ejpmr, 2019,6(2), 41-47

SJIF Impact Factor 4.897

EUROPEAN JOURNAL OF PHARMACEUTICAL AND MEDICAL RESEARCH

www.ejpmr.com

Research Article

ISSN 2394-3211

EJPMR

STUDY OF PROTECTIVE EFFECTS OF *PISTACIALENTISCUS* LEAVES AQUEOUS EXTRACT AGAINST GASTRIC ULCER AND RENAL STONE FORMATION IN RATS

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Article Received on 25/11/2018 **Article Revised on 15/12/2018** **Article Accepted on 05/01/2019**

ABSTRACT

Medicinal plants have been used by all societies since ancient times as medicines for the treatment of many diseases. In Morocco, the use of medicinal plants is an important part of the cultural diversity of population. The objective of this data is to investigate the protective effects of the aqueous extract of *Pistacialentiscus*leaves (AEPL) (50, 100 and 200 mg/kg) against kidney stones formation and gastric ulcer induced by ethylene glycol (EG) and by HCl/Ethanol respectively. In EG experiment, blood was collected to estimate calcium, phosphorus, creatinine and urea levels. The presence of stones was detected by microscopic observation of renal tissue sections. The obtained results showed that the higher amounts of calcium, phosphorus, urea and creatinine observed in EG/AC treated animals were decreased by AEPL treatment. Histology observation indicated that the number of calcifications in renal tissues of rats treated by AEPL given orally, were significantly reduced compared to the EG/AC group. On the other hand, the important antiulcerogenic effect observed in rat treated with AEPL is dose dependent. This data suggests that AEPL has a protective property against urolithiasisand gastric ulcer justifying its popular use in Morocco as an anti-urolithiasis and anti-ulcer agent.

KEYWORDS: *Plentiscus*; Urolithiasis; Ethylene glycol; stone formation; gastric ulcer.

INTRODUCTION

Nephrolithiasis is a complicated process that is due to a succession of various events such as supersaturation, growth, nucleation, aggregation and retention in the kidney tubules. The etiology of this disease would be related to the eating habits and lifestyle of the population.[1] It has a large effect on the value of life of those affected. Some epidemiological studies had reported that 80% of stones were made of oxalates calcium oxalate (CaOx).[2] It is important to note that urolithiasis is characterized by a high rate of recurrence, so preventive treatment is required. Extracorporeal lithotripsy by shock waves (ESWL) and drug treatment are among the treatments used, but some data suggest that exposure to shock waves at therapeutic doses can cause acute kidney damage, decreased renal function, and increased recurrence of stone formation.[3]

Gastric ulcer is a disease that affects a significant portion of the world's population. It is well known that the incidence of this disease is the result of disequilibrium between gastro-protective substances (mucus, prostaglandins) and aggressive substances such as gastric acid, bile salts. Unfortunately, despite significant progress in the field of medical therapy, the price and side effects of conventional drugs allows patients to look of an alternative remedies in natural substances. Thus, in Morocco, as in many countries, many patients are trying and using medicinal plants as an alternative therapy for many diseases, including lithiasis and gastric ulcer.

P. lentiscus L. commonly known as “Droo and Amgan” in Morocco[4], is largely used in lithiasis and stomach disease treatments. It is a shrub and / or evergreen tree belonging to the Anacardiaceae family producing bright red globular berries, widely distributed in the Mediterranean basin where it grows spontaneously.[5] Its height varies between 1 to 8 meters. Traditionally, the aerial parts have been used as hepatoprotective and antidiabetic agents[6], as a stimulant, to treat hypertension, due to their diuretic.[8] The phenolic compounds of its leaves possess an important antioxidant activity.[10],[9] The mastic gum of this plant contains substances that inhibit proliferation and induce the death of human colon cancer cells.[11] It has also been reported that *P. Lentiscus* contains phenolic acids (such as gallic acid) and flavonoids (such as myricetin derivatives).[12] His essential oil is used in industrial, food,

pharmaceutical and perfumery preparations[13], as flavoring in alcoholic beverages and chewing gum.[14] *P.lentiscus* oil is used in various industrial applications such as perfumery, food and pharmaceutical[13], and it has been evaluated as flavoring in alcoholic beverages and chewing gum.[14] Several studies have reported antifungal, antibacterial and antimicrobial effects of this essential oil.[15]

In the present study, the main goal was to evaluate the antiurolithiatic and the anti-ulcer properties of AEPL using ethylene glycol induced hyperoxaluria and HCl/Ethanol induced ulcer in rats.

