Original Article

Anti-inflammatory Activity of Methanolic Extract from *Pistacia atlantica* Desf. Leaves

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ABSTRACT

Introduction: The extracts of the *Pistacia* species are known for their anti-inflammatory activity, including fruits and oil of *P. atlantica*. However, the inflammatory effect of the methanolic extract of *P. atlantica* leaves has not been studied. This work aimed at assessing the anti-inflammatory and antioxidant activities of *P. atlantica* leaves extract in relation to phytochemical studies of flavonoids. **Methods:** The extract was obtained using sonication of leaves powder in 80 % methanol. The analysis of phenolic compounds was carried out using thin-layer chromatography (TLC). The antioxidant activity was evaluated using DPPH, ABTS and FRAP assays. The anti-inflammatory activity was determined by the reduction of carrageenan-induced hind paw edema in mice. **Results:** The TLC revealed 3 glycosylated flavonoids and gallic acid derivatives. The flavonoids identified corresponded to rutin, quercetrin and other heterosides of quercetin, kaempferol and myricetin. Total phenolics and flavonoids contents were comparable for the male and female trees. The antioxidant activity did not show a significative difference between the two sexes, except for that evaluated by the FRAP assay which was significantly greater for the male tree leaves extract. The leaves extract permitted significative reduction of the edema at h3 and 6 in a dose-dependent manner (100 and 250 mg/kg), while diclofenac used as control reduced the edema at h 1.5. This difference could be explained by the pharmacokinetic and pharmacodynamic properties of diclofenac and *P. atlantica* leaves compounds. **Conclusion:** *P. atlantica* has a strong anti-inflammatory activity and constitutes a potential source for the development of new treatments.

Key words: Antioxidant activity, Flavonoids, Methanolic extract, Plantar edema, TLC.

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INTRODUCTION

Over the last decade, great progress has been made in understanding the physiopathology of inflammation and the involvement of free radicals in its pathogenesis. The reactive oxygen species (ROS) produced from the action of free radicals on molecular oxygen increase abnormally during inflammation, causing an imbalance between the oxidizing molecules and the antioxidant system of the body. This oxidative stress causes inflammatory cascades that damage the cellular components.[1] The humoral and cellular mechanisms of inflammation are numerous and complex. They involve gene regulatory factors such as the nuclear factor-kappa B (NF-κB) and signaling substances synthesized by immune system cells such as cytokines and prostaglandins.[2]

Many factors may be at the origin of the inflammatory process; Infectious agents, ischemia, antigen-antibody interaction, thermal or physical shocks.[3] Steroid drugs, nonsteroidal anti-inflammatory drugs (NSAIDs) and immunosuppressants, usually used for the relief of inflammatory diseases, require long-term treatment and their use is often associated with serious side effects such as bleeding gastrointestinal and peptic

ulcers.[4] This has led to the search for alternative treatments. In this regard, secondary metabolites of various medicinal plants have been shown to be effective in the treatment of inflammation and pain.[5] Among these plants, the species of *Pistacia* genus are of interest. Indeed, this genus includes 11 species that are widely distributed on the different continents of the terrestrial globe. They are found in temperate forests, tropical hardwoods and boreal conifers.[6] *P. atlantica* is one of the most widespread wildlife species. It is the most characteristic plant of the arid and semi-arid zones of the northern part of Africa.[7] In traditional medicine, *P. atlantica* is used for the treatment of pain and other conditions such as upper abdominal discomfort, dyspepsia and peptic ulcer.[8] Several studies reported that the different parts of the plant as fruits, galls and oil, have biological effects including anti-inflammatory activities.[9-12] However, as far as we know, no work has been devoted to the study of the anti-inflammatory effect of the methanolic extract of *P. atlantica* leaves (MEPaL). Thus, this study aimed at assessing the anti-inflammatory activity of

the MEPal using carrageenan-induced hind paw edema in mice, in relation to the flavonoid composition evaluated by both thin-layer chromatography (TLC) and chemical assays.

