Safety and Gastro protective Activity of *Graviola* Seeds Extract against Acidified Aspirin Induced Gastric Ulceration

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Abstract

Peptic ulcer disease attacks millions of people yearly around the globe, affecting both quality and quantity of life. There are many different causes responsible for the etiology of this disease; one of the most common causes is non-steroidal anti-inflammatory drugs (NSAIDs) through different mechanisms. *Annona muricata* have been found to have antioxidant effects, along with a good protective effect against inflammation in rats, making it a good candidate as a potential gastro protective agent. So, this study is designed to evaluate this possible pharmacological activity. *Annona muricata* seeds were extracted; chemical analysis of the extract has been done, acute toxicity study has been performed using different doses of Ethanolic seed extract of *Annona muricata* (ESAM) 500, 2000, and 5000 mg/kg, the extract demonstrated relative safety at doses up to 2000mg/kg. The gastro protective effect of ethanol seeds extract of *Annona muricata* (ESAM) has been investigated using two doses of ESAM 250 and 500mg/kg of ESAM, using ranitidine 50mg/kg, as a reference, ulcer was induced by acidified aspirin solution after 7 days. The extract caused reduction in the gastric content volume, ulcer index and TNF-α, and a significant elevation in the gastric PGE2 and SOD levels in dose dependent manner showing a good preventive index in compare to the acidified aspirin group. Gastro protective effect of ESAM was prevented by pretreatment with sulphydryl alkylating group (NEM) and NOS inhibitor group (L-NNA) approving the involvement for sulphydryl (SH) group and endogenous nitric oxide (NO) in the gastro protective effect of *Annona Muricata* seeds. Immunohistochemical staining showed pretreatment with ESAM caused upregulation of HSP70. So, this study suggests that the seeds extract of *Annona muricata* is relatively safe. Its gastroprotective activity attributed to its antisecretory; cytoprotective; antioxidant and anti-inflammatory effects.

Keywords: Acute toxicity, *Annona muricata* seed extract, Gastro protective, Non-steroidal anti-inflammatory drugs.

Introduction

Peptic ulcer disease (PUD) is a common disease of the gastrointestinal tract; it can be defined as the soreness of the mucosa, with or without muscular is mucosa influence. A recent review article retrieved 178 full-text articles throughout the globe, found that annually the highest incidence of peptic ulcer was in Spain with 141.8 per 10⁵ individual, and the lowest was in the United Kingdom with 23.9 person.[1] The epidemiology of PUD is no longer caused primarily by helicobacter pylori (H. pylori). Aging has led to a rise in the use of non-steroidal anti-inflammatory drugs (NSAIDs) including aspirin™, this contributed to upper gastrointestinal hemorrhage, which becoming more common among the elderly [2]. These drugs disrupt the permeability of mucosal barrier, rendering the mucosa more susceptible to injury.

The pathogenesis of gastric ulcer through NSAIDs (including aspirin™ administration) can be associated partly with cyclooxygenase inhibition and on another part by cyclooxygenase-independent mechanisms, mainly from local direct actions [3]. Prostaglandin has many protective effects on
gastric mucosa, so NSAID induce ulcer and mucosal injury by suppression of this protective effect through prostaglandin synthesis inhibition. For instance, prostaglandin inhibits neutrophil activation and reactive oxygen species (ROS) release [4].

Also, NSAID tend to induce endothelial adhesion of activated neutrophil causing blockage of microvasculature that might lead to decrease mucosal blood flow and increase proteolytic enzymes, leukotriene and ROS release, and leading to focal mucosal necrosis augmented by low PH. Bicarbonate ion, act as a protective factor against low PH, is secreted in the mucosal mucus layer depending on cyclooxygenase derived prostaglandin by its receptor activation, thus NSAID contribute to gastric mucosal damage by inhibition of its secretion[5].

