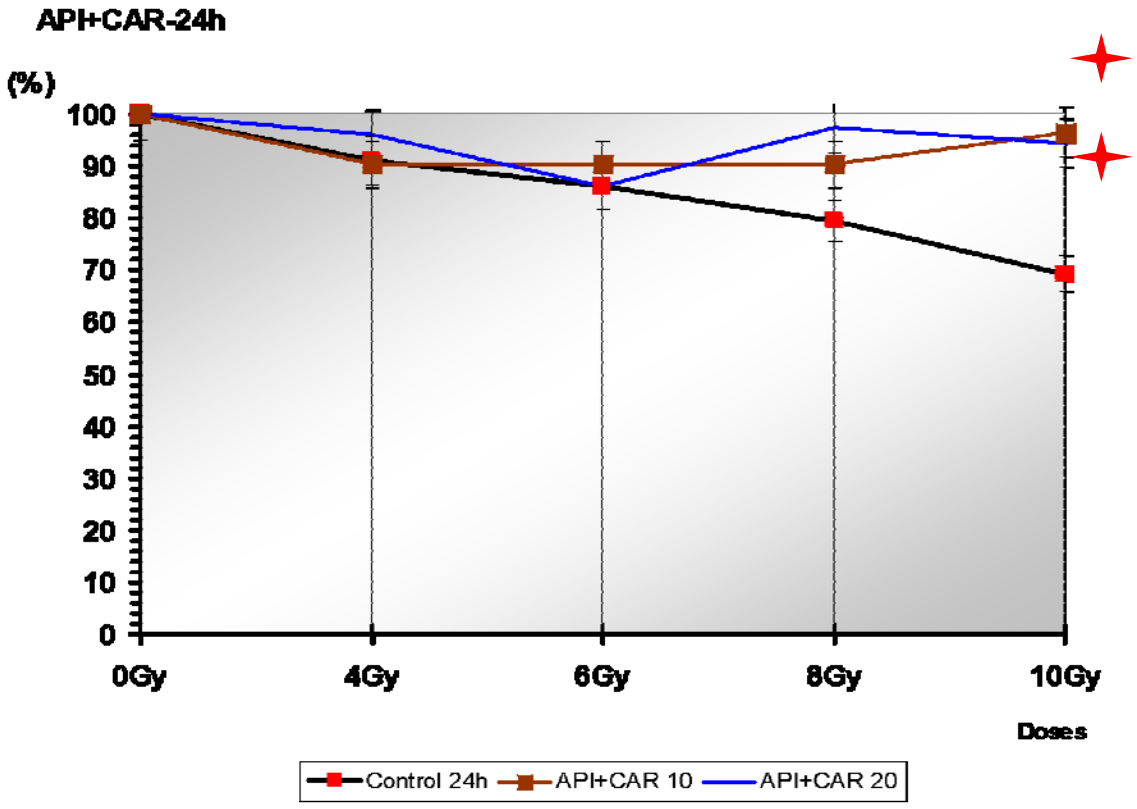


Figure M17. Cell survival (%) of normal human prostate epithelial PNT2 **cell cultures treated with 10 µM and 20 µM mixtures of (API + CARN) and exposed to different dosed of X-rays evaluated after 24 hours of incubation ((*): p<0.001) versus irradiated control cells).**

After exposure to the highest experimental radiation dose of 10 Gy and incubating for 24hours, the PF obtained with the cell cultures treated with the apigenin and CARN mixture was 88.6% at equimolar concentration of 10 µM and 81.8% for the 20 µM mixture.

When the cell cultures where treated with the highest experimental radiation dose of 10 Gy, and incubated for 48 hours, a DRF of 5.8 was obtained when the cultures were treated with the 10 µM mixture and 3.4 when treated with the 20 µM mixture.

When exposed to the highest experimental radiation dose of 10 Gy following 48 hours of incubation, the PNT2 cell cultures treated with the 10 µM and 20 µM mixtures, showed an increased cell survival of 38.8% for the higher equimolar mixture in relation

to the irradiated controls. At both concentrations, a significant increase in surviving cells was demonstrated compared with irradiated control cell cultures (**p<0.001**) (table M11; Figure M18).

Table M11. Percentage cell survival (%) of normal human prostate epithelial PNT2 cell cultures treated with 10 µM and 20 µM mixtures of (API + CARN) and exposed to different doses of X-rays evaluated after 48 hours of incubation.

Radiation dose	0 Gy	4 Gy	6 Gy	8 Gy	10 Gy
Control	100.0 ± 4.5	84.5 ± 8.2	77.3 ± 7.4	71.1 ± 5.6	61.2 ± 5.2
10 µM (API + CARN)	100.0 ± 8.2	70.5 ± 7.4	70.2 ± 9.1	70.8 ± 7.5	73.2 ± 8.9
20 µM (API + CARN)	100.0 ± 7.7	88.5 ± 7.3	90.9 ± 6.5	85.3 ± 2.1	100.0 ±12.4*

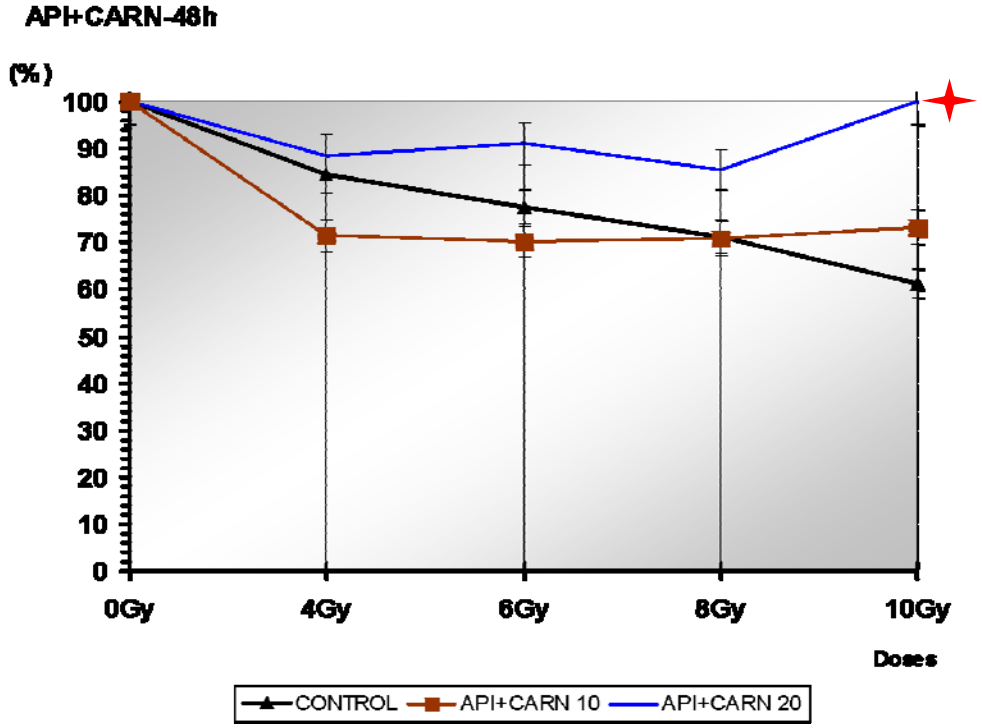


Figure M18. Cell survival curves of normal human prostate epithelial PNT2 cell cultures treated with 10 µM and 20 µM mixtures of (API + CARN) and exposed to different doses of X-rays evaluated after 48 hours of incubation ((*): (p<0.001) versus irradiated control cell cultures).

When cultured cells were exposed to the highest experimental radiation dose of 10 Gy and incubated for 48 hours, the PF obtained with samples treated with apigenin and carnosic acid mixture was 30.9% with the 10 µM mixture and 100% when the 20 µM mixture was used.

The DRF obtained in the samples treated with the equimolar mixtures at the highest experimental radiation dose of 10 Gy was 1.25 for the 10 µM mixture, however, at this same irradiation dose, a corresponding value could not directly be evaluated when the 20 µM mixture was used.

- **MIXTURES APIGENIN AND ROSMARINIC ACID (API + ROS)**

When apigenin and rosmarinic acid were co-administered at equimolar concentrations i.e., 25µl of (10µM API + 10 µM ROS) and (20µM API + 20 µM ROS), a decrease in percentage cell survival was observed after 24 hours of incubation. For the 10 µM mixture, cell survival reduced by 20.4% while the 20 µM mixture, yielded a 47.43% reduction in percentage cell survival both were in relation to the control cell cultures. After 48 hours of incubation and under the same test substance concentrations (10 µM and 20 µM mixtures), the decrease in percentage cell survival for the 10 µM mixture was 40.92%, while the 20 µM mixture produced a reduction of 62.52%, in percentage cell survival both cases were compared with the control cell cultures (Figure M19).

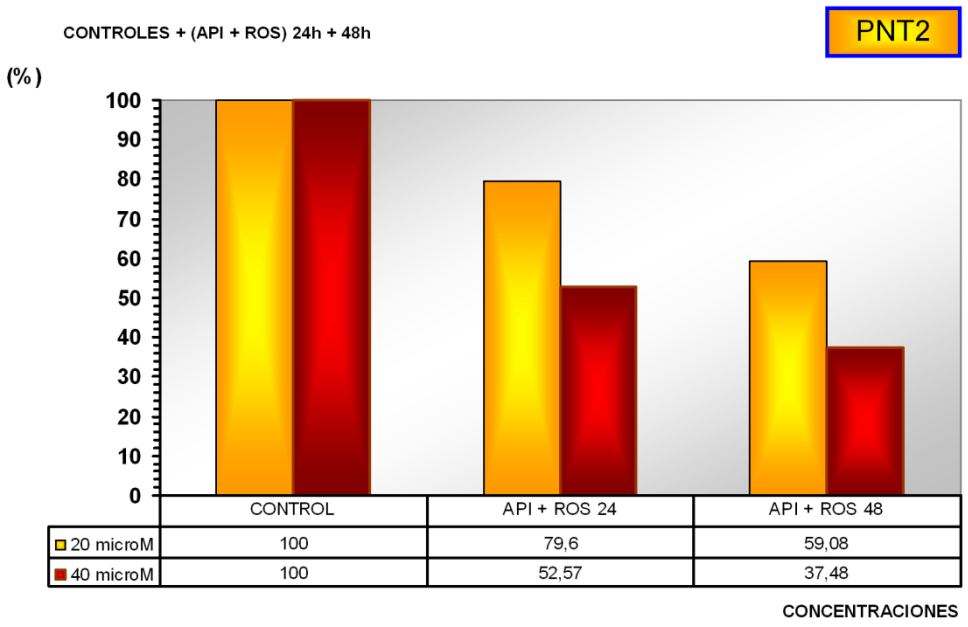


Figure M19. Effect of administration of different concentrations (10µM + 10 µM) and (20µM +20 µM) of (API + ROS) mixtures on PNT2 cell survival evaluated after 24h and 48h incubation periods.

In the PNT2 cell cultures treated with (API + ROS) mixtures and exposed to irradiation, the results obtained from 24 hour incubation cultures treated with the two equimolar mixtures (10 µM and 20 µM) showed an increased cell survival, in relation to the irradiated control cells cultures. An increase in cellular survival of 30.8% for both concentrations was observed when the cells were exposed to 10 Gy of radiation. At both concentrations assayed, a significant increase in the survival of PNT 2 cells was realized compared with irradiated control cell cultures (**$p<0.001$**) (TableM12; Figure M20).

Table M12. Percentage cell survival (%) of normal human prostate epithelial PNT2 cell cultures treated with 10 µM and 20 µM mixtures of (API + ROS) and exposed to different doses of X-rays evaluated after 24 hours of incubation.

Radiation dose	0 Gy	4 Gy	6 Gy	8 Gy	10 Gy
Control	100.0 ± 5.8	91.0 ± 7.1	86.2 ± 7.2	79.4 ± 5.4	69.2 ± 5.1
10 µM (Apigenin + ROS)	100.0 ± 8.9	87.5 ± 8.1	100.0 ± 12.4	93.8 ± 8.4	100.0 ± 8.5*
20 µM (Apigenin + ROS)	100.0 ± 8.2	87.5 ± 7.3	87.5 ± 7.9	94.6 ± 7.5	100.0 ± 7.8*

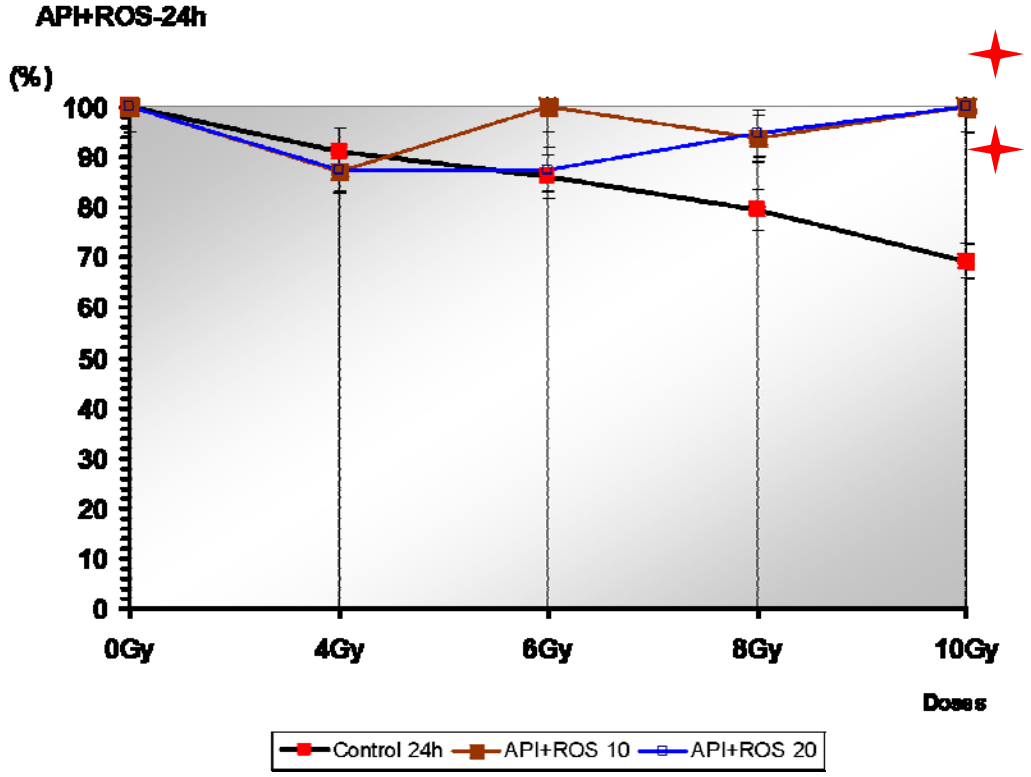


Figure M20. Cell survival curves of normal human prostate epithelial PNT2 cell cultures treated with 10 µM and 20 µM mixtures of (API + ROS) and exposed to different doses of X-rays evaluated after 24 hours of incubation. ((*): ($p<0.001$) versus irradiated control cell culture).

After 24 hours of incubation, the PF obtained with PNT2 cell cultures treated with both mixture concentrations of API and ROS and exposed to the highest experimental radiation dose of 10 Gy was 100%.

The study also evaluated DRFs at the different exposure doses and the two equimolar substance mixtures studied. At the experimental doses used and following a 48-hour incubation period, the DRF could not be evaluated from the cell culture results obtained upon administration of both 10 µM and 20 µM equimolar mixtures.

For the highest experimental radiation dose administered in this study (10Gy), and after 48 hours of incubation, the results obtained for the PNT2 cell cultures treated with the 10 µM and 20 µM equimolar mixtures of API and ROS showed an equal increase in cell survival rates in relation to irradiated control cell cultures. The cell survival increased by 38.8% for both equimolar mixtures at the highest radiation dose administered in this study (10Gy). At both concentrations studied, a significant increase in cell survival was determined compared with irradiated cell control cultures (**p<0.001**) (Table M13; Figure M21).

Table M13. Percentage cell survival (%) of normal human prostate epithelial PNT2 cell cultures treated with 10 µM and 20 µM mixtures of (API + ROS) and exposed to different doses of X-rays evaluated after 48 hours of incubation.

Radiation dose	0 Gy	4 Gy	6 Gy	8 Gy	10 Gy
Control	100.0 ± 4.5	84.5 ± 8.2	77.3 ± 7.4	71.1 ± 5.6	61.2 ± 5.2
10 µM (API + ROS)	100.0 ± 9.2	100.0 ± 9.3	82.8 ± 7.9	86.5± 6.9	100.0 ± 8.4*
20 µM (API + ROS)	100.0 ± 7.3	100.0 ± 8.2	100.0 ± 9.1	100.0 ± 10.0	100.0 ± 9.1*

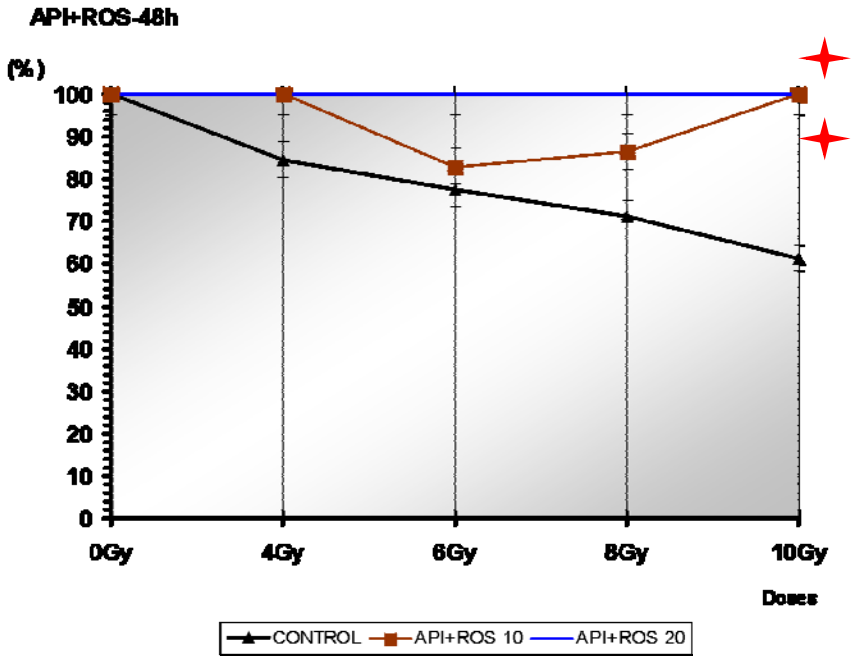


Figure M21. Cell survival curves of normal human prostate epithelial PNT2 cell cultures treated with 10 µM and 20 µM mixtures of (API + ROS) and exposed to different doses of X-rays evaluated after 48 hours of incubation. ((*): (p<0.001) versus irradiated control cell culture).

After exposure to the highest radiation dose of 10 Gy and following a 48 hour incubation period, the PF obtained in the cells treated with the 10 µM and 20 µM equimolar mixtures of API and ROS was 100%. However, at the radiation doses used, the experimental data obtained from cultures treated with both equimolar mixtures did not permit the evaluation of DRFs.

PYCNANTHUS ANGOLENSIS SEED EXTRACT (PASE)

The administration of 25 µl of 25µM of PASE to the cells did not produce any decline in the percentage cell survival with respect to cell proliferation values obtained in PNT2 control cell cultures. This volume (25 µl) and concentration (25µM) of PASE administered constituted the maximum tolerable concentration and volume of the test substance at which PNT2 cell survival was not altered (Figure M22).

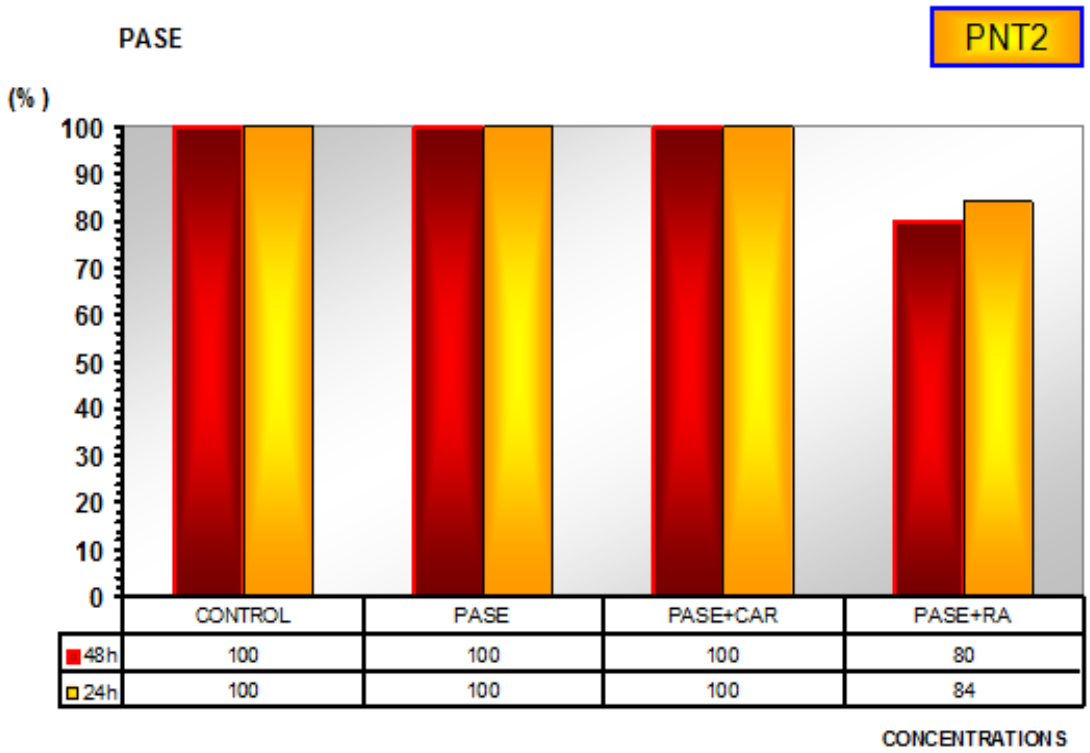


Figure M22. Effect of administration of PASE (alone and in combination with CARN and ROS) on the survival of normal human prostate epithelial PNT2 **cell cultures evaluated at two different incubation periods (24h + 48h).**

After 24-hour incubation, PNT2 cell cultures treated with PASE and exposed to graded doses of radiation, showed an enhanced cellular survival with respect to irradiated control cell cultures: The percentage cell survival in the test cultures exceeded those in the irradiated control cultures by up to 23.3% when the highest experimental radiation dose (10 Gy) was used. With the concentration of PASE used, a significant increase in the survival of PNT2 cells was realized compared with irradiated control cell cultures ($p<0.001$) (Table M14; Figure M23).