MATERIALS AND METHODS

Preparation of the methanolic extract

Leaves of male and female *P. atlantica* were collected from Tafilalt region in the south of Morocco. They were dried in the shade at 40°C and ground to a fine powder. Fifty mg of the fine powder were extracted with 1 ml of methanol-water solvent (80/20; v/v). After sonication for 15 min and centrifugation at 12000 rpm for 10 min at room temperature, the supernatant was recovered and kept at 4°C.

Phytochemical screening

It was performed using standard procedures to highlight the secondary metabolites. Extraction and revelation of these compounds involve a set of solvents and developers: methanol for extracting flavonoids, alkaloids and tannins; chloroform for cyanogenic compounds and coumarin; petroleum ether for free quinones; hexane for terpenes; and water for saponins. The developers used for the revelation were: 2-aminoethyl-diphenyl borate (Neu's reagent) 1% in methanol[13]for flavonoids; Dragendorff reagents, Mayer and iodoplatinate for alkaloids revelation;[14] iron chloride ($FeCl_3$) in 1% methanol for detecting tannins;[15]1% antimony trichloride in chloroformto reveal terpenes.

TLC was performed on a 10×20 cm TLC sheets coated with 0.2 mm layers of silica gel N-HR/UV$_{254}$ (Macherey-Nagel, Ref. 804023). After application of the extract and the standard solution (5 µl) the sheet was developed in paper-lined all-glass previously left for a preconditioning in the developing chamber for at least 30 min. The mobile phases used were: Ethyl acetate-Formic acid-Acetic acid (35%) -Water (100:11:11:26, v/v/v/v); Acetic acid 2%; Acetic acid 10% ; Ethyl acetate – Methanol-Water (100-13.5-10) ; Ethyl acetate - Formic acid -Water (65-15-20) ; Ethyl acetate – Methanol-Formic acid-Water (100-13.5-2.5-10) ; Toluene-Ethyl acetate - Acetic acid (70-30-1). The visualization of the flavonoids and phenolic acids was achieved by spraying the sheets with 1% methanolic diphenylboryloxyethylamine (Neu's reagent) followed by 5% ethanolic polyethylene glycol 4000.

The acidic hydrolysis, aiming to break C-O-C bonds involved in the glycosides, was conducted using chlorhydric acid (2N). One g of leaves powder was homogenized with 80 mL of 2N HCl and the mixturewas placed in a water bath at 100 °C for 40 min.

Determination of total phenols content[16]

To 25 µl of plant extract were added 110 µl of Folin-Ciocalteu solution. The mixture was stirred for 3 min and then 200 µl of sodium carbonate (Na_2C_{O3}) and 1.9 ml of distilled water were added. After incubation for 30 min at 60°C in a water bath in the dark, the absorbance was measured at 750 nm (IC 6400 visible spectrophotometer). The calibration range was made using gallic acid as standard. The results were expressed in terms of µg gallic acid equivalents (GAE)/mg of dry weight (DW).

Determination of total flavonoids content[17]

To 600 µl of plant extract were added 300 µl of $AlCl_3$. The mixture was incubated for 30 min at room temperature. Then, the absorbance was measured at 430 nm. Rutin was used as standard. The concentrations were expressed in terms of µg rutin equivalents/mg of dry weight (µg RE/mg of DW).

Determination of antioxidant activity

The evaluation of the antioxidant activity was carried out by free radical scavenging method (DPPH and ABTS) and ferric reducing antioxidant power (FRAP).

DPPH scavenging activity

The antiradical power of substances was measured by the decrease of absorption of DPPH (1,1-Diphenyl-2- picrylhydrazyl).[18] To 950 µl of a methanol solution of DPPH (0.1 mM) were added to 50 µl of the plant extract. After 30 min, the absorbance of the mixture was measured at 517 nm. The ability to scavenge DPPH radical was calculated using the following formula:

$$\% \text{ Inhibition of DPPH} = \frac{(A_c - A_s)}{A_c} \times 100$$

Ac: absorbance of control

As: absorbance of sample

ABTS scavenging activity

The technique is based on the scavenging of ABTS$^{•+}$ [(3-ethyl benzothiazoline 6-sulfonic acid) diammonium salt] radical cation which was generated by mixing solutions of ABTS (7 mmol/L) and potassium persulfate (2.45 mmol/L).[19] The mixture was then incubated in the dark at room temperature for 16h. The product was diluted for optimal absorbance of 0.7 at 734 nm. The decolorization of the ABTS$^{•+}$ solution by 100 µg/mL of the test sample or reference compound (Trolox) was monitored by a decrease in absorption at 734 nm during 30 min. The antioxidant activity expressed in µM trolox equivalent antioxidant capacity (TEAC)/mg dry weight (DW).