MATERIEL AND METHODS

Animals

All the experiments were carried out respecting the European community guidelines for the use of experimental animals, 1st February 2013, NOR: AGRG1238767A, and after obtaining prior approval from our institution. All efforts were made to minimize animal suffering and to reduce the number of animals. All measures have been taken to reduce the suffering and the number of animals.

Plant material and preparation of extract

The plant was collected in July 2016 in Rfala locality, a Moroccan Middle Atlas region and was identified by Professor Ahmed OUHAMMOU from herbarium of Faculty of Sciences Semlalia. A voucher specimen was deposited in this herbarium under number: 4229.

The method used in the preparation of the aqueous extract is that described by Ljubuncic et al., (2005).[16] Briefly, 25 g of dried leaves were stirred in 250 ml of distilled water for 15 min at 90°C, followed by a more delicate filtration on Whatman filter paper. The resulting filtrate was frozen and lyophilized. The yield of the powder was ~16% (w / w). Finally the powder was stored at –20°C in a desiccant until needed.

HPLC analysis

Separation of phenolic and flavonoids compounds of *Pistacia lentiscus* was carried out by HPLC method. The samples were filtered first through Whatman filter paper no.$_{42}$ and then through 0.22 mm membrane filters (Millipore). Chromatography separations were performed on a Reversed-Phase (RP- 18) Columns" Agilent Technologies (250 mm*4.6 mm, 5.0 µm), protected by an Agilent Technologies RP-18 (10 mm *4.6 mm) precolumn. Both columns were placed in a column oven set at 25° C. The HPLC system consisted of (Shimadu (Japan) SCL-10A series pumping system, SIL-10AD) automatic injector, (SPD 10A UV–visible detector set at spectrum begin 200 nm and spectrum end 700 nm), data collection and analysis were performed using Shimadu LC Solution chromatography data station software. Two solvents were used with a constant flow rate of (01 ml/min) injection volume (10 µL). Solvent A consisted of (5%) acetonitrile, (95%) water, solvent B is a phosphate buffer in water (pH 2.6). All the solvents used were of HPLC grade. For the elution program, the following proportions of solvent B were used:
HPLC analysis was performed first to the standards, followed by the lyophilized extract of *Pistacialentiscus* leaves, and finally spiking the samples with the standards. The phenolic compounds were identified by comparing their retention times with those of standards.

Acute toxicity test

Acute toxicity was determined following the experimental model described by Wilson and Hayes in 1994.[17] Mice were treated orally by different concentration (200, 400, 600, 800 and 1000 mg/Kg, po) of aqueous extract of *Pistacia lentiscus* (APLE), whereas the control group received normal saline only either by oral route. All animals were monitored to detect the number of dead animals and behavioral changes like changes fur, eyes, autonomic activity (lacrimation, piloerection, unusual breathing patter), and for presence of stereotypic activities like excessive grooming, repetitive circling, etc.

Observation was continued for 7 days to confirm that the number of animals per dose that remained alive did not alter. The LD50 values were determined by means of the method of Litchfield and Wilcoxon.[18]

Ethylene glycol-induced urolithiasis

Five groups of 6 rats each were used. Group 1 or negative group had free access to water and food and received oral saline. Groups 2, 3, 4 and 5 had free access to feed and water added by 0.75% ethylene glycol (EG) and 2% ammonium chloride (AC) with the aim to induce hyperoxaluria in the kidneys.[8] Maintained on ethylene glycol, three groups (2, 3and 4) of rats were also given daily, by gavage, *AEPL*, respectively at different concentrations (200 mg, 100mg and 50 mg). Animals were weighed every day of the experiment.

Analytical procedures

At the end of the experiments (10 days), the animals were sacrificed and the blood was collected and centrifuged for 10 minutes. In the serum obtained, the levels of calcium, urea, phosphorus and creatinine were analyzed. After sacrifice, both kidneys were removed. The left one was held at a temperature of 100° for 24 hours. After drying, the kidney was weighed and crushed into a container with 7 ml 0.5N nitric acid. After the mixture heating, the calcium level was determined by flame specftroscopy.[19] After fixation in the bouin liquid[20], the right kidney underwent histological sections of 3 to 4 µm thick and colored by hematoxylin and eosin.[21]

The presence of stone in the kidney tissues is quantified and classified into categories. The negative symbol (-) is assigned when there are no stones, (±) when the numbers is1 to 5, (+) 6 to 10 and (++) when the number declines 11 stones.

