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Anti-inflammatory Effects and Safety of Extracts and Essential Oil from *Clinopodium gilliesii* (muña muña)

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ABSTRACT

The chemical machinery of vegetables offers a great diversity of biological properties and plays a fundamental role in the field of pharmacology. The search for new drugs with fewer adverse effects represents a challenge for researchers. The objective of the present work was to carry out to investigate the medicinal properties and safety of extracts and essential oil from aerial parts of *Clinopodium gilliesii* (muña muña) and evaluate their antioxidant and anti-inflammatory activities *in vivo*. The extractions were carried out serially, using 96º alcohol (EE) and boiled distilled water (AE). The essential oil (EO) was obtained by steam dragging. The AE *in vivo* anti-inflammatory activity was determined by carrageenan induced plantar edema (acute inflammation) and granuloma formation by cotton disc (chronic inflammation) at oral doses of 250 and 500 mg/kg while that of the EO was established topically at doses of 50, 100µl/kg. *In-vitro* antioxidant activity was evaluated by DPPH depuration and inhibition of lipid peroxidation (β-carotene-linoleic acid method). Chemical study of the extracts was carried out by means of phytochemical screening and the essential oil was analyzed by GC-MS chromatography. The safety was evaluated with test of acute toxicity (48 h) and acute dermal toxicity (14 days). The results revealed that EE and EO had a significant acute and chronic anti-inflammatory activity, compared with positive patterns. EE (500 mg/kg), EO (100µl/kg), ibuprofen (100 mg/kg) and meprednisone (5 mg/kg) significantly reduced the weight of the exudate and cotton disc granuloma (24.17, 35.30, 45.56 and 57.17% respectively). The alcoholic and aqueous extracts presented important antioxidant activities with values higher than 90% (from 400µg/ml) in both methods and similar to the positive patterns (BHT and quercetin). The chromatographic profile of volatile oil compounds showed a great richness in terpene substances, pulegone, menthone and neomenthol, being its major constituents. The hydroalcoholic extracts revealed the presence of reducing compounds, polysaccharides, tannins, triterpenes, sterols and coumarins as major phytoconstituents. In the acute toxicity study, a single dose of 4000 and 8000 mg/kg b.w., produced no mortality and no clinical signs of disease were observed after 48 h. The essential oil at a single dose of 2000 and 5000 mg/kg of body weight did not produce treatment-related signs of toxicity or mortality in all rats tested during the 14 day observation period. These findings are encouraging to continue studies for the validation of popular use and development of a phytopharmaceutical with medicinal utility.

Keywords: *Clinopodium gilliesii* – *Satureja parviflora* – muña muña – Anti-inflammatory activity – essential oil.

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INTRODUCTION

Aromatic plants have been used since ancient times as medicinal therapy, as well as for preserving and aromatizing food, but only in the last decade has scientific research focused its interest on its essential oils and natural extracts as sources of antimicrobial, antioxidants and anti-inflammatory compounds. It has been reported that a large number of aromatic plants exhibit medicinal properties, including *Lippia graveolens, Clinopodium marostenum, Schinus molle, Artemisia ludica, Origanum vulgare* and *Laurus nobilis* and several species in Argentina and Latin America. [1-4] *Clinopodium gilliesii* (Benth) Kuntze, belongs to the Lamiaceae family (synonym of *Satureja parviflora*) and grows in Peru, Chile, Bolivia and Argentina. This species is popularly known as "muña-muña" in Argentina. [5] *C. gilliesii* is a shrubby, native plant, up to two meters high, that grows between 1200 and 4000 m high in the mountainous regions of the northwest, west and center of Argentina, in the provinces of Catamarca, Córdoba, Jujuy, La Rioja, Mendoza, Salta, San Juan and Tucumán. It is traditionally used in cooking, as an aromatic plant, and in traditional medicine, against colds, female sterility and altitude sickness; furthermore, it is also known as an aphrodisiac, and emmenagogue. Aerial parts in infusion are recommended against gastric troubles, stomachache, and haemorrhage. [6-8]

Previous investigations on the chemical composition of *Satureja* species revealed the presence of phenolic acids, flavonoids, amino acids, metals, sterols, diterpenes and triterpenes. [5] Some biological activities have also been reported like antioxidant and vasodilator. [5, 9]