Cyclooxygenase independent mechanism of gastric mucosal injury rely on the fact that most NSAIDs are acidic in nature. In gastric acidic environment, aspirin™ and other acidic NSAIDs can penetrate the cell membrane of the gastric epithelial cell remain trapped there, augmenting prostaglandin synthesis inhibition, uncouple mitochondrial oxidative phosphorylation, which decrease cellular Adenosine triphosphate (ATP) production, increase adenosine diphosphate (ADP) and adenosine monophosphate (AMP) levels inside the cell, which may increase intracellular calcium concentration, causing mitochondrial and cellular injury[6].

Various approaches have been made to study herbal drugs for treatment and prevention of peptic ulcer. So, many medicinal plants with anti-ulcer activity have been reported, like Adhatoda vasica [7]. Annona muricata Linn, is a tropical plant commonly known as graviola, soursop or guanabana, from Annonaceae family, several pharmacological uses and activities have been extensively studied using different parts extract of this plant including antioxidant,[8] anti-inflammatory, [9] wound healing, [10] antimicrobial, [11] anticancer,[12,13] and hypoglycemic activities[14].

The seed is about 4% of the whole fruit and has many ethno-medical uses such as carminative or as an insecticide, for skin parasites and lice,[15] and has many pharmacological activities.

So, this study is designed to evaluate the gastro protective effect of Annona muricata ethanolic seeds extract on aspirin- induced gastric ulcer along with the mechanism by which it can produce this proposed gastro protective effects and its acute toxic effects.

Materials and Methods

Materials

Plant: Annona muricata fruits were bought from local market in Baghdad in January 2017 that has been purchased fresh from Malaysia; these fruits had been identified by a professional plant taxonomist in the pharmacognosy department/ College of Pharmacy/ Al-Mustansiriya University.

Animals: Fifty four female albino rats weighing 180-230 g, obtained from the animal house of the College of Pharmacy/ Baghdad University and twenty four albino mice obtained from the animal house of the College of Pharmacy/ Al-Mustansiriya University, have weights between 15-25 grams, were permitted by the ethics committee for animal experimentation of College of Pharmacy/ Al-Mustansiriya University.

Before starting the study, the animals were positioned under controlled conditions of temperature 22±2o C and light of 12-12 hrs light/dark cycle by artificial lighting system, kept in plastic cages of 20x25x35 cm. Three animals per cage fed by free excess to standard rat pellets and water ad libitum.

Drugs: Aspirin powder (State Company for Drugs Industry & Medical Appliances Samarra, Iraq), N-Ethylmaleimide powder (Sigma-Aldrich, USA), N-Nitro-L-arginine powder (Sigma, USA), Ranitidine 150mg tablets (GlaxoSmithKline, UK).

Plant Extraction

The unripe fruits were washed with tap water, cut open, and the seeds were collected and air-dried for one week at room temperature, the dried seeds then pulverized by electrical mill (Clatronic, Germany) into coarse powder. About 100 gm of this powder was extracted with 500 ml of absolute ethanol in soxhlet’s apparatus, the extract then was concentrated using rotator evaporator (Heidolph, Germany) followed by oven drying at 45° C [16].
The oven dried extracts were prepared into concentration of 250 mg/kg, 500 mg/kg, 2000 mg/kg, and 5000 mg/kg by using 10% dimethyl sulfoxide (DMSO) as a vehicle, and kept in the fridge (4°C) until the time of the experiment.

**Photochemical Screening**

The crude ethanolic seeds extract of *Annona muricata* (ESAM) was analyzed qualitatively for the presence of alkaloids, Coumadin’s, anthraquinone and cardio active glycosides, flavonoids, saponin and tannins using standard procedures of analysis prescribed by Evans[17] and Harborne[18].

**Gas Chromatography-Mass Spectrometry (GC-MS)**

In this technique, an ionization system of electron with ionization energy of 70 eV was used with carrier gas (helium); this scan has been performed as a full scan mode. The sample was injected automatically in split less mode (10:1) and separated using temperature gradient program. Compounds were identified from their mass spectra by comparison of the retention times of peak with interpretation of mass spectroscopy fragmentation patterns. This work was achieved in the Medical Legal Institute/Ministry of Health.

