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Protective effect of zinc and Pine barks extract supplement against Indometacin-induced gastric ulcer in rats

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Abstract: *This investigation was aimed to study the effect of Pinus halepensis aqueous bark extract and zinc to prevent indomethacin induced gastric ulcer in rats. Thirty female albino Wistar rats were divided into 6 groups of 5 animals each (n=5); Group 1: normal control, Group 2: ulcer rats received normal saline, Group 3: ulcer rats treated with P. halepensis, Group 4: ulcer rats were treated with zinc, Group 5: ulcer rats were treated with P. halepensis + zinc and Group 6 ulcer rats were treated with Ranitidine for 15 days. Stomach ulcer was induced by a single oral administration of indomethacin (30 mg/kg). Various biochemical, physiologic and histologic parameters were estimated. Obtained results show that the ulcer index, pH and total acidity level were significantly reduced ($p < 0.05$) and Pepsin activity was significantly increased ($p < 0.05$) in ulcer induced rats pre-treated with extract of P. halepensis, zinc and ranitidine when compared with indomethacin treated rats. The MDA level was significantly decreased and GSH level was increased ($p < 0.05$) in rats treated with plant extract and zinc. Histopathology of gastric mucosa confirmed the gastro-protection by plant and zinc treatment. The study reveals anti-ulcer and antioxidant properties were observed in bark aqueous extract of P. halepensis groups with a benefic effect of zinc to reduce oxidative stress and gastric ulcer induced in the rat.*

Keywords: *Pinus halepensis*, zinc, stomach ulcer, oxidative stress, Wistar rats

Introduction

The stomach is a remarkable organ as it secretes digestive juices that can digest the various foods we eat, but seldom digests it-self. However, one pathological condition that has given the stomach serious problem is ulcer [1]. Gastric ulcer disease is a universal condition; its prevalence is estimated at 10% of the world's adult population [2]. Several factors are responsible for gastric ulcer disease of which 70% to 80% are due to infection of *Helicobacter pylori*, a spiral shaped and gram-negative bacterium. Oxidative damage is responsible for gastric mucosal injuries, including ulceration, erosion and hemorrhage. It increases the formation of superoxide radicals and hydrogen peroxide (H_2O_2), lipid peroxidation and protein oxidation, which can directly promote damage of DNA, membrane lipids, cellular proteins and organelles [3]. However, the use of non-steroidal anti-inflammatory drugs (NSAIDs) accounts for approximately 25% of gastric ulcer cases with an upward trend [4]. Non-steroidal anti-inflammatory drugs (NSAIDs) are some of the most commonly prescribed drugs in the world [5]. Indomethacin is a non-steroidal anti-inflammatory agent with antipyretic, analgesic properties and is an indole derivative designated chemically as 1-(p-chlorobenzoyl)-5-methoxy-2-methyl-1H-indole-3-acetic acid [6]. Natural products have a wide range of diversity of multi-dimensional chemical structures; in the meantime, the utility of natural products as biological function modifiers has also won considerable attention. Subsequently, they have been successfully employed in the discovery of new drugs and have exerted a far-reaching impact on chemobiology [7]. Pine (*Pinus halepensis*) is a large species of trees which grew widely in temperate regions, it has been used in traditional medicine in many parts of the world. Thus, this study was carried out to investigate the gastro-protective effect of aqueous extract of *P. halepensis* barks and Zinc against experimental gastric ulcer induced by indomethacin in rats.

Materials and methods

Plant materials

In this study, pinus barks (*P. halepensis*) were obtained from the market. These barks were powdered by a mechanical grinder until a fine powder was obtained. The powders of *P. halepensis* are stored at room temperature in airtight containers protected from bright light until the beginning of the experiment.

Aqueous extract preparation

Aqueous and organic extracts were prepared by putting 10 g of homogenized dried barks in 100 ml of distilled water at 50°C for 2 h then the extract was cooled and filtered after 24 h. After extraction, the solvents were removed using rotary evaporator, to get gel-like extracts.

