




Composition, antioxidant capacity, intestinal, and immunobiological effects of oregano (*Lippia palmeri* Watts) in goats: preliminary in vitro and in vivo studies

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Abstract

This study investigated *Lippia palmeri* Watt (oregano) phytochemical compounds, their antioxidant capacity, and immunological effects on goat peripheral blood leukocytes (PBL), and on the presence of intermediate polar compounds in goat feces fed dietary oregano. The polar and nonpolar fractions of *L. palmeri* W. were characterized and phytochemical contents and antioxidant capacity were determined. Twelve healthy Anglo-Nubian goats were used for the in vivo trials, which were randomly assigned to control fed with basal diet, or oregano group fed with basal diet + 2.6% (DM basis) dried oregano leaves. Goat peripheral blood leukocytes (PBL) were isolated for the in vitro study, and PBL were stimulated with oregano extracts at 100 and 150 µg/mL after 24 h. For the in vivo trial, dietary oregano (2.6% on DM basis) was evaluated in the goats for 90 days. Relatively high abundance of carvacrol and thymol phytochemical compounds was found in oregano. The highest antioxidant capacity of oregano extracts was detected at 100 and 150 µg/mL. Nitric oxide production, phagocytosis, and superoxide dismutase activities increased ($p < 0.05$) in stimulated PBL with oregano extracts, whereas the pro-inflammatory (TNF- α and IL-1 β) transcription and antioxidant (CAT and GPX-4) genes downregulated. In the in vivo experiment, dietary oregano enabled the detection of nine compounds found in goat feces, from which caproic (C6) was in a high relative quantity compared with the control group. Oregano has phytochemical compounds with strong antioxidant capacity that protect cells against oxidative stress damage and could modulate immune response and feces composition in goats.

Keywords Pro-inflammatory · Antioxidant activity · Immune system · Immunostimulants · Ruminants · Bioactive compounds

Introduction

Oregano is an aromatic plant appreciated for its organoleptic properties in many traditional and gourmet dishes. Several

therapeutic uses of oregano have been reported, including but not limited to the treatment of headache, diabetes, rheumatism, cough, and inflammatory disorder treatments (Dimayuga and Garcia 1991; Alonso-Castro et al. 2017; Gutiérrez-Grijalva et al. 2017). More than 60 species from 17 genera and different families are known as oregano, although the most relevant families are Verbenaceae and Lamiaceae (Calpouzos 1954). The medicinal effects of oregano have been related thus far to the presence and abundance of bioactive compounds of which polyphenols are the most widely studied. The bioactive compounds vary with species, subspecies, climate condition, geographic region, and edaphic characteristics (De Mastro et al. 2017). Some of the oregano species are well known for their antioxidant properties through their polyphenol content (Rodríguez-García et al. 2016). Antioxidant properties result from the capacity to reduce free oxygen radicals or induce the production of antioxidant enzymes in living organisms (Randhir et al. 2005; Paraskevakis 2015). Polyphenols are remarkable electron donors with

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relatively stable radical intermediates; thus, their main mode of action is encompassed in the type of free radical terminator or reducing agent, although some polyphenols can function as metal chelators and singlet oxygen eliminators (Shahidi et al. 1992). Currently, thymol, carvacrol, α -tocopherol, and 2-caffeoyl-oxy-3[2-(4-hydroxybenzoyl)-4,5-dihydroxy]-phenyl propionic acid have been reported in oregano as compounds responsible for antioxidant activities (Kulisic et al. 2004).

Oregano effects on modulating immune parameters have emerged as a novel approach (Gutiérrez-Grijalva et al. 2018). For instance, dietary oregano dried leaves in pigs (*Suis scrofa*) and earthworms (*Lumbricus terrestris*) modulated and enhanced immune responses, respectively (Stelter et al. 2013; Vatter et al. 2013). Similarly, some studies on leucocytes have reported pro-inflammatory, anti-inflammatory, or unaffected immune parameters after incubation with oregano extracts (Beltrán et al. 2018; De Santis et al. 2019). Although these inconsistencies might be related to many factors, it seems that the extract concentration has a critical impact on immune cell functions (Aytle et al. 2016). Nonetheless, the bioactive oregano compounds responsible for immunomodulatory activity are still unknown. For instance, most of the studies in animals, such as calves, poultry, and fish, have focused on evaluating the immunological effects of essential oils and whole extracts (Sabino et al. 2018; Beltrán et al. 2018; Stivanin et al. 2019).

Currently, to our knowledge, three studies on polyphenol content, antioxidant capacity, and immunomodulatory effects of the oregano *L. palmeri* W. have been published (Leyva-López et al. 2016; Gutiérrez-Grijalva et al. 2017, 2019). Therefore, the potential use of this oregano species remains almost elusive for its antioxidant and immunostimulant capabilities. Remarkably, the antioxidant and immunostimulant potentials of any oregano species have not been explored in goats. Under stress conditions, goats produce huge quantities of free radical oxygen species that can cause membrane cell disruption and a physiological redox imbalance named oxidative stress (Celi 2011). Additionally, oxidative stress also leads to immunosuppression and susceptibility to infectious diseases. Hence, this study aimed to determine oregano chemical composition, antioxidant capacity, intestinal, and immunostimulant effects on goats performing both in vitro and in vivo experiments.