Ferric reducing antioxidant power (FRAP)

The method is based on reduction of ferric tripyridyltriazine (Fe_3 – TPTZ) to ferrous complex tripyridyltriazine (Fe_2 – TPTZ) by an antioxidant in acidic pH. The ferrous Fe (II) complex -TPTZ develops a blue color with maximal absorbance at 593 nm. The methodology of Benzie and Strain[20] was used. FRAP mixture consists of 10 parts of acetate buffer solution (300 mM) at pH 3.6, 1 volume of 10 mmol/l 2,4,6-tripyridyl-s-triazine (TPTZ) in 40 mmol/ l HCl and 1 volumes of a solution of FeCl3 6H2O (20 mM). To 2 ml of the FRAP mixture were added 10 µl of the plant extract. After incubation of 15 min at room temperature, the absorbance was measured at 593 nm. The calibration range was prepared with Trolox. Results are expressed as µmol Trolox equivalent antioxidant capacities (TEAC)/mg DW.

Carrageenan-induced hind paw edema

Adult albino mice were obtained from "Office National de Securité Sanitaire des Aliments" (ONSSA), Agadir-Morocco, and shifted to the animal house of the Faculty of Sciences (Agadir-Morocco) one week earlier. Animals were kept on a 12-h light/12-h dark cycle at 22±2°C, and had free access to pallet chow and water. All the procedures were in strict accordance with "Guidelines for the care and use of laboratory animals" (Ministry of Agriculture, Law No. 28-7, Decree No. 2-10-473). Mice weighing 25-35 g were fasted for 24h prior to the experiment and deprived of water only during the experiment. According to the method of Winter *et al.,*[21] animals were subjected to sub plantar injection of 0.1 mL of λ-carrageenan (1% in NaCl 0.9%) into the right hind paw and were divided randomly into allocated groups (n=6). Then, they were subjected to a gavage with the following solutions; Group I: sterile saline 0.9% NaCl at 10 mL/kg; Group II: sodium diclofenac at 50 mg/kg as reference drug, Group III: extract of *P. atlantica* leaves at100 mg/kg; Group IV: the same extract at 250 mg/kg. *P. atlantica* extract and Diclofenac, both dissolved in 0.9% NaCl, were administered by oral dose 1h following carrageenan injection. The MEPaL was first evaporated under vacuum at room temperature to remove the methanol, and then the deposit was solubilized in a 0.9% NaCl solution.

The circumference of paw was measured at h1.5, 3 and 6 after carrageenan injection. Increases in the linear circumference of the right hind paw were taken as an indicator of paw edema. Percentage increase in edema (%IO) was estimated in terms of the difference in the zero time (C_0) linear circumference of the injected right hind paw, and its linear circumference at time t (C_t):

$$\% \, IO = \frac{(C_t - C_0)}{C_0} \times 100$$

Percentage inhibition of the inflammatory reaction produced by carrageenan was calculated following formula:

$$\% \, Inhibition = \frac{(IO_c - IO_t)}{IO_c} \times 100$$

Where IO_c and IO_t represented the mean increase in paw circumference in control and treated groups, respectively.

Statistical analysis

Statistica 6 software was used for statistical analysis. Differences between groups were determined using analysis of variance (ANOVA) followed by Student Newman-Keuls method for post hoc analysis.

RESULTS AND DISCUSSION

Table 1 and Figure 1 show the screening and the TLC profile of MEPaL respectively. Figures 2 and 3 correspond to the chromatographic analysis following an acid hydrolysis and an addition of flavonoid standards, respectively. The results showed the presence of flavonoids, terpenes, tannins, coumarin tannins, and the absence of alkaloids and saponins. In addition of gallic acid derivatives, 3 glycosylated flavonoids were identified. The compounds corresponded to rutin, quercetrin and heterosides of quercetin, kaempferol and myricetin (Figure 4).

