

Original Research Article

Chemical composition and pharmacological activity of a propolis extract from Ecuador

ABSTRACT

Aims: Propolis is a resinous substance accumulated by bees from resinous plants material, produced by different botanical processes. It has been used since ancient times for its therapeutic benefits. The chemical composition of propolis is mostly influenced by the geographic zone and also by botanic sources that the honey bee has used. In this sense, the aim of this study was to analyse the phytochemical profile and pharmacological activity of a sample of propolis from Ecuador.

Methodology: Chemical composition was analyzed by using gas chromatography coupled to mass spectrometry (GC/MS). The anti-inflammatory activity was determined by ear edema induced by 12-O-tetradecanoylphorbol-13 acetate and antinociceptive activity was analyzed by using writhing test induced by acetic acid.

Results: The chemical composition showed the presence of sugars, fatty acids, flavonoids and triterpenes in the sample. The analysis of the relative abundance of the detected signals suggested that triterpenes represent 25.38% of the total components of the mixture and 9,19-cyclo-9-beta-lanostane-24-on-3-beta-iloxy (cycloartenol), the majority compound. Propolis induced a maximum inhibition (77.2 %) in TPA model at a dose of 3 mg/ear, reducing dermal edema, cellular infiltration and ear thickness induced by TPA. Also, it produced a dose-related inhibition of acetic acid-induced writhing response with a maximal antinociceptive effect (49.5%) at dose of 500 mg/kg po.

Conclusion: The chemical analysis showed a predominant triterpene profile, being cycloartenol the majority compound in the sample and its anti-inflammatory and antinociceptive activities were confirmed by in vivo models.

Keywords: propolis, chemical composition, anti-inflammatory, antinociceptive, triterpenes.

1. INTRODUCTION

Despite many synthetic drugs are available for treating and managing common diseases, many of them are still associated with lack of efficacy or undesirable effect. Natural products represent an interesting and a promising strategy in health research since many of them have been used to treat different diseases in folk medicine. In particular, the developing market of natural products has a renewed interest in bee products. In this sense, propolis, a honey bee

product with a broad spectrum of biological activities, demonstrates to be a natural product with interesting profile (Alvarenga et al., 2021; Wieczorek et al, 2022).

Propolis is a resinous substance accumulated by bees from different botanical sources and bee secretions. It is used as a sealant material in beehives in order to maintain an adequate and aseptic internal environment. Also, as a cementing material, it could be capable to prevent weathering and invasion by predators (Casalone et al, 2020).

Propolis was used as a traditional medicine since 300 BC and demonstrate to possess an extensive spectrum of activities such as antioxidant, antibacterial, antiviral, antifungal, anti-inflammatory, and antiproliferative effects, representing promising an attractive strategy to treat different ailments (Ramírez et al. 2014, Villanueva et al. 2015, Salas et al. 2016, Anjum et al. 2018).

Although propolis has shown to possess a great versatility, the chemical composition could be diversified and depends on different factors, such as geographic area where the propolis is collected, the species of bee involved and also the collection time (Dantas et al. 2017, Huang, 2014). In this sense, chemical composition is an important point to be analyze in order to characterize the chemical profile that could be related to its pharmacological profile (Bankova et al. 2016).

Ecuador is a country in the north western part of South America and it is considered as a megadiverse country due to its high concentration of botanical and animal species. Considering that chemical diversity of propolis is related to phytogeographical conditions and the climatic characteristics, the aim of this study was to perform the chemical composition and pharmacological profile in relation to inflammation and nociception of an equadorian propolis extract.

2. MATERIAL AND METHODS

2.1 Material

The sample of propolis produced by *Apis mellifera* was collected in July 2015 from La Finca El Bosque located in La Parroquia Chacras (latitude: -3.562452, longitude: -80.056454, Provincia El Oro, Ecuador).