Materials and methods

Lippia palmeri (Oregano) characterization

Lippia palmeri Watts collection

Oregano (*Lippia palmeri* Watts) leaves were collected from the experimental station of the Universidad Autonoma de Baja California Sur located in La Matanza (Ranch “El Palmar de en

Medio”; 23° 38.040' N and 110° 17.088' W) in the surroundings of La Paz, Baja California Sur, Mexico. The weather of the site is arid with an annual mean temperature of 21.2 °C (BWhw, Köppen system). Annual precipitation is about 182 mm and close to 80% is recorded from July–September. The main soil types are alkaline, regosol, eutric, and calcareous, which are very permeable (Armenta-Quintana et al. 2011). Plants were harvested at a flowering state by cutting 65% of the plant during the end of the rainy season (November). Leaves were separated, dried, and stored in paper bags at 25 °C.

Ethanollic extraction

Dried leaves were pulverized to 500- μ m mesh, and 10 g was extracted with 100 mL of ethanol (100%) in a shaker at room temperature for 48 h. Thereafter, the sample was centrifuged (2000 rpm, 20 min), and the supernatant was dried in a rotovap at 45 °C (Nagappan 2012). The ethanollic extract was immediately used for further analysis.

Gas chromatography-mass spectrometry analysis

The polar and nonpolar oregano sub-fractions were prepared and analyzed by gas chromatography-mass spectrometry (GC-MS) in the Agilent GC-MS model 7890A/5977 (Agilent Technologies, Inc., Santa Clara, CA, USA) as previously reported (Reyes-Becerril et al. 2019).

Phytogenic contents

The total polyphenol content (TPC) was analyzed using the method of Singleton et al. (1999) using the Folin-Ciocalteu method. Gallic acid was used in a reference curve ranging from 0 to 150 μ g/mL and the TPC was expressed as mg GAE g⁻¹ DM.

Total flavonoid content was determined following the method based on aluminum chloride (AlCl₃) (Kamtekar et al. 2014). Optical density was read at 510 nm. Quercetin was used in a reference curve ranging 0–8000 μ g/mL and the TFC was expressed as mg QE g⁻¹ DM.

Antioxidant properties

Antioxidant activity of oregano extracts was quantified using the DPPH method (Brand-Williams et al. 1995). Absorbances were recorded at 515 nm, and the antioxidant values were reported as the extract quantity (mg) to attain 50% of activity in 1 mL reaction solution (EC₅₀, efficient concentration):

$$\text{DPPH scavenging activity (\%)} = \frac{[(A^0 - A^1)]}{A^0} * 100$$

where A^0 and A^1 are the control and sample absorbances, respectively.

Superoxide radical scavenging activity was determined as previously described (Martinez et al. 2001). Butylated hydroxyanisole (BHA) was the positive control. The inhibition percentage was determined as follows:

$$\text{Scavenging activity (\%)} = \frac{[(A^0 - A^1)]}{A^0} * 100$$

where A^0 and A^1 are the absorbances of blank or control and sample, respectively.

In vitro study on goat peripheral blood leukocytes incubated with *Lippia palmeri* W

Based on the antioxidant capacity results, two levels of oregano concentrations, i.e., 100 and 150 $\mu\text{L/mL}$, were used for the in vitro study on goat peripheral blood leukocytes (PBL). Goat PBL were isolated according to Angulo et al. (2018). The cells were adjusted to 1.2×10^6 cells mL^{-1} (TC20, Bio-Rad, Hercules, CA, USA). Then, 900 μL of PBL suspension were placed into flat-bottomed 24-well plates (Sigma, St. Louis, MO, USA). Thereafter, oregano extract was dispensed in PBL and kept at 37 °C with 5% CO_2 for 24 h. Samples consisting of cells with culture medium were used as control.

Immune parameters

Leukocyte viability was determined upon incubation with oregano extracts by the resazurin method (Riss et al. 2004) following modifications previously described (Angulo et al. 2018). Fluorescence was recorded (Varioskan, Thermo Scientific, Waltham, MA, USA) at 530 nm (excitation) and at 590 nm (emission). Control groups were PBL incubated without extracts and with DMSO (dimethyl sulfoxide, Sigma-Aldrich 472301, 10% final concentration). Values were corrected by fluorescence of RPMI medium with different treatments (oregano 100 and 150 $\mu\text{g/mL}$, DMSO, and water).

Phagocytosis was determined using a Phagocytosis Kit following the manufacturer's instructions (Vybrant, Invitrogen, Waltham, MA, USA). Sample readings were recorded at 484 nm for excitation and 535 nm for emission (Varioskan, Thermo Scientific, Waltham, MA, USA).

Nitric oxide levels were quantified using the method previously reported (Neumann et al. 1995). Absorbances were recorded at 562 nm, and nitric oxide production was reported μM of nitrites.

Superoxide dismutase (SOD) activity was measured by the percentage of xanthine oxidase activity inhibition in water soluble tetrazolium dye (WST-1) substrate with a SOD determination Kit (Sigma, St. Louis MO, USA)

following the recommended protocol. Catalase (CAT) activity was determined as described by Claiborne (1985). Absorbances were recorded at 240 nm; CAT activity (one unit) represents the quantity of enzyme needed to hydrolyze 1 mM of H_2O_2 in 1 min.

The mRNA expression patterns of two pro-inflammatory cytokines (TNF- α and IL-1 β) and two antioxidant enzymes (GPX-4 and CAT) were determined in PBL samples by reverse transcription quantitative real-time PCR (RT-qPCR) and the $2^{-\Delta\Delta\text{CT}}$ analysis (Livak and Schmittgen 2001). Primers are described in Supplementary Table 1. The mRNA transcripts were normalized with the 18S gene level in PBL stimulated with oregano extracts as indicated by Pfaffl (2001).

