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Flavonoid profile of *Lupinus mexicanus* germinated seed extract and evaluation of its neuroprotective effect

José de Jesús Uribe-Gómez[1], Juan Francisco Zamora-Natera[2], Jacinto Bañuelos-Pineda[1], Piotr Kachlicki[3], Maciej Stobiecki[4] and Pedro Macedonio García-López[2]
[1]Laboratory of Animal Morphophysiology, University of Guadalajara, Las Agujas Nextipac, Zapopan Jalisco, Mexico, [2]Laboratory of Biotechnology, Department of Botany and Zoology, University of Guadalajara, Las Agujas Nextipac, Zapopan Jalisco, Mexico, [3]Institute of Plant Genetics PAS, Poznan, Poland and [4]Institute of Bioorganic Chemistry PAS, Poznan, Poland

Summary. The aim of this study was to determine the flavonoid profile of *Lupinus mexicanus* germinated seed extract (PE) and to evaluate its effect as a phytoestrogen on the morphometric parameters of CA3 hippocampal neurons of ovariectomized rats (OVX). *L. mexicanus* seeds, germinated for 48 h, were homogenized and macerated using an 80% ethanol solution. The extract was analyzed by HPLC/MS-MS. Thirty young Wistar strain female rats (200±10 g) were randomly distributed into four groups: sham operated (S) treated with dimethyl sulfoxide (vehicle); ovariectomized and treated with 1250 μg of PE extract (OVX-PE); ovariectomized and treated with 5 μg estradiol benzoate (OVX-EB); and ovariectomized and vehicle treated (OVX). All substances were injected subcutaneously daily for 28 days. On day 29, the animals were sacrificed, perfused, and fixed to obtain the brains for histological processing. Each brain was cut and stained with hematoxylin and eosin. The thickness of the stratum oriens (SO), the nuclear diameter, and the neuronal density were measured in the hippocampus CA3 area. Nine different flavonoids and one non-identified compound were detected. The histological analysis demonstrated that the thickness of the SO was higher in the OVX-EB and S groups than in the OVX-PE and OVX groups ($p<0.05$); in addition, the nuclear diameters of the neurons in the OVX-EB and S groups were higher compared with the other groups ($p<0.05$). The OVX group had the highest cellular density among groups ($p<0.05$). Based on our results, the PE obtained did not have beneficial effects on CA3 hippocampal neurons.

Key words: *Lupinus mexicanus*, Flavonoids, Solid phase extraction, Chromatography-MS, Seedling

Offprint requests to: Dr. José de Jesús Uribe-Gómez, Laboratory of Animal Morphophysiology, University of Guadalajara, Las Agujas Nextipac, Zapopan, Jalisco, Mexico. e-mail: jpineda@cucba.udg.mx

Introduction

Phenolic compounds make up one of the major families of secondary metabolites in plants; they can be classified into non-soluble and soluble and include phenolic acids, phenylpropanoids, quinones, and flavonoids (Nicolas et al., 2005). According to their chemical structure, flavonoids are classified into several subclasses, such as chalcones, isoflavonoids, flavanones, flavones, dihydroflavonols, flavonols, anthocyanidins, and catechins based on the structure of the C-ring and the functional groups at C-3 and C-4. Generally, they exist as conjugates with sugars (glycosides) in vacuoles (Aoki et al., 2000). Flavonoids are common to most vascular plants and are particularly important because of the multiple roles they play, such as defending the plant against pathogenic microorganisms and as chemical signals in symbiotic nitrogen fixation. In addition, due to their biological activities, flavonoids have a positive impact on human health (Harborne, 1994; Harborne and Williams, 2000). In this regard, genistein and daidzein (isoflavonoids) have been employed as phytoestrogens to prevent both chronic degenerative and cognitive process-related diseases (Lephart et al., 2002; Cline et al., 2004; Wood et al., 2007; Xu et al., 2008). Previous studies in animal models have revealed that either the ingestion of diets rich in phytoestrogens or the repeated parenteral administration of these compounds improves

memory (Monteiro at al., 2008). Phytoestrogens also have a neuroprotective effect against the neurodegeneration caused by ovarian hypofunction (Xu et al., 2008), and likely also have neurotrophic properties (Pan et al., 1999; Jhamandas et al., 2001; Gleason et al., 2005).