2.2 Preparation of the extract

Propolis (26.702 g) was subjected to a maceration process in an erlenmeyer flask, using methanol as solvent (500 mL). The process was carried out for seven days with solvent replacement on the third and fifth days. The methanolic extracts were filtered by degreased cotton, pooled and concentrated to dryness in a Büchi rotary evaporator at 40 °C under reduced pressure. The dry extract of propolis was stored in an amber glass bottle, stored at 6 - 8°C until analysis.

2.3 Analysis by gas chromatography coupled to mass spectrometry (GC/MS)

Ten mg of the extract of propolis were dissolved in 1 mL of ethanol (90 %, v/v). One hundred µL of the solution was taken and evaporated to dryness under a stream of nitrogen. The residue was derivatized with 50 µL of piridin and 100 µL N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) in a sealed glass tube for 30 minutes at 100 °C. The gas chromatograph (Agilent model GC 6890) coupled to a quadrupole mass spectrometer (HP 5973N) was used. The injector was heated to 310 °C and was on split mode with a split ratio of 1:50. Sample was separated on a 30 m x 0.25 mm i.d., 0.25 µm film thicknesses, HP-5MS column. The temperature of the chromatograph was programmed at 60 °C for 2 min, increasing to 310 °C with a ratio of 3 °C per min⁻¹, followed by an isothermal process of 20 min. The final injection volume was 1 µL. The components of the sample were fractionated on the HP-5MS column (30m x 0.25 mm x 0.25 µm). The total run time was 100 min, operating by electronic ionization at 70 eV with a mass range of 35 - 700 uma. Helio was the carrier gas (Campo et al. 2008; Popova et al. 2010).

2.4 Anti-inflammatory activity

The anti-inflammatory activity of the propolis extract was determined by mouse ear edema test induced by 12-O-tetradecanoylphorbol-13 acetate (TPA). Groups of eight mice each were used. The animals were randomly distributed in six experimental groups. TPA (2.5 µg) dissolved in acetonic solution was placed topically on each side of the right ear of the mice in five groups, Topical acetone was placed only in control group. Propolis (0.3, 1 and 3 mg/ear) dissolved in acetone were applied in respective groups. The left ear was used as a control, applying only acetone. Indomethacin (0.5 mg/ear) was used as the reference drug. After 4 hours of administration of TPA disks of 6 mm diameter were removed from the two ears of the mice, determining the weight of them. Swelling was measured as the weight difference between

the right and left ear disks. Disk were fixed in 10% buffered formalin and embedded in paraffin. Sections of 5 μ m thickness were prepared and stained with hematoxylin and eosin and then examined under light microscopy (Riedel et al. 2014).

2.5 Antinociceptive activity

Nociception was induced by intraperitoneal injection (ip) of acetic acid 1.0 %, 0.1 mL/10 g body weight. Propolis (125, 250 and 500 mg/Kg) were administered by oral route (po) 60 min before acetic acid injection. Indomethacin (10 mg/kg ip) was used as reference drug. Control animals received a similar volume of saline solution. The animals were observed in experimental cages. Hand-operated counters and stopwatches were employed to score the number of abdominal writhes (full extension of both hind paws). The writhes were cumulatively counted over a period of 20 min immediately after the acetic acid injection (Collier et al. 1968).

3. RESULTS AND DISCUSSION

The gas chromatogram of the sample (Figure 1) showed 47 fundamental peaks. The analysis of the mass spectra obtained by electronic impact was used to characterize each peak. The structural proposal was obtained by comparison with the database (NIST 98). Twenty four compounds were summarized in table 1. Analyzing the fundamental fragments from mass spectra from 34 minutes and up to approximately 40 minutes, 8 compounds could be identified, suggesting flavones, flavanones and their iso variants as potential structures. The analysis of the relative abundance suggested that these compounds represent approximately 18.47% of abundance with respect to the total detected metabolites. According to the literature, the positions where the oxygenated substituents are generally located in 5, 7, 2', 3' and / or 4' positions. However, the use of NMR techniques is required to complete assignment of the structural features.

