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Research Article

Nutritional Value, Phytochemical Composition, Toxicological Properties and Antiulcer Action of the Aqueous Extract from *Pinus halepensis* Mill. Needles in Swiss Mice

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Abstract

Background and objectives: Pinus halepensis Mill. (Pinaceae) is commonly used in Tunisian traditional medicine to treat gastro-intestinal disorders, including diarrhea and ulcers. The aim of this study was to investigate the toxicological and gastroprotective properties of the aqueous extract from Pinus halepensis Mill. needles (AEPHN) against ethanol-induced gastric ulcer in mice.

Methodology: The phytochemical/antioxidant properties were analyzed by colorimetric/biochemical methods. The toxicological properties of AEPHN were tested with concentrations ranging from 50 to 3000 mg/kg b.w. To study the gastroprotective action of AEPHN, male mouse were divided into five groups of ten animals in each: control (C), ethanol (EtOH), and EtOH + various doses of AEPHN (75, 150, and 300 mg/kg, b.w., p.o.) were daily administrated for 15 days. The last day, animals were intoxicated by acute administration of absolute EtOH (500 µL/kg, b.w., p.o.). After EtOH intoxication, mice were sacrificed and various physiological and biochemical tests were conducted.

Results: The results demonstrated that AEPHN contained many bioactive compounds with a considerable free radicals scavenging activity. The in vivo studies showed that AEPHN protected against EtOH-induced macroscopic alterations and the secretory profile disturbances. AEPHN significantly corrected the depletion of both enzymatic and non-enzymatic antioxidants. Importantly, AEPHN also regulated the intracellular mediators levels in plasma and gastric mucosal.

Conclusion: Our findings clearly demonstrated that the AEPHN exerted an effective protective effect against EtOH-induced gastric ulcer. These effects could be associated with its antioxidant properties and by opposite effects to the components of the Fenton reaction.

Keywords: *Pinus Halepensis*; Peptic Ulcer; Oxidative Stress; Intracellular Mediators; Mice

Introduction

In humans, the digestive epithelial tissues are covered by layers of mucus. These perform multiple physiological functions. Mucus is secreted by goblet cells and by mucous glands. The glandular stomach has a two-layer system of internal and external mucus. The main constituents of mucus are glycoproteins (mucins), lipids, water and electrolytes. On average, mucus contains nearly 95% water, 0.5 to 5% glycoproteins and lipids, 0.5 to 1% mineral salts, and 1% of other secreted or trans-bonded proteins [1].

The alteration of this layer of mucus is the main cause of various digestive pathologies. In animal models, the gastroduodenal inflammations induced by castor oil/ethanol are accompanied by an alteration of the surface of the coating, the layer of mucus and loss of water and electrolytes [2,3].

Peptic ulcer (PU) is defined by a state of imbalance between the aggressive and defensive factors of the gastric and duodenal mucous membranes [4]. It has been also reported that *Helicobacter pylori* infection, alcoholism, excessive consumption of nonsteroidal anti-inflammatory drugs and smoking are the main causes of this pathology digestive [5].

Additionally, during the peptic ulcer, an inflammatory reaction has set in, via the activation of several possible mechanistic path-

ways: nuclear factor kappa B (NF-κB), mitogen-activated protein kinase (MAPK), Janus kinase signal transducer and transcription activator (JAK-STAT) and peptide transporter 1 (PepT1) [6]. These pathways regulate the release of libidal mediators of inflammation such as pro-inflammatory cytokines (TNF-α, IL-1β, IL-6 and IL-8) and the expression of lipid mediators such as cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) [7]. The gastrointestinal ulcers were accompanied by a state of oxidative stress and consequently an overproduction of reactive oxygen species (ROS). This state was confirmed by a lipoperoxidation and depletion of enzymatic antioxidants (SOD, CAT and GP_x) and non-enzymatic (GSH and –SH groups) [3,8]. ROS are responsible for the oxidation of many molecules, such as nucleic acids, lipids, carbohydrates and proteins, and consequently causing cellular alterations.

