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IN VITRO SCREENING OF *ANNONA CHERIMOLA* LEAVES AND BARK FOR THEIR ANTIOXIDANT ACTIVITY AND *IN VIVO* ASSESSMENT AS PROTECTIVE AGENTS AGAINST GASTRIC ULCER IN RATS

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Abstract

Gastric ulcer affect a large portion of the world population and are induced by several factors, including stress, alcohol consumption, nutritional deficiencies, and ingestion of non-steroidal anti-inflammatory drugs. The objective of this study is to evaluate the protective effect of *Annona cherimola* as anti- gastro ulcerative agent in rats. The evaluation was done through detection total phenols, flavonoids and alkaloids in different extracts and organs of *Annona cherimola*. The most pronounced extract with its different organs were subjected to *in vitro* antioxidant estimation. The most two effective organs of ethanol extract were *in vivo* investigated as anti-gastroulcerative agents. Severe drastic changes were observed in ulcerative stomach after ethanol induction to rats. Seven flavonoids compounds were identified from the leaves of ethanol extract. Rats protected with the leaves and bark ethanolic extract of *A. cherimola* showed improvement in ulcer index, oxidative stress markers, cell organelles marker enzymes as well as the histology of stomach. In conclusion, the bark and leaves ethanolic extract of *Annona cherimola* recorded the most *in vitro* antioxidant effect and served as protective gastroulcerative agents.

Keywords: *Annona cherimola*; gastric ulcer, antioxidants; enzymes, flavonoids

Abbreviations

Glutathione: GSH. Malondialdehyde: MDA. Superoxide dismutase: SOD. Nitric oxide: NO.Catalase: CAT. Succinate dehydrogenase: SDH. Lactate dehydrogenase: LDH. Glucose-6 phosphatase (G-6-Pase). Acid phosphatase: AP. Alkaline phosphatase: ALP. 5`-nucleotidase: 5`NT**.**

Introduction

Annona cherimola (Annonaceae), commonly known as cherimoya is a species of fruit found in different subtropical areas around the world. Its fruit used in traditional medicine as an antimicrobial agent and as an effective treatment for digestive disorders (Amoo *et al.*, 2008). Cherimoya contains different natural compounds having patent biological activity as alkaloids (Chen *et al.*, 2001).

Several factors induced gastric ulcer which affects a great world population. These factors include, stress, alcohol consumption, smoking, male nutrition and the administered of non-steroidal anti-inflammatory drugs (Júnior *et al.*, 2015). Gastric ulcer may be controlled by two approaches, the first one use to reduce the production of gastric acid and the second in reinforcing mucosal production (Moraes *et al.*, 2008). Modern approach of treatment includes proton pump inhibitors, histamine receptor blockers, drugs affecting the mucosal barrier and prostaglandin (Moraes *et al.*, 2008). This encouraged the scientists to discover new antiulcer drugs, which includes herbal ones.

Therefore, the aim of the present study is to counteracting gastric ulcer in rats by examine the protective action of *A. cherimola* leaves and bark ethanol extract. The evaluation was done through the estimation of the ulcer index, the oxidative stress markers and the cell organelles enzymes. The histopathological picture of stomach mucosa will be done for results confirmation.

Material and Methods

Plant Materials

A. cherimola trees were cultivated in a private farm at Mansoriya region, Giza Governorate, Egypt. They were identified by Dr. M.A. Gibali, Department of Taxonomy, Faculty of Science, Cairo University. Voucher specimens were deposited at the National Research Centre Herbarium under No. 522.

Determination of flavonoids, phenols and alkaloids

Total flavonoids, phenols and alkaloids were estimated in different extracts and for different organs by the methods of Singleton and Rossi (1965) and Kam *et al.* (1999).

As flavonoids, total phenolic and alkaloids contents were more pronounced in the ethanol extract, so further *in vitro* antioxidant effect of different organs of *A. cherimola* ethanol extract will be done. The most two pronounced organs will be investigated *in vivo* as gastro-ulcerative agents. As flavonoid content was more pronounced in leaves ethanolic extract, the flavonoid compounds were being isolated and identified.

Extraction of flavonoids from *Annona cherimola* leaves

Two and quarter Kg of Powdered *Annona cherimola* leaves were extracted with EtOH (70%, 3Lx5) by soaking at room temperature. The combined alcoholic extracts were concentrated under reduced pressure at 45°C using rotary evaporator which yield 655.75 g of residue. The crude

residue was suspended into water, left overnight, and was successively partitioned with methylene chloride (Me_2Cl_2, 127.52g), ethyl acetate (EtOAc, 18.71g) and n-butanol (BuOH, 280g). Elution solvent systems were used (BAW 4:2:1 and acetic acid 15%). After air-drying, the spots were visualized under UV light (Kam *et al.*, 1999).

Isolation of flavonoid compounds from BuOH fraction

The BuOH fraction was evaporated till free from solvent (280g) and then subjected to paper chromatography technique (whatmann No. 3MM) using 15% Acetic acid as eluting system yielding five bands. The bands were extracted with MeOH (70%).

Band (1) subjected for further purification using small Sephadex LH-20 column eluted with MeOH (70%) yielding two fractions, one of them subjected to PTLC using Butanol: Acetic acid: Water, (5:1:0.5) produce compound 1 semi purified, which was subjected to sephadex column LH-20 to afford one pure compound (1), Band (2) gave two compound 2,3), Band (3) gave one pure substrate (compound 4), Band (4) gave one pure substrate (compound 5) and Band (5) gave pure (compound 6,7) after purification using sephadex LH-20 column which eluted with 50% MeOH.

(i) Band(1):Quercetin 3-*O*-α-L-rhamnopyranosyl-(1→6))-β-D glucopyranoside(1)

Its structure was confirmed by ^{1}H NMR spectroscopic analysis (chemical shift δ in ppm, coupling constant *J* in Hz). The recorded spectrum (DMSO-d_6), exhibited signals at δ 6.20 (1H, d, *J*= 2.1 Hz, H-6), δ 6.39 (1H, d, *J*=2.1 Hz, H-8), δ 7.57 (1H,d, *J*=2.1Hz, H-2'), δ 6.89 (1H,d, *J* = 9 Hz, H-5'), δ 7.54(1H, dd, *J* =9, 2.1 Hz, H-6'), δ 5.35 (1H,d, J=7.5 Hz, H-1"), δ 4.40 (1H,d, J= 2.0 Hz, H-1'"), δ 1.01 (3H,d, J= 6.3 Hz, CH_3-rha) 3.16-3.65 (m, the rest sugar of glucose and rhamnose). The ^{1}H NMR of this compound revealed the chemical shift of protons identical with those reported in the literature for rutin. ^{13}C NMR spectral data displayed 27 carbons, 15 carbons of aglycone with 12 carbon resonances of the two sugar moieties.

