Antioxidant activity and gastro-protective effects of carob pod aqueous extracts on indomethacin-induced gastric ulcer in wistar rats

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Key words: Carob pods, Peptic ulcer, Gastric mucus, Ulcer index, antioxidants.

http://dx.doi.org/10.12692/ijb/12.6.48-69 Article published on June 14, 2018

Abstract

Gastroduodenal ulcer is a recurrent chronic disease aggravated by the use of non-steroidal anti-inflammatory drugs (NSAIDs), and long-term low-dose aspirin therapy. This study aimed at searching a natural anti-ulcer remedy without side effects. The carob pod antioxidant activity was tested on DPPH and its gastro-protective effect was investigated in Wistar rats submitted to a gastric ulcer, induced by a NSAID drug, indomethacin. The study was performed on twenty rats allocated into four groups. The negative control group received distilled water, the positive control group received indomethacin, the standard group received ranitidine, and group four received carob pod aqueous extract (CPAE). Animals were per-orally pretreated during 15 days, and the indomethacin was administrated the 16th day to all rats, except those of the negative control group. Carob exhibited important antioxidant activity (85%). Indomethacin-ulcerated rats showed congested, oedematous and fissured gastric mucus by numerous ulcer lesions, an increased ulcer index and significantly decreased adherent gastric mucus content. Pepsin activity and gastric juice volume increased, while pH diminished significantly. CPAE reduced the volume and increased the pH of the gastric juice, and lowered the pepsin activity. Ulcer lesions and ulcer index were strongly lowered. The gastric mucus was reconstituted and the mucosa appeared almost completely healed, with normal color and thickness. The carob protection percentage was similar to that of ranitidine. Carob therefore, undoubtedly has antiulcer potential, through an anti-secretory effect, possibly related to its strong antioxidant capacity. It can be proposed as a therapeutic opportunity in the management of peptic ulcers.

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Introduction
Gastric ulcer is a major and the most prevalent gastrointestinal disorders, that affects annually 5-10% of the world population. This disease concerns large population in all geographical regions, and is a major cause of mortality in developing countries (Lanas and Chan, 2017). Gastric ulcer is characterized by mucosal damage which may cause significant gastrointestinal bleeding (Lanas and Chan, 2017). It’s physiopathology is associated with the imbalance between protective and aggressive factors in the stomach. Defensive factors include sufficient mucus and bicarbonate secretion, normal blood flow, cells renewal, prostaglandins (PGs), adequate level of nitric oxide (NO), innate resistance of the mucosal cells, antioxidant enzymes and tight intercellular junctions (Backert et al., 2017). The aggressive factors include NSAIDs, Helicobacter pylori infection, excessive secretion of acid and pepsin, bile acids, oxidative stress, food ingredients, lipid peroxidation, inhibition of prostaglandin synthesis, diminished blood flow to the gastric mucosa, increased gastric motility and cell proliferation, free oxygen radicals and excess alcohol ingestion (Suzuki et al., 2012; Hunt et al., 2015). Two main factors are involved in this complex and multietiological disease, the bacterium H. pylori (Lanas and Chan, 2017) and long-term use of NSAIDs, and aspirin at low dose (Sostres et al., 2014; Shim and Kim, 2017). NSAIDs are extensively used throughout the world in analgesic, antipyretic and anti-inflammatory therapies, while aspirin at low dose is widely prescribed as an antithrombotic drug for the prevention of cerebrovascular and cardiovascular diseases (Lijima and Shimosegawa, 2015; Drini, 2017). Their use are associated with an increased risk for severe gastrointestinal mucosal damages as side effects which are generally thought to be mediated primarily through inhibition of mucosal cyclooxygenase-1 (COX-1) activity resulting in suppression of prostaglandin production (Sinha et al., 2013; Goldstein and Cryer, 2015). Many synthetic antiulcer drugs are used in the treatment of peptic ulcer, and unfortunately long-term treatment with these drugs is often associated with several undesirable adverse effects and poor gastric healing, leading to ulcer recurrence (Untersmayr, 2015). In addition, these drugs have low efficacy and are often costly, hence the high cost of managing the disease by the State and the patient himself. The prevention and treatment of peptic ulcers is one of the most important challenges confronting medicine nowadays. All these reasons lead us to orient ourselves to herbal medicine due to their cheapness and availability, particularly for the people of non-industrialized countries. In fact, fruits, vegetables and plants, are rich of bioactive compounds that can be used in the prevention and treatment of various diseases (Mota et al., 2009; Neselo et al., 2017).

