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Health-promoting potential of betalains *in vivo* and their relevance as functional ingredients: A review

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ABSTRACT

Background: Betalains are phytochemicals of relevance to the food industry not only for their natural presence in foods and beverages but also due to their utility as food colorants. However, the use of betalains as functional ingredients has not been extended despite their demonstrated health-promoting properties. The use of betalains as nutraceuticals is an emerging field thanks to the accumulation of scientific evidence on their beneficial effects on health in humans and animal models.

Scope and approach: In this review, all the health-promoting effects of betalains published to date are collected and discussed with a focus on their promising use as functional ingredients. All studies on animal models and humans are critically analyzed.

Key findings and conclusions: The bioactive properties of betalains have been manifested in rodents, fish, and nematodes. Chemopreventive, antioxidant, and anti-inflammatory activities are some of the effects produced by betalains *in vivo*. Assays performed in humans remain limited, but their conclusions are highly promising in terms of the health-promoting potential of betalains, supporting the use of these compounds as functional ingredients.

1. Introduction

Betalains are water-soluble, nitrogenous compounds that constitute the main pigments of plants belonging to the order Caryophyllales, where edible plants like beets, chards, or cactus pears are found. These pigments are produced in flowering petals, roots, stems, leaves, and fruits (Schliemann et al., 1996; Wang, Chen, & Wang, 2007) (Fig. 1A–G) and present betalamic acid as a structural and functional unit, being classified into the yellow betaxanthins and the violet betacyanins (Khan & Giridhar, 2015) (Fig. 1H). In addition to the physiological roles played by the pigments in plants, there is recent evidence on the beneficial effects produced *in vivo* after their administration to animals. In this sense, it has been claimed that betalains may produce beneficial effects on human health. Their effects may well be determined by their extraordinary antioxidant capacity, as these compounds are able to decrease oxidative stress by efficiently removing ROS (Cai, Sun, & Corke, 2003; Gliszczyńska-Swigło, Szymusiak, & Malinowska, 2006). Notably, betalains found industrial application as food colorants (red beetroot) (Rodriguez-Amaya, 2019) even before the first evidence of their antioxidant capacity was provided (Escribano, Pedreño, García--Carmona, & Muñoz, 1998). The bioactive properties described for betalains, coupled with their established industrial application, have fuelled the interest in their production, study, and characterization, with a recent special emphasis on their *in vivo* effects. Novel findings in *in vitro* assays have been followed by the study of betalains' effects in a variety of animal models and even in humans. Currently, there is a strong experimental evidence *in vivo* that supports the claimed biological activities of these pigments. Earlier reviews have focused on the bioavailability and bioaccessibility of betalains encompassing *in vitro* and *in vivo* studies (Rahimi, Abedimanesh, Mesbah-Namin, & Ostadrahimi, 2019) and on the biological effects and methods of extraction and processing of red beetroot betalains (Fu et al., 2020; Hadipour, Taleghani, Tayarani-Najaran, & Tayarani-Najaran, 2020). In general, previous reports reviewing the health-promoting activity of betalains

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have been focused on the bioactivity of one or a few pigments from selected natural sources (Joshi & Prabhakar, 2020) or in the prevention and treatment of a specific disease (Cheok, George, Rodriguez-Mateos, & Caton, 2020; de Oliveira, do Nascimento, Sampaio, & de Souza, 2020), without integrating or fully contemplating the broad spectrum of evidence available in the literature related to the *in vivo* protective effects of betalains. Other studies do not discriminate between *in vitro* and *in vivo* effects and, therefore, they do not take into account the consequences generated by particular characteristics of *in vivo* assays, providing a brief view on the biological properties of betalains (Sadowska-Bartosz & Bartosz, 2021). Few studies delve into the characterization of betalains from a therapeutic point of view and highlight their molecular targets (Madadi et al., 2020). The present work puts into context all the effects associated with betalains described *in vivo*. To this end, our aim is to provide a comprehensive, detailed, and complete view of the evidence reported in the promotion of health after the *in vivo* treatment with betalains. For the first time, all the studies that have described a biological activity for betalains using different animal models and those describing the effects on humans are reviewed in depth with a special emphasis on the search for common trends and mechanistic explanations of the health-promoting potential of betalains. Altogether, the new evidence strongly supports the use of betalains as nutraceutical compounds.

2. Betalains in nature

Betalains are found as secondary metabolites in many plants of the order Caryphyllales where they replace anthocyanins, one of the most abundant pigments in the plant kingdom. Anthocyanins have not been detected in betalain-producing plants, giving rise to a mutual exclusion relationship between these two families of plant pigments (Brockington, Walker, Glover, Soltis, & Soltis, 2011). There are many hypotheses about this fact, but the evolutionary reason behind is still unknown. Biochemical studies have shown that key enzymes involved in anthocyanin biosynthesis are not expressed in betalain-producing plants (Mabry, 1964; Timoneda et al., 2019). The presence or absence of betalains is therefore an aspect of high taxonomic relevance. Within the order Caryophyllales, only the families Molluginaceae, Kewaceae, Limeaceae, Macarthuriaceae, and Simmondsiaceae produce anthocyanins and therefore do not have betalains (Thulin et al., 2016). The coloration of the rest of the plants within the order is due to betalains. The main edible sources of these pigments in nature are red beetroot (*Beta vulgaris*) (Hempel & Böhm, 1997), the fruits of cacti of *Opuntia* (mainly *Opuntia ficus-indica*) (Felker et al., 2008), pitaya or dragon fruit (*Hylocereus polyrhizus*) (Wybraniec, Nowak-Wydra, Mitka, Kowalski, & Mizrahi, 2007), Swiss chards (*Beta vulgaris*) (Kugler, Stintzing, & Carle, 2004) and quinoa grains (*Chenopodium quinoa*) (Escribano et al., 2017). Some of the lesser-known sources are ulluco tubers (*Ullucus tuberosus*) (Svenson, Smallfield, Joyce, Sansom, & Perry, 2008), *Rivina humilis* berries (Khan, Denny Joseph, MuralidharaRamesh, Giridhar, & Ravishankar, 2011), Malabar spinach (*Basella alba*) (Lin et al., 2010), djulis grains (*Chenopodium formosanum*), a plant native to Taiwan (Tsai, Sheu, Wu, & Sun, 2010), the vegetable waterleaf (*Talinum triangulare*) (Swarna, Lokeswari, Smita, & Ravindhran, 2013) and some fruits of the

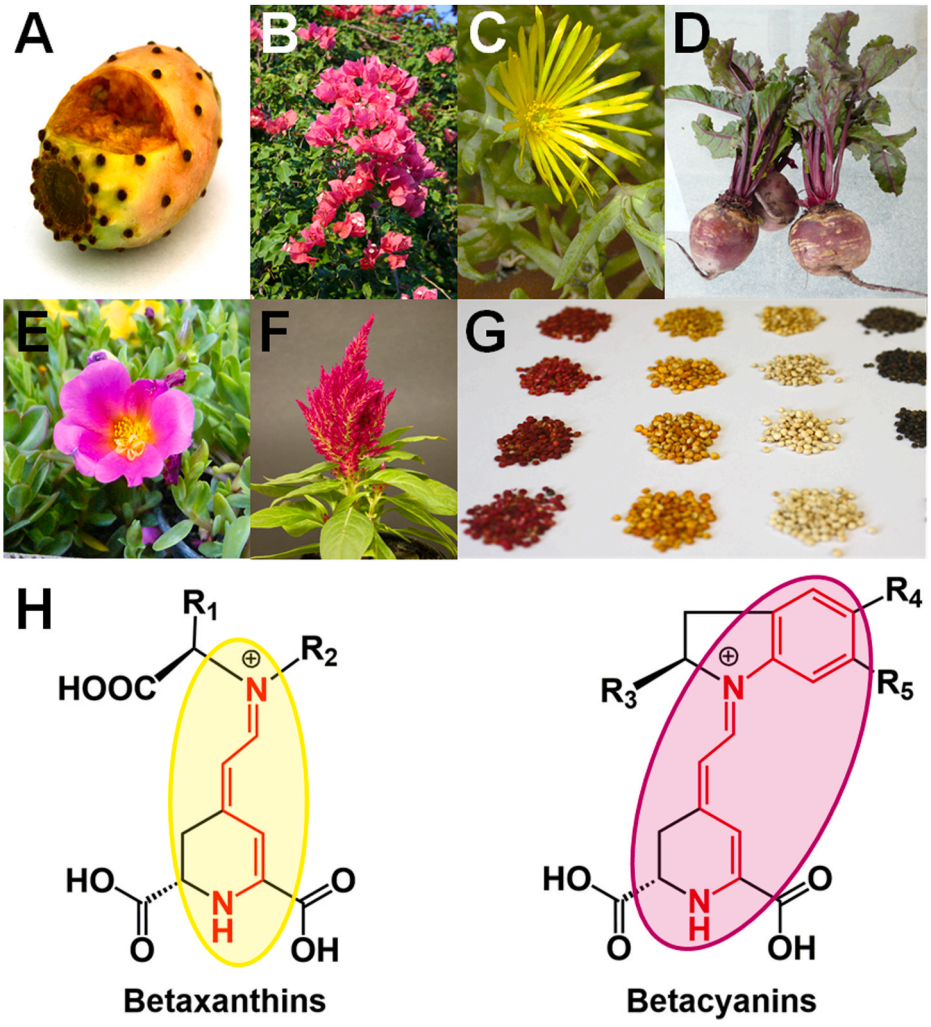

Fig. 1. Representative images of betalain-producing plants: (A) *Opuntia ficus-indica* fruit; (B) *Bougainvillea glabra* bracts; (C) *Lampranthus productus* flower; (D) *Beta vulgaris* roots; (E) *Portulaca oleracea* flower; (F) *Celosia argentea* inflorescence; (G) *Chenopodium quinoa* grains. (H) General chemical structures for betaxanthins and betacyanins. The resonance system implied in the color properties of betalains is highlighted in red. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Eulychnia cactus as the copao fruit (Masson et al., 2011). Within these examples, red beetroot is the most employed source of betalains. Betacyanins content in beetroot accounts for 75–95% of the total pigments while the remaining 5–25% corresponds to the yellow pigments betaxanthins (Delgado-Vargas, Jiménez, Paredes-López, & Francis, 2000). Its main pigment, the violet betacyanin betanin, is the best-known and most studied betalain. In addition, beetroot contains isobetanin, the epimer of betanin, and other betalains such as the betaxanthins vulgaxanthin I and miraxanthin V (Sawicki & Wiczkowski, 2018). Since the earliest works with these pigments, betalain production was assumed to be restricted to plants belonging to order Caryophyllales but recent studies have identified a bacterium capable of producing them (Contreras-Llano, Guerrero-Rubio, Lozada-Ramírez, García-Carmona, & Gandía-Herrero, 2019). Betalain-related compounds have also been found in some fungal genera such as *Amanita*, *Hygrocybe,* and *Hygrophorus* (Belhadj Slimen, Najar, & Abderrabba, 2017).