The carbon signals appeared at δ 104.8 and 102.4 which are assignable of the anomeric carbons of glucose and rhamnose. Assignments of the remaining carbons were aided by comparison with the chemical shift of the corresponding carbon resonances of quercetin 3-substituted (Kundakovic *et al.*, 2004). From the previous data, compound (1) was identified as, quercetin 3-*O*-α-L-rhamnopyranosyl-(1→6))-β-D-glucopyranoside (rutin) (Fig.1.).

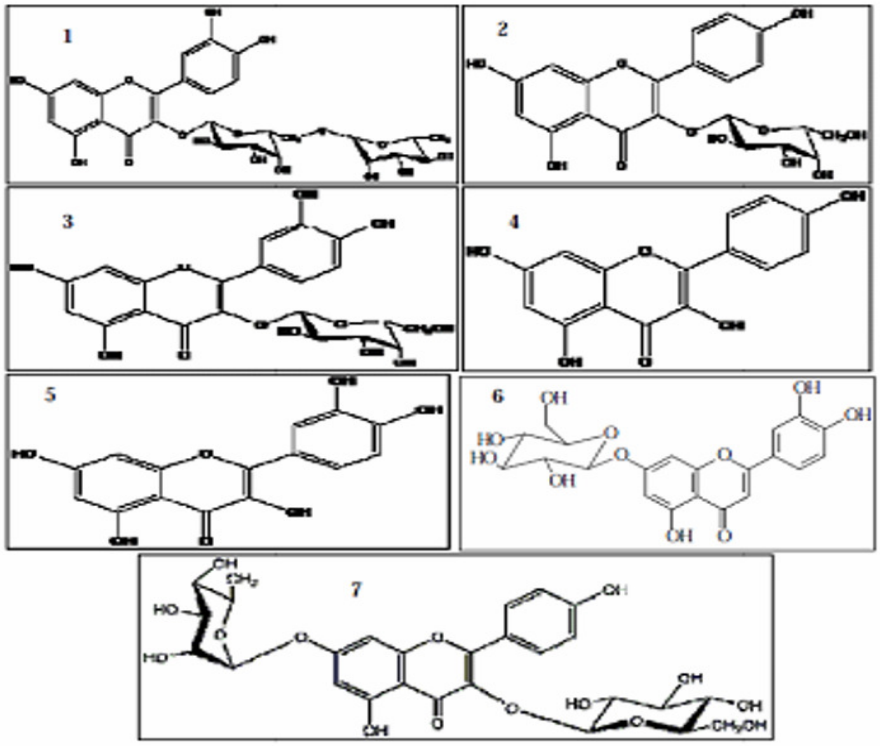

Fig. 1: Chemical structure of the isolated compounds (1-7)

(ii) Band (2) Kaempferol 3-*O*-*β*-D-glucopyranoside (2)

^{1}H NMR of compound showed aromatic signals at δ 8.1 (2H, d, *J* = 8.8 Hz) and 6.9 (2H, d, *J*= 8.8 Hz) assignable H-2', 6'and H-3', 5' respectively, together with two meta coupled protons at δ 6.3 (1H, *J* = 2.0 Hz) and δ 6.5 (1H, *J* = 2.0 Hz) assignable H-6 and H-8, respectively. The anomeric proton of the sugar appeared at (δ 5.57, d, *J* = 7.2 Hz) indicating its β configuration of glucose at position 3. ^{13}C NMR spectral data displayed 21 carbon, 15 of kampferol with 3-substituted and 6 carbon of glucose unit which confirmed by the anomeric carbon atom of glucose at δ101.29 and the other carbon signals appeared at their proper position (C-5'' at δ 77.83; C-3" at δ 74.59; C-4" at δ 76.80; C-2" at δ 70.28 and C-6" at δ 61.23) (Fig. 1).

From the previous data compound 2 is identified as kampferol-3-*O*-β-D-glucopyranoside(Santos *et al.*, 2000).

(iii) Band (2) Quercetin 3-*O*-*β*- D-glucopyranoside (3)

The ^{1}H NMR showed the aromatic protons of the B-ring appeared at δ 7.67 (1H, dd, *J* = 8.62 and 2.12 Hz); δ 7.53 (1H, d, *J* = 2.12 Hz); and 6.82 (1H, d, *J*= 8.62 Hz) assignable H-6', H-2' and H-5', respectively. The two aromatic proton of the A-ring showed as two doublet at δ 6.20 and δ 6.40with *J* = 1.8 Hz of each proton due to meta coupling of H-6 and H-8, respectively. The anomeric proton of the sugar appeared at (δ 5.37 with *J* =7.63 Hz) indicating its β configuration of glucose at position 3. ^{13}C NMR spectral data displayed 21 carbons, 15 of quercetin and 6 carbons of

glucose moiety. The anomeric carbon of glucose appeared at δ101.20 and the rest carbon signals of glucose appeared at their proper position. The carbon resonances of the quercetin were assigned by comparison with the corresponding signals in the published data. From the previous data compound 3 is identified as quercetin 3-O-β-D-glucopyranoside (Fig. 1) (Santos *et al.*, 2000).

(iii) Band (3) Kaempferol (4)

The ^{1}H NMR showed the aromatic proton of the B-ring as two doublets at δ 8.03, δ 6.93 with *J* = 8.5 due to ortho coupling of H-2', 6' andH-3', 5' respectively, two aromatic protons of the A-ring were revealed as two doublet at δ 6.41and δ 6.17 with *J* = 2.0 Hz due to meta coupling of H-6 and H-8 respectively. From these data this compound is identified as kampferol (Fig. 1) (Gonda *et al.*, 2000).

(iv) Band (4) Quercetin (5)

The ^{1}H NMR showed the aromatic proton of the B-ring as doublet at δ 7.69, *J* = 2.1 Hz of H-2' due to meta coupling of H-6'and doublet at δ 7.57, *J* = 2.1 Hz and 8.4 Hz of H-6'due to meta coupling with H-2'and ortho- coupling with H-5' respectively, a doublet at (δ 6.9, *J* = 8.4 Hz) for H-5' due to an ortho coupling with H-6' was observed , two aromatic proton of the A-ring showed as two doublet at δ 6.2 and δ 6.42with *J* = 1.8 Hz of each proton due to meta coupling of H-6 and H-8 respectively. ^{13}C NMR displayed 15 carbons which were found to be in accordance with the proposed structure of quercetin (Fig. 1) (Gonda *et al.*, 2000).

(v) Band (5) Luteolin-7-O-β-D-glucopyranoside (6)

Compound (6) is isolated as a faint yellow amorphous powder, m.p. 252-254oC, which possess chromatographic properties (dark brown spot on PC, turning bright green when fumed with ammonia vapor, changing yellow with $AlCl_3$ and λ_{max} (nm)as 254, 286, 352 identical with those given for luteolin 7-O-glucoside (Mabry *et al.*, 1970). This suggestion was supported by a molecular weight determination of (6) using positive ESI-MS technique (molecular weight 448, [M+H]+ at m/z: 449). Acid hydrolysis of (6) afforded glucose and luteolin, which compared with authentic markers.