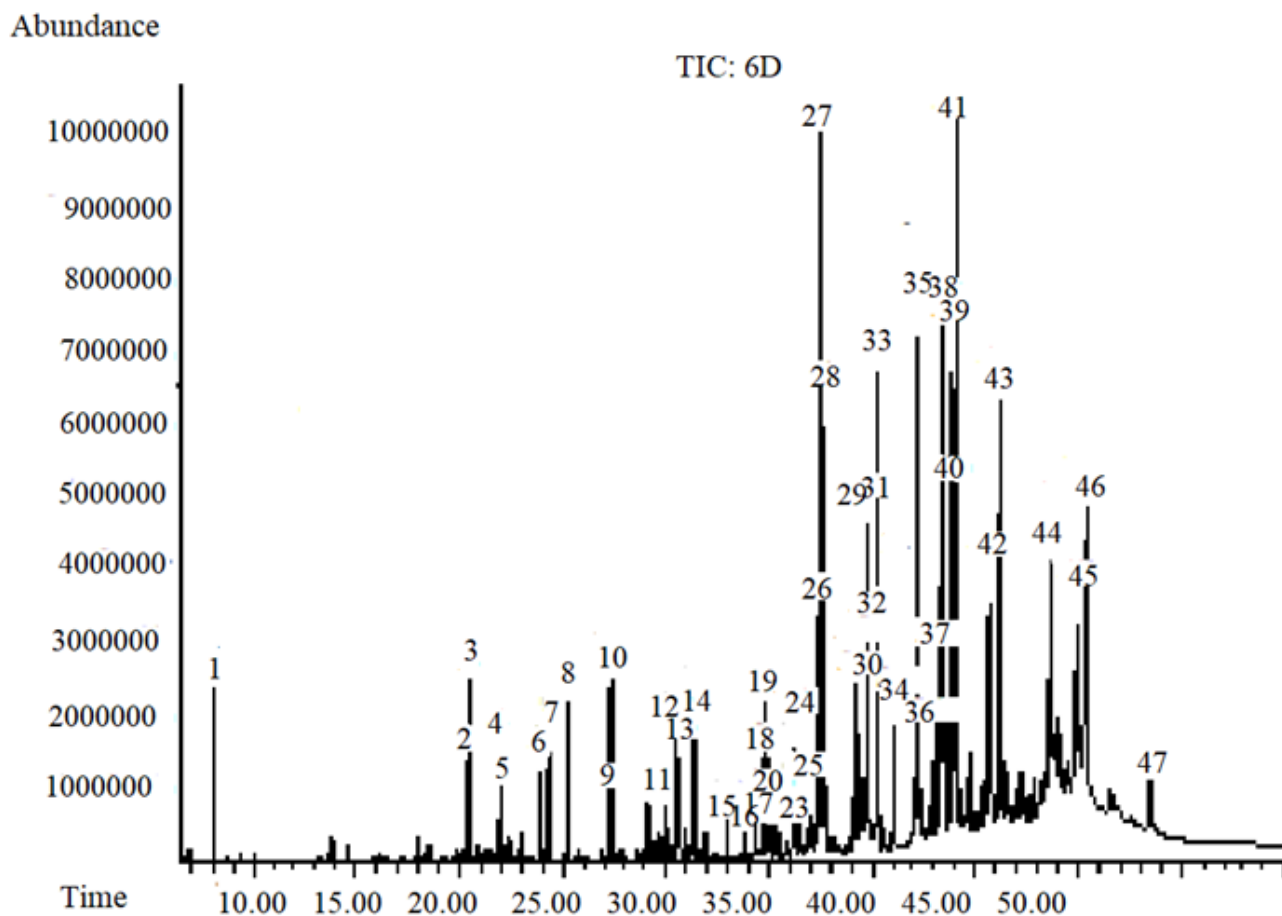


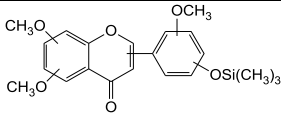
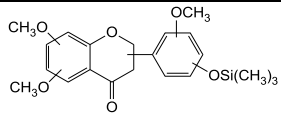
Figure 1: Gas chromatogram of sample