3. Biosynthetic pathway of betalains

Betalains are secondary metabolites and their biosynthetic pathway in plants derives from L-tyrosine, which is hydroxylated to give rise to L-DOPA (Fig. S1). Early studies stated that tyrosinase enzymes were responsible for this first step of the route (Steiner, Schliemann, Böhm, & Strack, 1999). However, it has recently been discovered that cytochrome P450 enzymes belonging to the CYP76AD family, which have tyrosine hydroxylase activity (Sunnadeniya et al., 2016), are responsible for this reaction. In addition, recombinant expression of CYP76AD genes has shown that some of these enzymes present an additional oxidase activity which yields *cyclo*-DOPA from L-DOPA (DeLoache et al., 2015; Suzuki et al., 2014). After hydroxylation of L-tyrosine to yield L-DOPA, oxidation and cleaving of the aromatic ring of L-DOPA take place, giving rise to 4,5-*seco*-DOPA. This step is catalyzed by 4,5-DOPA-extradiol-dioxygenase (4,5-DODA), the key enzyme in the biosynthetic pathway of betalains (Christinet, Burdet, Zaiko, Hinz, & Zrÿd, 2004; Sasaki et al., 2009). 4,5-*seco*-DOPA is an intermediate product that undergoes spontaneous intramolecular cyclization that results in the structural unit of betalains, betalamic acid (Fischer & Dreiding, 1972). The ability to synthesize betalamic acid due to the activity of a DODA enzyme was assumed to be exclusive to plants of the order Caryophyllales and some fungi (Belhadj Slimen et al., 2017) but recent studies have demonstrated the presence of enzymes with 4,5-DODA activity in some prokaryotes such as *Escherichia coli* (Gandía-Herrero & García-Carmona, 2014), *Gluconacetobacter diazotrophicus* (Contreras-Llano et al., 2019) or *Anabaena cylindrica* (Guerrero-Rubio, García-Carmona, & Gandía-Herrero, 2020).

Betalains are classified into two groups that differ in the nature of the compounds condensed with betalamic acid. The acid is able to spontaneously condense with *cyclo*-DOPA yielding the violet betacyanin betanidin, or with amines or amino acids to produce yellow betaxanthins by Schiff condensation (Khan & Giridhar, 2015). The production of betaxanthins does not imply additional enzymes once betalamic acid is synthesized. However, the production of betacyanins implies structural modifications by the action of additional enzymes, except for the above-mentioned betanidin. Betacyanins show glycosyl residues at C_5 or C_6 positions. These O-glycosidic bonds are formed enzymatically between a hydroxyl group of the betacyanin and a hydroxyl group of a sugar moiety, with the release of a water molecule (Harris et al., 2012; Sasaki, Adachi, Koda, & Ozeki, 2004). In betacyanins, there exist four main structural types depending on the position where the sugar molecules are linked to the *cyclo*-DOPA structure (Fig. S2). These four types, named betanin, gomphrenin, amaranthin, and bougainvillein, refer to the main species where they were first detected (*B. vulgaris, Gomphrena globosa, Celosia argentea,* and *Bougainvillea buttiana*). Betanin and gomphrenin only differ in the glycosylation position. Betanin shows a free hydroxyl group (-OH) at the C_6 carbon and a glucosyl residue linked to the –OH at C_5. In contrast, gomphrenin has a glucosyl residue linked to the hydroxyl group found at C_6 and a free one at C_5. On the other hand, amaranthin type betacyanins have a glucuronyl glucosyl residue at the –OH found at C_5 position. Finally, members of the bougainvillein group present a diglucosyl residue in C_5 or C_6 of *cyclo*-DOPA, and may or may not be carboxylated (Belhadj Slimen et al., 2017; Miguel, 2018).

4. Health-promotion in animal models

4.1. Mouse (*Mus musculus*)

4.1.1. Chemopreventive potential

The properties of betalains as antitumoral agents have been studied in animal models since 1996. An early study highlighted the chemopreventive power of these compounds (Kapadia, Tokuda, Konoshima, & Nishino, 1996) showing how tumors induced in the skin of mice were affected by the oral administration of beet (*B. vulgaris*) extracts. The results obtained showed a significant decrease in the number of papillomas after intake of these extracts. In the same study, the administration of beet extracts also decreased the number of mice with induced lung tumors while mice that still presented them, showed a reduction in their number by up to 30%. These pioneering studies began to highlight the possible health-promoting role of betalains. Kapadia et al. (2003) continued analyzing the inhibition of tumors produced by betalains' administration in their following studies. In particular, beet extracts were orally administered to mice with skin and liver cancer. Papillomas were induced by exposure to UV light and by carcinogenic chemical agents. In both cases, the intake of beet extracts significantly inhibited the incidence and number of topical tumors. On the other hand, liver cancer was chemically induced and, as in skin cancer trials, oral administration of extracts significantly reduced the growth and proliferation of liver tumors. The small dose of betanin (0.0025%) used in this study showed the great potential of these compounds as chemopreventive agents.

Due to the growing interest in finding new natural products with chemopreventive capacities, aqueous extracts of *O. ficus-indica* were injected in mice with ovarian cancer (Zou et al., 2005). The effects of their administration were compared with those obtained from the administration of *N*-(4-hydroxyphenyl) retinamide (4-HPR), a chemopreventive drug currently used in treatments against ovarian tumors. *Opuntia* extracts inhibited the growth of ovarian tumors with similar effects to those reported by 4-HPR. Therefore, evidence was found for the *in vivo* antitumoral activity of *Opuntia* aqueous extracts.

Later on, Zhang, Pan, Wang, Lubet, and You (2013) also showed the chemopreventive effects of betanin in mice. Two different carcinogens, vinyl carbamate and benzopyrene, were employed to induce the formation of lung tumors and after that, an aqueous solution of betanin was orally administered. Following treatment with betanin, a reduction of 39% was observed in vinyl carbamate-induced tumors and up to 65% in benzopyrene-induced tumors. The results indicated that inhibition of tumor growth is caused by the action of betanin on the apoptosis and angiogenesis processes. Specifically, it suggests that betanin acts by increasing the expression of caspase-3, a proapoptotic enzyme belonging to the cysteine-protease group (Alnemri et al., 1996), which increases cell apoptosis levels. In addition, betanin decreased the formation of endothelial microvases, resulting in an inhibition of angiogenesis. These molecular mechanisms seem to be responsible for the inhibition of the growth of lung tumors in mice. The anticancer effect of betalains has also been tested in mice with xenografts from human lung tumor cells (A549) (Yin, Yang, Guo, Veeraraghavan, & Wang, 2021), where intraperitoneal administration of pigments was able to decrease levels of pro-inflammatory cytokines, in addition to tumor markers such as lactate dehydrogenase activity. In this case, the authors did not disclose the source or purity of the pigments, making it difficult to study the pigment-activity relationship.

Indicaxanthin, the major pigment of yellow *O. ficus-indica* cactus pears, has also been able to inhibit the progression of melanomas in mice (Allegra et al., 2018). Melanomas were induced by subcutaneous

injection of B16/F10 cells, a murine melanoma cell line that causes tumors with similar characteristics to human melanomas (Dragsten et al., 1980). Following oral administration of indicaxanthin, the results showed significant inhibition of tumor development in mice. Taking these results into account, the authors suggested that the antitumoral properties of indicaxanthin might be an interesting tool for the treatment of melanomas.

Other researchers conducted revealed the cytotoxic activity of beet pulp in tumors induced in mice (Vulić et al., 2013). In this case, the animals were injected with Ehrlich Carcinoma (EAC) cells intraperitoneally. These cells are characterized by causing ascites, causing serous fluid to build up in the intraperitoneal cavity. Administration of different doses of beet extracts induced apoptosis of EAC cells. Measurements of the activity of antioxidant enzymes were made and a significant increase was observed following administration of the extracts. The authors claimed that the antioxidant activity of the betalains present in the extract was the reason for the death of EAC cells.

The radioprotective activity of betalains has also been a matter of study with mice exposed to gamma radiation (Lu, Wang, & Zhang, 2009). Relevant parameters were measured for the study of radioprotection, such as the number of leukocytes or the activity of antioxidant enzymes such as superoxide dismutase, catalase, and glutathione peroxidase. The results showed radioprotective activity in mice that was due to the administration of beetroot betalains. The radioprotective mechanism of these seems to be related to their antioxidant nature, and by producing a regulation at the immune system level and increased leukocyte activity.

4.1.2. Neuroprotective effects

Betalains' intake has shown health-promoting effects at the cognitive level in studies conducted with mice (Wang & Yang, 2010). Animals were treated with D-galactose, a neurotoxic agent that causes learning and memory problems, and given pure betacyanins extracted from *Portulaca oleracea*. Then, behavioral tests were performed and the activity of different antioxidant enzymes was analyzed together with the concentration of malondialdehyde (MDA). MDA is the final product of the lipid peroxidation process, which results in the destruction of cell membranes (Marnett, 1999). The behavioral test showed positive effects on the times required for mice to perform the activities when pigments were administered. This improvement correlated with increased antioxidant enzyme activities and a decrease in MDA. The results indicate that betacyanins successfully reversed the learning and memory problems caused by D-galactose in the animal model. In addition, comparative trials with vitamin C orally administered to mice showed that betacyanins were more efficient in improving cognitive decline.

A more recent study highlights the neuroprotective effect of betanin in mice with neurodegeneration induced by intraperitoneal administration of trimethyltin chloride (TMT) (Thong-asa, Prasartsri, Klomkleaw, & Thongwan, 2020). Neurodegenerative pathology consisted of increased memory loss and anxious behavior. The oral administration of betanin in the days before and during the development of the pathology managed to improve the behavior of the mice through an increase in spatial learning and a general cognitive improvement. In addition, the authors assessed oxidation levels in brain tissue, observing a significant increase in catalase and superoxide dismutase activity, as well as a reduction in MDA levels. The administration of the pigment also managed to increase the activity of choline acetyltransferase. With these results, the authors conclude that betanin protects mice from TMT-induced neurodegeneration, which opens new paths towards the use of betalains as neuroprotective agents. In a similar manner, the same researcher participated in a subsequent study in which the protective effect of betanin in mice with Parkinson's disease was evaluated (Thong-asa, Jedsadavitayakol, & Jutarattananon, 2021). In this case, neurodegeneration was induced by subcutaneous administration of rotenone. To assess the neuroprotective effects, various behavioral tests were performed, the brain oxidative state was analyzed, and immunohistochemistry assays were carried out. Sick mice treated with betanin showed significant improvements in their behavior, as well as an increase in antioxidant enzyme activity in brain tissue and a reduction in MDA concentration. The authors attribute the protective effects of betanin shown against Parkinson's to its great antioxidant capacity.

4.1.3. Antimalarial activity

Regarding antimalarial activity of betalains, a study was conducted that assessed the *in vivo* antimalarial activity of betalains (Hilou, Nacoulma, & Guiguemde, 2006). Extracts of *Amaranthus spinosus* and *Boerhavia erecta*, two plants traditionally used in humans with medicinal purposes as antimalarial treatment, were employed to ascertain if they actually inhibited the growth of the parasites in mice. Extracts from these plants were administered to animals previously supplied with *Plasmodium*-parasitized erythrocytes. The results showed significant inhibition of parasite growth, confirming the antimalarial activity of betalain extracts *in vivo*. Inhibition of the intracellular transport of choline in protozoa and the chelation of the cations required for their growth (Fe^{2+}, Ca^{2+}, and Mg^{2+}) seem to be the main mechanisms behind the antimalarial activity of the extracts.