The 1H-NMR spectrum (DMSO-d6) showed the structure of (6) to be luteolin 7-O-glucoside. ^{1}H-NMR revealed signals at δ ppm 7.45 (dd, J = 8.3 Hz, and J = 2.2 Hz, H-6'), 7.42 (d, J = 2.2 Hz, H-2'), 6.9 (d, J = 8.3 Hz, H-5'), 6.8 (d, J = 2.2 Hz, H-8), 6.7 (s, H-3), 6.4 (d, J = 2.2 Hz, H-6) and signal appeared as doublet at δ ppm 5.08 (d, J = 6.6 Hz, H-1" of glucose) assignable for the anomeric proton of the sugar moiety.

Further confirmation of the structure of (6) as luteolin 7-O-glucoside, was achieved through ^{13}C-NMR spectrum (DMSO-d6). The presence of β-glucopyranoside moiety in the compound of (6) confirmed from the anomeric carbon resonance at δ 99.82 ppm and from the chemical shift values of the remaining sugar resonances at δ 72.09, 77.14, 69.50, 76.36 and 60.58 ppm which assigned for (C-2"), (C-3"), (C-4"), (C-5") and (C-6") respectively. Resonances of the carbons of the flavonoid moiety were assigned by comparison with the corresponding signals in the published spectrum of Luteolin-7-O-β-D-glucopyranoside (Harborne and Baxter, 1999). Compound (6) was identified as Luteolin-7-O-β-D-glucopyranoside.

(a) Band (5) kaempferol 3-α-D-glucopyranoside-7-α-1-rhamnopyranoside (7)

Compound (7) was isolated as pale yellow crystals, UV analysis using shift reagents. Mabry *et al.*(1970) showed that compound (7) was a 3,7-di-0-glycoslde. Acid hydrolysis of the compound (7) yielded an equimolecular mixture of glucose, rhamnose and kaempferol. This suggestion was supported by a molecular weight determination of (7) using positive ion mode ESI(+) technique (molecular weight 594.1584, [M+H]+ at m/z: 595, 560, 449, 286 (Fig. 1).

The ^{1}H-NMR spectrum showed two doublets at δ 6.36 and δ 6.78 with J =2 Hz assigned to H-6 and H-8 respectively. The 4'-monosubstitution on the B-ring was indicated by two doublets (J =9 Hz) of two protons each at δ 6.89 (H-3' and H-5') and 8.06 (H-2' and H-6') The rhamnosylanomeric proton appeared as a doublet (J = 15 Hz) at δ 5.32 ppm while the rhamnosyl-methyl group appeared as an ill-shaped 3H doublet (J=4 Hz) at 1.23 ppm, a characteristic feature of 7-0-a-rhamnopyranosides (Mabry *et al.*, 1970). The glucosylanomeric proton appeared as a broadened ringlet (J = 1 Hz) at δ 5.65 ppm where the small value for the splitting of H-l" indicated that the glucose was α-linked to the aglycone (β-glucosides typically show the anomeric proton as a doublet with 517=Hz). A broadened doublet (J =24 Hz) at δ 4.37 ppm was assigned to H-2". The remaining sugar protons appeared as a complex multiplet between 32.4 1 ppm This glycoside was not affected by β-glucosidase but it was rapidly hydrolysed with α-glucosidase giving kaempferol 7- rhamnoside and glucose Therefore compound (7) was characterized as kaempferol 3-α-D-glucopyranoside-7-α-1-rhamnopyranoside.

***In vitro* antioxidant assay**

The antioxidant activity of serial concentrations (10:100µg) of the ethanol extracts of *A. cherimola* different organs were estimated by the method of Chen *et al.* (2007). As *A. Cherimola* ethanol extract of leaves and bark showed the most potent antioxidant effect, so we selected therefor further *in vivo* evaluation as anti-gastroulcerative agents in rats.

Animals & Ethics

Adult male healthy Wistar strain albino rats weighing 150-200 ±10g were obtained from the Animal House, National Research Centre, Dokki, Egypt. Rats were randomly divided into 7 groups of eight rats each. Animals were fed on standard diet and water *ad libitum*. Animals were acclimatized to the laboratory conditions for one week before starting the experiment. Temperature through the housing was adjusted to 24 ºC with relative humidity 65±5% and 12/12 h of light/dark cycles.

Anesthetic procedures and handling with animals were complied with the ethical guidelines of Medical Ethical Committee of the National Research Centre in Egypt and performed for being sure that the animals do not suffer at any stage of the experiment (Approval no: : 106/ 2012).

Acute toxicity

48 rats of 200 ±10g were divided into 2 groups (24 rats each). Each main group was subdivided into three subgroups (8 rats each) and received one oral dose of 250, 500, and 1000 mg/ kg body weight of bark and leaves of *A. cherimola* ethanol extract. Numbers of dead animals were counted

along 15 days. Mortality rate and LC_{50} were monitored. We noticed that the extracts were safety at doses 250 and 500mg/kg body weight; therefore we selected the dose of 500 mg/kg body for the biological determinations.

Doses and route of administration

Absolute ethanol was orally administrated at a dose of 0.5 ml/100g body weight on 24 hours empty stomach (Mard *et al.*, 2008). *Annona* species extracts were orally given at a dose of 500 mg/kg b.w/ day for one week (Gokhale *et al.*, 2002). Ranitidine as a reference antiulcer drug was orally administrated at a dose of 100 mg / kg b.w/ day for one week (Mard *et al.*, 2008).

Experimental groups

56 male Wistar strain albino rats were used in this study. Animals were divided into 7 groups (eight rats each).

Group 1: Normal healthy control rats.

Groups 2 and 3: Normal healthy rats orally treated with different alcoholic extracts of different plant organs (bark and leaves) daily for one week.

Group 4: received the ethanol dose on 24 hrs empty stomach, sacrificed after one hour later and served as the ulcerative rats.

Groups 5-7: Protective groups were administered with plant extracts or ranitidine daily for 7 days prior administration with one oral dose of absolute ethanol on 24 hrs empty stomach and sacrificed one hour later.

Sample preparations

Stomach tissue was homogenized in normal physiological saline solution (0.9% NaCl) (1:5 w/v). The homogenate was centrifuged at 4°C for 15 min at 3000 rpm and the supernatant was stored at -80°C for further estimations.

Estimation of gastric lesion counts

Stomach was removed, opened from the long curvature, washed with normal saline, expand and fixed on the dissection plate and lesion numbers were counted by magnifying lens (Szelenyi and Thiemer, 1978).

Gastric total acidity

Gastric content was collected and centrifuged at 3000 rpm for 15 min. The supernatant volume (ml) was measured and the total acidity was determined by titration with 0.1 N NaOH using 2% phenolphthalein as an indicator (Guedes *et al.*, 2008). The results were expressed as m Eq/L, where

mEq = Vol of NaOH x normality of NaOH x equivalent weight of HCl x 1000/ Sample volume.