Gastric disorders treatment and prevention with herbal medicine is now considered as a good option to manage the disease (Tripathy and Afrin, 2016; Bhoumik et al., 2017). The plants and herbs have long been recognized as a rich source of active ingredients that are widely used in the traditional medicine (Xu et al., 2017). They are free of side effects and very inexpensive compared to the syntetic pharmaceuticals (Almasaudi et al., 2017; Rasouli et al., 2017). Now, there is growing interest in natural products, well known for their gastroprotection, and a large research is being conducted on herbs to discover and identify remedies for the management of the malady (Bi et al., 2017; Aidi Wannes and Saidani Tounsi, 2017). Among these natural compound, carob is well known in traditional medicine for the treatment of gastrointestinal disorders (Rtibi et al., 2017a; Theophilou et al., 2017). The carob bean is the fruit of carob tree (Ceratonia siliqua L.) that belongs to the Leguminosae family and is widely cultivated in Mediterranean countries including those of the Arab Maghreb. It is one of the most useful trees for economic and environmental reasons (Battle and Tous, 1997; Bennmahioul et al., 2011). The pods have traditionally been used as animal and human food. In addition, because of its high carbohydrate content, carob pod is used in the production of bioethanol (Saharkhiz et al., 2013), and currently, it is the carob industrial waste that is valorized in the production of bioethanol (Bahry et al., 2017; Raposo et al., 2017).
Carob pods consist of two major parts: the pulp (90%) and the seeds (10%). Carob flour, because of its high fiber and nutritional ingredients content, is used in healthy food production such as gluten-free bread production for patients with celiac disease (Tsatsaragkou et al., 2014; Rozylko et al., 2017) and also as a chocolate substitute (Nasar-Abbas et al., 2016; Loullis and Pinakoulaki, 2017). Carob seeds are usually exploited for the production of a white to creamy powder extracted from seed endosperm, known as carob bean gum (CBG) or locust bean gum (LBG), which is a natural food additive used in the food industry as a thickener, stabilizer or flavoring in food. Now the gum is experiencing a novel use such as jelly foods, baby and children foods, and as novel barriers to improve organoleptic characteristics of food products (Goulas et al., 2016; Aydin and Ozdemir, 2017). The gum is also used in the cosmetic, pharmaceutical, textile, paint, oil drilling, and construction industries (Dionísio and Grenha, 2012; Barak and Mudgil, 2014). In addition, carob pods can be used as a cocoa substitute (Nasar-Abbas et al., 2016; Loullis and Pinakoulaki, 2017). Health benefits of carob have been revealed by several authors (Rtibi et al., 2017a). Leaves and pods of carob exerted diverse physiological functions such as antibacterial and antioxidant activities (Meziani et al., 2015; Custódio et al., 2015; Al-Olayan et al., 2017), antidiabetic properties (Macho-González et al., 2017; Rtibi et al., 2017b), and the nephrotoxicity (El-Sayyadet al., 2017). Furthermore, Carob has been reported in the literature as having antitumor, anti-proliferative, proapoptotic activity (Custodio et al., 2011; Nasar-Abbas et al., 2016), cholesterol lowering activity (Nasar-Abbas et al., 2016; El Rabeyet et al., 2017) and anti-inflammatory capacity (Lachkar et al., 2016; Rtibi et al., 2017a). It has also been reported that carob pod water extract ameliorated impairments in liver, kidney and lung functions induced by exposure to water pipe smoke and amiodarone in rats (Abdel-Rahman et al., 2017). More importantly again, the carob is traditionally well known for its performance on the gastric sphere, and according to several recent work, it was demonstrated that carob possesses significant laxative, anti-diarrheal, anti-colitis and antiemetic activities (Rtibi et al., 2016c, 2016d; Hamid and Janbaz, 2017; Theophilou et al., 2017). The present study was designed to investigate antioxidant potential of carob pods extract and its gastro protective effects on gastric ulcer induced by indomethacin in Wistar rats.

Materials and methods

Drugs and chemicals

Indomethacin and ranitidine were purchased from the central pharmacy in Mostaganem city. 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, quercetin, bovine hemoglobin, pepsin, Alcian Blue and MgCl$_2$ were obtained from Sigma-Aldrich chemie GmbH (Munich, Germany).

Plant material

The mature carob pods were collected during September 2016 from the region of Mostaganem (North-West Algeria). The plant material was authenticated and identified by the department of Agronomy at Abdelhamid Ibn Badis University of Mostaganem. The Voucher specimens have been deposited in the department of Biological Sciences, faculty of Natural and Life Sciences.

Preparation of carob pods aqueous extract (CPAE)

The preparation of the aqueous carob pods extract (CPAE) was carried out according to the method of Rtibi et al. (2015). Briefly, the plant material was dried in an incubator at 50 $^\circ$C during 72 h and powdered in an electric blender (Moulinex). Powder mixture containing carob pulp (90 %) and seeds (10 %) was dissolved in double distilled water and filtered through a colander (0.5 mm mesh size). Finally, the CPAE was immediately used for in vivo experiments.

Preparation of carob pods methanolic extract (CPME)

The methanolic extract of the carob powder was realized in a solvent methanol/water (90:10 v/v) acidified with HCl (100μl). Briefly, 50g of the carob powder was allowed to macerate in 100 ml of solvent
for 30 min at room temperature and in the dark. The filtrate was evaporated on a rotavapor. The extraction was performed in triplicate and the CPME was used for antioxidant activity analysis.

In vitro antioxidant activity of CPME

The DPPH (α, α-diphenyl-β-picryl-hydrazyl) is a free radical, stable at room temperature. It is reduced in the presence of an antioxidant. The reaction is based on electron transfer producing a violet solution in ethanol. The antioxidant yield hydrogen to DPPH and color of the solution changes from dark purple to bright yellow as the disappearance of DPPH. The absorbance decreased when the DPPH was scavenged by an antioxidant through donation of hydrogen to form a stable DPPH molecule.

