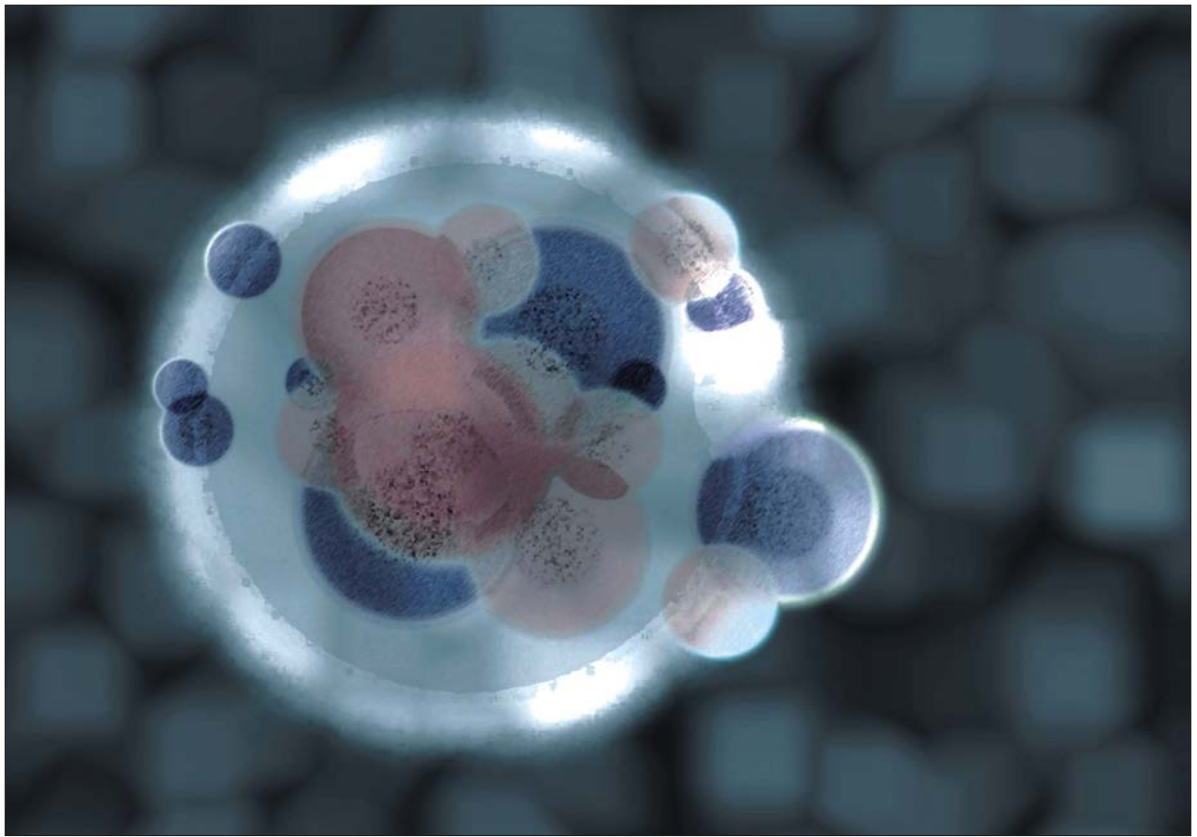




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Preliminary Evaluation of *Anethum graveolens* Fruit in Indomethacin-ulcer Induced Rats

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Abstract: *Anethum graveolens*, fruit has been used in the oriental medicine for the treatment of a variety of diseases including gastrointestinal disorders. The fruit powder of *Anethum graveolens* (AG) and its extracts were investigated for anti-secretory effects in rats with erosions on the glandular stomach induced by subcutaneous administration of 25 mg kg⁻¹ indomethacin, to rationalize the folkloric uses. The pretreatment of AG protected the rats against gastric effects induced by indomethacin in a dose-dependent manner. The aqueous (AG-A) and ethanolic (AG-E) extracts (equivalent to 2.0 g kg⁻¹) of the powder also attenuated the indomethacin-induced changes in gastric juice volume, pH, acid-output and ulcer index. AG and its extracts also showed significant acid buffering activities *in vitro*. The *in vitro* pepsin binding activity of AG was found significant while insignificant with AG-A and AG-E. These findings indicate that AG and its extracts protect the gastric mucosa against indomethacin-induced gastric changes, may result, from their antioxidant effect, which inhibits lipid peroxidation.