To treat this digestive tract diseases, synthetic drugs, such as anticholinergic agents (bethanechol), histamine H2-receptor antagonists, antacids and proton pump inhibitors omeprazole, esomeprazole, lansoprazole, dexlansoprazole, rabeprazole), antibiotics, and nonsteroidal anti-inflammatory drugs are usually used [9,10]. However, prolonged consumption and/or relatively high doses of these synthetic molecules can cause unpredictable side effects, such as excessive salivation, nausea and bradycardia and hypersensitivity reactions such as rashes, fever, intestinal microbiome disease and colorectal cancer [11-13].

In this respect, research is always oriented to find drugs that have the same properties as the standard drug, but without side effects on human health and that will serve as a powerful therapeutic agent. In this context, the WHO and the FAO have strongly encouraged investigations based on traditional practice for the treatment of digestive diseases. Plant-derived phenolic compounds are a good alternative to synthetic drugs, due to their safety, low cost, and ease of large-scale production [15].

Aleppo pine (*Pinus halepensis* Mill., Pinaceae Family) is a species that is characterized by great economic importance, they are used for the production of timber, resin and seeds [16]. Due to its multiple benefits, pine extracts are traditionally used for the treatment of dermatitis, ptilosis and toothache, liver disease, and hypertension [17]. Thanks to their richness in phenolic compounds and essential oils, the needles have anti-inflammatory, anti-cancer, and anti-aging properties [18,19]. They are also characterized by numerous biological properties including antioxidant, antibacterial, antiviral cytotoxic, phytotoxic, and larvicidal activities [20,21].

The present study aimed to investigate the toxicological and gastroprotective actions of Aleppo pine needles aqueous extract against ethanol-induced gastric ulcer in mice.

Materials and Methods

Plant collection and identification

Needles of *Pinus halepensis* Mill. were collected in Mars 2019 from the region of Tabarka (Northwestern Tunisia) and identified by Dr Foued Aloui, Associate professor in the Sylvo-Pastoral Institute of Tabarka-Tunisia. The voucher specimens (No. PH45) have been deposited in the herbarium of the Sylvo-Pastoral Institute of Tabarka, Tunisia. The needles were dried in an incubator at 60°C during 24 hours and powdered in an electric blender.

Determination of dry matter and mineral content

The levels of mineral matter (MM), total carbon (TC) and total nitrogenous matter (TNM) were analyzed according to the procedures of the AOAC [22]. The DM was determined after drying the samples at 105°C during 48 hours. The MM, organic matter (OM) and TC contents were determined following the calcination of the plant powders in a muffle furnace set at 550°C during 4 hours. The TNM were performed according to the Kjeldahl method.

Determination of parietal compounds

The neutral detergent fiber (NDF), acid detergent fiber (ADF) and acid detergent lignin (ADL) contents were determined using a Fibertest device according to the sequential method described by Van Soest., *et al*. [23]. True crude cellulose (TCB) and hemicellulose (HC) contents were estimated from the following equations:

% TCB = %ADF - %ADL

% HC = %NDF - % ADF.

Determination of total lipid content

The determination of total lipids was performed according to the method previously described by Bligh and Dyer [24]. The pwoder was ground in a mortar and the lipids were extracted with a chloroform-methanol mixture in the proportions 2/1 (v/v). The mixture is then filtered in test tubes and placed in a ventilated oven set at 60° C for 48 h. After evaporation, the total lipid yield was calculated.

Aqueous extract from *Pinus halepensis* needles pareparation

The aqueous extract from *Pinus halepensis* needles (AEPHN) was obtained by maceration. In fact, needles powder were macerated in bi-distilled water (1/20; w/v) during 24 h. The sample was centrifuged at 5 000 rpm for 10 min and the supernatant was recovered and lyophilized. The residue thus obtained was weighed and taken up in 3 ml of distilled water. Finally the product was stored at -40°C until use.