4.1.4. Anti-inflammatory properties

The anti-inflammatory properties of betalains were evident from the use of *B. vulgaris* betanin-rich dyes, which are used as additives in the food industry. In this case (Martinez et al., 2015), the effect of the dye on the edema induced by the injection of carrageenin into the mice leg was tested. Carrageenin is a compound widely used in anti-inflammatory studies due to its ability to trigger inflammatory processes (Vitalone, Torres Nieto de Mercau, Valdez, Davolio, & Mercau, 2000). Changes in the migration of leukocytes to the affected areas, superoxide anion levels, and cytokine production, were analyzed. After oral, subcutaneous, or intraperitoneal administration, betanin inhibited the growth of induced edema in mice. In addition, betanin significantly decreased the recruitment of leukocyte cells to the inflamed area. The results also showed a reduction in carrageenin-induced superoxide anion levels. In terms of cytokine production, levels of tumor necrosis factor-alpha (TNF-α) and interleukin 1-β (IL-1β), both stimulators of the inflammatory response (Landry & Oliver, 2001), were reduced. In addition, levels of IL-10, a cytokine with an anti-inflammatory activity that acts by inhibiting the production of pro-inflammatory cytokines (Mocellin, Panelli, Wang, Nagorsen, & Marincola, 2003), increased considerably. These results indicated that betalains show an important anti-inflammatory capacity, so the authors supported the use of these types of compounds to treat inflammatory disorders. From the molecular point of view, an additional underlying mechanism that could participate in the observed anti-inflammatory effects may be the demonstrated *in vitro* inhibition that betalains exert on the enzymes lipoxygenase and cyclooxygenase, involved in the formation of the inflammation-mediating compounds leukotrienes and prostaglandins (Reddy, Alexander-Lindo, & Nair, 2005; Vidal, López-Nicolás, Gandía--Herrero, & García-Carmona, 2014).

In addition, there are evidences on the role of betalains as autophagy stimulating agents as revealed by a study on mice with colitis (Macias-Ceja et al., 2017). Murine colitis was induced by intrarectal injection of 2,4,6-trinitrobencenosulfonic acid (TNBS). This compound altered the autophagy mechanisms of the mucosa, causing intestinal damage. After colitis induction, one group of mice was treated with betanin only, while another group was treated with betanin and 3-methyladenine (3 MA), an inhibitory agent of autophagy (Seglen & Gordon, 1982). In betanin-treated mice, the stimulation of autophagy in the intestinal mucosa was observed, in addition to a decrease in the synthesis of pro-inflammatory cytokines. In mice treated with betanin and 3 MA, the protective effects of betanin were masked by the autophagy inhibitor activity of 3 MA, resulting in a weaker protective response to colitis. Therefore, this study highlights the stimulating activity of autophagy presented by some betalains, promoting a decrease in inflammation and

an improvement in colitis in mice.

Furthermore, although the source or purity of the pigments was not disclosed, a recent study uses betalains to decrease airway inflammation in mice with allergic asthma (Dai, Wang, & Wang, 2021). The pathology was induced by treatment with ovalbumin, and the administration of betalains was able to reduce the migration of inflammatory cells into the affected respiratory tissues. In addition, the treatment managed to reduce the concentration of pro-inflammatory cytokines and reduce pulmonary oxidative stress. Gene expression analyses were carried out and showed that the pigment was able to reduce the expression of TGF-β and Smad proteins, suggesting that the action on this signaling pathway was responsible for the protective effects shown.

4.1.5. Antilipidic effects

Betalain-containing extracts of *Hylocereus undatus* have proven able to reduce weight gain in obese mice (Song, Chu, Xu, Xu, & Zheng, 2016). Obesity in mice was induced by a lipid-rich diet. In addition to weight reduction, the results showed an improved insulin resistance in mice, a reduction in adipose hypertrophy tissues, and an improvement in liver steatosis. Hepatosteatosis is caused by the accumulation of lipids in liver cells. After treatment with betacyanins, gene expression in hepatocytes was analyzed, showing an increase in the expression of lipid metabolism-related genes. The authors suggested that the improvement produced by betacyanins may be associated with the activation of lipid oxidation, although the mechanism of action was not entirely clarified. In an additional study, Song, Chu, Yan, et al. (2016) determined whether the beneficial effects of betacyanins could be related to the regulation of the gut microbiota. The study reaffirmed that betacyanins from *Hylocereus* protect mice from dietary-induced obesity by associating this effect with a regulation of the microbiota. Specifically, betacyanins decreased the proportions of *Firmicutes* and increased the abundance of bacteria belonging to the *Bacteroidetes* and *Akkermansia* groups. These proportions of gut microbiota appear to be associated with the protective effects that betacyanins produce in obese mice. Betanin extracted from *Hylocereus ocamponis* has also shown a protective effect in mice with hepatosteatosis (Lugo-Radillo et al., 2020). Sick mice treated with betanin showed a reduction in the negative effects caused by the lipid diet. Specifically, a decrease in liver inflammation was observed, as well as a significant reduction in hepatocyte necrosis levels. With these results, the authors consider betanin to have promising capabilities to deal with liver diseases like hepatosteatosis.

More recently, Yahaghi, Yaghmaei, Hayati-Roodbari, Irani, and Ebrahim-Habibi (2020) attempted to find out the molecular mechanisms underlying betanin action on mice with hepatosteatosis. In this assay, the disease was induced through a hyperlipid diet, producing excessive fat, increased blood cholesterol and triglyceride levels, increased MDA concentration, and insulin resistance. In addition, the activity of antioxidant enzymes was significantly reduced. At genetic level, an increase in the expression of sterol regulatory element-binding protein (SREBP-1c), a transcription factor involved in fatty acid synthesis (Shimano et al., 1999), was observed along with a decrease in the expression of peroxisome proliferator-activated receptor (PPAR-α), a nuclear receptor involved in fatty acid catabolism (Kersten, 2014). All these changes were effectively attenuated after the intake of betanin. The authors suggested that the pigment acts by positively regulating PPAR-α and by negative regulation of SREBP-1c, modifying lipid homeostasis in mice with hepatosteatosis.

4.1.6. Anti-nociceptive properties

In a different field, as is the management of pain by using animal models, it has been shown that betalains from *B. vulgaris* are able to soothe pain and reduce hyperalgesia in mice (Martinez et al., 2020). Various pain tests were conducted to evaluate the behavior and pain response developed by the mice. One of these tests was performed by intraperitoneal injection of the highly irritating acetic acid. Administration of betalains intraperitoneally was able to decrease abdominal contortions caused by acetic acid. In addition, a subplantation irritation test was performed by formalin injection. In this case, intraperitoneal administration of betalains decreased pain-associated behaviors. Finally, hyperalgesia in mice was also induced by the injection of carrageenin. Administration of betalains by subcutaneous and intraperitoneal pathways significantly reduced the induced hyperalgesia. Molecular analyses showed a decrease in levels of superoxide anions and cytokines in the mice treated with betalains. In addition, there was a reduction in lipid peroxidation. Inhibition of the feeling of pain caused by betalains appears to be mediated by mechanisms intended to control cytokine levels and oxidative stress. Therefore, this study demonstrates *in vivo* a novel analgesic activity of *B. vulgaris* betalains in different murine models of pain.

In relation to the above, a recent research evaluates the antinociceptive effect of the administration of beet extracts to mice with neuropathic pain (Kwankaew et al., 2021). To do this, locomotion and coordination tests were performed, as well as immunostaining tests to evaluate the expression of medullary glial markers. The results obtained showed that the administration of the extract, both orally and intraperitoneally, was able to decrease hypersensitivity in animals. In addition, the treatment reduced microglial activation in the spinal cord, involved in the development of neuropathic pathology. The authors point out that this microglial inhibitory mechanism is responsible for the protective effects observed in the treated mice, although the development of new research is necessary to clarify this point.

4.2. Rat (*Rattus norvegicus*)

Early research on the effects of betalains in rats aimed to evaluate the absence of toxicity and mutagenesis of these compounds for their use in the food industry. Thus, the idea arose that synthetic food dyes could be replaced by natural betalain dyes (Reynoso, Giner, & de Mejia, 1999; Schwartz, von Elbe, Pariza, Goldsworthy, & Pitot, 1983; von Elbe & Schwartz, 1981). Some years later, researchers changed their perspective and began to study the health-promoting properties of betalains, using rats as an animal model.

4.2.1. Hepatoprotective effects

In 2005, a study revealed the liver-protective role of the pigments (Galati et al., 2005). *O. ficus-indica* fruit extracts were administered orally to rats before and after inducing liver lesions. The hepatotoxic agent carbon tetrachloride (CCl_4) was used to produce liver damage. *Opuntia* extracts had a protective and curative effect at the liver level when administered both before and after the treatment with CCl_4. The results showed an improvement in the damaged liver at histological and biochemical levels, revealing the hepatoprotective effects of *O. ficus-indica* extracts *in vivo*. Two subsequent studies with betalain-rich extracts of *A. spinosus* also demonstrated hepatoprotective activity (Zeashan, Amresh, Singh, & Rao, 2008, 2009). Liver damage was also caused by the hepatotoxic agent CCl_4. The results of the studies confirmed the hepatoprotective capacity of betalain-containing extracts against liver lesions caused by CCl_4 in rats. It was also proposed that the protective effect was due to the antioxidant properties of the pigments.

B. vulgaris extracts have also proven to be positive against liver damage in rats (Krajka-Kuźniak, Szaefer, Ignatowicz, Adamska, & Baer-Dubowska, 2012). The lesions were induced with *N*-nitrosodietilamine, a potent hepatocarcinogen. Intake of beet extracts was able to reduce DNA damage in liver cells, as well as to minimize the activity of enzyme biomarkers of liver damage. This study demonstrated the protective role of beet extracts against liver lesions induced in rats. In addition, the hepatoprotective effects of betalain-containing extracts included increased activity of enzymes involved in drug metabolism and other xenobiotics. Specifically, the administration of beet juice has managed to reduce the damage induced by 7,12-dimethylbenzo(*a*) antracene (DMBA) in the liver and mammary glands of this animal model (Szaefer, Krajka-Kuźniak, Ignatowicz, Adamska, &

Baer-Dubowska, 2014). In both tissues, beet juice was able to significantly increase the activity of phase II enzymes in drug metabolism. In this phase, enzymes that catalyze conjugation reactions with endogenous substances help to detoxify and excrete DMBA.

Related to the above-mentioned study, another work evaluated the hepatoprotective property of pure betanin in rats. Liver damage was induced by intraperitoneal injection of paraquat, an agent used as an herbicide with high toxicity (Han, Zhang, et al., 2014). The injection of paraquat caused liver damage, increased oxidative stress, and mitochondrial damage. Liver lesions were evidenced by an increase in blood liver enzyme levels, such as aspartate aminotransferase and alanine aminotransferase. Moreover, mitochondrial damage was manifested by altering the mitochondria membrane and decreasing cytochrome *c* activity. Treatment with betanin led to an attenuation of the alterations caused by paraquat. The authors indicated that this hepatoprotective effect was mainly due to the mitochondrial protection that betalains produce in hepatocytes.

In another study, Shaban et al. (2020) evaluated the hepatoprotective effect of *B. vulgaris* juice administered to lead-intoxicated rats. This element is mainly present in the environment as a result of human activity (burning fossil fuels, mining, etc.). Lead is highly toxic and is considered one of the leading causes of liver problems in humans. In addition to the study of the potential liver protector of juice, this research also included 2,3-dimercaptosuccinic acid, a drug used to alleviate lead poisoning. To study the protective effects against this toxic element, rats were treated with *B. vulgaris* juice. The results showed that the juice managed to decrease the accumulation of lead in the liver, in addition to attenuating the alterations caused by poisoning. A combined treatment, containing *B. vulgaris* juice and 2,3-dimercaptosuccinic acid, showed a synergistic effect that boosted the protective effects against lead-induced liver toxicity. Based on these results, the authors claimed that *B. vulgaris* juice is able to reduce lead levels and their negative effects on intoxicated rats. In addition, its combination with 2,3-dimercaptosuccinic acid enhances the protective effects, highlighting the promising role they are able to play against this type of pathologies.

