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

Mona A. Mohammed, Manal A. Hamed, Souad E. El-Gengaihi, Ahmed M. Aboul Enein, Osama K. Ahmed and Emad M. Hassan

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 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-gastro-ulcerative 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 gastro-ulcerative agents.

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

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.

Abbreviations


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
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 1H NMR spectroscopic analysis (chemical shift δ in ppm, coupling constant J in Hz). The recorded spectrum (DMSO-d6), 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, CH3-rha) 3.16-3.65 (m, the rest sugar of glucose and rhamnose). The 1H NMR of this compound revealed the chemical shift of protons identical with those reported in the literature for rutin. 13C 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.).

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

1H 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. 13C NMR spectral data displayed 21 carbon, 15 of kaempferol 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).

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

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

Fig. 1: Chemical structure of the isolated compounds (1-7)
glucose moiety. The anomic 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 1H 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' and H-3', 5' respectively, two aromatic protons of the A-ring were revealed as two doublet at δ 6.41 and 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 kaempferol (Fig. 1) (Gonda et al., 2000).

(iv) Band (4) Quercetin (5)

The 1H 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 6.42 with J = 1.8 Hz of each proton due to meta coupling of H-6 and H-8 respectively. 13C 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. 252-254°C, which possess chromatographic properties (dark brown spot on PC, turning bright green when fumed with ammonia vapor, changing yellow with AlCl3 and λmax (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 ion mode ESI(+) technique (molecular weight 594.1584. [M+H]+ at m/z: 595, 560, 449, 286 (Fig. 1).

The 1H-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 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-O-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-1" indicated that the glucose was α-linked to the aglycone (β-glucosides typically show the anomic 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 3.2-4 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 °C 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 LC50 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 Group 4: received the ethanol dose on 24 hrs empty stomach, sacrificed after one hour later and served as the ulcerative.

Groups 5-7: Protective groups were administrated 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 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 (Banchoft 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>
<thead>
<tr>
<th>Different plant parts</th>
<th>Total Phenolics Acetate</th>
<th>Butanol</th>
<th>Total alcohol</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Cherimola</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leaves</td>
<td>2.36</td>
<td>16.68</td>
<td>20.68</td>
</tr>
<tr>
<td>Bark</td>
<td>1.68</td>
<td>14.68</td>
<td>15.64</td>
</tr>
<tr>
<td>Fruits</td>
<td>0.05</td>
<td>8.68</td>
<td>12.98</td>
</tr>
<tr>
<td>Seeds</td>
<td>1.03</td>
<td>10.75</td>
<td>8.32</td>
</tr>
</tbody>
</table>

**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-a-L-rhamnopyanosyl- (1→6))-β-D-glucopyranoside (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-a-D-glucopyranoside-7-a-L-rhamnopyranoside (7).

The mass spectrum of isolated flavonoids were summarized in table SI.

**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.

<table>
<thead>
<tr>
<th>Concentrations (µg/ml)</th>
<th><em>A. cherimola</em></th>
<th>Vitamin C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Leaves</td>
<td>Bark</td>
</tr>
<tr>
<td>10</td>
<td>66.66</td>
<td>59.45</td>
</tr>
<tr>
<td>50</td>
<td>70.00</td>
<td>64.28</td>
</tr>
<tr>
<td>100</td>
<td>72.72</td>
<td>87.61</td>
</tr>
</tbody>
</table>

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

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

**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 23.07% as compared with the control group. Gastroulcerative rats showed significant increase by 56.80, 72.28 and 71.56%, respectively. Malondialdehyde also 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 by 22.56, 16.99, 21.56, 32.36 and 30.71%, respectively (Table 3).

Regarding to the gastric total acidity, normal rats treated with *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 by 22.56, 16.99, 21.56, 32.36 and 30.71%, respectively (Table 3).

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.

<table>
<thead>
<tr>
<th>Different plant parts</th>
<th>Total Alkaloids</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Chloroform</td>
</tr>
<tr>
<td><em>A. Cherimola</em> Leaves</td>
<td>0.01</td>
</tr>
<tr>
<td>Bark</td>
<td>0.02</td>
</tr>
<tr>
<td>Fruits</td>
<td>0.01</td>
</tr>
<tr>
<td>Seeds</td>
<td>0.02</td>
</tr>
</tbody>
</table>

(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 (Table 3). 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 catalase 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 by 22.56, 16.99, 21.56, 32.36 and 30.71%, respectively (Table 3).

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

<table>
<thead>
<tr>
<th>Different plant parts</th>
<th>Total Flavonoids</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Chloroform</td>
</tr>
<tr>
<td><em>A. Cherimola</em> Leaves</td>
<td>0.07</td>
</tr>
<tr>
<td>Bark</td>
<td>0.03</td>
</tr>
<tr>
<td>Fruits</td>
<td>0.02</td>
</tr>
<tr>
<td>Seeds</td>
<td>0.04</td>
</tr>
</tbody>
</table>

Cell organelles marker enzymes

Concerning cell organelles marker enzymes, the present study revealed insignificant changes in SDH, LDH, G-6-Pase, AP and S'-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 S'-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.

