Serotonin (5-hydroxytryptamine; 5-HT) is an important neurotransmitter in the enteric plexus of the gut and an autocrine hormone when released from enterochromaffin (EC) cells in the gastrointestinal mucosa, pancreatic cells, and elsewhere. 5-HT released from EC cells by stimulations can be detected in intestinal lumen as well as systemic circulation. Apart from its motor and sensory functions in the gastrointestinal tract, 5-HT inhibits gastric acidity by increasing the gastric mucus secretion. It has been reported that 5-HT stimulates prostaglandin (PG) synthesis by enhanced activity of the cyclooxygenase pathway, which in turn stimulates mucosal blood flow and helps in the secretion of mucus along with bicarbonate. Mucus is regarded as a major component of the gastric mucosal barrier. Adherent mucus gel over the mucosal surface traps the bicarbonate secreted by the epithelium, thus neutralizing the luminal acid that diffuses toward the epithelium. This mucus also provides a diffusion barrier for certain low-molecular weight solutes and a physical barrier for microorganisms and their toxins. Moreover, mucus is capable of acting as an antioxidant, and so can reduce mucosal damage mediated by oxygen free radicals. The protective properties of the mucus barrier depend not only on the gel structure but also on the amount or thickness of the layer covering the mucosal surface. Furthermore, the thickness of the adherent mucus gel represents a dynamic balance between the rate of mucus secretion by the mucosal cells and the rate of mucus erosion by enzymatic degradation and mechanical shear forces.

Aegle marmelos (L) Corr. (Rutaceae) is a spiny tree sparsely distributed throughout India. Various parts of Bael tree (stem, bark, root, leaves, and fruits) have medicinal values and have a long tradition as herbal medicine. Pharmacological studies have shown that both fruits and roots have antiamebic and hypoglycemic activities as well as useful in treating diarrhea, dysentery, and stomachalgia. The alkaloid aegeline present in the leaf is a potent antiasthmatic agent. The antihistaminic effect of alcoholic extract of the leaves support the traditional use of Aegle marmelos (AM) in asthmatic complaints. Various

Original Article

Gastric Mucosal Protection by Aegle Marmelos Against Gastric Mucosal Damage: Role of Enterochromaffin Cell and Serotonin

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ABSTRACT

Background/Aims: Serotonin (5-hydroxytryptamine; 5-HT) released from enterochromaffin (EC) cells in gastric mucosa inhibits gastric acidity by increasing the gastric mucus secretion. In the present study, we evaluated the effect of aqueous extract of Aegle marmelos (AM) ripe fruit pulp (250 mg/kg body weight) on mean ulcer index (MUI), EC cells, 5-HT content, and adherent mucosal thickness of ulcerated gastric tissue in adult albino rats. Material and Methods: Ulceration was induced by using aspirin (500 mg/kg, p.o.), cerebellar nodular lesion and applying cold-restraint stress. Results: In all cases increased MUI in gastric tissue along with decreased EC cell count was observed with concomitant decrease of 5-HT content and adherent mucosal thickness (P < 0.05). Pretreatment with AM for 14 days decreased MUI, increased EC cell count, and 5-HT content as well as adherent mucosal thickness in all ulcerated group (P < 0.05).

Conclusion: AM produces gastric mucosal protection mediated by increased EC cell count and 5-HT levels.

Key Words: Aegle marmelos, adherent gastric mucosa, enterochromaffin cells, serotonin, ulceration

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chemical constituents, including alkaloids, coumarins, and steroids have been isolated and identified from different parts of bael fruit tree.\textsuperscript{[26-28]}

The above literature suggests the involvement of neuronal mechanism in the gastroprotective activities of AM. Thus, this study aimed to investigate the involvement of EC cell and 5-HT in the antiulcer efficacy of AM against different models of experimental gastric ulceration. The study also aimed to quantitatively evaluate, through the microscopic examination of gastric mucosal tissue, the damage induced by various physical and chemical ulcerogenic agents in rats with and without pretreatment with AM. The objectives of study were extended to investigate the influence of AM on mucus-producing cells and on mucus production in stomachs both exposed and not exposed to the damaging effect of experimental ulcerogenic agents.