Essential oils (EOs) are liquid mixtures of volatile compounds obtained from aromatic plants. They constitute what is called the "essence" of a plant and usually have pleasantly scented fragrances. Some of their purported antiseptic, antioxidant, and anti-inflammatory beneficial properties have been supported by recent scientific investigation. [10-11] Additionally, several essential oils have been attributed good antioxidant properties that play a fundamental role in the protection of other materials like food against such pathologies as rancidity. [12-13] The essential oil from *C. gillesii* has been studied by several authors and the results in composition have shown remarkable differences. Other authors have described the presence of piperitone, piperitenone, piperitenone oxide and *cis*-piperitenone epoxy and menthol, carvacrol and carvacryl acetate. [7, 14-17] Two triterpenic acids (ursolic and oleanolic) and two flavonoids (luteolin and eriodictyol) have also been found. [18] Exceptionally other monoterpenoids: (E)-Isocitral, pulegone, isopulegyl acetate, ρ-cymene, menthol, are also present. [19-20] Additionally, Hernandez *et al*., 2006 demonstrated the insect repellent, antifungal and antibacterial activities of the muña muña essential oil.

Differences in the chemical composition of essential oils are associated with potential toxicity risks and also with different healing properties of plants and plant extracts. This latter is a fact that so far has not been taken into account. Considering that this plant species is highly used in ethnomedicine in different countries, for treatments of discomforts and diseases, its potential toxicity is urgently required in order to improve its management for the treatment of human health.

The present study we reports for first time, the pharmacological screening in vivo of extracts and essential oil from *Clinopodium gilliesii*. We evaluate its antioxidant and anti-inflammatory properties, as well as its acute toxicity by oral administration of extracts and topical administration of essential oils.

MATERIALS AND METHODS

Plant material

We worked with the aerial parts of *Clinopodium gilliesii* (Benth.) Kuntze specimens, collected in Famatina, province of La Rioja, Argentina between 2000 and 3000 m ASL, in the month of February 2014. The plant material was identified by Lic. Gloria Jaime. Voucher specimens have been deposited in Herbarium of Cátedra de Farmacobotánica (Facultad de Bioquímica, Quimica y Farmacia, Universidad Nacional de Tucumán, Argentina). The species was determined with reference material from the Miguel Lillo Institute Herbarium, LIL. Nº 420243. Its identity and update (scientific name) was made with specific bibliography. [22-24]

Preparation of extracts

Dried and finely divided plant material was extracted in serial form with two solvents of different polarity. The first extraction of the aereal parts is performed with 96% ethanol after 5 contact days. Then, the residue was extracted with boiled distilled water during 20 min, for obtaining the ethanol (yields of 10.55%) and aqueous extracts (yields of 25.87%), respectively. The extracts were filtered through Whatman paper no. 1 and

evaporated to dryness. All dry extracts were stored in sterile eppendorf at 4°C until used.

Isolation procedure of essential oil

For essential oil extraction, 200 g of dried aerial parts of *C. gilliesii* were subjected to hydro-distillation for 3 h using a Clevenger-type apparatus. The oil was obtained in yields of 2.51%. Anhydrous sodium sulphate was added to the oil to eliminate remaining water being then stored in an amber flask at −4°C until chemical analyses.

Phytochemical analysis of ethanol and aqueous extract of *C. gilliesii*

The aqueous and ethanol extracts were evaluated by phytochemical qualitative reactions for usual plant secondary metabolites. The intensity of color or the precipitate formation was used as analytical responses to these tests. [25-26]

Determination of total phenol content

Total phenolic content was estimated by the Folin–Ciocalteu method. [27] The absorbance at 765 nm was measured. Gallic acid (0-10 mg/l) was used for the standard calibration curve. The results were expressed as mg gallic acid equivalent (GAE)/100g dry weight of vegetable material, and calculated as mean value ± SD (n = 6).

Total flavonoid content

Total flavonoids content was determined by the colorimetric). [28] The absorbance was measured at 430 nm. Quercetin (0-10 mg/l) was used for the standard calibration curve. Total flavonoids contents were calculated as mg quercetin equivalent (mg QE /100g dry weight of vegetable material and calculated as mean value ± SD (n = 6).

Gas chromatography - mass spectroscopy (GC-MS) analysis of Essential oil *C. gilliesii*

Mass spectra were recorded on a 5973 Hewlett Packard selective mass detector coupled to a Hewlett Packard 6890 GC using Perkin Elmer Elite-5MS capillary column (5% phenyl methyl siloxane, length 30 m, inner diameter 0.25 mm, film thickness 0.25µm). The injector, GC-MS interphase, ion source and selective mass detector temperatures were maintained at 250°C, 280°C, 230°C and 150°C, respectively; ionization energy, 70 eV; injection size: 0.1µL (10% solution in ethyl acetate) (split mode). He was used as carrier gas at a flow rate of 1.0 $mLmin^{-1}$. The oven was programmed as follows: 60°C for 0 min, 60-300°C at 3.0°C min^{-1} and 300°C for 10 min. The identification of components of the oil was based on comparison of their mass spectra with those found in the literature and mass spectrometry data bank commercial (NBS75K, NIST 98, WILEY275). The retention index was determined by co-injection of the homologous n-hydrocarbon C_8-C_{18} mixture with the oil sample. Percentages are the mean of three runs and were obtained from electronic integration measurements using selective mass detector. [29]