In vivo study on goats fed *Lippia palmeri* W: effects on chemical composition in feces

Animal feeding, experimental design, and sampling

In order to know the oregano effect on chemical composition of feces, an in vivo study was conducted. Twelve adult lactating Anglo-Nubian female goats (47 ± 8.48 kg (mean \pm SD), 4th lactation, 3 years old) located at the sheep and goat education facilities at Universidad Autonoma de Baja California Sur were used. Animals were maintained under stabled conditions and divided into four pens (floor space 4×4 m) with 3 goats per pen. Two pens were randomly allocated to one of the two experimental diets. Diets were formulated to be isocaloric and isonitrogenous according to the NRC (2007):

- (1) Control group. Basal diet ($n = 6$ goats);
- (2) Oregano group. Basal diet plus 2.6% oregano (dry matter) ($n = 6$ goats).

Oregano nutritional composition consisted of 11.2% crude protein (CP), 49.04% total digestible nutrients (TDN), 44.9% neutral detergent fiber (NDF), 33.4% acid detergent fiber (ADF), 7.3% ether extract (EE), and ash (11.1%). The chemical composition of oregano and experimental diets were determined following standard methods of the AOAC (2000) and Van Soest et al. (1991). Nutritional diet content has been described in Table 3. Animals were fed daily with 1.4 kg dry matter for 90 days with a previous diet adaptation period of 14 days. Clean and fresh water was given to the animals ad libitum. Pens and water drinkers were cleaned twice a week. At the end of the experiment (day 90), feces (5 g) were obtained directly from the rectum of each goat and placed into 50-mL Falcon tubes for further analysis. Animal handling was conducted following the national guide of ethical care of experimental animals (NOM 0062-ZOO-1999) avoiding excessive distress for the animals.

Chemical composition of goat feces by gas chromatography-mass spectrometry

After collection, feces were frozen at -70°C until the GC-MS analysis was performed. To analyze the low molecular weight compounds of feces composition via GC-MS, a medium polarity extract was prepared as follows: 100 mg of feces were resuspended in 500 μL of phosphoric acid (0.5%) and 500 μL of ethyl acetate. Then, samples were sonicated at 40°C for 30 min, vortexing every 10 min. Samples were centrifuged (10,000 rpm, 10 min) and 200 μL of ethyl acetate phase was evaporated under nitrogen flow. Once dried, samples were derivatized with 80 μL of N,O-bis(trimethylsilyl)-trifluoroacetamide plus trimethylchlorosilane (1%) and 20 μL of pyridine at 85°C for 25 min. Once the reaction period ended, the samples were allowed to cool, and 100 μL of isooctane was added. Then, samples were immediately analyzed by GC-MS in an HP-5 column (30 m \times 250 μm \times 0.25 μm , Agilent Technologies, Santa Clara CA, USA) at a rate of 1 mL/min as follows: GC injector setup at 280°C was gradually ramped for 13 min. The MS operated at 70 eV in the scan mode from 40 to 550 m/z .

Statistical analysis

Descriptive statistics were performed and means \pm S.D. were generated. One-way ANOVA was performed on the biological variables by the oregano extract effects in the in vitro studies. For the in vivo study, the Student's t test was used to compare control and treatment (oregano) groups. Means were separated by Tukey's test with a significance of $p < 0.05$ (SPSS v.19.0 software, Richmond, VA, USA).

Results

Biochemical composition of *Lippia palmeri* W. extracts

The biochemical composition of oregano ethanolic extracts determined by GC-MS is expressed as the relative area mean of each identified compound, which is based on specific standards. The nonpolar extract fraction indicated that carvacrol (52.85%) and thymol (16.53%) were the most abundant compounds (Table 1). Similarly, carvacrol (30.56%) and thymol (19.77%) were the highest representative compounds in the polar fraction, but high quantity of carbohydrates, such as fructose (8.76%) and glucose (5.39%), were also identified (Table 2).

Total phenolic and flavonoid contents of *Lippia palmeri* W. extracts

Polyphenol and flavonoid contents in extracts are displayed in Fig. 1a. Polyphenol concentration in the sample was approximately 1 mg GAE g^{-1} DM, whereas flavonoids in the sample accounted for almost 4 mg QE g^{-1} DM.

Antioxidant capacity of *Lippia palmeri* W. extracts

The antioxidant capacity of different extract concentrations was determined by the ability to scavenge superoxide and free radicals (Fig. 1b, c). The highest free radical scavenging activity of the extracts was found at 100 $\mu\text{g/mL}$, whereas the highest for superoxide was observed at 150 $\mu\text{g/mL}$. Therefore, both concentrations (100 and 150 $\mu\text{g/mL}$) were further used for the in vitro bioassays with goat peripheral blood leukocytes (PBL) (Table 3).

In vitro study

Immune parameters

Cell viability remained unaffected in PBL incubated (24 h) with oregano extracts at 100 (90%) or 150 $\mu\text{g/mL}$ (80%) with respect to the control cells (Fig. 2a). The results indicated that oregano extracts at 100 $\mu\text{g/mL}$ enhanced ($p < 0.05$) both phagocytosis and nitric oxide production in PBL compared to the control and 150 $\mu\text{g/mL}$ treated groups (Fig. 2b,c).

Superoxide dismutase activity was higher in leukocytes treated with oregano extracts with respect to the enzyme activity level in control leukocytes (Fig. 2d, $p < 0.05$), whereas CAT activity remained unaffected in cells treated with oregano extracts when compared with the control group (Fig. 2e).