**MATERIAL AND METHODS**

**Preparation of AM ripe fruit extract**
The ripe fruit of AM was purchased from the local market. The pulp was taken out from ripe fruit of AM after identification and authentication by the Botanical Survey of India, Howrah (No. CNH/I-1239/2008/Tech.II/278). It was then strained through wire-mesh, sun dried, and powdered in an electric grinder. The crushed powder that was obtained was soaked in double distilled water for 24 h. The extract obtained was filtered through Whatman filter paper and vacuum dried at 40-50°C to get a dry powder and it was stored at −4°C for further use.\textsuperscript{[29]}

**Drugs and chemicals**
Aspirin (SRL Ltd, Mumbai, Maharashtra, India) was used. Silver nitrate, neutral red, Xylol, and all other chemicals were obtained from Merck Ltd, Mumbai, Maharashtra, India.

**Animal use and maintenance**
Holtzman strain adult albino rats (of either gender) weighing 175-210 g were used throughout the experiment. They were maintained under standard laboratory conditions (22-28°C, 60-70% relative humidity, 12:12 h light/dark cycle) with standard pellet diet (M/s. Hindustan Lever Ltd., India) and water ad libitum. Food intake (g/day/rat) and body weights of the rats were recorded everyday and maintained throughout the experimental period. The experiments were carried out as per the regulation of the Institutional Animal Ethical Committee.

**Experimental design and animal grouping**
The experiments were done in three different schedules for each of the three different models used for ulceration. Each of the three schedules comprised of 24 rats. The rats were divided into four different groups having six animals in each group.

- **Group 1:** Control group: Rats were treated with double distilled water (2 ml/kg body weight), once daily for 14 consecutive days orally by using an orogastric cannula between 10:30 am and 11:30 am
- **Group 2:** AM group: Rats were treated with aqueous extract of ripe fruit pulp of AM at a dose of 250 mg/kg (standardized in laboratory), once daily for 14 consecutive days orally by using an orogastric cannula between 10:30 am and 11:30 am
- **Group 3:** Ulcer group: Pretreated with double distilled water (2 ml/kg body weight), once daily for 14 consecutive days orally by using an orogastric cannula before producing ulcer
  - (a) Aspirin-induced ulcerated rats (Acute model)
  - (b) Cold-restraint stress–induced ulcerated rats (Chronic model)
  - (c) Cerebellar lesion–induced ulcerated rats (Neurogenic model)
- **Group 4:** AM + Ulcer group: Pretreated with AM ripe fruit extracts
  - (a) Aspirin-induced ulcerated rats pretreated with AM ripe fruit extracts
  - (b) Cold-restraint stress–induced ulcerated rats pretreated with AM ripe fruit extracts
  - (c) Cerebellar lesion–induced ulcerated rats pretreated with AM ripe fruit extracts

**Preparation of ulcerated animal models**

- **Aspirin-induced ulcer model**
  After the experimental period, animals were fasted for 24 h and were given a single dose of aspirin (500 mg/kg body weight) dissolved in distilled water orally by an orogastric cannula.\textsuperscript{[30]} The animals were sacrificed after 4 h.

- **Cold-restraint stress–induced ulcer model**
  Animals were subjected to cold-restraint stress by placing them in perplex glass restraining cages at 4°C for 3 h daily for seven consecutive days.\textsuperscript{[31,32]}

- **Cerebellar lesion–induced ulcer model**
  Prior to surgery all the animals were fasted overnight but had free access to water. For induction of ulceration by stereotaxic surgery, rats were injected with sodium pentobarbitone (40 mg/kg, i.p., Abott India Ltd, Verna Salcette, Goa, India), until a level of anesthesia was achieved where the fore-paw withdrawal reflex was abolished.