A further study attempted to clarify the molecular mechanisms behind the hepatic protective effect of betanin administered orally to rats with cisplatin-induced hepatotoxicity (El Shaffei, Abdel-Latif, Farag, Schaalan, & Salama, 2021). This agent, used as an antitumor drug, causes oxidative damage in the DNA decreasing the activity of sirtuin 1, which is part of the SIRT1/PGC-1α signaling pathway, and whose alteration produces deficiencies in mitochondrial functioning. In this sense, it is known that the miRNA-34a is capable of interfering with the correct functioning of the route (Yamakuchi, 2012), which increases hepatic dysfunction. The results of the research showed how betanin is able to reduce the expression of miRNA-34a and, therefore, to minimize cisplatin-induced liver damage in animals.

4.2.2. Chemopreventive potential

Oral administration of *B. vulgaris* extracts to rats has also shown the remarkable chemopreventive ability of the betalains as described above in mice (Lechner et al., 2010). Esophageal tumors were induced by treatment with *N*-nitrosometilbencilamine (NMBA) and beet extracts were orally supplied in water. In those rats given the aqueous extract containing betalains there was a reduction of 45% in the number of esophageal tumors. In addition, immunohistochemistry techniques determined the effects that the extract produced on angiogenesis, inflammation, and cellular apoptosis in esophageal tissue. The results showed a decrease in angiogenesis and in inflammatory processes, while an increase in apoptosis was recorded. These effects, together with the reduction of cell proliferation, seemed to be responsible for the anticancer activity of *B. vulgaris* extracts and suggested that the chemopreventive capacity of betalains in rats is determined by their strong antioxidant activity, thus blocking the transduction of ROS-induced signals and preventing the stimulation of uncontrolled cell proliferation.

4.2.3. Anti-inflammatory properties

The anti-inflammatory properties of betalains have also been widely studied in rats. One of these studies uses carrageenin to induce pleurisy (pleura inflammation) in these animals and to examine the anti-inflammatory power of pure indicaxanthin from *O. ficus-indica* fruits to reverse the inflammation produced (Allegra et al., 2014). To this end, indicaxanthin was orally administered to rats that had been induced pleurisy by injecting carrageenin into the pleural cavity. To evaluate the function of indicaxanthin, inflammatory indicators such as nitric oxide (NO), prostaglandin E2, and cytokine levels were measured. In addition, the number of leukocytes recruited in the inflamed area was measured. Results showed that the treatment with indicaxanthin significantly reduced the production of NO, prostaglandin E2, and pro-inflammatory cytokines. In addition, the number of cells recruited in the pleura decreased by up to 95%. With these data, the authors suggested that indicaxanthin shows a high anti-inflammatory potential as it is the pigment-containing the potentially beneficial for health fruits of *Opuntia*. Some additional mechanisms responsible for the effects shown could be related to the strong *in vitro* inhibition of the expression of the intercellular adhesion molecule ICAM-1 exerted by indicaxanthin (Gentile, Tesoriere, Allegra, Livrea, & D'Alessio, 2004). A further investigation assessed the protective effects of betanin against kidney damage (Tan, Wang, Bai, Yang, & Han, 2015). Kidney injuries were induced by the intraperitoneal injection of paraquat. This toxic agent produced an increase in the level of renal proteins in the blood, oxidative stress, and an inflammatory reaction, evidenced by increased expression of the enzyme cyclooxygenase. Rats treated with betanin showed parameters closer to the normal ones: the markers of renal damage in the blood were reduced, oxidative stress decreased, and the expression of the enzyme cyclooxygenase was minimized. These results demonstrated the healing effect of betanin against kidney damage.

An additional study showed that betanin purely administered to rats also appears to be beneficial against lung injuries, such as interstitial pneumonia (Han, Ma, Zhang, Yang, & Tan, 2015). This pathology can be induced by intraperitoneal injection of paraquat. Rats treated with this toxic agent suffered many changes at histological and biochemical levels. Specifically, paraquat increased pulmonary permeability, leading to increased infiltration of leukocyte cells. In addition, there was an increase in the levels of MDA, the final product of lipid peroxidation, an increase of myeloperoxidase activity, and a reduction of antioxidant enzymes activity. Treatment with pure betanin was able to reduce inflammatory processes and oxidative stress, leading to the reversal of the above-mentioned parameters. In addition, betanin acted at the genetic level by activating the expression of antioxidant genes through the nuclear erythroid factor 2 (Nrf2) pathway. At the same time, treatment with betanin inactivated the pro-inflammatory pathway NF-kB, contributing to protection against the pathology. At this point, the authors recognized that the bioactive properties of betanin and its mechanism of action should be studied further.

Like mice, rats have been used as an animal model to analyze the effect of betalains in those affected by colitis. A recent study used *O. dillenii* fruit juice, rich in betanin, to address this aspect (Babitha, Bindu, Nageena, & Veerapur, 2019). Colitis was induced by the injection of acetic acid intrarectally. After oral administration of the juice, a significant reduction in myeloperoxidase and MDA levels and an increase of glutathione were observed. These results showed that the positive effect of *O. dillenii* juice is due to its antioxidant properties. However, without pure betanin treatment, the observed effects may result from additional bioactive compounds present in the extracts used, thus limiting the conclusions.

Betalains may also be effective against rat-induced gastric ulcers. Specifically, fruit juice from *O. ficus-indica* has been used to examine this protective function by itself and administrated along with maltodextrin (Kim et al., 2012). Both treatments improved gastric lesions by maintaining mucosal integrity. The results indicated that the main effect of *O. ficus-indica* fruit juice was the decrease of myeloperoxidase activity, a

ROS-generating enzyme.

The increased interest in betalains has also led to the analysis of their health-promoting capacities in rats with induced intestinal ischemia-reperfusion (Toth et al., 2019). Betanin was intraperitoneally supplied and immunohistochemical and tissue morphology analyses were performed to analyze its effects. The results showed an improvement in jejunal tissue after the administration of betanin. In particular, a significant increase in the height of intestinal mucosa microvilli was observed, as well as a reduction in mast cell levels. The administration of betanin also had positive effects on pulmonary parenchyma. Specifically, it decreased pulmonary oxidative and inflammatory stress manifested in a decrease in myeloperoxidase activity and leukocyte infiltration in the pulmonary parenchyma. In addition, after the administration of betanin, pulmonary morphometric analyses showed an increase of up to 20% in the alveolar surface area. Therefore, the healing effects of the administration of betanin at the intestinal and pulmonary levels are highlighted. All these results show the protective role that betanin is able to exert against these pathologies.

Tural et al. (2020) also used the same pathology to examine the beneficial effects of betalains, but in this case, the ischemia-reperfusion injury occurred at the aorta level and the administration of betanin occurred intraperitoneally before inducing the injury. Cardiac and pulmonary studies were conducted to analyze the effects of betanin. In those rats treated with the pigment, reductions in MDA and myeloperoxidase levels were detected in both tissues, thus alleviating injury-induced oxidative stress. More recently, the same research group used rats with ischemia-reperfusion to evaluate the protective effects of betanin treatment on the spinal cord (Tural, Ozden, Bilgi, Kubat, et al., 2021). Again, the pigment was administered intraperitoneally, and biochemical analyses related to antioxidant capacity were carried out. The results obtained showed a significant reduction in myeloperoxidase activity and MDA levels, adding new evidence that points to betanin as a protective agent against this type of pathology.

4.2.4. Cardioprotective effects

The cardioprotective effects of beetroot chips administered to models of dyslipidemia rats have also been studied. Dyslipidemia is characterized by increased plasma concentration of cholesterol and other lipids and was induced in rats through a high-fat diet. Blood cholesterol and triglycerides levels decreased following oral administration of beetroot chips, protecting rats from metabolic changes induced by dyslipidemic disorder (Wroblewska, Juskiewicz, & Wiczkowski, 2011). A more recent study assessed protection against induced myocardial lesions in rats. Researchers used betalain-rich beetroot juice to analyze its possible beneficial effects (Raish et al., 2019). The induction of myocardial damage in the animal model was achieved by subcutaneous administration of isoproterenol. This procedure caused myocardial ischemia in animals, resulting in increased heart weight, higher levels of blood heart damage indicators, and modifications in myocardial histology. The main effect that oral administration of beet juice promoted was the decrease in oxidative stress, due to an increased activity of cardiac antioxidant enzymes. Isoproterenol also increased the levels of pro-inflammatory cytokines, myeloperoxidase activity and promoted the activation of the transcriptional factor NF-kB. This factor is involved in the synthesis of proteins in response to oxidative stress and inflammation. Treatment with beetroot juice managed to significantly reduce the above parameters. These results showed the cardioprotective potential of the extract in rats and suggested that the mechanism of action of betalains is based on the reduction of inflammatory processes and oxidative stress. More recently, the first study using rats with heart failure to evaluate the cardioprotective role of betalains has been conducted, (Gao, Liang, Tian, Ma, & Sun, 2021). Pathology was induced by the administration of isoproterenol and, although the purity and nature of the pigments were not revealed, betalains were administered orally. To study the pigments effects, the activity of antioxidant enzymes was measured, and inflammatory markers and heart failure were monitored. The results showed an increase in the activity of antioxidant enzymes and a significant reduction in the levels of pro-inflammatory cytokines in those animals treated with betalains. In addition, a significant decrease in the expression of miR-423 and miR-27, involved in heart failure, was observed. This fact suggests that the cardioprotective capacity of the betalains used may be due, in addition to their antioxidant and anti-inflammatory potential, to modulations of microRNA activity.

4.2.5. Effect on blood cells

O. ficus-indica fruit extracts also have a protective effect on erythrocitary damage induced by oral administration of ethanol in rats (Alimi, Hfaeidh, Bouoni, Sakly, & Ben Rhouma, 2012). Treatment with ethanol triggered numerous negative effects on the red blood cells of rats. These include increasing the concentration of MDA and oxidized proteins, as well as reducing glutathione amounts. Ethanol also increased hemolysis levels and caused alterations in the morphology of these cells. Oral administration of *O. ficus-indica* extracts was able to counteract alterations induced by ethanol. The observed healing effect was dependent on the dose of extract supplied. The authors suggested that this protection was due to the presence of antioxidant compounds in the fruits of *O. ficus-indica*.

Another effect attributed to extracts rich in betalains is the induction of hematopoiesis (Pandey, Ganeshpurkar, Bansal, & Dubey, 2016). To study this effect, phenylhydrazine was injected intraperitoneally to induce anemia in rats. The extracts of *A. cruentus* were then administered orally. The results showed a recovery in blood cell levels, as well as an increase in hematocrit. The authors concluded that the extracts in this plant have hematopoietic capabilities but indicated that it is difficult to assert which compounds are responsible for the effect in the complex extract. Further analyses with purified components were suggested to study the described hematopoiesis-promoting activity.