Animal

Animal and husbandry condition

In this study, 30 female Wister rats aged 8 weeks old and weighing 117-182 g, were obtained at the Animal Service of the Pasteur Institute, Algeria. The animals were carried under the same conditions, photoperiod (12 h of light/12 h of black) with a relative humidity of 65.3% and an ambient temperature of ($25 \pm 2^{\circ}\text{C}$) for four weeks. Animals have free access to water and food by a standard diet. The experiment was conducted over a period of 15 days.

Ulcer induced

P. halepensis extracts and drugs were administered to the animals in their water for 15 days. After 15-day treatment, the animals have fasted for 24 h. the ulcer was induced by gavage using indomethacin (dissolved in 2% NaCO_3) and brought to a volume of 1.0 ml with saline ($\text{pH}=6.8$) at a dose of 30 mg/kg on the day of sacrifice.

Treatment animals

After a period of adaptation, the animals were divided into six experimental groups of 5 animals each as follows:

Group 1 (Control groups): were normal rats received 1ml of normal saline oral.

Group 2 (IND treated groups): were gastric ulcer induced rats which received normal saline, oral for 15 days and then received a single oral dose of indomethacin (IND, 30 mg/kg).

Group 3 (IND+*P. hal* treated rats): were rats received an oral dose of aqueous extract of *P. halepensis* (300 mg/kg) for 15 days and then received a single oral dose of IND.

Group 4 (IND+Zinc treated rats): were rats received an oral dose of zinc (1 mg/kg) for 15 days and then received a single oral dose of IND.

Group 5 (IND+*P. halepensis*+zinc): were rats received a concomitant dose of both aqueous extract of *P. halepensis* and zinc using the same doses and routs as above and then received a single oral dose of IND.

Group 6 (IND+Rant treated rats): were rats received an oral dose of Ranitidine (20 mg/kg) for 15 days and then received a single oral dose of IND.

Sacrifice, blood and Gastric juice sampling and tissues collection

After 6 h of indomethacin administration these animals were sacrificed under slight anesthesia by chloroform (94%) by inhalation. Gastric juice was collected from each stomach to measure; there volumes, pH, total acidity, pepsin and Catalase (CAT) activity. Then the stomachs were cut open along the greater curvature and washed in normal saline. Then it was laid flat and the number and degree of erosions were counted and scored. Stomach and duodenal stored at -20°C for oxidative stress and in 10% formaldehyde for histological analysis.

Ulcer determination parameters

Photos of stomach epithelium of Rats

The stomach's epithelium photos were tacked for each rat with perfection camera (Canon version 600).

Determination of Volume and pH of Gastric Juice

The animals were sacrificed, the stomach was dissected out, and the gastric juice collected was centrifuged for 5 min at 2000 rpm. The volume of the supernatant was expressed as ml and pH was measured using pH meter.

Determination of preventive index

The procedure of Ulcer Score was performed according to the method described by Kulkarni [8]. The ulcer index is measured or registered using the following scores involving the number and severity of ulcers as shown in Table 1.

Table 1. Ulcer index measured

Score	Description
0.0	Normal colored stomach
0.5	Red coloration
1.0	Spot ulcers
1.5	Hemorrhagic streaks
2.0	Ulcers with area > 3 but ≤ 5mm
3.0	Ulcers > 5mm

The ulcer index and percentage protection given by the following equation:

Ulcer index (UI) = UN + US + UP / 10,

UI = ulcer index,

UN = average number of ulcers per animal,

US = average of severity score,

UP = percentage of animals with ulcer.

$$\% \text{ Protection} = \frac{\text{Ulcer Index (control)} - \text{Ulcer Index (test)}}{\text{Ulcer Index (control)}}$$