Determination of total polyphenols

The determination of total polyphenols was carried out using the Folin-Ciocalteu reagent [25]. The total content of phenolic compounds was expressed in mg of gallic acid equivalents per gram of dry matter (mg GAE/g DM).

Quantification of total flavonoids

The flavonoids were determined according to the method described by Hussain., *et al*. [26]. The total flavonoid contents were expressed as quercetin equivalents (mg QE/g DM).

Dosage of condensed tannins

The concentration of condensed tannins in AEPHN was determined according to the method previousley described by Price., *et al*. [27]. The total tannin content was expressed in catechin equivalent (mg CE/g of DM).

In vitro antioxidant capacity

The free radical scavenging activity of AEPHN was determined according to the method previously described by Elfalleh., *et al*. [28]. Briefly, 500 µL at different increasing concentrations of extract (from 10 to 400 µg/mL) were prepared and each concentration was added to 375 µL of an ethanolic solution of DPPH (96%). The mixture was kept in the dark at room temperature for 60 minutes. Absorbance was recorded at 517 nm. Finally, the percentage inhibition of free radical DPPH was calculated and the IC_{50} value was determined from the graph curve.

Animals and pretreatment

Adult male Swiss Albino mice (weighing approximately 25 g; housed ten per cage) were purchased from the Society of Pharmaceutical Industries of Tunisia (Ben- Arours, TN). They were provided with standard food (standard pellet diet-Badr, Utique, TN) and water ad libitum and maintained in an animal house at a controlled temperature (22 ± 2°C) with a 12 h light–dark cycle.

All the experiments on animals were used following the local ethics committee of Tunis University of the use and care of animals and in accordance with the NIH recommendation. The protocol was approved by the "Comite d'Ethique Bio-medicale (CEBM)" (JORT472001) of the "Institut Pasteur de Tunis".

Toxicological activity

The acute toxicity of AEPHN was studied according to the method previously described by Olorunnisola., *et al*. [29]. AEPHN were administered orally to different groups of mice (n = 6) at increasing doses as follows: 50, 100, 200, 400, 600, 800, 1000, 1500, 2000, 2500 and 3000 mg/kg b.w. Animals were examined every 30 minutes for up to 4 hours, then occasionally for an additional 8 hours. After 24, 48 and 72 h, mortality was recorded. The mice were also observed for other signs of toxicity, such as motor coordination, righting reflex, respiratory changes, and digestive disorders.

Antiulcerogenic activity of AEPHN

The animals were divided into five groups of 10 mice and were pretreated during 15 days as follows: Groups 1 and 2 received bidistilled water (5 ml/kg, b.w., p.o.) and served as controls. Groups 3, 4, and 5 were pretreated with various doses of AEPHN (75, 150 and 300 mg/kg, b.w., p.o.). Animals were fasted for 20 hours before the last administration of AEPHN. After 2 hours, each animal, except those of group 1, was intoxicated by acute administration of absolute EtOH (500 µL/kg, b.w., p.o.). One hour later, all animals were sacrificed. Blood was collected in heparinized tubes. After centrifugation at 3000 rpm during 15 min, plasma was treated for biochemical analysis.

Evaluation of gastric mucosal damage

The stomach of each animal was removed and opened along its greater curvature. The tissues were gently rinsed in NaCl 0.9 %. The lesions in the gastric mucosa were macroscopically examined and the photographs of hemorrhagic erosions were acquired with a Canon EOS1100D digital camera. Ulcer indexes were determined as the sum of the lengths of the whole gastric lesions (in mm^2). The percentage of ulcer inhibition or/and AEPHN protection was calculated according to the following formula:

Inhibition (%) = [(GUI EtOH group − GUI AEPHN group)/(GUI EtOH group)] × 100.