4.2.6. Antidiabetic effect

The effects of betalains on hyperglycemia in diabetic rats have also been studied. Diabetes was induced in rats by intraperitoneal injection of STZ and nicotinamide (Dhananjayan, Kathiroli, Subramani, & Veerasamy, 2017). Following oral treatment with betanin, biochemical and histological analyses of the liver and pancreas were performed. The results showed how the administration of betanin was able to restore blood glucose and insulin levels to normal ranges. Biochemical analysis showed that this effect is a consequence of the activation induced by betanin on liver glycolytic enzymes. In addition, there was a significant reduction in the activity of gluconeogenic enzymes. Regarding the pancreas, immunohistochemical analyses showed that betanin was able to increase the number of insulin-producing cells. However, the authors claim that orally administered betanin is able to regulate carbohydrate metabolism, producing protective effects on diabetes induced in rats. A similar study conducted by Indumathi, Sujithra, Srinivasan, and Vinothkumar (2018) reaffirmed the antihyperglycemic capabilities of betanin. Diabetes induction was performed by intraperitoneal injections of the same drugs (STZ and nicotinamide). This pathology caused an increase in blood glucose and resulted in reduced blood insulin levels. Treatment with betanin managed to restore normal blood glucose and insulin values. These two studies reveal the more than possible antihyperglycemic effect of betanin.

The protective effect of betalains against diabetic heart fibrosis has also been demonstrated in rats. Han, Tan, Wang, Yang, and Tan (2015) attempted to assess whether pure betanin improves this pathology in rats in which cardiac fibrosis was induced by the administration of fructose. In those rats treated only with fructose, an increase in blood levels of glucose, insulin, and glycosylated hemoglobin was observed. Besides, there was a significant accumulation of collagen in the ventricle of these rats and an increase in the activity of proteins related to the synthesis of connective tissue. It is therefore claimed that the administration of fructose produces diabetic heart fibrosis in rats. After treatment with betanin, the above-mentioned parameters were reversed in a

very effective way, thus revealing the protective effect of this betalain against diabetic heart fibrosis. The authors pointed out that this protective function was due to betanin being an antifibrotic compound and protein glycosylation inhibitor. A later study revealed the protective effect caused by pure betanin from *O. elatior* fruits against diabetic fibrosis in the kidney (Sutariya & Saraf, 2017). Diabetes was induced by intraperitoneal injection of streptozotocin (STZ), a drug commonly used to cause this pathology in rats. Treatment with pure betanin was able to mitigate the alteration of the parameters of renal damage induced by STZ. Specifically, betanin lowered blood glucose and proteinuria levels and increased the activity of renal antioxidant enzymes. In addition, after histological analysis of renal tissue, a significant decrease in fibrosis was observed. The molecular mechanism of action behind these effects is not entirely clear. However, the results show how the pigment was able to downregulate the expression of mRNA and the proteins TGF-β, type IV collagen, a marker of myofibroblasts alpha-smooth muscle actin (α-SMA) and E-cadherin in kidney tissue. Therefore, the protective effect of betanin against this pathology seems to occur through the modulation of the TGF-β signaling pathway.

4.2.7. Neurophysiological effects

The neurophysiological potential of indicaxanthin extracted from *O. ficus-indica* has also been highlighted (Allegra et al., 2015). The pigment was orally administered to rats and studies were conducted to locate the compound within the animal's body. Surprisingly, 1 h after intake, indicaxanthin appeared in the brains of the rats. The highest amount of this pigment in the brain was reached 2.5 h after intake. This shows that indicaxanthin is able to cross the blood-brain barrier and accumulate in the brain. In addition, using microiontophoretic techniques, very small amounts of indicaxanthin were released into neurons in the cerebral hippocampus. In this way, the effect that indicaxanthin causes on neural activation could be studied. Indicaxanthin was observed to cause a decrease in neural discharge. Specifically, the compound produced an inhibition of glutamate-induced neuronal arousal. With these results, the authors demonstrated that indicaxanthin administered in rats can lead to modifications in neural activation, which warrants further research in this field.

A study recently conducted with rats evaluates the protective capacity of betanin against alterations caused by excessive consumption of two well-known anti-inflammatory drugs: acetaminophen and diclofenac (Motawi, Ahmed, El-Boghdady, Metwally, & Nasr, 2019). Tests were performed administering the drugs separately, as well as supplemented with betanin. Levels of thyroid hormones and sex hormones were measured as was the release of neurotransmitters. Different markers of oxidative stress were also analyzed. In those rats treated only with the drugs, a significant decrease in hormone and neurotransmitter levels was observed as well as an increase in oxidative stress markers. However, those rats that were supplemented with betanin showed an improvement in many of the alterations produced by the drugs. These results demonstrate the corrective role of betanin against endocrine and oxidative disorders caused by the overconsumption of these drugs.

4.2.8. Neuroprotective effects

The neuroprotective effects of betalains have also been studied in rats. A cognitive improvement has been observed in rats with Alzheimer's disease after treatment with a diet based on beetroot extract (Olasehinde, Oyeleye, Ibeji, & Oboh, 2020). The disease was induced by the injection of scopolamine. To evaluate the neuroprotective capacity of the extract orally administered, different behavioral tests were carried out, as well as the analysis of the activity of purinergic enzymes, cholinesterases, and antioxidant enzymes. The results obtained showed an improvement in cognitive abilities in the treated animals, as well as a decrease in the enzymatic activity of enzymes involved in pathological development. Similarly, beetroot extract induced an increase in the activity of antioxidant enzymes. With these results, the authors point out that the neuroprotective power of betalain-rich beets in this animal model is due to the modulation of the aforementioned families of enzymes. In this sense, a recent research used betalains whose origin was not disclosed to protect rats from neurodegeneration caused by Alzheimer's disease induced by $AlCl_3$ (Shunan, Yu, Guan, & Zhou, 2021). To do this, the behavior of the animals was analyzed together with biochemical parameters related to oxidative stress. The neuroprotective effect of orally administered betalains resulted in an improvement of cognitive processes and learning. In addition, the treatment reduced MDA levels and increased the activity of antioxidant enzymes such as superoxide dismutase and catalase. The authors conducted gene expression studies and observed a significant decrease in NF-kB activation after treatment, so they suggest that the mechanism of action of the betalains used is based on the modulation of this signaling pathway.

An additional study conducted in 2021 shows how oral treatment with beet extract is able to induce a protective effect against neurotoxicity caused by lead administration in rats (Shaban, Abd El-Kader, Mogahed, El-Kersh, & Habashy, 2021). In this case, parameters related to antioxidant, anti-inflammatory, and anti-neurotoxic activity were evaluated. Rats treated with beetroot extract showed a lower concentration of lead in plasma and brain tissue. The authors suggest that this decrease is due to the metal sequestration capacity described for betalains (Guerrero-Rubio, Martínez-Zapata, Henarejos-Escudero, García--Carmona, & Gandía-Herrero, 2020), among other compounds present in the extracts. In addition, the treatment managed to reduce inflammation and oxidative stress in the animals. The authors suggest that antioxidant and anti-inflammatory capacities play a major role in the observed neuroprotection.

4.2.9. Antioxidant activity

A study with rats highlighted the antioxidant effects of betalains *in vivo* (Da Silva et al., 2019). To induce oxidative stress, rats underwent an over-fat diet and pure betanin was orally administered. For the study of the antioxidant effect of betanin, analysis of biochemical and enzymatic blood markers was carried out. In addition, liver levels of lipid peroxidation, as well as changes at the enzymatic level, were analyzed. The excessive fat in the diet induced an increase in plasma concentration of glucose, insulin, and markers of liver damage, such as aspartate aminotransferase and alanine aminotransferase. In addition, oxidative stress was increased in rats, evidenced by a significant decrease in the activity of antioxidant enzymes such as glutathione peroxidase, catalase, and superoxide dismutase. The diet also increased the lipid peroxidation levels. Under these altered conditions, the administration of betanin had significant protective effects. The pigment enabled the recovery of the parameters to normal values and considerably decreased dietary-induced oxidative stress in rats. The antioxidant and hepatoprotective capabilities that betanin is able to offer *in vivo* were unambiguously demonstrated with this animal model.

A study conducted in 2021 also shows how betanin induces protective effects on oxidative stress in rats with femoral artery vasospasm (Tural, Ozden, Bilgi, Merhan, et al., 2021). The animals were treated orally with betanin and parameters indicative of oxidative stress such as MDA and nitric oxide plasma levels were studied, showing a reduction to normal values. In addition, for the evaluation of the vasodilatory effect of the pigment, the diameter of the lumen and the thickness of the wall of the femoral artery of the animals were analyzed. The results showed an increase in lumen diameter and a decrease in arterial wall thickness in rats treated with betanin. Thus, the pathology was attenuated by betalains treatment in rats, apparently thanks to the high antioxidant power of the pigments.

4.3. Carp (*Cyprinus carpio*)

Few studies have used fish as an animal model to examine the health-promoting properties of betalains. Han, Gao, Yang, Wang, and Tan (2014) showed the protective capabilities of pure betanin against liver damage in common carp (*Cyprinus carpio*). The fish were fed with

betanin before the liver injury was promoted by injecting CCl_4 intraperitoneally. Once the damage was performed, biochemical and histological analyses were carried out to detect any change produced. Control groups, treated with the harmful agent only, showed an increased activity of hepatic CYP2E1, a member enzyme of the cytochrome P450 complex involved in phase I metabolism. Oxidation, reduction, and hydrolysis reactions are carried out in this phase in an attempt to transform the harmful agent that may result in the inactivation of the drug or in the formation of toxic by-products. In addition to the increased CYP2E1 activity, higher blood levels of aspartate aminotransferase and alanine aminotransferase were detected in the control groups - evidence of the liver damage caused by CCl_4. In betanin-treated fish, significant recovery was observed in many of the altered parameters. Specifically, betanin significantly reduced CYP2E1 activity and the above-mentioned blood markers of liver damage. Lower levels of hepatic oxidative stress were detected in fish treated with betanin, by decreasing the concentration of MDA and by increasing the activity of antioxidant enzymes, such as superoxide dismutase and catalase. Based on these results, the authors stated that betanin was able to protect carp from induced liver damage and suggested that this was a direct consequence of the great antioxidant power of these pigments.

4.4. Zebrafish (Danio renio)

A recent study has used pitaya (*H. polyrhizus*) to examine the anxiolytic effects that it induces in adult specimens of zebrafish (*Danio rerio*) (Lira et al., 2020). This plant belongs to the family Cactaceae and its fruit, commonly named pitaya or dragon fruit, is characterized by a high content of betalains and phenolic compounds (Fathordoobady, Mirhosseini, Selamat, & Manap, 2016; Luo, Cai, Peng, Liu, & Yang, 2014). To develop the trials, the pulp and the fruit peel were used separately to detect differences between the effects produced by both parts of the fruit. To measure anxiolytic activity, the fish were subjected to a standard light/dark test. This test is often used to measure anxiety in rodents and is based on the rejection that these animals show towards brightly lit areas. In this case, the test was performed using an aquarium that contained a light area and a dark one. The fish were placed in the illuminated area of the aquarium and, to study the anxiolytic effect, the percentage of treated fish remaining in that area with respect to the group of untreated fish was measured. Between 82.8% and 85.2% of fish orally treated with pitaya pulp remained in the illuminated area, compared to 3.6% of untreated fish. Similarly, between 78.1% and 95.6% of fish treated with freeze-dried pitaya peel remained in the illuminated area. Thus, both parts of the fruit showed similar effects on the permanence of fish in the illuminated area. Experiments using diazepam as anxiolytic showed similar permanence percentages. These findings showed that pitaya is capable to produce diazepam-like anxiolytic effects in zebrafish. Given these results, the authors suggested that pitaya peel, which is discarded by the food industry, should be exploited as a source of betalain-rich extracts due to its anxiolytic properties. In addition, a combined treatment with the drug flumazenil was realized prior to pitaya administration in the same research showing a lower anxiolytic effect of the pulp and pitaya peel, compared to experiments without flumazenil. This drug is an inhibitor of benzodiazepines that works by blocking receptors of gamma-aminobutyric acid (GABA). Based on this fact, the authors suggested that the pulp and peel of pitaya are anxiolytic agents that exert their action through GABA.