The mRNA transcription of IL-1 β and TNF- α in PBL treated with oregano extracts is shown in Fig. 3. The IL-1 β gene expression was similar for the control PBL and cells treated with oregano extracts at 100 $\mu\text{g/mL}$; however, this gene downregulated in PBL incubated with the extracts at 150 $\mu\text{g/mL}$ (Fig. 3a). The TNF- α mRNA gene showed a dose-dependent downregulation effect ($p < 0.05$) in PBL incubated with oregano extracts compared to leukocytes in the control group (Fig. 3b).

Antioxidant enzyme gene expression of CAT and glutathione peroxidase-4 in PBL incubated with oregano extracts downregulated in a dose-dependent manner compared with the expression of these genes in the control group (Fig. 3c, d).

In vivo study

The profile of compounds extracted and detected by GC-MS from feces of goats fed with control and supplemented

Table 1 Nonpolar fraction of *Lippia palmeri* W. as determined by using GC-MS and expressed as relative percentage

R.T.	Name	Area mean	Relative % area
14.096	α -Terpinene	6,514,270.9	0.04
14.424	o-Cymene	27,067,550.0	0.16
17.486	β -Linalool	19,461,210.3	0.12
24.65	Thymol	2,782,003,317.7	16.53
25.15	Carvacrol	8,894,021,320.9	52.85
25.857	5-Isopropenyl-2-methylcyclopent-1-enecarboxaldehyde	15,151,122.3	0.09
26.677	O-Acetylthymol	17,462,625.1	0.10
26.814	3-Allyl-2-methoxyphenol	55,835,091.9	0.33
27.215	Thymol acetate	15,428,369.7	0.09
27.602	NI	32,898,309.1	0.20
28.228	Eugenol methyl ether	27,128,172.1	0.16
28.689	β -Caryophyllene	498,769,271.0	2.96
29.092	α -Bergamotene	4,743,608.0	0.03
29.729	NI	2,061,417,024.0	12.25
30.933	Eremophilene	6,960,164.6	0.04
31.346	β -Bisabolene	10,605,903.4	0.06
31.471	Phenol, 2,4-di-tert-butyl-	22,550,060.1	0.13
31.77	δ -Cadinene	11,589,330.9	0.07
32.866	NI	8,228,456.1	0.05
33.739	Caryophyllene epoxide	363,766,960.9	2.16
34.574	12-Oxabicyclo[9.1.0]dodeca-3,7-diene	103,846,007.8	0.62
35.36	Calarene epoxide	12,953,762.3	0.08
37.095	Caryophyllene epoxide isomer 2	38,633,508.1	0.23
39.624	NI	9,245,089.53	0.05
43.493	NI	36,323,906.3	0.22
43.969	NI	49,723,462.6	0.30
45.135	NI	27,621,397.8	0.16
45.594	NI	61,733,309.9	0.37
47.046	NI	84,966,019.7	0.50
47.126	Retinoic acid	129,693,554.8	0.77
47.837	i-Propyl 9-octadecenoate	77,906,930.1	0.46
48.359	NI	62,290,481.50	0.37
51.874	NI	11,815,621.37	0.07
53.054	Heptacosane	36,062,968.0	0.21
54.28	Squalene	311,918,420.3	1.85
54.512	Nonacosane	17,275,389.6	0.10
56.496	Tetratriacontane	96,742,642.3	0.57
56.901	α -Tocopherol	155,989,644.3	0.93
57.121	NI	19,614,321.31	0.12
57.36	Dotriacontane	22,900,654.5	0.14
57.846	Campesterol	10,438,627.5	0.06
58.183	Stigmasterol	31,189,291.6	0.19
58.441	Trtriacontane	268,151,113.2	1.59
58.856	Clionasterol	129,680,612.7	0.77
59.274	β -Amyrin	17,370,703.2	0.10
59.532	Tetratriacontane	28,911,546.9	0.17
59.839	α -Amyrin	9,770,185.8	0.06
59.983	Simiarenol	30,275,833.8	0.18
60.931	Pentatriacontane	53,604,861.5	0.32

NI unidentified, R.T. retention time

Table 2 Polar fraction of *Lippia palmeri* W. as determined by using GC-MS and expressed as relative percentage

R.T.	Name	Mean	Relative % area
18.67	Glycerol	23,468,552.3	0.40
19.416	Thymol	1,161,362,553	19.77
19.911	Carvacrol	1,795,126,114	30.56
22.014	Caryophyllene	23,737,017.6	0.40
22.862	α -Humulene	11,045,785.5	0.19
24.051	NI	25,334,552.54	0.43
24.193	NI	21,147,853.11	0.36
24.516	NI	900,749,064.1	15.34
25.987	Caryophyllene oxide	24,172,625.5	0.41
26.585	1,5,5,8-Tetramethyl-12-oxabicyclo[9.1.0]dodeca-3,7-diene	7,897,063.18	0.13
28.067	Ribitol	18,460,338.5	0.31
31.505	1-Cyclohexene-1-carboxylic acid, 3,4,5-trihydroxy	139,578,041	2.38
34.69	Galactose	17,816,440.1	0.30
33.292	Glucose	316,480,810	5.39
36.995	Inositol	47,422,770	0.81
38.505	Linoleic acid	20,837,645	0.35
38.646	α -Linolenic acid	68,580,824.4	1.17
41.589	NI	24,673,257.6	0.42
42.771	Thymol- β -d-glucopyranoside	11,868,069.4	0.20
43.145	NI	62,515,462.5	1.06
43.219	Carvacrol- β -d-glucopyranoside	13,927,747.9	0.24
47.682	Squalene	20,719,461.9	0.35
48.836	NI	31,508,000.7	0.54
49.303	Catechin	270,161,399	4.60
49.634	Sucrose	514,531,704	8.76
49.751	NI	66,909,696.9	1.14
50.331	α -Tocopherol	23,773,278.4	0.40
51.275	Melibiose	59,377,606.3	1.01
51.5	Stigmasterol	30,217,562.2	0.51
52.007	β -Sitosterol	64,503,107	1.10

NI unidentified, R.T. retention time

oregano diets after 90 days are shown in Table 4 and Fig. 4. The GC-MS analysis enabled the detection of nine compounds, from which caproic acid (C6) was found relatively high in goats fed with oregano compared with the control group (6.87 vs 0.54%, respectively).