Table M14. Percentage cell survival (%) of normal human prostate epithelial PNT2 cell cultures treated with PASE and exposed to different doses of X-rays evaluated after 24 hours of incubation.

Radiation dose	0 Gy	4 Gy	6 Gy	8 Gy	10Gy
Controls	100.0 ± 5.8	91.0 ± 7.1	86.2 ± 7.2	79.4 ± 5.4	69.2 ± 5.1
25 µl of PASE	100.0 ± 6.7	94.4 ± 7.1	87.4 ± 7.9	86.4 ±7.6	85.3 ± 8.1*

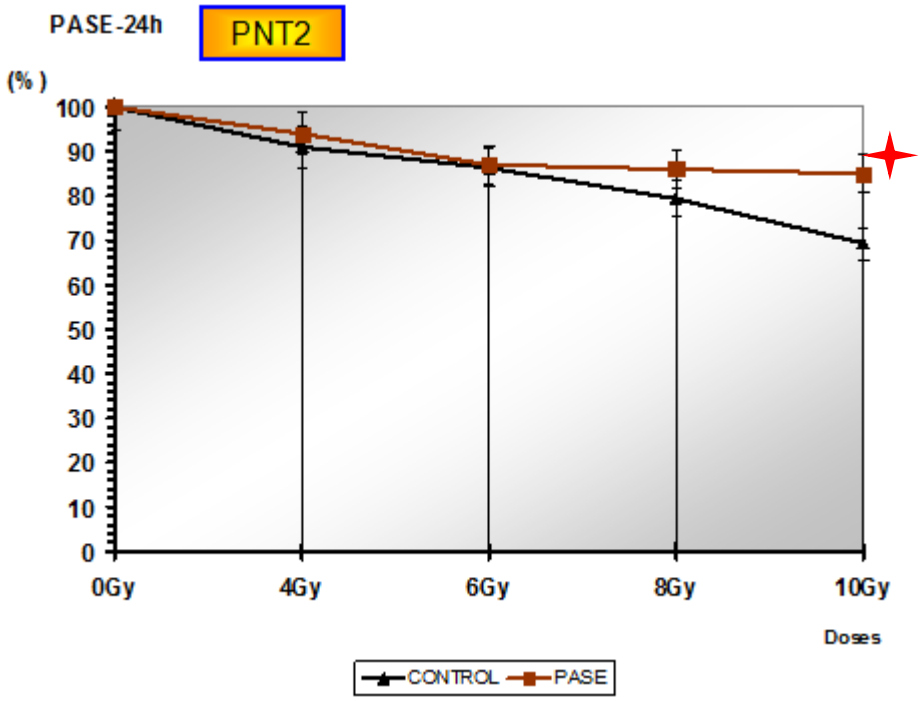


Figure M23.: Cell survival curves of normal human prostate epithelial PNT2 cell cultures treated with PASE and exposed to different doses of X-rays evaluated after 24 hours of incubation ((*): (p<0.001) versus irradiated control cell cultures).

The **Protection Factor** (**PF**) obtained from irradiated PASE-treated PNT2 cell cultures exposed to the highest experimental radiation dose (10 Gy) and incubated for 24 hours at the tested concentration was 17.3%.

The **Dose Reduction Factor** (**DRF**) obtained from 24-hour PASE-treated PNT2 cell cultures exposed to the highest experimental radiation dose (10 Gy) and the test substance concentration (25 µl of 25µM) was 2.0.

After 48 hours of incubation, PNT2 cell cultures treated with the concentration of PASE (25 µl of 25µM PASE solution) studied, showed an improved survival compared with irradiated controls; achieving an increase of 23.8% over control cultures at the highest radiation dose. This concentration of PASE showed a significant increase in the PNT2 cell survival compared with irradiated control cell cultures ($p<0.001$) (Table M15; Figure M24).

Table M15. Percentage cell survival (%) of normal human prostate epithelial PNT2 cell cultures treated with PASE and exposed to different doses of X-rays evaluated after 48 hours of incubation.

Radiation doses	0 Gy	4 Gy	6 Gy	8 Gy	10Gy
Controls	100.0 ± 4.5	84.5 ± 8.2	77.3 ± 7.4	71.1 ± 5.6	61.2 ± 5.2
25 µl of PASE	100.0 ± 6.5	100.0 ± 7.5	95.0 ± 8.9	90.0 ± 7.5	83.0 ± 5.6*

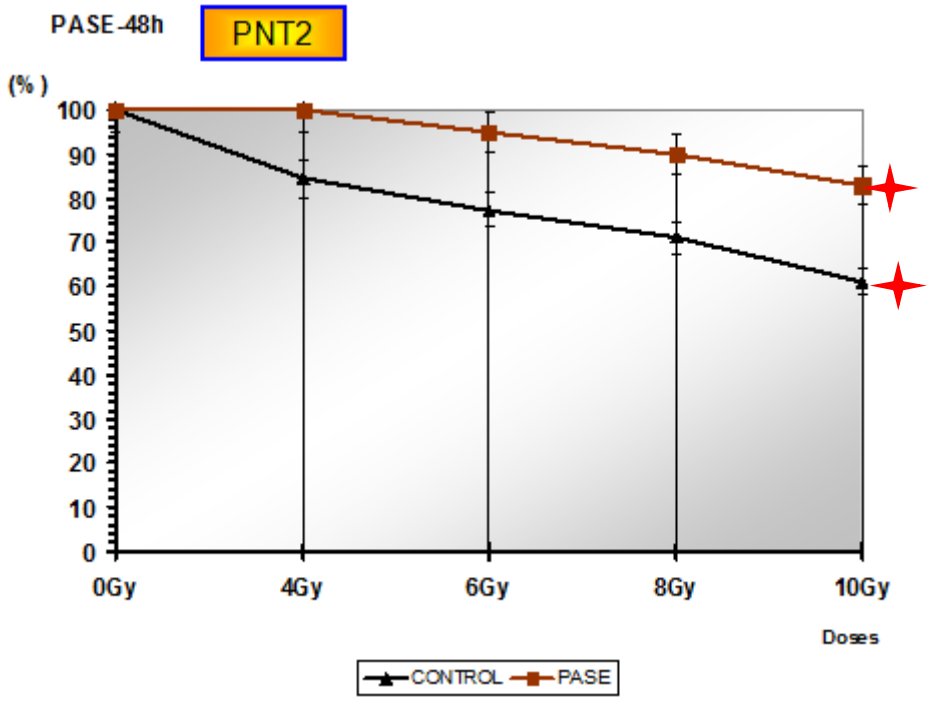


Figure M24. Cell survival curves of normal human prostate epithelial PNT2 cell cultures treated with PASE and exposed to different doses of X-rays evaluated after 48 hours of incubation ((*): (p<0.001) versus irradiated control cell cultures).

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A Protection Factor (PF) of 35.5% was obtained from the 48-hour incubation PNT2 cell cultures treated with 25µlof 25µM PASE and exposed to the highest irradiation dose (10 Gy) used.

The **Dose Reduction Factor** (**DRF**) obtained with the PNT2 cell cultures treated with 25 µl of 25µM PASE at the highest experimental dose of the study (10 Gy) was 2.5.

- **MIXTURES PASE AND CARNOSIC ACID (PASE + CARN)**

The co-administration of PASE and CARN at concentrations of 25 µl of 25µM PASE and 20µl of 20 µM CARN respectively, did not lead to a decrease in the percentage of proliferating PNT2 cells when compared to that obtained from control PNT2 cell cultures (Figure M18).

After 24-hours of incubation, PNT2 cell cultures co-treated with PASE and CARN (25 µl+ 20 µl of 10 µM respectively), demonstrated an increase in cellular survival, with respect to irradiated control PNT2 cell cultures. The percentage cell survival of test cell culture was 27.3% higher than what was measured for control cell cultures at the highest experimental dose of 10 Gy and the test substance concentration used. At the concentration of PASE and CARN studied, a significant increase in PNT2 cell survival was observed compared with irradiated control cell cultures ($p<0.001$) (Table M16; Figure M25)

Table M16. Percentage cell survival (%) of normal human prostate epithelial PNT2 cell cultures treated with (PASE + CARN) and exposed to different doses of X-rays evaluated after 24 hours of incubation.

Radiation doses	0 Gy	4 Gy	6 Gy	8 Gy	10 Gy
Controls	100.0 ± 5.8	91.0 ± 7.1	86.2 ± 7.2	79.4 ± 5.4	69.2 ± 5.1
PASE and CARN (20 µl+10 µM)	100.0 ± 7.8	93.0 ± 9.4	91.3 ± 9.8	90.3 ± 7.5	96.5 ± 7*

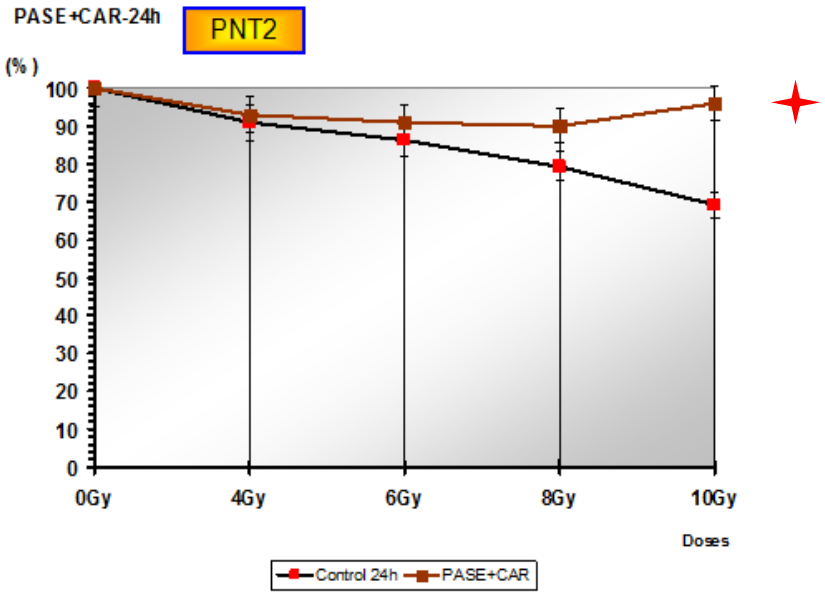


Figure M25. Cell survival curves of normal human prostate epithelial PNT2 cell cultures treated with (PASE + CARN) and exposed to different doses of X-rays evaluated after 24 hours of incubation (*): ($p<0.001$) versus irradiated control cell cultures.

The **Protection Factor** (**PF**) obtained from 24-hour PNT2 cell cultures co-treated with PASE and CARN and exposed to the highest experimental radiation dose (10 Gy) was 88.6%.

The **Dose Reduction Factor** (**DRF**) obtained from PNT2 cell cultures co-treated with PASE and CARN, exposed to the highest experimental radiation dose of 10 Gy and incubated for 24 hours was 5.8.

After 48 hours of incubation, PNT2 cell cultures co-treated with PASE and CARN at the studied concentrations also showed an improved cellular survival rate compared with irradiated PNT2 control cell cultures. The survival rate in the test cell cultures was 12% higher than that obtained in the irradiated control PNT2 cell cultures at the highest experimental radiation dose (10 Gy) and the test substance concentrations used ($p<0.01$) (Table M17; Figure M26).

Table M17. Percentage cell survival (%) of normal human prostate epithelial PNT2 cell cultures treated with (PASE + CARN) and exposed to different doses of X-rays evaluated after 48 hours of incubation.

Radiation doses	0 Gy	4 Gy	6 Gy	8 Gy	10 Gy
Controls	100.0 ± 4.5	84.5 ± 8.2	77.3 ± 7.4	71.1 ± 5.6	61.2 ± 5.2
PASE and CARN (25 µl + 10 µM)	100.0 ± 8.2	90.0 ± 7.4	81.2 ± 9.1	75.8 ± 7.5	73.2 ± 8.9*

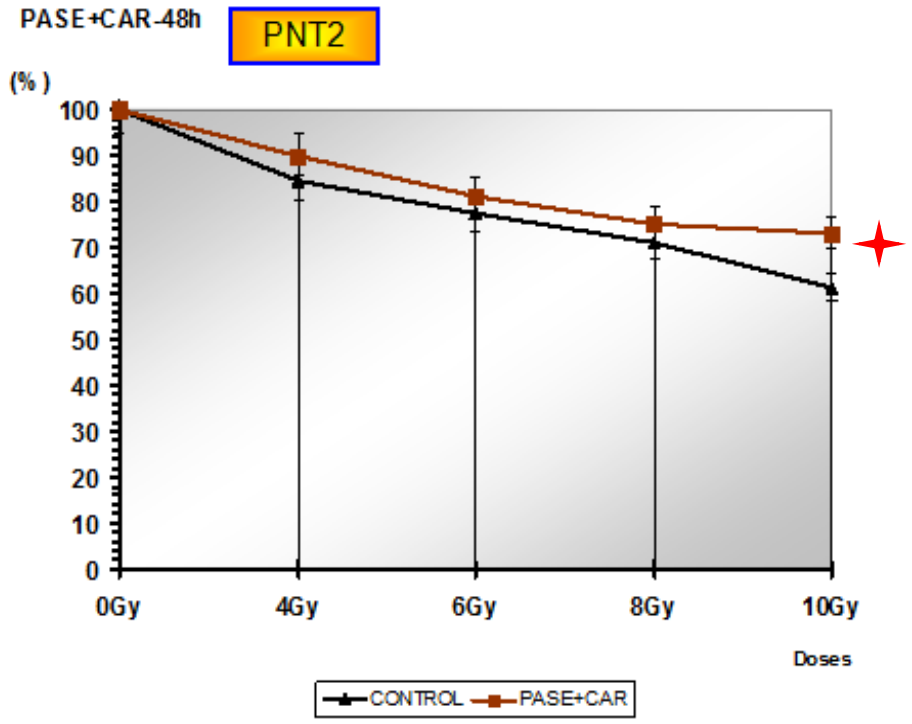


Figure M26. Cell survival curves of normal human prostate epithelial PNT2 cell cultures treated with (PASE + CARN) and exposed to different doses of X-rays evaluated after 48 hours of incubation ((*): (p<0.001) versus irradiated control cell cultures.

The **Protection Factor** (**PF**) obtained in the PNT2 cell culture samples treated with PASE and CARN and exposed to the highest experimental radiation dose (10 Gy) was 30.9 when the cells were incubated for 48 hours.

The **Dose Reduction Factor** (**DRF**) obtained in the PNT2 cell culture samples treated with PASE and CARN and exposed to the highest dose (10 Gy) used in this study was 1.25.

- **MIXTURES PASE AND ROSMARINIC ACID (PASE + ROS)**

The co-administration of PASE and Rosmarinic Acid at respective concentrations of 25 µl of 25µM PASE+ 20µl of 10 µM of ROS produced a decrease of 16% in cellular survival of PNT2 cells after 24 hours and a 20% decrease after 48h (figure M18).

24-hour incubation PNT2 cell cultures co-treated with PASE and ROS at the concentrations studied and subsequently exposed to radiation, demonstrated an increased cellular survival with respect to irradiated control cells. The survival rate in the cell cultures treated with PASE and ROS was 30.8% greater than that observed in the irradiated control PNT2 cell cultures at the tested concentrations and a radiation dose of 10 Gy. At the concentrations of PASE + ROS studied, a significant increase in the survival of PNT2 cells was observed compared with irradiated control cell cultures ($p<0.001$) (table M18; Figure M27).

Table M18. Percentage cell survival (%) of normal human prostate epithelial PNT2 cell cultures treated with (PASE + ROS) and exposed to different doses of X-rays evaluated after 24 hours of incubation.

Radiation doses	0 Gy	4 Gy	6 Gy	8 Gy	10 Gy
Controls	100.0 ± 5.8	91.0 ± 7.1	86.2 ± 7.2	79.4 ± 5.4	69.2 ± 5.1
25µM PASE and 10µM ROS (25 µl + 20 µl)	100.0 ± 8.9	87.5 ± 8.1	100.0 ± 12.4	93.8 ± 8.4	100.0 ± 8.5*

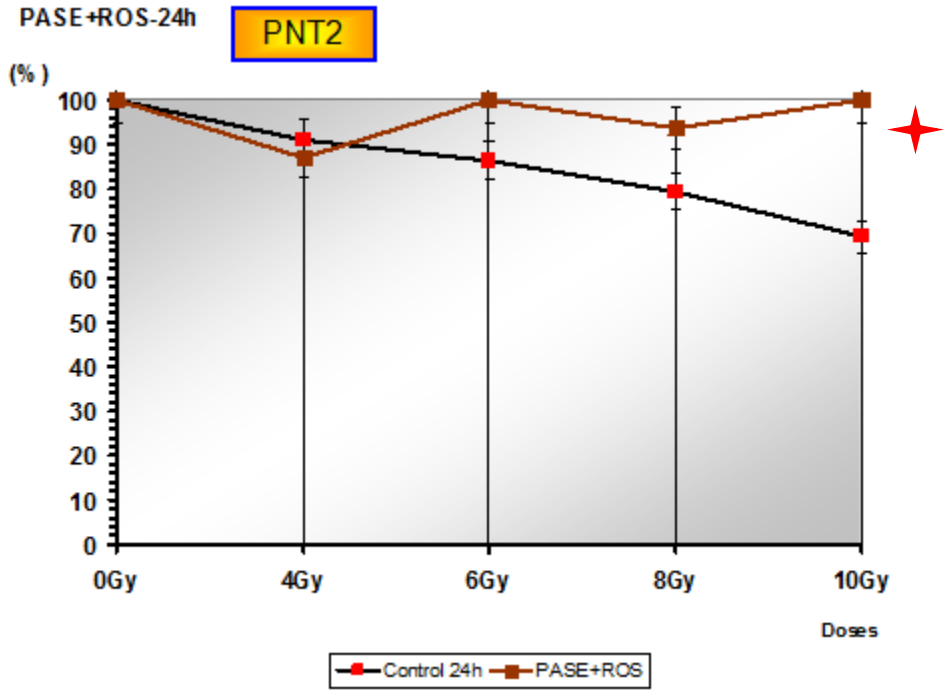


Figure M27. Cell survival curves of normal human prostate epithelial PNT2 cell cultures treated with (PASE + ROS) and exposed to different doses of X-rays evaluated after 24 hours of incubation ((*): ($p<0.001$) versus irradiated control cell cultures).

The **Protection Factor** (**PF**) obtained from 24-hour PNT2 cell cultures co-treated with PASE and ROS after exposure to the highest experimental radiation dose of 10 Gy was 100%. From the data point obtained in this assay, it was not possible to evaluate a **Dose Reduction Factor (DRF**) from PNT2 cell cultures co-treated with PASE and Rosmarinic Acid and exposed to the highest radiation dose (10 Gy) after 24 hours of incubation.

After 48 hours of incubation, PNT2 cell cultures co-treated with PASE and ROS at the studied concentrations showed an increased cellular survival rate compared with irradiated PNT2 control cell cultures. There was a 38.8% increase in cell survival in the test cultures compared with the irradiated control cultures at the test concentrations used and the highest experimental radiation dose administered in this study (10Gy). At the concentrations of PASE + ROS studied, a significant increase in PNT2 cell survival was observed when compared with irradiated control cell cultures ((*): (**p<0.001**) versus irradiated control cell cultures (Table M19; Figure M28).

Table M19. Percentage cell survival (%) of normal human prostate epithelial PNT2 cell cultures treated with (PASE + ROS) and exposed to different doses of X-rays evaluated after 48 hours of incubation.

Radiation doses	0 Gy	4 Gy	6 Gy	8 Gy	10 Gy
Controls	100.0 ± 4.5	84.5 ± 8.2	77.3± 7.4	71.1 ± 5.6	61.2 ± 5.2
PASE + ROS (20 µl + 10 µM)	100.0 ± 9.2	100.0 ± 9.3	82.8 ± 7.9	86.5 ± 6.9	100.0 ± 8.4*

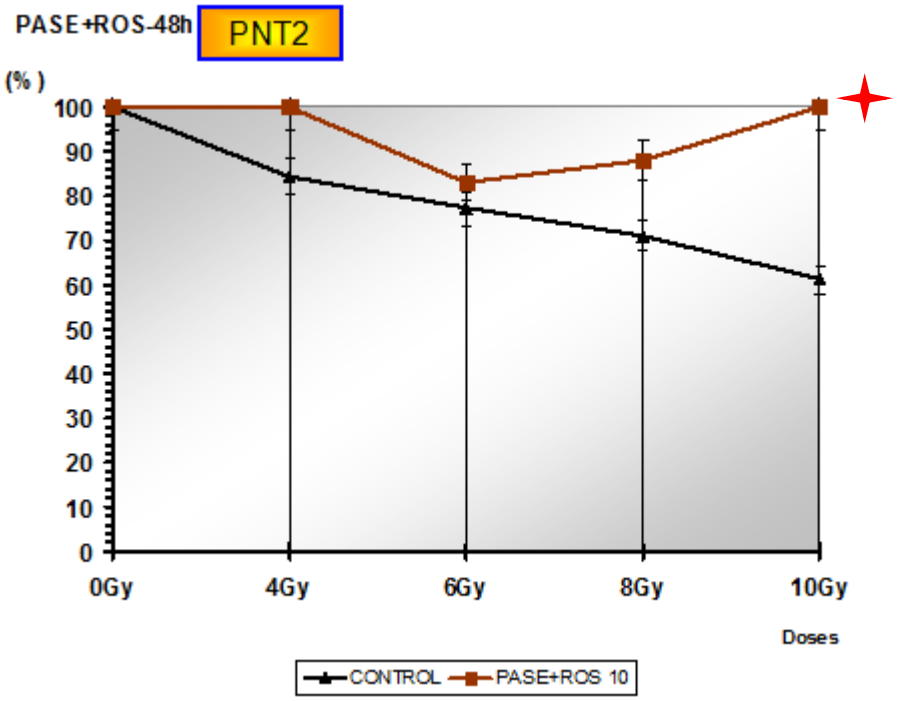


Figure M28. Cell survival curves of normal human prostate epithelial PNT2 cell cultures treated with (PASE + ROS) and exposed to different doses of X-rays evaluated after 48 hours of incubation ((*): (p<0.001) versus irradiated control cell cultures).

The **Protection Factor** (PF) obtained from the PNT2 cell culture samples treated with PASE and ROS and irradiated with the highest experimental dose (10 Gy) was 100% when the cell cultures were incubated for 48 hours.

It was not possible to directly obtain a **Dose Reduction Factor** (DRF) from the PNT2 cell cultures treated with PASE and ROS under the experimental conditions employed.