Determination of pH and gastric juice volume

After gastric opening, the stoamch contents were collected and centrifuged at 2000 rpm for 10 min. The total volume of the gastric content was expressed with mL. The pH values were measured using an ADAT type pH meter.

Gastric mucosa preparation

The gatric mucosas were rapidly excised and homogenized in phosphate buffer saline. After centrifugation at 10 000 rpm for 10 min at 4°C, supernatants were used for biochemical determination of protein, intracellular mediators, sulfhydryls groups (-SH), and antioxidant enzyme activities (SOD and CAT).

Assessment of superoxyde dismutase activities

The spectrophotometric method was used to evaluate the SOD activity in stomach mucosa using the epinephrine/adenochrome system and using bovine catalase (CAT, 0.4 U/mL) and epinephrine (5 mg/mL) as enzymes. Changes in absorbance were evaluated at 480 nm [30].

Stomach mucosa catalase (CAT) activities

The activity of CAT was assessed by measuring the rate of hydrogen peroxide disappearance by spectrophotometry at 240 nm, which will be degraded into H_2O and O_2. CAT activity is expressed in µmoles H_2O_2/mg protein [31].

Gastric groupements sulfhydriles levels

The thiol groups (-SH) content in stomatch mucosa was determined according to the method of Ellman [32]. The concentration of thiol groups was calculated by subtraction operation between two absorbances (A_2 and A_1) using a molar extinction coefficient of $13.6\times103\ M^{-1}cm^{-1}$. The results were expressed In nmol of thiol groups/mg proteins.

Intracellular mediators

The gastric and plasma H_2O_2 levels were determined according to the method of Dingeon., *et al*. [33]. Briefly, the hydrogen peroxide reacts with p-hydroxybenzoic acid and 4-aminoantipyrine in the presence of peroxidase leading to the formation of quinoneimine that has a pink color detected at 505 nm.

The levels of free iron and clacium in gastric mucosa and plasma were measured colorimetrically using commercially available diagnostic kits (Biomaghreb, Ariana, TN, Tunisia).

Protein determination

The gastric and plasma total protein concentrations in plasma were determined using commercially available diagnostic kits (Biomaghreb, Ariana, TN, Tunisia).

Statistical analysis

The data were analyzed by one-way analysis of variance (ANOVA) and expressed as means +/- standard deviation (S.D.). All analysis was performed using the SAS (Statistics Analysis System). All statistical tests were two-tailed, and a $P < 0.05$ was considered significant.

Results

Phytochemical properties, nutritional value and in vitro antioxidant activity of AEPHN

The results of the analysis carried out on the powder showed that the needles of *Pinus halepensis* are provided with abundant contents of mineral matter, organic matter and total carbon. On the other hand, Aleppo pine has a high level of fibers (parietal constituents) such as NDF (48.27 ± 3.32% of DM), ADF (32.03 ± 2.84% of DM), cellulose (16.93 ± 1.26% of DM) and hemicelluloses (14.93 ± 1.26 % of DM). In addition, the needles contain a high concentration of total lipids (11.29 ± 0.83% of DM) (Table 1).

On the other hand, AEPHN also contains high levels of total polyphenols, flavonoids and tannins, and a moderate concentration of condensed tannins.

For the antioxidant activity, we showed that the percentages of inhibition of AEPHN and ascorbic acid (AA) against the DPPH• radical were increased in a dose-dependent manner. However, AEPHN is characterized by a significant activity antiradical (IC_{50} = 78.45 ± 2.86 µg/mL), but lower than that of AA (IC_{50} = 22.64 ± 0.22 µg/mL) used as reference antioxidant molecule (Table 1).