Key words: *Anethum graveolens*, anti-ulcer, ulcer index, anti-secretary, indomethacin

INTRODUCTION

Anethum graveolens, Linn. (Syn: Dill, Soya) [Umbelliferae] has long been used in the oriental system of medicines for the treatment of gastrointestinal (GIT) disorders^[1,2]. The plant is native to Europe/Mediterranean region and is also found in Pakistan, India, Sudan, U.S.A., Bahamas, Belgium, Dominican Republic, Great Britain, Iraq, Nepal, Peru, Spain, Turkey, Venezuela and West Indies^[3]. *A. graveolens* fruit (AG) has been allegedly used to treat flatulence, cough, dropsy, jaundice, sclerosis, scurvy, sores, tumours, stomach pain, dyspepsia and as gastro-intestinal antiseptic and anti-hyperlipidaemic^[4-6]. It has also been prescribed in vomiting and as digestive, diuretic, laxative, narcotic, resolvent, sedative, stimulant and stomachic^[7-9]. Coumarins have been reported to be present in leaf, fruit and aerial parts, aesculetin in fruit and aesculetin 4-methyl in fruit essential oil^[10,11]. Anethofuran (monoterpene), dill furan, apigenin and apigenin-7 O-β-D-glucuronide have been found in the essential oil^[12,13]. The presence of sterols in stem, tannins, apigenin in leaf, flavonoids in leaf and aerial parts, alkaloids in fruits and seeds have also been reported^[14-16].

MATERIALS AND METHODS

Plant drug and extracts: AG was purchased locally from herbal dealer in Bahawalpur, Pakistan. The plant material

was authenticated and compared with its standard in the herbarium maintained by Department of Botany, University of Agriculture, Faisalabad and a sample was preserved in the Pharmacognosy laboratory, Department of Pharmacy, Islamia University Bahawalpur. The shade-dried plant material was finely powdered to the mesh size 200 and stored in well-closed cellophane bags at 4°C^[17,18]. The aqueous and ethanolic extracts were prepared by maceration; 1.00 kg of powdered plant was soaked in about 2.00 l of distilled water and absolute ethanol separately for 24 h. The extracts were decanted; remaining materials were re-soaked in the respective solvents twice. The collective extracts were dried completely by Rotavapour at 37°C. All the test substances were suspended in aqueous 2.5% gum tragacanth solution or dissolved in the normal saline solutions before their administration^[19,20].

Chemicals: All chemicals used were of analytical grade and obtained from E. Merck (Darmstadt, FRG), BDH Poole (England) and Sigma Chemical Co. (USA).

Test animals: Adult healthy Sprague-Dawley albino male rats weighing 150-250 g each were used in this study. The animals were housed the standard conditions of temperature (23±12°C), humidity (55±15%) and 12 h light (7.00-19.00)^[2]. The rats were provided with a free access to a standard feed provided by M/S Lever Brothers, Rahim

Yar Khan-Pakistan and water *ad libitum*. The animals were fasted for 24 h prior to their use in the experiments^[22,23]. The use of animals was in accord to the guidelines of National Policy.

Acid buffering capacity: 500 mg AG and AG-A, AG-E (Equivalent to 500 mg of powder) dissolved in 2.0 ml of distilled water each treated with 10 ml of HCl solution, pH 1.0 separately. 2.0 ml of distilled water was added in the controlled test tubes in addition to above 10 ml of HCl solution. The final pH values were recorded after 1 and 30 minutes^[21].