Gastric ulcer induced by HCL/Ethanol

The anti-ulcerogenic activity of *P.lentiscus* leaves extract was studied using 150mM Hcl/Etoh solution. Five groups of rats were used. A control group wish received an oral saline solution, tested groups which receiving aqueous extract (50, 100 and 200mg/kg) and the standard group received omeprazole (30 mg/kg) by the same route. One hour after necrosis agent administration, animals were sacrificed and their stomachs were excised and opened along the greater curvature in order to examine the existence of lesions and to determine the index of ulceration. In this experiment, all the animals were deprived of food for 36 hours but had free access to the water.

Statistical analysis

All the results were presented as the means +SEM. The difference between the groups was determined by one-way ANOVA test. Values with $p<0.05$, were statistically significant.

RESULTS

Acute toxicity test

All administered doses (200, 400, 600, 800 and 1000 mg/kg) of AEPL, did not cause toxicity, lethality or behavioral changes in mice.

HPLC analysis results

On the basis of retention time (min) of the standard compounds the polyphenols identified in AEPL were Gallic Acid (R_t=3.9), Caffeic Acid (R_t=16.1), Ferulic Acid (R_t=23.3), Naringebin(R_t=27), Sorbic Acid (R_t=27.8). The chromatogram showing polyphenolcompounds in the extracts is represented in **Fig.1.**

Ethylene glycol-induced urolithiasis

Serum analysis

In this study, levels of biochemical factors (urea, creatinine) were very higher in all groups receiving AEPL and treated by the EG (group 2, 3, 4 and 5) compared to the control group (table 1). The increase of those factors indicates the occurrence of renal damage caused by the EG treatment. The results also indicate thatcreatinine, urea, calcium and phosphorus levels were significantly lower in rats receiving AEPL(100 and 200 mg/kg) compared to rats treated with EG/AC alone.

Kidney levels of calcium

the analyzes showed the existence of a high amount of calcium (3.11±0.73 mmol/l) in the kidneys of animals treated with EG compared to control animals (2.34±0.14mmMol/l) while the oral administration of 200 mg/kg of AEPL reduced this calcium accumulation compared to the positive group (Table 2). This dose of AEPL also reduces the amounts of phosphorus, urea and creatinine.

Observations of histological kidney sections revealed the existence of significant calcium depositions in the majority of the kidneys of the animals treated with EG alone (Fig. 2, e). In contrast, after the AEPL treatment (100 and 200 mg/kg) a very significant reduction in the number of formed stones was noted (Fig.2b, 2c). In the kidneys of rats given especially 200 mg/kg dose of AEPL, the CaOx depositions were very limited (Fig.2b). No CaOx depositions were found in renal tissues of control group (Fig. 2a).

HCL/Ethanol induced gastric ulcer

Obtained results showed that administration of HCl/EtOH to the control animals induces an important ulceration with an ulcer index of 17,517± 0,1662. The omeprazol groups showed significant reduction ($p<0.001$) in ulcer index with 6,550± 0,1522 and a percentage of inhibition of 62,04%. The rats pre-treated orally with 3 doses (50, 100 and 200mg/kg) of the extract, showed a dose dependant reduction of the HCl/Ethanol induced gastric ulcerationscompared to control group. The percentages of inhibition are 35, 49%, 44, 92% and 54, 62% respectively (table 4).

