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Salix babylonica a source of active compounds for the treatment of inflammatory diseases in animals

Salix babylonica una fuente de compuestos activos para el tratamiento de enfermedades inflamatorias en animales

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ABSTRACT

The wellbeing of terrestrial animals describes society's expectations for the conditions animals should experience when under human control. These principles include that animals must be free from pain, injury and disease. Inflammation is a host's defensive mechanism against bacterial or viral infection and physical or chemical stimulus to the host. Metabolites from plants are an efficient alternative for treatment of inflammatory diseases. The purpose of this research was to evaluate the anti-inflammatory capacity of a hydro-alcoholic extract from *Salix babylonica* leaves (HESB) employing the auricular edema induced by the TPA model in mice. The HESB showed good anti-inflammatory activity without significant difference from the reference drug, indomethacin. For this reason, a bipartition of HESB was conducted, obtaining an aqueous fraction (AFSB) with slight activity (30.64 ± 3.03 %) and an organic fraction (EAFSB), which showed the best anti-inflammatory effect (67.08 ± 7.15 %). Previously, we identified anti-inflammatory flavonoids luteolin and luteoloside as the major components of EAFSB. In addition, histopathological analysis showed that mouse ears treated with *Salix babylonica* suppressed neutrophil infiltration into the inflammation site. These results support the ethno-medical use of this plant and evidenced that *Salix babylonica's* applicability and value as an anti-inflammatory treatment for animals.
Keywords: *Salix babylonica*, anti-inflamammatory effect, neutrophils.

RESUMEN

El bienestar de los animales terrestres describe las expectativas de la sociedad sobre las condiciones que los animales deberían experimentar cuando están bajo el control humano. Estos principios incluyen que los animales deben estar libres de dolor, lesiones y enfermedades. La inflamación es el mecanismo de defensa de un huésped contra una infección bacteriana o viral, o una lesión física o química al huésped. Los metabolitos secundarios de las plantas son una alternativa eficaz para el tratamiento de enfermedades inflamatorias. El propósito de esta investigación fue evaluar la capacidad antiinflamatoria de un extracto hidroalcohólico de hojas de *Salix babylonica* (HESB) empleando el modelo de edema auricular inducido por TPA en ratones. El HESB mostró una buena actividad antiinflamatoria sin diferencia estadística significativa con el fármaco de referencia, indometacina. Por este motivo, se realizó una bipartición de

HESB, obteniendo una fracción acuosa (AFSB) con actividad farmacológica leve (30.64 ± 3.03%) y una fracción orgánica (EAFSB) que mostró el mejor efecto antiinflamatorio (67.08 ± 7.15%). Anteriormente, identificamos los flavonoides antiinflamatorios luteolina y luteolosido como los componentes principales de EAFSB, por lo que el efecto antiinflamatorio de *Salix babylonica* puede ser atribuido a estos compuestos. Además, el análisis histopatológico mostró que las orejas de los ratones tratados con *Salix babylonica* suprimieron la infiltración de neutrófilos en el sitio de inflamación. Estos resultados apoyan el uso etnomédico de esta planta y evidencian el posible uso de *Salix babylonica* en el tratamiento del proceso inflamatorio en animales.
Palabras clave: *Salix babylonica*, efecto antiinflamatorio, neutrófilos.

INTRODUCTION

Currently, there is growing public concern about the treatment of captive and domesticated animals, including wild, laboratory, farm, work, zoo and companion animals (Webb *et al.,* 2019). Animal welfare is a complex and multifaceted issue with scientific, ethical, social, religious, cultural, political and economic dimensions. According to the American College of Animal Welfare: “Animal welfare refers to the state of the animal. Assessment of welfare includes consideration of the animal’s health, behavior, and biological function;” therefore, anything that contributes to or demerits the normal state of an animal affects its well-being (Castle *et al*., 2016). On the other hand, health refers to the state of the body's systems that combat pathogens, tissue damage or physiological disorders; in other words, it is the state of an animal in relation to its attempts to confront pathology. As such, it is an important component of animal welfare (Broom, 2011).