**Acute Toxicity Study in Mice**

*Annona muricata* seeds acute toxicity study was performed as described by Souza Brito.[19] Twenty four female mice were divided into four groups (n=6 for each group).

First group received 10% DMSO (10 mL/kg) by gavage and kept as normal control. A single dose of *Annona muricata* seeds extract was administered orally to group 2, 3 and 4 at doses of 500, 2000, and 5000 mg/kg, respectively.

The mice then were observed for possible mortality or other signs of toxicity like the change in behavior, movement, and body weight during the period of 14 days. On day 15, the mice were euthanized by xylazine and ketamine overdose, the liver, kidney and the heart have been harvested to get the relative organ weight, and to be tested for histological examination.

**Experimental Design**

Thirty rats were divided randomly into five groups (n=6 for each group), as shown in table 1, all 30 female rats pretreated orally through gavage needle for 7 days according to Table 1. At the seventh day, all the rats were fasted for 24 hours (water accessible).

To induce gastric lesion, 1 hour after pretreatment, all rats (except the control group who received 10% DMSO) orally administered acidified aspirin solution that was prepared by dissolving 2g of aspirin powder in 100 ml of 0.2 M HCl to get 2% solution of acidified aspirin. The rats euthanized four hours later by high dose of xylazine and ketamine, and their stomachs removed and prepared for ulcer scoring and further analysis.

**Table 1: Experimental Design**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Pretreatment (oral)</th>
<th>Treatment (oral)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I: Control group</td>
<td>10% DMSO (10 mL/kg)</td>
<td>10% DMSO (10 mL/kg)</td>
</tr>
<tr>
<td>II: Acidified aspirin group</td>
<td>10% DMSO (10 mL/kg)</td>
<td>acidified aspirin (200 mg/kg)</td>
</tr>
<tr>
<td>III: Low dose ESAM group</td>
<td>ESAM (250 mg/kg)</td>
<td>acidified aspirin (200 mg/kg)</td>
</tr>
<tr>
<td>IV: High dose ESAM group</td>
<td>ESAM (500 mg/kg)</td>
<td>acidified aspirin (200 mg/kg)</td>
</tr>
<tr>
<td>V: Ranitidine group</td>
<td>Ranitidine (50 mg/kg)</td>
<td>acidified aspirin (200 mg/kg)</td>
</tr>
</tbody>
</table>

DMSO: Dimethylsulfoxide, ESAM: ethanolic seed extract of *Annona muricata*

**Role of Sulphhydryl Group (SH) and Nitric Oxide (NO) in the Gastro Protective Effect of Esam**

Having fasted for 24 hrs, twenty four female Wistar rats were distributed into four groups (n=6 for each group). The first group of rats was treated orally with 10% DMSO (10 ml/kg) and the other three groups treated with ethanolic seeds extract of *Annona muricata* (ESAM) (250 mg/kg, P.O.), the third and the fourth groups treated with specific inhibitors 30 minutes before ESAM administration to identify the proposed gastro protective mechanism of ESAM. The third group was intraperitoneally treated with N-ethylmaleimide (NEM, 10mg/kg), a sulphhydryl alkylating agent,[20] while the fourth group was orally treated with N-Nitro-L-arginine (L-NNA, 50 mg/kg), which is nitric oxide synthase inhibitor that has been dissolved in water (50 mg/ml).[21] One hour later, all the rats orally administered acidified aspirin (200 mg/kg) for gastric ulcer induction and their stomachs had been extracted 4 hours later to be evaluated histologically.