Discussion

Oregano is one of the most commonly used medicinal plants in Mexico, especially the species that belong to the genus *Lippia* (Verbenaceae) (Alonso-Castro et al. 2017). The presence and concentration of bioactive compounds in oregano species are influenced by environmental and other factors (De Mastro et al. 2017). In this study, ethanolic extracts of oregano collected in Baja California Sur, Mexico, showed the presence of many bioactive compounds, including carvacrol

and thymol (most abundants). The presence of β -caryophyllene, caryophyllene epoxide, and squalene was also identified. In methanolic and chloroform extracts of *L. palmeri* W., Leyva-López et al. (2016) identified several bioactive compounds, such as thymol and carvacrol by LC-DAD-ESI-MS/MS, which confirmed that these compounds were commonly found in this species and may be extracted by different solvents.

Polyphenols and flavonoids are the main compound groups in oregano with biological functions. In this study, oregano ethanolic extracts had 1 mg GAE g⁻¹ DM of total polyphenols and 4 mg QE g⁻¹ DM of total flavonoids. In *L. palmeri* W. methanolic extracts, total polyphenol concentration varied from 22.87 to 1232.28 mg GAE g⁻¹ DM, whereas flavonoids ranged from 10.44 to 1090.46 mg QE g⁻¹ DM (Gutiérrez-Grijalva et al. 2017, 2019). Interestingly, the presence and quantity of polyphenols have been associated with antioxidant

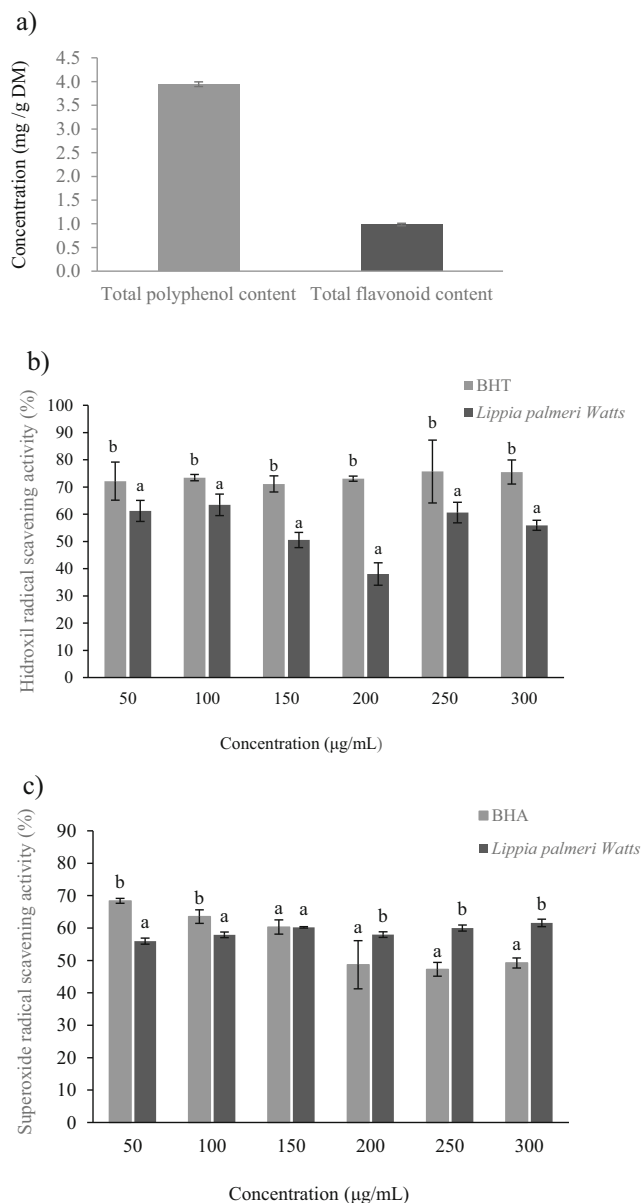


Fig. 1 Total polyphenol and total flavonoid contents in ethanolic extracts (a), hydroxyl radical (b), and superoxide radical scavenging (c) activities of *Lippia palmeri* Watts. Butylated hydroxytoluene (BHT) and Butylated hydroxyanisole (BHA) were used as positive controls. The results are means \pm standard deviation (S.D.) of three separate experiments, each in triplicate. Different letters indicate significant ($p < 0.05$) differences among groups

capacity in several oregano species, such as those commonly found in America (Arcila-Lozano et al. 2004); moreover, this antioxidant capacity is a desirable feature that relies on the power to donate or accept electrons to counteract reactive oxygen species (ROS). In the current study, high DPPH free radical and superoxide scavenging activities were found in oregano extracts. Notably, this high antioxidant capacity of oregano extracts has been recently reported (Gutiérrez-Grijalva et al. 2018, 2019).