Acute toxicity of AEPHN

There were no visible signs of toxicity and death in animals treated with AEPHN with doses of increasing order (50 to 3000 mg/kg) for 3 days. In acute toxicity tests, oral administration of AEPHN to mice dit not result in any significant alterations in behavior, respiration, sensory responses of the nervous system or gastrointestinal effects during manipulation. Additionally, there was no apparent change in body weight and no change in water and food consumption. Finally, no mortality or toxic reactions in any animal were observed in any group throughout the study period (72 h). The LD_{50} is therefore greater than 3000 mg/kg of body weight for the AEPHN.

Parameters		Contents
Mineral and organic composition	Mineral matter (MM %)	10.44 ± 0.02
	Organic matter (OM %)	99.56 ± 0.08
	Total carbon (TC %)	56.12 ± 1.25
	Total nitrogenous matter (TNM %)	16.40 ± 0.32
Parietal composition	Neutral Detergent Fiber (NDF %)	48.27 ± 3.32
	Acid Detergent Fiber (ADF %)	32.03 ± 2.84
	Acid Detergent Lignin (ADL%)	13.23 ± 0.25
	True Crude Cellulose (TCB %)	16.93 ± 1.26
	Hemicelluloses (HC %)	14.24 ± 0.68
	Soluble fraction (SF %)	51.73 ± 2.94
Total lipids (TL %)		11.29 ± 0.83
Phenolic compounds	Total polyphenols (mg GAE/g DM)	110.25 ± 3.42
	Flavonoids (mg QE/g DM)	47.24 ± 1.64
	Total tannins (mg TAE/g DM)	34.42 ± 2.92
	Condensed tannins (mg CE/g DM)	4.52 ± 0.23
DPPH• (CI50, µg/mL)		78.45 ± 2.86
Ascorbic acid (CI_{50}, µg/mL)		22.64 ± 0.22

Table 1: Phytochemical composition, nutritional quality and IC_{50} values of anti-radical activities of the aqueous extract from *Pinus halepensis* needles (AEPHN) and ascorbic acid (AA) against DPPH• radical; IC_{50}: the inhibitory concentration of AEPHN and AA which inhibits 50% of the DPPH concentration.

Data are expressed as mean ± SD (n = 3); SEM: Standard Error of the Mean; DM: Dry Matter; GAE: Gallic Acid Equivalent; QE: Quecetin Equivalent; TAE: Tannic Acid Equivalent; CE: Catechin Equivalent.

Qualitative and quantitative macroscopic evaluation of AEPHN anti-ulcer activities

Animals intoxicated with ethanol showed an extensive elongated thick, dark red and black band of hemorrhagic lesions on the glandular part of the stomach as shown in figure 1. However, AEPHN pre-treatment significantly protected the gastric mucosa against EtOH induced injury.

Effect of AEPHN on stomach ulcer index

As shown in table 2, gastric mucosa in the EtOH group is characterized by a significant increase in ulcer index. AEPHN pre-treatment restricted the alcohol-induced gastric deregulation in a dose-dependent manner.

Effect of AEPHN and EtOH on pH and gastric juice volume

The stomach ulceration was accompanied by a decrease of pH values as well as an increase of the gastric juice volume. AEPHN administration significantly protected against the secretory profile disturbance in a dose-dependent manner (Table 3).

Parameters/ Groups	Ulcer Index (Mean ± SD)	Percentage of protection (%)
Control	0	--
EtOH	8.44 ± 0.08*	--
AEPHN 75 + EtOH	5.56 ± 1.32#	34.12
AEPHN 150+ EtOH	3.76 ± 0.63#	55.54
AEPHN 300+ EtOH	2.48 ± 0.12#	70.12

Table 2: Effect of the aqueous extract from *Pinus halepensis* needles (AEPHN) on gastric macroscopic alterations induced by EtOH: ulcer index and percentage of protection (%). Animals were pretreated with various doses of AEPHN (75, 150 and 300 mg/kg, p.o.) or vehicle (H_2O). The data are expressed as mean ± SD. (n = 10) $^*p < 0.05$ compared to control group and $^\#p < 0.05$ compared to EtOH group.

