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Cytoprotective Effects of Honey and Methanol Extracts from *P. granatum* L. Fruit Peel and *N. sativa* L Seeds on Ethanol-induced Gastric Damage in Rats

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Abstract: The gastroprotective effects of honey and methanol extract of *P. granatum* L. fruit peel and *N. sativa* L seeds were investigated in the rat against ethanol-induced gastric damage. 5 groups of adult *Sprague-Dawley* rats were used throughout the experiment. Oral administration of absolute ethanol (5 ml kg^{-1} body weight) to fasted rats produced extensive lesions of gastric mucosa (Group 2). Pretreatment with honey (2.5 g kg^{-1} body weight) alone (Group 3) or honey in combination with each of *P. granatum* L. (0.1 g kg^{-1}) (Group 4) or *N. sativa* L. (0.1 g kg^{-1}) extracts (Group 5) orally 30 min before administration of absolute ethanol (5 ml kg^{-1}) decreased or prevented the formation of such lesion. The observed protection was more pronounced when honey combined with each extract than honey alone. Honey in combination with *N sativa* L. completely ablated gastric lesions. Although the mechanism of gastric cytoprotection is unknown, honey in combination with each plant extracts appears to increase the resistance of gastric mucosal cells to the necrotizing effect of strong irritants (absolute alcohol). These results suggest that honey in combination with *N. sativa* L. or *P. granatum* L. extracts maintain the cellular integrity of the gastric mucosa, and might be beneficial in the treatment of a variety of diseases in which gastric mucosal injury is present.

Key words: *P. granatum* L, *N. sativa* L., honey, rats, ulcer

Introduction

Honey is elaborated by honey bees from sugars present in the nectar of various plants. Besides carbohydrates which are constituents (70-80%), honey contains, in low amounts, various substances such as organic acids, proteins, amino acids, vitamins, enzymes, minerals and different other molecules (pigments, flavonoids, antibacterial factor, etc. (White, 1979). Since ancient times, people knew the curative properties of honey. The ancient Greeks, Romans, Chinese and Egyptians used honey to heal wounds and to cure gut diseases (Zumla and Lulat, 1989). Several reports have shown the effectiveness of natural honey in the treatment of wound ulcers and skin burns (Kingsley, 2001; Natarajan *et al.*, 2001; Fox, 2002 and Molan, 2002). The healing properties of honey have been attributed to its ability to absorb edema fluid around wounds (Efem, 1988 and Gupta *et al.*, 1992), to inhibit the growth of major-infecting species of bacteria (Efem *et al.*, 1992 and Willix *et al.*, 1992) and to promote formation of healthy granulated tissues in and around wounds (Suguna *et al.*, 1992). It has been reported that natural honey is able to protect the rat stomach against acute ethanol- and indomethacin-induced lesions and to accelerate healing of rat antral ulcers induced by indomethacin (Ali *et al.*, 1990 and Ali, 1995) .

Punica granatum L. fruit shell are widely used as a medicinal herb. A decoction of this plant is usually prescribes against gastric discomfort and diarrhea (Duke and Ayensu, 1985 and Das *et al.*, 1999) and as cardioprotective and anti-inflammatort medicament for internal and external applications (Schubert *et al.*, 1999 and Gharzouli *et al.*, 1999). Polyphenols present in this plant is regarded as the active compounds. Numerous data are available showing a wide array of biochemical and pharmacological actions of flavonoids (Schubert *et al.*, 1999). The Crude tannin-rich aqueous extract of *Cistus incanus* prevented gastric lesion formation induced in rats by HCl, ethanol, indomethacin, reserpine and serotonin (Attaguile *et al.*, 1995). Several polyphenols including tannic acid, ellagic acid, flavone, flavanoneand quercetin have been reported to inhibit gastric acid secretion and protect the stomach against necrotizing agents (Gharzouli *et al.*, 1999 and Ares *et al.*, 1995). The extracts of root of *P. granatum* (Carraz *et al.*, 1978), rind (Zafar and Singh, 1990) and flowers (Jafri *et al.*, 2000) of this plant have been reported to exert some sugar lowering action in animals. Pomegranate peel contains in fact a large amount of tannin (20%) (Duke and Ayensu, 1985), have been used anti-diarrheal herb empirically and are recommended as a primary health care (Das *et al.*, 1999). Tannins are responsible for protein denaturation producing protein tannate, which reduces secretion from intestinal mucosa (Das *et al.*, 1999 and Tripathi, 2003). Previous reports of antimicrobial activity have been carried out for *P. granatum* L (Caceres *et al.*, 1987 and Navarro *et al.*, 1996) *N. sativa* L. is an annual of the Ranunclacea herbaceous plant growing in countries bordering the Mediterranean sea, Pakistan and India, the seeds of this plant are called black seeds or black cumen. It is of the native plants that are widely distributed in Egypt and also other parts of the world (Jansen, 2003). This plant is one of the most extensively studied (both phytochemically and pharmacologically (El-Sayed and El-Din, 1998; Ghosheh *et al.*, 1999; Burits and Bucar, 2000; Enomoto *et al.*, 2001; Kumara and Huat, 2001; Al-Jishi and Abuo Hozaifa, 2003).