Antioxidant activity

DPPH scavenging activity

The antioxidant activity was assessed by the measurement of the scavenging ability of extracts towards the stable free radical 1,1 biphenyl -2-picrylhydrazyl (DPPH). [30] Absorbance at 517 nm was measured versus ethanol as a blank. Quercetin (1.50µg/ml-10 mg/ml), a natural antioxidant and butylated hydroxytoluene (BHT) (1.56µg/ml-10 mg/ml), a synthetic antioxidant one, were used as reference solutions (n=6). The degradation of DPPH was evaluated against a control (0.25 ml of DPPH solution and 0.75 ml ethanol 96 %). Antioxidant activity was expressed as percentage inhibition of the DPPH radical and was determined by the equation:

AA % = (Abs control – Abs simple/ Abs control) × 100

β-carotene bleaching method

The antioxidant activity of extracts and essential oil of *C. gilliesii* were evaluated using β–carotene–linoleate model system, as described by Sun & Ho (2005). Quercetin or BHT was used as positive control and ethanol was the negative control. The absorbance at 470 nm, which was regarded as to, was measured immediately against a blank consisting of the emulsion without β–carotene. The antioxidant activity (AA) was measured in terms of successful bleaching of β–carotene by using the equation:

$$AA = [1- (A_0 – At / A_{00} – At_0)] \times 100$$

Where A_0 and A_{00} were the absorbance values before incubation for test sample and control respectively. At and At0 were the respective absorbances of the test sample and the control after incubation for 120 min. The results were expressed as % of the prevention of bleaching of β–carotene.

Anti-inflammatory activity study

Animals

Male Wistar rats (weighing 190–240 g) used for this study were obtained from the Bioterio de la Facultad de Bioquímica, Química y Farmacia, Instituto de Biología (INSIBIO), Universidad Nacional de Tucumán. The rats were first left for 7 days to acclimatize to laboratory conditions. All animals were kept under normal laboratory conditions of humidity, temperature (25 ± 1°C) and light (12 h dark/light cycle), and allowed free access to food and water *ad libitum*. The studies were conducted in accordance with the internationally accepted principles for laboratory animal use and care (EEC Directive of 1986; 86/609/EEC). All the experimental protocols were approved by the institutional committee for the care of laboratory animals of the National University of Tucumán (CICUAL).

Carrageenan-induced hind paw edema in rats

Paw edema was induced in rats by carrageenan injection 0.1 ml of 1.5 % (w/v) into the sub plantar region of the right hind paw of the rats according to the method described by Winter *et al* (1962). All rats (six per group) were given free access to food and water after the sub plantar injections. Control group rats received saline solution [0.9% (w/v) NaCl] (2 ml/kg) and the reference group received 100 mg/Kg

ibuprofen, orally. The test groups of rats were treated orally with 250 and 500 mg/kg of the ethanol and aqueous extracts of *C. gilliesii,* orally and 50 and 100µl/kg of essential oil topically, 30 min before the carrageenan injection. The paw volume was measured before administering carrageenan (Vo) and 1, 2, 3, 4 and 6 h after (Vt). Inflammation was calculated as the increase in volume (ml) of the paw after treatment subtracted of the basal volume. Results were expressed as percentage of inhibition of edema, calculated according to the following formula [(Vt−Vo)/Vo] × 100. [33]

Cotton pellet-induced granuloma formation

Male rats weighing 180–200 g were randomly divided into seven groups of six rats each. Two sterilized cotton pellets (20 mg) were implanted subcutaneously, one on each side of the abdomen in all groups, under light ether anaesthesia. Rats in groups I (control group) received vehicle. Rats in groups II and III received ibuprofen and meprednisone, at the dose of 100 and 5 mg/kg/day orally, respectively. Rats in groups IV to VII received ethanol and aqueous extracts of *C. gilliesii* at the dose of 250 and 500 mg/kg/day orally respectively. Rats in groups VIII y IX received essential oil of *C. gilliesii* at the dose of 50 and 100µl/kg/day topical respectively. Each test substance was administered for 7 d. On the eighth day, each rat was anesthetized. The rat was then sacrificed and the implanted pellets as well as the thymus were dissected out and determined for their wet and dry weights (dried at 60 1C for 18 h). The granuloma and transudative weights and the percent inhibition of granuloma formulation of the test compounds were calculated. [34]