Oxidative stress and cell organelles markers

Glutathione (GSH), malondialdehyde (MDA), superoxide dismutase (SOD), nitric oxide (NO), catalase (CAT), succinate dehydrogenase (SDH), lactate dehydrogenase (LDH), glucose-6 phosphatase (G-6-Pase), acid phosphatase (AP), alkaline phosphatase (ALP), 5`-nucleotidase (5`NT) and total protein were assayed in stomach tissue by colorimetric Kits (Biodiagnostic Co., Cairo, Egypt).

Histopathological analysis

Stomach tissues slices were fixed in 10% paraformaldehyde and embedded in paraffin wax blocks. Sections of 5 μm thick were stained with hematoxylin & eosin (H&E) and Masson's trichrom, then examined under light microscope for determination of pathological changes (Banchroft *et al.*, 1996).

Statistical analysis

All data were expressed as mean ± S.D. of eight rats in each group. Statistical analysis was carried out by one-way analysis of variance (ANOVA), Costat Software Computer Program. Significance values between groups were at $P< 0.05$.

Results

Phenolic, flavonoids and alkaloids contents

Table (1) revealed the total phenols in bark, fruit and seed extracts were lower than its content in leaves. The highest concentration of total phenolic was found with butanol and total alcohol extract of leaves of *A. cherimola* (20.68, 21.45 mg/g, respectively). The highest flavonoid content was found in leaves of *A. cherimola* (4.6 mg/g) with total EtOH followed by bark ethanol extract (1.5 mg/g). The results showed low presence of total alkaloids. Moreover, no significant difference was observed between the two solvents EtOAc and BuOH. The total alkaloid contained found in the leaves and bark of *A. Cherimola* was 0.03 and 0.05 mg/g, respectively.

Table 1 : Totalphenolics content (mg/g) in *A. cherimola* using different extracting solvents.

Different plant parts		Total Phenolics			
		Chloroform	Ethyl Acetate	Butanol	Total alcohol
A. Cherimola	Leaves	2.36	16.68	20.68	21.45
	Bark	1.68	14.68	15.64	9.85
	Fruits	0.05	8.68	12.98	10.98
	Seeds	1.03	10.75	8.32	9.68

Identification of flavonoidal compounds from *A. cherimola* leaves

Seven known flavonoids (Fig. 1) were identified by comparing their spectral data with published data as Quercetin 3-*O*-α-L-rhamnopyranosyl- (1→6))-β-Dglucopyranoside (1), Kaempferol 3-*O*- β-D-glucopyranoside (2),Quercetin3-*O*- β -D-glucopyranoside(3), Kaempferol (4), Quercetin (5), Luteolin-7-O-β-D-glucopyranoside (6), kaempferol 3-α-D-glucopyranoside-7-α-1-rhamnopyranoside (7).

The mass spectrum of isolated flavonoids were summarized in table *S1*.

Antioxidants effect

The *in vitro* antioxidant evaluations of *A. cherimola* against vitamin C as a standard are seen in table 5. The results revealed that the leaves and the bark of *A. Cherimola* ethanol extract showed the highest antioxidant effect than fruits and seeds through the inhibition of the DPPH free radicals.

Table 5: *In vitro* antioxidant activity of different plant parts of *Annona cherimola* ethanol extract.

Concentrations (µg/ml)	*A. cherimola*				Vitamin C
	Leaves	Bark	Fruit	Seeds	
10	66.66	59.45	29.09	18.12	43.47
50	70.00	64.28	25.00	21.15	80.85
100	72.72	87.61	37.5	27.56	95.63

Data are inhibition percentages (IP) of DPPH free radicals at different concentrations.

$$\% \text{ IP} = \frac{\text{Mean of control (3 reading) - Mean of sample (3 reading)}}{\text{Mean of control}}$$

***In vivo* study**

(i) Acute toxicity study

The results revealed that the ethanolic extract of leaves and bark of *A. cherimola* were safe in 250 and 500mg/kg b.wt. At 1000 mg/kg b.wt., we observed 12.5% mortality rate after treatment with leaves of *A. cherimola*. Therefore, we selected the median dose of 500mg/kg body weight for the biological parameters.

(ii) Gastric ulcer markers

Regarding to the PH level in control rats protected with different plant parts extracts, the results revealed insignificant changes revealing extracts safety on the PH level (Table 2). Gastroulcerative rats recorded significant decrease by 23.07% as compared with the control group. Gastroulcerative rats protected with leaves and bark extracts and ranitidine drug showed significant increase in the pH level by 34.80, 34.00 and 44.80%, respectively as compared with the ulcer group. So, improvement in the pH levels by 26.76, 26.15 and 34.64% were observed after protection with leaves, bark extracts and ranitidine drug, respectively.

Gastric volume in control rats protected with different plant extract and ranitidine drug recorded insignificant changes revealed extract safety (Table 2). Ulcerative stomach showed significant increase in its volume content by 2260.08% as compared with the control group. Protected rats with leaves of bark of *A. cherimola* and ranitidine drug recorded significant decrease in gastric volume by 56.80, 59.50 and 34.30%, respectively as compared with the ulcer group. Therefore, the improvement levels reached to 1340.93, 1397.59 and 812.11%, respectively.

Regarding to the gastric total acidity, normal rats protected with different extracts recorded insignificant changes as compared with the control group (Table 2). The gastric ulcerative rats showed significant decrease in the total acidity value by 76.65% as compared with the control group. Protective rats with leaves, bark of *A. cherimola* and ranitidine drug recorded significant increase in the total acidity level by 362.68, 192.23 and 171.64%, respectively as compared with the ulcer group. Ameliorated levels amounted to 84.66, 44.94 and 40.06%, respectively.

Concerning to the lesions counts, the stomach ulcerative group showed nearly eleven ulcer lesions per stomach (Table 2). In protected rats with leaves, bark of *A. cherimola* and ranitidine drug, the gastric lesions count decreased 84.44, 76.71 and 70.84%, respectively as compared with the ulcerative group.

Table 2 : Total flavonoid content (%) in *A. cherimola* using different extracting solvents.

Different plant parts		Total Flavonoids			
		Chloroform	Ethyl Acetate	Butanol	Total alcohol
A. Cherimola	Leaves	0.07	1.24	2.9	4.6
	Bark	0.03	0.99	1.2	1.5
	Fruits	0.02	0.63	1	1
	Seeds	0.04	0.45	0.26	0.36

(iii) Oxidative stress markers

With respect to the oxidative stress markers, protective rats with different plant extracts and ranitidine drug recorded insignificant changes in catalase, NO, SOD, glutathione, malondialdehyde and total protein level as compared with the control group (Table3).Significant increase in catalase, NO, SOD, glutathione, malondialdehyde and total protein levels in gastroulcerative rats by 141.22, 280.00, 805.90, 269.01, 64.10 and 55.36%, respectively as compared with the control group.