The seeds of *N. sativa* L. are used in folk medicine all over the world for the treatment and prevention of a number of diseases and conditions that include asthma, dirrhoea and dyslipidaemia. The seeds contain both fixed and essential oils, proteins, alkaloids and saponin. The beneficial effects of the use of the seeds and thymoquinone might be related to their cytoprotective and antioxidant actions, and to their effect on some mediators of inflammation (Ali and Blunden, 2003).

Thymoquinone (TQ), the main active constituent of the volatile oil of the black seeds (*N. sativa* L. family: Ranunculacea) has been demonstrated to possess strong antioxidant properties (Aboutable *et al.*, 1986 and Houghton *et al.*, 1995). In addition, Hassan and El-Dakhakhny (1992) reported that *N. sativa* oil possesses a protective effect on chemical induced carcinogenesis and retard the carcinogenic process. Moreover, (Houghton *et al.*, 1995) found that the oil has an anti-inflammatory action with inhibition of eicosanoids generation and membrane lipid peroxidation. TQ has been shown to protect non-tumor tissues from chemotherapy-induced damage (Badary *et al.*, 1997 and Al-Shabanah *et al.*, 1998).

There is little information about the effect of honey in combination with *N. sativa* L. or *P. granatum* L extracts on gastrointestinal system. Studies have proved that *N. sativa* L. oil have a cytoprotective effect (El-Kadi *et al.*, 1987) an antioxidant and an inhibitory effect on lipid peroxidation (El-Tahir *et al.*, 1993). Experimental studies have demonstrated that the oxygen free radicals and lipid peroxidation play important roles in the pathogenesis of acute gastric ulcer (Ligumsky *et al.*, 1995). Both *N. sativa* oil and thymoquinone possess gastroprotective effect against gastric lesions in rats that may be related to the conservation of the gastric mucosal redox state (El-Abhar *et al.*, 2003).

The aim of this study was to investigate the cytoprotective properties of honey alone or in combination with each plant extract against ethanol-induced gastric damage in rats.

Materials and Methods

Honey: Pure, unprocessed, unboiled commercial honey was obtained from Faculty of Agriculture, University Putra Malaysia, Malaysia, was used for the present study.

Plant Materials: *P. granatum* L. mature fruits were purchased from a cultivator in Jordan. The fruit peels were separated from the fruits. *N sativa* L seeds were purchased from cultivator in Yamen. Fruit shells, were cut into small pieces, and *N sativa* seeds were labeled, wash with distilled water and air dried in dark at room temperature. The fruit peels and seeds were ground to a fine texture or become powder form separately using a grinder.

Preparation of Extracts: The dried and powdered materials from each of *P. granatum* L and *N sativa* L were extracted by maceration in methanol (100 g/1500 ml) in a conical flask for 5 days at 37°C. Afterwards, the solvents were filtered using filter funnel and the solvents were distilled under reduced pressure in a EYELA rotary evaporator until the extract become completely dry. For N. sativa L extracts freeze-dryer was used for completely dry. All the extracts were labeled and stored at -20°C in scintillation vials.

Experimental Animals: 40 adult male *Sprague-Dawley* rats were obtained from animal house, Faculty of Medicine University Malay, Malaysia. Animals weighing between 180-225 g were deprived of food for 48 h, but they were allowed free access to tap water until 2 h before the experiment. During the fasting period, the animals were placed individually in cages (Bollman cages) with wide-mesh wire bottoms to prevent coprophagy. On the day of experiment, the rats were randomly divided into 5 groups of eight animals each.