The chemical structures of isoflavonoids allow them to interact with both α and β estrogenic receptors (ER), although with lower relative affinity than estradiol. They act as natural selective estrogen receptor modulators (SERMs) (Kuiper at al., 1997, 1998; Brzezinski and Debi, 1999). It has been shown that estrogens mediate the hippocampal function through ERα and ERβ (McEwen and Alves, 1999), which are involved in learning and behavioral processes (O'Neil et al., 1996). Hence, the neurons in the hippocampus are particularly sensitive to blood estrogen levels. Therefore, any alteration in the levels of this hormone in the brain induces significant physiological changes on the hippocampus, particularly in the CA3 area, which receives sensorial information from the external and internal environments through two principal pathways: mossy fibers and the perforant path (Amaral and Witter, 1989; Dolorfo and Amaral, 1998). Furthermore, the CA3 area is considered an auto-association network for the storage and retrieval of information, particularly in working memory processes (Treves and Rolls, 1992; Rolls and Treves, 1998; Stupien et al., 2003).

The mechanism by which isoflavonoids improve memory and learning capacity is still unknown; although it has been suggested that isoflavonoids increase mRNA levels of neurotrophic factors derived from the brain, choline acetyltransferase, and nerve growth factors in the cerebral cortex and hippocampus (Pan et al., 1999; Jhamandas et al., 2001). On the other hand, these compounds have shown antioxidant activity in hippocampal neuron cell cultures through the inhibition of lactate dehydrogenase (Zhao et al., 2002).

Most studies involving phytoestrogenic effects have been done employing soybean flavonoids; however, other leguminous plants rich in these compounds have not been studied, including species of the genus *Lupinus*, which are widely distributed in Mexico. Profiles of flavonoids synthesized in tissues of the Old World lupines, as well as the glycosylation and malonylation pattern of these compounds, have been thoroughly investigated using HPLC-MS techniques (Kachlicki et al., 2005). However, knowledge of the presence of these compounds in the New World lupine species is rather limited, and a systematic analysis of isoflavonoid glycoconjugates has only been reported for a few species from North America and only the flavonoid profiles from the vegetative organs (leaves, roots, stem, and inflorescence) of these species have been reported (Garcia et al., 2006; Stobiecki et al., 2010; Wojakowska et al., 2013).

To the best of our knowledge, the extraction and characterization of flavonoids from germinated lupin seeds has not been done in any of the Mexican wild lupin species. *L. mexicanus*, one of the 15 lupin species that grow in the Mexican state of Jalisco, represents a potential source of flavonoids due to its abundance and wide distribution. However, knowledge of the biological activity of this species is limited, although it can be supposed to be estrogenic. Therefore, the aim of this study was to determine the flavonoid profile of germinated seed extract from *L. mexicanus* and to evaluate it.

Materials and methods

Plant material

Seeds of *Lupinus mexicanus* were harvested in March 2008 from a native population growing in the municipality of Matanzas, Ojuelos, Jalisco, Mexico. The collection site is located at 2,205 masl, with an annual pluvial precipitation of 473.5 mm, an average annual temperature of 17.1°C, a maximum of 25°C, and a minimum of -3.9°C. The seeds were cleaned and then stored in a sealed container at room temperature. The identification of herborized plants was carried out by curators of the Instituto de Botanica, Departamento de Botanica y Zoologia, Universidad de Guadalajara.

Seed germination

Healthy and clean seeds (50 g) were scarified using sulfuric acid (98%) for 30 min. After scarification, the acid was decanted and the seeds were washed ten times with distilled water. Seed germination (defined as having the seeds' radicle protruding through the seed coat) was conducted under laboratory conditions at 25°C using a glass container (20x30x5 cm) on filter paper moistened with distilled water.

Isolation of phenolic secondary metabolites from plant tissue

For the analyses of flavonoid glycoconjugates, the germinated seeds were homogenized in 80% MeOH, and the suspension was placed in an ultrasonic bath for 30 min. The extract was centrifuged and the alkaloids were removed from the supernatant using SPE SCX (10 g, Supelco) columns. The loaded SPE columns were washed with 10 mL of 10% MeOH and 10 mL of MeOH; in addition, an aliquot of the eluate was evaporated and stored at -80°C prior to the LC-MS analyses. The rest was freeze-dried in a lyophilizer (LAB CONCO Corporation, USA). The lyophilized extract sample was kept at -80°C until its use in animal experiments.