The content of polyphenols and flavonoids is reported in Table 2. The results showed a total polyphenol content of 65.47 ± 6.80 and 58.82 ± 3.43 mg GAE/g DW in the male and female trees respectively. The level of flavonoids was 110.62 ± 15.79 and 100.10 ± 4.50 g RE/g DW for the 2 sexes respectively. However, the variations noted in the 2 sexes were not significantly different. The values obtained for total phenolics compounds are comparable to those previously reported for the same species.[22,23] For the total flavonoids content, lower values have been reported for *P. atlantica* from the south-west of Morocco and Iran.[22,24] This could be explained by different factors as the genetic variability of the plant and the effect of the climat which is more tempered in the south of Morocco thanks to the oceanic influence.

Table 1: Screening of secondary metabolites in *P. atlantica* leaves extract

| Secondary metabolites classes		Female	Male
Gallic tanins		+	+
Saponins		-	-
Terpens		+	+
Coumarines		+	+
Flavonoïdes	Aglycone flavonoids	+	+
	Draggendorf test	-	-
Alcaloïdes	Iodoplatinate test	-	-
	Mayer test	-	-

+: presence, -: absence
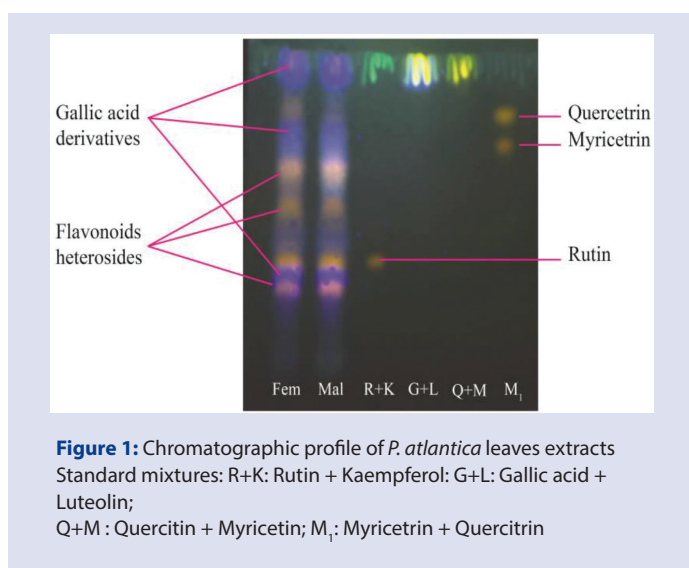
Figure 1: Chromatographic profile of *P. atlantica* leaves extracts
Standard mixtures: R+K: Rutin + Kaempferol: G+L: Gallic acid + Luteolin;
Q+M : Quercitin + Myricetin; M₁: Myricetrin + Quercitrin

Table 2: Total phenolics and flavonoids in *P. atlantica* leaves extract

Extracts	Total phenolics (mg GAE /g DW)	Flavonoids (mg ER/g DW)
Female	58.82 ± 3.43[a]	100.10 ± 4.50[a]
Male	65.47 ± 6.80[a]	110.62 ± 15.79[a]