- **Electrode implantation and lesion**
  Each rat was placed on a David Kopf Stereotaxic instrument equipped with ear bar that prevents damage to the tympanic membrane. The incisor bar was kept 3.3 mm down to bring the skull surface in horizontal position. Head was fixed to keep the
bregma and the lambda sutures in the same horizontal plane. Strict aseptic conditions were maintained during surgery. Care was taken to prevent any damage to the tympanic membrane. Surgery was performed by a midline incision on the back of the head. The scalp was incised posteriorly in the midline, and the adjacent pericranial muscles were retracted laterally. According to the stereotaxic coordinates of nodulo-uvular areas of posterior cerebellar-vermis\textsuperscript{[15,14]} (AP = 12.8 mm posterior to bregma, L = 0.4 mm, D = 6.8 mm ventral to the dura), a burr hole of 1-2 mm was made on the posterior aspect of the skull so that the electrode could penetrate the required area of the cerebellum. Bleeding if any was controlled by aseptic bone wax. A 0.2 mA DC current for 40 s was passed through conventional bipolar electrode (insulated by epoxylite with 0.5 mm tip exposed) to produce lesion. Following recovery from surgery, all the animals were carefully maintained with all the necessary precautions and aseptic measures. Routine antibiotic injection (i.m.) of Benzyl Penicillin (10,000 IU) was given in all the animals for three consecutive days after surgery.\textsuperscript{[35]}

**Postoperative care**
Following surgery, all the animals were carefully maintained with all the necessary precautions and aseptic measures to recover from surgical stress. Particular care was taken for feeding until the animals recovered from surgical stress. For the first two days, animals were given intraperitoneal injection of dextrose-saline until the animals became capable of taking either liquid milk or standard diet.

**Ulcer scoring**
After termination of the experiment, the animals were sacrificed by an overdose of sodium pentothal following overnight fasting. The stomach was cut opened along the greater curvature, and ulcer was indexed accordingly.\textsuperscript{[36]} After the collection of the tissues, those were rinsed in normal saline and spread over paraffin tray and numbers of bleeding spots were examined using a magnifying glass. The length of bleeding spots (in mm) was also measured using a scale and scoring of ulcer index was done by the following grade for a single animal.

- 0 = normal, i.e. no pathology
- 1 = small ulcer (bleeding spot with 1-2 mm in length)
- 2 = medium ulcer (bleeding spot with 3-4 mm in length)
- 4 = large ulcer (bleeding spot with 5-6 mm in length)
- 8 = large ulcer (bleeding spot with > 6 mm in length).

The sum of the total severity scores in each group of rats divided by the number of rats was expressed as the mean ulcer index (MUI).

**Determination of percentage protection (P%) by AM**
Percentage protection (P%) by aqueous extract of ripe fruit pulp of AM against peptic ulceration was determined\textsuperscript{[37]} using the following formula: 

$$P = \left( \frac{MUI_{\text{ulcer}} - MUI_{\text{extract}}}{MUI_{\text{ulcer}}} \right) \times 100.$$  

**Spectrofluorometric estimation of serotonin**
Stomach tissues were dissected out, weighed, and washed in ice cold saline and homogenized in 10 mL acidified butanol. Homogenate (4 mL) was mixed with 10 mL of 10% heptane and 5 mL 0.003 (N) HCl and then shaken for 5 min and centrifuged at 2000 rpm for 10 min. Acid layer (4.5 mL) was eluted and mixed with 200 mg alumina and 1 mL of 2 (M) sodium acetate. The mixture was shaken for 5 min and centrifuged at 2000 rpm for 10 min. Supernatant was taken for estimation of 5-HT. Supernatant was mixed with 3 volumes of 10% isobutanol and shaken twice with equal volume of salt saturated buffer at pH 10. Then 2 volumes of 10% heptane was added to be butanol phase and 5 mL of 0.1 (N) HCl was added and shaken well and then the 1 mL of 0.3 (N) HCl was added finally to the mixture. This was taken for estimation of 5-HT. The fluorescence of 5-HT was measured in the Perkin Elmer MPF 44B Fluorescence spectrophotometer with activation and emission wavelength set at 295 and 550 nm.\textsuperscript{[12,36-40]}

**Histomorphological studies**
To observe significant structural and functional correlations, morphological data were required, which were obtained by histological studies. After termination of the experiment, all animals were sacrificed by cervical dislocation and immediately stomach of each animal was dissected out, washed with normal saline, and stored in 4% formalin solution for 24 h. The tissues were then prepared for paraffin sections, which were cut at 4-5 μm. Paraffin sections of stomach were examined using the following stains to assess histomorphological changes due to ulceration.

