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Gastro-protective activity of green mussel *Perna viridis* extracts on ethanol-induced ulcer model in rats

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Abstract

The mussel *Perna viridis*, commonly known as the Indian green mussel, is a widely distributed edible mytilid bivalve seen all along India's east and west coasts. The mussel, which is abundantly found in most Asian regions, is an inexpensive source of protein with high biological value, essential minerals and vitamins. It is also been reported to have antioxidant, anti-inflammatory, and anti-bacterial properties. The present study evaluates the gastro-protective nature of methanol and aqueous/ethanol (7:3) extracts of the Indian green mussel *Perna viridis*. Gastric ulcer was induced in rats by administering 80% ethanol after 36 hours of food deprivation. Methanol and aqueous/ethanol extracts of *P. viridis* were given orally (100, 500 and 1000 mg/kg) 1 h prior to the ethanol challenge and Ranitidine (50 mg/kg body weight) was given as the reference drug. The results showed that gastric ulcer was significantly and dose dependently reduced by methanol and aqueous/ethanol extracts of *P. viridis*. Methanol extract showed a significant and dose dependent inhibition of ulcer index ranging from 1.93±0.29, 1.47±0.18 and 0.55±0.16, respectively, for the doses 100, 500 and 1000 mg/kg. Antioxidant enzymes like SOD, CAT and GSH levels were also elevated in the gastric mucosal tissue on treatment with mussel extracts. Overall, the *P. viridis* extracts ameliorated ethanol induced gastric ulcer, showing a visible reduction in ulcer lesions and antioxidant defence against the ethanol induced tissue damage.

Keywords: *Perna viridis*, Gastroprotective, Ethanol, Ulcer index, Antioxidant enzymes

1. Introduction

Gastric ulcer represents a significant public health problem, and its therapy seems to be challenging due to the unpredictable side effects of long-term uses of commercially available drugs (Bandyopadhyay et al., 2002; Lehne, 1998). The drugs used to treat ulcers include receptor blockers, proton pump inhibitors, drugs affecting the mucosal barrier and drugs acting on the central nervous system (Rao et al., 2004). Many products are available for treating this disease, and most of them are thought to act by either neutralizing acid secretion or blocking H_2 receptors, proton pump or acting as anti-cholinergics (Bafna and Blaraman, 2004). Unfortunately, they produce several adverse reactions conferring simpler to severe side effects like arrythmias, gynaecomastia, enterochromaffin like cell (ECL) hyperplasia and hematopoietic changes (Akhtar et al., 1992). Many synthetic compounds, such as ranitidine and omeprazole, which block acid secretion, are now used as antiulcer drugs. Still, their precise mechanism of action for gastroprotection at the molecular level is not fully known yet. Hence there is a need for a drug with fewer side effects to have a better and safer alternative for the treatment of this disease and the search extends to novel natural products

The Asian green mussel, *Perna viridis*, is a widely distributed edible mytilid bivalve seen all along both east and west coasts of India (Jones and Alagarswami, 1973). The species is considered to be an economically important bivalve mollusc, which is used as a food source due to its exceptionally fast growth and high nutritional properties (Kripa and Mohamed**,** 2008). In addition to being a highly nutritious species, many potential bioactive properties have also been reported on this species, such as anti-HIV, antibacterial, anti-microbial, anti- angiogenic, anti-inflammatory, antioxidant etc. (Mitra and Chatterji, 2004; Annamalai et al., 2007; Chandran et al., 2009: Mirshahi et al., 2009; Sreejamole et al., 2011; Sreejamole and Radhakrishnan, 2016). The present study was intended to evaluate the gastro protective activity of extracts from *P. viridis* using ethanol induced gastric ulceration in rats.

2. Materials and Methods

2.1. Extraction

The procedure mentioned in the extraction of invertebrates by Cannell (1998) was adopted. The whole mussel tissue (300 g) was macerated in a blender and extracted twice with 600 ml of methanol (MeOH) by mechanical stirring overnight. The suspension was centrifuged at 8,000 rpm for 20 min at 4° C. The resultant residue was successively extracted in the same way with 600 ml water: ethanol, 7:3 (Aq/EtOH). The two supernatants were evaporated to dryness in a rotary evaporator (35–55° C) under reduced pressure. The extracts were stored in airtight glass vials at -25° C until use.