The inflammatory process is a common syndrome to numerous pathological situations of mammals. It results in the local or systemic response of the organism against harmful external stimuli of a microbial, chemical or physical type. The purpose being to eliminate the causative agent, as well as, repair damaged tissue and maintain homeostasis. This process can be divided into two phases: acute, characterized by local vasodilation and increased capillary permeability; and chronic, characterized by a greater immune response, tissue degeneration and fibrosis (Kumawat *et al*., 2012). During the inflammatory response, pro and anti-inflammatory mediators are synthesized and secreted. These are substances that have a direct effect on inflammatory cells and blood vessels or that intervene in reactions that generate compounds that act on these cells. Among the inflammatory mediators and cell pathways are the cytokines (e.g., interferons, interleukins and tumor necrosis factor α), chemokines (e.g., monocyte chemo-attractant protein 1), eicosanoids (e.g., prostaglandins and leukotrienes) and the potent inflammation-modulating transcription factor nuclear factor κB (Azab *et al*., 2016). Most of these molecules are derived from the phospholipid components of cell membranes, which are released as a result of their destruction (Kumawat *et al*., 2012).

Throughout the millennia, medicinal plants have been used for the care of human and animal health, because they have a great diversity of secondary metabolites such as terpenes, alkaloids and flavonoids that have several biological properties (Mayer *et al*., 2014; Miara *et al*., 2019; Starlin *et al*., 2019). In addition, the preference for the use of medicinal plants or phytopharmaceuticals obtained from them, continues to increase because of they lack side effects or these are minimal (Mayer *et al*., 2014; Miara *et al*., 2014; Starlin *et al*., 2019; Laudato & Capasso, 2013).

The genus *Salix*, belonging to the Salicaceae family, has been used since ancient times for the treatment of fever, pain and inflammation. For these purposes, the bark of *Salix alba* and *Salix nigra*; flowers of *Salix caprea* and aerial parts of *Salix canariensis*, demonstrate a counter inflammatory response (Drummond *et al*., 2013; Gutiérrez *et al*., 2017; Sharma *et al*., 2011; Gyawal *et al*., 2013; Ahmed *et al*., 2011).

Salix babylonica, commonly known as the weeping willow tree, is the most acknowledged species of the willows, distributed in some areas of Asia, Europe, and America. It has been used as an ornamental and medicinal plant (González-Alamilla *et al*., 2019). In traditional medicine, the intake of an infusion of weeping willow leaves allows pain relief, whether rheumatic, muscular, head, ears, or toothache, among others (Waizel-Bucay, 2011). Thus, the objective of the present work was to evaluate the anti-inflammatory activity in-vivo of a hydro-alcoholic extract of *Salix babylonica* leaves and identify the compounds responsible for this activity.

MATERIALS AND METHODS

General considerations

Solvents were eliminated with a rotary evaporator Büchi (Flawil, Switzerland). Acetone, Ethyl acetate, Ethanol, 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and Indomethacin were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

Plant material

Leaves of *Salix babylonica* were collected during the period of June–August 2018 in Tulancingo de Bravo, Hidalgo, Mexico (20°50’09” N 98°21’48” W). For plant identification, the Herbarium of UNAM (Universidad Nacional Autonoma de Mexico, Mexico City, Mexico) was consulted, and the vegetal specimen was identified as *Salix babylonica* L. (IBUNAM: MEXU: 9744). Plant material was dried under dark conditions at room temperature for three weeks. Afterwards, the plant material was ground in an electric blender.

Preparation of extract

The dried, ground material (1.5 kg) was extracted by maceration with ethanol: water (60:40 v/v) for a 1 d period and was repeated three times. All extractions were performed

using 1:3 plant material/solvent proportions. The solvent was eliminated under reduced pressure distillation with a rotary evaporator. The extract was stored at 4° C, until its use.

Anti-inflammatory activity

The anti-inflammatory activity of extract and fractions was studied by the method of acute inflammation in mouse ears induced with TPA as described by Rivero-Pérez *et al*., (2016). Adult male CD-1 mice with a body weight of 20–25 g were grouped (five individuals per group). Mice were maintained under standard laboratory conditions at 22°C ± 3°C, 70% ± 5% humidity, 12-h light/dark cycle, and food/water ad-libitum. The mice were allowed at least 1 week to adapt to the laboratory environment before initiating the experiments. Experiments were performed according to the Official Mexican Rule NOM-062-ZOO-1999 Guidelines (Technical Specifications for the Production, Care, and Use of Laboratory Animals), and the protocol was approved by the Institutional Committee on Ethical Guidelines for the Care and Use of Experimental Animals of the Universidad Autónoma del Estado de Hidalgo (ICSa/CIECUAL/001/2018).