**Staining of enterochromaffin cells**
Tissues were stained by silver staining method to study enterochromaffin cell.\textsuperscript{[41,42]} According to this method, stomach tissues were collected, rinsed in 0.9% saline, and fixed in Bouin’s fluid. After dehydration and embedding, all tissues were sectioned at 4-5 μm thickness and stained to study EC cells. For staining of EC cells, 10% aqueous silver nitrate solution was prepared, and concentrated ammonia solution was added to it drop by drop until it formed a clear solution. To this solution, 10% aqueous silver nitrate solution was added drop-by-drop carefully until a faint opalescence was seen. It was kept at 60°C in an incubator. Rehydrated sections were placed in that preheated silver solution for 30 min until light brown color was seen. The sections were removed, washed well in distilled water, and immersed in 1% aqueous sodium thiosulphate for 1 min. Sections were then washed well in distilled water, counterstained using 0.5% aqueous neutral red, dehydrated, and mounted.
**Enumeration of EC cell count**

EC cell density was determined by counting the total number of EC cells in each section with visible nuclei in the sections (objective × 10, magnification × 200). The values thus obtained were referred to as cell density.

**Periodic acid – Schiff–Alcian blue staining of adherent mucus**

Tissues were stained using periodic acid – Schiff (PAS)–Alcian blue to study the mucous epithelium. (43) According to this method, rehydrated sections were treated with 3% acetic acid solution for 5 min and washed with water. Then it was treated with 1% alcian blue solution for 10 min followed by thorough washing with tap and distilled water. The sections were then treated with 1% periodic acid solution for approximately 5 min and rinsed with distilled water. Then Schiff’s reagent was applied on those sections for another 5 min and washed in running tap water for 5-10 min. The nuclei were then stained with hematoxyline and washed thoroughly in distilled water. The sections were then dehydrated, cleared using xylol, and mounted for microscopical observation.

**Measurement of mucus thickness**

Each section was positioned transversely on a microscopic slide and observed under the microscope using light illumination. The adherent mucus layer appears as a lightly pink-stained layer between the dense mucosa and the clear bathing solution. The thickness of the mucus layer is measured using an eyepiece graticule. Individual mucus thickness values were measured on at least six sections per mucosal specimen. Sections were examined with objective × 10 (visual field diameter, 2.5 mm) and eyepiece × 10 (with scale bar inserted). Measurements were taken at 200 mm intervals all along the section, and a minimum of 10 readings were made on each section. Measurements were not made when there was evidence of distortion of the mucosa or of mucosal lesions.

**Statistical analysis**

All the data were expressed as mean ± standard error of mean. The one-way analysis of variance followed by multiple comparison t test was used for statistical analysis. Difference below the probability level 0.05 was considered statistically significant.

**RESULTS**

There was no mortality during the experimental period, and food intake was similar in all the groups. The following results were obtained.

**Effect of AM on ulcer index**

Oral administration of aqueous extract of ripe fruit pulp of AM at a dose of 250 mg/kg body weight daily, for 14 consecutive days protected the gastric mucosa from ulcer induced by aspirin [nonsteroidal anti-inflammatory drugs (NSAIDs)], cerebellar lesion and exposure of rats to cold-restraint stress [Table 1]. Administration of aspirin, electrolytic lesion of cerebellar-nodular area and exposure of rats to cold-restraint stress produced significant ulceration in stomach of rats of untreated ulcerated group as evidenced by increased MUI [Table 1]. The severity of ulceration was however reduced in the gastric tissues on pretreatment with aqueous extract of ripe fruit pulp of AM at a dose of 250 mg/kg body weight daily, for 14 days, and the inhibition of ulceration was about 93.09% in aspirin-induced ulcer model, 87.06% in cerebellar-nodular lesion-induced ulcer model, and 66.39% in cold-restraint stress-induced ulcer. No significant gastric mucosal lesion was observed in control and AM group of rats.

**Effect of AM on serotonin content (ng/g tissue) and EC cell count (cells/mm²) in stomach in different ulcer models**

The 5-HT content and EC cell count of stomach are decreased significantly (P < 0.001) in different ulcer models but pretreatment with AM markedly prevented this decrease (P < 0.001), thus rendering neuroprotection to the gastrointestinal tract [Table 2a-2c]. The decrease in 5-HT content was 44% by aspirin, 47% by cerebellar-nodular lesion, and 48% by cold-restraint stress. The decrease in EC cell count was 34% by aspirin, 48% by cerebellar-nodular lesion, and 47% by cold-restraint stress.