Parameters/Groups	Gastric volume (mL)	pH of gastric juice
Control	1.14 ± 0.22	4.75 ± 0.85
EtOH	0.75 ± 0.08*	2.12 ± 0.33*
AEPHN 75 + EtOH	0.96 ± 0.06#	3.75 ± 0.75#
AEPHN 150+ EtOH	1.03 ± 0.13#	3. 83 ± 0.16#
AEPHN 300+ EtOH	1.08 ± 0.05#	4.09 ± 0.25#

Table 3: Effect of the aqueous extract from *Pinus halepensis* needles (AEPHN) on EtOH-induced disturbance on pH and volume of gastric juice. Animals were pretreated with various doses of AEPHN (75, 150 and 300 mg/kg, p.o.) or vehicle (H_2O). The data are expressed as mean ± SD. (n = 10) $^*p < 0.05$ compared to control group and $^\#p < 0.05$ compared to EtOH group.

In vivo antioxidant activity of AEPHN

On other hand we examined the effect of AEPHN and acute EtOH administration against antioxidant enzyme activities and -SH groups (Figure 2). As expected, stomach injuries were accompanied by a depletion of superoxide dismutase (A) and catalase (B) activities and -SH groups levels (C). More importantly, AEPHN treatment restored the depletion of both enzymatic and non-enzymatic antioxidants.

Effects of AEPHN on intracellular mediators

We further looked at the EtOH and AEPHN on intracellular mediators such as hydrogen peroxide, free iron and calcium levels in plasma and gastric mucosa (Table 4). Alcohol group showed a significant decrease in free iron, H_2O_2 and ionizable calcium levels in plasma and gastric mucosa when compared to negative control group. However, subacute pretreatment AEPHN (75, 150 and 300 mg/kg, p.o.) protected the alteration of those intracellular mediators.

Parameters/Groups	H_2O_2 (mmol/mg protein)		Free iron (µmol/mg protein)		Calcium (µmol/mg protein)	
	Plasma	Stomach	Plasma	Stomach	Plasma	Stomach
Control	0.12 ± 0.02	0.25 ± 0.08	8.56 ± 0.56	20.18 ± 1.06	25.56 ± 4.65	40.34 ± 4.65
EtOH	0.26 ± 0.08*	0.63 ± 0.02*	20.18 ± 2.08*	43.58 ± 3.22*	74.29 ± 7.96*	98.55 ± 6.3 6*
EtOH + AEPHN 75	0.17 ± 0.09#	0.49 ± 0.07#	15.20 ± 1.86#	35.45 ± 2.17#	52.64 ± 4.55#	71.37± 8.49#
EtOH + AEPHN 150	0.14 ± 0.04#	0.34 ± 0.03#	12.56 ± 1.74#	28.86 ± 2.06#	47.86 ± 6.46#	57.24 ± 6.88#
EtOH + AEPHN 300	0.13 ± 0.07#	0.32 ± 0.03#	10.95 ± 0.88#	23.76 ± 1.13#	35.73 ± 5.54#	46.66 ± 4.32#

Table 4: Effect of the aqueous extract from *Pinus halepensis* needles (AEPHN) on EtOH-induced disturbance in gastric mucosa and plasma H_2O_2, free iron and calcium levels. Animals were pretreated with various doses of AEPHN (75, 150 and 300 mg/kg, p.o.) or vehicle (H_2O). The data are expressed as mean ± SD (n = 10) $^*p < 0.05$ compared to control group and $^\#p < 0.05$ compared to EtOH group.

Discussion

In the present reserach study, the nutritional value, chemical composition of Aleppo pine needles and the gastroprotective actions of AEPHN against EtOH-induced gatric ulcer in mice were evaluated.