Animal ear inflammation was induced with 2.5 µg TPA dissolved in 20 µL of acetone applied to the internal and external surface of the right ear to cause oedema. Sample doses of 3.2 and 1 mg/ear of the extracts and fractions, respectively, were applied topically. The anti-inflammatory drug Indomethacin was used as a positive control and was administered at 0.5 mg/ear. SBHE and AFSB were dissolved in distilled water, AEFSB in EtOH:water (1:1 *v/v*) and Indomethacin in acetone. A negative control group received acetone, and other EtOH:water (1:1 *v/v*) as a vehicle. All treatments were applied topically to the right ear immediately after TPA application. Six hours after application of the doses, the animals of each treatment were sacrificed by cervical dislocation. Circular sections of 6 mm in diameter were taken from both the treated (t) and the non-treated (nt) ears, which were weighed to determine the inflammation.

Percentage of inhibition was determined using the following equation:

$$Inhibition\% = \left(\Delta w\ control - \ \Delta w \frac{treatment}{\Delta w}\right) x100$$

Where Δw = wt − wnt, with wt being the weight of the section of the treated ear and wnt being the weight of the section of the non-treated ear.

Histological analysis

After euthanizing the mice, a circular sample was taken from each ear (6 mm) and were fixed by immersion in 3.8% formaldehyde, in aqueous solution, phosphate buffered, by 24 hours. Subsequently, the samples were processed by the paraffin inclusion method using

an automated Microm tissue processor, model TP1020, cut into a Leica model 2125RT microtome at 4 µm thick and colored with the hematoxylin-eosin technique (HE) (Prophet *et al*., 1995). The resulting histological preparations were observed and analyzed with an Olympus clear field compound microscope, model BX41. A representative area was selected and inflammatory severities were subsequently qualitatively assessed based on inflammatory cell infiltration. The selected images were captured with a MediaCybernetics digital camera, Model Evolution VF, using Image-Pro Express 6.0 software (MediaCybernetics), installed on a Vaio brand computer with Pentium 4 processor and 1 GB of RAM.

Statistical Analysis

The results obtained from the pharmacological test were submitted to analysis of variance, followed by Tukey testsusing the SAS program, version 9.0 (SAS, 2006). $P < 0.01$ was considered significantly different.

RESULTS

In vivo anti-inflammatory activity

The anti-inflammatory capacity of *Salix babylonica* leaves hydro-alcoholic extract (HESB), and its less complex fractions AFSB and EAFSB were evaluated in TPA-induced auricular oedema model in mice at a dose of 3.2 and 1 mg/ear, respectively. All treatments were significantly different ($P <0.01$) with the negative group control, and SBHE and EAFSB did not show significant statistical difference with reference drug (indomethacin) or between them (table 1).

Table 1. Anti-inflammatory activity of extracts and fractions from *Salix babylonica* leaves

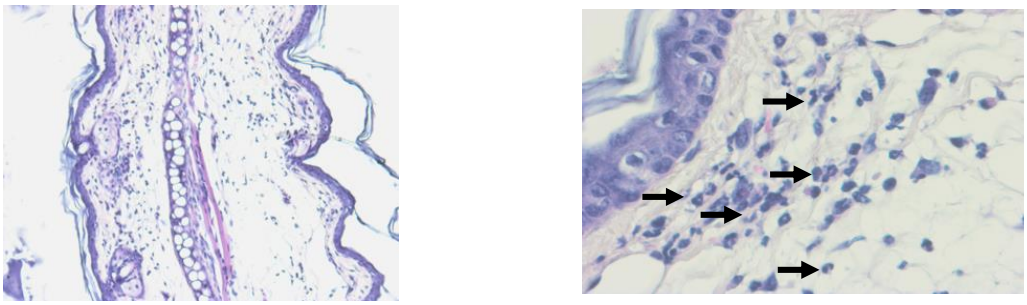
Treatment	Dose (mg/ear)	% inhibition of inflammation ± SEM
Hydroalcoholic extract of *S. babylonica* leaves (SBHE)	3.2	66.92 ± 3.20 [a]
Aqueous fraction from SBHE (AFSB)	1	30.64 ± 3.03 [b]
Organic fraction from SBHE (EAFSB)	1	67.08 ± 7.15 [a]
Indomethacin	0.5	79.54 ± 6.16 [a]
Negative control	0	0 [c]

Different literals in the column showed significant differences ($P \leq 0.01$) among the compounds evaluated. SEM: Standard error of the mean.