Pepsin binding capacity: The AG powder 500 mg and its equivalent AG-A and AG-E extracts were added into separate test tubes to 1 ml of pepsin solution, 2 mg ml⁻¹ each. 4 ml of 0.2 N HCl buffered with 1 ml of 0.2 N sodium citrate solution to ensure the pH 1.6 was added to the tubes. The excess pepsin was treated with protein by adding 1ml bovine serum albumin (5 mg ml⁻¹) except controlled test tubes preincubation at 37°C for 30 min. The well shaken contents were further incubated at 37°C for 30 minutes. 1.0 ml of biuret reagent was added to each test tube and read their absorbance at 546 nm following the alkalization by adding 5 ml 0.2 N NaOH. The values obtained were expressed as %age binding of pepsin^[21,22].

Induction of gastric ulcer: The experimental gastric ulcer in rats was produced by the modified method of Yoshikawa *et al.*^[20]. The pylorus of 24 h fasted rats were ligated under light ether anaesthesia. The test substances were given orally, immediately following pylorus-ligation and indomethacin was injected *s.c.* in the treated groups of rats. All the test drugs were given to the animals by oral route t.d.s for 14 consecutive days while pylorus was ligated on the 15th day^[20,24].

The animals were divided randomly into seven groups, six rats each. The normal (untreated) and treated control groups received 3 ml kg⁻¹ of 2.5% tragacanth vehicle orally. Rats of different treated groups received AG powder 1.0, 1.5 and 2.0 g kg⁻¹ while AG-A and AG-E (equivalent to 2.0 g kg⁻¹ of powder) orally. The treated control and treated animals were administered indomethacin 25 mg kg⁻¹.

The operative procedure adopted was that of Shay *et al.*^[21] as modified by Takeuchi *et al.*^[25]. The rats were anesthetized with ether and the abdomens were opened through a midline incision. The pylorus were secured and ligated with silk suture, after which the abdominal wound were closed and the animals were allowed to recover from anesthesia. After ligation of pylorus, drinking water was withheld and gastric juices were allowed to collect for a

period of 4 h. The rats were then killed by an overdose of chloroform vapors and the stomachs were removed after clamping the oesophagus. The gastric contents were collected through the oesophagus. The gastric mucosae were washed with 3 ml of lukewarm distilled water. The gastric contents and washings were homogenized and centrifuged at 4000 rpm for 10 min. The stomachs were inflated with 10 ml of 1% formalin and the oesophagus ligated. The stomachs were then immersed in 10% formalin for 10 min. to fix the inner and outer walls. The stomachs were then examined for gastric ulcers following the incisions along the greater curvatures of stomachs^[23,26-28].

Determination of gastric juice volumes, pH and acid-outputs: Volumes and pH of centrifuged gastric secretion were measured by pipette and pH meter respectively while acidities were determined by titration to pH 7 with 0.1 N sodium hydroxide solutions^[26,27]. The acid outputs were calculated by using the following equation according to the method of Ishizuka *et al.*^[23].

$$\text{EqH}^+ / 100\text{g}/4\text{h} = 1 / \text{antilog pH} \times 1000 \times \text{Volume of gastric juice (ml)} \times 100 / \text{body weight of animal (g)}.$$

Calculation of gastric ulcer index: All the stomachs were examined under a simple microscope. The gastric damages in the glandular regions were located in the gastric mucosae as elongated black-red lines parallel to the long axis of stomachs. The length (mm) of each lesion was measured and lesion index was calculated by adding the length of all the lesions in the fundic region^[26].

Statistical analysis: The data was expressed as means±S.E.M (standard error of means) and analyzed statistically by the application of SPSS (Statistical Package for Social Science) for Windows version 7.5. The Student's "t" test was applied and "P" values were determined. Differences were considered non-significant at P>0.05, significant at P<0.05 and highly significant at P<0.001^[29].