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**Gastric Mucosal Lesions**

After rinsing the rats' stomachs with saline to remove any blood clots and other gastric content residues, they were fixed to examine the gastric lesion index macroscopically under supervision of a professional pathologist, according to the following scoring: edema or hemorrhage, loss mucosal folding, mucosal discoloration (score: 1 each), ulcers less than 1 mm/cm² (score: number of ulcers ×2), ulcers more than 1 mm/cm² (score: number of ulcers ×3), perforated ulcers (score: number of ulcers ×4).[22] Ulcer index (UI) for each group taken as the mean lesion score of all the rats in that group.

\[
\text{UI} = \frac{\text{total ulcer score}}{\text{number of animals ulcerated}}
\]

The preventive index (PI) of *Annona muricata* seed extract calculated by the equation

\[
\text{PI} (%) = \left( \frac{\text{UI. of aspirin group} - \text{UI. of each pretreated group}}{\text{UI. of aspirin group}} \right) \times 100\%
\]

**Gastric Tissue Homogenate Preparation**

Gastric tissue homogenate preparation was done by using ice cold 0.01M phosphate buffered saline (PBS) as a lysis buffer, with a ratio of 1:10 (net weight of tissue : volume of PBS) using basic ULTRA-TURRAX tissue homogenizer in cold environments. Then, the supernatant of the tissue homogenate was separated using micropipette after centrifugation at 4,000 rpm for 15 min at 4°C and used to estimate superoxide dismutase (SOD), prostaglandin-E2 (PGE-2) and tumor necrosis factor alpha (TNF-α) levels, using commercial kits (mybiosource, USA) by special protocol according to manufacturer.

**Tissue Preparation for Histopathological Examination**

Stomach tissues were prepared for histopathological examination using paraffin sections method according to the technique prescribed by Junqueira et al.[23] Blocks were cut by microtome (KARL KOLB, Germany) into 5μm thickness for haematoxylin and eosin staining (H&E) and 4μm thickness for immunohistochemistry (IHC).

**Immunohistochemical staining (IHC)**

After processing of gastric tissue, 4 μm sections were fixed on positively charged slides and supplemented with heat shock protein (Hsp70) and proliferating cell nuclear antigen (PCNA) (mybiosource, USA), for IHC staining using ImmunoCruz® ABC Kit (Santa-Cruz biotechnology, USA) according to the manufacturer datasheet, then digital images of the slides were captured using Leica® DM4000B LED Microscope (Germany) with built-in capturing software LAS (v.4.5) with brown color representing positive IHC staining.

**Statistical Analysis**

Data were presented in simple measures of mean ± standard error of mean (M±SEM) or percentage. Analysis of data was performed using the analysis of variance test (ANOVA) according to the statistical package of SPSS (16.0). Whenever the P-value was less than 0.05, statistical significance was considered.

**Results and Discussion**

**Chemical Analysis of ESAM**

Preliminary phytochemical screening of the seeds extract of *Annona muricata* (ESAM) in this study showed the presence of coumarins, flavonoids, saponin, and tannins, while GC-MS analysis approved the presence of sesquiterpenes like alpha-copaene and longifolene (Figure 1), chemical screening was in agreement with the results obtained in other study by Vijayameena et al. who found that *Annona muricata* ethanolic extract contain carbohydrates, flavonoids, proteins, saponin, and tannins, while alkaloid presence was only proved by Mayer’s and Wagner's lests and not by Dragendorff's test[24].

Coumarins are promising compounds as a potential source of exogenous antioxidants,[25] coumarin isolated from *Mikania laevigata* were confirmed to have antisecretory action against acid hypersecretion induced by histamine, pentagastrin and bethanechol.[26] Flavonoids has a wide range of biological effects, including anti-ulcer activity.
Flavonoids can increase mucosal prostaglandin, [27] decrease histamine secretion from mast cells by histidine decarboxylase inhibition, [28] also it can inhibits formation of acid by parietal cells, [29] and has a free radical scavenging properties [25].