Protection of gastro-ulcerative rats with leaves, bark of *A. cherimola* and ranitidine drug recorded significant decrease in catalse activity by 79.36, 41.91 and 71.70%, respectively. Also, nitric oxide significantly decreased by 62.10, 58.94 and 63.15%, respectively, while superoxide dismutase significantly decreased by 81.65, 83.79 and 80.72%. In addition, glutathione level decreased by 72.27, 72.28 and 71.56%, respectively. Malondialdehyde also decreased by 35.93, 39.84 and 39.84%, while the total protein content was decreased by22.56, 16.99, 21.56, 32.36 and 30.71%, respectively (Table3).

Improvement levels were reached to 152.08, 236.00, 739.75, 266.70, 58.97 and 33.5% for CAT, NO, SOD, GSH, MDA and total protein levels after protection with leaves extract. Protection with bark extract showed improvement by 101.11, 224, 759.07, 266.74, 65.38, and 51.78%, respectively. Ranitidine showed improvement by 172.98, 240.00, 731.32, 764.07, 65.38 and 47.72%, respectively.

Table 3 : Total alkaloids content (mg/g) in *A. cherimola* using different extracting solvents.

Different plant parts		Total Alkaloids			
		Chloroform	Ethyl Acetate	Butanol	Total alcohol
A. Cherimola	Leaves	0.01	0.001	0.009	0.03
	Bark	0.02	0.002	0.01	0.05
	Fruits	0.01	0	0.008	0.02
	Seeds	0.02	0.001	0.007	0.03

Cell organelles marker enzymes

Concerning cell organelles marker enzymes, the present study revealed insignificant changes in SDH, LDH, G-6-Pase, AP and 5`-NT in normal rats protected with different plant extracts and ranitidine drug as compared with the control group (Table 4).

Gastroulcerative rats showed significant increase in SDH, LDH, G-6-Pase, AP and 5`-NT activities by 209.27, 390.29, 102.03, 172.04 and 116.56%, respectively as compared with the control group.

SDH showed significant decrease by 71.15, 59.70 and 71.60% after protection with leaves of *A. cherimola*, bark of *A. cherimola* and ranitidine drug, respectively. LDH recorded inhibition by 79.66, 79.58, and 79.23%, while G-6-Pase inhibited by 37.56, 32.84 and 37.16%, respectively. In addition, AP enzyme diminished by 58.22, 57.01 and 52.85%, while 5`-NT decreased by 47.12, 44.68, 35.06, 36.16 and 50.27%, respectively.

Thereafter, leaves extract recorded improvement by 75.88, 220.02, 390.60, 75.57 and 390.60% for G6P, SDH, LDH, 5`NT and AP, respectively. The bark extract showed enhancement by 66.35, 184.81, 390.18, 77.95 and 390.18%, respectively, while ranitidine drug improve the enzymes by 75.06, 221.45, 388.50 and 108.36%, respectively.

Table 4 : Mass spectrum of fragmentation ion of isolated flavonoidal compounds.

metabolite identification	Molecular weight	Chemical formula	Chemical formula from ESI	exact mass of $[M-H]^-$ or $[M+H]^+$		Δ ppm	Fragmentation pathway		λ max [nm]	ID
				measured	caculated		negative ion mode ESI(-)	positive ion mode ESI(+)		
Hirsutrin (Quercetin 3-O-beta-D-glucopyranoside)	464.09547	$C_{21}H_{20}O_{12}$	$C_{21}H_{19}O_{12}$	463.0885	463.0877	1.7700	463, 201, 255, 151	465, 303, 165, 229	227.8, 278.9	2
Rutin	610.15338	$C_{27}H_{30}O_{16}$	$C_{27}H_{29}O_{16}$	609.1462	609.1456	1.1176	609, 301, 178, 254	611, 465, 367, 303, 249, 272, 202, 153, 110	256, 352	2
Kaempferol 3-glucoside-7-rhamnoside	594.1584	$C_{27}H_{30}O_{15}$	$C_{27}H_{29}O_{15}$	593.1515	593.1506	1.4236	593, 413, 277, 241, 153	595, 560, 449, 286		2
Astragalin Kaempferol 3-O-beta-D-glucoside	448.1005	$C_{21}H_{20}O_{11}$	$C_{21}H_{19}O_{11}$	447.0927	447.0932	-1.0289	447, 401, 383, 297, 221, 163, 123	449, 639, 569, 491, 438, 392, 279, 472	211.5, 284.8, 322.9	2
Cynaroside	448.3802	$C_{21}H_{20}O_{11}$	$C_{21}H_{19}O_{11}$	447.3769	447.1005	-3.254	447, 285, 241, 267, 257, 243, 217, 199, 178, 151, 133		254, 286, 352, 348,	2
Kaempferol	286.0477	$C_{15}H_{10}O_6$	$C_{15}H_{10}O_6$	285.0399	285.0405	-1.9120		287, 153, 133, 165, 121		2
Quercetin	302.0426	$C_{15}H_{10}O_7$	$C_{15}H_9O_7$	301.0348	301.0354	1.8144	301, 153, 149, 165, 137	303, 179, 121, 151, 273, 257, 229		2

Histopathological picture

Concerning to the histopathological picture of normal rats stomach, Fig. 2a showed no injuries of the gastric mucosa with normal mucosal and submucosal layers.

The histological section of gastric mucosa in normal rat treated with leaves extract showed very mild disruption to the surface epithelium with mild edema and no leucocytes infiltration of the submucosal layer (Fig. 2b). The gastric mucosa in normal rat treated with bark extract showed no disruption to the surface epithelium with no edema and no leucocytes infiltration of the submucosal layer (Fig.2c).

The histological section of ulcerative stomach showed erosion of surface epithelium with moderate edema and moderate leucocytes infiltration of the submucosal layer with hemorrhage (Fig. 2d, e).

The ulcerative gastric mucosa prophylactic with leaves and barks extract showing no disruption to the surface epithelium (healed) with no edema and mild leucocytes infiltration of the submucosal layer (Fig. 2f, g). The histological section of ulcerative gastric mucosa prophylactic with drug showed no disruption to the surface epithelium (healed) with no edema and mild leucocytes infiltration of the submucosal layer (Fig. 2h).