RESULTS

Acid-buffering and pepsin-binding effects: The powdered AG increased significantly (P<0.05) the pH of acidic solution from 1.0 up to 1.618±0.009. AG-A and AG-E extracts were also significantly increased the pH levels of acidic solution. AG-A raised the pH from 1 up to 1.589±0.057 and AG-E from 1 upto 1.12±0.0115 (Fig. 1). However, AG exhibited highly significant pepsin binding capacity (70.40±1.13% while AG-A and AG-E 0.61±0.053%

Table 1: Effects of *A. graveolens* fruit powder and its aqueous and ethanolic extracts on gastric secretion volume, pH, acid-output and ulcer index in rats

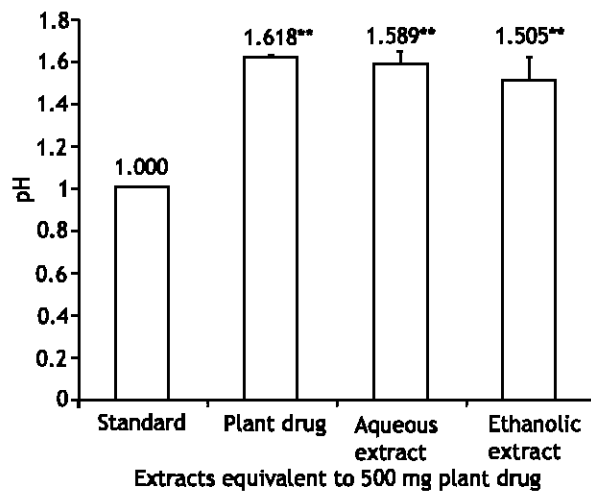
Group No.	Treatment(s)	Gastric secretion			
		Volume (ml)	pH	Acid output (μ Eq/100 g/4 h)	Ulcer index (mm)
		Mean $\pm$ S.E.M	Mean $\pm$ S.E.M	Mean $\pm$ S.E.M	Mean $\pm$ S.E.M
1	Normal (Untreated control)	2.20 $\pm$ 0.06	2.82 $\pm$ 0.01	2.82 $\pm$ 0.15	2.62 $\pm$ 0.26
2	Indomethacin (25 mg kg ⁻¹ s.c.) (Treated Control)	8.6 $\pm$ 0.45**	1.08 $\pm$ 0.20**	340.23 $\pm$ 14.21**	49.83 $\pm$ 2.47**
3	<i>A. graveolens</i> fruit (1.0 g kg ⁻¹ p.o.)+Indomethacin (25 mg kg ⁻¹ s.c.)	7.60 $\pm$ 0.06*	1.13 $\pm$ 0.02	312.99 $\pm$ 13.22	44.97 $\pm$ 2.32
4	<i>A. graveolens</i> fruit (1.5 g kg ⁻¹ p.o.)+Indomethacin (25 mg kg ⁻¹ s.c.)	4.42 $\pm$ 0.05**	1.35 $\pm$ 0.04*	141.02 $\pm$ 4.52**	28.99 $\pm$ 3.30**
5	<i>A. graveolens</i> fruit (2.0 g kg ⁻¹ p.o.)+Indomethacin (25mg kg ⁻¹ s.c.)	3.20 $\pm$ 0.08**	2.51 $\pm$ 0.06**	8.66 $\pm$ 0.67**	18.90 $\pm$ 1.01**
6	Aqueous extract of <i>A. graveolens</i> fruit (Equivalent to 2.0 g kg ⁻¹ p.o.)+ Indomethacin (25 mg kg ⁻¹ s.c.)	2.65 $\pm$ 0.06**	1.88 $\pm$ 0.04**	22.62 $\pm$ 1.40**	31.17 $\pm$ 1.34**
7	Ethanol extract of <i>A. graveolens</i> fruit (Equivalent to 2.0 g kg ⁻¹ p.o.)+ Indomethacin (25 mg kg ⁻¹ s.c.)	2.20 $\pm$ 0.08**	2.05 $\pm$ 0.09**	14.64 $\pm$ 1.26**	27.60 $\pm$ 1.56**