V. Results.

RESULTS OF CYTOPROTECTION ASSAYS B16F10 MELANOMA CELL LINE

V. Results.

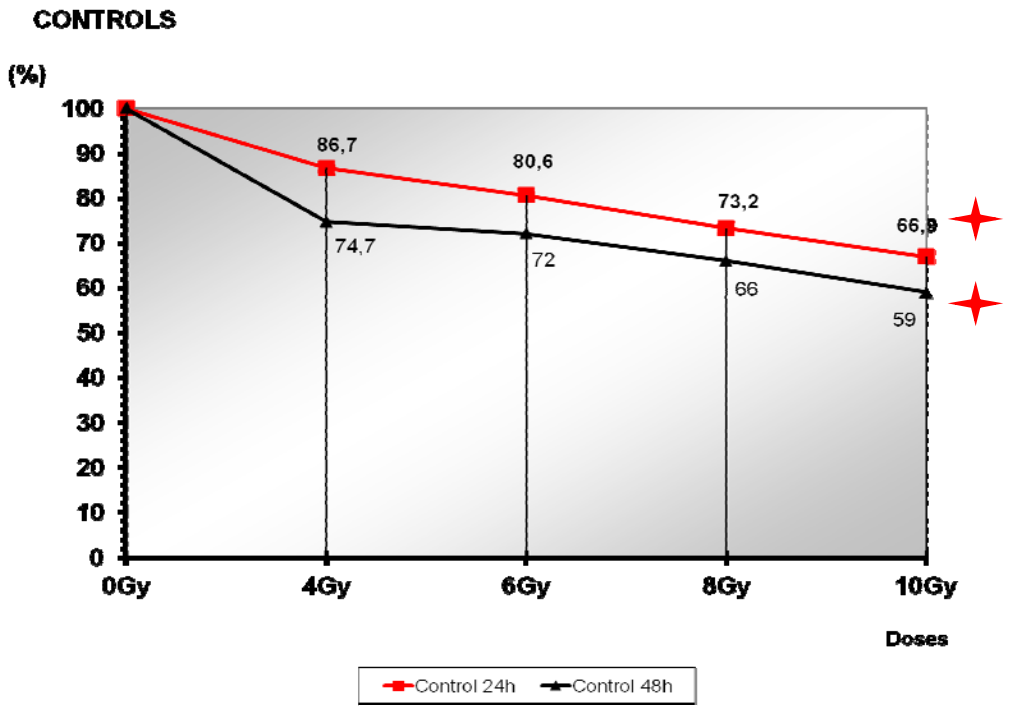
CELL SURVIVAL CURVES WITH B16F10 MELANOMA CELL LINE.

- **Percentage cell survival in B16F10 control cultures after exposure to X-rays**

The cell survival results of B16F10 cells not treated with any of the test substances but irradiated with various X-rays doses i.e., 0 Gy, 4 Gy, 6 Gy, 8 Gy and 10 Gy showed a progressive decrease in the number of viable cells which varied inversely as the radiation dose and post-irradiation incubation period (**p<0.001**). That is, cell viability decreased with increasing radiation dose and post-irradiation period of incubation; Cell survival after 24 hours incubation reduced by 33.1% at the highest radiation dose of 10 Gy and 41% after 48 hours incubation at the same radiation dose (**p<0.001**)(Table M20; Figure M29).

TableM20. Percentage cell survival (%) of control B16F10 cells cultures.

Time/ Radiation doses	0 Gy	4 Gy	6 Gy	8 Gy	10Gy
24 hours	100 ± 9.5	86.7 ± 7.4	80.6 ±8.2	73.2 ± 6.8	66.9 ± 7.7*
48 hours	100 ± 0	74.7± 8.3	72 ± 6.8	66.0 ± 7.4	59.0 ± 5.9 *



FigureM29. Cell survival curves of metastatic B16F10 melanoma control cell cultures ((*): (p<0.001) versus irradiated control cells.

- **SUBSTANCES ASSAYED**

Figure M26 shows the radioprotective effect of the administration of different substances and mixtures on metastatic B16F10 melanoma cells (except the effect elicited by *Pycnanthus angolensis* seed extract (PASE) which is shown in Figure M39). Different substance and mixture concentrations (20µM and 40 µM) were used in this quest for their cellular radioprotection. The results obtained after treating B16F10 cell cultures with these test substances and incubating the cell cultures for 24h have been summarized in Figure M30.

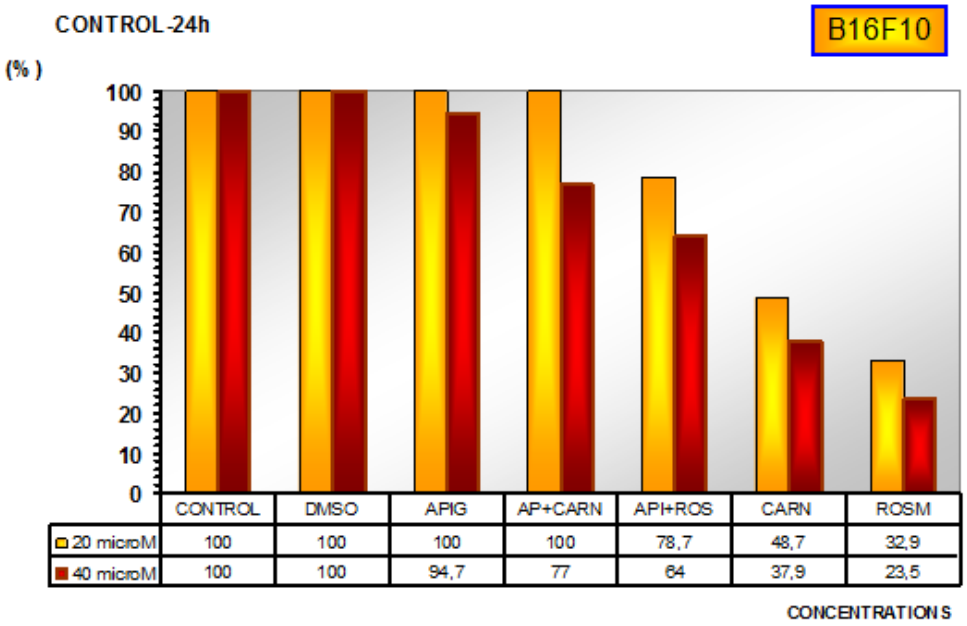


Figure M30. Percentage cell survival of control cell cultures after the administration of the different substances studied on B16F10 melanoma cells following a 24h incubation period (DMSO: Dimethyl sulfoxide; API: Apigenin; CARN: carnosic Acid; ROS: Rosmarinic Acid).

Figure M27 shows the radioprotective effect of the administration of different substances and mixtures on metastatic B16F10 melanoma cells (except the effect elicited by *Pycnanthus angolensis* seed extract (PASE) which is shown in Figure M40). Different substance and mixture concentrations (20µM and 40 µM) were used in this quest for cellular radioprotection by different substances. The results obtained after treating B16F10 cell cultures with these test substances and incubating the cell cultures for 48h have been summarized in Figure M31.

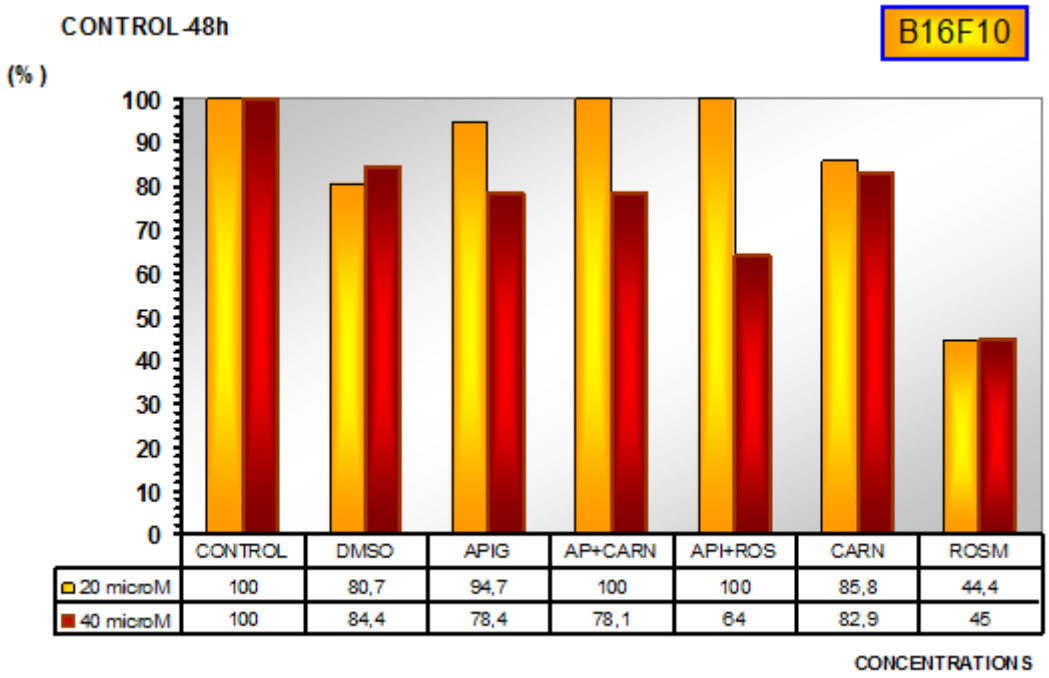


Figure M31. Percentage cell survival of control cell cultures after the administration of the different substances studied on B16F10 melanoma cells following a 48h incubation period (DMSO: Dimethyl sulfoxide; API: Apigenin; CARN: carnosic Acid; ROS: Rosmarinic Acid).

- **APIGENIN**

Exposure of B16F10 cells to 25µl of a 20 µM solution of apigenin over a 24-hour incubation period did not produce a decrease in the rate of cell proliferation when compared to control cultures. However, the administration of apigenin to these cells at a concentration of 40 µM for the same incubation period (24 hours) produced a small decrease (5.3%) in the rate of cellular proliferation (Figure M30). After 48 hours incubation at the same concentrations i.e., 20 µM and 40 µM, used above, the rate of cellular proliferation reduced by 5.3% and 21.6% respectively when compared with the control cultures (Figure M31).

After 24 hours incubation of B16F10 cell cultures treated with the two concentrations (20 µM and 40 µM) of apigenin and exposed to X-rays, the results obtained demonstrated an increased cell survival, with respect to irradiated control cells. At the highest radiation dose of 10Gy there was a noticeable decrease in cell survival of 33.1% in the control irradiated cells when compared with cells treated with 40 µM of apigenin. At both concentrations studied, a significant increase in the cell survival of

metastatic B16F10 melanoma cells was established when compared with irradiated control cell culture values (**p<0.001**) (Table M21; Figure M32)

Table M21. Percentage cell survival (%) of B16F10 cell cultures treated with 20 µM and 40 µM of Apigenin and exposed to different doses of X-rays evaluated after 48 hours of incubation.

Radiation doses	0 Gy	4 Gy	6 Gy	8 Gy	10Gy
Control	100.0 ± 9.5	86.7 ± 7.4	80.6 ± 8.2	73.2 ± 6.8	66.9 ± 7.7
Apigenin 20 µM	100.0 ± 7.4	100 ± 7.1	91.4 ± 6.5	88.9 ± 5.3	85.7 ± 5.2*
Apigenin 40 µM	100.0 ± 7.1	100 ± 8.1	100.0 ± 5.7	100.0 ± 6.2	100.0 ± 6.9*

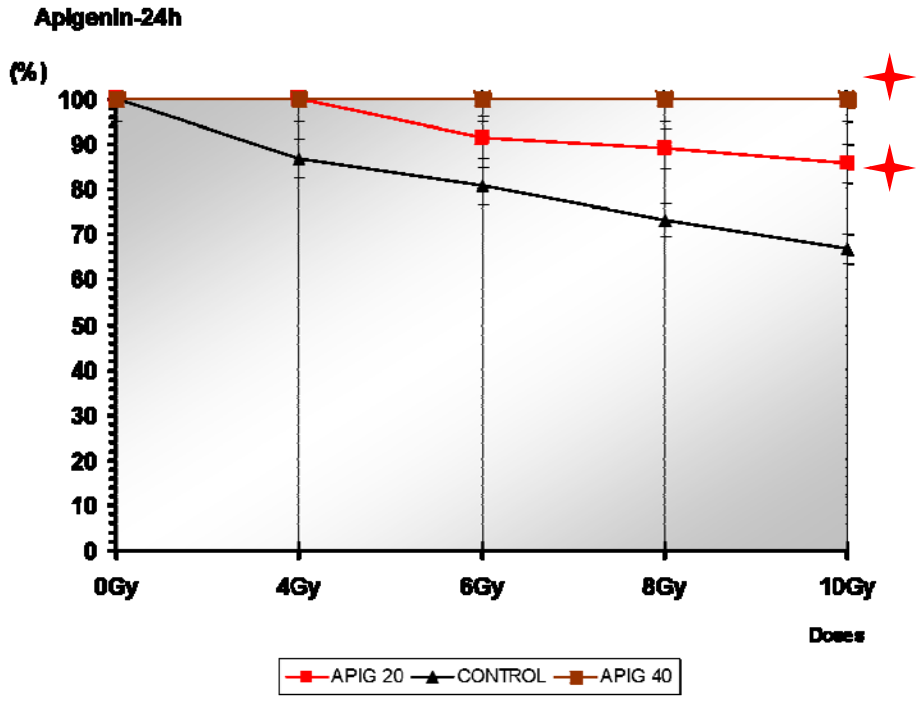


Figure M32. Cell survival curves for irradiated Apigenin (20 to 40 µM) treated B16F10 cell cultures evaluated after 24 hours of incubation ((*) :(p<0.001) versus irradiated control cell cultures).

The radiation **Protection Factor** (**PF**) obtained with 24-hour incubation cultures of B16F10 cell cultures treated with 20 µM apigenin and exposed to the highest experimental radiation dose of 10 Gy was established to be 56.79% and 100% when the cells were treated with the 40 µM apigenin solution and incubated for the same period.

The radiation **Dose Reduction Factor** (**DRF**) in 24-hour B16F10 cell cultures treated with 20 µM apigenin at the highest experimental radiation dose (10 Gy) used was found to be 2.3. However, when treated with a higher concentration (40 µM)

of apigenin and exposed to the same radiation dose of 10 Gy and incubation period, a DRF could not be directly evaluated from the experimental data points available.

After 48 hours of incubation following the administration of the two concentrations of apigenin (20 to 40 µM) assayed, the results showed an increase in cell survival when compared with control irradiated cell cultures. An increase of 14.5% in cell survival was obtained for the higher concentration of apigenin (40 µM) at the highest radiation dose employed (10 Gy) while a 24.3% increase in cell survival was noticed for the lower concentration (20 µM) of apigenin at the same radiation dose (10 Gy). At both concentrations of apigenin studied, a significant increase in the survival of B16F10 melanoma cells was established when compared with irradiated control cell cultures (**p<0.001**) (Table M22; Figure M33).

Table M22. Percentage cell survival (%) of B16F10 cell cultures treated with 20 µM and 40 µM of Apigenin and exposed to different doses of X-rays evaluated after 48 hours of incubation.

Radiation doses	0 Gy	4 Gy	6 Gy	8 Gy	10Gy
Control	100.0 ± 0	74.7 ± 8.3	72.0 ± 6.8	66.0 ± 7.4	59.0 ± 5.9
Apigenin 20 µM	100.0 ± 9.1	100.0 ± 8.1	83.2 ± 7.8	74.6 ± 7.5	83.3 ± 7.9*
Apigenin 40 µM	100.0 ± 8.4	100.0 ± 8.7	90.7 ± 8.1	82.9 ± 7.5	73.5 ± 7.9*

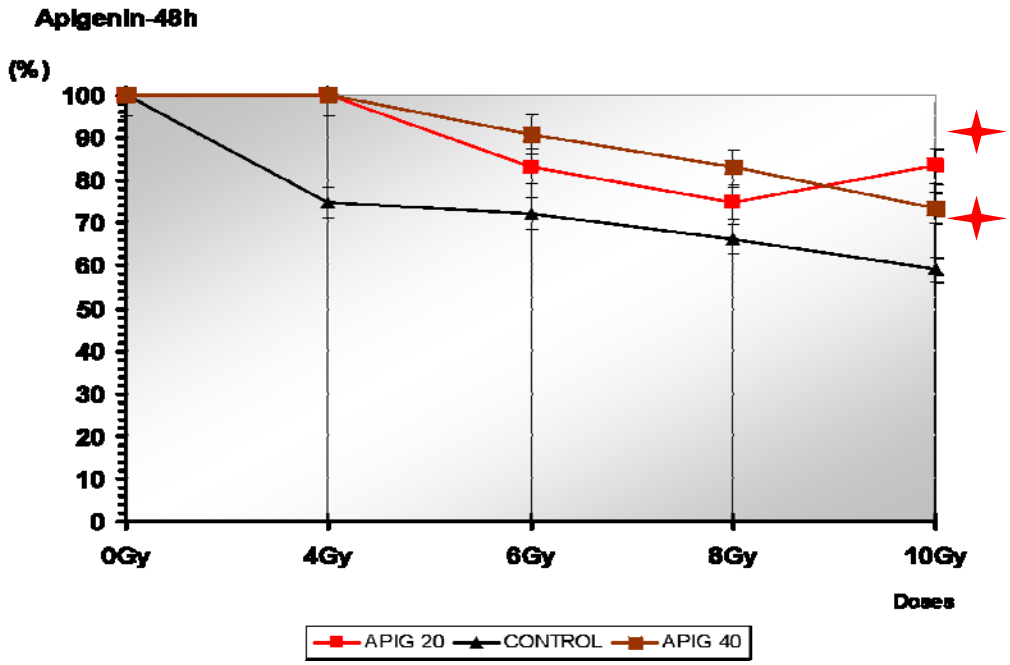


Figure M33. Cell survival curves of B16F10 cell cultures treated with Apigenin (20µM and 40µM) and exposed to different doses of X-rays evaluated after 48 hours of incubation. ((*): (p<0.001) versus irradiated control cell cultures.

The radiation **Protection Factor (PF)** obtained from 48-hour B16F10 cell cultures treated with 20 µM apigenin after exposure to the highest experimental radiation dose (10 Gy) was 59.2% and 35.3% when a 40 µM concentration of apigenin was used.

A **Dose Reduction Factor (DRF)** of 4 was obtained with cell culture samples conditioned with 20 µM solution of apigenin at the highest radiation dose used in this study (10 Gy) while a DRF of 2 was obtained when a 40 µM apigenin solution was used.

- **DIMETHYL SULFOXIDE (DMSO)**

Administration of **DMSO** at concentrations of 0.1% and 0.2% did not decrease the percentage of surviving B16F10 cells after 24 hours of incubation compared with control cultures (Figure 30). However, after 48 hours of incubation at the same concentrations of DMSO (i.e., 0.1% and 0.2%), cellular survival reduced by 19.3% and 15.6% respectively compared with control cultures (Figure 31).

After 24 hours incubation of the B16F10 cells with the two concentrations of DMSO studied (i.e., 0.1% and 0.2%) and exposure to the highest experimental radiation dose (10 Gy), a higher cell survival of 21.8 % with respect to irradiated control cells was obtained for the lower DMSO concentration tested (0.1%) while a less superior cell survival of 10.1% with respect to irradiated control cultures was obtained for the higher DMSO concentration (0.2%) and the same dose (10 Gy). At both concentrations of DMSO studied, a significant increase in the survival of B16F10 melanoma cells was established when compared with irradiated control cell cultures (**$p<0.01$**) (Table M23; Figure M34).

Table M23. Percentage cell survival (%) of B16F10 cell cultures treated with 0.1% and 0.2% of DMSO and exposed to different doses of X-rays evaluated after 24 hours of incubation.

Radiation doses	0 Gy	4 Gy	6 Gy	8 Gy	10Gy
Control	100.0 ± 9.5	86.7 ± 7.4	80.6 ± 8.2	73.2 ± 6.8	66.9 ± 7.7
DMSO 0.1%	100.0 ± 0	100.0 ± 0	94.7 ± 0	88.0 ± 0	88.7 ± 0*
DMSO 0.2%	100.0 ± 0	100.0 ± 0	89.0 ± 0	87.6 ± 0	77.0 ± 0Δ

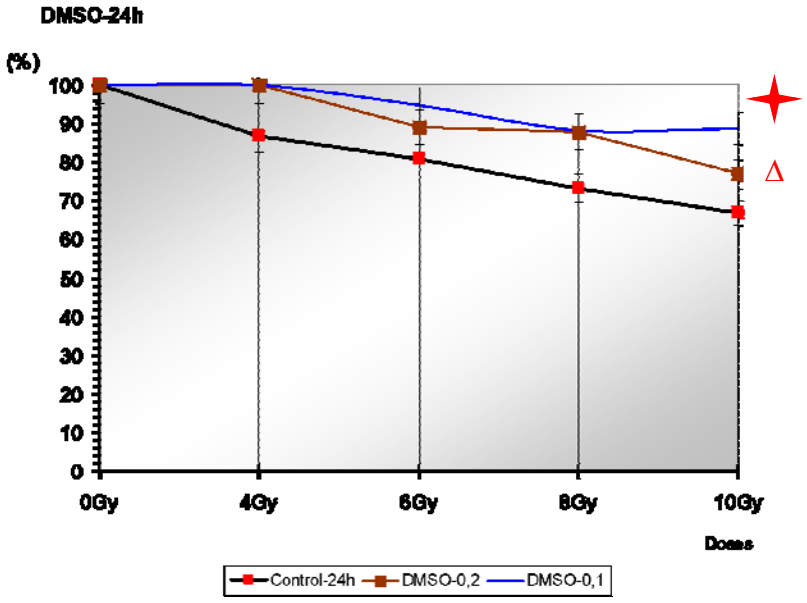


Figure M34. Cell survival curves of B16F10 cell cultures treated with 0.1% and 0.2% of DMSO and exposed to different doses of X-rays evaluated after 24 hours of incubation ((+): ($p<0.01$) versus irradiated control cell cultures.

The radiation **Protection Factor (PF**) obtained from 24-hour cultures of B16F10 cells conditioned with 0.1% DMSO and exposed to 10 Gy of radiation was 65.8% while a PF of 30.5 % was established for cultures treated with the higher DMSO concentration (0.2%) and the same radiation dose and incubation period.

Dose Reduction Factor (FRD) obtained from 24-hour cultures of B16F10 cells conditioned with 0.1% DMSO and exposed to the highest radiation dose (10 Gy) was 2.7., but reduced to 1.3 when a higher DMSO concentration (0.2%) was used.

After 48 hours of incubation following exposure of B16F10 cells to both concentrations of DMSO (0.1% and 0.2%) assayed, a higher cell survival of 6.1% relative to irradiated controls was established with the 0.1% DMSO solution at a radiation dose of 10 Gy, while the higher concentration of DMSO (0.2%) produced a cell survival of 7% less than the irradiated controls at 10 Gy (Table M24; Figure M31).