2.2. Ethanol induced gastric ulcer in rats

Male Wistar rats (180-200 g) purchased from the Small Animal Breeding Station of Kerala Agricultural University, Mannuthy, India, were used for the experiment. Animals were grouped into nine, containing six animals

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in each. After 36 h of food deprivation, they were administered 1 ml of 80% ethanol (control). Other groups were given MeOH and aqueous/EtOH extracts orally (100, 500 and 1000 mg/kg) 1 h before the ethanol challenge. A normal group was maintained with saline without any treatment. Ranitidine (50 mg/kg body weight) was given as the reference drug. All the animals were sacrificed after 4 h of ethanol administration (Paiva et al., 1998).

The stomach of the animals was cut open along the greater curvature, gastric juice was collected, and the volume was measured. After washing with normal saline, the lesions on the stomach mucosa were examined using a hand lens. The ulcer index was calculated by the severity of gastric mucosal lesions, graded as in table 1.

Table 1. Scoring of gastric mucosal lesions

Erosions	Score
1mm or less	1
1–2 mm	2
More than 2 mm	3

Ulcer Index (UI) was calculated using the formula:

UI = 1× (number of lesions of grade 1) + 2 × (number of lesions of grade 2) + 3 × (number of lesions of grade 3). When divided by a factor 10, the overall score was designated as the Ulcer Index (Main and Whittle, 1975).

2.3. Determination of antioxidant enzymes in rat stomach mucosa

After measuring gastric lesions, stomachs were excised and rinsed with cold saline. The corpus mucosa was scraped, weighed and 10% homogenate was prepared using Tris-HCl under ice cold conditions. The supernatant obtained after centrifugation of the homogenate was used for the enzymatic analysis. All biochemical measurements were carried out using Hitachi U-2001, UV–VIS spectrophotometer.

2.3.1. Estimation of superoxide dismutase (SOD)

SOD activity was measured according to McCord and Fridovich (1969). A known quantity of tissue homogenate was mixed with 0.2 ml of 0.1M EDTA A known volume of tissue homogenate mixed with 0.2 ml of EDTA (0.1M), 0.1 ml of NBT (1.5 mM) and phosphate buffer (67 mM, pH 7.8) to form a volume of 2.6 ml. The absorbance of this solution was measured at 560 nm after adding 0.05 ml of riboflavin, against distilled water as blank. All the tubes were illuminated uniformly for 15 minutes and the absorbance of the blue colour formed was measured again. The percentage of inhibition was calculated after comparing the absorbance of the sample with the absorbance of the control (the tube with no enzyme activity). The superoxide anion generated was expressed as 1 unit of enzyme activity and expressed in U/mg protein.

2.3.2. Estimation of catalase (CAT)

Decomposition of H_2O_2 in the presence of catalase was measured at 240 nm (Aebi, 1984). The standard assay was performed in 3 ml of 50 mM potassium phosphate buffer at p^H 7 (1.9 ml) containing 10 mM H_2O_2 and 0.1 ml of tissue homogenate. CAT activity was defined as the enzyme required to decompose 1 nanomole of H_2O_2 per min, at 25 ^oC and p^H 7.8. Results are expressed as units per minute per milligram of tissue.

2.3.3. Estimation of lipid peroxidation (LPx)

Gastric mucosal LPx was determined by estimating malondialdehyde (MDA) levels using the TBA method (Ohkawa et al., 1979).To 0.4 ml of tissue homogenate, 1.5 ml of 0.8% TBA, 1.5 ml of acetic acid, along with distilled water was added and kept in a boiling water bath for 1 h at 95 °C. After 1 hour, the reaction mixture was removed from the water bath, cooled and added 1 ml of distilled water was followed by pyridine: butanol mixture (1:15). This was mixed thoroughly and centrifuged at 3000 rpm for 10 minutes, and the absorbance of the supernatant was measured at 532 nm against pyridine: butanol mixture. The activity was expressed as nmol of MDA/ mg protein