Indomethacin: significant from untreated control (Normal) * P<0.05; ** P<0.001

Test drugs: significant from treated control (Indomethacin) * P<0.05; ** P<0.001

All the other values are N.S. (P > 0.05) from treated control (Indomethacin)

Mean $\pm$ S.E.M = Mean values $\pm$ Standard error of means of six experiments



Test drugs: significant from standard ** P<0.001

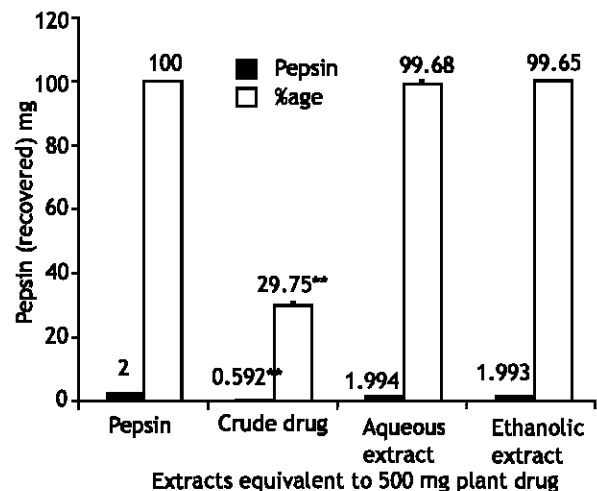
Mean $\pm$ S.E.M = Mean values $\pm$ Standard error of means of ten experiments.

Fig. 1: Acid buffering activities of *A. graveolens* and its extracts (*in vitro*)

and 0.35 $\pm$ 0.032%, pepsin binding capacities, respectively (Fig. 2).

Effect on gastric secretion volume: Oral administration of AG in 1.0-2.0 g kg⁻¹ doses inhibited the increase in gastric juice volume induced by indomethacin in a dose-dependent manner (Table 1). The effect was highly significantly (P<0.001) more with 2.0 g kg⁻¹ dose than in the treated controls. AG-A and AG-E equivalent to 2.0 g kg⁻¹ of the powder also caused highly significant (P<0.001) inhibition of gastric secretion volume in the treated rats.

Effects on gastric pH: Oral administration of 1.0-2.0 g kg⁻¹ of AG inhibited decrease in gastric pH induced by



Test drugs: significant from standard ** P<0.001

Mean $\pm$ S.E.M = Mean values $\pm$ Standard error of means of ten experiments.

Fig. 2: Pepsin binding capacities of *A. graveolens* and its extracts (*in vitro*)

indomethacin in a dose-dependent manner (Table 1). The inhibitory effect was highly significantly (P<0.001) more with the 2.0 g kg⁻¹ dose. The AG-A and AG-E equivalent to 2.0 g kg⁻¹ of the powder also caused a highly significant decrease in gastric pH in the treated rats.

Effect on gastric acid-output: Oral administration of 1.0-2.0 g kg⁻¹ of AG, prevented increase in gastric acid-output induced by indomethacin in a dose-dependent manner (Table 1). The inhibitory effect was highly significantly (P<0.001) more with 2.0 g kg⁻¹ dose. The AG-A and AG-E equivalent to 2.0 g kg⁻¹ of the powder produced similar highly significant inhibition in the rats.

Effect on gastric ulcer index: Oral administration of 1.0-2.0 g kg⁻¹ of AG inhibited gastric ulcer formation induced by indomethacin in a dose-dependent manner (Table 1). The gastric ulcer index decreasing (anti-ulcerogenic effect) was highly significantly higher ($P < 0.001$) with the 2.0 g kg⁻¹ dose. The AG-A and AG-E equivalent to 2.0 g kg⁻¹ of the powder also caused a highly significant reduction of ulcer index in treated rats.