After 48h of incubation, the cell survival values obtained for B16F10 melanoma cells cultures treated with two levels of DMSO (0.1% and 0.2%) did not show significant differences compared with the cell survival value obtained with irradiated control cell cultures (Table M24; Figure M35).

Table M24. Percentage cell survival (%) of B16F10 cell cultures treated with 0.1% and 0.2% of DMSO and exposed to different doses of X-rays evaluated after 48 hours of incubation

Radiation doses	0 Gy	4 Gy	6 Gy	8 Gy	10Gy
Controls	100.0 ± 0	74.7 ± 8.3	72.0 ± 6.8	66.0 ± 7.4	59.0 ± 5.9
DMSO 0.1%	100.0 ± 6.1	65.0 ± 7.5	71.9 ± 7.1	72.5 ± 6.4	65.1 ± 6.9
DMSO 0.2%	100.0 ± 7.4	58.4 ± 7.0	61.9 ± 6.4	60.1 ± 8.0	52.0 ± 8.1

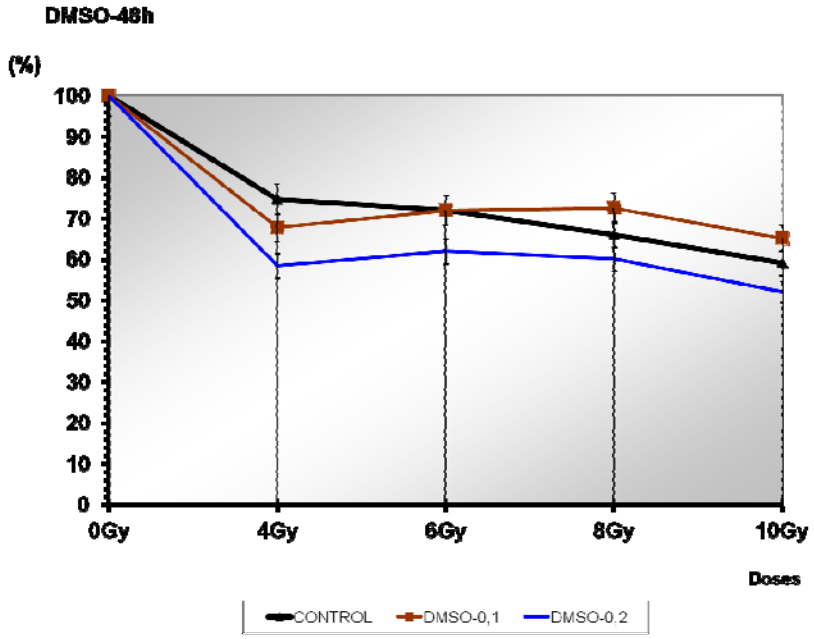


Figure M35. Cell survival curves of B16F10 cell cultures treated with 0.1% and 0.2% of DMSO and exposed to different dosed of X-rays evaluated after 48 hours of incubation ((*): (p<0.001) versus irradiated control cell cultures.

Protection Factor (PF) obtained for 48-hour B16F10 cell cultures conditioned with 0.1% DMSO and exposed to the experimental highest dose (10 Gy) was 14.8% and zero (i.e. , a lack of protective capacity) when 0.2 % DMSO was used.

The **Dose Reduction Factor (DRF)** obtained for 48-hour cell cultures sensitized with 0.1% DMSO after exposure to the highest dose used in this study (10 Gy) was 1.2, whereas it was not practical to obtain a DRF value when a 0.2% solution of DMSO was used as this concentration was without radioprotective capacity.

- **CARNOSIC ACID (CARN)**

Sensitization of B10F16 cell cultures with 10 µM and 20 µM solutions of carnosic acid for 24 hours produced a decreased percentage of 51.3% and 62.1% in proliferating cells respectively with respect control cultures (Figure M30). After 48

hours of cell incubation at the same concentrations of carnosic acid (i.e., 10 µM and 20 µM), percentage cellular proliferation further decreased by 14.2% and 17.1% respectively when compared with control cell cultures (Figure M31).

After 24 hours of B16F10 cell incubation at both concentrations of carnosic acid (10 and 20 µM) tested**,** cell survival was observed to increase by 15.5% for the lower concentration (10 µM) and 33.1% for the higher concentration (20 µM) of carnosic acid used when compared with irradiated control cell cultures; in both cases when the highest radiation dose (10 Gy) in this study was used. At both concentrations of CARN used, a significant increase in the survival of B16F10 melanoma cells was established when compared with irradiated control cell cultures (**p<0.001**) (Table M25; Figure M36).

Table M25. Percentage cell survival (%) of B16F10 cell cultures treated with CARN (20µM and 40 µM) and exposed to different doses of X-rays evaluated after 24 hours of incubation.

Radiation doses	0 Gy	4 Gy	6 Gy	8 Gy	10Gy
Controls	100.0 ± 9.5	86.7 ± 7.4	80.6 ± 8.2	73.2 ± 6.8	66.9 ± 7.7
Carnosic Acid 10 µM	100.0 ± 8.4	100.0 ± 8.1	100.0 ± 7.6	100.0 ± 7.9	82.3 ± 9.2*
Carnosic Acid 20 µM	100.0 ± 7.9	100.0 ± 8.4	100.0 ± 8.2	100.0 ± 9.1	100 ± 10.2*

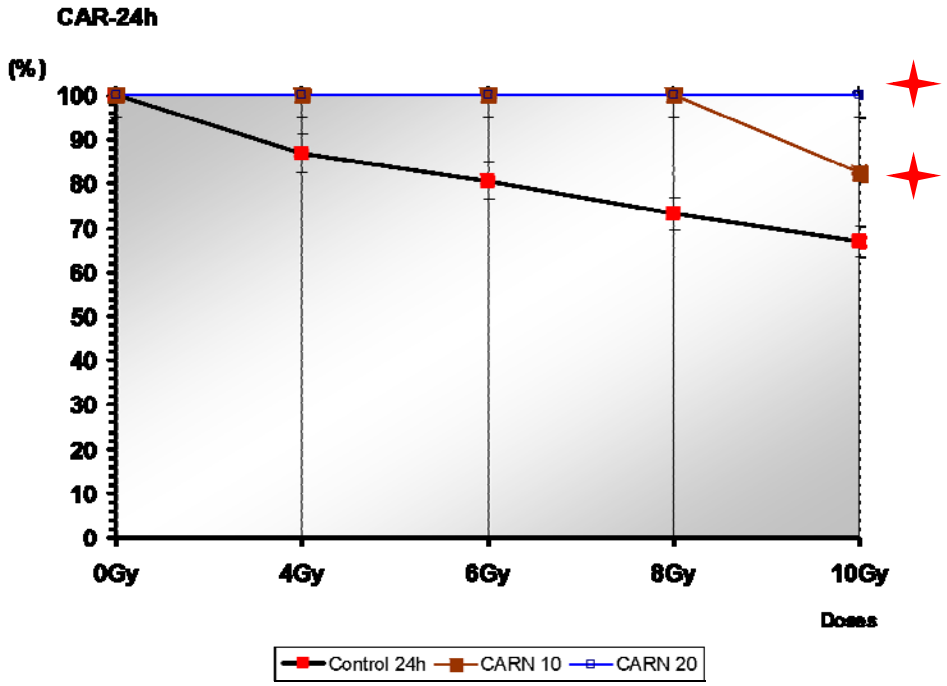


Figure M36. Cell survival curves of B16F10 cell cultures treated with CARN (20µM and 40 µM) and exposed to different dosed of X-rays evaluated after 24hours of incubation ((*): (p<0.001) versus irradiated control cell cultures).

Protection Factor (PF) obtained from 24-hour B16F10 cell cultures treated with 10 µM carnosic acid and exposed to the highest dose (10 Gy) was 46.5% and 100% when 20 µM apigenin solution was used.

Dose Reduction Factor (DRF) obtained for 24-hour cultures treated with 10 µM carnosic acid and exposed to the highest experimental radiation dose (10 Gy) was 1.8 but an equivalent value could not be computed directly from the results obtained with the 20 µM carnosic acid concentration and the radiation dose used.

After 48 hours of B16F10 cell culture following administration of the two carnosic acid concentrations and exposure (10 and 20 µM) to 10 Gy radiation, cell survival decreased by 19.2% and 2.5% compared to controls cell irradiated for the 10 µM and 20 µM solutions respectively. At both concentrations of CARN used, there was no significant radioprotective differences when compared with those obtained from the irradiated control cell cultures (**p<0.001**) (Table M26; Figure M37). Contrarily, the administration of CARN showed a radiosensitizing effect (Fig M37).

Table M26. Percentage cell survival (%) of B16F10 cell cultures treated with CARN (20µM and 40 µM) and exposed to different doses of X-rays evaluated after 48 hours of incubation.

Radiation doses	0 Gy	4 Gy	6 Gy	8 Gy	10Gy
Controls	100.0 ± 0	74.7 ± 8.3	72.0 ± 6.8	66.0 ± 7.4	59.0 ± 5.9
Carnosic acid 10 µM	100.0 ± 6.9	100.0 ± 7.9	77.7 ± 7.1	62.3 ± 6.2	39.8 ± 6Δ
Carnosic acid 20 µM	100.0 ± 8.4	100.0 ± 9.1	76.1 ± 7.4	71.2 ± 6.2	56.5 ± 6.7

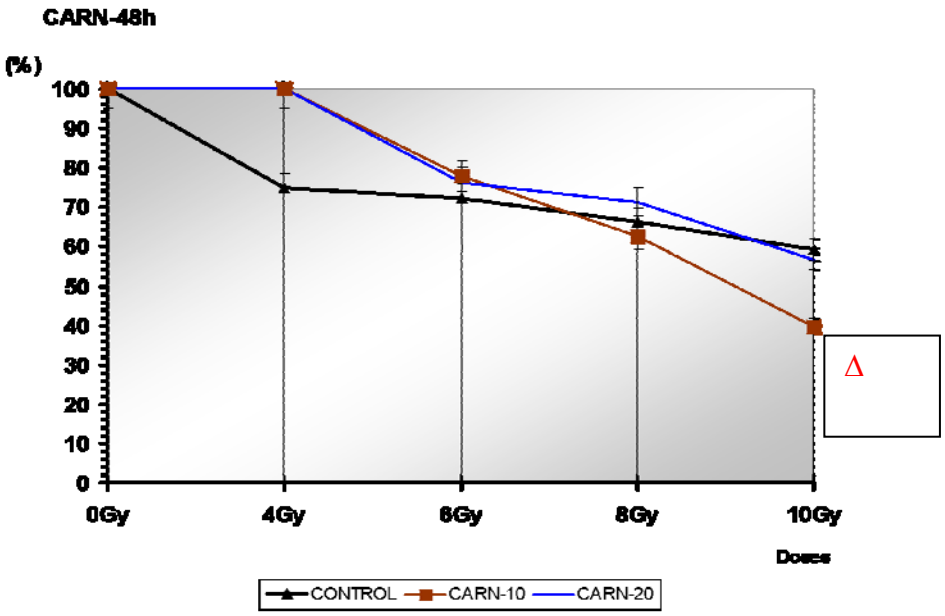


Figure M37. Cell survival curves of B16F10 cell cultures treated with CARN (20µM and 40 µM) and exposed to different doses of X-rays evaluated after 48 hours of incubation. ((+): (p<0.001) versus irradiated control cell cultures).

Protection Factor (**PF**) obtained from 48-hour B16F10 cell cultures treated with the two carnosic acid concentrations tested (10 µM and 20 µM) and exposed to the highest radiation dose (10 Gy) was zero, i.e. no radiation protective capacity.

It was not possible to compute the **Dose Reduction Factor (DRF**) for 48-hour carnosic acid-treated B16F10 cell cultures. This means that the two concentrations used (10 µM and 20 µM) shows no dose reducing effect.

- **ROSMARINIC ACID (ROS)**

Rosmarinic acid administration at two concentrations (20 µM and 40 µM) and incubated for 24 hours was found to produce a significant decrease in the percentage of cell proliferation compared to control cultures. Cell survival decreased by 67.1% when the lower concentration (20 µM) and 76.5% when the higher concentration (40 µM) of ROS was administered (Figure M30). After 48 hours of incubation, and at the same concentrations of ROS used (i.e., 20 µM and 40 µM), there was an improvement in cell proliferation (slightly higher than at 24 hours) with percentage survivals of 55.6% and 55% for the 40 µM and 20 µM solutions respectively relative to controls cultures (Figure M31).

The 24-hour B16F10 cell cultures treated with both admixture concentrations of rosmarinic acid (20 µM and 40 µM) and irradiated with 10 Gy of ionizing radiation portrayed a decrease of 18% in cell survival in the 20 µM admixture whereas the 40 µM admixture displayed a slight increase of 6.6% in cell survival compared with irradiated controls. Significant differences were not established when percentage cellular survival values were compared with those from the irradiated control cell cultures, however, the results obtained showed a radiosensitizing effect of 20µM ROS solution on B16F10 (Figure M38).

Table M27. Percentage cell survival (%) of B16F10 cell cultures treated with ROS (20µM and 40 µM) and exposed to different doses of X-rays evaluated after 24 hours of incubation.

Radiation doses	0 Gy	4 Gy	6 Gy	8 Gy	10Gy
Controls	100.0 ± 9.5	86.7 ± 7.4	80.6 ± 8.2	73.2 ± 6.8	66.9 ± 7.7
Rosmarinic Ac. 20 µM	100.0 ± 6.1	61.0 ± 6.3	46.5 ± 5.5	48.9 ± 7.1	48.9 ± 6.8
Rosmarinic Ac. 40 µM	100.0 ± 9.2	70.0 ± 7.1	73.0 ± 4.7	73.0 ± 9.2	73.5 ± 6.9*

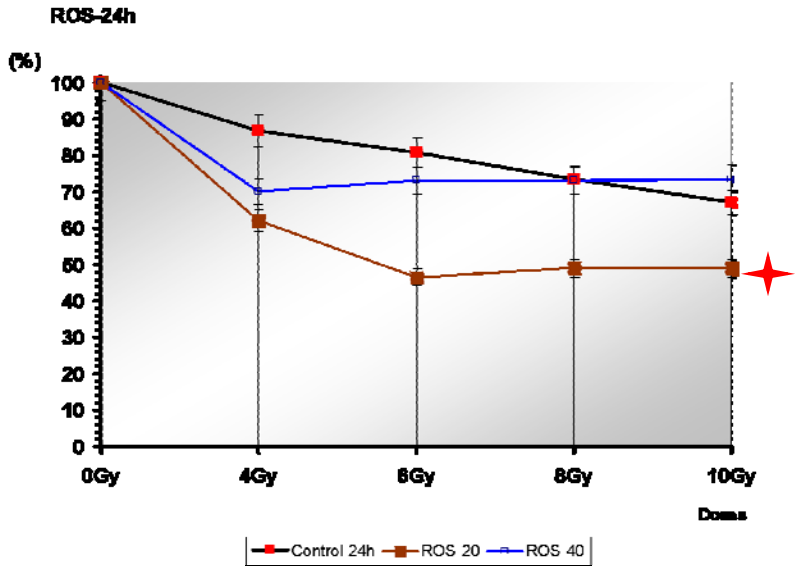


Figure M38. Cell survival curves of B16F10 cell cultures treated with ROS (20µM and 40 µM) and exposed to different doses of X-rays evaluated after 24 hours of incubation ((*): (p<0.001) versus irradiated control cell cultures).

Protection Factor (**PF**) obtained from the 24-hour B16F10 cell cultures treated with 20 µM rosmarinic acid and exposed to highest experimental radiation dose (10 Gy) was zero, i.e. 20 µM ROS shows no radiation protective capacity however, a PF of 19.9% was obtained when cells were conditioned with 40µM rosmarinic acid, exposed to 10 Gy of radiation and incubated for 24 hours.

A **Dose Reduction Factor (DRF) o**f 1.2 was obtained with cell cultures treated with 40 µM rosmarinic acid and exposed to the highest experimental radiation dose (10 Gy), while it was not possible to evaluate a DRF at the lower concentration (20 µM) of ROS used as it was not possible to obtain the same dose effect without a radioprotective capacity, i.e., it showed no dose-lowering effect.

After 48 hours of cell incubation following the administration of both concentrations (20 to 40 µM) of Rosmarinic acid studied and exposure to 10 Gy of radiation, there was an improvement in the cell survival compared with irradiated control cultures. The 20 µM concentration inched-up by 0.2%, however, a decrease of 25% cell survival was recorded for the 40 µM concentration compared to irradiated control cultures. No radioprotective effect was determined (Table M28; Figure M39) in

contrast, the results obtained portrayed a radiosensitizing effect of the 40 µM solution of ROS on B16F10 melanoma cells.

Table M28. Percentage cell survival (%) of B16F10 cell cultures treated with ROS (20µM and 40 µM) and exposed to different doses of X-rays evaluated after 48 hours of incubation.

Radiation doses	0 Gy	4 Gy	6 Gy	8 Gy	10Gy
Controls	100.0 ± 0	74.7 ± 8.3	72.0 ± 6.8	66.0 ± 7.4	59.0 ± 5.9
20 µM ROS	100.0 ± 8.5	81.0 ± 9.4	63.0 ± 7.8	56.8 ± 7.3	59.2 ± 6.9
40 µM ROS	100.0 ± 10.3	58.3 ± 9.5	63.2 ± 8.4	36.4 ± 8.1	34.0 ± 7*

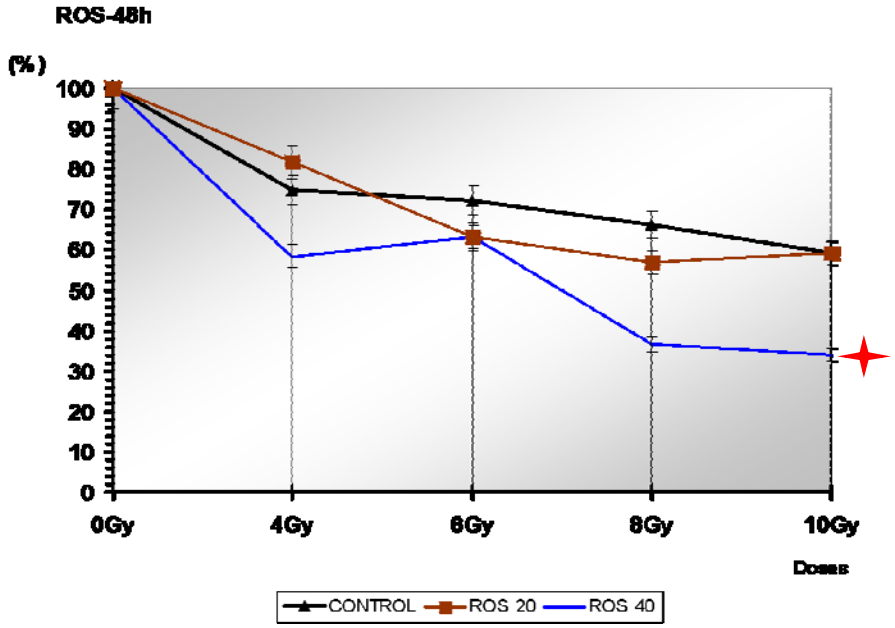


Figure M39. Cell survival curves of B16F10 cell cultures treated with ROS (20µM and 40 µM) and exposed to different dosed of X-rays evaluated after 48 hours of incubation. ((+): (p<0.001) versus irradiated control cell cultures).

The 48-hour cell cultures treated with 20 µM rosmarinic acid and irradiated with the highest radiation dose of 10 Gy produced a radiation **Protection Factor** (**PF**) of 0.48% and zero (0%) when treated with the 40 µM ROS and exposed to 10 Gy radiation dose i.e., it lacks protective capacity.

A **Dose Reduction Factor** (**DRF**) of 1.0 was recorded for cell cultures treated with 20 µM rosmarinic acid and exposed to the highest dose used in this study (10 Gy), whereas a DRF could not be computed for the higher concentration of RO (40 µM) used in this study as it lacks radioprotective capacity.

MIXTURES APIGENIN AND CARNOSIC ACID (API + CARN)

Admixtures of apigenin and carnosic acid (API + CARN) at concentrations of 10 µM (10 µM API + 10 µM CARN) and 20 µM (20 µM API + 20 µM CARN) were administered to cell cultures and their effect on radioprotection assessed.

Twenty-four (24)-hour cultures of B16F10 cells simultaneously exposed to both apigenin and carnosic acid at the two concentrations stated above did not display any decrease in percentage cell proliferation with the 10 µM mixture, while a reduction of 23% in cell proliferation was observed with the 20 µM mixture (Figure M30). Similarly, 48-hour B16F10 cell cultures exposed to the same concentrations of admixture solutions above (i.e., 10 µM and 20 µM) did not demonstrate any decrease in cellular proliferation with the lower admixture concentration (10µM) but showed a 21.9% decrease in percentage of proliferating cells with the 20 µM admixture solution when compared with irradiated control cultures (Figure M31).

After 24 hours of B16F10 cell incubation with co-treatment of apigenin and carnosic acid at (10 µM and 20 µM) concentrations, and exposure of cell cultures to the highest experimental radiation dose (10 Gy), a decrease in cell survival was shown compared with irradiated control cell cultures. A decrease of 46.9% was observed for the 10µM combination while the 20µM combination produced only 3% decrease in cell. No radioprotective effect was determined for this mixture (Table M29; Figure M35), however, contrarily, the results obtained showed a radiosensitizing effect of the 10µM (API + CARN) mixture on B16F10 melanoma cells (**$p<0.001$**) (Table M29; Figure M40) similar to the effect shown by administration of 10 µM CARN alone.

Table M29. Percentage cell survival (%) of B16F10 cell cultures treated with 10 µM and 20 µM mixtures of (API + CARN) and exposed to different doses of X-rays evaluated after 24 hours of incubation.