The antioxidant capacity of the CPME was performed using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging activity as previously described by Brand-Williams et al. (1995). Briefly, 200 µl of CPME of increasing concentrations (20, 50, 100, 150, and 200 µg/ml) were added to 2 ml of 0.1 mM methanol solution of DPPH. The reaction mixture was left in the dark at room temperature for 1 hour. The absorbance was recorded at 517 nm using a spectrophotometer. A methanolic solution of DPPH (0.1mM) was used as control and the percentage of DPPH radical scavenging activity (RSA) was calculated according to the following equation:

\[
\text{RSA} \% = \frac{(\text{absorbance of the control} - \text{absorbance of the sample})}{\text{absorbance of the control}} \times 100.
\]

IC₅₀ was calculated as the concentration of extract causing a 50% inhibition of DPPH radical; Quercetin was used as a reference molecule in the same concentration as the test extract. All tests were performed in triplicate.

Animals

Adult male Wistar albino rats of 8 weeks old and weighing between 200–250g and approximately of the same age were obtained from the Pasteur Institute of Algiers, Algeria. The experimental animals were housed in plastic cages with good aerated covers at 25°C±0.5°C, 45-75% relative humidity and kept under standard laboratory conditions with alternating light and dark cycles of 12 h each. They were maintained under observation for about 15 days before the onset of the experiment. During the study they were allowed to take standard laboratory food pellets and water ad libitum.

Experimental design

The experimental protocol was approved by the Algerian Association of Sciences in Animal Experimentation (AASEA) of the University of Science and Technology Houari Boumediene (USTHB), Algiers, Algeria. The animals received humane care in accordance with the Guide for the Care and Use of Laboratory Animals, published by Ethics of the association.

The experiment consisted to pretreat healthy rats with the CPAE for 15 days then administered the ulcerogenic agent (indomethacin) and the animals were sacrificed 6 hours after. Two control groups were processed in parallel, the negative control group receiving only physiological saline (no treatment) and the positive control group receiving only the ulcerogenic agent (indomethacin). A standard group treated with a reference drug (ranitidine) to treat gastric ulcers was also followed to compare the gastro-protective effect of the CPAE to a standard.

Rats were randomly allocated into four groups of five rats each as shown in table 1. Before the first day of treatment, rats were deprived of food for 48 hours to obtain an empty stomach, with free access to water. If water is also eliminated with the deprivation of food and to avoid dehydration during fasting, animals were supplemented with 8% sucrose solution (w/v) prepared in 0.2% Nacl solution (w/v). This solution should be removed 1 hour before the experiment. All the test agents or saline were administrated by oral feeding tube once daily for consecutive 15 days. Groups 1 (negative control) and 2 (positive control) received normal saline (5 ml/kg/day, p.o.)
and served as normal control and peptic ulcer control groups respectively.

In the first day:
Group 1 (negative control): received 2 doses of distilled water (5ml/kg/day, p.o) with 6 hours in intervals.
Group 2 (positive control): received 2 doses of distilled water (5ml/kg/day, p.o) with 6 hours in intervals.
Group 3: standard treated with ranitidine: received 2 doses of ranitidine (100 mg/kg orally) with 6 hours intervals. Ranitidine was dissolved in distilled water (5 ml/kg).
Group 4: treated with CPAE: received 2 doses of CPAE (2g/kg) (5 ml/kg) with 6 hours in intervals.

In the second day:
Group 1 (negative control): received 1 dose of distilled water (5ml/kg/day, p.o).
Group 2 (positive control): received 1 dose of distilled water (5ml/kg/day, p.o).
Group 3: standard treated with ranitidine: received 1 dose of ranitidine (100 mg/kg orally).
Group 4: treated with CPAE: received 1 dose of CPAE (2g/kg) (5 ml/kg orally).

The duration of treatment is 15 days and during the 15 days of treatment, the rats were treated as the second day. We took the initial and final body weight of each rat.

The 16th day, we administered orally to all animals except those in Group 1 (negative control or normal), indomethacin (30 mg/kg) dissolved in distilled water. Six (06) hours after gastric ulcer induction, animals were anesthetized with chloroform by inhalation using a soaked cotton placed in the nose. Blood was collected by cardiac puncture with a 5 ml syringe and placed in dry and heparinized tubes for biochemical and hematological analysis, and conserved at –20°C. After blood collection, all animals were sacrificed by decapitation. Serum was prepared by allowing the blood to clot for one hour at room temperature to remove coagulation factors, then centrifuged at 2500-3000 rpm for 15 min. The resulting clear serum was stored at -20°C for subsequent biochemical assays.