Values are expressed in mean ± SEM. Means in each column followed by the same letter are not significantly different (P<0.05)
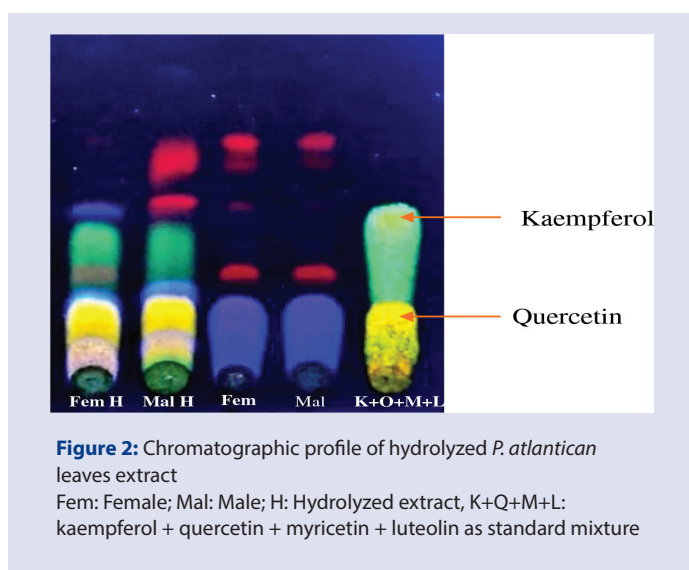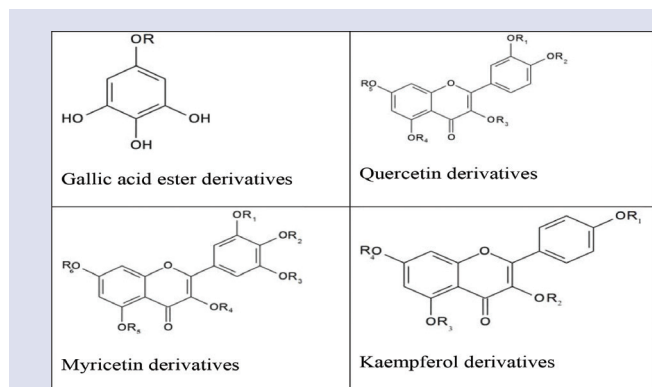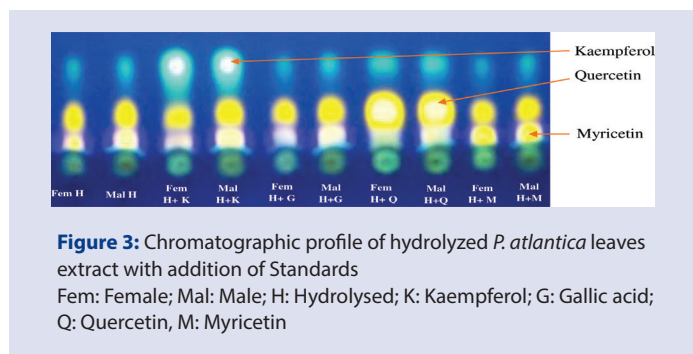

Figure 2: Chromatographic profile of hydrolyzed *P. atlantican* leaves extract
Fem: Female; Mal: Male; H: Hydrolyzed extract, K+Q+M+L: kaempferol + quercetin + myricetin + luteolin as standard mixture

Table 3 shows the antioxidant activity evaluated by the three techniques. The IC₅₀ of the DPPH radical scavenging was 162.6 ± 17.26 and 135.6 ± 16.07 µg/ml respectively for the male and female plants. For the ABTS test, the male and female trees exhibited values of 1633.08 ± 33.29 and 1448.06 ± 25.41 µM TEAC/mg DW respectively. Whether for the DPPH test or the ABTS test, differences between the male and female trees were not significant. With respect to FRAP, the MEPaLs of male and female trees recorded significantly different values (86.64 ± 3.79 and 66.55 ± 4.52 µM TEAC/mg DW respectively). The literature reports that the antioxidant activity of plant extracts depends on both the nature of the
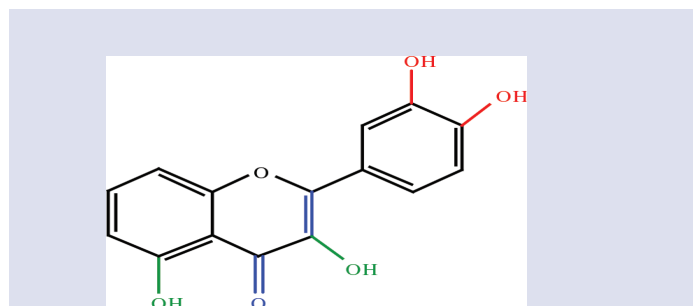
Table 3: Antioxidant activitie of *P. atlantica* leaves extract assessed by DPPH, ABTS and FRAP methods