Table 1. Chemical compounds identified by GC/MS

No.	Tr (min)	Chemical compounds	%
1	08.041	Glycerin	0.95
2	20.336	D-fructose	0.73
3	20.505	Isomero D-fructose	1.30
4	21.961	Methyl ester of hexadecanoic acid	0.63
5	22.046	Glucose	0.41
6	23.888	Glucose isomer	0.66
7	24.315	Hexadecanoic acid	0.81
8	25.221	9-octadecenoic acid methyl ester	1.19
9	27.338	Oleic acid	1.34
10	34.282	Flavone (iso)	0.34
11	34.646	Flavone or flavanone (iso)	0.71
12	34.951	Flavanone (iso)	0.32
13	35.272	Tetracosanoic acid methyl ester	0.35
14	37.372	Flavone (iso)	1.89
15	37.516	Flavone (iso)	7.32
16	37.711	Flavone or flavanone (iso)	3.24
17	37.753	Flavanone (iso)	0.49
18	40.277	Flavone (iso)	4.16
19	42.868	Beta amirone	0.57
20	43.195	((3-beta) -lanosta-8,24-dien-3-yl) -oxi	1.60
21	43.384	Beta amyirin	5.80
22	43.804	Alpha amyirin	4.58
23	43.892	Triterpene isomer of amyirin	2.21
24	44.087	9,19-Cyclo-9-beta-lanostane-24-on-3-beta-iloxil	10.62

The molecular ions and the base peaks obtained in the mass spectrum and the possible structures for the identified flavonoids are shown in table 2.

Table 2: Molecular ions, base peaks and structures of flavonoids analyzed by mass spectrum

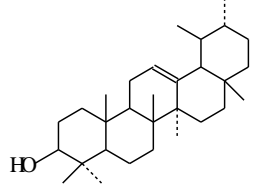
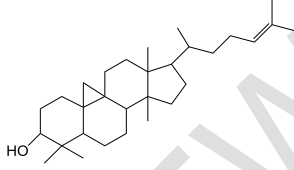
N°	Chemical compounds	M/Z molecular ion-base peak	Structures
10	Flavone (iso)	400 - 180	
11	Flavonone (iso)	402 - 180	

12	Flavonone (iso)	462 - 268	
14	Flavone (iso)	488 - 268	
15	Flavone (iso)	430 - 180	
16	Flavonone (iso)	490 - 268	
17	Flavonone (iso)	492 - 268	

The molecular ions and the base peaks obtained in the mass spectrum and the possible structures for the identified triterpenes are shown in table 3. The final signals of the gas chromatogram corresponded to natural triterpenes of the oleanane, ursane and lanostane groups. The comparison of mass spectrum fragment ions with mass spectral library allowed to identify some of the compounds. The analysis of the relative abundance of the detected signals suggested that the described structures represent 25.38% of the total components of the mixture (Table 3).

Table 3: Molecular ions, peaks and structures of triterpenes analyzed by mass spectrum

N°	Chemical compounds	Molecular ion- peaks	Structures
19	Beta amirone	424- 218, 203, 189	
20	((3-beta) -lanosta-8,24-dien-3-yl) -oxi	498- 483, 393	
21	Beta amyirin	498- 218, 279, 205, 203, 189, 133	

22	Alpha amyrin	498- 218, 279, 205, 203, 189, 133	
24	9,19-Cyclo-9-beta-lanostane-24-on-3-beta-iloxil	498 - 408, 393, 365, 339	