4.5. Caenorhabditis elegans

The bioactive properties of betalains have also been tested in worms by using the model organism *C. elegans*. In a preliminary study, 4 pure betalains were tested for antioxidant and health-promoting capabilities (Guerrero-Rubio, Hernández-García, García-Carmona, & Gandía-Herrero, 2019). The study sought to obtain results about the effects that indicaxanthin, phenylethylamine-betaxanthin, betanin, and indoline-betacyanin were able to exert on the worms' welfare. Two different strains of *C. elegans* were used for testing. For the study of oxidative stress, the mutant strain TJ375 was used because it contains the green fluorescence protein (GFP) coupled to the promoter of the *hsp-16.2* gene. In situations of oxidative stress, HSPs increase their expression, resulting in an increase of the fluorescence emitted for TJ375 due to the expression of GFP. In this study, the nematodes of the fluorescent lineage were fed with individual betalains. Then, worms were exposed to the chemical agent juglone to induce oxidative stress to study the protective and antioxidant capacity of each individual pigment *in vivo*. The results showed that all betalains used were able to reduce the intensity of fluorescence as a result of a decrease in the expression of GFP and therefore a decrease in the oxidative stress. The highest decrease was obtained in worms pre-treated with indicaxanthin, the major compound of the *O. ficus-indica* fruits (Castellanos-Santiago & Yahia, 2008).

The wild-type N2 strain was also employed for the measurement of its lifespan. The mean lifespan is a parameter widely used to analyze the effect of bioactive compounds in these nematodes. For this purpose, the mean lifespan of worms fed with the above-mentioned individual betalains was analyzed in the Lifespan Machine, a device for the automatic control of the organism *C. elegans* (Guerrero-Rubio, Hernández-García et al., 2019; Stroustrup et al., 2013). The results showed that all betalains significantly increased the lifespan of nematodes, with indicaxanthin being the most potent. It is noteworthy that the concentrations of tested betalains are much lower than those used for other antioxidant molecules (Guerrero-Rubio, Hernández-García et al., 2019), showing the great antioxidant and health-promoting capacity of betalains. Given the interesting effect of the four betalains analyzed, the study was expanded to seventeen pure betalains, thus showing the extraordinary potential of these molecules in the increase of the *C. elegans* lifespan and in the protection against oxidative stress (Guerrero-Rubio, Hernández-García et al., 2020). Microarray analyses performed with worms fed with pure molecules showed that the health-promoting effects of betalains in *C. elegans* are also due to the modulation of the expression of genes involved in longevity pathways. Betalains are able to modulate the expression of *hsp* genes, small peptides with chaperone-like activity, which maintain proteome stability. Altered patterns in the expression of sHSPs have been reported in diseases like Alzheimer's, Parkinson's, or cancer (Bakthisaran, Tangirala, & Rao, 2015) so betalains may be of interest in the fight against these diseases.

C. elegans as an animal model has also been used to test the antitumoral effect of betalains (Henarejos-Escudero, Hernández-García, Guerrero-Rubio, García-Carmona, & Gandía-Herrero, 2020). Betalain-rich extracts and pure compounds were applied to a tumor-induced mutant strain of *C. elegans*. To study the effect of betalains on tumors, pigments were administered to the worms and tumor sizes were measured. Both, extracts and individual betalains reduced the size of the tumors, with tryptophan-betaxanthin proving to be the most active compound, reducing the tumor size by up to 56.4% and increasing the mean lifespan of the worms by up to 9.3%. In addition, intracellular location studies showed that the mechanism responsible for the observed effects was the activation of DAF-16, a transcription factor involved in resistance to oxidative stress. Betalains antioxidant capabilities, with their ability to regulate factors involved in longevity pathways, appear to be primarily responsible for the health-promoting effect of betalains.

5. Health-promotion in humans

Even though betalains are safe molecules for their consumption (Khan et al., 2011), research on their biological activity carried out in humans is scarce. However, there are few studies that highlight the health-promoting activities of betalains and confirm the expectations raised by the results obtained in animal models. Published experiments

have mainly been focused on the protective effects that betalains have on cardiovascular diseases and on the beneficial effects related to physical activity and sport. In this case, the administration of betalains results in a decrease in muscle damage and in increased athletic performance.

Regarding the protective effect of betalains against cardiovascular diseases, Hobbs, Kaffa, George, Methven, and Lovegrove (2012) showed that the administration of juice and bread enriched with beetroot to normotensive males resulted in a reduction in blood pressure. Consumption of both types of food managed to significantly reduce systolic and diastolic blood pressure. However, the authors suggested that the hypotensive effect observed after administration of tested foods may be due to the high nitrate content of beetroot, since, after digestive processes, this compound can be transformed into nitric oxide (NO), a molecule with vasodilator properties. However, the hypotensive effect observed may be partially due to the inhibition of angiotensin-converting enzyme I (ACE I), involved in vasoconstriction by converting angiotensin I to angiotensin II, as its activity is reduced in the presence of *B. vulgaris* betalains (Sawicki et al., 2019).

Rahimi, Mesbah-Namin, Ostadrahimi, Abedimanesh, et al. (2019) also highlighted these protective effects in males with coronary artery disease. Individuals were fed with supplements rich in *O. stricta* betacyanins and *B. vulgaris* betanin. After two weeks of treatment, blood and urine samples were collected from each patient and the results showed that betalain-rich supplements were able to reduce blood levels of glucose, homocysteine, cholesterol, triglycerides, and low-density lipoproteins (LDL), components that increase the risk of serious cardiovascular disease. In addition, intake of the supplements was also able to reduce the systolic and diastolic blood pressure of the patients. This study, therefore, shows that betalains can have protective effects in patients with cardiovascular disease, decreasing the risk of atheroma formation. The same group of researchers sought the molecular mechanisms underlying this protective effect of betalain-rich supplements (Rahimi, Mesbah-Namin, Ostadrahimi, Separham, et al., 2019). To do this, 24 men with coronary artery disease received a supplement rich in beet betalains and a supplement rich in *O. stricta* betacyanins daily for two weeks. Gene expression analyses showed how betalain supplementation induced a significant decrease in the expression of oxidized lectin-type LDL receptor 1 (LOX1), whose elevated expression is associated with human atherosclerotic lesions. In addition, an increase in the expression of SIRT1 and blood levels of the protein was observed, which is related to protection against cardiovascular diseases. Finally, the study showed a drop in levels of C-reactive protein (CRP), used as a marker of inflammation. Thus, betalains seemed to be able to modulate the expression of the described genes and to protect patients with coronary artery disease.

Regarding the effect of betalains on physical activity, a study conducted by Clifford, Bell, West, Howatson, and Stevenson (2016) showed that beet juice supplementation is capable of reducing muscle damage following eccentric exercises. In this case, male patients took different doses of beetroot juice immediately after performing 100-drop jumps. In addition, supplements were also taken 24 and 48 h after doing exercise. Following the measurement of different rates of muscle damage, the results obtained showed a significant reduction in muscle pain in those individuals who had ingested the beet juices, with respect to trials without supplementation. A year later, the same researchers conducted a similar study comparing the effects of beet juice supplementation with the consumption of nitrate-rich beverages (Clifford, Howatson, West, & Stevenson, 2017). The results reaffirmed the protective effects of beet juice against muscle damage, as well as demonstrated that these effects are more beneficial than those obtained from nitrate-rich beverages. The protective effects of betalains were also measured after a marathon (Clifford, Allerton, et al., 2017). Specifically, 34 professional runners consumed beetroot juice for the next 3 days after this sporting event. In addition to the muscle damage rates measured in the previous study, blood tests were performed to reveal information on markers of inflammation, such as blood levels of cytokines, leukocytes, creatine kinase (CK), CRP, and aspartate aminotransferase. The measurement of the different markers was made before the marathon, immediately after, and 2 days after the marathon. However, the results showed that beet juice did not significantly reduce muscle damage or inflammation after the marathon, with respect to tests without supplementation. The authors suggested that this result may be due to the fact that the study was conducted with expert runners, and the muscle damage was virtually non-existent after the marathon. It was concluded that beetroot juice taken after a marathon has no protective effects against muscle damage, at least in professional runners. However, the authors call for future research to be conducted with prior treatments, rather than with post-marathon treatments.

In relation to the above studies, there is also research that directly evaluates the improvement in the sport performance of athletes after the intake of supplements rich in betalains. Special reference should be made to a study conducted by Montenegro et al. (2017) in which 22 triathletes had supplements enriched in beetroot during the seven days prior to the physical test, consisting of 40 min of cycling prior to a 10 km race. The following day, the athletes ran a distance of 5 km so the recovery could be analyzed. As a control, the same athletes completed the same physical tests the week before, but without taking the betalain-rich supplementation. To perform the analyses, the times when athletes completed both races, the heart rate, and blood levels of CK were measured. The results showed that, in trials with supplements rich in betalains, athletes completed the 10 km race in less time. However, despite being faster, the heart rate did not differ significantly from the non-supplementation trial, suggesting that the supplement improved athletes' performance. In addition, the subjects who received the supplement improved their resilience as they needed less time to complete the 5 km race. CK levels also decreased, resulting in reduced muscle damage. These results support those obtained in a similar study by Van Hoorebeke, Trias, Davis, Lozada, and Casazza (2016) to demonstrate the role of betalain-rich concentrates in improving athletic performance, in this case in competitive runners. The experiments conducted were similar and led to the same results. In this case, to assess muscle damage the enzyme lactate dehydrogenase was analyzed as a marker, with significant decreases in its activity observed in those runners who took the supplement. Beetroot betalain supplementation has also been shown to improve sport performance in cyclists following a 30-min physical test (Mumford et al., 2018).

Recently, a study conducted by Aliahmadi et al. (2021) demonstrated that the intake of red beets improves glucose metabolism and has beneficial effects on cognitive function in people with type 2 diabetes. To this end, 44 patients were selected and consumed 100 g of red beets per day for eight weeks. After the measurement of markers of carbohydrate metabolism and cognitive function parameters, the results obtained showed a significant improvement in both, demonstrating the health-promoting capacity of the consumption of beet rich in betalains on patients with type 2 diabetes. In short, the reported results in these types of studies have shown that supplements rich in betalains are able to significantly improve sport performance and recovery in healthy humans, which may boost conducting additional future research. However, the information obtained showed that young male subjects were the main target to conduct experiments (Table 1). This limits the applicability of the results since they may not be considering gender and age variability. For future research, it would be interesting to conduct studies with wider age ranges, or by using elderly groups that complement the results of the studies already conducted. This, coupled with a greater inclusion of women in the experiments, would clarify whether age and sex are influential factors in the body's responses to the administration of betalains. Even so, the results in humans, although scarce, support the evaluation of bioactive properties performed with animal models and anticipate a renewed interest of the food and pharmaceutical industries in betalains.

Table 1
Number of male and female individuals used for different studies on the bioactivity of betalains in humans. The average age of the total number of subjects in each study is also shown. As noted, the use of male individuals predominates. The young age of the volunteers is also highlighted.