Histological analysis

Topical application of TPA for 6 hours, induced an acute inflammatory response at the level of the dermis of the mice ear and a marked increase in the neutrophil population was noted (Figure 1). No changes in the epidermis were observed. Furthermore, when only acetone (TPA vehicle) was applied, this reaction was not observed, but rather sustained

the normal appearance of the dermis particularly among the collagen fibers and fibrocytes/fibroblasts. Similarly, the application of indomethacin, hydroalcoholic extract of *Salix babylonica* (HESB) and ethyl acetate fraction (EAFSB) obtained from HESB, inhibited an acute inflammatory response, evidenced by the poor population of inflammatory cells, particularly neutrophils, associated with the dermis of the skin of the ear (Figure 2).



(a) (b)

Figure 1. Mouse ear skin. H-E. 100x (a) and 400x (b). Effect of TPA, without the addition of anti-inflammatory. There is a marked increase in neutrophils in the dermis (arrows, representative of at least 40 cells in the field)

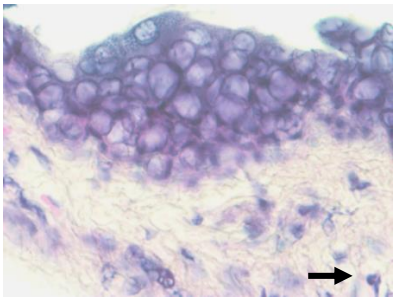


Figure 2. Mouse ear skin. H-E. 400x. Application of the organic fraction of the hydroalcoholic extract of *S. babylonica*. As can be seen, the presence of inflammatory cells in the dermis is minimal (arrow)

DISCUSSION

According to the World Organization for Animal Health (OIE) Terrestrial Code, animal welfare means the physical and mental state of an animal in relation to the conditions in which it lives and dies. The guiding principles which inform the OIE's work on the welfare of terrestrial animals include the 'Five Freedoms'; which describe society's expectations

for the conditions animals should experience when under human control, namely: freedom from hunger, malnutrition and thirst; freedom from fear and distress; freedom from heat stress or physical discomfort; freedom from pain, injury and disease; and freedom to express normal patterns of behavior (OIE, 2019).

Inflammation, an organism's response to irritation or injury, is characterized by redness, heat, swelling and pain. Non-steroidal anti-inflammatory drugs (NSAIDs) are used for the treatment of inflammatory diseases. These compounds affect the function or production of prostaglandins. In veterinary medicine, NSAIDs are used to control, among other ailments, pain and inflammation associated with osteoarthritis in dogs and horses; as well as, for postoperative pain in dogs and cats. However, there are potential risks associated with the use of NSAIDs such as gastrointestinal ulcers/perforations and liver and kidney toxicity (Mathews *et al*., 2014). Therefore, the search of new compounds with a counter inflammatory reaction, without the side effects of NSAIDs, is of vital importance for the maintenance of animal welfare. The use of medicinal plants is an alternative for the treatment of pain and inflammation in animals.

Plants have been proven very rich sources of structurally and biologically interesting metabolites. Less than 10% of the world's biodiversity has been evaluated for potential biological activity, many more useful natural leader compounds await discovery (Gyawali *et al*., 2013). In addition, the world-wide interest in herbal products has grown significantly. Cattle, horses, sheep, goats and pigs represent about 70% of the animals treated with herbal remedies, followed by poultry (9.1%), dogs (5.3%) and rabbits (4.3%) (Laudato & Capasso, 2013).

Regarding the use of plants for the treatment of the inflammatory process in animals, *Geranium robertianum*, *Artemisia abrotanum*, *Brassica oleracea*, *Avena sativa*, *Anagallis arvensis*, *Linum usitatissimum*, *Scrophularia canina*, *Hypericum perforatum*, *Achillea millefolium* and *Buxus sempervirus* are used to treat or prevent mastitis in cattle because of their anti-inflammatory effect (Mayer *et al.*, 2014; Laudato & Capasso, 2013). What's more, *Aloe spp.* has been used for treatment of inflammation, pains and itching in several animals; while dandelion and linseed have been used for perianal gland inflammation treatment in dogs (Laudato & Capasso, 2013). *Rosa canina*, *Matricaria chamomilla, Glycyrrhiza glabra*, *Aegle marmelos*, *Asparagus racemosus* Wild, *Argemone mexicana* Linn, *Datura metal* Linn, *Eclipta prostrata* Linn, *Moringa oleifera* Lamk, *Syzygium cumini*, *Allium cepa*, *Nerium oleander* L, *Anayclus davatus*, *Mentha pulegium* L, *Lawsonia inemis* L, *Olea europaea* L, *Ziziphus lotus* L, *Ruta graveolens* and *Ziziphus jujuba* Linn, are used for the treatment of different inflammatory diseases in sheep, cattle, goats, rabbits and poultry (Miara *et al*., 2019; Laudato & Capasso, 2013; Verma, 2014).