Taking into account the results, the chemical study showed that during the first 30 minutes of the chromatographic run, chemical composition is similar to other propolis collected in other geographical areas. Mainly, polyhydroxy compounds, sugars and organic acids were observed. The presence of polyols in general and sugars in particular, is an inherent characteristic of propolis of any geographical origin. Although sugars are not fundamental constituents of the plant exudates that bees collect to produce propolis, they are found mainly in the nectar that the insect collects to produce honey. Therefore, samples of propolis always contain different kind of sugar. It is important to note that the manipulation by human being collecting bee products (honey and propolis) can also modify the content of these kind of compounds (Huang 2014, Elnakady et al. 2017).

From the identified triterpenes in the sample, 9,19-cyclo-9-beta-lanostane-24-on-3-beta-iloxil (cycloartenol) were recognized as the major compound with 10.62% relative abundance. Meanwhile, within the flavonoids, flavone (iso) M⁺430 showed a relative abundance of 7.32%.

Triterpenes and flavonoids are secondary metabolites widely distributed in the plant kingdom, so it is common to find them in samples of propolis from other geographic latitudes and also with other ecuadorian propolis from different areas in the country (Cuesta et al. 2017). So, it could be possible to suggest that the bee collects resins of plant species that is closely related to each other, regardless of the geographical latitude in which it is found. In particular, cycloartane type triterpenes such as cycloartenol, the main compound described in this study, have been used as chemical marker of plant source from, considering that its presence could be indicating that *Mangifera indica* L is one of the plant source (Popova et al., 2021).

Considering the chemical profile showed in this work, it could be possible that the studied sample could belong to type III or Cuban yellow propolis, nevertheless further studies should be carried out to elucidate the structures of the unsolved compounds and confirm its classification.

The chemical profile could be different with other propolis samples obtained in the country, reaffirming the need to study a greater number of propolis samples in order to classify them according to their chemical structure and establish an adequate and reproducible strategy to obtain a reliable classification.

Since propolis has been used in folk medicine by different civilizations over centuries and particularly it has attracted interest for its anti-inflammatory and analgesic properties. the aim of this research was to study the characteristics related to these pharmacological activities of the studied propolis.

In particular, its topical anti-inflammatory activity was tested in the ear edema in mice and the results are shown in Figure 2. The maximal anti-inflammatory activity (inhibition of 77.2 %) was obtained at a dose of 3 mg/ear. Meanwhile, the anti-inflammatory control drug, indomethacin (1 mg) exhibited anti-inflammatory activity with an inhibition of 95.7 %.

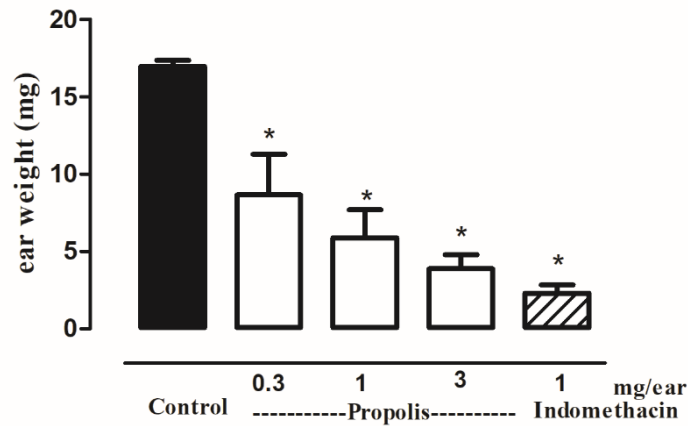


Fig. 2. Effect of propolis (0.1, 1, 3 mg/ear) and indomethacin (1 mg/ear) on ear weight.
Results represent means \pm SEM. * $P = .05$ compared to control group (saline group)

Histological analysis showed that TPA induced intense dermal edema, cellular infiltration of inflammatory cells and increased thickness compared with the control group, treated with the vehicle. But, these events were markedly reduced in the ear's tissue of the animals treated with propolis and indomethacin (Figure 3).