Radiation doses	0 Gy	4 Gy	6 Gy	8 Gy	10 Gy
Controls	100.0 ± 9.5	86.7 ± 7.4	80.6 ± 8.2	73.2 ± 6.8	66.9 ± 7.7
10 µM (API + CARN)	100.0 ± 6.9	79.0 ± 9.2	45.2 ± 7.8	34.3 ± 8.4	20.0 ± 9.1*
20 µM (API + CARN)	100.0 ± 8.1	92.8 ± 7.9	90.9 ± 5.3	77.0 ± 6.2	63.9 ± 7.5

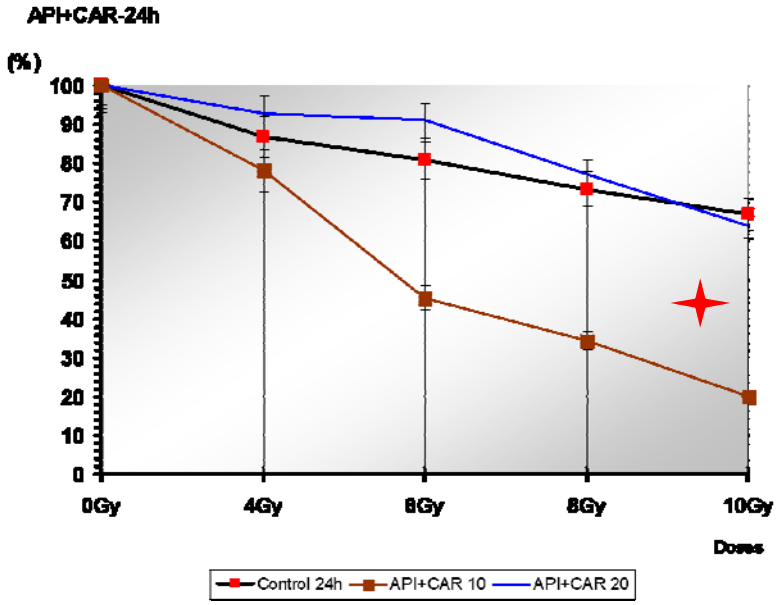


Figure M40. Cell survival curves of B16F10 cell cultures treated with 10 µM and 20 µM mixtures of (API + CARN) and exposed to different doses of X-rays evaluated after 24 hours of incubation. ((*): (p<0.001) versus irradiated control cell cultures).

24-hour B16F10 cell cultures simultaneously treated with 10 µM and 20 µM each of carnosic acid and apigenin and exposed to a radiation dose of 10 Gy produced a radiation **Protection Factor** (**PF**) of zero, i.e. no radiation protection capacity for both combinations of the test substances.

It was not possible to obtain a **Dose Reduction Factor** (**DRF**) for the 24-hour B16F10 cell cultures simultaneously treated with any of the combinations (10 µM or 20 µM) of carnosic acid and apigenin mixture and exposed to the highest dose of radiation (10 Gy) since neither solutions showed no radioprotection.

The 48-hour B16F10 cell cultures simultaneously treated with both combinations of apigenin and carnosic acid (10 µM and 20 µM) and exposed to the highest experimental radiation dose (10 Gy), showed a decrease in cell survival with respect to the irradiated control cell cultures. Cell proliferation in cultures exposed to the 10 µM combination decreased by 8.3% while those exposed to the 20 µM combination produced a decrease of 45.8% in cell survival. No radioprotective effect was established for this mixture (Table M30; Figure M41). However, conversely the results obtained showed a radiosensitizing effect of the 20µM (API + + CARN) mixture on B16F10 melanoma cells (**P <0.001).**

Table M30. Percentage cell survival (%) of B16F10 cell cultures treated with 10 µM and 20 µM mixtures of (API + CARN) and exposed to different doses of X-rays evaluated after 48 hours of incubation.

Radiation doses	0 Gy	4 Gy	6 Gy	8 Gy	10 Gy
Controls	100.0 ± 0	74.7 ± 8.3	72.0 ± 6.8	66.0 ± 7.4	59.0 ± 5.9
10 µM (API + CARN)	100.0 ± 7.9	98.5 ± 8.1	81.3 ± 9.2	64.9 ± 7.5	50.7 ± 8.3
20 µM (API + CARN)	100.0 ± 7.7	91.8 ± 6.9	61.9 ± 5.3	24.0 ± 7.4	13.12 ± 12*

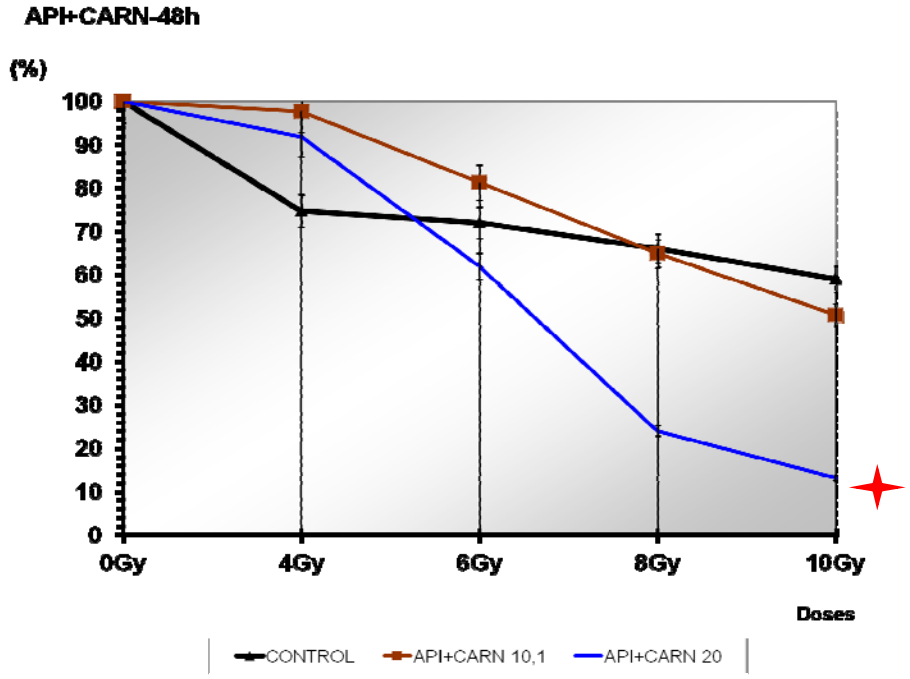


Figure M41. Cell survival curves of B16F10 cell cultures treated with 10 µM and 20 µM mixtures of (API + CARN) and exposed to different doses of X-rays evaluated after 48 hours of incubation. ((*): (p<0.001) versus irradiated control cell cultures).

A **Protection Factor (PF)** of zero was obtained when 48-hour B16F10 cell cultures simultaneously treated with both combinations of carnosic acid apigenin (10 µM, as for the 20 µM) and irradiated with the highest experimental radiation dose (10 Gy) i.e., no protective capacity was shown by either test substance combination (10 µM or 20 µM).

It was not possible to evaluate a **Dose Reduction Factor** (**DRF**) for either of the 48-hour B16F10 cell cultures co-treated with the two combinations of apigenin and carnosic acid (10 µM and 20 µM) and exposed to the highest radiation dose used in this study (10 Gy). This was so because the radiation dose that produces the same effects could not be determined without a radioprotective property of the test compounds.

- **APIGENIN AND ROSMARINIC ACID (API + ROS)**

Admixtures of apigenin and rosmarinic acid at concentrations of 10 μM (10 μM API + 10 μM ROS) and 20 μM (20 μM API + 20 μM ROS) were administered to cell cultures and their effect on radioprotection assessed. Twenty-four (24)-hour cultures of B16F10 cells exposed to both mixture concentrations displayed decreases in percentage cell proliferation. Cells exposed to the 10 μM mixture showed a reduction of 21.3%, while cells exposed to the 20 μM mixture registered a 36% reduction in cell proliferation compared with control cultures (Figure M30). After 48-hour of incubation, B16F10 cell cultures exposed to the same concentrations of apigenin and rosmarinic acid mixture as above (i.e., 10 μM and 20 μM) did not lead to any noticeable change in percentage cell proliferation at the lower concentration (10 μM), however, a 36% decrease in percentage cell proliferation was observed when the cells were exposed to the 20 μM mixture compared with control cultures of the same cells (Figure M31).

Twenty-four (24)-hour cultures of B16F10 simultaneously exposed to both concentrations of apigenin and rosmarinic acid (10 μM and 20 μM) studied and irradiated with 10 Gy of ionizing radiation showed an increased percentage cell survival with respect to irradiated controls only in the 10μM combination mixture. The percentage survival shown by cultures in the 10 μM mixture increased by 9.1%, on the other hand, a reduction of 0.4% in cell proliferation was shown by their counterparts conditioned with the 20 μM combination (Table M31; Figure M42). No radioprotective effect was established for this treatment.

Table M31. Percentage cell survival (%) of B16F10 cell cultures treated with 10 μM and 20 μM mixtures of (API + ROS) and exposed to different doses of X-rays evaluated after 24 hours of incubation.

Radiation doses	0 Gy	4 Gy	6 Gy	8 Gy	10 Gy
Controls	100.0 ± 9.5	86.7 ± 7.4	80.6 ± 8.2	73.2 ± 6.8	66.9 ± 7.7
10 μM (API + ROS)	100.0 ± 8.1	79.0 ± 8.7	74.5 ± 11.1	68.5 ± 9.2	76.0 ± 8.9
20 μM (API + ROS)	100.0 ± 8.5	86.2 ± 7.9	67.5 ± 7.6	81.0 ± 8.1	66.5 ± 9.5

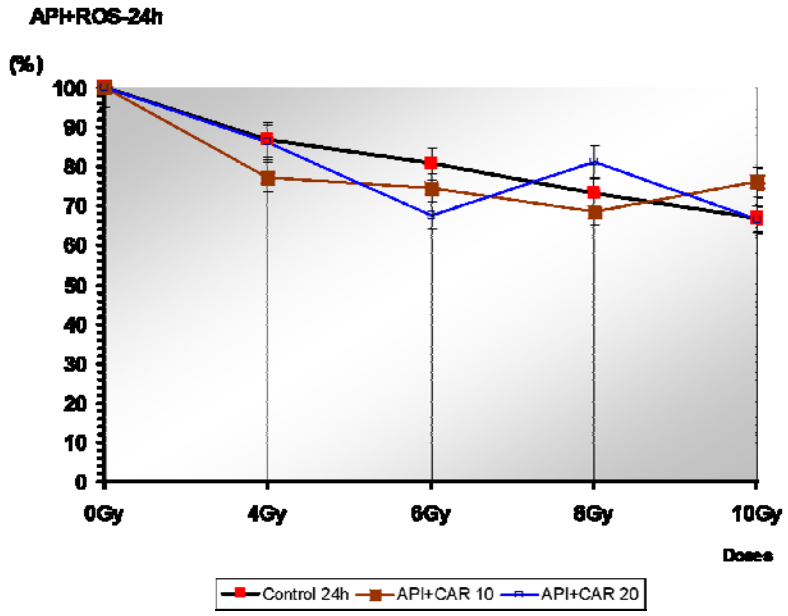


Figure M42. Cell survival curves of B16F10 cell cultures treated with 10 µM and 20 µM mixtures of (API + ROS) and exposed to different doses of X-rays evaluated after 24 hours of incubation.

A **Radiation Protection Factor (PF)** of 27.5% was obtained with 24-hour B16F10 cells cultures treated with 10 µM combination of apigenin and rosmarinic acid and exposed to the highest radiation dose (10 Gy), while a PF of zero was recorded for cells administered with the 20 µM combination i.e., no protection capacity was noticed at this concentration.

At a 10 µM combination of apigenin and rosmarinic acid, 24-hour cultures of B16F10 cells exposed to the highest experimental radiation dose of 10 Gy produced a radiation **Dose Reduction Factor (DRF)** of 1.3 while it was not possible obtain a DRF value for the 20 µM combination as the PF for this combination was zero (i.e., it showed no radioprotection). This implies that at this concentration, the test mixture shows no dose-reducing effect.

In the 48-h B16F10 cell cultures, a decrease in percentage cell survival compared with irradiated control cell cultures was recorded with both concentrations of apigenin and rosmarinic acid (10 µM and 20 µM) studied and exposed to 10 Gy. Percentage cell survival decreased by 22.6% when cells were conditioned in the 10 µM admixture and 13.1% in the 20 µM admixture. No radioprotective effect was observed, however, conversely, the results obtained showed a radiosensitizing effect of the

mixture (API + ROS) concentration used on B16F10 melanoma cells (Table M32; Figure M43).

Table M32. Percentage cell survival (%) of B16F10 cell cultures treated with 10 µM and 20 µM mixtures of (API + ROS) and exposed to different doses of X-rays evaluated after 48 hours of incubation.

Radiation doses	0 Gy	4 Gy	6 Gy	8 Gy	10 Gy
Controls	100.0 ± 0	74.7 ± 8.3	72.0 ± 6.8	66 ± 7.4	59.0 ± 5.9
10 µM (ApI + ROS)	100.0 ± 9.1	60.0 ± 8.3	46.3 ± 7.5	36.6 ± 7.6	36.4 ± 8.2*
20 µM (API + ROS)	100.0 ± 7.5	72.4 ± 8.2	46.2 ± 8.1	40.1 ± 6.9	45.9 ± 8.3*

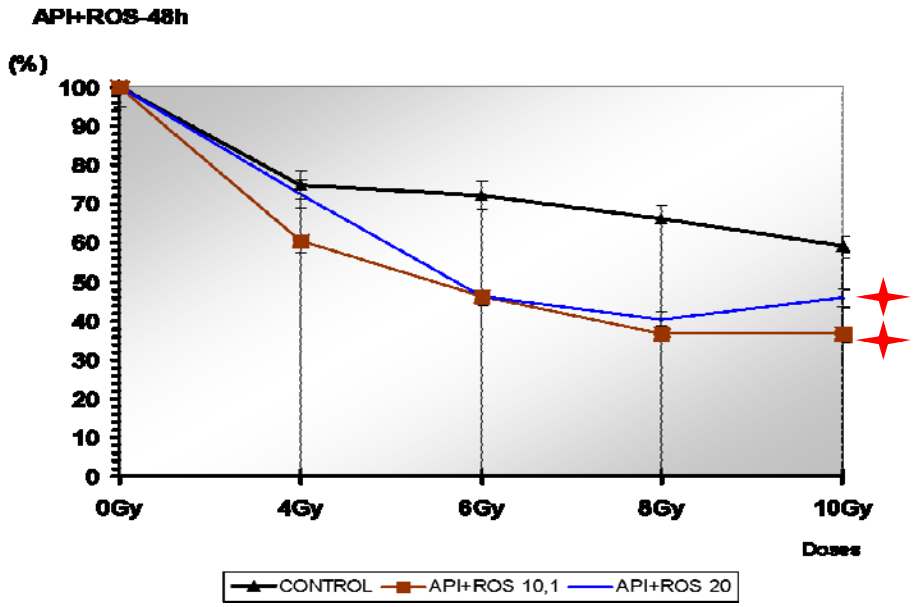


Figure M43. Cell survival curves of B16F10 cell cultures treated with 10 µM and 20 µM mixtures of (API + ROS) and exposed to different doses of X-rays evaluated after 48 hours of incubation. ((*): (p<0.001) versus irradiated control cell cultures.

A radiation **Protection Factor (PF)** of zero was recorded in 48-hour B16F10 cell cultures exposed to both admixture concentrations of apigenin and rosmarinic acid (10 µM, and 20 µM) and exposed to the highest experimental radiation dose (10 Gy) i.e., no both admixtures produced no radiation protective effect..

It was not possible to measure **Dose Reduction Factors (DRF)** in 48-hour B16F10 cell cultures treated with both admixture concentrations of apigenin and rosmarinic acid (10 µM and 20 µM) and exposed to 10 Gy of ionizing radiation since both showed no radioprotective effect (i.e., radiation protection factors were zero).

PYCNANTHUS ANGOLENSIS SEED EXTRACT (PASE)

The administration of 25µl of 25µM PASE did not lead to a decrease in the percentage of proliferating cells compared to control cultures of these cells after 24h or 48h of cell culture (Figure M44).

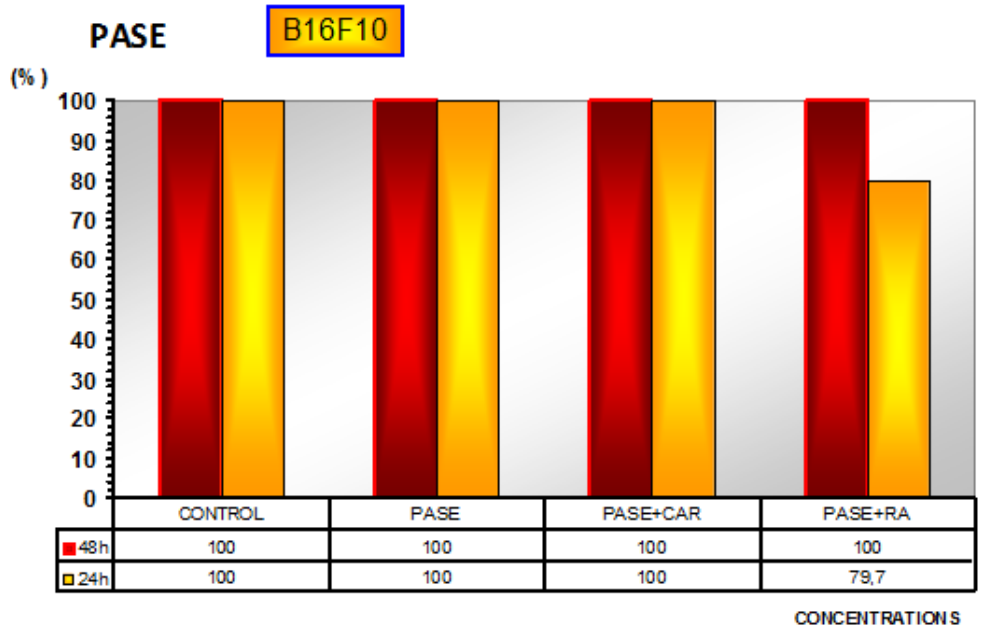


Figure M44. Effect of administration of PASE (alone and in combination with other substances studied) on the survival of B16F10 melanoma cell cultures evaluated at two different incubation periods (24h + 48h).

After treatment with PASE and exposure to radiation, 24-hour B16F10 cell showed a higher cell survival with respect to irradiated control cells. At the highest radiation dose employed (10 Gy), survival of cells treated with PASE was 18.8% more than the control cells. This concentration of PASE used portrayed a significant increase in the survival of B16F10 cells when compared with those obtained from irradiated control cell cultures ($p<0.001$)(Table M33; Figure M45).

Table M33. Percentage cell survival (%) of B16F10 cell cultures treated with PASE and exposed to different doses of X-rays evaluated after 24 hours of incubation.

Radiation doses	0 Gy	4 Gy	6 Gy	8 Gy	10Gy
Controls	100±5.8	86.7±7.2	80.6±6.3	73.2±6.7	66.9±5.1
PASE 25 µl	100±7.4	100±5.8	91.4±6.6	88.9±6.5	85.7±6.7 (*)

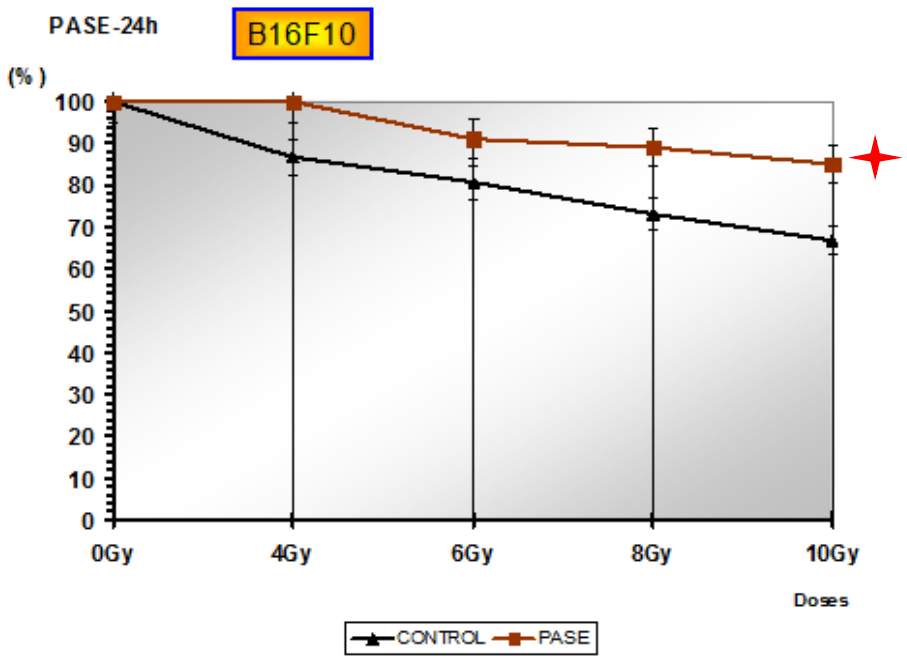


Figure M45. Cell survival of B16F10 cell cultures treated with PASE and exposed to different doses of X-rays evaluated after 24 hours of incubation. ((*): (p<0.001) versus irradiated control cell cultures).

The **Protection Factor** (PF) obtained in 24-hr incubation cultures treated with PASE after receiving the highest experimental radiation dose (10 Gy) was 28.1%.

The **Dose Reduction Factor** (**DRF**) obtained from 24 hour B16F10 cultures treated with PASE at the highest dose (10 Gy) was 2.3.

After 48 hours of cell incubation, , the results of B16F10 cell cultures treated with PASE showed improved survival compared to irradiated control culture. The percentage cell survival for cells treated with the highest radiation dose of 10 Gy was 24% higher than what was observed for the irradiated control cultures. This concentration of PASE evaluated also portrayed a significant increase in the survival of B16F10 cells when compared with cell survival values obtained from irradiated control cell cultures (**p<0.001)** (Table M34; Figure M46).

Table T34. Percentage cell survival (%) of B16F10 cell cultures treated with PASE and exposed to different doses of X-rays evaluated after 48 hours of incubation.

Radiation doses	0 Gy	4 Gy	6 Gy	8 Gy	10Gy
Controls	100.0 ± 4.5	74.7 ±7.5	72.0 ± 6.5	66.0 ± 5.6	59.0 ± 7.6
PASE 25 µl	100.0 ± 4.7	100.0 ± 4.5	83.2 ± 6.6	74.6 ± 6.7	83.3 ± 5.2*

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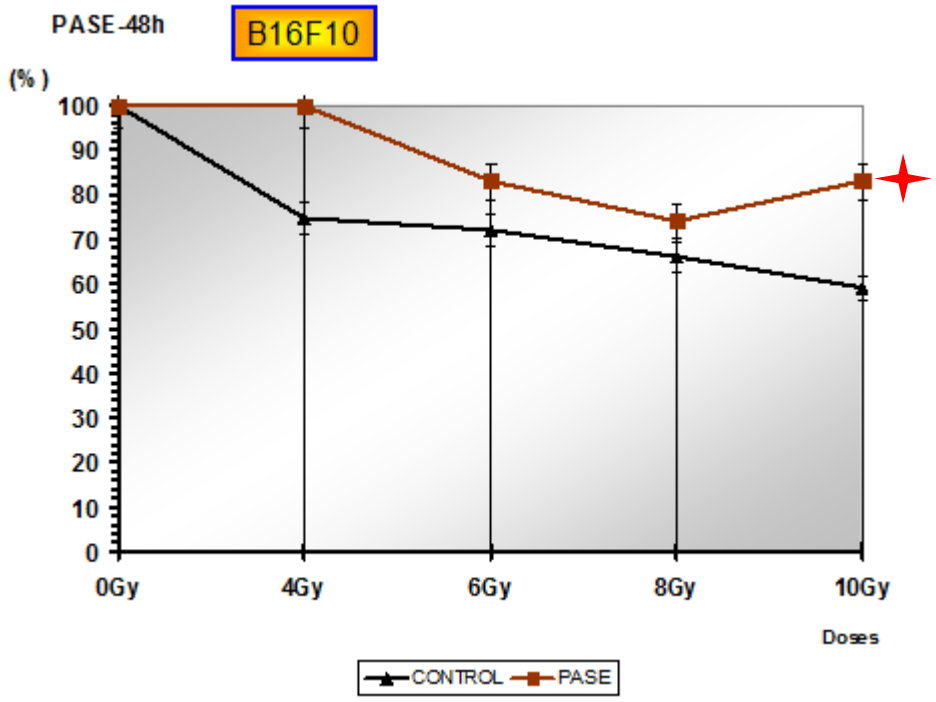

Figure M46. Cell survival of B16F10 cell cultures treated with PASE and exposed to different doses of X-rays evaluated after 48 hours of incubation. ((*): (p<0.001) versus irradiated control cell cultures.