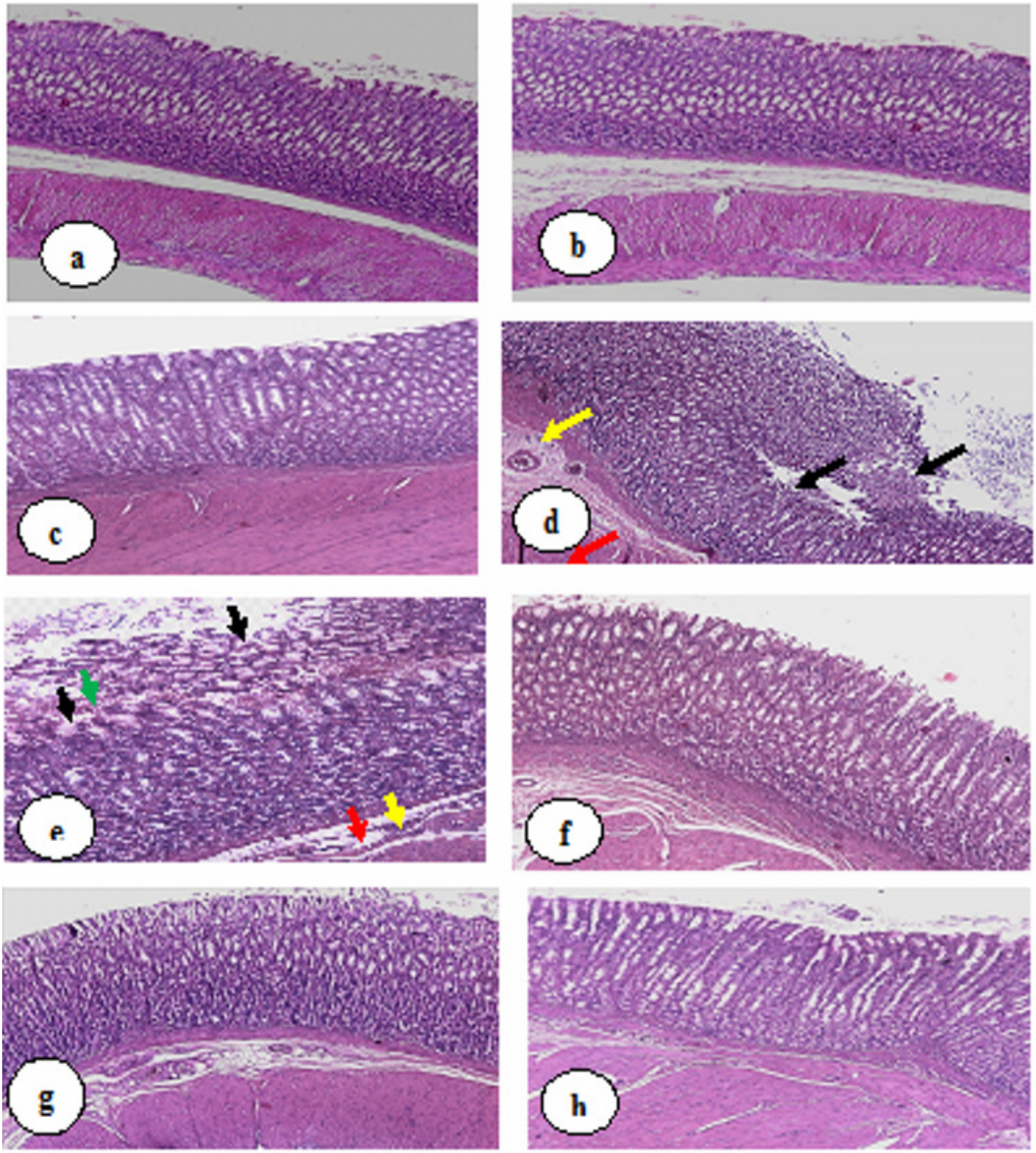

Fig. 2(a): Photomicrograph of normal stomach showing normal mucosal and submucosal layers (H&Ex100), (b) Gastric mucosa in normal rat treated with leaves of *A. cherimola* showing very mild disruption to the surface epithelium with mild edema and no leucocytes infiltration of the submucosal layer (H&E stain 10x). (c) Gastric mucosa in normal rat treated with bark of *A. cherimola* showing no disruption to the surface epithelium with no edema and no leucocytes infiltration of the submucosal layer (H&E stain 10x). (d) Gastric mucosa with ethanol induced ulcer for one hour, showing erosion of surface epithelium (black arrow) with moderate edema (red arrow) and moderate leucocytes infiltration of the submucosal layer (yellow arrow) (H&E stain 10x). (e) Gastric mucosa with ethanol induced ulcer for one hour, showing erosion of surface epithelium (black arrow) with mild edema (red arrow) and mild leucocytes infiltration of the submucosal layer (yellow arrow), hemorrhage (green arrow) (H&E stain 20x). (f) Ulcerative gastric mucosa prophylactic with leaves *A. cherimola* showing no disruption to the surface epithelium (healed) with no edema and mild leucocytes infiltration of the submucosal layer (H&E stain 10x). (g) Ulcerative gastric mucosa prophylactic with barks of *A. cherimola* showing no disruption to the surface epithelium (healed) with no edema and mild leucocytes infiltration of the submucosal layer (H&E stain 10x). (h) Ulcerative gastric mucosa prophylactic with drug showing no disruption to the surface epithelium (healed) with no edema and mild leucocytes infiltration of the submucosal layer (H&E stain 10x).

Table 6 : Protective effect of *A. cherimola* extract on gastric ulcer markers in rats.

Parameters	Control	Control+ leaves extract	Control + bark extract	Ulcer	Ulcer protected with leaves extract	Ulcer protected with bark extract	Ulcer protected with Rantidine
pH	3.25 ^{b} ± 0.46	3.38 ^{b} ± 0.51	3.25 ^{b} ± 0.46	2.50 ^{a} ± 0.53	3.37 ^{b} ±0.51	3.37 ^{b} ±0.51	3.62 ^{b} ±0.74
Gastric Volum(µl)	132.37 ^{e} ± 18.18	135.00 ^{e} ± 14.14	134.62 ^{e} ± 10.91	3125.00 ^{a} ± 183.22	1350.00 ^{c} ± 292.77	1275.00 ^{cd} ± 223.60	2050.00 ^{b} ± 297.60
Total Acidity (m Eq/L)	2.87 ^{ab} ± 0.50	2.72 ^{abc} ± 0.27	2.72 ^{abc} ± 0.22	0.67 ^{e} ± 0.22	3.10 ^{a} ±0.70	1.96 ^{cd} ±0.95	1.82 ^{d} ±0.66
Ulcer index (number)	--	--	--	11.25 ^{a} ±1.98	1.75 ^{c} ±148	2.62 ^{bc} ±1.30	3.25 ^{b} ±0.88

- Data are mean ± SD of eight rats in each group.
- Statistical analysis is carried out by one way analysis of variance (ANOVA) – Costat Software Computer Program accompanied by post hoc (LSD) between groups at $p<0.05$.
- Unshared letters between groups are significant values at $p<0.0001$.

Table 7 : Protective effect of *A. cherimola* extract on antioxidant levels and protein content of gastric ulcer in rats.