**Effect of AM on gastric adherent (gel) mucus**

Figure 1a and 1b shows the gastric mucosa of control and AM only treated rat, respectively, as observed under the stereomicroscope with bright illumination. In all preparations, the surface adherent gel layer was optically distinct from the dense mucosa. The mucus gel layer is

| Parameters | Mean ulcer index | % P |
|------------|-----------------|-----|
| Group 1 (control) | No lesion |    |
| Group 2 (AM) | No lesion |    |
| Group 3a (ASP) | 38.67±2.74* | -   |
| Group 4a (AM+ASP) | 2.67±0.67** | 93.09 |
| Group 3b (CNL) | 37.33±2.36* | -   |
| Group 4b (AM+CNL) | 4.83±1.08** | 87.06 |
| Group 3c (CRS) | 21.33±1.56* | -   |
| Group 4c (AM+CRS) | 7.17±0.70** | 66.39 |

Values are mean ± standard error of mean from six animals in each group;

*P<0.001 as compared with control group, **P<0.001 as compared with ASP group;

Table 1: Effect of Aegle marmelos (AM) on mean ulcer index and percentage of protection (% P) in stomach

| Parameters | Mean ulcer index | % P |
|------------|-----------------|-----|
| Group 1 (control) | No lesion |    |
| Group 2 (AM) | No lesion |    |
| Group 3a (ASP) | 38.67±2.74* | -   |
| Group 4a (AM+ASP) | 2.67±0.67** | 93.09 |
| Group 3b (CNL) | 37.33±2.36* | -   |
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| Group 4c (AM+CRS) | 7.17±0.70** | 66.39 |
The optical comparison among samples of stomach from control ulcerated and AM pretreated ulcerated rats showed a decrease in the thickness of the adherent mucus gel layer in the experimental ulcerated groups and prevention of the ulcerated condition in AM-pretreated rats.

Table 3 shows the quantitative results obtained after measuring the adherent mucus layer overlying the surface epithelial layer, as described in Materials and Methods section. The mean mucus gel layer thickness was expressed in microns. The mean adherent mucus gel layer thickness in stomach obtained from the AM group is significantly increased ($P < 0.05$) compared with the corresponding value from the control group. The mean adherent mucus gel layer thickness in ulcerated stomach obtained from ASP, CNL, and CRS groups are significantly decreased ($P < 0.001$) compared with the corresponding value from the control group. The percentage increase in the adherent mucus layer thickness after AM treatment is higher in cold-restraint stress (62%) and aspirin-induced ulcer (50%) than in cerebellar-lesion–induced ulcer (37%).

**DISCUSSION**

It is evident from our results that the severity of ulceration as measured by MUI was increased, along with the decrease in the amount of 5-HT, EC cell density, as well as gastric mucosal thickness in ulcerated conditions produced by different experimental agents used in the study. Administration of AM orally to the rats protected the stomach from these changes, mainly by increasing the mucus secretion, which remains adhered as continuous sheath to the epithelial lining of gastrointestinal tract.[32]

The present findings provide evidence that AM by itself causes an increase in the thickness of the adherent mucus gel layer in the stomach, thus strongly confirming our previous findings, in which we reported through conventional histological method (hematoxylin and eosin staining) and quantitative examination that the cytoprotective action of AM is related to the ability of the herb to stimulate the epithelial lining of gastrointestinal tract. The adherence of mucus gel layer was easily recognized as an optically distinct, light-pink–stained material covering the surface epithelium. The stomach in all three ulcerated conditions produced similar secretory response to the herbal extract as evidenced by similar increase from the mucosal surface. A variety of surface epithelial changes in shape, size, and orientation, accompanied by marked loss of surface mucus epithelial cell were found. Marked disorganization and atrophy of glands were invariably noted. This seems to be prevented by AM pretreatment, which maintains the adherent mucus lining resisting the erosion of glandular cells as seen in Figure 1h.

The present findings also provide evidence that AM by itself causes an increase in the thickness of the adherent mucus gel layer, thus strongly confirming our previous findings, in which we reported through conventional histological method (hematoxylin and eosin staining) and quantitative examination that the cytoprotective action of AM is related to the ability of the herb to stimulate the epithelial lining of gastrointestinal tract. The present findings also provide evidence that AM by itself causes an increase in the thickness of the adherent mucus gel layer in the stomach.
in mucus thickness after AM treatment. The presence of a number of coumarins, alkaloids, lignan glucosides, triterpenoids, sterols, carbohydrates, anthraquinones, and lactones\cite{45} in AM may account for its antiulcer activity.