References	Males ♂	Females ♀	Age in years (mean ± standard deviation)
Hobbs et al. (2012)	32	0	28 ± 2.5
Clifford et al. (2016)	30	0	21.5 ± 4.5
Van Hoorebeke et al. (2016)	13	0	25.3 ± 5.4
Clifford, Howatson, et al. (2017)	30	0	22.15 ± 2.8
Clifford, Allerton, et al. (2017)	21	13	40.5 ± 11
Montenegro et al. (2017)	9	13	38 ± 11
Mumford et al. (2018)	28	0	29 ± 10
Rahimi, Mesbah-Namin, Ostadrahimi, Abedimanesh, et al. (2019)	48	0	49.47 ± 2.05
Rahimi, Mesbah-Namin, Ostadrahimi, Separham, et al. (2019)	24	0	50.34 ± 2.9
Aliahmadi et al. (2021)	No data	No data	57 ± 4.5

6. *Beta vulgaris* betanin, the most tested betalain *in vivo*

The present work summarizes decades of work on the characterization of bioactivities of betalains *in vivo*. To the best of our knowledge, all the available references have been considered and altogether show the biological activity of betalains *in vivo* and how they are able to induce improvements in animal physiological functions. Limitations are mainly due to the lack of variations in the exact type of betalain used. It is noteworthy that in 63% of all collected trials *B. vulgaris* was used as a source of pigments to study their properties (Fig. 2A). Besides, the animals chosen for the majority of the *in vivo* trials conducted were mice and rats (Fig. 2B), classical animal models widely used by the scientific community due to their genetic proximity to humans (90% and 80% homology in rat and mouse respectively) (Gibbs et al., 2004; Waterston et al., 2002).

Thus, multiple health-promoting effects have been recorded after the administration of betalains extracted from *B. vulgaris*, as Table 2 summarizes, with betanin being the main compound tested. Table 2 does not cite the properties described that were a direct consequence of those already reported, or when the mechanism of action is not entirely clear. For example, the radioprotective activity of beetroot betalains in mice is assumed to be a direct consequence of their antioxidant properties (Lu et al., 2009). Similarly, the reduction of hyperalgesia in mice after beet treatment may also be caused by its anti-inflammatory and antioxidant properties (Martinez et al., 2020).

Most of the biological effects attributed to betalains, all of which are treated in this work, are not independent. The connection between the different mechanisms of action of betalains gives rise to the numerous health-promoting properties based on common characteristics. This fact is explained very well by analyzing one of the most studied bioactivities of betalains: the antitumor capacity of *B. vulgaris* betanin (Lechner et al., 2010; Zhang et al., 2013). This property is the result of numerous molecular mechanisms triggered by this pigment (Fig. 3). The bibliography suggests that the antitumor capacity does indeed derive from the anti-inflammatory and antioxidant properties of this betalain. The anti-inflammatory power of betanin is able to reduce the production of pro-inflammatory cytokines that increase the migration of leukocytes to the inflamed area (tumor) (Martinez et al., 2015; Tan et al., 2015). In addition, the antioxidant properties of the pigment increase the activity of antioxidant enzymes that neutralize ROS produced by immune cells (Da Silva et al., 2019; Han, Gao, Yang, Wang, & Tan, 2014; Han, Ma, et al., 2015; Lira et al., 2020; Vulić et al., 2013). Finally, the antitumor power of the compound causes the formation of blood vessels (angiogenesis) around the tumor area, while increasing levels of apoptosis in

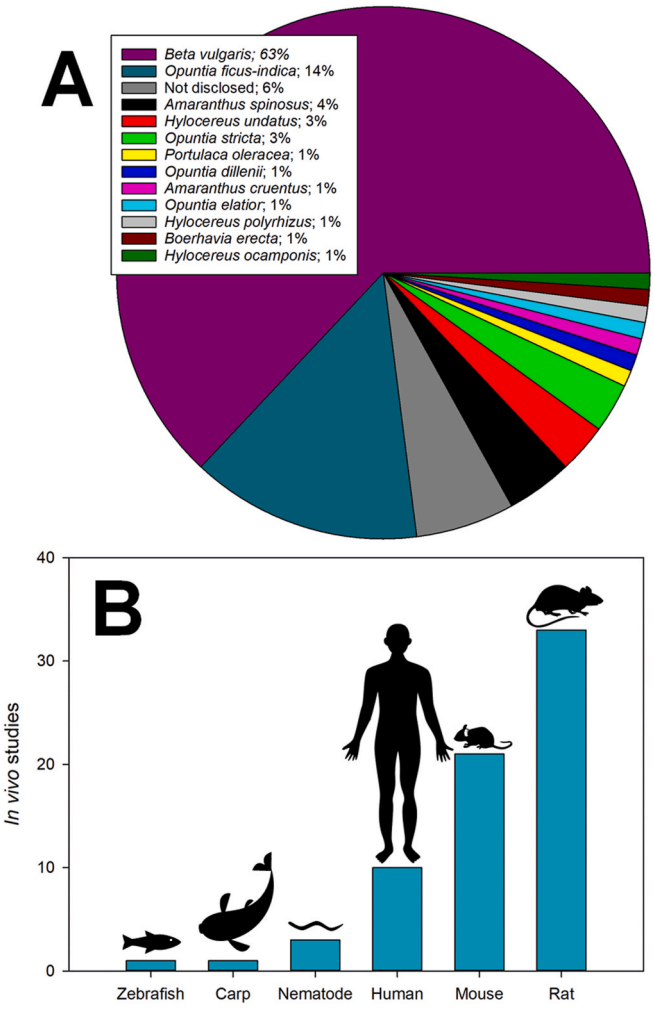

Fig. 2. Betalain sources and animal systems most used in the *in vivo* studies. (A) Plants used as a source of betalains with respect to the total studies analyzed. *Beta vulgaris* is the most common source of pigments used (63%), followed by *Opuntia ficus-indica* (14%). (B) *In vivo* research conducted with each animal model considered.

tumor cells (Lechner et al., 2010; Zhang et al., 2013). In this sense, it has been pointed out that betalains act by regulating the expression of some genes related to cell development and apoptosis (Zou et al., 2005). The joint action of all these mechanisms described in the literature and summarized in Fig. 3 seems to be responsible for the antitumor activity of betanin. However, the mechanism of action of betalains against cancer may not be clear. Although most of the authors directly relate the antioxidant activity of betalains to their antitumor property, it is interesting to note that dopaxanthin, the betalain with the greatest antiradical capacity, did not show an inhibitory effect on tumor development in *C. elegans*, which may question the direct link between both bioactive properties (Henarejos-Escudero et al., 2020). In fact, in that study, the molecules that showed the greatest antioxidant effect had little effect on the reduction of tumors. In addition, tryptophan-betaxanthin, with a weak antiradical activity, was the molecule with the best results in the inhibition of tumor development (Henarejos-Escudero et al., 2020). A similar situation was also reported when an excess of vitamin C was unable to improve symptoms and survival of patients with advanced cancer (Creagan et al., 2010). Other studies show how antioxidants can prevent apoptosis, thus promoting the proliferation of tumor cells (Gal et al., 2015). Therefore, the anticancer effects should not be directly linked to the antioxidant properties of the betalains, as other factors such as the type of molecule and its chemical structure, the type and

Table 2
Properties and most relevant biological effects of betalains extracted from *Beta vulgaris*.

Property	Biological effect	Animal system	References
Antitumoral	Stimulation of apoptosis Inhibition of angiogenesis	Mouse, rat and nematode (*C. elegans*)	(Henarejos-Escudero et al., 2020; Lechner et al., 2010; Zhang et al., 2013)
Anti-inflammatory	Decreased leukocyte migration Lower production of pro-inflammatory cytokines Stimulation of autophagy	Mouse, rat and human	(Clifford, Allerton, et al., 2017; Macias-Ceja et al., 2017; Martinez et al., 2015; Tan et al., 2015)
Antioxidant	Reduction of myeloperoxidase activity Reduces ROS levels Increased activity of antioxidant enzymes Decreased lipid peroxidation	Mouse, rat, carp and nematode (*C. elegans*)	(Da Silva et al., 2019; Guerrero-Rubio, Hernández-García et al., 2019; Han, Ma, et al., 2015; Han, Tan, et al., 2015; Vulić et al., 2013)
Hepatoprotective	Reducing mitochondrial damage Reducing expression of miRNA-34a Increased phase II enzyme activity of drug metabolism Upregulating PPAR-α Downregulating SREBP-1c	Rat and carp	(El Shaffei et al., 2021; Han, Gao, et al., 2014; Han, Zhang, et al., 2014; Krajka-Kuźniak et al., 2012; Szaefer et al., 2014; Yahaghi et al., 2020)
Cardioprotective	Reduction of serum cholesterol and triacylglycerides Reduction of LDL levels Reduction of serum homocysteine Reduction of systolic blood pressure Reduction of serum glucose Reduction of isovaleric acid level Inhibition of the synthesis of short-chain fatty acids	Rat and human	(Rahimi, Mesbah-Namin, Ostadrahimi, Abedimanesh, et al., 2019; Raish et al., 2019; Wroblewska et al., 2011)
Antifibrotic	Inhibition of the glycosylation of proteins involved Decreased advanced glycation end products (AGEs) Downregulated the receptors for AGEs (RAGEs) Downregulate the expression of mRNA and proteins of TGF-β, type IV collagen, α-SMA and E-cadherin	Rat	(Han, Tan, et al., 2015; Sutariya & Saraf, 2017)
Antihyperglycemic	Increased activity of liver glucolytic enzymes Increased activity of β pancreatic cells	Rat and human	(Aliahmadi et al., 2021; Dhananjayan et al., 2017; Indumathi et al., 2018)
Neuroprotective	Increased activity of choline acetyltransferase Reduction of brain oxidative stress Decreased activity of purinergic enzymes Decreased monoamine oxidase activity Decreased activity of ACE I Neurotoxic metals chelation	Mouse and rat	(Olasehinde et al., 2020; Shaban et al., 2021; Thong-asa et al., 2020, 2021)

properties of the tumor, or the molecular mechanisms involved come into play.

Besides *B. vulgaris*, other betalain-producing plants have also been used in the bibliography as sources of betalains. One of the most commonly used pigments is indicaxanthin, the major betaxanthin present in the edible fruits of *O. ficus-indica*, which exhibits *in vivo* effects similar to those described for betanin. Although *B. vulgaris* and *O. ficus-indica* are the most representative species of the studies focused on the properties of betalains, other studies have been able to introduce novel molecules through alternative betalain-producing plants belonging to the order Caryophyllales. This is the case of the extracts of *A. spinosus* and *B. erecta* used in research on the antimalarial properties of betalains (Hilou et al., 2006). Studies of cognition, learning, and memory improvement have been conducted with extracts of *P. oleracea* (Wang & Yang, 2010). Other sources, such as *A. cruentus* have been used to study the induction of hematopoiesis in rats (Pandey et al., 2016), while the anxiolytic effects in zebrafish have been reported by using the pulp and peel of *H. polyrhizus* (Lira et al., 2020). In the latter case, pitaya is also characterized by a high content of phenolic compounds (Fathordoobady et al., 2016; Luo et al., 2014), which may interfere in the health-promoting properties described. These properties have been reported with extracts of such a variety of plants and are now supported by the discovery of the capacity of betalains to act at the transcriptional level by modulating the expression of certain genes. The capacity of betalains to modulate the expression of key genes in *C. elegans* promotes an extension in the worms' lifespan and promotes protection against oxidative stress, with the modulation of the *hsp* genes and the longevity pathways serving as the underlying mechanisms which induce the health-promoting effects of the pigments.