Parameters	Control	Control+ leaves extract	Control + bark extract	Ulcer	Ulcer protected with leaves extract	Ulcer protected with bark extract	Ulcer protected with Rantidine
CAT µmol/mg protein	3.59 ^{cd} ± 1.39	2.20 ^{de} ± 1.04	1.40 ^{e} ± 0.33	8.66 ^{a} ± 1.10	3.20 ^{cd} ±0.81	5.03 ^{b} ± 1.50	2.45 ^{de} ± 0.55
NO µg/mg protein	0.25 ^{cd} ± 0.03	0.28 ^{bcd} ± 0.04	0.29 ^{bcd} ± 0.05	0.95 a ± 0.09	0.36 ^{bcd} ±0.20	0.39 ^{bc} ± 0.07	0.35 ^{bcd} ± 0.10
SOD µmol/mg protein	16.22 ^{b} ± 4.94	21.38 ^{b} ± 1.87	16.23 ^{b} ± 3.17	147.21 ^{a} ± 1.71	27.00 ^{b} ±7.50	23.86 ^{b} ± 4.71	28.37 ^{b} ± 6.84
GSH µg/mg protein	25.50 ^{b} ± 2.41	24.06 ^{b} ± 2.00	25.94 ^{b} ± 0.81	94.10 ^{a} ± 12.00	26.09 ^{b} ±1.78	26.08 ^{b} ± 1.69	26.76 ^{b} ± 1.16
MDA µmol/mg protein	0.78 ^{bcd} ± 0.02	0.68 ^{cd} ±0.03	0.86 ^{b} ±0.10	1.28 ^{a} ± 0.15	0.82 ^{bc} ±0.09	0.77 ^{bcd} ± 0.04	0.77 ^{bcd} ± 0.21
T. protein mg/g tissue	24.62 ^{de} ± 7.4	27.25 ^{cd} ± 1.83	22.37 ^{e} ± 1.06	38.25 ^{a} ± 4.49	30.00 ^{bc} ±3.38	25.87 ^{d} ± 3.09	26.50 ^{d} ± 2.20

- Data are means ± SD of eight rats in each group.
- Statistical analysis is carried out by one way analysis of variance (ANOVA) – Costat Software Computer Program accompanied by post hoc (LSD) between groups at $p<0.05$.
- Unshared superscript letters between groups are the significance values at $p < 0.0001$.

Table 8 : Protective effect of *A. cherimola* extract on cell organelles marker enzymes of gastric ulcer in rats.

Parameters	Control	Control+ leaves extract	Control + bark extract	Ulcer	Ulcer protected with leaves extract	Ulcer protected with bark extract	Ulcer protected with Rantidine
G6P µmol/mg protein	242.03 fg ± 23.23	271.40 ^{def}±7.07	279.62^{cde}±19.04	488.93^{a} ±45.37	305.26 ^{bcd} ± 11.65	328.34 ^{b} ± 61.77	307.24 ^{bcd} ± 15.74
SDH µmol/mg protein	74.94 ^{c} ± 12.82	64.51 ^{c} ± 6.70	71.18 ^{c} ± 9.42	231.77^{a} ±27.84	66.86 ^{c} ± 9.56	93.27 ^{b} ± 3.53	65.81 ^{c} ± 8.69
LDH µmol/mg protein	54.61 ^{b} ±4.21	58.28 ^{b} ± 5.56	60.69 ^{b} ± 6.57	267.75^{a} ± 4.66	54.44 ^{b} ± 8.89	54.67 ^{b} ± 9.49	55.59 ^{b} ± 10.92
5\NT µmol/mg protein	52.12 ^{c} ± 5.30	53.29 ^{c} ± 4.02	52.02 ^{c} ± 6.07	112.35^{a} ± 6.61	72.96 ^{b} ± 15.49	71.72 ^{b} ± 14.39	55.87 ^{c} ± 6.82
AP µmol/mg protein	73.32 ^{c} ± 5.96	75.01 ^{c} ± 6.18	73.66 ^{c} ±7.36	199.46^{a} ± 3.90	83.32 ^{bc} ± 9.55	85.74 ^{bc} ± 11.17	94.03 ^{b} ± 15.26

- Data are means ± SD of eight rats in each group.
- Statistical analysis is carried out by one way analysis of variance (ANOVA) – Costat Software Computer Program accompanied by post hoc (LSD) between groups at $p<0.05$.
- Unshared superscript letters between groups are the significance values at $p < 0.0001$.

Discussion

Our results revealed the presence of phenols, flavonoids and alkaloids in *A. cherimola* ethanol extract. Many studies revealed that medicinal plants are containing phenols, flavonoids and alkaloids which have different biological activities (Hassan *et al.*, 2016; Hassan *et al.*, 2008). The choice of the proper solvents controlled the properties of phenolic components of the concerned plants, where the highest extractable values have been attained using alcohol compared with the other solvents investigated.

These data may show some lights on the polar properties of the phenolics characterized in *Annon*a species, and this may be confirmed by the less efficiency of chloroform for extracting phenolics. The best extraction efficiency was achieved by ethanol 100%, then80% followed by 50% EtOH.

From phytochemical study the plants included in this investigation focused on the isolation two groups of natural product; flavonoids and alkaloids. Seven flavonoids are identified as Rutin, Kaempferol 3-glucoside-7-rhamnoside, Astragalin, Kaempferol, Quercetin, Hirsutrin and Luteolin-7-O-β-D-glucopyranoside from the extracts are mentioned.

Pathogenesis of peptic ulcers, secretion of gastric acid is still recognized as a central component of this disease. Therefore, the main therapeutic target is the control of this secretion using antiacids, H_2 receptor blockers like ranitidine, famotidine, anticholinergics like pirenzepin, telezipine or proton pump blockers like omeprazole, lansoprazole (Qadeer *et al.*, 2006). However, gastric ulcer therapy faces nowadays major drawbacks because most of the drugs currently transition metal ions chelation, inhibition of oxidizing enzymes, increase of proteic and nonproteic antioxidants and reduction of lipid peroxidation. These effects are correlated with presence in the structures of an *o*-dihydroxy in the ring B (catechol), and additionally a 2,3 double bond in conjugation with a 4-oxo function, as well as the presence hydroxyl groups in positions 3, 5 and 7. Besides the gastroprotective activity, sofalcone (a chalcone), quercetin and naringenin (flavanones) accelerate the healing of gastric ulcers (Abdelwahab *et al.*, 2013). In addition, flavonoids are able to decrease ulcerogenic lesions by promoting the formation of gastric mucosa which inhibit the production of pepsinogen and diminish acid mucosal secretion (La-Casa *et al.*, 2000). Mota *et al.*(2009)have summarized the literature on 95 flavonoids with varying degrees of antiulcerogenic activity, confirming that flavonoids have a therapeutic potential for the more effective treatment of peptic ulcers. The most important effect of flavonoidsis their antioxidant properties. This is seen in garcinol, rutin and quercetin, which involves free radical scavenging effect.

These results go parallel with the data obtained by Anand *et al.* (2015). They found the extraction of phenolic and flavonoid compounds from a plant depends on the methods and type of extracting solvent.