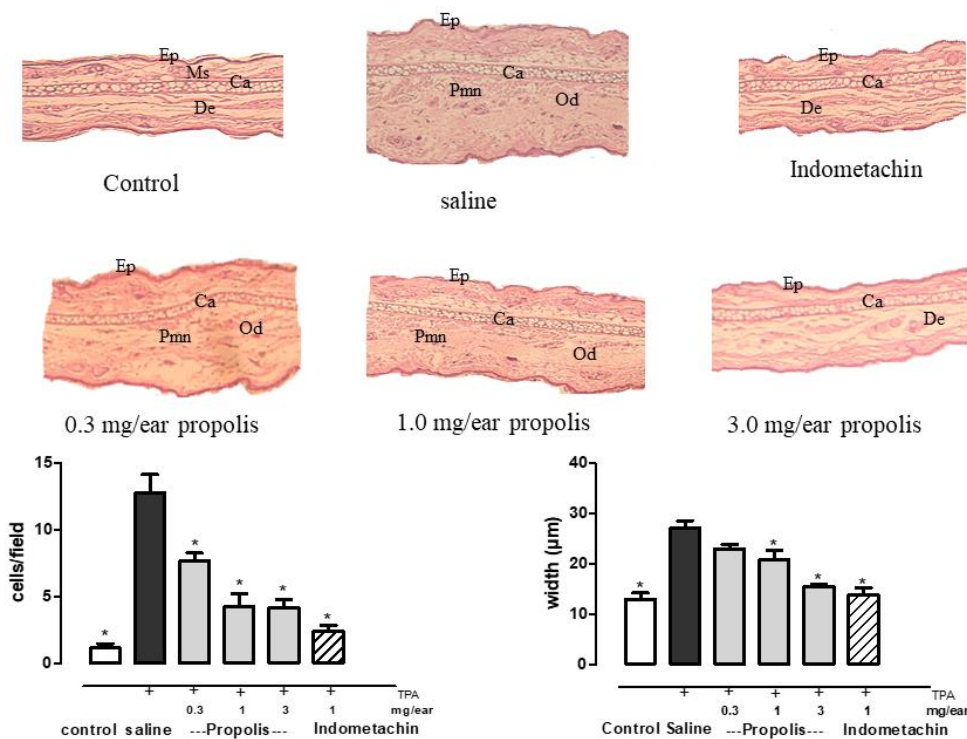


Fig. 3. Histological study: effect of propolis (0.1, 1, 3 mg/ear) and indomethacin (1 mg/ear) on ear
Results represent means \pm SEM. * $P = .05$ compared to saline group

TPA induce irritant effect on the skin, producing edema and migration of leukocytes into the dermis. In these events protein kinase C (PKC) pathway is involved. PKC comprises a family of serine/threonine kinase, that regulate different cellular processes such as proliferation, migration and cell survival. The topical application of propolis were able to reverse the edema formation and reduce the polymorphonuclear migration to the inflamed tissue, demonstrating that it could possess a direct action on PKC activity, reducing signs of inflammation induced by flogogen agent. Taking into account the chemical composition of the studied propolis, it could be possible that triterpenes could be responsible of the

anti-inflammatory activity observed. Numerous authors have demonstrated the anti-inflammatory capacity of pentacyclic triterpenes, metabolites present the studied sample (Bracho et al. 2009; Ouviaña, et al. 2009; Riedel et al. 2014). Particularly, β -amyrin identified in studied sample EP but also isolated from other natural sources showed anti-inflammatory activity in preclinical studies. In the same sense, α,β -amyrone showed to possess anti-inflammatory activity in phenol-induced ear edema model, being as potent as dexamethasone (Krishnan et al. 2014).