The *Salix* genus has been used in the treatment of arthritis; menstrual, dental and back pain; fevers and inflammation. Willow trees belong to the Salicaceae family and records of its medicinal use go back about 6,000 years (Drummond *et al*., 2013; Gutiérrez *et al*., 2017; Gyawali *et al*., 2013). *Salix babylonica* has demonstrated antihelmintic, antiseptic, analgesic, antipyretic, antimalarial, antioxidant, anticarcinogenic, astringent, antifungal and antibacterial activity (González-Alamilla *et al*., 2019; Abdel Wahab *et al*., 2018). In this work, the anti-inflammatory activity of the hydroalcoholic extract of *S. babylonica* leaves was evaluated and we found that it inhibited inflammation by 66.92 ± 3.20%, without significant statistical differences with respect to the reference drug, indomethacin (table 1).

There are some reports regarding the anti-inflammatory effect of extracts obtained from other species of *Salix*; for example, Gyawali *et al*. (2013), evaluated the anti-inflammatory capacity of an methanolic extract from *Salix alba* bark using the formalin induced paw oedema model in rats, and found an inhibition of inflammation of 74% at a dose of 93.5 mg/Kg. Likewise, an aqueous extract from aerial parts of *S. canarensis*, obtained by infusion, at a dose of 105 mg/Kg, caused an 78% inhibition of the inflammation in the same model (Gutiérrez *et al*., 2017). On the other hand, Ahmed *et al*., (2011) evaluated the anti-inflammatory effects of methanolic and hydroalcoholic extracts of flowers of *S. caprea* at 400 µg/mL, using the Human Red Blood Cell (HRCB) membrane stabilization method, and found that this extract showed 66.78 and 60.49 % of membrane protection, respectively. However, these results cannot be directly compared with that obtained in this work for the hydroalcoholic extract of *S. babylonica* (HESB), because the extracts evaluated are of different polarity, obtained by different methods and evaluated in different pharmacological models.

In order to identify the chemical nature of the active compounds in the hydroalcoholic extract from *S. babylonica* leaves, a bipartition procedure with water an ethyl acetate solvents was performed. The aqueous fraction from SBHE (AFSB) and organic fraction from SBHE (EAFSB) were evaluated in their capacity to inhibit inflammation *in-vivo*. As shown in table 1, EAFSB (66.92 ± 3.20 % inhibition of inflammation (% II)) was most active than AFSB (30.64 ± 3.03 %II), without significant statistical difference with respect to the extract SBHE, and the effective dose was three times lower. In addition, EAFSB had no significant statistical differences with the reference drug Indomethacin. These results suggested to us that the anti-inflammatory compounds from *Salix babylonica* were in the organic fraction.

Previously, we identified the flavones luteolin (Figure 3a) and luteoloside (luteolin 7-*O*-glucoside) (Figure 3b) as the major components of ethyl acetate fraction obtained by bipartition from hydroalcoholic extract of *Salix babylonica* leaves (González-Alamilla *et al*., 2019).

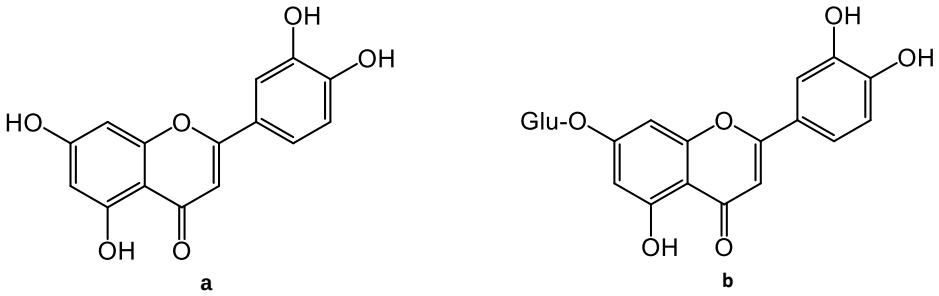


Figure 3. Chemical structure of flavonoids identified as major components in EAFSB. (a) luteolin (3′,4′,5,7-tetrahydroxyflavone) and (b) luteoloside (3′,4′,5,7tetrahydroxyflavone-7-*O*-glucoside)