Activity	DPPH (%)							ABTS (µM TEAC/ mg DW)	FRAP (µM TEAC/mg DW)
	62,50 µg/ml	156,25 µg/ml	312,50 µg/ml	468,75 µg/ml	562,50 µg/ml	625,00 µg/ml	IC$_{50}$ (µg/ml)		
Femelle	31.91 ± 0.57	53.92 ± 1.64	72.97 ± 0.58	80.02 ± 0.57	82.53 ± 0.68	84.80 ± 0.65	135.6 ± 16.07[a]	1448.06 ± 25.41[a]	66.55 ± 4.52[b]
Male	17.62 ± 1.65	41.21 ± 1.15	64.96 ± 1.73	76.16 ± 1.63	78.30 ± 1.42	78.88 ± 1.62	162.6 ± 17.26[a]	1633.08 ± 33.29[a]	86.64 ± 3.79[a]

Values are expressed in mean ± SEM. Means in each column followed by different letters are significantly different (P<0.05)

Figure 3: Chromatographic profile of hydrolyzed *P. atlantica* leaves extract with addition of Standards
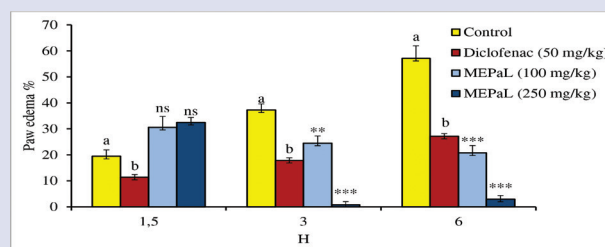Fem: Female; Mal: Male; H: Hydrolysed; K: Kaempferol; G: Gallic acid; Q: Quercetin, M: Myricetin

Figure 4: Phenolic compounds identified in *P. atlantica* leaves extract

Figure 5: Structure of aglycone flavonoid.
In red: ortho-dihydroxy structure in the B ring. In blue: double bond in conjugation with a 4-oxo function in the C ring. In green: hydroxyl groups at positions 3 and 5.

Figure 6: Reduction of paw edema by oral administration of Diclofenac and methanolic extract of *P. atlantica* leaves (MEPaL) (100 and 250 mg/kg) at 1.5, 3 and 6 h after the induction of edema. Results are expressed as mean ± SEM (n=6).
Diclofenac group versus control group: results with same letters are not significantly different at P=0.05. MEPaL group versus diclofenac group: ** significant difference at p <0.01, *** significant difference at p<0.001

test and the phenolic compounds.[25] For example, quercetin showed very low antioxidant activity in the FRAP test but very high activity in the DPPH and ABTS assays.[26] Indeed, polyphenols are compounds which have more than one hydroxyl group attached to one or more benzene rings. They are usually encountered as esters or glycosides rather than as free compounds. Then, the antioxidant activity of the polyphenols depends on the arrangement and the number of hydroxyl groups in the phenolic rings and their connections with the saccharides. Polyphenols could act as reducing initiators, chelating agents or by the prevention of oxidative reactions caused by active singlet oxygen.[27,28] For our study, the antioxidant activity could be explained by the presence of gallic acid and its derivatives and the flavonoids rutin, quercetrin and quercetin heterosides. Indeed, the glycosides of quercetin, despite the presence of osides, contribute to the antioxidant power thanks to the ortho-dihydroxy structure in the B ring, and the double bond in conjunction with the 4-oxo function in the C ring (Figure 5). As a result, the antioxidant activity of the aglycone flavonols is relatively high. Finally, kaempferol possesses a lower activity due to the presence of a single hydroxyl in the B ring.[29-31] These data make it possible to conclude that the antioxidant activity recorded in our work results from the synergy of the various phenolic compounds, even with other non-phenolic antioxidants.[32,33]

The results of the anti-inflammatory activity are shown in Figure 6. The inflammation caused by carrageenan increased with time and reached a maximum after 6 h (57.16 ± 4.82%). Administration of Diclofenac (100 mg/kg body weight) significantly reduced plantar edema at 1.5, 3 and 6h following the carrageenan administration (44.31%, 52.09% and 52.48% respectively). Administration of MEPaL decreased plantar edema significantly later, starting at h 3 (34.28 and 97.88%) and 6 (44.31 and 92.02% at doses of 100 mg/kg and 250 mg/kg body weight respectively). However, the anti-inflammatory effect obtained with the extract was more