Determination of Total acidity of Gastric Juice

The procedure of Gastric Juice acidity determination was performed as described by Card and Marks [9]. The supernatant of gastric juice was taken and diluted 10 times and a few drops of phenolphthalein were

added to the solution. Titration was done using (0.01 M) NaOH solutions until the color of the test solution changed to light pink, indicating pH=7.0. The volume of sodium hydroxide (NaOH) needed for titration was used in the calculation to derive the hydrogen ion concentration. The total acidity is expressed as m equiv/L using the following formula:

$$\text{Total acidity (m equiv /L)} = n \times 0.01 \times 40 \times 1000,$$

Where, n = volume of NaOH quantified;

40 is the molecular weight of NaOH;

0.025 is normality of NaOH;

1000 is the factor represented in liter.

Determination of Pepsin Activity and Oxidative stress parameters

Pepsin activity was measured according to the method of Northrop [10]. Malondialdehyde (MDA) was measured according to the method described by Sastre et al. [11] and reduced glutathione (GSH) concentration was performed with the method described by Ellman's reagent [12]. The assay of the catalase enzyme activity consists in measuring the catalase-induced loss of H₂O₂ contained in the sample by the measurement of H₂O₂ absorbance at 560 nm using a spectrophotometer according to the Aebi method [13].

Histopathological study of stomach tissues

After rats were sacrificed, stomach tissues were removed and immersed in fixative (solution 36% formaldehyde) Intel the time of slices preparation. Whish dehydrated in ascending graded series of ethanol, cleaned with toluene, immersed in paraffin, and colored with hematoxylin and eosin. Histopathological evaluation was performed with a light microscope.

Ethical information

The study protocol of all animal experiments was approved by the Animal Ethical Committee of Natural sciences and life Faculty, El Oued University, Algeria (Protocol Number 25 CMB/130/ 2018).

Statistical analysis

Our statistical study is carried out by the Minitab software using (Student t test) to compere means among our different experimental groups; Differences were considered statically significant at p<0.05.

Results

Macroscopic evaluation of gastric lesions

Ulcerative indices and gastro protection percentage were determined by measuring ulcerative lesion area. Results shown in Figure 1 indicated that no macroscopic in control and Indo + *P. halepensis* groups, but on the contrary Indo group shows a deep and large lesions in whole stomach compared with the control, the

rest of the experimental groups (Indo + *P. halepensis* + Zn, Indo+ Zn, Indo+ Raniti) shows a little lesions in separated regent of the stomach.

Figure 1. The representative macroscopic appearance of gastric mucosa in control rat (A); Indomethacin treated rats (B); rats treated with *P. halepensis* alone (C) or combined with Zn (D); stomach of rats treated with Zn alone (E); stomach of Ranitidine treated rats (F)

Determination of preventive index

As shown in Table 2, ulcer scoring was significantly increased ($p < 0.001$) in the INDO group compared to the control. The results show also that treatment with *P. halepensis*, zinc and ranitidine decrease significantly ($p < 0.001$) the Ulcer scoring compared to the INDO group. Results of Protection percentage of experimental group show a high protection is appearing in the group treated with *P. halepensis* against stomach ulcer compared to the other group. Although there is a significant decrease in pH and an increase of acidity levels in INDO group compared to control. Treatment with extract of plant, zinc or ranitidine increase significantly of pH ($p < 0.05$) and decrease of acidity levels compared to INDO group. Also, the results show that there is a big decrease in juice volume in INDO group compared to the Control, as for treatment group, the results show that there is a significant increase ($p < 0.05$) of juice volume in the group treated with *P. halepensis* compared to the INDO group and decrease in other treatment groups. The results of pepsin activity show that a significant decrease ($p < 0.001$) in pepsin activity in the INDO group compared to control, treatment with *P. halepensis* and ranitidine increase significantly ($p < 0.05$) of pepsin activity compared to the INDO group, and no change activity of pepsin in the other experimental group.