Gastric lesions estimation
After the blood collection, the rats were pinned on a dissection board, and then dissected by abdominal opening. The stomachs were carefully removed and weighed. The stomach was opened along the greater curvature and washed gently with cold normal saline to remove blood contaminants. The stomach was then spread and attached on a cleaned support. Ulcerative lesions localized in the glandular part of the stomach were observed with the naked eye or using a magnifying glass. The ulcers were photographed with a Sony digital camera. We performed the measurement of the length of ulcerous bands which cover the glandular part of the stomach for calculating the ulcer number, ulcer index and the preventive or curative percentage. The length of long lesions (elongated) in mm was measured with a ruler and the number of petechial lesions (red spots of color or purple) was counted. Each five petechial lesions are considered as a 1 mm ulcer. The sum of the total length of the long ulcers and petechial lesions in each group of rats were divided by its number to calculate the ulcer index (mm) (Al-wabel et al., 2012). In each rat, the macroscopic injury of each ulcer was scored by an independent observer according to a scale ranging from 0 to 5 as follows (table 2): (0) no lesion (normal), (1) 5 petechiae, (2) Ulcer of less than 1 mm, (3) Ulcer between 1 and 2 mm, (4) Ulcer between 2 and 4 mm, (5) Ulcer of more than 4 mm. When the width of erosion was larger than 1 mm, the score was multiplied by 2.

The ulcer index was calculated as follow: Total ulcer score / number of animals ulcerated

The protection percentage was calculated according to the following formula:

Protection percentage = [(Control ulcer index – Test ulcer index) / (Control ulcer index)] × 100.
Estimation of gastric adherent mucus content

Quantification of adherent gastric mucus was assessed according the method described by Corne et al. (1974) using the Alcian Blue which reacts with mucopolysaccharides of the gastric mucosa. Alcian Blue solution prepared in 1% sucrose solution 0.16 M (0.16 mol / L) buffered with sodium acetate 0.05 M, pH = 5.8 (0.05 ml) was used. Adjust the pH to 5.8 with HCL.

The stomach was opened along the greater curvature, weighed, and immersed in 10 ml of 0.1% Alcian Blue (0.16 M sucrose in 0.05 M sodium acetate, pH 5.8) for 2 h. The stomach was then rinsed twice in 0.25 M sucrose solution (30 min each) to remove the excessive dye. Dye complexed with gastric wall mucus was extracted with 10 ml of 0.5 M MgCl₂ by shaking intermittently for 1 min after every 30 min intervals for 2 h. The resulting blue solution was shaken vigorously with an equal volume of diethyl ether and the resulting emulsion was centrifuged at 3000 rpm for 10 min. The absorbance of the aqueous layer was read at 580 nm against blank MgCl₂ solution. The concentration of gastric wall mucus was calculated through a standard curve of Alcian Blue, and the results were expressed in µg of Alcian Blue per gram of wet glandular tissue.

Measurement of pepsin activity

The quantification of pepsin activity was performed in gastric content as described by Anson (1938). Briefly, 2 ml of 2.5% bovine hemoglobin plus 0.5 ml of 0.3 N HCl and 0.5 ml of diluted gastric juice (0.1ml juice / 9.9ml normal saline) were mixed and the mixture was incubated for 10 min at 37°C. The reaction was stopped by adding 5 ml 0.3 N trichloroacetic acid. The resulting solution was stirred and filtered, and its optical density was measured at 280 nm by using a spectrophotometer. The amount of pepsin was determined using a standard curve prepared under identical conditions with known concentrations of porcine pepsin (1 µg = 3 peptic units), and was expressed as micrograms of pepsin (Shahrokhi et al., 2015; Parvan et al., 2017).

Statistical analysis

All in vitro tests were performed in triplicate and data were expressed as mean ± standard deviation (SD). Analysis of variance (ANOVA) with the software R (R version 3.3.3) was employed to analyse the results. Statistical significance between groups was assessed by Student's test, p values less than 0.05 were considered as statistically significant.

Results

Determination of antioxidant activity

In the present study, the antioxidant activity of CPME was measured using DPPH assay which evaluated free radical scavenging activity of the carob. Based on this test, CPME showed a dose dependent scavenging activity and the highest antioxidant activity was found to be equal to 85% for a maximum concentration of CPME of 200 µg / ml (Fig. 1). The IC₅₀ value was equal to 225.19 ± 3.22 µg/ml.

Measurement of gastric juice volume and pH

The content of the stomach (gastric juice), after its drainage into centrifuge tubes, T was centrifuged at 3000 rpm for 10 min to remove insoluble materials. The supernatant was after measured with graduated tubes. The pH of gastric content was measured using a digital pH meter.

Table 1. Experimental design.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Day 1 Doses</th>
<th>Ingredient</th>
<th>Interval</th>
<th>Day 2 to Day 15 Doses</th>
<th>Ingredient</th>
<th>Day 16 Doses</th>
<th>Ingredient</th>
<th>Sacrifice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>2 doses</td>
<td>Distilled water</td>
<td>6 h</td>
<td>1</td>
<td>Distilled water</td>
<td>6 h later</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 2</td>
<td>2 doses</td>
<td>Distilled water</td>
<td>6 h</td>
<td>1</td>
<td>Distilled water</td>
<td>6 h later</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 3</td>
<td>2 doses</td>
<td>Ranitidine</td>
<td>6 h</td>
<td>1</td>
<td>Ranitidine</td>
<td>6 h later</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 4</td>
<td>2 doses</td>
<td>CPAE</td>
<td>6 h</td>
<td>1</td>
<td>CPAE</td>
<td>6 h later</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Macroscopic evaluation of gastric lesions