Liquid chromatography with UV and mass spectrometric detection

Analyses of the seed extract were performed with two LC-UV-MS systems. The first system consisted of

an Agilent 1100 HPLC (Waldbronn, Germany) combined with an ion trap (IT) mass spectrometer, model Esquire 3000 (Bruker Daltonics, Bremen, Germany); the second was an Agilent RR1200 SL system connected to a micrOTOF-Q spectrometer from Bruker. An X-Bridge C-18 column (150x2.1 mm; 3.5 µm grain diameter, Waters) and Zorbax Eclipse XDB-C18 column (100x2.1 mm; 1.8 µm grain, Agilent) were used. Chromatographic runs in the Agilent 1200 system were performed at a 0.5 mL/min flow rate using mixtures of two solvents: A (99.5% H_2O, 0.5% HCOOH v/v) and B (99.5% MeCN, 0.5% HCOOH, v/v). The column effluent was split 3:2, so 0.2 mL/min was delivered to the ESI ion source. The elution steps were as follows: 0-8 min linear gradient from 5 to 30% B, 8-10 min linear gradient to 95% B; 10-12 min isocratic at 95% B; and the equilibration was achieved 3 min after returning to the initial conditions. The same solvents were used in the Agilent 1100 system at a 0.2 mL/min flow, and the gradient was: 0-35 min linear gradient from 5-30% B; 35-45 min up to 95% B; 45-52 min isocratic at 95% B; and re-equilibration occurred 60 min after the return to initial conditions. The settings of the IT mass spectrometer were: electrospray ion source (ESI), voltage +4 kV (positive ion mode), nebulization with nitrogen at 1.7 bar, dry gas flow of 7 L/min, gas temperature of 310°C, skimmer 1 voltage +12.4 or -11.2 V, and collision energy set to 1 V and ramped within 40-200% of this value. The number of ions accumulated in the trap was set to 10000 and the maximum accumulation time was 200 ms. According to results of preliminary experiments, spectra were recorded in the targeted mode in the mass range m/z 50-1500. The instrument operated under Esquire Control, version 5.1, and the data were analyzed using the Data Analysis package, version 3.1 (Bruker). The micrOTOF-Q spectrometer consisted of ESI operating at a voltage of +4.5 kV, nebulization with nitrogen at 1.6 Bar, dry gas flow of 8.0 L/min at a temperature of 220°C. The system was calibrated externally using a mixture containing sodium formate clusters. An additional internal calibration was performed for every run by injecting the calibration mixture during the LC separation using the diverter valve. All calculations used the HPC quadratic algorithm. This calibration gave an accuracy of at least 5 ppm. MS/MS spectra were acquired at the frequency of 1 scan per second for ions chosen on the basis of preliminary experiments, with argon used as the collision gas. Collision energy depended on the molecular masses of the compounds and was set between 15 and 30 eV in positive ion mode. The instrument operated at a resolution higher than 10000 (FWHM-full width at half maximum) under the control of the micrOTOF Control Program, ver. 2.3. The data were analyzed using the Data Analysis package, ver. 4 (Bruker). Metabolite profiles were registered in positive and negative ion modes. For the identification of compounds, the instrument was operated in the targeted MS/MS mode and single-ion chromatograms for exact masses of [M+H] + ions (+ 0.005 Da) were recorded.

Surgical procedure

Thirty young Wistar strain female rats (200±10 g), previously anesthetized with an intramuscular injection of ketamine sodium (50 mg/kg) and xylazine (10 mg/kg), were ovariectomized; while, 10 rats underwent the surgical procedure but the ovaries were not removed (sham group). The protocol followed for animal usage in our study was approved by the institutional ethics committee, and all animal procedures were conducted in accordance with the production, care, and use of laboratory animals established in the Mexican Official Standard (Secretaría de Agricultura, Ganadería, Desarrollo Rural, Pesca y Alimentación, 1999).