Flavonoids have an anti-inflammatory effect by inhibiting both cyclooxygenase and lipoxygenase activity diminishing inflammatory mediator formation.[30] Saponins a particular form of glycosides. Escin which is a mixture of saponins obtained from the seeds of Aesculus hippocastanum has been shown to have an anti-ulcer and gastro protective effect in different ulcer models due to inhibition of gastric acid and pepsinogen secretion [31]. When a low concentration of tannins is applied to the mucosa, the outermost layer becoming less permeable, these provide an additional protection to the subjacent layers against the harmful action of bacteria, chemical irritation, and against mechanical irritation [32].

Several plants contain flavonoids; saponin and tannins have been reported to have an anti-ulcer effect including the seed extract of Annona squamosa, from Annonaceae family.[33] Sesquiterpenes has been demonstrated to have several biological activities such as antibacterial, [34] and antioxidant effect, protecting mitochondrial enzymes activity against oxidative stress[35]. Alfa-copaene was found to have an antioxidant effect increasing total antioxidant capacity in cell cultures [36].

![Figure 1: GC-MS chromatogram of seeds extract of Annona muricata.](image)

**Acute Toxicity**

Several plants with different extracts have been found to be toxic or harmful in high or even moderate or low doses, in this study, no mortality in mice was recorded with three concentrations of the ethanolic seed extract of Annona muricata (0.5, 2 and 5g/kg). After 2 weeks of ESAM administration, the mice did not manifest significant changes in body weight, behavior, nor relative organ weights (Table 2), doses up to 2000mg/kg didn’t cause any organic tissue damage to the heart, kidney and liver this indicate that ESAM is quite safe in low and moderate doses, renal tissue was looking normal apart from mild vascular congestion and stromal hemorrhage at doses higher than 2000mg/kg (Figure 2). Hepatic microscopic evaluation showed vascular congestion, portal inflammation and inflammation inside the hepatic parenchyma in dose of 5000mg/kg (Figure 2), which is not compatible with a previous study on the safety of Annona muricata seed extracts (ethanol and n-hexane) that showed the seed extract could be considered safe within doses up to 5000mg/kg[16].
Table 2: percent of body weight change and relative organs weight in three groups of mice that administered different doses of Annona muricata seed extract

<table>
<thead>
<tr>
<th>Groups</th>
<th>n</th>
<th>%Body weight change</th>
<th>%Relative kidney weight</th>
<th>%Relative liver weight</th>
<th>%Relative heart weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>10%DMSO</td>
<td>6</td>
<td>+1.36±1.84</td>
<td>0.647±0.043</td>
<td>6.43±0.33</td>
<td>0.46±0.014</td>
</tr>
<tr>
<td>10ml/kg</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ESAM</td>
<td>6</td>
<td>+2.86±3.79</td>
<td>0.621±0.062</td>
<td>6.16±0.13</td>
<td>0.49±0.07</td>
</tr>
<tr>
<td>500mg/kg</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ESAM</td>
<td>6</td>
<td>+0.61±3.20</td>
<td>0.601±0.037</td>
<td>6.00±0.35</td>
<td>0.42±0.07</td>
</tr>
<tr>
<td>2000mg/kg</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ESAM</td>
<td>6</td>
<td>-4.61±3.87</td>
<td>0.638±0.009</td>
<td>5.99±0.14</td>
<td>0.50±0.11</td>
</tr>
<tr>
<td>5000mg/kg</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data are reported as means ± SEM

N: number of mice per group, DMSO: dimethylsulfoxide, ESAM: ethanolic seed extract of Annona muricata, SEM: standard error of them

Figure 2: Histopathology analysis of the heart, kidney and liver of the control and different doses of ESAM-treated groups

Control, B: ESAM at 500mg/kg, C: ESAM at 2000mg/kg, D: ESAM at 5000mg/kg. staining: haematoxylin & eosin.