7. *In vitro* vs *in vivo* activity

The establishment of the *in vivo* structure-activity relationship of betalains is highly limited by the widespread use of *B. vulgaris* and *O. ficus-indica* as the main sources of pigments. Due to this, betanin and indicaxanthin, the main pigments of the above-mentioned edible plants, have been associated with a greater amount of biological effects. Even though they are different betalains from the structural point of view, they have similar bioactive profiles (high antioxidant, anti-inflammatory, and antitumor capacity), making it even more difficult to establish a structure-activity relationship *in vivo*. Complexity is increased because most of the studies performed to analyze the *in vivo* bioactive properties of betalains were performed with extracts rather than with pure compounds (Fig. 4). This limits the understanding of the underlying biochemical processes and may even lead to ambiguous adscriptions of recorded protective effects, since they may result from the synergistic action of several compounds in addition to betalains. On the other hand, it is also possible that the action of certain substances present in complex extracts may mask the beneficial effects of the pigments. Other bioactive components such as flavonoids, phenolic compounds, and ascorbic acid may also be present in betalain-containing extracts, in addition to betalains (Galati et al., 2005). Therefore, studies using extracts may provide results that cannot be attributed exclusively to the effect of betalains. For this reason, the production of pure betalains is essential in order to develop experiments able to show that the effects described are an exclusive consequence of betalains' properties, thus giving support to health claims.

Purification of betalains from whole plants was the usual procedure but it is ineffective due to the complexity of the extracts, the low

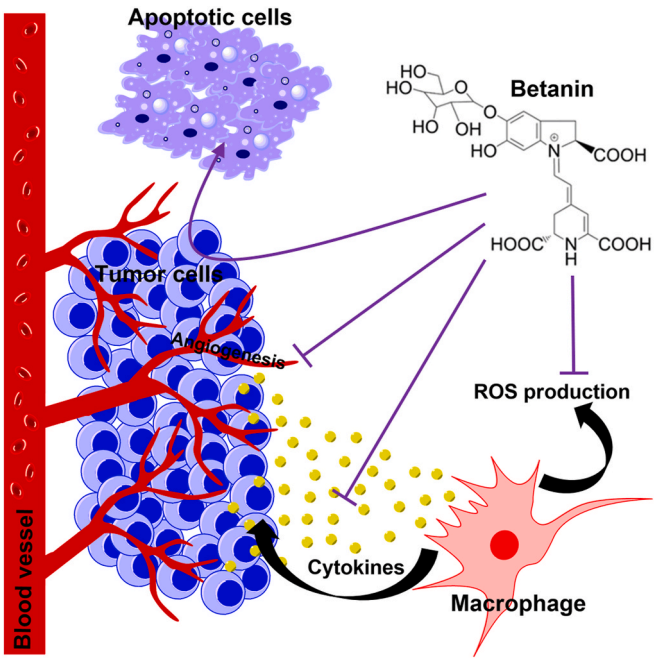

Fig. 3. Main molecular and physiological effects that confer antitumor properties to *Beta vulgaris* betanin.

quantities of certain pigments and the influence of different environmental factors on betalain levels in plants (Khan, Sri Harsha, Giridhar, & Ravishankar, 2012). For this reasons, various techniques have been developed to obtain betalains through semisynthetic methods, consisting of the degradation of betanin to obtain betalamic acid (Gandía-Herrero, García-Carmona, & Escribano, 2006); or by *in vitro* culture of plant cells (Guadarrama-Flores, Rodríguez-Monroy, Cruz-Sosa, García-Carmona, & Gandía-Herrero, 2015). Although these methods have provided enough material to develop *in vitro* studies, the efficiency of these techniques is low, making it difficult to obtain pure betalains in the quantities necessary to perform studies in animal models. This has led to the search for alternative methods that improve the production of betalains. In this sense and taking into account the simple biosynthetic pathway of betalains, different biotechnological approaches have led to the obtention of betalains thanks to the heterologous expression of DODA enzymes. The key step in the biosynthesis of betalains is the formation of betalamic acid thanks to the activity of the 4,5-DOPA-extradiol-dioxygenase (DODA) enzyme and thus it can be used to start the formation of natural pigments and analogs. These methods involve the development of microbial factories. *Saccharomyces cerevisiae* cultures (Grewal, Modavi, Russ, Harris, & Dueber, 2018) have been used as a host to produce betalains thanks to the expression of the DODA enzyme from *Mirabilis jalapa*. Different betalain-producing *E. coli* cultures have also been studied and betalains produced thanks to the expression of the DODA enzyme from the bacterium *G. diazotrophicus* (Contreras-Llano et al., 2019; Guerrero-Rubio, López-Llorca, Henarejos-Escudero, García-Carmona, & Gandía-Herrero, 2019). In both techniques, the supply of the substrates necessary for the correct activity of the enzymes allows the production of betalains in a purer form. Hence, it is possible to develop studies using purified compounds instead of extracts or mixtures, in order to minimize artifacts and to increase the reliability of the results.

Although the *in vivo* structure-activity relationships have been little studied due to the limitations mentioned above, the *in vitro* activity has been characterized with a structural approach, although the number of works remains scarce. The first studies focused on betanin and showed a direct relationship between the antioxidant power of the pigment and its ability to donate H and electrons (Gliszczyńska-Swigło et al., 2006).

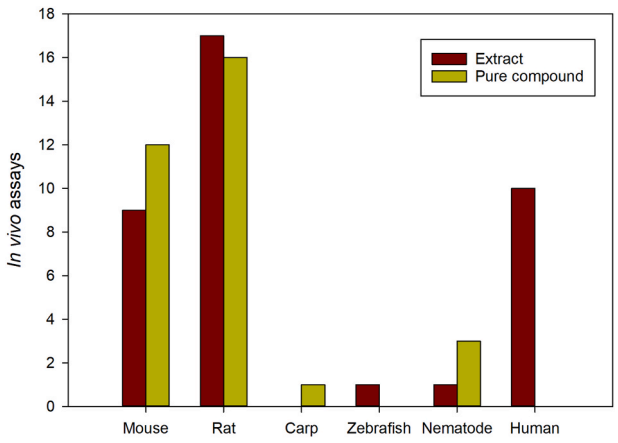

Fig. 4. Betalain administration methods in each animal model. A distinction is made between research where plant extracts are administered and that in which betalain compounds are supplied in pure forms. No *in vivo* studies with pure compounds have been performed in humans and zebrafish.

Subsequently, it was pointed out that the existence of antiradical activity was not exclusive to the presence of hydroxyl groups, although it was enhanced when the betalain presented this kind of group (Gandía-Herrero, Escribano, & García-Carmona, 2009). In addition, a strong correlation was demonstrated between the presence of charges in betaxanthins derived from secondary amines and the reduction of the antioxidant power (Gandía-Herrero, Escribano, & García-Carmona, 2010). Similarly, it has been suggested that the existence of an aromatic ring capable of extending the resonance system of betalamic acid results in a significant improvement in the ability to eliminate ROS, giving enhanced results when the structure formed is of the indoline type. In contrast, the addition of carboxyl groups is not linked to an increase in the antioxidant potential of betalains (Gandía-Herrero et al., 2010). The available data suggest that, from a structural point of view, betacyanins are candidates to show greater antioxidant potential than betaxanthins *in vitro*. However, *in vitro* results should not serve as a starting point for the characterization of *in vivo* activities, as there are examples of highly antioxidant betalains *in vitro* but irrelevant in reducing oxidative stress *in vivo* (Guerrero-Rubio, Hernández-García et al., 2020). The presence of positive charges in betalains had a negative effect on reducing oxidative stress in *C. elegans*, while positively charged molecules showed high antioxidant and health-promoting effects *in vivo*. The differences between the *in vitro* activities and the *in vivo* potential may result from the different bioavailability, assimilation, accumulation, and metabolization of the different pigments once administered to living organisms. In this sense, a labile and unstable pigment will hardly exert its potential *in vivo*, while it could be active in a short-time *in vitro* assay. The variability is even greater if the wide repertoire of methods of administration of extracts/pure pigments used in the declared studies is taken into account (Fig. 5). With studies mainly focused on betalains used as possible nutraceuticals, it is observed that oral treatment is the most commonly used method for the administration of betalains. However, in studies with classical mammalian models, the selection of different routes of administration for the distribution of the pigments and the administration of different doses is observed. As a result, the assimilation and bioavailability of betalains may differ between trials, increasing the heterogeneity of the studies analyzed. This fact, coupled to the variability of forms of administration described (Fig. 4), makes it difficult a quantitative summary of the doubtless *in vivo* health-promoting effects induced by betalains. In any case, it is necessary to critically observe any correlation between *in vitro* activities and the biological effects observed in animals, putting into context the total benefits of betalains *in vivo*.

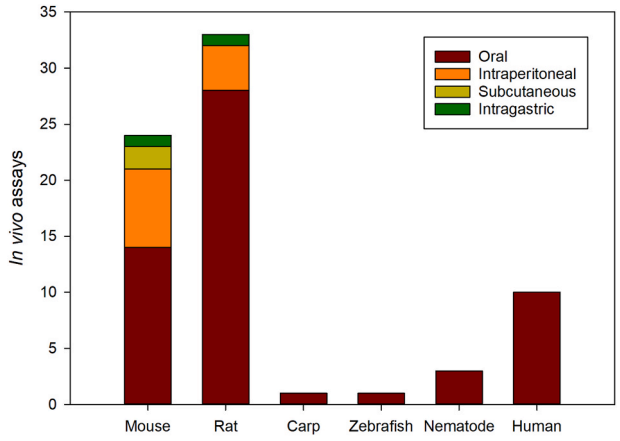

Fig. 5. Routes of administration of betalains, both pure and in the form of extracts, used in studies for each animal model. Oral treatment is the most wide form of administration used. In addition, other routes of administration, such as intraperitoneal, subcutaneous or intragastric, have been considered in mouse and rat.

8. Conclusions

Many studies have demonstrated the health-promoting effects of betalains, both naturally occurring ones and synthetic derived pigments. These studies have focused on multiple properties, such as their antioxidant, antitumoral or anti-inflammatory capacities. Even though the majority of these studies have been performed with extracts, the latest assays performed with pure, individual compounds are a significant proof of the extraordinary potential of these compounds. However, the molecular mechanisms associated with the reported effects on disease prevention and treatment are difficult to clarify when extracts are used. This fact increases the complexity in establishing unequivocal *in vivo* structure-activity relationships further influenced by the bioavailability of compounds once incorporated into living organisms. Despite the limitations, currently, there is enough *in vivo* evidence to state that betalains are not only non-harmful pigments but also phytochemicals with high potential as bioactive compounds.

On analyzing the information obtained on the biological activity of betalains in humans, it is observed that the properties are similar to those recorded in animal models. However, these effects have been primarily focused on the pursuit of benefits at the physical and sporting levels. In fact, the protective effects of betalains have not been exploited sufficiently in medical or pharmaceutical contexts. However, the numerous studies *in vivo* described here in animal models, provide a sufficiently solid base to discuss health claims and the health-promoting biological activities of betalains in humans. Altogether, the promising results summarized in this work describe betalains as compounds of great potential for application as functional ingredients.

Author contributions

P. M.-R.: Data curation, Formal analysis, Investigation, Resources, Writing - original draft. M. A. G.-R.: Conceptualization, Data curation, Supervision, Validation, Methodology, Writing – original draft. P. H.-E.: Data curation, Investigation, Resources, Writing- original draft. F. G.-C.: Conceptualization, Funding acquisition, Project administration, Validation, Writing – review and editing. F. G.-H.: Conceptualization, Funding acquisition, Methodology, Project administration, Supervision, Validation, Writing – review and editing.

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Declaration of competing interest

The authors declare no competing financial interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tifs.2022.02.020.

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