The macroscopic examination of gastric mucosa is shown in Fig. 1. The experiment revealed that
indomethacin application resulted in extensive gastric lesions on gastric mucosa which appeared as visible black elongated bands of hemorrhagic erosions on gastric mucosa parallel to the long axis of the stomach (Fig. 2c). Rats pre-treated with CPAE for fifteen days before receiving indomethacin had a significantly reduced area of hemorrhagic lesions with a bright translucent gastric mucosa and almost completely healed from the ulcers (Fig. 2d). The significant inhibition of gastric injuries in CPAE administrated rats was comparable to ranitidine. Ranitidine suppressed the ulcerous lesions and the macroscopic appearance of gastric mucosa is similar to that of healthy rats in the negative control group (Fig. 2b) in which no erosion was observed (Fig. 2a). These results indicated that CPAE impedes indomethacin-induced gastric damages formation as do the ranitidine.

Table 2. Scoring criteria for the macroscopic ulcer index according to Al-wabel et al. (2012).

<table>
<thead>
<tr>
<th>Ulcer (mm)</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (no pathology)</td>
<td>0</td>
</tr>
<tr>
<td>5 Petechial lesions = 1mm</td>
<td>1</td>
</tr>
<tr>
<td>Ulcer of less than or equal to 2 mm</td>
<td>2</td>
</tr>
<tr>
<td>Ulcer between 2 and 4 mm</td>
<td>3</td>
</tr>
<tr>
<td>Ulcer between 4 and 6 mm</td>
<td>4</td>
</tr>
<tr>
<td>Ulcer more than 6 mm</td>
<td>5</td>
</tr>
</tbody>
</table>

Ulcer number, ulcer index, and protection percentage of CPAE

The number of ulcers and ulcer indexes of rats subjected to indomethacin administration were very high compared to those of the other groups of rats, and the difference was highly significant (P<0.001) (Fig. 3). These quantitative data were in harmony with the macroscopic findings. Pretreatment with ranitidine and CPAE drastically diminished ulcer number, and ulcer index and significantly prevented the incidence of gastric injuries as compared to ulcer control group. Preventive ratio of the CPAE was 79% and that of ranitidine was 96% (Fig. 4). According to these values, the protective capacity of ranitidine against indomethacin-induced gastric ulcers is slightly higher without reaching significance than that of CPAE, which is normal to a reference standard drug used to cure ulcers. So, a significant improvement in the level of inhibition against ulceration was however observed in the CPAE treated animals.
Effects of carob pods aqueous extract on adherent gastric mucus content

The results of the gastric mucus show a highly low value in the mucus content of the positive control group compared to other groups (Fig. 5). There is a highly significant decline in the concentration of gastric mucus in the positive control group (1.09μg / g glandular tissue) compared to the negative control group (9.15μg / g glandular tissue), the ranitidine-treated group (8.5μg / g glandular tissue) and the carob pods aqueous extract pretreated rats (7.92μg / g glandular tissue) (Fig. 5). This considerable decrease is highly significant and clearly show the sharp deterioration of the mucus barrier of the stomach wall when taking anti-inflammatory.

Fig. 2. Effects of carob pods aqueous extract (CPAE) on the macroscopic appearance of the gastric mucosa in indomethacin-induced gastric ulcer in male Wistar rats. The negative control group (a) pretreated with 5 ml/kg distilled water, displayed an intact stomach with a bright, translucent and ticker mucosa devoid of any injuries. The reference control group (ranitidine, 100 mg/kg) (b) showed a gastric mucosa without ulcerative lesions. The positive control group or indomethacin-ulcerated group (c) had a gastric mucosa covered with severe ulcerous lesions in the form of elongated hemorrhagic bands of variable length and width, with many dispersed petechial lesions; the mucosa exhibited a thin dehydrated and less translucent surface. The CPAE pretreated rats (2g/kg, 5 ml/kg) (d), showed an almost completely healed gastric mucosa, with moderate to mild injuries. The mucosa appeared with a similar brightness and thickness to that of the normal control group.

The values obtained allowed us to conclude that carob pods aqueous extract caused a significant increment in gastric wall mucus content and that the effect on mucus production of the CPAE was equivalent to ranitidine at 100mg/kg.
Effects of carob pods aqueous extract on pepsin activity
Pepsin activity was significantly increased in indomethacin injured rats (Fig. 6). The CPAE significantly diminished pepsin activity and the value was close to that of the other groups (normal control and ranitidine pretreated groups) (Fig. 6).

Effects of carob pods aqueous extract on volume and pH of gastric content
Gastric juice volume was significantly increased in indomethacin ulcerated rats when compared with the others tree groups (normal control, ranitidine and CPAE pretreated groups).

Fig. 3. Effects of carob pods aqueous extract (CPAE) on ulcer number and ulcer index of indomethacin ulcerated rats (n=5, X ± SEM). *Significantly different from normal control group, ranitidine and CPAE administrated rats value at \( P < 0.001 \). (Statistically analysed by ANOVA followed by Student’s test).