The **Protection Factor** (**PF**) obtained in the 48 hour B16F10 cell samples treated with PASE after exposure to the highest experimental irradiation dose (10 Gy) was 41.2%

The **Dose Reduction Factor (DRF**) obtained in the PASE treated B16F10 samples at the highest dose used in this study (10 Gy) was 4.

- **PASE AND CARNOSIC ACID MIXTURE (PASE + CARN)**

The co-administration of PASE and carnosic acid at 25 µl of 25µM PASE + 20 µl of 10 µM CARN respectively, did not lead to an observed decrease in the percentage of cell proliferation compared to cell proliferation values in control cell cultures (Figure M44).

In 24-hour irradiated B16F10 cell cultures simultaneously exposed to both PASE and carnosic acid (25 µl of 25 µM PASE + 20 µl of 10 µM CARN the highest experimental radiation dose (10 Gy) used showed a decrease in cell survival, compared to irradiated control cells, decreasing by 46.9% when referenced with control cell

culture survival values. No radioprotective effect was determined. The results obtained demonstrates a radiosensitizing effect of the simultaneous administration (PASE + CARN) on B16F10 melanoma cells **(p <0.001**) (Table M35, Figure 47).

Table M35. Percentage cell survival (%) of B16F10 cell cultures treated with (PASE + CARN) and exposed to different doses of X-rays evaluated after 24 hours of incubation.

Radiation doses	0 Gy	4 Gy	6 Gy	8 Gy	10 Gy
Controls	100.0 ± 5.8	86.7 ± 9.1	80.6 ± 7.7	73.2 ± 7.5	66.9 ± 7.5
25 µl of 25 µM PASE + 10 µl of 10 µM CARN	100.0 ± 7.6	79.0 ±7.8	45.2± 5.8	34.3 ± 9.4	20.0 ± 5*

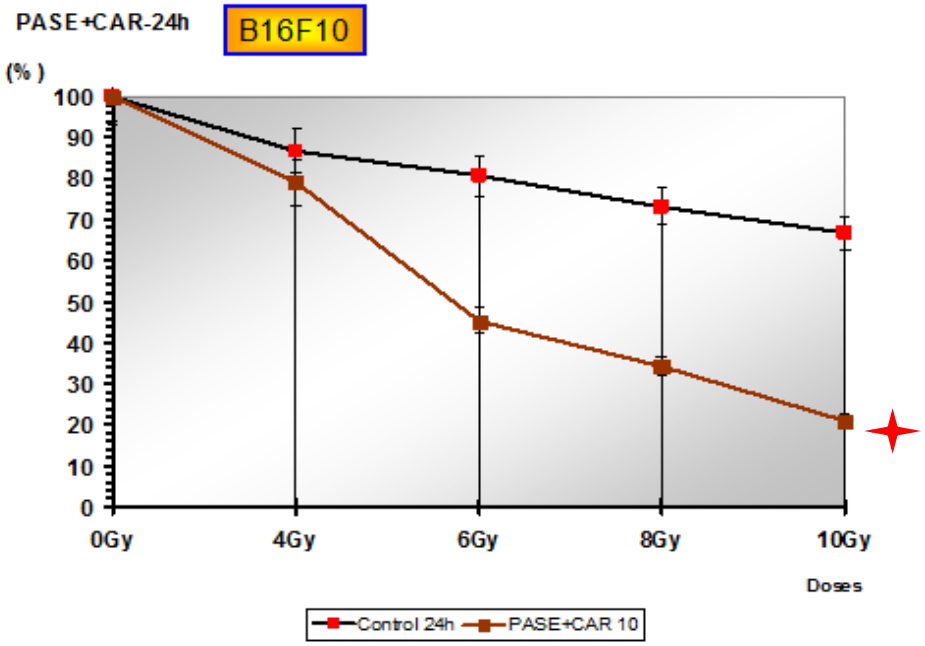

Figure M47. Cell survival curves of B16F10 cell cultures treated with (PASE + CARN) and exposed to different doses of X-rays evaluated after 24 hours of incubation. ((*): (p<0.001) versus irradiated control cell cultures).

The **Protection Factor** (**PF**) obtained from 24-hour B16F10 cultures treated with PASE and CARN after exposure to the highest irradiated dose (10 Gy) used in this study was zero (0) i.e., it lacked radioprotective capacity.

It was however, not possible to compute the **Dose Reduction Factor** (**DRF**) for the 24-hours B16F10 cell cultures treated with PASE and carnosic acid and exposed to the highest radiation dose of 10 Gy since it had no radioprotective capacity. This implies that it presents no dose-lowering effect.

In 48-hour B16F10 cell cultures co-treated with PASE and CARN at (25 µl of 25 µM PASE + 20 µl of 10 µM CARN) respectively and exposed to the highest experimental radiation dose (10 Gy), there was a decrease in percentage cell survival when compared with irradiated control cell cultures. The percentage cell survival of the irradiated control cohort was 8.3% superior to cell cultures that were co-treated with PASE and carnosic acid.

Table M36. Percentage cell survival (%) of B16F10 cell cultures treated with (PASE + CARN) and exposed to different doses of X-rays evaluated after 48 hours of incubation.

Radiation doses	0 Gy	4 Gy	6 Gy	8 Gy	10 Gy
Controls	100.0 ±	74.7 ±	72.0 ±	66.0 ±	59.0 ±
25 µl of 25 µM PASE + 20 µl of 10 µM CARN	100.0 ±	98.5 ±	81.3 ±	64.9 ±	50.7 ± 4

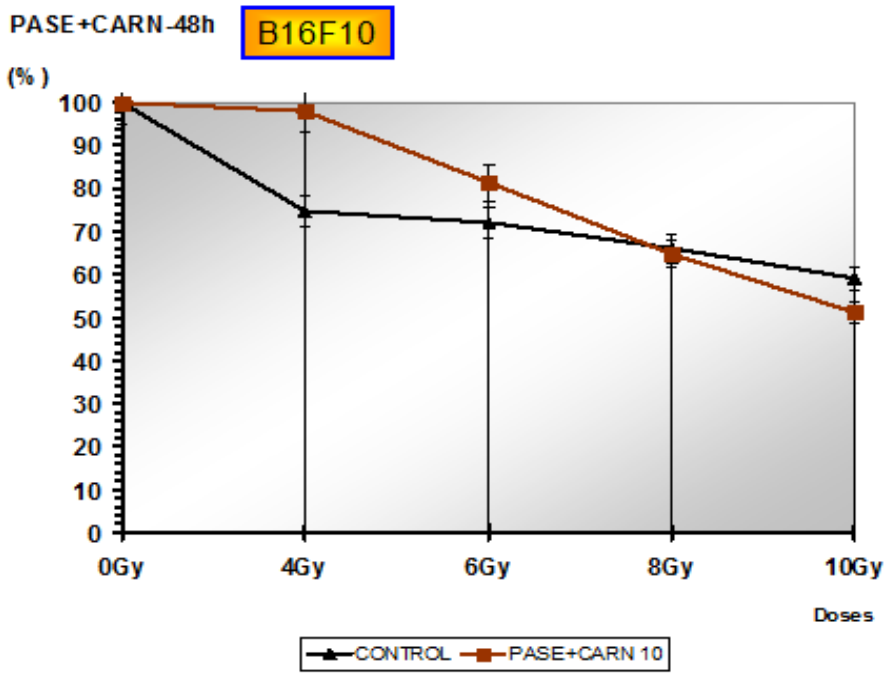

Figure M48.Cell survival curves of B16F10 cell cultures treated with (PASE + CARN) and exposed to different doses of X-rays evaluated after 48 hours of incubation. ((*): (p<0.001) versus irradiated control cell cultures).

The **Protection Factor** (**PF**) obtained in the 48 hours B16F10 cell culture samples treated with PASE and carnosic acid and exposed to the highest experimental radiation dose (10 Gy) was zero i.e., it had no protective capacity.

It was not possible to evaluate the **Dose Reduction Factor** (**DRF**) in the samples treated with PASE and carnosic acid and exposed to the highest dose used in

this study (10 Gy) since it did not show any radioprotective capacity. This suggests that the treatment presents no dose-lowering effect.

- **PASE AND ROSMARINIC ACID MIXTURE (PASE + ROS)**

The joint administration of PASE and rosmarinic acid at concentrations of 25 µl of 25µM PASE + 20µl of 10 µM ROS to metastatic B16F10 melanoma cells showed a decrease in the percentage of cell proliferation after 24 hours of incubation. When a 10 µM was used, the reduction in cell survival was 21.3% with respect to cell proliferation in control cultures obtained from same cells. After 48 hours of incubation and at the same concentrations used above, i.e., 25 µl of 25µM PASE + 20µl of 10 µM ROS, there was no decrease in the percentage of proliferation (Figure M44).

In 24-hour irradiated B16F10 culture treated with both PASE and Rosmarinic acid (25 µl of 25µM PASE + 20µl of 10 µM ROS) and irradiated with a dose of 10 Gy, there was an increased cell survival with respect irradiated controls, with the control cell expressing a 9.1% increase in survival over the control cultures. No radioprotective effect was observed when this mixture was added to B16F10 cells in culture (Table M37; Figure M49).

Table M37. Percentage cell survival (%) of B16F10 cell cultures treated with (PASE + ROS) and exposed to different doses of X-rays evaluated after 24 hours of incubation.

Radiation doses	0 Gy	4 Gy	6 Gy	8 Gy	10 Gy
Controls	100.0 ± 5.8	86.7±7.2	80.6 ± 7.3	73.2 ± 7.7	66.9±5.4
25 µl of 25µM PASE + 20µl of 10 µM ROS	100.0 ± 8.1	79.0 ± 8.3	74.5 ± 6.1	68.5 ± 5.1	76.0 ±5.9

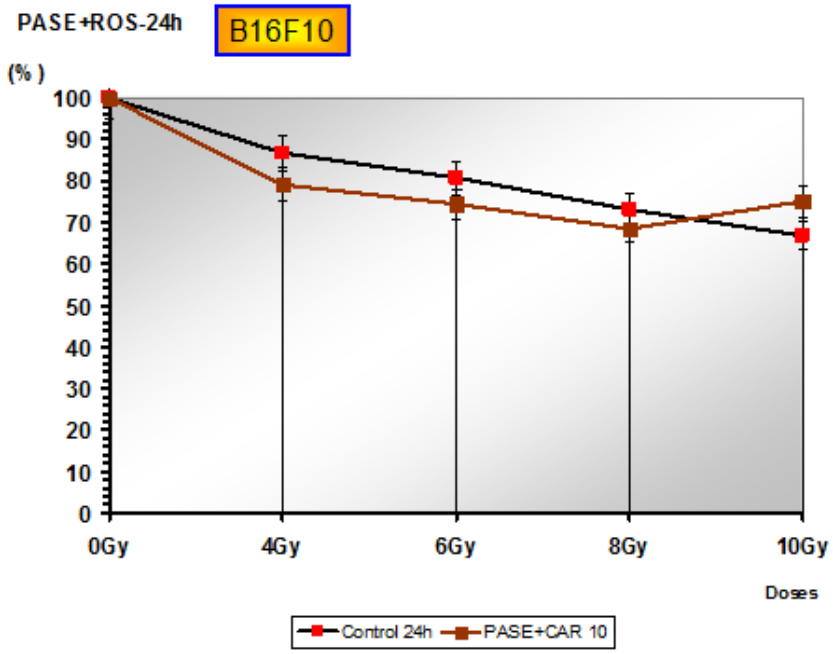

Figure M49. Cell survival curves of B16F10 cell cultures treated with (PASE + ROS) and exposed to different doses of X-rays evaluated after 24 hours of incubation. ((*): (p<0.001) versus irradiated control cell cultures).

The **Protection Factor** (**PF**) obtained from 24-hour cultures treated with PASE and ROS after exposure to highest experimental radiation dose (10 Gy) was 8.6% when 10 µl was administered. The **Dose Reduction Factor** (**DRF**) obtained from 24-hour incubation cultures treated with PASE and Rosmarinic Acid at and at the highest radiation dose (10 Gy) was 1.3.

After 48 hours of incubation, B16F10 cell cultures treated with both concentrations of PASE and ROS (25 µl + 10 µM respectively) and exposed to a radiation dose of 10 Gy, showed a decreased cell survival compared with irradiated control cells cultures; decreasing in percentage cell survival by some 22.6%. No radioprotective effect was observed when this mixture was administered. Contrarily, the results obtained showed a radiosensitizing effect of the administration (PASE + CARN) mixture on B16F10 melanoma cells **(p <0.001**) (Table M38, Figure M50).

Table M38. Percentage cell survival (%) of B16F10 cell cultures treated with (PASE + ROS) and exposed to different doses of X-rays evaluated after 48 hours of incubation.

Radiation doses	0 Gy	4 Gy	6 Gy	8 Gy	10 Gy
Controls	100.0 ± 9.3	74.7 ± 7.7	72.0 ± 6.7	66.0 ± 7.4	59.0 ± 4.5
25 µl of 25µM PASE + 20µl of 10 µM ROS	100.0 ± 10.0	60.0 ± 6.9	46.3 ± 6.9	36.6 ± 7.8	36.4 ± 4.9*

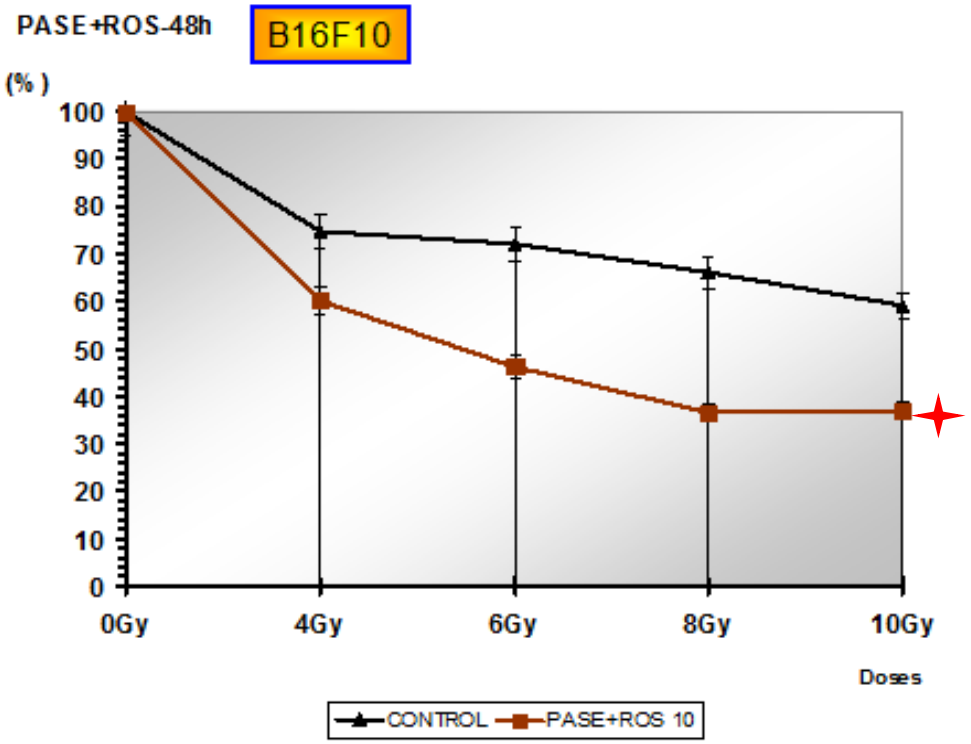

Figure M50. Cell survival curves of B16F10 cell cultures treated with (PASE + ROS) and exposed to different doses of X-rays evaluated after 48 hours of incubation.((*): ($p<0.001$) versus irradiated control cell cultures).

The Protection Factor (PF) obtained in 48-hour B16F10 cell culture samples treated with PASE and Rosmarinic acid exposed and to highest radiation dose (10 Gy), was zero, i.e., it lacked radioprotective capacity.

It was not possible to evaluate the **Dose Reduction Factor** (**DRF**) in 48-hour incubation samples of B10F16 cells treated with PASE and Rosmarinic acid and subsequently exposed to irradiation, since the treatment lacked radioprotectant properties, which means it presents no dose-lowering effect.

VI. Discussion.

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Ionizing radiation finds very important applications in medicine including diagnostic radiology, nuclear medicine and radiation therapy (Radiation Oncology), however, the harmful biological effects induced by overt exposure of body tissue to ionizing radiation has long been recognized. Thus there is always a risk-benefit balance when ionizing radiation is applied in medical settings. This radiobiological challenge has spurred interest in the development of chemical agents that could be used as protective agents for normal tissues against the damaging effect (radioprotective) or enhance radiation damage to tumors (radiosensitizers) while sparing normal tissues during the during radiotherapy and other radiation exposure scenarios. Therefore, the increased industrial use of nuclear energy, the risk of nuclear accidents/events and terrorist mediated nuclear attacks, and increased professionals exposed to radiation has increased interest in this aspect of radiobiology (WEISS, 1997; PRASAD et al., 2004; ALCARAZ *et al*., 2011).

Numerous studies have attempted to provide guides on how to reduce the damage caused by ionizing radiation to patients receiving raditherapy, professionally exposed radiation workers and victims of radiation accidents (ALCARAZ *et al.,* 2010, 2011). Furthermore, there are many papers in scientific literature that endeavour to demonstrate the protective capacities of various substances against damage induced by exposure to ionizing radiation (KONOPACKA and REZESZOWSKA-WOLNY, 2001; GREENROD AND FENECH, 2003, LEE *et al.,* 2004).

The absorption of ionizing radiation by the body depends on the radiation type and quality but also on the sensitivity of tissue being irradiated. Radiation is random in nature and the energy deposited per track length in biological tissue creates a multitude of ionizations and excitations in which water molecules are always involved. These cause at least two types of molecular alterations: by unpredictable or stochastic direct effects on many different molecular constituents of cells and the extracellular matrix ; and by transfer their energy to charged particles (two-step process) number of molecular species leading to the formation of reactive oxygen species (ROS) generated from the hydrolysis of water (ALCARAZ *et al.,* 2009a).

When ionising radiations (IR) passes through biological tissues high amounts oxygen-centred free radicals also known as reactive oxygen species (ROS) are generated *in vivo* by the homolytic cleavage of body water (radiolysis). These reactive oxygen species include the hydroxyl radicals·OH, superoxide anions, $O2^{\cdot -}$, HO_2·or RO_2· and hydrated electrons $e_{(aq)}$. Endogenous hydrogen peroxide is also formed by reduction of the superoxide anion through two mechanisms: the Haber-Weiss and Fenton models. The hydroxyl radical is the most cytotoxic of all these radicals mentioned, with an estimated half-life of 10^{-9} s. the high reactivity of these radicals implies immediate reaction at site of generation (SCHWARTZ and TERNS, 1992; MUNNÉ-BOSCH *et al*, 1999; MASUDA *et al*., 2001;). Thus, in the event of a massive generation of hydroxyl radicals as pertains during X-irradiation, the cytotoxic effect is enhanced through the interaction of these radicals with cellular structures rich in poly-unsaturated fatty acids (PUFAs), such as cell membrane phospholipids, where radiation exposure can induce lipid peroxidation chain reactions that trigger reactions both within and beyond of the membrane (LAMAISON *et al.,* 1991; MUNNÉ-BOSCH *et al.,* 1999; SÁNCHEZ-CAMPILLO *et al.,* 2009; ALCARAZ *et al.,* 2009a; KIANG *et al.,* 2012;).

High amounts of ROS in any biological system leads to oxidative stress and under such conditions of oxidative stress, when the endogenous antioxidant systems are overwhelmed or defective, exogenous agents with strong radical-scavenging capacities may be used as complements. The scavenging capacities of these proposed agents depend on high specific affinity for the different free radicals and a high degree of stability of the intermediate radicals formed which requires that both os and ws antioxidants are related through their molecular structure, physical properties and bioavailability (ALCARAZ *et al.,* 2010; ZAFIROV *et al*., 1990; GÁBOR, 1986; TERENCIO *et al*., 1991).

The ability of different substances to prevent genotoxic and cytotoxic damage is measured in terms of their ability to mop-up excess ROS generated by the radiolysis of water (ALCARAZ *et al.,* 2009). We previously used the MN test to evaluate the genoprotective capacities of several compounds. Furthermore, we also demonstrated that some pure flavonoids (e.g., diosmin and apigenin) and some polyphenolic extracts from plant origin expressed higher radioprotective capacities than some traditional radioprotectors, for example sulphydryl compounds (DMSO and AMF), against both X-

rays *in vivo* (CASTILLO *et al.,* 2000; BENAVENTE-GARCÍA *et al.,* 2002; 2005) and γ-irradiation *in vitro* (BENAVENTE-GARCÍA *et al.,* 2005). We described how these protective capacities depended on the degree of polymerization and solubility (hydrophobicity) of the substances assayed, since both modify their bioavailability (CASTILLO *et al.,* 2001; 2000). Reports from other authors demonstrates that polyphenolic antioxidant extracts sourced from different plants such as olive leaf (*Olea europaea*) and citrus fruits (citroflavonoids) (CASTILLO *et al.,* 2000; 2002; 2010; BENAVENTE *et al*, 2002; BENAVENTE-GARCÍA *et al.,* 2005;) show high protective promise when administered alone.

This study attempted to obtain an extract from a suitable part of African nutmeg *Pycnanthus angolensis* (PASE) as well as quantify it radioprotective capacity when administer before (pre-) and immediately after (post-) exposure to ionizing radiation in line with results of our previous studies. In those studies, different test substances were evaluated for radioprotection some of which expressed higher degrees of protection against harmful damage induced by ionizing radiation when compared with reference radioprotective compounds. In order to achieve these objectives and to enable effective comparisons, the same experimental protocols used in those previous studies were adapted for this study.