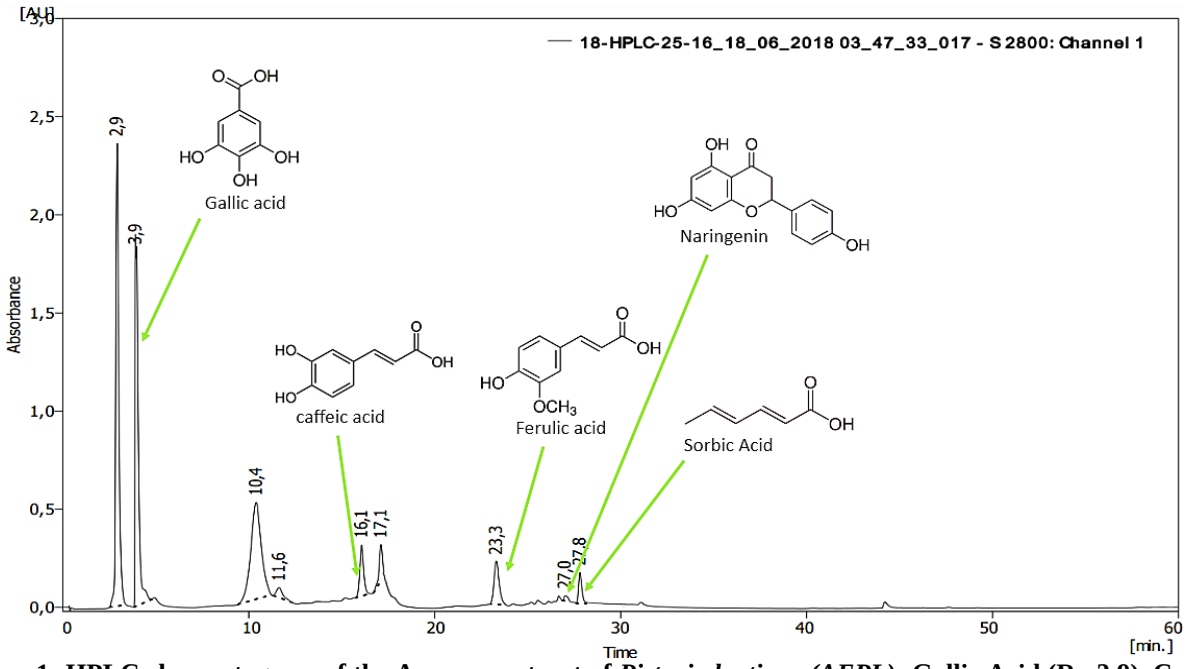

Figure 1: HPLC chromatogram of the Aqueous extract of *Pistacia lentiscus(AEPL):* Gallic Acid (R_t=3.9), Caffeic Acid (R_t=16.1), Ferulic Acid (R_t=23.3), Naringebin (R_t=27), Sorbic Acid (R_t=27.8).

Table 1: Effect of aqueous extract of *P. Lentiscus leaves* on serum parameters in control and experimental animals.

Parameters	Calcium	Phosphorus	Urea	Creatinine
Groups	**mmol/l**	**mmol/l**	**mmol/l**	**µmol/l**
Negative control	2.34±0.14	2.33±0.14	4.43±0.63	42.67±1.86
200 mg/kg	2.41±0.47[a]	3.18±0.63[**a]	16.83±1.09[***b]	107.23±14.19[***b]
100 mg/kg	3.03±0.88	4.02±0.82[***]	18.04±1.31[***a]	113.17±16.34[***a]
50 mg/kg	3.14±0.56[**]	4.11±0.37[***]	20.99±3.03[***]	133.00±15.74[***]
Positive control	3.11±0.73[*]	4.22±0.57[***]	19.92±1.51[***]	136.67±16.68[***]

The values are expressed as means ± S.E. for six animals in each group.
[*]p< 0.05; [**] p< 0.01; [***] p< 0.001: significantly different compared with the control group.
[a]p<0.05; [b]p< 0.01; [c]p< 0.001: significantly different compared with the untreated group.

Table 2: Effect of aqueous extract of *P.lentiscus* on contents of calcium in dry rat papillary left kidney tissue (µg /g).

Groups	Calcium (µg/g)
Negative control	255.90±34.94
200 mg/kg	273.64±26.65[b]
100 mg/kg	292.23±40.05[a]
50 mg/kg	348.42±53.27[**]
Positive control	367.75±66.38[**]

The values are expressed as means ± S.E. for six animals in each group.
[*]p<0.05; [**] p< 0.01; [***] p< 0.001: significantly different compared with the control group.
[a]p< 0,05; [b]p< 0.01; [c] p< 0.001: significantly different compared with the untreated group.

Table 3: Effect of aqueous extract of *P.lentiscus* on crystal deposits on the surface of the kidney tissue.