The phytochemical compositions at the plant level provide nutritional value. Physico-chemical analysis has shown that Aleppo pine needles contain high levels of mineral matter and total nitrogenous matter. Indeed, previous work has shown that feeds, which contain high levels of total nitrogenous matter, have positively affected digestibility parameters in ruminants [34]. Other studies have shown that total nitrogen is an essential player in the synthesis of parietal compound. It is also involved in the processes of expression of defense genes. In addition, nitrogenous constituents are involved in the synthesis of phytoalexins, and protection against oxidative stress [35]. On the other hand, *Pinus halepensis* powder has a high fiber content. These parietal constituents are necessary and in great demand in medicine. Indeed, a high fiber content (NDF) in functional foods can be used as a laxative agent in people who suffer from constipation. Fiber has been shown to act, via physical action, as a major laxative causing the acceleration of the gastrointestinal transit process [36]. These constituents are also valued in the field of animal nutrition [34].

Phytochemical screening showed that AEPHN exhibits strong scavenging action against DPPH• radical. This antioxidant activity could be attributed to high levels of phenolic compounds such as flavonoids and total and condensed tannins at the AEPHN. Our results are in line with those published by Bhardwaj., *et al.* [37]. In addition the results published by Refifa., *et al.* [38] showed that the contents of total polyphenols, flavonoids and condensed tannins

varied significantly depending on the collection region and the the part of the plant. In addition, the HPLC analysis of AEPHN allowed to identification of eighteen phenolic compounds [38], including phenolic acids (gallic, procatchuic and caffeic acids) and flavonoids such as apigenin, luteolin 7 glucoside and resveratrol. These compounds are characterized by significant anti-inflammatory properties and can protect against the onset of oxidative stress and inflammatory reactions [8,39].

In vivo, the toxicicological properties showed that the LD_{50} is significantly higher than 3000 mg/kgb.w., p.o. Indeed, no mortality or behavioral changes were recorded during the period of observation. Generally the bioactive molecules isolated from Aleppo pine are characterized by low toxicity. Indeed, the study evaluated by Atmane., *et al*. [40] showed that cold pressed oil of *Pinus halepensis* seeds (COPHS) is relatively non-toxic and has a large margin of safety (LD_{50} > 5000 mg/kg).

In the present study, the acute alcohol administration altered the gastric mucosa and submucosa resulting in the appearance of elongated bands of dark red coloration. Our data are in line with previous reports that have used EtOH as an inducer of gastric/duodenal lesions in rats [3,41]. Other studies have also shown similar effects using a combination of HCl/EtOH to induce gastric ulcer in mice [42]. Subacute treatment with AEPHN during 15 days protects against alcohol-induced injury.

The deficiency of prostaglandins in the gastroduodenal mucosa is defined as the major pathogenic mechanism of diseases of the digestive system induced by ethanol. However, this deficit makes the digestive mucosa more vulnerable to different types of attack [43]. Alcohol intoxication caused lesions of the vascular endothelial cells in gastric mucosa and induced microcirculatory disorders and hypoxia, linked to the massive production of free radicals [44].

The AEPHN also contributed to the reduction of observed macroscopic damage. Indeed, the phenolic compounds of the aqueous extract of Aleppo pine act according to many mechanisms of action, such as the chelation of metal ions (Fe^{2+}, Cu^{2+}), membrane stabilization [45], inhibition of pepsinogen production and increased mucus production, which traps bicarbonates and delays the penetration of H^+ endoluminous ions [46].

The consumption of AEPHN corrected these disturbances in a dose-dependent manner. The state establishes a pH gradient ranging from less than 3 at the luminal side of this layer, to more than 7 at the surface of the mucosa. In this context, we have shown that alcohol intoxication leads to a decrease in pH values and volume of gastric juice. On the other hand, pretreatment with AEPHN significantly regulated these disorders in the secretory profile in a dose dependent manner. These results are in line with some previous reports using other medicinal plants such as *Myrtus communis* [41] and *Diospyros mespiliformis* [47].