Acute oral toxicity study

The animals were divided into three groups, with six animals each. They were treated orally with a single dose of ethanol extract of *C. gilliesii* dissolved in distilled water and doses of 4000 and 8000 mg/kg in 10 ml/kg volume. The control group received distilled water as a single dose. All animals were observed after treatment. The parameters evaluated were: death, alertness, sedation, ptosis, dyspnea, urination, diarrhea, convulsions, spontaneous motor activity, postural reflex, piloerection, response to touch, among others. The total number of deaths in each group was quantified by the end of the period of 48 h. [35]

Acute dermal toxicity study

The study was carried out according to the proposal to the new guide 434 of the OECD (Organization for Economic Co-operation and Development). [36]

Ten Wistar rats (5 of each sex) were used for each group, the back of the animals was shaved 24 hours before starting the procedure taking care not to damage the skin, in an area of the back approximately 10% of the corporal surface. 10 mL of essential oil was applied to the treated group and equal amount of distilled water to the control group in the shaved area, covered with a gauze dressing and fixed with tape. After 24 h, the bandage was removed, cleaning any residue from this area. During this at the time, symptoms and clinical signs of toxicity were observed. The weight of the animals was recorded at the beginning, at 7 and 14 days after the experiment. After this time the animals were sacrificed and a macroscopic study of the main organs was carried out.

Statistical analysis

Data obtained from animal experiments were expressed as the mean and standard error of the mean (mean ± S.E.M.). Statistical differences between the treated and the control groups were evaluated by ANOVA and Dunnett's tests. The criterion for statistical significance was $p < 0.05$.

Table 1: Phytochemical screening of aqueous and ethanol extracts of *Clinopodium gilliesii*

Phytoconstituents of *Clinopodium gilliesii*	Aqueous extract	Ethanol extract
Amides	-	-
Mucilage	-	-
Reducing compounds	+	+
Polysaccharides	+	+
Catechistic Tannins or Proanthocyanins	+	+
Alkaloids	-	+
Saponins	-	-
Leucoanthocyanins	-	-
Anthacenoids	-	-
Coumarinc	+	-
Flavonoids	-	-
Triterpenic Sterols	-	+

(+) Presence (-) Absence

RESULTS

Phytochemical analysis of *C. gilliesii* extracts

The phytochemical analysis of ethanol and aqueous extracts of *C. gilliesii* confirmed the presence of reducing compounds, polysaccharides, catechistic tannins or proanthocyanins, coumarins and triterpenic sterols (Table 1).

Quantification of phytoconstituents

Total polyphenol content was higher in the alcoholic than in the aqueous extract, 129.40 ± 7.90 and 297.55 ± 12.95 mg GAE / 100 g m.v. respectively. The same was observed in the flavonoid content, the alcoholic extract being richer in these compounds than the aqueous extract, 974.41 ± 15.50 and 266.05 ± 10.80 mg QE / 100 g m.v. respectively.

Analysis of *C. gilliesii* essential oil

Twenty three components were identified in the essential oil of *C. gilliesii.* The major components were pulegone (57.1 %), menthone (13.8 %), neomenthol (6.0 %), cis-dihydrocarbone (4.2 %) and caryophyllene (2.7 %). The oil chemical composition may be seen in Table 2.

Biological activity evaluation

Antioxidant activity

The antioxidant activity of muña muña extracts and essential oil was examined by comparing it with the activity of known antioxidants such as BHT and quercetin using in vitro inhibition of DPPH radical and the β-carotene bleaching method. These results showed

that EE and AE of *C. gilliesii* were able to reduce the stable free radical DPPH with an IC_{50} of 226.00 and 201.42µg/ml respectively, whereas the reference antioxidants Quercetin and BHT showed an IC_{50} of 2.00 and 80.00µg/ml respectively (Figure 1). The muña muña essential oil, presented a very low antioxidant activity and the IC_{50} value in the range of concentrations tested could not be determined.

Figure 2 shows the decrease in absorbance of β-carotene emulsion in presence of the extracts, essential oil and the reference antioxidants (BHT and Quercetin). The addition of 2 mg/ml of EE, AE and 15 mg/ml of EO of *C. gilliesii* was significatively effective in inhibiting the oxidation of linoleic acid and subsequent bleaching of β- carotene, in comparison with the control ($p < 0.05$). Activity percentages were EE (67.03%), AE (55.02%), EO (52.85%), BHT (95.00%) and Quercetin (93.00%). The results indicated that the extracts and essential oil of muña muña were effective antioxidants in a β-carotene linoleic acid model system at the doses tested.