The development of gastroduodenal ulcers is mainly attributed to the consumption of alcohol. When alcohol was administered to human body it penetrates rapidly to the gastro intestinal mucosa causing damage and erosion (Repetto and Llesuy, 2002). The increase in the permeability in mucosal together with active products from mast cells, macrophages and blood cells will produce vascular injury, necrosis and hence formation of ulcer (Kvietys *et al.*, 2009).

Ethanol and excess of reactive oxygen together are critical factors loading to mucosal damage (Repetto and Llesuy, 2002). An increased in the parameters characterizing gastric ulceration such asgasteric volume, pH and total acidity were recorded. Gracioso *et al.* (2002) attributed the rate of mucosal defensive mechanisms to the hydrochloric acid released from the surface of epithelial cells.

The present study revealed significant increase in malondialdehyde, superoxide dismutase, catalse, NO and glutathione. Demir *et al.* (2003) mentioned that high gastric mucosal MDA levels in patients with peptic ulcer and gastritis are thought to reflect free radicals mediated gastric mucosal damage. In agreement with our results, it was observed significant elevation of SOD in gastric ulcer state (Tandon *et al.*, 2004). The first authors explained this observation according to stress causes stimulation of stomach leading to local hypoxia or actual "ischemia". An increase in the level of H_2O_2 by SOD action which in conjugation with O2 generate OH in caused by ischemic condition. In this cortex OH radicals generated important constituents like structural and functional proteins and lipids membrane. Hydroxyl radicals thus generated oxidizes important cellular constituents such as structural and functional proteins and membrane lipids. Lipid peroxidation causes loss of membrane fluidity, impaired ion transport and membrane integrity and finally loss of cellular functions. It has been firmly established that oxidative stress and impaired prostaglandin synthesis contribute to gastric mucosal damage in experimental models of gastric lesions induced by ethanol (Kwiecien *et al.*, 2002).

It was reported that, a reduction in gastric glutathione can occur following ethanol consumption and glutathione pretreatment could subside the gastric damage (Loguercio *et al.*, 1993). In contrast to many investigations, GSH in the present study recorded significant elevation in gastric ulcer group. Indeed, glutathione status is dependent on relative activity of many other enzymes (Malmezat *et al.*, 2000). The increased activity of enzymes involved in GSH synthesis (γ-glutamyl-cysteine synthetase) and GSH reduction (glutathione reductase) can lead to an increase of GSH concentration. Conversely increased the activity of GSH peroxidase (the enzyme responsible for catalyzing the formation of oxidized glutathione) and GSH transferase (the enzyme responsible for the conjugation of toxic compounds with GSH) led to decrease in GSH concentration. This was in accordance with Koc *et al.*(2008)who observed decrease of glutathione peroxidase and glutathione transferase in indomethacin induced gastric ulcer, ethanol induced mucosal injury and in stress ulcer which may give an additional support to our results.

In the present investigation, it is revealed that total protein content can be used as a useful index for the severity of cellular disorder for many diseases. The amelioration of protein synthesis has been considered as a contributory self-healing mechanism which in turn can accelerates the regeneration process (Sharma and Shukla, 2011).

Ethanol treatment recorded severe elevation of mucosal enzymes. This was in parallel with the observation of Ozeki *et al.* (1987) who showed marked activation of SDH in gastric ulcer mucosa. They attributed this elevation to

increase of mitochondrial permeability and depolarization. The same observation was recorded in case of increase LDH in gastric mucosa. Brzozowski *et al.* (2005) consider LDH elevation as a sensitive indicator of mucosal damage in ulcerative and ischaemic conditions.

Lysosomal membrane stability plays a very important role in the inflammatory process (Rodrigues *et al.*, 1998). Erosive gastropathy and gastroduodenal ulcerative showed a great liability of lysosomal membranes and autoaggressive enzymes release (Rodrigues *et al.*, 1998). This was in accordance with the observed elevation of acid phosphatase enzyme, which is mainly localized in lysosomes. Glusose-6-phosphatase and 5`-nucleotidase also recorded significant increase after ethanol ulceration. This was in parallel with the results of Ozeki *et al.* (1987)who mentioned that gastric ulcer mucosa is mediated *via* endoplasmic reticulum and plasma membrane stress response following enzymes leakage and damage to their membranes. Disturbance of cell membranes in ulcer is one of the main pathogenetic components of ulcer genesis. Alcohol consumption leads to a change of the membrane phase state, which significantly affects membrane transport processes and systems of transmembrane information transfer that led to enzyme disturbance (Yakubtsova *et al.*, 2008).

Ulcer healing is a complex process and entails several distinct repair mechanisms. Epithelial cell proliferation and migration from the ulcer edge across the ulcer bed is accompanied by maturation of granulation tissue beneath the ulcer base. Within this tissue, vascular endothelial cells form new capillaries to restore the microvasculature, while fibroblasts restore the *lamina propria* (Berenguer *et al.*, 2002). Ulcer healing is associated with regulation of pH at the gastric surface. Some plant-derived substances have been shown to attenuate ethanol-and stress-induced gastric lesions *via* activation of prostaglandin, nitric oxide and sensory nerve pathways and thus improving the microcirculation (Brzozowski *et al.*, 2005). These observations are in line with our results through the recorded decrease in gastric volume, acidity, lesion counts, antioxidant levels and mucosal enzymes by the actions of the selected extracts.

The presented decrease in gastric volume, lesions and acidic value reinforced the presence of antisecretory and antiulcerogenic effects of the selected extracts. Ranitidine also recorded a protective potential role in gastric ulcer. This was attributed to the antisecretory and mucosal strengthening effects beside its cicatrisation action (Moraes *et al.*, 2008).

Regarding to the histological changes in gastric mucosa upon ulceration by ethanol, deep ulcer reached to the basement membrane lined the *lamina propria* was recorded. The thickened ulcer base recorded some polymorphous lymphocytes fibrin. The gastric glands are hyperplastic and surrounded the ulcer. The *lamina propria* contains few lymphocytes and polymorphonuclear leucocytes with high degree of fibrosis (Gracioso *et al.*, 2002; Moraes *et al.*, 2008). This was in accordance with our recorded histological observation of ulcerated mucosa.

Rats submitted to protection by the selected plant extracts present well-develop degenerative epithelium at the ulcer margin and throughout the wide area where the ulcer had been implanted. The stomach also showed the simple columnar epithelium and *lamina propria* presenting simple branched tubular glandules with dilated lumen. Ulcer healing takes place either by a regeneration process that starts from the neck cells of the glands or by a rapid process involving the migration of cells towards the luminal surface and their deposition on the area stripped by the ulcerogenic agent (Galati *et al.*, 2002).

In conclusion, rats subjected to protection by *A. cherimola* showed improvement in ulcer index, oxidative stress markers and cell organelles markers enzymes as well as the improvement of the histopathological pattern in gastric mucosa. The presence of phenolics and flavonoids improved the selected parameters due to their antioxidant effects as well as its role as anti-ulcerative agent. Further studies are needed for their pharmacological and clinical applications.

Conflict of interest

The authors confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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