pronounced in comparison to Diclofenac. The rapid anti-inflammatory effect of Diclofenac is due to its pharmacokinetic properties represented by a 1h Tmax and a bioavailability of 65% with oral administration.[34] The later action of the MEPaL could be explained both by the pharmacokinetic and pharmacodynamic behaviour of the active secondary metabolites. Indeed, the digestion and absorption of flavonoids are slow processes especially for glycosylated forms because of the resistance of the osidic bonds resulting in decrease of bioavailability.[35] On the pharmacodynamic level, the inflammatory process is complex and involves several cell signalling pathways in addition to the free radicals production which is responsible for tissue degeneration. One of the important mechanisms is an inhibition of eicosanoid-generating enzymes including phospholipase A2, cyclooxygenases (1 and 2), lipoxygenases and nitric oxide synthase (NOS), thereby reducing the concentrations of prostanoids and leukotrienes.[36-39] In addition, flavonoids may modulate the expression of enzymes involved in inflammation and pro-inflammatory molecules by inhibiting transcription factors, such as NF-κB. As example, kaempferol may modulate NF-κB signal pathways during inflammation and alter the expression of genes involved in the inflammatory process.[40,41] In fact, the chromatographic analysis of MEPaL showed the presence of rutin and derivatives of quercetin, myricetin, kaempferol and gallic acid. The literature reported that rutin exerts intestinal anti-inflammatory effects in experimental models of colitis[42] and methyl gallate, one of the main compounds of the active fraction of the methanolic extract of the same family of *Anacardiaceae*, *Lithrea molleoides*, would explain the anti-inflammatory activity observed in the model of ear edema.[43] Similarly, quercetin has been described as anti-inflammatory in several study models[44] and kaempferol reported as an effective compound against inflammation.[45] These data make it possible to attribute the anti-inflammatory activity of the MEPaL to the presence of gallic acid derivatives and flavonoids such as the derivatives of quercetin, myricetin and kaempferol. However, further investigations should be carried out to determine the structure and content of *P. atlantica* glycosylated flavonoids.

CONCLUSION

Our study identified rutin, gallic acid derivatives and heterosids of quercetin, myricetin and kaempferol as flavonoids of *P. atlantica* leaves. The results showed that polyphenols and flavonoides contents were comparable in male and female trees. The same was true for the antioxidant activity except for the FRAP test which showed a greater activity in the male tree. The extract exhibited a strong anti-inflammatory activity which was expressed 3 and 6h after the induction of the edema by injection of carageenan. These results allow us to conclude that *P. atlantica* leaves have potential for the development of new treatment against inflammatory conditions.

ACKNOWLEDGEMENT

CONFLICT OF INTEREST

There is no conflict of interest.

ABREVIATIONS USED

ABTS: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid); **DPPH:** 2,2-diphenyl-1picrylhydrazyle; **FRAP:** Ferric reducing antioxidant power; **iNOS:** Inducible nitric oxide synthase; **MEPaL:** Metanolic extract of *Pistacia atlantica* leaves; **NF-κB:** Nuclear factor-kappa B; **NSAIDs:** Nonsteroidal anti-inflammatory drugs.

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GRAPHICAL ABSTRACT
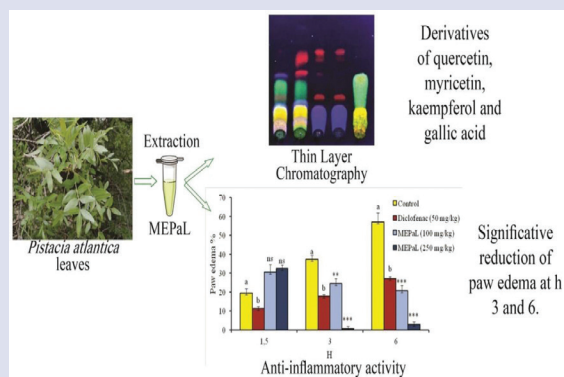
SUMMARY

- This paper reported an important anti-inflammatory activitie of methanolic extract of *Pistacia atlantica* leaves in mice plantar edema model.

- The flavonoids identified were rutin, quercetrin and heterosides of quercetin, kaempferol and myricetin.

- These compounds are known for their anti-inflammatory properties and may explain the results obtained in the experiment.

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