Evaluation of the estrogenic activity of the PE extract

After a post-operatory period of 4 days, all ovariectomized animals were randomly distributed into the following groups: Ovx (OVX), treated with 0.04 mL of dimethyl sulfoxide (vehicle); Ovx+estradiol (OVX-EB) treated with 5 μg/animal/day of estradiol benzoate; Ovx+PE (OVX-PE), treated with 1250 μg/animal/day of PE (in accordance with Xu et al., 2007); and the sham group (S), treated with vehicle. All four groups of rats were injected subcutaneously daily at 8:00 A.M. for 28 days. On day 29, all animals were sacrificed and perfused intracardially with 100 mL of warm heparinized saline (37°C) for 4 min, followed by 250 mL of 4% paraformaldehyde in 0.1 M of phosphate buffer, pH 7.4. The brain of each animal was removed and post-fixed for 24 h in 4% paraformaldehyde in 0.1 M of phosphate buffer, pH 7.4.

The brains were then washed in a phosphate buffered saline solution, dehydrated in increasing concentrations of alcohol, and embedded in paraffin. All brains were cut in coronal sections using a rotatory microtome (10 μm) and were subsequently stained with hematoxylin and eosin. Then, the sections were analyzed histologically, followed by a quantification of the thickness of the SO, the nuclear diameter of pyramidal neurons, and the neuronal density of the pyramidal stratum, using a Leica light microscope connected to an image analyzer (QWin500).

Statistical analysis

The data were analyzed by one-way variance analysis and the mean values were compared, with significance set at $p<0.05$. The SPSS 10.0 software was used.

Results

Flavonoid profile

The analytical technique employed in this study to determine the flavonoid profile of germinated seeds of *L. mexicanus* was able to unambiguously establish the presence of 10 flavonoid glycoconjugates (mono- and

diglucosides), acylated or not. The aglycones present in these compounds were the flavones acacetin, apigenin, and chrysoeriol; the isoflavones genistein and 2'-hydroxygenistein; and an unknown compound with a molecular weight of 694 (Table 1). Based on the molecular weight and some fragments observed in the MS/MS spectra, the unknown compound could be acacetin O-diglucoside malonylated; although, the spectral data were not sufficient to unequivocally identify this metabolite.

The identified acylated flavonoid glycoconjugates were chrysoeriol malonylated C-glucoside; two malonylated diglucosides of 2'-hydroxygenistein; and two malonylated diglucosides of genistein, but one of these contained two malonic acid moieties with the genistein 4',7-O-diglucoside dimalonylated. In contrast, acylation was absent in the following flavonoid glycoconjugates: genistein 8-C-glucoside, acacetin 8-C-glucoside, and chrysoeriol C glucoside. Apigenin 6,8-*di*-C-glucoside-7-O-apioside, a compound found in seeds of several lupin species that is not present in plant organs, was also found.

Histological description

The microscopic analysis of the histological preparations of the hippocampus CA3 area indicated the neurons found in the OVX-EB group were the largest; while, the smallest were found in the OVX-PE group. In the S and OVX-EB groups, the neurons showed rounded nuclei, were slightly basophilic, with one or occasionally two prominent nucleoli in their central part. In the OVX and OVX-PE groups, the nuclei were oval in shape. The nucleoli were clearly defined with granular chromatin in the OVX group; while, they were not evident and the chromatin was condensed in the OVX-PE group.

In addition, in the OVX-PE and OVX groups, the neurons showed degenerative changes such as pyknosis, cavitation, and eosinophilia; while, these changes were only occasionally observed in the S and OVX-EB groups. The neuronal cytoplasms were dense in all groups, with the exception of the OVX-EB group, which showed a pallid pink color. This dense cytoplasm was eosinophilic in the OVX and OVX-PE groups, and slightly eosinophilic in the S group. The largest part of the neuropil was uniform in color and texture in the S and OVX-EB groups; although, there were small areas with lumpy texture. On the other hand, the OVX and OVX-PE groups showed a lumpy-textured neuropil. The features described above are shown in Fig. 1.