<table>
<thead>
<tr>
<th>Metabolite identification</th>
<th>Molecular weight</th>
<th>Chemical formula</th>
<th>Chemical formula from ESI</th>
<th>Exact mass of [M-H]⁻ or [M+H]⁺ measured</th>
<th>Δ ppm</th>
<th>Fragmentation pathway negative ion mode ESI(-)</th>
<th>Fragmentation pathway positive ion mode ESI(+)</th>
<th>λ max [nm]</th>
<th>ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hirsutrin (Quercetin 3-O-beta-D-glucopyranoside)</td>
<td>464.09547</td>
<td>C₂₁H₂₀O₁₂</td>
<td>C₂₁H₁₉O₁₂</td>
<td>463.0885</td>
<td>463.0877</td>
<td>1.7700</td>
<td>463, 201, 255, 151</td>
<td>465, 303, 165, 229</td>
<td>227.8, 278.9</td>
</tr>
<tr>
<td>Rutin</td>
<td>610.15338</td>
<td>C₂₇H₃₀O₁₆</td>
<td>C₂₇H₂₉O₁₆</td>
<td>609.1462</td>
<td>609.1456</td>
<td>1.1176</td>
<td>609, 301, 178, 254</td>
<td>611, 465, 367, 303, 249, 272, 202, 153, 110</td>
<td>256, 352</td>
</tr>
<tr>
<td>Kaempferol 3-glucoside-7-rhamnoside</td>
<td>594.1584</td>
<td>C₂₇H₃₀O₁₅</td>
<td>C₂₇H₂₉O₁₅</td>
<td>593.1515</td>
<td>593.1506</td>
<td>1.4236</td>
<td>593, 413, 277, 241, 153</td>
<td>595, 560, 449, 286</td>
<td>2</td>
</tr>
<tr>
<td>Astragalin</td>
<td>448.1005</td>
<td>C₂₁H₂₀O₁₁</td>
<td>C₂₁H₁₉O₁₁</td>
<td>447.0927</td>
<td>447.0932</td>
<td>-1.0289</td>
<td>447, 401, 383, 297, 221, 163, 123</td>
<td>449, 639, 569, 491, 438, 392, 279, 472</td>
<td>211.5, 284.8, 322.9</td>
</tr>
<tr>
<td>Quercetin</td>
<td>302.0426</td>
<td>C₁₅H₁₀O₇</td>
<td>C₁₅H₇O₇</td>
<td>300.0348</td>
<td>301.0354</td>
<td>1.8144</td>
<td>301, 153, 149, 165, 137</td>
<td>303, 179, 121, 151, 273, 257, 229</td>
<td>2</td>
</tr>
</tbody>
</table>

**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 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).
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

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.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>Control+ leaves extract</th>
<th>Control + bark extract</th>
<th>Ulcer</th>
<th>Ulcer protected with leaves extract</th>
<th>Ulcer protected with bark extract</th>
<th>Ulcer protected with Rantidine</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>3.25 ± 0.46</td>
<td>3.38 ± 0.51</td>
<td>3.25 ± 0.46</td>
<td>2.50 ± 0.53</td>
<td>3.37 ± 0.51</td>
<td>3.37 ± 0.51</td>
<td>3.62 ± 0.74</td>
</tr>
<tr>
<td>Gastric Volum(µL)</td>
<td>132.37 ± 18.18</td>
<td>135.00 ± 14.14</td>
<td>134.62 ± 10.91</td>
<td>3125.00 ± 183.22</td>
<td>1350.00 ± 292.77</td>
<td>1275.00 ± 223.60</td>
<td>2050.00 ± 297.60</td>
</tr>
<tr>
<td>Total Acidity (m Eq/L)</td>
<td>2.87 ± 0.30</td>
<td>2.72 ± 0.27</td>
<td>2.72 ± 0.22</td>
<td>0.67 ± 0.22</td>
<td>3.10 ± 0.70</td>
<td>1.96 ± 0.95</td>
<td>1.82 ± 0.66</td>
</tr>
<tr>
<td>Ulcer index (number)</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>11.25 ± 1.98</td>
<td>1.75 ± 0.148</td>
<td>2.62 ± 1.30</td>
<td>3.25 ± 0.88</td>
</tr>
</tbody>
</table>

- 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.

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

- 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.

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

- 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 *Annona* species, and this may be confirmed by the less efficiency of chloroform for extracting phenolics. The best extraction efficiency was achieved by ethanol 100%, then 80% 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 antacids, H2 receptor blockers like ranitidine, famotidine, anticholinergics like pirenzipen, telzipine 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, sofalone (a chalcone), quercetin and naringenin (flavanones) accelerate the healing of gastric ulcers (Adelwahab 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 asgastric 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, catalase, 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 H2O2 by SOD action which in conjunction 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 (Loguerio 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
increased 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. Glucose-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 antulcerogenic 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 surrounding 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.

References