Effect of Ethanolic Seeds Extract of Annona Muricata on Gastric Content Volume, Ulcer Index and Preventive Index

Gastric acidity is one of the various factors that are involved in the intensification of ulcers’ lesions; it is known as an aggressive factor, which can break down the gastric mucosal barrier and causes an increase in the calcium levels, leading to free radical generation [37]. In this study the gastric content volume significantly elevated in the acidified aspirin group in compare to the apparently normal control group. Low and high dose of ESAM showed significant reduction in the gastric content volume in compare to acidified aspirin group, the anti-secretory effect of ESAM suggested being due to the coumarins, flavonoids and saponin component.

This anti-secretory effect of ESAM minimizing gastric content volume is consist with Moghadamtousi et al. study who found that the ethanol extract of Annona muricata leaves also has an anti-secretory effect on ethanol induced ulcer rats, [38].
Macroscopic mucosal damage and hemorrhage was found to be significantly alleviated by pretreatment with ESAM at 250 and 500 mg/kg and ranitidine, ESAM in low and high dose has a decent antiulcer effect that is, in a dose-dependent manner, decreasing the ulcer and increasing the percentage of the preventive index (26.4% and 59.7%; respectively) against aspirin-induced gastric injury. Previous study have showed that methanolic leaves extract of *Annona muricata* significantly reduce the mean number of ulcers in aspirin-induced ulcer model.[39] This antiulcer and possible strengthening of gastro protective effect is multifactorial and may be attributed to antioxidant and anti-inflammatory elements in these extracts. Macroscopic mucosal evaluation between the groups is showed in Figure 3, gastric content volume; ulcer index and preventive index have been demonstrated in Table 3.

![Figure 3: Effect of ethanolic seed extract of *Annona muricata* on gross appearances of stomach in acidified aspirin ulcerated rats](image)

Table 3: Effect of ethanolic seeds extract of *Annona muricata* on gastric content volume, ulcer index, and preventive index in acidified aspirin ulcerated rats

<table>
<thead>
<tr>
<th>Groups</th>
<th>n</th>
<th>Gastric content volume (ml/100mg)</th>
<th>UI (score)</th>
<th>PI (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6</td>
<td>1.60 ± 0.07</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Acidified aspirin</td>
<td>6</td>
<td>2.10 ± 0.11*</td>
<td>12.0 ± 0.89*</td>
<td>0</td>
</tr>
<tr>
<td>Low dose ESAM</td>
<td>6</td>
<td>0.91 ± 0.03*</td>
<td>8.83 ± 1.10***</td>
<td>26.4</td>
</tr>
<tr>
<td>High dose ESAM</td>
<td>6</td>
<td>0.83 ± 0.03*</td>
<td>4.83 ± 0.54**</td>
<td>59.7</td>
</tr>
<tr>
<td>Ranitidine</td>
<td>6</td>
<td>1.02 ± 0.03*</td>
<td>6.33 ± 0.33**</td>
<td>47.2</td>
</tr>
</tbody>
</table>

Data are reported as means ± SEM or percentage.

* n: number of samples per group, UI: ulcer index, PI: preventive index, DMSO: dimethylsulfoxide, ESAM: ethanolic seed extract of *Annona muricata*, SEM: standard error of the mean.

significant difference when compared with control group.

* Significant difference when compared with acidified aspirin group.

# Significant difference when compared with high dose ESAM.

* *P*-value <0.05 considered significant difference.

**Effect of Ethanolic Seeds Extract of *Annona muricata* on the Gastric Levels of PGE2, SOD, and TNF-α**

Prostaglandins (PGs) have been implicated in modification of mucosal integrity and regulation of different functions, such as acid and bicarbonate secretion, mucus production and mucosal blood flow.[40] In this study, acidified aspirin ulcerated group showed a considerable decrease in the gastric prostaglandin E2 when compared to the apparently normal control group. High dose ESAM significantly increased gastric PGE2 when compared to acidified aspirin and ranitidine groups, this may reveal that ESAM at high dose can reduce ulcer incidence by prostaglandins compensatory level elevation. Pretreatment with low dose ESAM also led to an increment in PGE2 level, although it didn’t reach to the
statistical significance. These results agree with Moghadamtousi et al., who had demonstrated that *Annona muricata* ethanolic leaves extract augmented PGE2 level in the stomach tissue [38]. This finding can be explained by mucosal prostaglandin increasing property of flavonoids. Lipid peroxidation mediated by reactive oxygen species is known to be an important reason of destruction and damage to cell membranes, leading to changes in its fluidity and permeability, enhance the rates of protein degradation, causing ultimately to cell lysis [41].