Animal Treatment: Group 1 and Group 2 rats each received 1 ml of PBS orally (5 ml kg^{-1}); Group 3 animals each received 2.5 g kg^{-1} honey orally; Group 4 animals each received honey (2.5 g kg^{-1} body weight) mixed with *P. granatum* L. extract (0.5 g kg^{-1}); Group 5 rats each received honey (2.5 g kg^{-1}) mixed with *N. sativa* L. extract (0.5 g kg^{-1}) by the same route.

Thirty minutes later, all animals (except Group 1 which received also 1 ml of PBS) were administered orally with 1 ml of 100% absolute ethanol (5 ml kg^{-1}) each. After 1 hour, all animals were sacrificed with an over-dose of ether and their stomach were then removed and inflated with 10 ml of 1% formalin solution and immersed in the same solution to fix the outer layer of the stomach. Each stomach was opened along the greater curvature, rinsed with tap water to remove gastric contents and blood clots and the mucosa was examined under dissecting microscope grossly (X20) with a square-grid eyepiece to assess the formation of ulcers (hemorrhagic lesions). The sum for the number of gastric lesions for each stomach was used as ulcer index (UI), and the inhibition percentage was calculated by the following formula:

$$\text{Inhibition (\%)} = [UI_{Control} - UI_{Treated})/UI_{Control}] \times 100$$

Statistical Analysis: Results were expressed as mean and ± S.E.M. The statistical differences between the mean ulcer index of treated groups and that of the controls were calculated by using Student's *t*-test.

Results

Gross Lesion Evaluation: No lesions were observed in stomach of rats received PBS twice (Group 1) Table 1 and 2). Severe gastric damage was observed in rats received PBS and administered 100% absolute alcohol (Group 2) (Table 2).

Rats pretreated with honey in combination with *P. granatum* L extract (Group 4) significantly ($P<0.05$) reduced induction of gastric lesions (79.8 % inhibition) compared to that pretreated with honey alone (Group 3) (Table 1). There were no gross gastric lesions (99.18 % inhibition) observed in animals pretreated with honey in combination with *N. sativa* L extracts (Group 5) compared to animals pretreated with honey alone Group 3 (Table 1).

Animals pretreated with honey alone (55.09% inhibition) (Group 3) or in combination with *P. granatum* L extract (Group 4) (90.78% inhibition)or *N. sativa* L (Group 5) (99.63% inhibition) showed significantly ($P<0.05$) reduced induction of gastric necrotic lesions compared to animals pretreated with PBS and received absolute alcohol (Group 2) (Table 2).

In animals pretreated with PBS (Group 2), administration of absolute alcohol produced severe gastric damage visible from the outside of stomachs as a thick red lines. After opening the stomachs, hemorrhagic lesions were found in the mucosa. They were located mostly in the corpus (the portion of the stomach secreting acid and pepsin). No gross lesion developed in the fore stomach (the non-secreting part covered with squamous epithelium) in all animals.

Microscopical Lesion Evaluation: No gastric lesions were observed in rats administered PBS twice (Group 1). Animal pretreated with PBS 30 min before administration of absolute ethanol (Group 2) showed severe acute gastric ulcerations with extensive damage. The lesions consisted of hemorrhagic necrotic patches involved the full mucosal thickness. The muscularis mucosa was intact. The submucosa was markedly thickened by edema and contains few inflammatory cells. Animals pretreated with honey in combination with each plant extract showed reduction of gastic lesions compared to animals pretreated with honey alone (Group 3).

Oral administration of honey alone (Group 3), or in combination with each plant extracts (Group 4 and Group 5) 30 min before administration of absolute ethanol showed reduction of gastric lesions compare to rats pretreated with PBS and administered with absolute alcohol (Group 2)

Rats pretreated with honey in combination with N. sativa extract the histology of the stomach showed a normal mucosa (no hemorrhagic lesion, intact mucosal cells) and normal submucosa (no edema, no infiltration with leucocytes).