In CPAE administrated rats, gastric juice volume diminished to almost reach that of normal and ranitidine treated rats, and the difference is not significant \( (P > 0.05) \) (Fig. 7). Analysis of the gastric content pH or gastric content acidity displayed a remarkable increase of gastric acidity in indomethacin injured group compared with normal control, ranitidine and CPAE treated groups (Fig. 8).

The acidity of the gastric juice in CPAE pretreated group exhibited no significant differences \( (P > 0.05) \) when compared with the normal control and ranitidine treated groups. It is interesting to note that the carob pods aqueous extract produced lower acidity with higher pH than ranitidine used as reference drug but the difference is not significant.
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Fig. 4. Carob pods aqueous extract (CPAE) protection percentage against indomethacin-induced gastric ulcer in Wistar rats (n=5, X ± SEM).  *Non-significant difference between CPAE, ranitidine and normal control groups value at P< 0.05. (Statistically analysed by ANOVA followed by Student’s test).

Discussion

Antioxidant activity of carob pods methanolic extract

Carob pod is a natural resource rich in nutritional and functional ingredients (El Batal et al., 2016; Goulas et al., 2016; Rtibi et al., 2017a).

It is essentially rich in mineral compounds (El Bouzdoudi et al., 2017a) mostly the calcium, iron and potassium (El Hajaji et al., 2013), and total sugars and reduced sugars (Rtibi et al., 2016a; Petkova et al., 2017; Mahtout et al., 2018) hence its sweetness power. On the other hand, the protein content is moderate followed by a good level of dietary fibers (Khlifa et al., 2013; Durazzo et al., 2014; Goulas et al., 2016) and polyphenols (El Bouzdoudi et al., 2017b; Ydjedd et al., 2017a).

Carob pods contain low level of fat (Rababah et al., 2013; Youssef et al., 2013).

Fig. 5. Effects of CPAE on adherent gastric mucus content, (n=5, X ± SEM). *Significantly different from normal control group, ranitidine and carob pods aqueous extract administrated groups values at P < 0.001. @ Non-significant difference between CPAE, ranitidine and normal control groups values at P > 0.05. (Statistically analysed by ANOVA followed by Student’s test).
Recently, it has been shown that carob pods are very rich in oleic acid, but does not contain sufficient amounts of Ω-3 polyunsaturated fatty acids to be considered as good nutritional oil (Nguyen et al., 2017). Additionally, high amount of anti-nutrients such as trypsin inhibitor and phytic acid was found in the seed and leaves and thus the pulp which offers the highest nutritional power among the carob tree products. The latters could be used in medical applications and food industry because of their antioxidant power (Mahtout et al., 2018).

Fig. 6. Effects of CPAE on pepsin activity, (n=5, X ± SEM). *Significantly different from normal control group, ranitidine and carob pods aqueous extract administrated groups values at P < 0.001. (Statistically analysed by ANOVA followed by Student’s test).

In the present study, the percentage of DPPH radical scavenging activity of carob pods extract was 85%, that of quercetin was equal to 96,45%.

Our results are consistent with those obtained previously by El Hajaji et al. (2011), Akkaya and Yilmaz (2012) and Sebai et al. (2013) with variable values. Our value is greater when compared to that recorded by Sebai et al. (2013) in Tunisia (64, 7%) and by El Hajaji et al. (2011) in Morocco (61,7%), and was low when compared to that obtained by Akkaya and Yilmaz (2012) in Turkey (93,81%).

The IC50 value (0,22 mg/ml) was similar (0,28 mg/ml) to that obatined by Boufadi et al. (2015). The variability of percentage inhibition value could result from geographical and climatic conditions as well as the solvent and the extraction mode as it has already been suggested by Rtibi et al. (2016a). It has been demonstrated that flavonoid and phenolic contents of immature carob cultivated in Tunisia dependent on the solvent used (Sebai et al., 2013). Add to that, the genotype, the variety of the tree and the ripening stage of the carob (Benchikh and Louaileche, 2014; Ydjedd et al., 2017a; El Hajaji et al., 2011).

It is well known in the past, and has also been recently demonstrated that the antioxidant activity of a product is principally related to its polyphenol content (Adefegha, 2017; Benchikh et al., 2014, 2016; Petkova et al., 2017). In fact, the carob’s richness in nutritional and functional ingredients has been reported by several authors (El Batal et al., 2016; Goulas et al., 2016; Rtibi et al., 2017a). It is essentially rich in mineral compounds (El Bouzdoudi et al., 2017a) mostly the calcium, iron and potassium (El Hajaji et al., 2013), and total sugars and reduced sugars (Rtibi et al., 2016a; Petkova et al., 2017; Mahtout et al., 2018) hence its sweetness power. On the other hand, the protein content is moderate
followed by a good level of dietary fibers (Khlifa et al., 2013; Durazzo et al., 2014; Goulas et al., 2016) and polyphenols (El Bouzdoudi et al., 2017b; Ydjedd et al., 2017a). Additionally, several studies have coincided the carob bioactive components with its antioxidant capacity (Petkova et al., 2017; Mahtout et al., 2018). Concerning the Algerian carob, the work of Boufadi et al. (2015) demonstrated that the carob ethanolic extract had the highest amount in total polyphenols and flavonoids contents, and the best antiradicalar capacity. While, Benchikh and Louaileche (2014) and Benchikh et al. (2016) found that the highest amounts of phenolics and flavonoids, and consequently the best antioxidant activity were obtained in the 70% aqueous acetone and ethyl acetate extracts. El Hajaji et al. (2010) reported that the ethyl acetate extract displayed a higher reducing activity compared to the ethyl ether and dichloromethane extracts. Continuing their study, a year later El Hajaji et al. (2011) found that the methanolic extracts showed a very good scavenging effect compared with ethyl acetate extracts.