During inflammatory condition, several factors could reduce tissue pH and it is known that the protons are capable to stimulate cutaneous neurons and activate nociceptors, such as TRPV1 receptors and acid-sensing ion channels on sensory free nerve endings, being able to develop chemogenic inflammatory pain (Muley et al. 2016). Therefore, acetic acid writhing test, a classical animal model of inflammatory pain, was used to analyze the activity of propolis in this experimental condition. In this sense, Propolis (125-500 mg/kg, po) produced a dose-related inhibition of acetic acid-induced writhing response with a maximal antinociceptive effect (49.5%) at dose of 500 mg/kg po. Meanwhile indomethacin (reference drug) produced a significant inhibition (77.1%) of the writhing response (Figure 4).

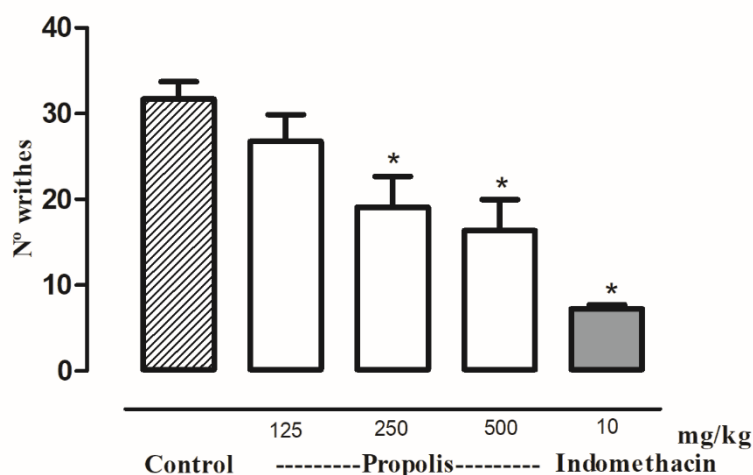


Fig. 4. Antinociceptive effect of propolis (125, 250 and 500 mg/Kg) and indomethacin as reference drug (10 mg/Kg) on acetic acid writhing test. Results represent means \pm SEM. * $P = .05$ compared to control group (saline group)

Since prostaglandins, cytokines and bradykinins could be released by acetic acid administration and sensitize visceral nociceptive afferents, it could be possible that propolis interfere in the release of these mediators involved in order to induce antinociceptive action. Additionally, alfa and beta amyrin identified in the extract could be responsible for the antinociceptive activity, since it was reported that these compounds induced a significant antinociception in the visceral model pain used in this investigation (Otuki et al. 2005). However, other structures could be involved in the effect described in the whole extract.

Taking together, the results of the current study suggest that propolis produced by *Apis mellifera* exert anti-nociceptive and topical anti-inflammatory effect, supporting the potential use of this propolis as a therapeutic agent for the alleviation of inflammation and/or inflammatory pain.

Flavonoids and terpenoids could be responsible of the beneficial profile observed in the equadorian propolis sample studied, since anti-inflammatory and analgesic activities of other propolis are attributed to the presence of pentacyclic triterpenes such as α -amiryn and β -amyrin, identified in the studied sample (Yam-Puc et al. 2019) and also seems to be associated with the presence of flavonoids in propolis, that reduce the cyclooxygenase activity in order to decrease prostaglandins and produce a reversion of inflammation process and also pain (Araujo et al., 2012, Valenzuela-Barra et al., 2015).

4. CONCLUSION

The present work showed predominant triterpene profile for the studied sample that could explain, at least in part, the anti-inflammatory and antinociceptive activities of it. Although flavonoids, sugars, polyols and fatty acids have been also identified. The chemical composition of a specific propolis that support the pharmacological activity could help to obtain standardized preparations and make safer and more efficient use of the beneficial properties of propolis.

ETHICAL APPROVAL

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed. The experiments were approved by the local Ethics Committee (Exp-FyB: 06517813)

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