Table 1: Anti-ulcerogenic activity of honey alone or in combination with each plant extract against ethanol-induced ulcerogenesis

Tested sample	No. of rats	Extract dose per Os (g kg^{-1})	Ulcer index (mean ± S.E.M)	Inhibition (%)
PBS (Group 1)	8			
Control (honey)	8		91.5 ± 0.11	
P. granatum L. + honey	8	0.5	18.38 ± 0.51*	79.48
N. sativa L. + honey	8	0.5	0.75 ± 0.25**	99.18

*$P<0.001$ significant from control (honey)(Group 3)
**$P<0.0001$ significant from control (honey)(Group 3)

Table 2: Anti-ulcerogenic activity of PBS alone, honey alone or honey in combination with each plant extract against ethanol-induced ulcerogenesis

Tested sample	No. of rats	Extract dose per Os (g kg^{-1})	Ulcer index (mean ± S.E.M)	Inhibition (%)
PBS (Group 1)	8			
Control (PBS) (Group 2)	8		203.75 ± 4.09	
Honey (Group 3)	8		91.5 ± 2.11*	55.09
P. granatum L. + honey	8	0.5	18.38 ± 1.51**	90.78
N. sativa L. + honey	8	0.5	0.75 ± 0.25**	99.63

*$P<0.001$ significant from control (Group 2)
**$P<0.0001$ significant from control (Group 2)

Discussion

The present results demonstrate that pretreatment of rats with honey in combination with methanol extracts of each *P. granatum* L. or *N. Sativa* L. extracts significantly protect the rat gastric mucosa against hemorrhagic lesion induced by absolute ethanol compare to control rats. Also pretreated rats with honey alone or in combination with each plant extract showed significantly anti-ulcerogenic activity of gastric mucosa compared to control. *N. sativa* L. showed prominent effect against ethanol-induced ulcerogenesis in rats, which may be indication of the cytoprotective activity.

P. granatum L and *N. sativa* L extracts protect the stomach against the necrotizing effect produced by absolute alcohol. Thymoquinone (TQ), the main active constituent of the volatile oil of the black seed (*N. sativa* L) (Aboutable *et al.*, 1986), has been demonstrated to possess strong antioxidant properties (Houghton *et al.*, 1995). TQ has been shown to protect non-tumor tissues from chemotherapy-induced damage (Badary *et al.*, 1997 and Al-Shabanah *et al.*, 1998). TQ protects stomach against ethanol-induced damage through an antioxidant mechanism. TQ is known to possess strong antioxidant activity, as it has been shown to inhibit eicosanoid generation of leucocytes and inhibit lipid peroxidation of ox brain (Houghton *et al.*, 1995). TQ were observed to be metabolized by liver diaphorase to dihydrothyoquinone (DHTQ), a phenolic metabolite that acts as a radical scavenger and inhibits lipid peroxidation *in vitro*. This finding strengthens the potential of TQ as a cytoprotective agent against oxidative damage in many tissues. In this context, *N. sativa* seed extract was shown to protect against cisplatin-induced myelosuppression in mice (Nair *et al.*, 1991). It seems that TQ may be the main cause of *N. sativa* extract-mediated protection.

The protective effect of *N. sativa* against ethanol-induced ulcer may be explained by different mechanisms. First of all, the increased glutathione level in gastric mucosa by two different ways. Firstly through a glutathione depletion, gastric damage is caused (Jimmy *et al.*, 1997) and the increase in glutathione level caused a decreased in the ethanol-induced gastric damage as reported by (Mutoh *et al.*, 1990). Secondly, glutathione being a cofactor in some steps of PGs synthesis, so it will help the conversion of PGG2 to PGH2 and the subsequent conversion to PGE2 (Shen, 1979). This will lead to formation of different protective prostaglandin. Prostaglandin synthetase is being nearly incapable of synthesizing PGE2, after depletion of glutathione from the medium (Wallach and Daniele, 1971). This postulation of increased PGE2 by *N. sativa* oil is in agreement with the study of (El-Sayed and N.S. El-Din, 1998) who found the treatment of normal and sensitized animal with *N. sativa* oil provoked a marked increase in PGE2 in the perfused guinea pig lung.

Prostaglandin had an established role in the protection of gastric mucosa against different types of gastric lesions. Of particular interest is the fact that the anti-secretory prostaglandin can protect the mucosa at non-secretory dosage. Moreover, the non-antisecretory PGs, such as PGF2 are also protective (Alarcon *et al.*, 1993). Several flavonoids have been found to be free radical scavengers (Baumann *et al.*,1980). Some of them have been shown to increase the mucosal content of *P. granatum* in tissues and to protect the gastric mucosa against various ulcerogens (Konturek *et al.*, 1986).

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