Table 2. Preventive index parameters in control and experimental groups

Parameters	Control	INDO	INDO+AEPh	INDO+ Zn	INDO+AEPh+Zn	INDO+RANT
Gastric Juice (ml)	359±131	197.5±88,3***	275±143b*	151.1±22.7**	130.0 ± 60.5**	152.5 ±5.8**
Ulcer scoring	0.5±0.003	2.2±0.05***	0.5±0.002 c	0.9±0.07 c	0.7±0.03 c	1.02 ±0.05*c
Ulcer Reduction (%)	-	0.02	99	60	80	60
Gastric Juice PH	6.7±.004	5.1±0.002*	6.6±0.008 a	6.8±0.006 b	6.1±0.07 a	6.5±0.005 a
Total acidity (m Eq/L)	500±6.25	670±3.65***	550±4.65 c	540±5.68*c	620 ±1.89*a	560±8.36**c
Pepsin activity (mg tyr/ml gastric juice)	6.5±0.12	4.01±0.18*	5.01±0.25a	4.4±0.28	4.2±0.34	6.5±0.16a

Data are expressed as Mean ± SEM, (n=5 animals/group)

* p<0.05, **p<0.01, ***p<0.001: significantly different from control group

a p<0.05, bp<0.01, cp<0.001: significantly different from INDO group

Oxidative stress parameters

Stomach and duodenal MDA levels show in table 3. for duodenal results, no change of the MDA level in INDO group compared to the control, but treatment with *P. halepensis*, Zn and Ranitidine decrease the MDA level compared to the INDO induced ulcer group. Also, results indicated that, a high signification increase of stomach MDA levels in drugs group compared to the control but in *P. halepensis* alone or with zinc groups MDA level was decreased significantly compared to the INDO group. On the other hand, Compared to the INDO group, Stomach GSH levels show a significant increase in *P. halepensis* (p<0.01), *P. halepensis* + Zn (p<0.01), Zn (p<0.05) and RANT (p<0.05) groups. In duodenal, no change of the GSH level in INDO group compared to the control, however, the results (Table 3) show an increase in GSH level in all different treatment groups compared to Endo group. The results of catalase enzyme activity in gastric juice illustrate a very high signification increase in CAT activity in the INDO group when compared to the control. Also, the treatment rats by *P. halepensis* extract alone or combined with Zn significantly decrease (p<0.01 and p<0.05) the CAT activity respectively when compared to the INDO group.

Table 3. Oxidative stress parameters levels in control and experimental groups

	Control	INDO	INDO+ AEPh	INDO+Zinc	INDO+ AEPh+Zinc	INDO+RANT
Stomach MDA (μmol/g tissus)	1.40±0.002	2.5±0.005**	1.7 ±0.001 ^b	2.01 ±0.001 ^c	1.5±0.002 ^b	3.02 ±0.001 ^{***c}
duodenum MDA (μmol/g tissus)	2.02±0.002	2.7±0.001**	1.5 ±0.003 ^{***c}	1.7 ±0.002 ^{***c}	0.8 ±0.007 ^{***c}	1.6 ±0.004 ^{***c}
Stomac GSH (μmol/g tissus)	0.19±0.02	0.10±0.05***	0.16 ±0.01 ^b	0.20±0.009 ^a	0.11 ±0.02 ^{**b}	0.20±0.03 ^a
Duodenum GSH (μmol/g tissus)	0.13±0.001	0.06±0.004**	0.23 ±0.008 ^{*c}	0.30 ±0.01 ^{***c}	0.34 ±0.02 ^{***c}	0.30 ±0.06 ^{***b}
Gastric juce Catalase (U/min/g prot)	11.2±0.2	36.04±0.08***	9.13±0.1 ^b	21.17 ±0.5 ^{***a}	22.09±0.4 ^{**}	46.01±1.02 ^{***}

Data are expressed as Mean $\pm$ SEM, (n=5 animals/group).