Superoxide dismutase which catalyzes dismutation of O$_{2}^{-}$ into less harmful H$_{2}$O$_{2}$ is found to be significantly lowered in the acidified aspirin group when compared to normal control group. Pretreatment with ESAM in both doses (250 and 500 mg/kg) significantly increased gastric superoxide dismutase levels in compare to the acidified aspirin group this consist with Nordin N. et al. who found that the leaf and stem extracts of *Enicosanthellum pulchrum* (*Annonaceae* family) augment gastric SOD level, [42] this might be due to the antioxidant action of the extract, as free radicals can damage the cellular antioxidant enzymes such as catalase (CAT), SOD and others that acting as the first line of cellular defense against oxidative injury. The TNF-α, which is acting via TNF receptor-1 initiate inflammation and tissue destruction by recruiting leucocytes, especially neutrophils and monocytes, leading to their activation, thus activated neutrophils may induce injury through microvascular occlusion or through the following ischemia, leading to release of ROS and proteases [43].

In this study, gastric level of TNF-α was found to be markedly higher in the ulcer group than the normal group as prostaglandins inhibition by NSAIDs is responsible for the TNF-α rise. Pretreatment with high dose ESAM group resulted in reduced gastric level of TNF-α in compare to the acidified aspirin group, In previous study on mice, ethanolic leaves extract of *Annona muricata* was associated with an attenuation in the TNF-α protein expression, manifesting that the extract can be used against both acute and chronic inflammation, [44] this may be attributed to the anti-inflammatory activity of the flavonoids content of the extract. Prostaglandin E2, superoxide dismutase, and tumor necrosis factor-alpha in the studied groups have been demonstrated in Table 4.

| Table 4: Effect of ethanolic seeds extract of Annona muricata on the levels of tissue biomarkers |
|-----------------|--------|---------|---------|--------|
| Groups          | n      | PGE2 (pg/ml) | SOD (U/ml) | TNF-α (pg/ml) |
| Control         | 6      | 421.62±14.02 | 10.16±0.56 | 37.95±8.42 |
| Acidified aspirin | 6     | 344.99±16.96* | 3.76±0.48* | 114.06±11.17* |
| Low dose ESAM   | 6      | 376.47±10.76 | 9.04±0.58*a | 100.56±8.45* |
| High dose ESAM  | 6      | 413.73±12.26c | 13.33±0.66b | 74.76±4.05c* |
| Ranitidine      | 6      | 358.81±10.78*a | 7.58±0.67c | 43.57±8.75a* |

Data are reported as means ± SEM.


* significant difference when compared with control group.

*o significant difference when compared with acidified aspirin group.

*Δ significant difference when compared with low dose ESAM group.

*# significant difference when compared with high dose ESAM group.

p-value &lt;0.05 considered significant difference.

Effect of Ethanolic Seeds Extract of *Annona Muricata* on Histological Examination of the Gastric Tissue

Histopathological studies revealed that acidified aspirin induced mucosal ulceration that might be manifested as epithelial necrosis, hemorrhage, and leukocyte infiltration (Figure 4B and C). This effect on mucosal oxidative stress and histological perturbation was mitigated by high dose ESAM (Figure 4E) and ranitidine (Figure 4F). These findings go in harmony with other study that manifested *Annona muricata* leaves ethanolic extract had minimized gastric ulcer picture with reduced necrosis, hemorrhage and oedema against ethanol induced ulcer.[44] Ethanolic seed extract of *Annona muricata* had minimized the gastric mucosal lesions showing cytoprotective effects due to the antioxidant and anti-inflammatory action of its phytochemicals.
Role of Sulfhydryl Group (SH) and Nitric Oxide (NO) in the Gastro Protective Effect of ESAM