Table 3 Nutritional composition of the diets supplemented with *Lippia palmeri* W. fed to goats

	Control	Experimental
Ingredient (%)		
Alfalfa hay	9.0	0.50
Ground corn grain	44.0	46.0
Green bean Straw	45.9	49.5
<i>L. palmeri</i> Watts	0	2.6
UREA	1.1	1.4
Nutritive value		
DM (%)	89.44	89.21
CP (%)	11.17	11.01
ME	3.38	3.38

Ingredients (%) = Percent of ingredients in the diet

DM (%) = Percent of dry matter

CP (%) = Percent of crude protein

ME = Metabolizable energy Mcal/kg of dry matter

To explore oregano immunomodulatory potential, an in vitro study was performed using goat peripheral blood leukocytes. The first condition of any immunostimulant for goats is that it must be safe. The PBL incubated with oregano extracts demonstrated that cell viability was not compromised, especially at 100 µg/mL. In murine macrophages (RAW 264.7), *L. palmeri* W. extracts at 100 µg/mL had a similar result on cell viability to that obtained using goat PBL (Leyva-López et al. 2016).

Phagocytosis is a key cellular response involved in particle recognition and uptake by several cells, such as macrophages, dendritic, neutrophils, and B cells (Gordon 2016). Oregano extracts promoted phagocytosis and nitric oxide production in goat PBL, an effect that depended on the extract dose. In contrast, Leyva-López et al. (2016) found that murine macrophages had reduced nitric oxide production when incubated for 5 h with oregano methanolic or chloroform extracts and then stimulated with lipopolysaccharide (LPS) for 19 h. In addition, the cellular antioxidant activity promoted by plant extracts in leukocytes as an immune indicator was mainly related to the respiratory burst activity (ROS production). The SOD and CAT are enzymes that catalyze ROS (superoxide anion and hydrogen peroxide, respectively) to maintain cellular homeostasis. This study found that SOD activity was higher in PBL incubated with oregano extracts, but CAT activity remained unaffected among groups compared to the control cells. Murine macrophages incubated with oregano extracts and challenged with LPS reduced ROS production (Leyva-López et al. 2016). Similar to these results, SOD activity increased in rats supplemented with an aqueous oregano (*Origanum vulgare* L.) extract (Srihari et al. 2008). In another investigation, Park et al. (2015) reported that ducks fed diets containing oregano enhanced serum SOD activity.

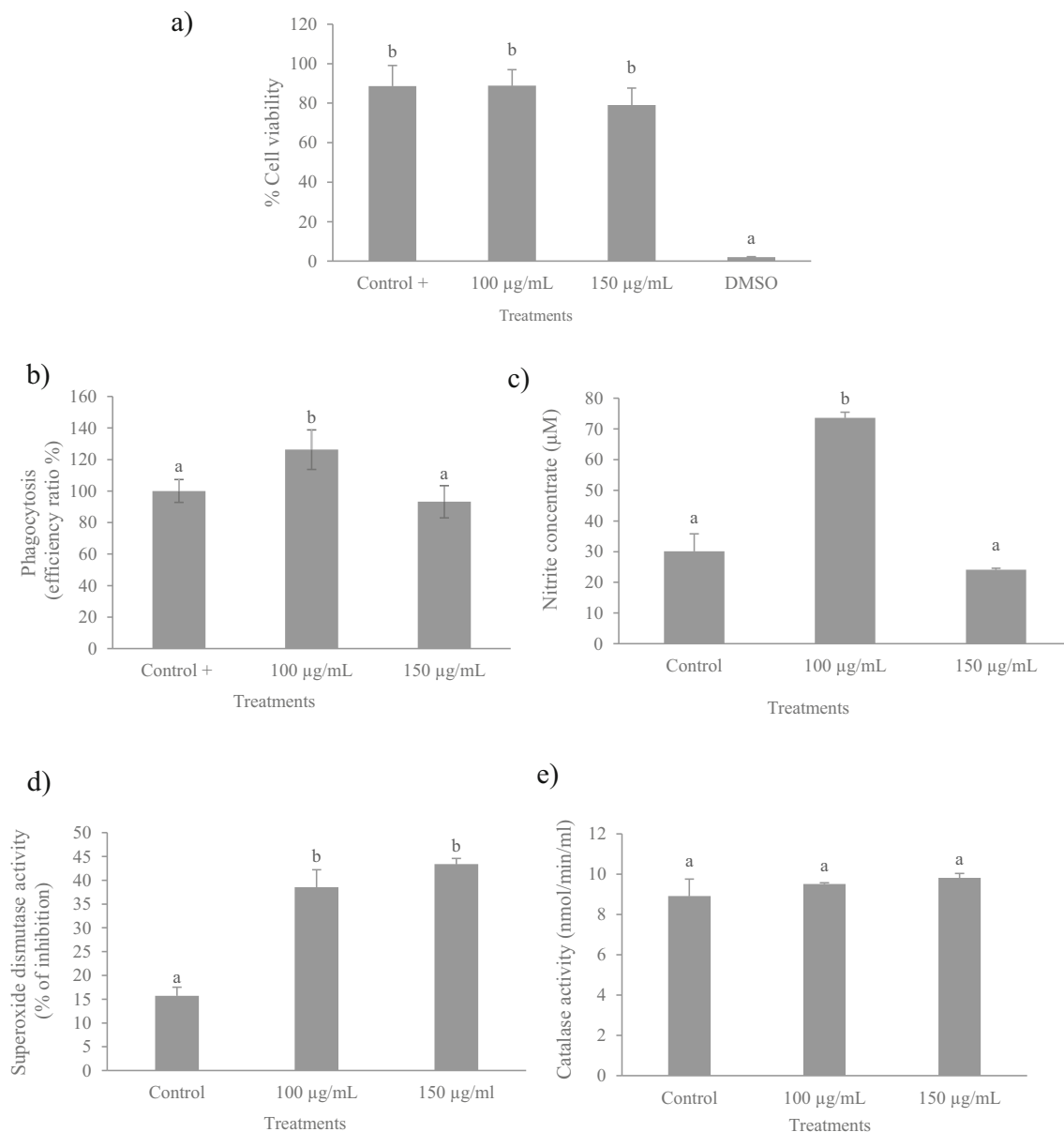


Fig. 2 Resazurin assay to measure cell viability (a) (dimethyl sulfoxide (DMSO) was used as cellular toxic control), efficiency ratio of phagocytosis response (b), nitric oxide production (c), superoxide dismutase activity (d), and catalase activity (e) in goat peripheral blood leukocytes

stimulated with *Lippia palmeri* Watts (100 and 150 µg/mL) at 24 h. Bars represent the mean \pm standard deviation (S.D.) ($n = 9$). Different letters indicate significant ($p < 0.05$) differences among groups