Luteolin is a 3',4',5,7-tetrahydroxyflavone, a kind of flavonoid with a double bond between C2 and C3, and a carboxyl group in position 4 of ring C. Luteolin has been isolated from vegetables, fruits and medicinal plants. There are reports that evidenced its antioxidant, antimicrobial, antidiabetic, chemoprotective, chemotherapeutic, neuroprotective, anti-allergic and anti-inflammatory activity. Additionally, this molecule is considered non-toxic (González-Alamilla *et al*., 2019; Aziz *et al*., 2018; Zhang *et al*., 2018). Regarding anti-inflammatory responses, luteolin suppresses pro-inflammatory cytokines expression including interleukin (IL)-6, IL-1β, IL-2, IL-8, IL-12, IL17, TNF-α, interferon (INF)-β, granulocyte-macrophage colony-stimulating factor and increase the level of anti-inflammatory cytokine IL-10. It also inhibits the induction of nitric oxygen synthase (iNOS) and it's active expression, as well as the production of NO, ROS and chemokines. These aid in controlling the migration and positioning of immunity cells, such as chemokine (C-X-C motif) ligand 2 (CXCL2), chemokine (C-Cmotif) ligand 2 (CCL2), CXCL9 and CXCL8 (IL-8). Further, it also inhibits the production and release of eicosanoids, prostaglandins, and leukotrienes, as well as, pro-inflammatory adhesion molecules such as monocyte chemo-attractant protein-1 (MCP-1), intercellular adhesion molecule-1 (ICAM), and vascular cell adhesion molecular-1 (VCAM). Similarly, it prevents the NF-κB pathway, protein kinase B (AKT), and the mitogen-activated protein kinase (MAPK) pathway; hyaluronidase and elastase activity; stabilization of mast cell; reduction of vascular permeability; modulation of cell membrane fluidity, active antioxidants enzymes and prevents adenosine triphosphate (ATP) from binding to spleen tyrosine kinase (Syk) or proto-oncogene tyrosine-protein kinase (Aziz *et al*., 2018; Zhang *et al*., 2018; Li *et al*., 2019). Moreover, luteolin 7-*O*-glucoside (luteoloside) possesses anti-inflammatory activity too, this flavonoid inhibits the release of β-hexosaminidase (a marker of degranulation in mast cells), leukotriene C4 (LTC4), NO and PGE2 production; as well as, COX-2 and iNOS enzymatic activity. Furthermore, luteoloside is a potent inhibitor of JNK3, a protein kinase of the MAPK family that is potently activated by a variety of environmental stress and pro-inflammatory cytokines (Aziz *et al*., 2018).

On the other hand, topical application of TPA in mice ears produced oedema with its typical symptoms: swollen, increased thickness and infiltration of pro-inflammatory cells such as neutrophils, leukocytes and lymphocytes. These cells are the key components that infiltrate the site of inflammation first and promote host defense functions and are aggregated and bonded to the walls of blood vessels on the range of 4 to 6 h after application of TPA (Hernandez-Valle *et al*., 2014; Mendes *et al*., 2016; Silva *et al*., 2017). In the present study, samples for histological analysis were collected 6 h after stimulating with TPA, as shown in figure 1. TPA generated oedema, extravasation of fluids and increase of neutrophils in the dermis. When Indomethacin, a steroidal anti-inflammatory drug widely used as the validation for the pharmacological mode, was applied, local inflammation decreased; a similar result was observed in animals receiving treatment with hydroalcoholic extract SBHE or EAFSB (fig 2). In other words, SBHE and EAFSB induced the same response compared to the control drug. In conclusion, the histological analysis of H-E-stained mouse ears show that *Salix babylonica* suppresses the cells infiltration into the inflammation site induced by TPA.

CONCLUSIONS

The hydroalcoholic extract SBHE from leaves of *Salix babylonica* and its fractions AFSB and EAFSB display anti-inflammatory activity, EAFSB being the most active fraction. Previously, we reported that the chemical analysis of EAFSB fraction by HPLC revealed the presence of flavonoids luteolin and luteoloside (luteolin-7-*O*-glucoside) as major compounds. As a consequence, the anti-inflammatory effect shown by *S. babylonica* leaves could be attributed to the presence of those anti-inflammatory compounds. The histological analysis of the ears treated with *S. babylonica* suggest that these down-regulate the migration of pathogenic neutrophils into the site of inflammation decreasing the production of inflammatory cytokines. Finally, our results show that *S. babylonica* are able to decrease inflammation and could serve as a valuable alternative for the treatment of inflammatory processes in animals.

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Conflicts of Interest: The authors declare no conflict of interest.

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Errata Erratum

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