Table 2: Percentage composition of the essential oil of *Clinopodium gilliesii*.

Compounds	SP16JUN %MS	RI#	Identification$^{\Phi}$
α-pinene	0.1	932	MS, RI, co-GC
Sabinene	0.1	969	MS, RI, co-CG
p-cymene	0.1	1020	MS, RI, co-GC
Limonene	0.1	1024	MS, RI, co-GC
1,8 cineole	1.1	1026	MS, RI, co-GC
γ-terpinene	Tr	1054	MS, RI, co-GC
p-mentha-3,8-diene	0.5	1068	MS, RI, co-CG
Terpinolene	Tr	1086	MS, RI, co-CG
Linalool	0.5	1095	MS, RI, co-CG
menthone	13.8	1148	MS, RI
neo-menthol	6.0	1161	MS, RI
Menthol	0.4	1167	MS, RI
cis-dihydrocarvone	4.2	1191	MS, RI
Pulegone	57.1	1233	MS, RI
menthyl acetate	0.9	1294	MS, RI
dihydro carveol acetate	0.4	1306	MS, RI
piperitenone	0.3	1340	MS, RI
piperitenone oxide	0.8	1366	MS, RI
β-caryophyllene	2.7	1417	MS, RI, co-GC
α-humulene	Tr	1452	MS, RI, co-CG
germacrene-D	1.3	1484	MS, RI, co-GC
byciclogermacrene	1.5	1500	MS, RI
δ-cadinene	0.3	1522	MS, RI, co-GC
Total	92.2		

tr: trace <0.05; RI# the retention index ADAMS; $^{\Phi}$Co-GC: co-injection with an authentic sample. Percentages are the mean of three runs and were obtained from electronic integration measurements using a selective mass detector

Table 3: Effect of extracts and essential oil of *Clinopodium gilliesii* on edema carrageenan-induced rat paw edema.

Group (n=6)	Dose		Paw edema vol in ml (Mean ± S.E.)						
	mg/kg bw (oa)	µl/kg/bw (ta)	0 H[a]	1 H[a]	2 H[a]	3 H[a]	4 H[a]	5 H[a]	6 H[a]
Control	s.s.		1.30 ± 0.10	1.60 ± 0.05	1.75 ± 0.10	1.80 ± 0.05	1.80 ± 0.10	1.95 ± 0.10	2.07 ± 0.12
Ibuprofen	100		1.40 ± 0.05	1.45 ± 0.15 (*)	1.40 ± 0.10 (*)	1.40 ± 0.10 (*)	1.45 ± 0.15 (*)	1.45 ± 0.05 (*)	1.55 ± 0.15 (*)
AE	250		1.40 ± 0.05	1.65 ± 0.10	1.68 ± 0.26	1.75 ± 0.20	1.78 ± 0.15	1.98 ± 0.30	1.95 ± 0.40
	500		1.30 ± 0.10	1.55 ± 0.10	1.65 ± 0.10	1.70 ± 0.10	1.75 ± 0.10	1.80 ± 0.20	1.79 ± 0.16
EE	250		1.30 ± 0.10	1.45 ± 0.10 (*)	1.60 ± 0.20	1.65 ± 0.15	1.70 ± 0.15	1.85 ± 0.15	1.90 ± 0.15
	500		1.30 ± 0.05	1.40 ± 0.05 (*)	1.40 ± 0.10 (*)	1.40 ± 0.10 (*)	1.50 ± 0.15 (*)	1.65 ± 0.25	1.70 ± 0.10 (*)
EO		50	1.43 ± 0.15	1.63 ± 0.06	1.70 ± 0.20	1.75 ± 0.20	1.80 ± 0.10	1.90 ± 015	1.95 ± 0.15
		100	1.40 ± 0.05	1.60 ± 0.05	1.40 ± 0.05 (*)	1.45 ± 0.05 (*)	1.45 ± 0.05 (*)	1.73 ± 0.32	1.80 ± 0.20

SS: saline solution; oa: oral administration; ta: topical administration; Values are expressed in mean ± S.E.M. ($n = 6$). [a] Time after carrageenan injection (h).
* Statistically significant from control group: $p < 0.05$.

Table 4: Effect of extracts and essential oil of *Clinopodium gilliesii* on cotton pellet-induced granuloma formation in rats.