The supplementation of oregano also demonstrated another important role in ruminants. Simitzis et al. (2008) found that dietary incorporation of oregano essential oil exerted strong antioxidant effects retarding lipid oxidation (LPO by MDA formation) in raw sheep meat. Supporting this result, Gumus et al. (2017) observed that oregano essential oil decreased the LPO rate in lamb meat tissue. The contribution of intrinsic antioxidant capacity of oregano bioactive compounds in the extracts should be considered into the redox balance outcome, and also why these effects may have been observed in different studies (Leyva-López et al. 2016; Gutiérrez-Grijalva et al. 2019). For instance, the expression of catalase and glutathione

peroxidase-4 genes downregulated in PBL incubated with oregano extracts, which supported our hypothesis.

Immunomodulation is also related with the pro-inflammatory response expression (Gordon 2016). Many oregano species have anti-inflammatory effects that are related with up and downregulation of anti- and pro-inflammatory gene expression, respectively (Wei et al. 2015). The current results indicated a potential anti-inflammatory effect based on the main pro-inflammatory IL-1 β and TNF- α gene downregulated expression in PBL incubated with oregano. Similarly, Ocaña-Fuentes et al. (2010) observed a reduced expression level of pro-inflammatory IL-1 β , IL-6, and TNF- α cytokines in human

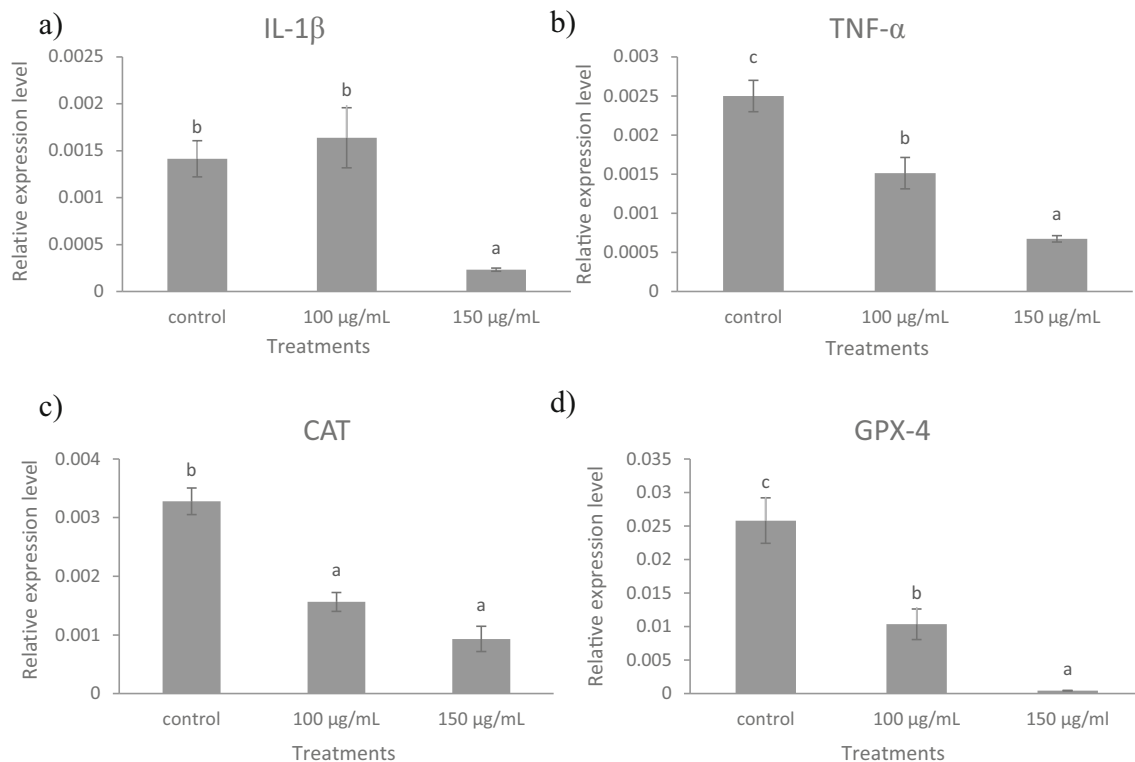


Fig. 3 Relative mRNA expression of pro-inflammatory cytokines (IL-1β (a) and TNF-α (b)); antioxidant enzymes (catalase (CAT (c)) and glutathione peroxidase-4 (GPX-4 (d))) by quantitative real-time PCR in goat peripheral blood leukocytes stimulated with *Lippia palmeri* Watts (100

and 150 μg/mL) at 24 h. Data are shown as mean ± standard deviation (S.D.) Different letters denote significant differences among treated groups ($p < 0.05$)

macrophage THP-1 cells stimulated with *O. vulgare* essentials oils. Other molecules have been also associated to oregano *L. palmeri* W. anti-inflammatory effect in leukocytes (Leyva-López et al. 2016). In other studies, the anti-inflammatory effects of several oregano species have been reported, highlighting it as a potential helper in inflammatory modulation and therapeutic

effects on gut disorders (Zou et al. 2016). Finally, the profiling of compounds extracted from goat feces fed with control and oregano diet was analyzed by GC-MS. Remarkably, goat feces from the oregano group had greater caproic acid compared to the control group. This short-chain fatty acid has shown microbicidal activity, intestinal health improvements, and stimulatory