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$: significantly different from control group.

a $p < 0.05$, bp $p < 0.01$, cp $p < 0.001$: significantly different from INDO group

Stomach histological

As shown in (Figure 2), results indicated normal epithelium muscular layer structure with clear around gastric pits appeared in the control group, conversely in indomethacin (INDO) group the histological results show a deep lesion in whole epithelium layer with hemorrhagic necrosis and a huge destruction in the gastric pits in whole epithelium and muscular layer too appeared in Figure 2B. Histological observations of the stomach body of the rats treated with *P. halepensis* extract show normal morphological appearance of the different components of the mucosa layer with few gastric pits lesion (Figure 2C). In *P. halepensis* + Zn and Zn group the histological results show a partial correction in morphological a deep lesion in gastric pits compared to the INDO group (Figure 2D, 2E). Finally, in Ranitidine treated group the results show a deep lesion in the epithelium and muscular layer with gastric pits totally destroyed compared to the INDO group (Figure 2F).

Figure 2. Histological examinations indicate stomach sections of control rat (A); Indomethacin treated rats (B); Rats treated with *P. halepensis* alone (C) or combined with Zn (D); Stomach sections of rats treated with Zn alone (E); Stomach sections of Ranitidine treated group (F). Haematoxylin and Eosin x 400

★ = deep lesions in epithelium cells, ➡ = gastric pits destruction)

Discussion

The results obtained show that indomethacin induces elevation of ulcer score, a lesion in the gastric epithelium, an increase in the acidity of gastric juice and a decrease of pepsin activity compared with control, which causes a state of gastric ulcer in rats. Our study agrees with several studies that Indomethacin at a dose (30 mg/ kg) induced gastric ulcers, such as Uzma et al. and Alkushi et al. [14, 15]. Indomethacin induces its gastrointestinal toxicity via several mechanisms such as an increase in gastric acid secretion; interfere with mucosal cell regeneration via inhibition of PGE2 synthesis, production of free radicals, reduction of gastric nitric oxide level and invasion of activated neutrophils as well as induction of gastric cells apoptosis [16]. On the other side, the results of our study show that aqueous extract of bark *P. halepensis* shows a remarkable anti-ulcer activity induced by indomethacin. This results it so remarkable at all levels; macroscopic, microscopic, stress and ulcer parameters. These results refer to their phytochemical compounds of aqueous extract of bark *P. halepensis* especially; polyphenols. Where flavonoids, terpenoids, tannins [17], are highly gastroprotective, through different cellular and biochemical pathways [18, 19], flavonoids, tannins, saponins are thought to increase mucosal prostaglandin content, decrease histamine secretion from mast cells by inhibition of histidine carboxylase. Flavonoids are among the cytoprotective active compounds for which antiulcer property has been extensively confirmed. The flavonoids are suggested to be able to stimulate the secretions of mucus, bicarbonate, and prostaglandins [20]. Our results have shown also that zinc treatment partially protect gastric cell from ulcer with. Zinc is physiologically a very important metal and act as a cofactor for many enzymes [21]. The mechanism of the Zn^{2+} induced gastric protection process is not fully explained in this study and further studies are needed to determine whether potent gastro protective and ulcer healing mediators such as endogenous PG and NO could be involved in the ulcer healing action of zinc [22].

Ranitidine is in the same class as cimetidine with the only difference that it contains a furan ring in place of the imidazole ring of cimetidine. Any agents with anti-histaminic property may thus have some form of antiulcer property [23]. This study revealed a significant increase in MDA level and CAT activity and decrease in GSH level in indomethacin group compared to the control. Our results are according to the study of Hakan Dursun shown that gastric mucosal MDA level was increased in the group of rats treated by indomethacin [24]. Increased lipid peroxidation expresses the state of oxidative stress induced by indomethacin by several mechanisms, on the one hand, the Indomethacin inhibited the mitochondrial oxidative phosphorylation leading to the release of cytochrome c from mitochondrial inter membranous space into cytosol and to the release of ROS such as superoxide anion and H_2O_2 [25]. Oxidative stress induced generation of ROS and its overproduction is one of the prime an etiologic factor that causes gastric ulcer [26]. While *P. halapensis* group shows no significant effect of catalase activity compared to control, which this is refer to the high protection of *P. halapensis* barks to cell damage probably due to their second metabolic