The findings obtained by Benchikh et al. (2016) are consistent with those of Ydjedd et al. (2017a) for a carob harvested in the same region (Bejaia-Algeria). Additionaely, Ydjedd et al. (2017b) suggested that the microencapsulation of carob polyphenols showed a protective effect against pH changes and enzymatic activities along digestion, thereby promoting a controlled release and targeted delivery of the encapsulated compound, which contributed to an increase in its bioaccessibility in the gut.

Macroscopic examination of gastric ulcers (ulcer number, ulcer index, protection percentage)

The macroscopic examination of the gastric mucosa revealed the presence of large patches of petechiae, several ulcerated elongate zones of varying lengths and widths and hyperemia in the glandular stomach of rats ulcerated by indomethacin. The necrotizing effect of indomethacin on the gastric mucosa was shown in many studies (Tarique et al., 2016; Ibrahim et al., 2017; Katary and Salahuddin, 2017). Indomethacin became the first-choice drug to produce an experimental ulcer model as a result of having a higher ulcerogenic potential than other NSAIDs (Akpanu et al., 2013).

The ulcer profile observed here is of the same magnitude when compared to that obtained in rats in

<table>
<thead>
<tr>
<th>Normal control</th>
<th>Indomethacin</th>
<th>Ranitidine</th>
<th>Carob pods aqueous extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gastric juice volume (ml)</td>
<td>5</td>
<td>4</td>
<td>3</td>
</tr>
</tbody>
</table>

*Significantly different from normal control group, ranitidine and carob pods aqueous extract administrated groups values at P < 0.05. *Non-significant difference between CPAE, ranitidine and normal control groups values at P > 0.05.(Statistically analysed by ANOVA followed by Student’s test).
a gastric ulcer model induced by absolute ethanol, and other NSAIDs such as aspirin, diclofenac and naproxen (Bakhtaoui et al., 2014; Sebai et al., 2014; Choi et al., 2016; El-Deen et al., 2016; Kim et al., 2016; Paulrayer et al., 2017; Yuniarto et al., 2017). Our study showed that in CPAE pretreated rats, the area of gastric lesions was significantly diminished and that the gastric mucosa was almost similar to that of normal rats and treated with ranitidine.

**Fig. 8.** Effects of CPAE on gastric content pH, (n=5, X ± SEM). *Significantly different from normal control group, ranitidine and carob pods aqueous extract administrated groups values at P < 0.05. *Non-significant difference between CPAE, ranitidine and normal control groups values at P > 0.05. (Statistically analysed by ANOVA followed by Student’s test).

The efficacy of carob on gastric ulcer has already been demonstrated in rats by Bakhtaoui et al. (2014) and Rtibi et al. (2015) who reported that CPAE pre-treatment significantly reversed EtOH-induced gastric mucosa macro- and microscopic lesions in a dose-dependent manner. A highly elevated number of ulcers and ulcer indexes was noted in ulcerated rats with indomethacin, and CPAE provoked a significant decrement in these parameters. In addition, protection percentage of carob was 79% while Bakhtaoui et al. (2014) reported a value equal to 50.6% in aspirin injured rats, and in ethanol intoxicated rats carob protection percentage was 92.2%, higher than that of famotidine (68.1%) (Rtibi et al., 2015). These quantitative results correlated perfectly with the macroscopic examination. Analogous observations were mentioned in another case of gastric ulcer model induced by indomethacin in rats (Pradeep kumar et al., 2017; Katary and Salahuddin, 2017; Alkushi and Elsawy, 2017; Ibrahim et al., 2017). In addition to its gastro protective effect, the CPAE displayed a beneficial effect on dextran sulfate sodium-induced ulcerative colitis in rats and that this action is related to the antioxidant and anti inflammatory properties of its polyphenol-rich content (Rtibi et al., 2016d). In the literature, a high level of evidence confirmed the bioefficacy and safety of dietary polyphenols in the management of peptic ulcer (Farzaei et al., 2015). We showed in the present study, that the gastroprotective effect of ranitidine was slightly higher compared to that of CPAE, which is normal to a standard reference product. However, our findings were not in line with those obtained by Rtibi et al. (2015) in ulcerated rats by ethanol and aspirin, who found that curative ratio was superior for carob extract than famotidine. Previously, it has been demonstrated by our laboratory the richness of carob extract in total polyphenols and flavonoids, coinciding with good in vitro and in vivo antiradical capacities (Boufadi et al., 2015). This is what has been
suggested for Leathery Murdah, *Terminalia coriacea* (Roxb.) in aspirin injured rats (Ali Khan et al., 2017). So, it could be concluded that CPAE protected the gastric mucosa against ulcer induced by indomethacin with an efficiency comparable to that of ranitidine, and that this antiulcer effect is due to its content in functional ingredient such as polyphenol and flavonoids. This natural food product inexpensive, easily accessible and without adverse effects would replace the expensive medications to treat certain ulcers and avoid unnecessary expenses.