Non protein-SH compounds can bind to the free radicals, minimizing lipid peroxidation, and are also involved in the production and nature of mucus formed and in antioxidants recycling.[45] Nitric oxide found to possess anti-apoptotic, anti-necrotic properties, promoting angiogenesis and increase PGE2, also NO has been shown to inhibit gastric acid secretion.[46] In the current study, the SH-alkylating group (Figure 5C) and NOS-inhibitor one (Figure 5D) cancelled the gastro protective effects of ESAM against acidified aspirin-induced ulcer and this clarified by the histological changes of gastric mucosa (necrosis, edema, sloughing of mucosa and hemorrhage). These agree with another study that showed the strong involvement of endogenous SH group in the gastro protection effects of Annona muricata hydro alcoholic extract,[47] while disagree with another previous study showed that pretreatment with NO-synthase inhibitor did not alter the ethanolic leaf extract of Annona muricata cytoprotection in induced gastric lesions, excluding the role of endogenous NO in mediating cytoprotective effect [44].
Effect of Ethanolic Seeds Extract of Annona Muricata on HSP70 and PCNA Immunohistochemical Staining

Heat shock protein 70 (HSP70) is constitutively expressed in gastric mucosal cells, this protein plays a defensive role by stimulation of the apoptotic pathway through mitochondria, playing a principle role in protecting mucosal cells against NSAID-induced damage,[48] the HSP70 positive cells were found at the superficial two thirds of the gastric pits and also scattered among basal parts. Most control and acidified aspirin treated cases stained negative for HSP70, although control group showed a higher level of Hsp70 expression, compared to the acidified aspirin group, it was statistically non-significant.

Pretreatment with both doses of ESAM has resulted in upregulation of HSP70, this is compatible with Moghadamtousi et al. who found that Ethanolic leaves extract of Annona muricata can cause HSP70 upregulation in the gastric tissue.[38] HSP70 immunohistochemical staining is demonstrated in Figure 6. Proliferating cell nuclear antigen (PCNA) is a marker of proliferation activity that is used to evaluate cell proliferation and DNA repair.[49] Non-steroidal anti-inflammatory drugs is found to inhibit cell growth, suggested that inhibition of COX activity and the resulting inhibition of prostaglandin synthesis may play a role in NSAID-induced inhibition of epithelial cell proliferation.[50]

Proliferating cell nuclear antigen (PCNA) presence was limited to opposing cell groups at the necks and middles of the gastric pits, Basal PCNA-positive cells were only seen in the acidified aspirin-treated group.

Most PCNA positive cells appeared metabolically active with well-defined nuclei and nucleoli and multiple cytoplasmic granules, the immunohistochemical analysis showed that there was no increase in the PCNA staining in the gastric mucosa of rats pretreated with ESAM for 7 days, indicating that ESAM can't induce proliferative activity in the gastric cells, while Moghadamtousi demonstrated that Annona muricata leaf crude ethyl acetate extract downregulate the expression of PCNA protein in treated cell lines.[51] PCNA staining among the different groups is represented in Figure 7.

![Figure 6: Microphotograph of immunohistochemical (IHC) staining of HSP70 in rat gastric tissue](image)

A: control, B: acidified aspirin group, C: low dose of ESAM, D: high dose of ESAM, and E: Ranitidine group.

Magnification: 400X.

![Figure 7: Microphotograph of immunohistochemical (IHC) staining of PCNA in rat gastric tissue](image)

A: control, B: acidified aspirin group, C: low dose of ESAM, D: high dose of ESAM, and E: Ranitidine group.

Magnification: 400X.
Conclusion
The seeds extract of Annona muricata is relatively safe. Its gastro protective activity attributed to its antisecretory; cytoprotective; antioxidant and anti-inflammatory effects.

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References


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