The AEPHN contains a high level of total tannins (34.42 ± 2.92 mg TAE/g DM). These phytoactives molecules are known for their protective actions against peptic ulcer development through vasoconstrictor effects or by precipitating their proteins into the site of ulceration, producing an impermeable coating on the mucosa that defends the underlying mucosa from injury. We also observed a high content of flavonoids, known for their anti-ulcer and gastroprotective properties [47].

It was also exhibited in the present study that EtOH intoxication induced the depletion of the activities of antioxidant enzymes such as SOD and CAT, as well as a decrease in the levels of thiol groups. Deregulations of these parameters are defined as an indicator of ROS generation in tissues and plasma. However, it is well known that acute administration of EtOH induces a state of oxidative stress through several pathways such as generation of reactive oxygen species [8]. Indeed, SOD catalyzes the superoxide radical by dissipation into H_2O_2, which has been reduced in the gastric mucosa. When this intracellular mediator was not recovered by CAT, this could lead to lipo-peroxidation after generation of hydroxyl radical ($OH^{\bullet}$), known for its high reactivity.

More importantly, the administration of AEPHN at increasing doses (75, 150 and 300 mg/kg, p.o.) suppressed EtOH-induced oxidative stress in the gastric mucosa. These results are similar to those published in previous reports that reported the benefits of plant-derived bioactive compounds against the deleterious effects of EtOH, such as *Ceratonia seliqua* [3] and *Salvia officinalis* [8].

The protection offered by PHNAE is attributed to its richness in total polyphenols, flavonoids, condensed tannins and a high level of gallic, procatchuic and caffeic acids and flavonoids such as luteolin 7 glucoside, apigenin and resveratrol [38]. These molecules are the main source of the antioxidant capacity of this aqueous fraction studied and participate in the elimination of free radicals such as the superoxide anion ($O_2^{\bullet-}$) and the hydroxyl radical ($OH^{\bullet}$). In addition, sulfydryl groups are also involved in gastric cytoprotection, as well as in maintaining the integrity of the mucosal barrier and the elimination of ROS [48].

The obtained results demonstrated a significant increase in intracellular mediators such as hydrogen peroxide, calcium, and free iron in plasma and gastric mucosa in response to EtOH-induced oxidative stress. These data are consistent with several previous studies [41]. This deregulation of EtOH-induced intracellular mediators has previously been observed in the liver and kidneys [49]. Interestingly, our plant extract exerted a beneficial effect by chelating free iron and scavenging H_2O_2 and regulating calcium homeostasis. It has been also suggested that pretreatment with AEPHN protects against the overload of gastric mucosal cells with free iron and H_2O_2 caused by the single administration of ethanol. Howver, these two agents are involved in the generation of the hydroxyl radical ($OH^{\bullet}$) via the Fenton reaction, which plays a major role in

oxidative damage by affecting molecular structures [50]. In this respect, Pisoschi and Pop [51] reported that living organisms create a complex endogenous and exogenous antioxidant defense system to protect against ROS.

Conclusion

The results showed that *Pinus halepensis* needles were characterized by a chemical composition that could be adapted to feed animals. In addition, our findings demonstrated that AEPHN exerted protective actions against ethanol-induced gastric ulceration owing in part to its antioxidant properties primarily related to the presence of high amount of phenolic compounds. As a whole, these findings confirm the beneficial effects of AEPHN and their use as a strategy in the treatment of gastrointestinal physiological disorders. More importantly, the phytoactive compounds of Aleppo pine can be used as an alternative to marketed antiulcer drugs as well as in the formulation of phytomedicines.

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Conflict of Interest Statement

Authors declare that there are no competing interests.

Ethical Statement

All the experiments on animals were used following the local ethics committee of Tunis University of the use and care of animals and in accordance with the NIH recommendation. The protocol was approved by the "Comite d'Ethique Bio-medicale (CEBM)" (JORT472001) of the "Institut Pasteur de Tunis".

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