	Cotton pellet-induced granuloma formation					
	Dose		Transudative	Granuloma	Granuloma	Dry Thymus
Groups (n=6)	mg/kg/d (oa)	µl/kg/d (ta)	Weight (mg)	Weight (mg)	Inhibition (%)	Weight (mg/100 g BW)
Control	ss		594.35 ± 25.60	135.40 ± 13.30	--	30.49 ± 2.06
Ibuprofen	100		178.90 ± 15.50 (*)	76.20 ± 2.80 (*)	43.72	33. 24 ± 3.40
Meprednisone	5		165.10 ± 14.85 (*)	55.80 ± 9.00 (*)	58.78	22.61 ± 2.95 (*)
AE	250		497.15 ± 19.77	123.15 ± 16.86	9.04	26.87 ± 5.93
	500		445.48 ± 46.02	119.20 ± 11.95	11.85	27.72 ± 6.50
EE	250		313.05 ± 14.60 (*)	94.15 ± 10.99 (*)	30.46	26.87 ± 6.13
	500		310.03 ±15.23 (*)	82.33 ± 11.46 (*)	39.19	27.68 ± 5.65
EO		50	384.80 ± 27.2 (*)	122.90 ± 13.60	9.23	31.15 ± 1.50
		100	290.00 ± 29.20 (*)	87.60 ± 1.60 (*)	35.30	33.11 ± 2.80

Values are expressed as mean ± S.E.M. (n=6). TrW: Transudative weight, GrW: Granuloma weight, GI: Granuloma inhibition, BW: Body weight, TW: Thymus weight. * Significantly different from the control group, $p<0.05$. SS (saline solution); oa: oral administration; ta: topical administration

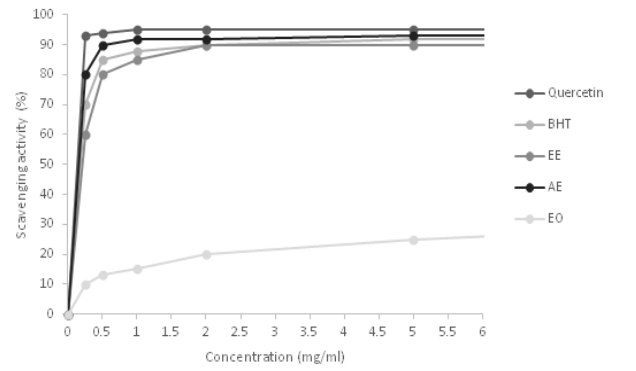

Fig. 1: DPPH radical scavenging activity of the Ethanol extract (EE), Aqueous extract (AE) and Essential oil (EO) of *Clinopodiun gilliesii*. Quercetin and BHT were usedas reference antioxidants. Values represent the mean ± S.E.M. (n=6).

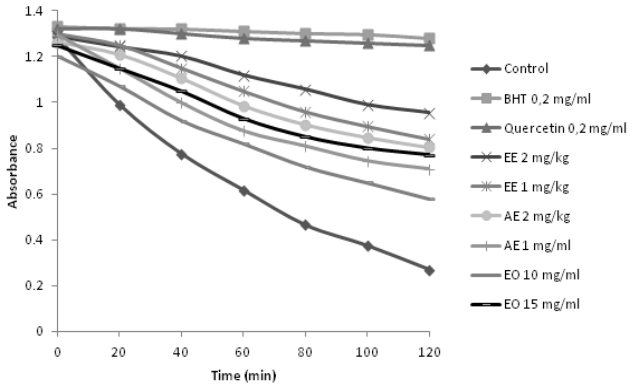

Fig. 2: Antioxidant activities of the Ethanol extract (EE), Aqueous extract (AE) and Essential oil (EO) of *Clinopodiun gilliesii* measured by the b-carotene bleaching method. Quercetin and BHT were used as reference antioxidants. Values represent the mean ± S.E.M. (n=6).

Anti-inflammatory activity

Carrageenan-induced rat paw edema

In the carrageenan-induced edema test, the average right back paw volumes by the extracts and standard drug are shown in Table 3. The injection of the phlogistic agent started localized edema in the control group at 1.0 h after injection. The swelling increased progressively to a maximum volume of 2.07 ± 0.12 ml at 6.0 h after the carrageenan injection. Orally pre-treated rats with the ethanol extract of *C. gilliesii* and topical application of the essential oil, presented a significant edema reduction 2.0 h post-dosing at 500 mg/kg and 100µl/ml respectively. This behaviour was similar to that of the standard, ibuprofen (100 mg/kg, *p.o*). The aqueous extract, did not show any activity in this test.

Cotton pellet-induced granuloma formation

Ibuprofen and meprednisone at a dose of 100 and 5 mg/kg/d, ethanol extract at a dose of 500 mg/kg/d orally and essential oil (100µl/kg/d, topical), significantly reduced transudative and granuloma weights as shown by their granuloma inhibition of 45.56%, 57.10%, 24.17% and 35.30% respectively (Table 4). Dry thymus weights were not significantly different in the control, ibuprofen, essential oil, aqueous and ethanol extracts groups, except in the meprednisone group which revealed a significant decrease. The aqueous extract did not show any activity in this test.