Table 4 Chemical compounds found in feces of goats fed *Lippia palmeri* W. after 90 days as determined by GC-MS and expressed as a relative percentage

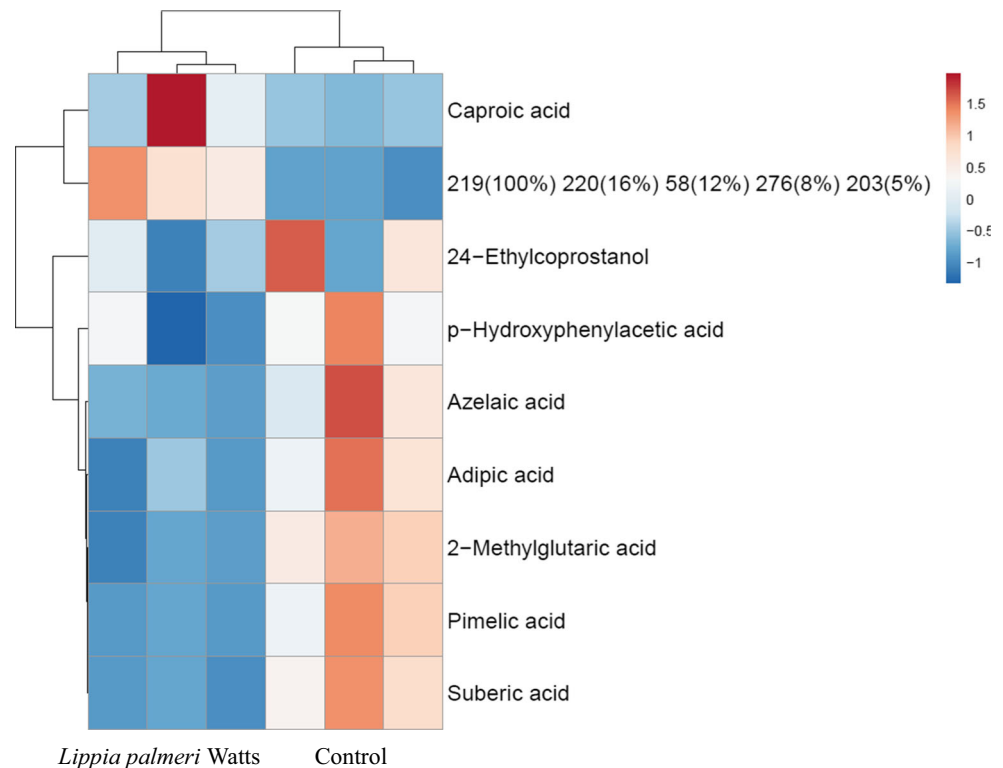
R.T.	Compound	Relative area (%)	
		Control	<i>Lippia palmeri</i>
12.915	Caproic acid	0.54543332 ^a	6.87667512 ^b
22.084	2-Methylglutaric acid	0.45735575 ^a	0.34174372 ^a
24.3	Adipic acid	6.53161833 ^a	4.90183393 ^a
26.589	Pimelic acid	1.30516863 ^a	0.86423506 ^a
27.353	p-Hydroxyphenylacetic acid	3.14793459 ^a	1.93664905 ^a
28.699	Suberic acid	4.46462756 ^a	3.5874772 ^a
30.774	Azelaic acid	11.0683609 ^a	7.87674229 ^a
32.388	219(100%) 220(16%) 58(12%) 276(8%) 203(5%)*	5.30507377 ^a	9.18113866 ^b
52.8	24-Ethylcoprostanol	67.1744272 ^a	64.433505 ^a
	Total	100	100

Different letters denotes significant differences between groups at $p < 0.05$

R.T. retention time

*Not identified

Fig. 4 A representative heat map of chemical compounds determined by gas chromatography-mass spectrometry (GC-MS) in feces of goats fed with *Lippia palmeri* Watts at 90 days of experimental trial



effects on several organisms (Llorens et al. 2013; De Keyser et al. 2019). For instance, caproic acid has controlled the pathogen *Alternaria alternata* in mandarin and the pathogenic *Escherichia coli* in pigs (Llorens et al. 2013; De Keyser et al. 2019). Short-chain fatty acids in feces, such as caproic acid, are the result of the fermentation of gut microbiome and their high levels in gut could be related with an improved inflammatory balance (De Preter et al. 2015).

The preliminary studies demonstrated that (1) *L. palmeri* W. has common phytochemical compounds found in other oregano species that account for its strong antioxidant capacity; (2) its extracts promote cellular antioxidant and anti-inflammatory responses in goat leukocytes; and (3) its dietary supplementation (2.6% dry matter based on the total formulated diet) in adult Anglo-Nubian goats increased the production of caproic acid and compounds associated with the improvement of intestinal health. The beneficial effects of oregano for ruminants remain almost elusive, so this study opens the path for further investigation and potential applications of *L. palmeri* Watts on animals.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This study used the National Guide of Ethical Care of Experimental Animals (NOM 0062-ZOO-1999) avoiding excessive distress for the animals.

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