2.3.4. Estimation of reduced glutathione (GSH)

The activity of GSH in the gastric mucosa was determined according to the method of Moron *et al.* (1979)..Tissue homogenate was mixed with 25% TCA (0.1 ml) and cooled with ice for a few minutes, followed by centrifugation at 3000 rpm. The supernatant was mixed with 0.2 M sodium phosphate buffer (0.7 ml) and 2 ml 0.6 mM DTNB (prepared in 0.2 M buffer pH 8). The absorbance of the resultant yellow colour solution was measured at 412 nm against a blank. The amount of GSH was calculated with the help of the standard graph plotted using different concentrations of GSH in 0.3 ml of 5% TCA and expressed as nmol/mg protein.

2.4. Statistical analysis

All data were represented as mean ± S.D (n=6). The data were analysed using one way ANOVA followed by Dunnett's t-test to compare the treated groups with control. Statistical analysis was done using SPSS® version 14. $p<0.05$ was considered statistically significant.

3. Results

3.1. Effect on ethanol induced gastric ulcer in rats

The current investigation clearly showed that the aqueous/ EtOH and MeOH extracts of *P. viridis* could significantly and dose dependently reduce the rat mucosal gastric injury induced by ethanol. Oral administration of 80% ethanol in the control animals brought severe gastric mucosal damage with visibly thick reddish–black lines. The gastric lesions found in the mucosa have consisted of elongated bands, 1–10 mm long, usually parallel to the long axis of the stomach. There were characteristic lesions in the glandular portion of the stomach with an Ulcer Index of 4.1 ± 0.17 in the control group. In contrast, the normal rats treated with saline produced no lesions at all. Methanolic and comparatively to a lesser extent, aqueous/ EtOH extract ameliorated the severity of gastric ulceration induced by ethanol (Table 2).

MeOH extract of *P. viridis* produced a significant and dose dependant inhibition of UI ranging from 1.93±0.29, 1.47±0.18, 0.55±0.16, respectively, for the doses 100, 500 and 1000 mg/kg (Table 2). Even though the effect was slightly lower, the aqueous/EtOH extract also effectively cured the stomach lesions. The standard drug ranitidine (50 mg/kg) treated rats showed a gastric index of 1.23±0.04. Post Hoc Dunnett's test indicated that the ulcer

Table 2. Effect of MeOH and aqueous/EtOH extracts of *P. viridis* on ethanol induced gastric mucosal damage in rats. a- p=0.001, b- =0.01, c=0.05

Treatment	Dose (mg/kg)	Ulcer Index	Gastric volume (ml)
Control	-	4.1± 0.17	2.63±0.78
MeOH	100	1.93± 0.29[a]	2.15±0.44[c]
MeOH	500	1.47± 0.18[a]	1.53±0.41[b]
MeOH	1000	0.55± 0.16[a]	1.17±0.52[a]
Aq/EtOH	100	2.37± 0.19[a]	2.22±0.25[N.S]
Aq/EtOH	500	1.7± 0.15[a]	1.7±0.76[c]
Aq/EtOH	1000	0.85± 0.10[a]	1.38±0.35[b]
Ranitidine	50	1.23±0.40[a]	2.05±.74[c]
Normal	-	-	1.2±0.51[a]

index of *P. viridis* extract treated groups was significantly different ($p<0.001$) compared to control.

Ethanol treatment considerably increased gastric secretion volume in the control group (Table 2). An overall significant difference in gastric volume was observed $p<0.001$, across the groups. Aqueous/EtOH and MeOH extract treated groups showed significant ($p<0.01$) reduction in the gastric volume of 1.38±0.35 and 1.17 ±0.52 ml, respectively for higher concentrations (1000 mg/kg).