Acute oral and dermal toxicity

No deaths or toxic symptoms were observed in any of the animals after oral and topical administration of the different doses of the ethanol extract and essential oil. There were no changes in body weight or food and water intake between the control and the treated groups. The treated rats did not present any behavioral alterations during the assessment period. These results suggest that single oral doses of 4000 and 8000 mg/kg b.w. and single topical doses of 2000 and 5000 mg/kg b.w safe to use in rats.

DISCUSSION

C. gilliesii has an important ethnobotanical value, emphasizing its use as condiment as well as its applications as a medicinal plant. [37] Essential oils are plant secondary metabolites extensively used in aromatherapy and traditional medicine for their different pharmacological properties. Generally, essential oils of the Satureja genus have a significant variability in their chemical composition depending on climate, soil composition, plant organ, age and vegetative cycle stage. [38-39] In some species, like *Satureja montana,* variability in chemical composition leads to the definition of two chemotypes, namely A and B, depending on the prevalence of phenolic compounds (thymol and carvacrol) or terpenic alcohols (linalool, ρ-cymene and α-terpineol), respectively. [40] The high percentages of terpenes of essential oil from *C. gilliesii* reported in this study indicate that the plant could match chemotype A. Other authors agree that the major class of identified compounds in *S. parvifolia* was that of monoterpenes. [20] The results obtained for *Satureja macrantha* were similar. [41] Additionally, the ethanolic and aqueous extracts showed different qualitative and quantitative compositions (Table 1). The total phenolic contents of the two analyzed extracts ranged between ca. 129 and 297 mgGAE/gr, the aqueous extract being the richest, a fact similar to the results obtained by Cabana *et al*. (2013). The differences observed are due to the different extraction techniques and solvents used. The use of different extraction techniques modulates the chemical composition of *S. parvifolia* extracts and, consequently, their biological activities. The ethanol extract yielded the highest amount of flavonoid compounds and evidenced a significant anti-inflammatory activity. The qualitative analysis revealed that the ethanol extract contained distinct phytoconstituents, highlighting the presence of triterpenes (absent in the AE), responsible for the anti-inflammatory activity observed.

The DPPH assay has been extensively used for screening plant extracts because it is sensitive enough to detect active ingredients at low concentrations. [42] The antioxidant activity of *C. gilliesii* essential oil and extracts is summarized in figures 1 and 2. Among all the test samples, the ethanol extract of *C. gilliesii* was found to be the most potent antioxidant (IC_{50} 201µg/mL), followed by the aqueous extract (IC_{50}

values 226µg/mL). The essential oil of *C. gilliesii* showed a poor radical scavenging activity (IC_{50} values not determined). BHT and Quercetin were taken as reference antioxidants (IC_{50} 80 and 2 µg/mL, respectively). Results were similar with the lipid peroxidation inhibition method and the muña muña ethanol and aqueous extracts presented the highest activity. Essential oil showed a similar activity, but with a dose 5 times higher than that of the extracts, evidencing a lower antioxidant power. According to the results, it may be said that the essential oils rich in terpenes showed better values for the antioxidant activity in the β-carotene/linoleic acid system oxidation assay. This test can be especially useful for investigations of lipophilic antioxidants and is appropriate for the investigation of antioxidant activity of essential oils.

There is an ample scientific background regarding the correlation between antioxidant activity and flavonoid content in plant extracts. [5, 43-44] This group of compounds is also present in the extracts of the present study and other authors relate this activity to the content of polyphenols in the same way. [30, 45]

The antioxidant activity of aromatic plants has been a target of investigation due to their potential as preservatives, cosmeceuticals or nutraceuticals in the food and cosmetic industries. The low antioxidant activity found in the essential oil can be explained as a result from the absence of compounds such as flavonoids. [44] However, the presence of terpenes and sesquiterpenes is related to a low antioxidant activity. [46] These results, may explain the low antioxidant activity of *C. gilliesii* essential oils.

Inflammatory diseases are generally treated with steroidal and non-steroidal anti-inflammatory drugs. However, both of them have significant negative side effects, reducing their use in certain segments of the population. [1] Hence, there is a need to develop new drugs with novel modes of action and fewer side effects.