Quantitative microscopic analysis

The SO, which also includes the stratum alveus, was significantly thicker (85.68 μm) in the brains of the OVX-EB group than in the brains of other groups ($p<0.05$). The descending order of thicknesses in the other groups were: S (71.75 μm), OVX-PE (63.66 μm), and OVX (59.32 μm). With regard to the pyramidal neurons, the nuclear diameter in the OVX-EB group was 14.25 μm; this value was larger ($p<0.05$) than that found in the S (11.82 μm), OVX (9.95 μm), and OVX-PE

Table 1. Flavonoids and their derivatives identified in the *Lupinus mexicanus* germinated seed extract using UPLC/ESI/qToF-MS.

No	RT (min)	Compound	MW	Elemental composition of [M+H]+ ion	Exact mass of [M+H]$^+$ ion			Fragmentation pathway (product ions obtained from precursor ion	
					Calculated	Observed	Error (ppm)	Positive ions [M+H]$^+$ (m/z)	Negative ions [M-H]$^-$ (m/z)
1	2.5	Apigenin 6,8-di-C-glucoside-7-O-apioside	726	$C_{32}H_{39}O_{19}$	727.2080	727.2076	-0.6	727, 709, 595, 577, 559, 499, 481, 409, 391	725, 605, 455, 335
2	3.8	2'-hydroxygenistein C, O-diglucoside malonylated	696	$C_{30}H_{33}O_{19}$	697.1611	697.1617	0.9	697, 535, 491, 449, 431	695, 489, 447
3	3.9	Genistein 4',7-O-digluco-sidemalonylated	680	$C_{30}H_{33}O_{18}$	681.1661	681.1670	0.2	681, 519, 433, 287	nr
4	4.0	Genistein 6-C-glucoside	432	$C_{21}H_{21}O_{10}$	433.1129	433.1136	-1.6	433, 415, 397, 379, 313	431, 311, 283
5	4.1	2'-hydroxygenistein 4',7-O-diglucoside malonylated	696	$C_{30}H_{33}O_{19}$	697.1611	697.1611	0.0	697, 535, 449, 287	651, 489
6	4.4	Chrysoeriol 8-C-glucoside	462	$C_{22}H_{23}O_{10}$	463.1235	463.1227	-1.7	463, 445, 427, 409, 397, 385, 381, 373, 367, 353, 343	461, 341, 326
7	4.8	Unknown	694						
8	5.4	Acacetin 8-C-glucoside	446	$C_{22}H_{23}O_{10}$	447.1286	447.1282	-0.9	447, 429, 411, 381, 351, 327, 397	445, 355, 325, 297, 282
9	5.6	Chrysoeriol -O-glucoside malonylated	548	$C_{25}H_{25}O_{14}$	549.1239	549.1237	-0.4	549, 463, 301	547, 299
10	5.6	Genistein 4',7-O-diglucoside dimalonylated	766	$C_{33}H_{35}O_{21}$	767.1665	767.1646	-2.5	767, 681, 519, 433, 271	765, 473, 431, 269/268

(8.89 μm) groups, respectively. Finally with respect to neuronal density, the OVX group had the highest value (10.34 neurons/0.01 mm^2) and the OVX-PE group (6.0 neurons/0.01 mm^2) the lowest value. Intermediate neuronal density values were recorded in the OVX-EB (8.21 neurons/0.01 mm^2) and S (7.75 neurons/0.01 mm^2)

Table 2. Effect of estradiol benzoate and PE extract on the CA3 hippocampal brain region of ovariectomized rats.

Treatment (group)	Nuclear diameter (μm)	Neuronal density (neurons/0.01 mm^2)	Stratum Oriens thickness (μm)
S	11.82±2.30[b]	7.75±2.41[b]	71.75±4.57[b]
OVX	9.95±4.74[c]	10.34±2.61[a]	59.32±12.98[c]
OVX-EB	14.25±3.40[a]	8.21±2.85[b]	85.68±15.73[a]
OVX-PE	8.89±4.02[d]	6.0±2.32[c]	63.66±13.02[c]

Different letters in columns indicate a statistical difference ($p < 0.05$), n=10