It can be speculated that the antiulcer activity of AM is due to increased production of PG\cite{12,46} possibly by altering the activities of serotonin and/or muscarinic receptors in the gastroduodenal mucosa.\cite{47} However, we have not measured the levels of PG or the receptor activities in this study. Aspirin and related NSAIDs inhibit mucus secretion by preventing the synthesis of PG.\cite{2,8} Experiments on electrical lesion of cerebellum suggested a decrease in gastric mucus release possibly mediated by inhibition of vagal or splanchnic nerve.\cite{2,29,48} Stress also produces severe gastric erosion through the activation of central vagal discharge.\cite{49-51} Available literature suggests that in cold-restraint stress, where the vagal overactivity was prolonged, both petechiae and severe hemorrhagic ulcers were found.\cite{52,53} As cold stress causes the damage of the mucosa by decreasing the blood flow, involvement of PG is also responsible for the mechanism.\cite{54} The biochemical quantification of serotonin content and measurement of adherent mucus thickness in gastric tissues suggests that this enormous mucus production after treatment with AM is mediated through the activation of serotonergic cells. Serotonergic terminals located in the gastroduodenal mucosa liberate 5-HT from EC cells (through paracrine action) under response to acetylcholine, noradrenergic

Figure 1: Representative histomorphological picture depicting the changes in the gastric mucosal layer in ulcerated condition induced by aspirin (ASP), cerebellar lesion (CNL), and cold-restrain stress (CRS), and the protection rendered by Aegle marmelos (AM) pretreatment (arrows show the damage to the mucosal lining). (a) presents the control group, (b) represents the only Aegle marmelos–treated group (AM), (c) represents the aspirin-induced ulcerated group (ASP), (d) represents the Aegle marmelos–pretreated aspirin-induced ulcerated group (AM+ASP), (e) represents the cerebellar-lesion–induced ulcerated group (CNL), (f) represents the Aegle marmelos–pretreated cerebellar-lesion–induced ulcerated group (AM+CNL), (g) represents the cold-restraint stress–induced ulcerated group (CRS), and (h) represents the Aegle marmelos–pretreated cold-restraint stress–induced ulcerated group (AM+CRS)
Table 3: Effect of Aegle marmelos (AM) on adherent mucosal thickness in stomach

| Parameters            | Adherent mucosal thickness (µm) |
|-----------------------|---------------------------------|
| Group 1 (control)     | 81.67±1.97                      |
| Group 2 (AM)          | 88.17±2.09*                     |
| Group 3a (ASP)        | 50.5±1.77**                     |
| Group 4a (AM+ASP)     | 77.5±2.53*                      |
| Group 3b (CNL)        | 55.83±2.93**                    |
| Group 4b (AM+CNL)     | 75±1.95##                       |
| Group 3c (CRS)        | 47.83±2.17**                    |
| Group 4c (AM+CRS)     | 75.5±2.09##                     |

Values are mean ± standard error of mean from six animals in each group; *P<0.005, **P<0.001 as compared with control group, ***P<0.001 as compared with ASP group, ****P<0.001 as compared with CNL group.

stimulation, and vagal influence. Guha and Ghosh have also shown that 5-HT itself inhibits hyperacidity in gastric mucosa by increasing the synthesis of mucus. This observation reinforces the conclusion that protection of ulceration by AM is due to 5-HT and EC cell activity as EC cell count is also found to be enhanced in AM treated rats. The presence of 5-HT in the gastroduodenal tract has already been demonstrated immunohistochemically in EC cells. Further studies are obviously required to assess the significance of this hypothesis.

We conclude that AM induces an increase in the thickness of the adherent mucus gel layer. This fact confirms that mucus secretion is one of the main mechanisms involved in the cytoprotective action of AM. We also consider it important to emphasize that the present study efficiently serves to evaluate the effects of AM on mucus secretion. The aqueous extract of ripe fruit pulp of AM used in the present study is purely herbal in origin and is thought to possess significant gastroduodenal cytoprotective activity through the release of 5-HT from EC cells.

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