Crystal deposits	Control rats (Group 1n=6)	200mg /kg (Group 2 n=6)	100mg/kg (Group 3n=6)	50 mg/kg (Group 4n=6)	Positive control (Group 5 n=6)
None	6	5	4	-	-
Crystals: +	-	1	2	2	-
Crystals ++	-	-	-	4	-
Crystals +++	-	-	-	-	6

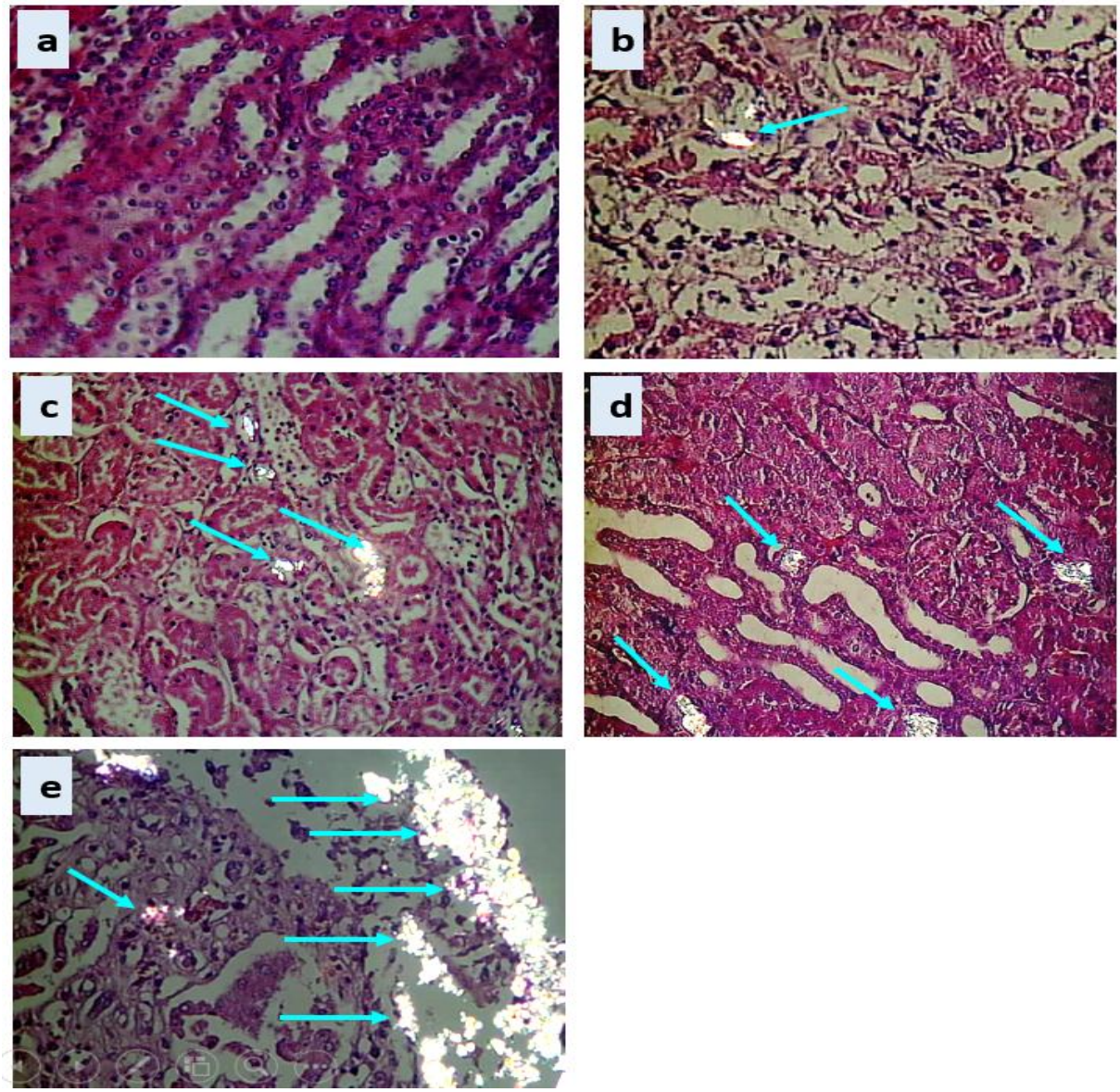

Figure 2: Paraffin sections viewed under polarized light of (a) a control rat kidney 'negative control group', (b) a kidneys from a rat that received EG and 200 mg/kg, (c) 100 mg/kg and (d) 50 mg/kg of aqueous extracts of *Pl* 'treated group' and (e) a kidney from a rat that received EG only 'positive control group' where numerous crystals can be seen. (× 400).