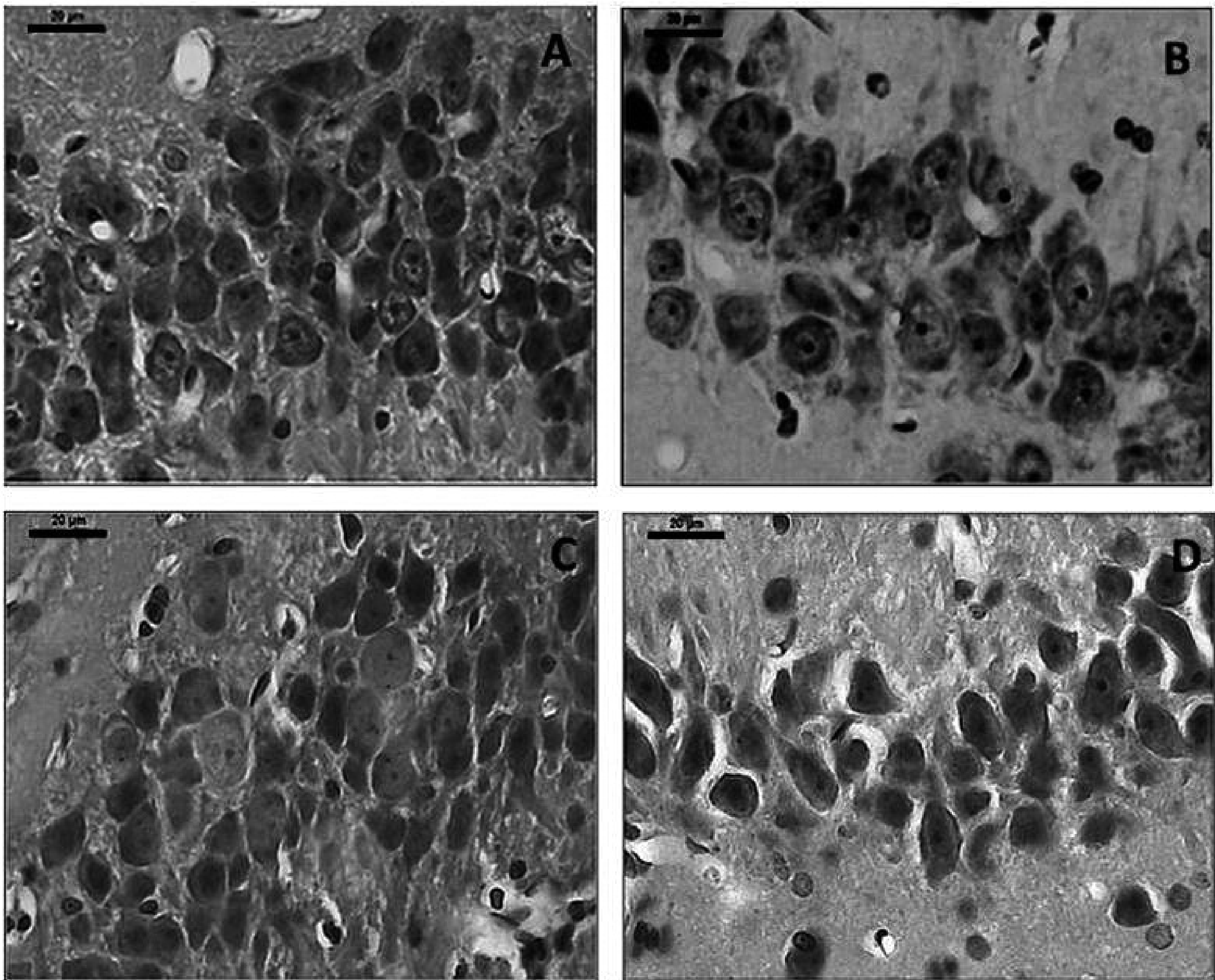

Fig. 1. Hippocampal CA3 neurons. **A.** S group; morphologically normal neurons, slightly eosinophilic, with high cellular density. **B.** OVX-EB group; large neurons with normal morphological features. **C.** OVX group; high neuronal density with many eosinophilic neurons showing slight pyknosis and cavitation. **D.** OVX-PE group; a reduction in both neuronal density and size can be observed, with perineuronal cavitation and intense eosinophilia associated with pyknosis. HE. Bar: 20 μm.

groups ($p<0.05$), as shown in Table 2.