DISCUSSION

Normally the aggressive/corrosive activities of gastric acid and pepsin are well protected by defensive/cytoprotective factors of the gastric mucosa, i.e. blood flow and mucus, bicarbonate and prostaglandin secretions. The mucosal concentration of prostaglandins has been found directly related to the gastric blood flow, mucus and bicarbonate secretions. Indomethacin pretreatment has been found to reduce the cytoprotective action^[30,31]. It has been reported that indomethacin is a potent inhibitor of cyclo-oxygenase^[32]. In addition, Maria *et al.*^[33] has reported that indomethacin causes drastic changes in arachidonic acid metabolism particularly during first hour of its treatment. Further mucosal contents of PGI₂, PGE₂ and TxA₂ have been shown to decrease progressively following indomethacin treatment while leukotrienes (LTs) increase dramatically^[34]. Indomethacin has been found not to inhibit the lipoxygenase pathway of arachidonic acid while it strongly inhibits the cyclo-oxygenase pathway. The increased level of LTs in gastric mucosa has potent action on mucosal vasculature^[34]. The lipid mediators play important role in causing inflammation and pain. Indomethacin potentiates the secretory response elicited by histamine due to the inhibition of PGE₂ synthesis. Therefore, induction of gastric ulcers and erosions is accompanied by progressive decrease in the level of PGE₂^[35,36]. It has also been indicated that programmed cell death (apoptosis) is an intrinsic part of organismal development and aging^[37]. Indomethacin and many other non-steroidal anti-inflammatory drugs (NSAIDs) cause apoptosis. The recent evidences have been shown that the damage in the stomach is activated acid secretion by a stimulatory pathway in addition to a PGs, NO and Ca⁺² dependent inhibitory mechanism. PGs may have a dual role in the regulation of acid secretion in the damaged stomach: an inhibitory effect at the parietal cell and an excitatory effect, probably through enhancing the release of mucosal histamine. Therefore, in the present study the raised gastric secretion volume and acid out-put were observed while decreased pH was found highly

significantly in the treated control group of rats in comparison to control group animals. Similarly gastric ulcer index was also induced by indomethacin highly significantly (Table 1). These changes in gastric secretions, ulcer indices and pepsin activities have reported due to its apoptosis activity^[38-41].

AG and its extracts showed highly significantly acid-buffering activities (*in vitro*). AG also bound to pepsin highly significantly while AG-A and AG-E did not show such *in vitro* pepsin binding activities (Fig. 1 and 2). In the rats having indomethacin-induced ulcerations, AG in 1.0-2.0 g kg⁻¹ doses inhibited the gastric effects induced by indomethacin in a dose-dependent manner and 2.0 g kg⁻¹ dose exhibited maximum preventive effects. AG-A and AG-E also showed similar gastric effects (Table 1). This attenuation indicated that the test drugs may interfere with the indomethacin-induced apoptosis activity. Yoshikawa *et al.*^[20] have been indicated that the lipid peroxidation induced by oxygen radicals plays an important role in the pathogenesis of indomethacin-induced gastric mucosal changes as well as in gastric injuries. Moon *et al.*^[42] have further shown that oxygen free radicals serve as second messengers in pro-inflammatory signal transduction pathways. The oxygen active species, such as O₂⁻¹, H₂O₂, HO⁻¹ and lipid radicals, such as ROO⁻¹, RO⁻¹ and hydroperoxides, are generated during lipid peroxidation and metabolism^[43]. Therefore, it may further be speculated that the anti-secretory and anti-ulcer or mucosal cytoprotective effects of AG, AG-A and AG-E exerted due to the antioxidant activities. The data has been suggested that the anti-ulcer active principle(s) of AG is/are both water and ethanol extractable (Table 1).

In conclusion, the reported results have validated the folkloric use of the drug tested for use in the therapy of gastric ulcer disease. In particular, the present studies have pointed out possible gastroprotective effects of the AG and its extracts. Nevertheless, detailed chemical studies followed by pharmacological investigations and toxicity evaluations are still required to isolate the pure active principle(s) of the AG and to elucidate their precise mode(s) of anti-ulcer actions studies are also needed.

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