A set of *in vitro* and *in vivo* micronucleus test was used as tools for genotoxicity assessment while the MTT cell viability assay was used to develop cell survival dose-response curves for cytoprotective evaluation. The *in vivo* mutagenicity assay of mouse bone marrow micronucleus (TAKECHI *et al*., 1985) and the *in vitro* cytokinesis-block micronucleus assay with irradiated human lymphocytes (GÁBOR, 1986) are two cytogenetic assays based on an increase in the frequency of micronuclei. These assays are the most common analytical tools employed to evaluate the mutagenic capacity of a chemical or physical genotoxic agents.

The cytokinesis-blocked micronucleus assay performed on direct cultures of human lymphocytes described by Fenech and Morley (1985) which was founded on some initial observations made by Carter (1967), seems, at present to be one the best MN tests for the purposes of establishing absorbed radiation doses in humans (KORMOS and KOTELES, 1988; RAMALHO et al., 1988; PROSSER et al., 1988; GATENBERG et al., 1991; BALASEM and ALI, 1991; BAN *et al*., 1993, KIM et al.,

1993; WUTTKE *et al*., 1993; FENECH, 2000; IAEA, 2011). The *in vivo* mouse bone marrow micronucleus assay is one of the most widely used assays to evaluate genotoxicity and mutagenicity in short-term toxicology studies (SCHMID, 1976; MAZUR, 1995b).

Using these two assays, the protective effect of different compounds can be evaluated in the experimental models by analyzing the extent to which they reduce the frequency of MN produced when test models are exposed to different genotoxic agents, such as ionizing radiation, as carried out in this study (STONE *et al*., 2004; IAEA, 2011; OECD, 2004;). Previously, our team used the MN test to show that genotoxic lesions were not induced in nuclear medicine patients following diagnostic and therapeutic procedures, and also to identify the genotoxic effects at higher doses compared with doses used in ablation treatments for thyroid cancer or complex radiodiagnostic examinations.

On the other hand, the MTT assay has been employed extensively to assess the cytotoxic effect of several compounds and agents on cells. These assays provide a means of measuring the activity of living cells via mitochondrial dehydrogenases. In this test, yellow 3-(4, 5-dimethythiazol- 2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) is metabolically reduced by mitochondrial succinate dehydrogenase to an insoluble, dark purple compound (formazan) which is quantities colorimetrically. Any factor that inhibits dehydrogenase activity will affect the associated colour reaction. It has been amply demonstrated that activated cells produce more formazan than resting cells and therefore permits the measurement of cell activity (MOSMANN, 1983; CARMICHAEL, *et al*, 1987a; 1987b)

Recently, several researchers have accepted the evaluation of micronuclei frequency by cytokinesis-block micronucleus method in human lymphocytes as a fairly accurate tool as a biological dosimeter of *in vivo* ionising radiation exposure (ARMERO *et al*., 2000; ALCARAZ *et al.* , 2000; IAEA, 2011). This became particualry eminent after technical changes that led the acceptance of the recommendation of the micronucleus study after the first mitotic division (FENECH, 1993a, 1993b), and also from the work of various authors who establisshed dose-effect realationships upon the

exposure of cell cultures to different doses of ionizing radiation (ALMASSY *et al.*, 1987a; BAN *et al.,* 1993).

In this study, the spontaneous or basal micronucleus frequency found in non-irradiated peripheral blood lymphocytes was 0.02 MN /binucleated cell. This spontaneous rate is consistent to that observed by some authors (BALASEM and ALI, 1991; KIM et al., 1993), but somewhat higher than those reported by others (KREPINSKY and HEDDLE, 1983; AGHAMOHAMMADI *et al.,* 1984; FENECH and MORLEY, 1985a, 1985b). However, most of the studies reviewed recommend that micronuclei be counted in at least 500 cytokinesis-blocked cells in order to minimize these differences (GATENBERG *et al*., 1991). In this study the spontaneous MN frequency in the control samples was 10 MN/500 BC, consistent with that described in several previous studies (KIM *et al.*, 1993; FENECH, 2000).

In this study, two pro-oxidants substances (eriodictyol and quercetin) and one genotoxic substance (zoledronic acid; previously identified by our team as a genotoxin) were incorporated in the test as positive controls along with a mutagenic agent (X-rays). By and large this inclusion was to assist in the validation of the technical quality of the tests under the experimental conditions in the laboratory and serve as an essential quality assurance tool to obtain reliable and reproducible results, as the proposed radioprotective substances were evaluated (LENARZYK and SLOWIKOWSKA, 1995).

Regarding the spontaneous or basal MN frequencies established for *in vivo* polychromatic erythrocytes, the MN frequencies reported are always less than 5 MN/1000 PCE in non-irradiated control animals and is often considered as the quality control parameter for such preparations (SCHMID, 1976; ABRAHAM *et al*., 1993; SARMA and KESAVAN, 1993; MAZUR, 1995a; SINGH et al., 1995a; HENNING *et al.,* 1996; BISHT and UMA DEVI, 1995). The basal MN rate determined in non-irradiated control animals in this study was 3 MN/1000 PCE counted which is therefore within accepted limits of this technique.

An extensive review of the literature on *in vivo* MN assays portrays both dose- and time-dependent responses for micronuclei induction by irradiation. Thus, one can find previous work on bone marrow cells of mice irradiated with different doses of gamma radiation (UMA DEVI and SARMA, 1990, JAGETIA, 1990 and ABRAHAM

et al., 1993), others in polychromatic erythrocytes of pig (LUDEWIG *et al.,* 1991) and mouse bone marrow (LENARCZYK and SLOWIKOWSKA 1995; MAZUR, 1995b) irradiated with X-rays.

Generally, the radiation doses used in the experimentation are different, but are usually relatively high; 0.25 Gy (SINGH *et al*., 1995a, HENNING *et al.,* 1996), 0.5 Gy (SINGH *et al.,* 1995b; LENARCZYK and SLOWIKOWSKA, 1995), 1 Gy (SARMA and KESAVAN, 1993; BISHT and UMA DEVI, 1995; SHIMOI *et al.,* 1996), 2 Gy (SINGH *et al.,* 1995b; MAZUR, 1995b), 6 Gy (MAZUR, 1995a) and even 8 Gy (HENNING *et al.,* 1996).

In most reviewed studies, significant increases in the frequency of micronuclei induced by exposure to both X- (MAZUR, 2000; 1995a; 1995b), and γ-rays (BROWN *et al*., 1982, ABRAHAM *et al.,* 1993; SARMA and KESAVAN, 1993; SINGH *et al*., 1995; BISHT and UMA DEVI, 1995; SHIMOI *et al.,* 1996) have been demonstrated.

The second objective was to determine the radioprotective capacity of PASE against cytotoxic and genotoxic damage induced by ionizing radiation. The results showed a protection factor of 23% to 35% against genotoxic damage and 28% to 41% against the cytotoxic damage induced by ionizing radiation on normal non-tumor cells. Under these experimental circumstances, PASE may be consider to have demonstrated a significant radioprotective effect. However, the true assessment of the radioprotective capacity measured in PASE needs to be put into the proper perspective by comparing the mneasured effects with the same effects of other substances assayed under similar conditions.

Thus the third objective of this study was the comparison of the protective capacity of PASE with that of numerous other antioxidant substances known to possess radioprotective capacities. Our analysis revealed the following genoprotective trend amongst the various substances assayed and showed significant differences between pre-and post- treatment periods of the substances.

- Before X-irradiation:

Rosmarinic acid (ROS)> Procianidin (P90) > Carnosic acid (CAR) = Apigenin (API) = Vit. E (E) > Carnosol (COL) = Soluble Citrus Extract (CE) > Vit (C) =

Amiphostine (AMIF) > P. Angolensis extract (PASE) > Green Tea Extract (TE) = Rutin (RUT) = Dimethyl Sulphoxide (DMSO**) (p<0.001)**

- After X-irradiation:

Carnosol (COL) > Carnosic acid (CAR) > Procianidin (P90) = > P. Angolensis extract (PASE) = Apigenin (API) = Vit. E (E) > = Soluble Citrus Extract (CE) > Vit C (C) > Green Tea Extract (TE) = Rutin (RUT) = Rosmarinic acid (ROS) = Dimethyl Sulphoxide (DMSO) (**p<0.001**)

Significant differences were identified between pre-and post- treatment application periods of the test substance. The extract tested (PASE) probably due to its lipophilic character, showed an increased genoprotective activity when it was administered after the exposure to ionizing radiation and probbably indicate that this lipophilic capacity demonstrated provide an effective radioprotective barrier, as previously described by our group (ALCARAZ *et al* 2009; 2010).

Using the *in vivo* micronucleated polychromatic erythrocytes (MNPCEs) in mice bone marrow as a complementary tool, the *in vitro* reuslts obtained with the cytokinesis-blocked micronuclues assay of irradaiated human lympocytes were further authenticated. The *in vivo* tecchnique permits the *in vivo* evaluation of the protective capacities of the tested substances, taking into account biological responses in living organisms and bioavailability issues. The *in vivo* micronucleus test was devised primarily for screening chemicals for their chromosome-breaking effects. The test substances are normally applied sub-acutely to small mammals and their effects were read from direct smears in bone marrow preparations. It was previously used to determine the genotoxic effects of physical agents such as IR (ALMASSY *et al.,* 1987) and magnetic fields (VIJAYALAXMI and OBE, 2005). The MN assay in bone marrow polychromatic erythrocytes (PCEs) in mice which was originally developed by Schmid (1975), is currently probably the most frequently used *in vivo* short-term genotoxicity test. Micronucleated polychrmatic erythrocytes (MNPCEs) in bone marrow cells provide a simple and rapid method for the detection of chromosomal damage induced by chemical and physical agents (HEDDLE and SALAMONE, 1981; ALMASSY *et al.,* 1987; MAVOURNIN *et al.,* 1990; MAZUR, 1995a) and results obtained from this test are probably the most significant in terms of potential human hazard (ASHBY *et al.,* 1997). An increase in the frequency of MNPCEs is an indication of aneuploidy or clastogenic induction. For this reason, micronuclei have been widely used *in vivo* and *in*

vitro to detect chromosomal breakage and chromosome lagging (MORITA *et al.*, 2011; KIERSCH-VOLDERS *et al.,* 1997).

For this reason, the results obtained with regard to the magnitude of protection offered by different treatments given pre- and post X-ray exposure suggest the probable existence of different radioprotective mechanisms in each case. In the pre-X-irradiation treatments, the radioprotective effects (anti-mutagenic activity) of phenolics is theoretically based, as mentioned earlier, on the scavenging capacity against the ROS generated during radiation exposure especially the hydroxyl radical ($^{\cdot}OH$), whose generation is massive during X-ray irradiation. From the above structural considerations, the anti-mutagenic activity of the tested compounds given as pre-X-irradiation treatments is consistent with their antioxidant properties and specific affinities to scavenger free radicals.

When the substances assayed were added post-X-irradiation, the only ROS likely to be present in the cells, according to the half-life of superoxide anion and hydroxyl radicals, are the lipoperoxy radicals ($R^{-}OO^{\cdot}$), which are mainly known to be responsible for continuous chromosomal oxidative damage. Furthermore, ionizing radiation enhances lysosomal enzyme secretion and arachidonic acid release from membranes through lipo-oxygenase, cyclo-oxygenase and phospholipase activities thereby increasing cellular inflammatory responses. Under these complex oxidative stress conditions, it is very difficult to determine the structural elements responsible for the experimental data obtained for the anti-mutagenic activity of different post-X-irradiation treatments, although some considerations are possible (ALCARAZ *et al.*, 2009a).

In this study, the genoprotective capacity of Amiphostine, an approved radioprotective drug was evaluated. Preclinical studies demonstrated that amifostine can selectively protect almost all normal tissues from the cytotoxic effects of some chemotherapeutic agents and radiation therapy even though its use is restricted by cytotoxicity issues (HENSELEY *et al.,* 1999; 2009; LINKS *et al*., 1999; MORAIS *et al*., 1999). Amifostine is an inactive pro-drug that cannot protect until dephosphorylated to the active metabolite, WR-1065, by alkaline phosphatase in the plasma. The pro-drug (WR2721) accumulates preferentially in the kidneys and salivary glands, where it is

metabolized to the active WR1065. Once inside the cell, WR-1065 scavenges free radicals like O_2^- as well as $^{\cdot}OH$ and lipoperoxyl ($LOO^{\cdot}$), protecting cellular membranes and DNA from damage.

Amifostine needs to be present at time of radiation and in sufficient quantities (BACQ, 1953; TRAVIS, 1979); the results of this study were consistent with those enumerated in these previous studies. The absence of protective capacity when Amifostine was administered immediately after irradiation (post-irradiation treatment of Amifostine) and the much lower protective capacities of pre-irradiation application of some of the substances tested in this study is noteworthy.

Based on the pre-irradiation results obtained in this study amifostine is a radioprotective substance which presented with a protection factor (PF) of 38% and a Dose Reduction Factor (DRF) of 3.3. The radiation protection factor of Amifostine noted was significantly lower than that obtained with more radioprotective substances in this study and significantly lower than that obtained for PASE.

All the substances tested (flavonoids and organosulfur compounds) seem to act through their antioxidant action by scavenging free radicals in the medium induced by ionizing radiation as applies to organosulfur substances especially those with disulfide bridges (S-S).

The selective protection to non-cancer cells against radiation damage (radioprotective effects) found has been attributed to several factors. The precise mechanisms by which amino-thiols exert their protective action on normal tissues are not well understood, although these mechanisms may be multiple. Thus, there are various proposed mechanisms or combinations of mechanisms to explain the protective effect of the thiol agents:

- At the molecular level it exerts its cytoprotective effect scavenging oxygen free radicals, donating hydrogen ions to free radicals or thiols, binding to and inactivating cytotoxic drugs (MAZUR, 1995a).
- Tumors are relatively hypovascular thus resulting in comparative hypoxia thus at the physiological level, induces the depleting oxygen (hypoxia), hypothermia or shock, low interstitial pH and lower expression of alkaline phosphatase.

- At the cellular level, stimulate the recovery of haematopoietic stem cells (MAZUR, 1995a; CULY and SPENCER, 2001).

Other organosulfur substances evaluated were the thiourea drug thiourea drug, 6-*n*-propylthiouracil (PTU) and dimethyl suphoxide (DMSO). The antioxidant capacity of DMSO has widely been recognized and is known to be a potent neutralizer and scavenger of free radicals induced by radiation, especially hydroxyl radicals (˙OH). Its radioprotective properties are also thought to be mediated through facilitation of DNA double-strand break repair (KASHINO *et al.,* 2010). DMSO turns out is more "liquid" than water and it can therefore penetrate to places in the body nothing else can reach so fast. The DMSO-water bond is 1.3 times stronger than the water-water bond. This attribute of bonding with water better than water molecules themselves is highly significant. DMSO substitutes for water and moves rapidly through cell membranes and therefore can readily scavenge free radicals, even at the intracellular level (SZMANT *et al.,* 1975).

In general, over 300 different substances sulfur containing compounds have been shown to posses some level of radioprotection. These compounds possess certain characteristic features with similar characteristics that probably account for their ability to protect against damage induced by ionizing radiation. These features include but not limited to:

1. They are chemical analogues of cysteine (a thiol group containing amino acid). Similar to cysteine with the sulfhydryl group protected by a phosphate.
2. These substances have their SH group separated by two to three carbon atoms from the basic amino group.
3. Having another amino group separated from the first by three carbon atom.
4. Actual average dose 600 mg / kg.
5. Soluble in distilled water at pH 6, which is indicative of a high pK value. (BROWN *et al.,* 1982; Brown, 1967).

However DMSO and PTU do not portray all these properties which probably account for their significantly lower protection factors. Unfortunately,

effective radioprotective doses are usually toxic to animal tissues at recommended doses and must be administer prior to exposure to radiation. Furthermore, significant differences in radioprotection were found in the post-irradiation administration of substances. For instance, when amifostine was administered immediately after exposure to X-radiation, it had no protective effect and no DRF.

In principle, all tested substances appear to act as antioxidants by neutralizing free radicals induced by ionizing radiation. In agreement with our results, Sarma and Kesavan (1993) reported that α-tocopherol consistently afforded higher radioprotection than ascorbic acid in both pre- and post-irradiation treatments. The protective effects observed has been explained by the scavenging of ROS but since both vitamins are present in the system during exposure to the clastogen (pre-irradiation), the mechanism of action of the vitamins as protective agents observed when they were administered post irradiation is rather obscure.

Although some researchers have attributed the radioprotective effects of vitamin E to increased immune response, they also suggest that further studies be conducted to delineate the possible mechanism by which peroxyl radicals generated *in vitro* are more efficiently repaired by a-tocopherol than by ascorbic acid; a mechanism that protects liposomal membranes against radiation-induced lipid peroxidation. Other authors are of the opinion that this reduction of the peroxy radicals generated during radiation by the vitamins applied suggests that some other type of protective mechanism is operational (SALAH, *et al*., 1995; RICARDO da SILVA *et al*., 1991)

The results obtained suggest that this pathway of lipoperoxy radical elimination is quantitatively greater when the antioxidant compounds are administered immediately after exposure to ionizing radiation. The distinction between anti-radical (vs superoxide anion and hydroxyl radicals) and anti-lipoperoxidant (vs lipoperoxy radicals) activities proposed by Pincemail et al (1989) seems reasonable, according to the data obtained in the pre- and post-c-irradiation models, and could be invoked to explain the different behaviours of the compounds assayed in terms of the lipid peroxidation processes. The anti-lipoperoxidant activity of phenolics and flavonoids depends in a complex way on various factors, including the nature of the lipid substrate susceptible to oxidation, cell culture conditions and even the method used for the evaluation. The results obtained

show that the only compounds with a significant radioprotective–antimutagenic capacity were the liposoluble compounds (CARN, COL, E and PASE).

Figure D1 below is a graphic representation depicting some possible sites of action of some of the substances assayed.

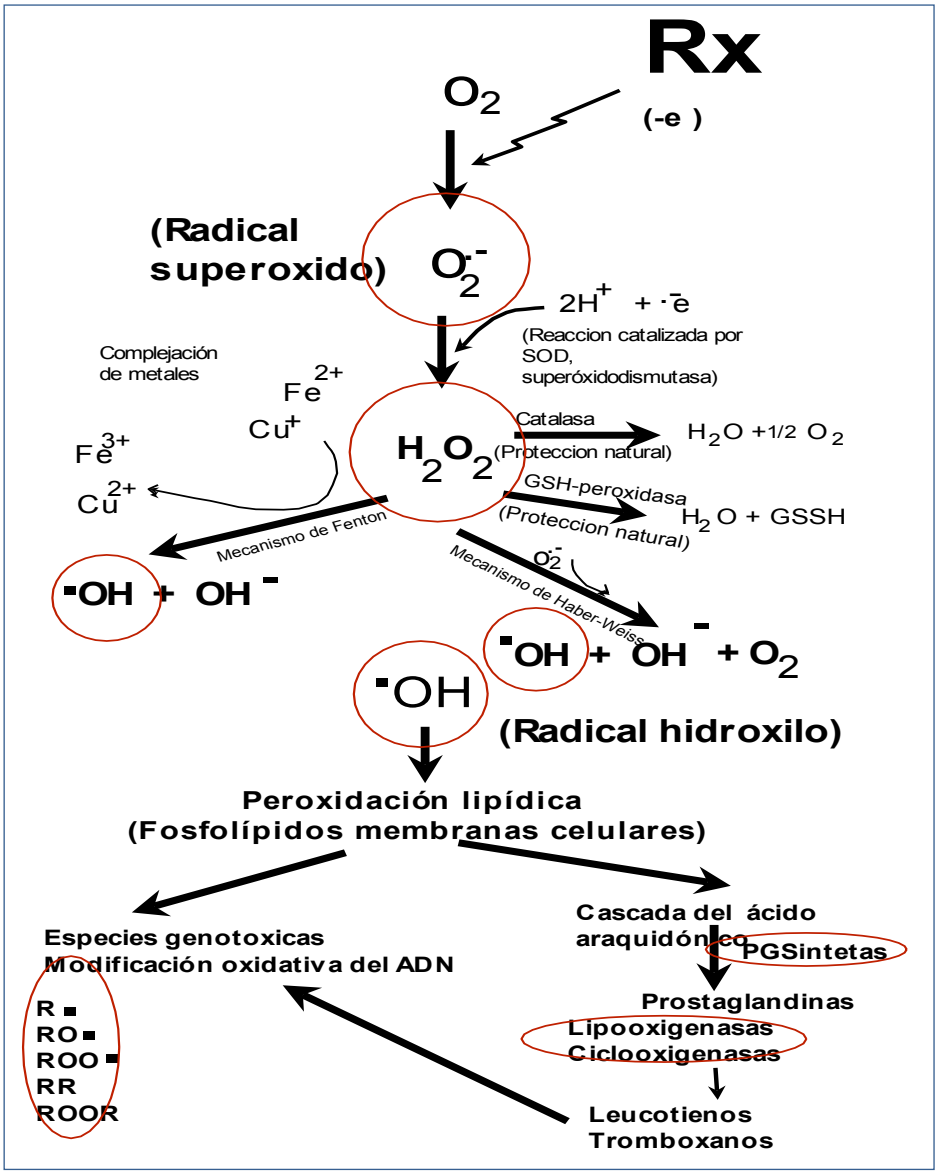

Figure D1. Schematic representation of the mechanism by which radiation causes damage and possible sites of action of antioxidants (ALCARAZ *et al.*, 2013)

Similar results with regard to reduction of chromosomal damage induced by ionizing radiation by these substances have been described in the literature using the same bone marrow micronucleus test with the administration of antioxidants such as vitamin E (SARMA and KESAVAN, 1993) β-carotene (ABRAHAM *et al.,* 1993), fresh garlic extracts (SINGH *et al.,* 1995b) and even DNA groove-binders (MAURYA and

DEVASAGAYAM, 2011; LOBACHEVSKY *et al*., 2011). Recent developments in regard to radioprotective development has demonstrated in the radioprotective capacity of luteolin, a flavonoid derived from tea plants using the micronucleus assay in peripheral blood reticulocytes (MnRETs) which is a variation of the regular bone marrow micronucleus test.

Radioprotection afforded by substances after exposure to ionizing radiation was traditionally not been considered possible (TRAVIS, 1979) since none of the then known radioprotective compounds (basically the amino-thiols) were able to demonstrate this ability. Some of our results are consistent with reports by Konopacka and Rzeszowska-wolny (2001), which showed a radioprotective effect of vitamins C and E towards lymphocytes exposed to X-ray radiation whether administered before or after irradiation. However, the maximum effect is observed when administered within the first hour after irradiation. The radioprotective effect was also found to be dependent on the concentration of vitamins in the lymphocyte culture; the pre-irradiation adminstration of vitamins C at 1µg/ml and Vitamin E at 2µg/ml was found to decrease radiation induced micronucleus frequency.