Table 4: Effect of P.*lentiscus* leaves extract againstulcer induced by HCl/Ethanol.

treatment	Dose (mg /kg)	Ulceration index (mm)	Pourcentage of inhibition par rapport au control
Contol	-	17,517±0,1662	-
Aqueous Extract	50	11,300± 0,1862 ***	35,49%
	100	9,650± 0,3085 ***	44,92%
	200	7,950± 0,1176 ***	54,62%
omeprazole	30	6,550± 0,1522 ***	62,04%

Values are expressed as Mean ± S.E.M, n= 6 rats per group.
***: P<0,001: very significant from control.

DISCUSSION

Our results demonstrate that *P.lentiscus*possess important antilithiac activities in rats. It's often used in Moroccan traditional medicine and is rich in compounds that have a large spectrum of pharmacological actions in various areas. In response to 10 days of treatment with EG, rats develop renal calculing composed mainly of calcium oxalate.[22,6] In this work, the weight of the positive control and treated (AEPL*)* groups is lower than control group at the end of experiments. Indeed, other studies revealed that Ethylene glycol presents other effects, such as the fall of weight observed in rats and

mice exposed by oral way.[23] In the positive control group, high rates of creatinine and urea could be related to kidney damage. In this way, Cheraft-Bahloul et al., (2017)[7] have reported that the *P. lentiscus*leaves extract reduces the damage induced by calcium oxalate monohydrate crystals on kidneys cells.[7] The AEPL treatment might cause an important diuresis resulting in dissolution and excretion of the formed stones and prevention against formation of new stones in the urinary system. Various studies, demonstrated that the alkaloids of *P. lentiscus*have a high antioxidant capacity.[24] In this context, the decrease of serum levels of waste products can be attributed to the improvement of the glomerular filtration and to the protective effects of *P. lentiscus* against low-density lipoprotein oxidation. The increase rat of calcium in the kidney tissues induced by EG was prevented by AEPL treatment. The examination by microscope of renal sections from EG rats, showed a greater amount of deposit stones compared to treated animals by the plant leaves extract (200 mg/kg). Preliminary phytochemical analysis of the AEPL revealed that there is presence of phenols and flavones. The same result was reported by other authors[24] and the important drop in the number and size of urinary stones induced by AEPL treatment may be due to those compounds. The antiurolithiasic activity of AEPL may also be to the flavonoid compound as reported by some works.[25,26] The occurring mechanism in this activity is accomplished probably, through antioxidant, nephroprotective property, and by decreasing the concentration of constituents contributed in the formation of the urinary stones and by its diuretic properties of AEPL.[10] In this way, Cheraft-Bahloul et *al., (*2017)[7], have reported that the action of *P.lentiscus* compounds could interfere with calcium oxalate monohydrate crystals by preventing adherence to the epithelial tubular cell surface.[7]

The investigation of the antiulcerogenic effect of *AEPL*, have shown the presence of important lesions in control group compared to the *P. lentiscus* groups and to the omeprazole group. Our results indicate that pre-treatment with aqueous extract of this plant, decreased the size of gastric damage compared to the control. In this study, the protective effect of *AEPL* can be attributed to his bioactive substances as flavonoids, tannins, and terpenoids.[26] Those compounds are known for their gastroprotective and antiulcer effects as reported by several authors.[27,28]

In conclusion, our results demonstrated that aqueous extract of *P. lentiscus* leaves display potent antiurolithiasicand anti-ulcer activities in rats. This study, a first in vivo investigation of this species on those diseases, provides additional support for the popular and traditional use of this plant as an effective remedy against urolithiasis and gastric ulcer.

Conflict of interest

Theauthors declare that there are no conflicts of interest.

ACKNOWLEDGEMENT

The authors are thankful to the Department of pathology, Avicene military Hospital of Marrakech for their assistance to carry out the histopathological evaluation. We also thank Abderrazak Regragui for the expert animal handling required in this study and Prof Ahmed Ouhammou for the plant determination.

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