**Adherent gastric mucus**
Our results revealed a greatly significant low value in the content of gastric adherent mucus in the injured rats by indomethacin (positive control group) compared to the other groups. There is a net decline in the concentration of gastric mucus in the indomethacin-ulcerated rats compared to that of the negative control group, the ranitidine-pretreated group and the CPAE pretreated rats. This considerable reduction is highly significant and clearly show the sharp deterioration of the mucus barrier of the stomach wall when taking indomethacin. A similar weakening was noted in different modele of gastric ulcers: indomethacin and ethanol ulcerated mice (Jaccob, 2016; Silva et al., 2016; Al-Wajeeh et al., 2017), pylorus ligation ulcerated rats (Zakaria et al., 2016; Wang et al., 2018), aspirin ulcerated rats (Ali Khan et al., 2017) and cold restraint stress–induced ulcer model (Singh et al., 2015).

A significant diminution in the gastroduodenal mucosal thickness was observed in rats injured by aspirin (Das and Roy, 2012; Singh et al., 2015). Our finding demonstrated the chancing effect of carob pods extract on the mucus layer that covers the gastric mucosa, probably through the stimulation of its synthesis and secretion. Similar results were recently found in rats injured by aspirin and treated with *Biochanin A* and *Aegle Marmelos* respectively (Singh et al., 2015). The partipation of mucus in the gastroprotection has also been suggested before (Zanatta et al., 2009; Martins et al., 2014; Halim et al., 2017).

The main biological effect of NSAIDs is the inhibition of prostaglandin synthesis (Lanas and Chan, 2017). PGE2 play an important role against various gastric injuries in modulating mucosal integrity via mucus production (Luiz-Ferreira et al., 2012; Jaccob, 2016; Halim et al., 2017). Previously, in rats injured with ethanol and treated with *Anacardium humile* St. Hil, it has been stipulated that the increase in the amount and thickness of adherent gastric mucus was mediated by PGE2 production, stimulating the mucus secretion (Luiz-Ferreira et al., 2010; Elimam and Baragob, 2014). So, it can be speculated that the potential mechanisms of the gastroprotective effect elicited by CPAE could result from enhancement of the gastric mucosal defense action such as PGE2 that generates mucous production, and that adherent mucus may be an important mechanism of gastric cytoprotection.

**Pepsin activity, volume and pH of gastric content**
Our results showed that pepsin activity was significantly recovered in CPAE pretreated rats when compared to the injured rat by indomethacin. A similar results were reported by da Silva et al. (2015) in rats injured by acetic acid and treated by *Maytenus robusta* Reissek. Additionally, CPAE restored the volume and pH of the gastric juice, which were significantly altered in the indomethacin ulcerated rats. Studies reported by Parvan et al. (2017) indicated that both hydroalcoholic and aqueous extracts of quince exert their activity by acid reducing and decreasing pepsin activity similar to CPAE. Also, it has been postulated a physicochemical interaction between quince extracts and pepsin enzyme (Parvan et al., 2017). Arago et al. (2018) stipulated that *Ximenia americana* L. aqueous extract exerted a gastroprotective action, mediated in part by antisecretory activity. Several studies delineated that mechanisms involved in anti-ulcer activity of plants are related to a decrease in gastric secretion and an increase in gastric mucus content (Boeing et al., 2016; Katary and Salahuddin, 2017; AlKushi and
Elsawy, 2017; Wang et al., 2018), and that these effects may be attributed, at least in part, to the presence of some alkaloids and other bioactive phytoconstituents such as tannins, saponins and flavonoids (Zakaria et al., 2014; Martins et al., 2014; Ibrahim et al., 2017). Aragon et al. (2018) argued that the antiulcer activity of Ximenia americana L. aqueous extract is correlated to its major constituents, procyanidins B, C, and catechin/epicatechin. This hypothesis has already been issued for the carob where the presence of alkaloids, saponins, tannins, flavonoids and phenols in C. siliqua would have been responsible for spasmylytic, anti-diarrheal and antiemetic activities (Hamid and Janbaz, 2017).

By its action on the pepsin, the volume and the pH of the gastric juice, we can attribute to the carob extract an antisecretory effect. It could exert an antiacid effet and could slow the hypersecretion of pepsin and increase the production of mucus.

Conclusion
Based on these data, carob pods extract displayed a gastroprotective capacity on a gastric ulcer induced by an anti inflammatory drug, indomethacin. It could have as mechanism of action an antisecretory effect. It is likely thanks to its antioxidant potential that this natural food product have demonstrated its beneficial effects on the gastric mucosa. Consequently, it can therefore be proposed as a promising drug in the natural treatment and management of peptic ulcers.

Conflicts of interests
The authors declare that they have no conflict of interest.

Funding
This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Acknowledgments
We are grateful to the Dean of the Faculty of Nature and Life Sciences (Mr Lotmani Brahim) for providing us with the means of transportation of animals from Algiers to Mostaganem.

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