Discussion

PE flavonoid profile

It should be noted that the flavonoid profiles of conjugates present in the leaves and roots of different lupin species are distinct and they differ from those observed in non-germinated seeds (Garcia et al., 2006; Stobiecki et al., 2010; Siger et al., 2012; Wojakowska et al., 2013). Compounds abundant in seeds, such as apigenin 6,8-di-C-glucoside-7-O-apioside, are still present at early stages of germination and completely disappear in the leaves and roots of 3-week old plants. In contrast with European lupin seeds (*Lupinus albus*) that contain 2'-hydroxygenistein and genistein as free aglycones (Stobiecki et al., 2010), there were no free flavonoid aglycones in *L. mexicanus* PE. However, 2'-hydroxygenistein and genistein were detected as free aglycones in the roots, stems, leaves, and inflorescences of *L. exaltatus* (Garcia et al., 2006). As with the European lupine flavonoids, malonylation is an important structural feature in some Mexican lupins. In this regard, genistein 4',7-O-diglucoside malonylated has been found in the roots, stems, and inflorescences of *L. exaltatus* and in the roots of *L. reflexus*. There are also other dimalonylated flavonoids in these species, i.e. 2-hydroxygenistein 4' and 7-O-diglucoside malonylated (Garcia et al., 2006; Stobiecki et al., 2010). It is important to indicate that when studying *L. exaltatus* (Garcia et al., 2006), we were not aware of the existence of these compounds. However, it was later determined that the compounds identified as biochanin derivatives in *L. reflexus* (Stobiecki et al., 2010) were acacetin derivatives. This was corrected in newer research (Wojakowska et al., 2013).

It is interesting that genistein, as a free aglycone, was absent in *L. mexicanus* PE; although, it is well known that this isoflavonoid is found in lower amounts in lupin seeds than in soybeans (Nakamura et al., 2001). This aglycone and other flavonoids found in leguminous plants (lupins, chickpeas, beans, clover, mung beans) possess estrogenic and anticarcinogenic activities, and there are also reports about their roles in the prevention of cardiovascular diseases and osteoporosis.

Histological findings

In this study, it was evident that the PE treatment had an unfavorable effect on hippocampus CA3 neurons, which were smaller in size and showed signs of neuronal degeneration (a great number of shrunken neurons with pyknotic nuclei). However, the stressful conditions of the surgical procedure and the manipulation of the animals (daily treatment and handling) during the experiment should be considered.

On the contrary, the EB treatment showed a beneficial effect on the CA3 brain area, which showed an increased cell nuclear diameter due to a direct enhancement of the nuclear activity of the neurons (Srebro et al., 1988). The thickness of the SO was also greater, which is directly related to the growth of dendrites (Woolley and McEwen, 1994). These parameters are indicative of a neurotrophic effect of the EB on the neurons of the hippocampus (McEwen, 2002). Thus, the anticipated negative effect from the absence of steroids after the ovariectomy was overcome by the administration of EB.

The negative effects obtained with the PE treatment could be explained by the chemical structure of their flavonoids (glycoconjugates), and the subcutaneous administration. It is well known that phytoestrogens must undergo a chemical transformation in order to have high estrogenic activity, and this occurs after the oral administration of phytoestrogens (Casanova et al., 1999). Therefore, it is possible that after the subcutaneous administration of PE no chemical transformation or good absorption occurred. Additionally, it has been demonstrated that the flavonoids genistein and daidzein have a lower affinity for estrogenic receptors in the brain and uterus than the endogenous estrogens and 17ß estradiol (Wink, 2005). In the present study, it was evident that PE was not capable of protecting the CA3 neurons after ovariectomy, as the BE treatment and endogenous estrogen could.

Conclusion

The flavonoid profile of the PE evaluated showed the presence of 10 flavonoid glycoconjugates, with 5 compounds being isoflavone derivatives. However, these compounds did not have neurotrophic or neuroprotective effects, based on the small size and signs of neuronal degeneration of the hippocampal CA3 neurons of OVX rats. Further studies are being undertaken in our laboratory to elucidate the effectiveness of extracts from germinating seeds of different Mexican lupine species as phytoestrogens.

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