The results obtained with the procyanidins (P90) is consistent with the results obtained by other researchers and affirm that the main phenolic components in alcoholic wine (P90) can reduce DNA damage induced by ionizing radiation and hydrogen peroxide (GREENROD and FENECH, 2003).

In the reviewed studies conducted, similar reductions of radiation-induced chromosome damage, using the *in vitro* micronucleus and *in vivo* mouse bone marrow assays due to the administration of antioxidants such as vitamin E (SARMA and KESAVAN, 1993), β-carotene (ABRAHAM *et al*., 1993), garlic extracts (SINGH *et al*. 1995b) and even DNA binders (DENISON *et al.,* 1992; MARTIN *et al*., 2004; 1996; WANG and ZHENG, 1992; ZIMMER and WAHNERT, 1986) have been found. In these reports, different degrees of reduction in the occurrence of the frequency micronuclei in polychromatic erythrocytes (MnPCEs) from mouse bone marrow and human peripheral blood lymphocytes (CBMN) have been amply demonstrated.

Some flavonoids such luteolin, quercetin and rutin, which are known to elaborated some antioxidant activities have been reported to be capable of inhibiting or

minimizing the detrimental effects caused by the ionizing radiation through scavenging of the free radicals induced by the radiation (SHIMOI *et al*., 1996). More recently using micronucleus assay in peripheral blood reticulocytes (MnRETs) of rats, the radioprotective effect of luteolin, a flavonoid derived from tea, has been reported (LEE *et al*., 2004).

The mechanisms of action of these compounds are influenced by structural factors such as the presence or absence of sugar on one of the hydroxyl groups (glycosylated forms), the total number of hydroxylated positions, and the existence of methylated hydroxyl groups. These structural elements of the flavonoids are essential for the activation and/or inhibition of multiple enzyme systems involved in the enzyme cascades in metabolic regulation; e.g. cyclooxygenase, lipoxygenase, phospholipase and prostaglandin synthases (MARTINEZ *et al*., 2002b).

Obviously, the degree of effectiveness ROS, Disomin, RUT, CE and TE as radioprotectors depends on their structures. It is known that the capacity to scavenge hydroxyl radicals is principally based on the combination of conjugated structures in the polyphenolic skeleton, mainly the o-dihydroxy-phenol or catecol structure; thus the presence in CARN and ROS of two catechol groups conjugated with a carboxylic acid group, increases its their antioxidant activities in aqueous media. In fact, going by the above mentioned structural considerations, the antimutagenic activity of ROS and CARN administered before exposure X-rays is consistent with its antioxidant properties and high specific activities for free radicals. These would explain the higher antimutagenic activity of ROS over those of AMF and DMSO (ALCARAZ *et al*., 2011).

The antioxidant properties and free radical scavenging ability of flavonoids have generally been primarily attributed to presence of three main chemical structural elements:

i. The presence of ortho-diphenol structure in the B ring of the flavonoid skeleton (catechol group), which confers greater stability to the aroxyl ($ArO^{\cdot -}$) radical formed.

ii. The double bond between carbons 2 and 3 of the C ring in conjugation with the 4-oxo function. The presence of two catechol rings, conjugated to a carboxylic

acid group is, probably the most important structural element in the antioxidant activity of this compound (DEL BAÑO *et al.,* 2003b).

iii. The presence of hydroxyl groups at positions 3 and 5 of the rings C and A, respectively.

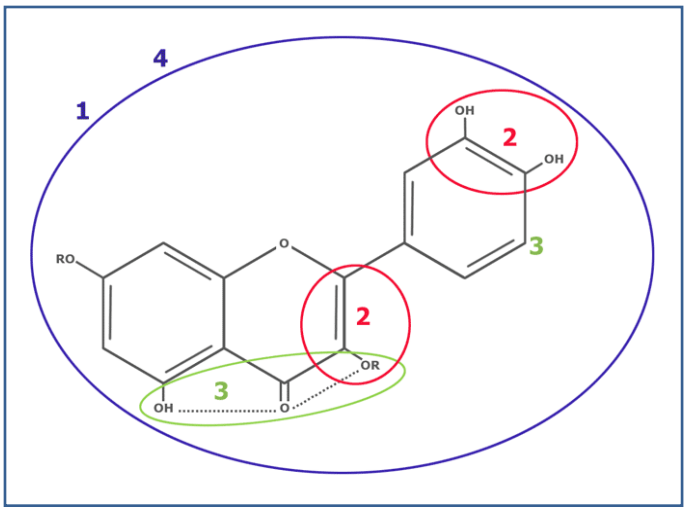

Figure D2. Possible mechanisms of action of the flavonoids assayed:
1. The electron cloud may interrupt free radical chain reaction by capturing free radicals.
2. Catechol group in ring B and double bond at C2-C3 can capture free radicals.
3. Hydroxyl groups at positions 3 and 5 of ring C and A respectively may facilitate or promote complexation of metals.
4. Some flavonoids and phenolic compounds are known to inhibitor the activity of prostaglandin synthetase.

The free radical scavenging capacities, metal complexing abilities and general antioxidant properties of flavonoid would thus be dictated by a combination of these three basic structural elements. Furthermore, other structural elements may participate as the presence of three adjacent hydroxyl groups in flavonoid structure allows the conjugation of adjacent flavonoid skeletons to form polymers.

The results suggest that no single chemical structure can be related solely to the genoprotective capacities of the compounds assayed. Its further suggest that the antimutagenic effects observed in a way, is proportional to the antioxidant capacity, even though it is also dependent on the bioavailability properties of the test substance in the medium assayed. Accordingly, it was observed that the flavan-3-ols showed the highest protective capacity of all the polyphenols assayed (CASTILLO *et al.,* 2000, 2001); while other flavonoids with a high antineoplastic and antiproliferative capacities

showed a lower antimutagenic capacity (BENAVENTE-GARCÍA *et al.,* 2005; MARTÍNEZ-CONESA *et al.,* 2005a; YAÑEZ *et al.,* 2004). Continuing the search for compounds with high antioxidant capacities, our team described other substances with a different chemical structure, ROS and CARN (DEL BAÑO *et al.,* 2006) which showed higher genoprotective capacity. However, the present study shows that the substance (PASE) assayed in the post-X-irradiation treatment do not follow the trend observed above. Indeed their protective capacities were less than when used in the pre-X-irradiation treatment, underscoring the importance underlying the time of administration.

From the studies conducted it has been established that PASE elaborates radioprotective capacity albeit with a different chemical structure. Thus the third objective of this study was to determine the chemical structure and relate it with the radioprotective activity against damage induced by ionizing radiation. It could be inferred that to greater or lesser extent, the radioprotective effectiveness of the extract may not be due to a specific chemical structure, but a combination of structural elements present in the extract.

When protective agents are administered prior to irradiation of blood samples the radioprotective capacity seems to be dictated by the antioxidant capacity of the compound, irrespective of the structural parameters that confer this property to the compound. However, when treatment is administered after the irradiation of blood samples, the radioprotective effectiveness seems to be due to the lipophilicity of the compound, in addition to its antioxidant properties.

The basic requirements of an ideal radioprotectors include them being less toxic and highly effective at non-toxic dose levels, effective antioxidant and free radical scavenging capacity along with good bioavailability and solubility properties. They should be capable of acting through multiple mechanisms and should there for not be limited by time of application.

When these substances are administered after exposure to ionizing radiation, the hydroxyl and superoxide radicals would have disappeared from the biological environment due to their extremely short half-lives by causing a cascade of events

leading to radiation injury, hence some additional features of radioprotectors are sought. The results obtained shows that lipid antioxidants are capable of trapping peroxy radicals formed in cellular membranes and thereby producing a higher degree of protection against the damage induced by ionizing radiation. This way, these substances may work against eliminate lipid peroxidation product and reduce their deleterious effects by producing less harmful adducts when they react with the free radicals associated with lipid peroxidation.

From the results obtained it may be essential to in the immediate future conduct genoprotective analysis on the simultaneous administration of PASE and other substances to evaluate their potential protective synergies. In this quest, the mixture of radioprotective substances may afford an enhancement or a modification of the radioprotective capacities of the test substances according the time of administration (i.e., whether they are administered pre or post radiation), or even reduce the cytotoxic effect caused by the administration of only one of the substances and allowing the a higher concentration of the radioprotector to be administered. This would lead to the unearthing different radioprotective substances as per the desired goals of radioprotective drug development i.e. as a prophylactic (prevention) administered before exposure to radiation; as radiation mitigator (protection) against a known exposure or even as a treatment and administered after hours following accidental exposure to radiation.

Lipid peroxidation may be prevented by free radicals scavengers or by singlet oxygen quenchers. It has been shown in TBARS assay that, the addition of CAR leads to a decrease in oxidative compounds during the autoxidation of linoleic acid. Also the results obtained from the evaluation of CARN´s ability scavenge the $ABTS^{\cdot+}$ radical cation, confirmed the antioxidant capacity of a CARN rich concentrate.

When polyphenolic compounds are added after X-irradiation treatment, the only ROS that will be present in the cells according to the half-life of superoxide anion and hydroxyl radicals are the lipoperoxy radicals $R\text{-}OO^{\cdot}$, which are responsible for continuous chromosomal oxidative damage. In addition, ionizing radiation enhances lysosomal enzyme secretion and arachidonic acid release from membranes through lipo-oxygenase, cyclo-oxygenase and phospholipase activities, increasing the inflammatory

cell response. The lipoxgenases and cycloxygenase are involved in other processes such as endoperoxide formation, prostaglandins, leukotrienes etc.

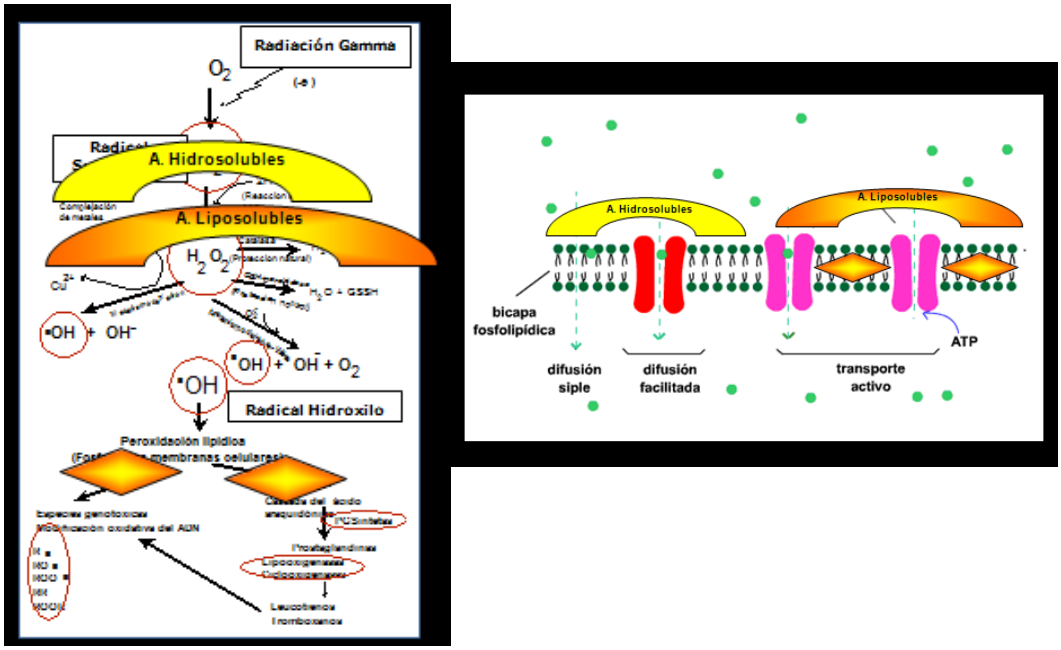

Figure D3. Schematic representation of the locations and mechanism by which antioxidants substances reduce the damage caused by ionizing radiation.

In this study radioprotective effect of the substances assayed was complemented by studying the cytotoxic effects of these compounds using the MTT cell viability assay. In the genotoxicity studies, amiphostine was evaluated as a radioprotective-antimutagenic agent, but cross-reactions in the MTT technique did not permit its use in the cell survival assay. Instead, DMSO, a free radical scavenger, with a high *in vitro* hydroxyl radical scavenging capacity was used. However, it is known to be highly toxic to animals when applied at radioprotective (MURRAY *et al.,* 1988; WEISS *et al.,* 1990).

The results obtained from the cytotoxicity studies, showed significant radioprotective effects of CARN and ROS on prostate epithelial cells. It has been reported elsewhere that these substances have minimal effects on non-tumorigenic prostate epithelial cells when treated with increasing concentrations (JOHNSON, 2011). Thus a protective effect on cell survival curves against damage induced by IR similar to that obtained in the study of genoprotection is hypothesised with this substance.

Evidently, the results of this study demonstrates that when normal human prostrate epithelial cells (PNT2) are exposed to PASE, ROS, CARN, DMSO and the different mixtures tested, the substances act as antioxidants which can eliminate the excess free radicals induced by IR in conjunction with the intracellular redox defensive system (ALCARAZ *et al*., 2013).

Surprisingly, an absence of radioprotective capacity was established with the metastatic melanoma cell line B16F10, which even showed a radiosensitizing effect induced by CAR, ROS and different mixtures thereof of these tested substances. The radioprotective capacity PASE to the melanoma cells decreased only slightly when administered alone. This response is considered to be appropriate when dealing with melanoma cells that are highly resistant to radiation and confirming its radioprotective capacity. However, this radioprotective capacity disappeared when PASE was mixed with CARN and ROS. This strange result has forced us to analyze the possible causes of these contradictory effects of the radioprotective capacity of PASE.

In this regard, the antitumor capacities of ROS, CARN and COL have previously been established in different human and non-human tumour cell lines (NGO *et al.,* 2011, YESIL-CELIKAS *et al.,* 2010, JOHNSON, 2011), in human melanoma cells (RUSSO *et al.,* 2009) and also in the B16F10 cell line (YOSHIDA *et al.,* 2005, HUANG *et al.,* 2005, YESIL-CELIKAS *et al.,* 2010; JOHNSON, 2011). COL has been reported to inhibit the invasion of highly metastatic mouse melanoma B16F10 cells *in vitro* leading to the conclusion that COL targets MMP-mediated cellular events in cancer cells and provide a new mechanism for its anticancer activity (HUANG *et al.,* 1994). Additionally, Visanji *et al* (2006) postulated that the induction of G2 / M phase cell cycle arrest by carnosol and carnosic acid is associated with alteration of cyclin A and B1 levels.

From our experience, in the study of growth inhibition of different types of melanoma cells induced by polyphenolic substances (BENAVENTE-GARCÍA *et al.,* 2005; MARTÍNEZ-CONESA *et al.,* 2005; YAÑEZ *et al.,* 2004), we had not expected a complete loss of radioprotective capacity of the tested substances. Also unclear is the mechanism by through CARN loses its radioprotective capacity in tumour cells. This could offer a new application to selectively protect healthy cells from IR without

protecting tumour cells when both types of cells are jointly exposed to IR in the same field. In this case, normal cells may benefit from their radioprotective effects; whereas in contrast, the tumour cells suffer radiosensitizing effect. Therefore, according to Yesil-Celiktas *et al.,* (2010), CARN alone or in combination with other anticancer drugs or IR may offer a good strategy for the treatment of a variety of human cancers that are resistant to chemotherapy and/or radiotherapy.

Although the mechanism by which CARN and ROS lose their radioprotective capacities in mouse melanoma B16F10 cells is obscure, one possible mechanism could be related to melanogenesis in these cells. The effect of some botanical extracts on melanogenesis has been documented (KIM *et al.*, 2006) and studies have shown that some flavonoids such as naringin (OHGUCHI *et al.,* 2006), caffeoyl compounds such as caffeic acid (KUDUGUNTI *et al.*, 2011) and its dimer rosmarinic acid (ROS) (LEE *et al.*, 2007, SANCHEZ-CAMPILLO *et al*., 2009) stimulate melanogenesis in mouse B16F10 melanoma cells. We have previously described the increase in thyrosinase activity and expression in mouse melanoma B16F10 cells after 48 h stimulation with ROS and compared these with corresponding levels in negative controls. In fact, upon visual inspection of cell pellets from samples of B16F10 melanoma cells treated with ROS, a darker colour is noticed compared with the control samples which may be explained by increased melanin content in the treated cells (SANCHEZ-CAMPILLO *et al.,* 2009). According to our description, ROS has been postulated to induce melanogenesis (increased melanin content and thyrosinase expression) in a concentration-dependent manner, probably through protein kinase A activation signalling (LEE *et al.,* 2007).

It is believed that the GSH redox system may be essential for the production and for the attenuation of abnormal melanin production, with glutathione thought to be involved in the regulation of melanin synthesis during melanogenesis. Furthermore, gluthatione (GSH) and GSH-related enzymes including gamma-glutamate cysteine lyase (gamma-GLC) and glutathione S-transferase (GST) are important antioxidant defences responsible for maintaining cellular redox balance with capacity to eliminate the ROS induced by IR (PANICH *et al*., 2012; ALCARAZ *et al.,* 2013)

It has been reported that the administration of Caffeic acid ester, to participate in melanogenesis, results in the intracellular GSH depletion, increased ROS in B16F10 melanoma cells, induction of apoptosis in B10F16 cells and *in vivo* B16F0 tumour size growth inhibition. Also there is a concomitant decrease in alanine aminotransferase activity and an increase in the level of malondialdehyde (MDA) showing a decreased capacity of lipid peroxidation and also a decrease in the free thiol content in liver and kidney (KUDUGUNTI *et al.,* 20011). Similarly, it is well known that the highly metastatic B16F10 cell line has a lower SOD activity (superoxide radical scavenging) than other less metastatic lines (KWEE, *et al.,* 1991). All these mechanisms are modified during melanogenesis and they are also responsible for the "endogenous" radioprotective capacity of cells exposed to IR.

Therefore, in this study, in the normal prostrate epithelial cells (PNT2), ROS and CARN act as antioxidants which eliminate the excess free radicals induced by IR in conjunction with the intracellular redox defensive system. However, in the B10F16 melanoma cells, melanogenesis is activated leading to redistribution of the enzymatic balances of glutathione and cysteine lyase to facilitate melanin production which could compromise the intracellular redox defence system. Since this involves a reduction in the scavenging levels of the superoxide radicals together with a loss of part of the antioxidant capacities of ROS/CARN, it causes an elevation of intermediate elements such as intracellular hydrogen peroxide which leads to higher amount of cell damage. The effect of this would be presented as an increase in the damage capacity induced by IR, and therefore, portray a paradoxical protective effect of the antioxidant substances towards the melanoma cells (ALCARAZ *et al*, 2013).

Most of the substances assayed in this study are antioxidants that have been consumed in human diet for several decades within certain dose ranges where no toxicity has been reported (HERTOG *et al*., 1993; HOLLENBERG, *et al*., 2004, COOK and SAMMAN, 1996; AHERNE and O'BRIEN, 2002). Several authors have proposed that a combination of dietary antioxidants could be useful in protecting normal tissues against radiation damage no matter how small that damage might be. The amounts of individual antioxidants to be included in the mixture will depend on the diagnostic doses of radiation to be applied, the bioavailability and liposolubility properties as well

as the concentration of the substances, as some of them may show pro-oxidant activity thereby, increasing the damage induced by ionizing radiation.

According to Prasad *et al* (2002), a clinical study to evaluate the radioprotective value of antioxidants should be conducted among patients receiving diagnostic radiation, using oxidative stress and chromosomal damage as biological end points.

In conclusion, no single chemical structure may account for the ability of some of the compounds assayed reduce the mutagenic effects of ionizing radiation. Antioxidant capacity appears to dictate the degree of protection of the compounds administered before radiation. However, it has also been demonstrated that the liposolubile properties of the compound provides greater protection if the substance is administered immediately after ionizing radiation. PASE evaluated can be considered as a good radioprotective substance whether in the biological medium before exposure to ionizing radiation or if administered immediately after radiation exposure.

VII. Conclusions.

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The **findings** of these **investigations** are briefly summarized below:

1. An extract was obtained from the **seeds** of ***Pycnanthus angolensis*** (**PASE**) which was used for the cellular radioprotection assays at concentrations similar to other substances tested.

2. A **significant decrease** in the chromosomal damage and radiation-induced cytotoxicity was determined after administration of **PASE** in "*in vivo*" and "*in vitro*" assays which shows a significant **radioprotective capacity** of **PASE** against DNA damage induced by ionizing radiation.

3. The **seed extracts** of ***Pycnanthus angolensis*** (**PASE**) has a radioprotective capacity greater than some of the radioprotective substances currently used in Radiation Oncology, although lower than other antioxidants tested, at least at the non-toxic concentrations of the extract used in this study.

VIII. Abstract.

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Medicinal plants and plant products have been used as traditional treatments for numerous human diseases, and remain a repository for obtaining new actives principles for clinical use.

This study aims at obtaining an extract from seeds of *P. angolensis* (PASE) suitable for use in cytoprotection and genoprotection assays in order establish its radioprotective capacity against chromosome damage induced by X rays, as well as compare it with other radioprotective antioxidants used in radiotherapy and different radiobiological models.

The genoprotective effect of PASE was studied by means of two micronucleus tests for antimutagenic activity in which the reduction in the frequency of micronuclei was evaluated *in vitro* using cytokinesis-blocked human lymphocytes and *in vivo* using polychromatic erythrocytes from mouse bone marrow. The radioprotective effect of PASE was studied via the MTT cell viability test in normal human prostate PNT2 epithelial cells and B16F10 mouse melanoma cells. The test extract was administered both prior and after exposure to a range of X-ray doses.

PASE showed a significant genoprotective capacity ($p<0.001$) against X-rays with a protection factor of 25-35% and a dose reduction factor of 2.5-3.7, depending on the time of administration. Cell survival obtained with PASE after exposure to 10 Gy of X-rays showed a protection factor of 86 %, thus eliminating 23 % of radiation-induced cell death in normal prostate epithelial cells (PNT2) ($p<0.001$) and a protection factor of 41 % ($p<0.001$) in the metastatic B16F10 melanoma cells after 48h of incubation and exposure to 10 Gy of X-rays.

An extract with suitable properties for use in radiation protection assays has been accomplished. The said extract demonstrates a significant radioprotective capacity and higher comparable genoprotective property relative to other radioprotective agents used in radiation oncology and similar to other substances tested in different radiobiological models.

IX. Bibliography.

IX. Bibliography.

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