compounds. The result of the effect of aqueous extract of *P. halapensis* on oxidative stress parameters appeared a high decrease of MDA in gastric homogeneous compared to Indo group, and show a very high decrease in MDA and increase the reduced glutathione (GSH) level compared to control and the IND group in stomach and duodenal homogenate. The plant extract can improve the oxidative stress by polyphenols and flavonoids contained a hydroxyl group in their structure wish make them have a very important antioxidant activity [27], many flavonoids chelate free Fe and Cu that could otherwise increase ROS generation, and also reduce ROS such as O^{2-} , and $HO\cdot$ [28]. In our study *P. halapensis* level compared to drugs disease group. GSH is probably the most important antioxidant present in the cells [29]. Therefore, other enzymes that help to generate GSH are critical to the body's ability to protect itself against oxidative stress. The functions of GSH are removal of oxygen free radical species and maintenance of membrane protein thiols [30]. Zinc could inhibit oxidative stress via the protection and stabilizer of lipids and proteins. Thus, Zn protects cellular membranes, macromolecules and preservation of sulfhydryl groups for proteins and preventing oxidation by forming strong thiolate complexes. Moreover, these protective and anti-apoptotic actions of zinc were shown to be mediated by the SMAC/DIABLO (mitochondrial protein that potentiates some forms of apoptosis) signaling in indomethacin-induced apoptosis of gastric cells [31]. In the present study, indomethacin causes a total destruction and deep injury in gastric epithelium cells compared to control. We can explain this effect as follows: Cytokines are proteins that play an important role in the inflammatory response. Interleukin (IL)-1B and tumor necrosis factor (TNF)- α are two of the main cytokines involved in the inflammatory response [32]. On the other side histological results of *P. halapensis* show a high protection against tissue lysis induced by indomethacin. our in-vitro result appeared that *P. halapensis* barks containing different second metabolic compound which they have anti-inflammatory property such as flavonoids, according to flavonoids appear to be important modulators of pro-inflammatory cytokines, such as IL-1 β , IL-6 and TNF- α . However, the effect of flavonoids on intracellular signaling pathways and on other inflammatory mediators remains to be investigated, since it would depend on the type of cells, the studied disease and the applied stimulus [33]. The high protection property of our plant *P. halapensis* is due to the inhibition of pro-inflammatory cytokines that refer to their second metabolic. The beneficial effect of zinc at the histological level can be explained as follows: zinc and zinc-containing proteins are involved in nearly every stage of cutaneous wound repair due to their profound role in the modification of the ECM component, cell migration, protein synthesis and anti-inflammatory properties [34]. This healing action by zinc could also be attributable to its protective action on visceral organs by demonstrating that zinc, exerts inhibitory effects on pro-inflammatory NF- κ B activation [35]. Miriam et al. reported that zinc limits iNOS-derived high output NO production in endothelial cells by inhibiting NF- κ B-dependent iNOS cells and protects endothelial cells against H_2O_2 -mediated damage via zinc-mediated increase of cellular glutathione de novo synthesis [36].

Conclusion

Based on the above results, aqueous extract of *P. halapensis* is an excellent source of phytochemical compounds which that are known to be antioxidants. Notably, the aqueous extract of *P. halapensis* shows inhibitory effects of indomethacin-induced acute stomach injury by enhancing anti-oxidative and anti-ulcer activities. Additionally, the findings may have therapeutic value in the treatment or prevention of stomach diseases.

Conflict of interest statement

All authors have approved the manuscript with no conflict of interest.

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Authors' Contribution

D. S. substantially contributed to the conception and design, analysis of the laboratory parameters, drafting the manuscript and analysis and the interpretation of the data K.A. was responsible for the designing of the study, revising and drafting the manuscript.

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