The antiinflammatory effect of *C. gilliesii* extracts and essential oil was investigated in the present study. The carrageenan test was selected because of its sensitivity in orally detecting active antiinflammatory agents, particularly in the acute phase of inflammation. [35] The intraplantar injection of carrageenan in rats leads to paw edema. Its first phase (0 – 3 h after injection) results from the concomitant release of mediators: histamine, serotonin and kinins in the vascular permeability. The second phase is correlated with the elevated production of prostaglandins, oxygen-derived free radicals, and inducible cyclooxygenase. [47] Oral administration of the ethanol extract and topical application of essential oil of *C. gilliesii* suppressed the oedematous response after 1 h and this effect continued up to 6 h. The observed effect was similar to that of Ibuprofen. We evaluated the anti-inflammatory properties of essential oil by topical route. The cutaneous or external route is the way that gives more security (the skin acts as a filter), the fastest and offers us a prolonged action. The essential oils have affinity with fats and easily penetrate the different skin layers to reach the peripheral microcirculation and the general circulation to exert their therapeutic action.

The inflammatory granuloma is a typical feature of chronic inflammatory reaction. [48] The cotton pellet granuloma method has been widely used to assess the transudative, exudative and a proliferative phase of chronic inflammation. The fluid adsorbed by the pellet greatly influences the wet weight of the granuloma, whereas the dry weight correlates well with the amount of granulomatous tissue formed. Most of the NSAIDs like ibuprofen show only a slight inhibition of the granuloma formation while the steroidal drug, on the contrary, exhibits a profound granuloma reduction. The ethanol extract and essential oil of muña muña, at a dose of 500 mg/kg and 100µl/kg, respectively elicited a significant inhibitory activity on the wet weight of granuloma and the proliferative phase of inflammation (inhibition of granuloma formation). The muña muña ethanol extract and essential oil were able to reduce paw edema and inflammatory cell infiltration in a chronic inflammation. These results suggest that the anti-inflammatory activity may be brought about by the biosynthesis inhibition of inflammation mediators such as prostaglandin, histamine or serotonin. Similar results were obtained by Perez *et al*., (2005) and Rungqu *et al*. (2016). The antinociceptive properties of the major constituent of the essential oil (pulegone) were studied in chemical and thermal models of nociception. [49-50] da Silveira e Sá *et al*., 2013 [51] determined the anti-inflammatory activity of 32 bioactive monoterpenes found in essential oils including menthone and neo menthol, second and third major constituents in muña muña. The oil data demonstrate the anti-inflammatory pharmacological potential of this group of natural chemicals.

Pulegone (57.1%) was the major component in the essential oil of *C. gilliesii* evaluated in this work. Carvajal *et al*., 2017 [52] reported the amount of pulegone (93.87%) in the essential oil of leaves of *C. gilliesii*, collected in the Chilean highlands, showed remarkable differences with those reported by other authors. (+)-Pulegone is a p-menthane monoterpene ketone [p-menth-4(8)en-3-one] found in essential oils from many mint species (e.g. *Minthostachys mollis* (Kunth) Griseb., *Minthostachys verticillata* (Griseb.) Epling *Hedeoma pulegioides* (L.) Pers. and *Mentha pulegium* L.). [53-55] Woolf, 1999 reported that this monoterpene was hepatotoxic and potentially, carcinogenic in male and female mice, and female rats. In general there is a concept that medicinal herbs are very safe and have no side effects, so the use of herbal medicine products have become universally popular in primary health care, especially in developing countries.

However, there are many very toxic compounds of natural origin such as cytotoxic drugs, anticancer drugs derived from plants, digitalis, pyrrolizidine alkaloids, ephedrine, phorbol esters, etc. [56] The adverse effects of most herbal drugs are moderately less frequent when the drugs are used appropriately compared with synthetic drugs. [57] A toxic substance might elicit interesting pharmacological effects at a lower non-toxic dose. Toxicity results from animals will be crucial in definitively judging the safety of medicinal plants if they are found to have sufficient potential for development into pharmacological products. Acute oral and dermal toxicity testing in laboratory animals were used to evaluate ethanol extract and essential oil of *C. gilliesi* natural remedies for antiinflammatoy activity. The extract and essential oil did not produce any mortality or alter the behavioral patterns of the rats during the acute toxicity testing; similar results were observed by Reynoso *et al*. (2013) and Lopez Barreiro *et al*. (2014) with other plant using the same toxicological method. [58]

This work reports the antioxidant activity and anti-inflammatory effect of ethanol extract and essential oils of *Clinoposium gilliesi*. These effects represent important pharmacological evidences for the medicinal use of *Clinoposium gilliesi*. Our research continues in order to establish the chemical structure of the compounds responsible for the observed pharmacological properties and deepen our understanding of their mechanism of action and sub chronic toxicity.

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