3.2. Antioxidant enzymes in rat stomach mucosa

3.2.1. Effect on superoxide dismutase

Superoxide dismutase activity in the control group (ethanol treated) decreased to 3.56±0.96 U/mg protein, whereas the activity in the untreated normal rats was 10.04±0.75 U/mg protein (Fig 1). *P. viridis* extract treated rats showed SOD activity almost comparable to the normal group. An overall significant ($P<0.001$) difference in SOD activity was observed between the groups and the Dunnett's t-test showed a significant ($p<0.001$) increase in SOD level for the *P. viridis* extract treated groups compared to the control.

3.2.2. Effect on catalase

A dose dependent increase in CAT activity was observed for both the *P. viridis* extracts treated groups. The catalase level of MeOH extract (1000 mg/kg) and ranitidine treated rats were almost comparable to the normal group, whereas in the control group the activity was reduced to 13.3±0.76 U/mg protein (Fig 2). Catalase activity in rats pre-treated with mussel extracts was significantly different ($p<0.01$) from the control group and was dose dependent.

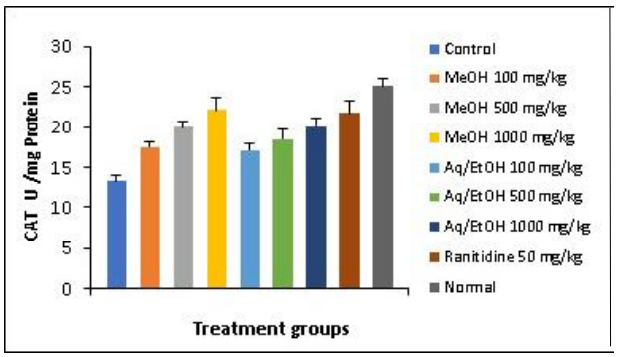

Fig. 2. Effect of *Perna viridis* extracts on the gastric mucosal catalase activity in ethanol induced ulcer in rats

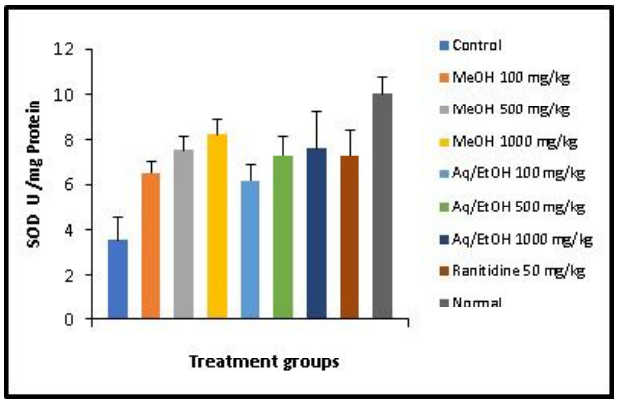

Fig. 1. Effect of *Perna viridis* extracts on the gastric mucosal superoxide dismutase activity in ethanol induced ulcer in rats

3.2.3. Effect on lipid peroxidation

The increase in lipid peroxidation due to ethanol administration was dose dependently reduced in the *P. viridis* treated groups (Fig.3). Analysis by one way ANOVA showed significant difference ($p<0.001$) in the lipid peroxidation level between groups. All doses of *P. viridis* extracts and ranitidine significantly reduced the lipid peroxidation (LPx) in rat stomach ($p<0.001$) whereas it was considerably high (20.04 ±0.88) in the control group.

3.2.4. Effect on glutathione reductase

The rats pre-treated with aqueous/EtOH and MeOH extracts of *P. viridis* showed subsequent increase in GSH levels compared to control. The GSH levels in control rats were reduced to 2.78±0.13 nmol/mg protein whereas in the untreated normal rats it remained at 4.5±0.43 (Fig. 4). It was observed that the GSH activity of the medium and higher concentrations of both the extracts were significantly ($p<0.001$and $p<0.01$) improved compared to control.

4. Discussion

The results of the current study reveal that pre-treatment with aqueous/EtOH and MeOH extracts significantly reduced the ulcer index and the formation of gastric lesions induced by absolute ethanol. Ulcer number, score and index are the parameters which may help to assess the anti-ulcerogenic efficacy of the treatments. Nevertheless, ulcer score is a reliable and relevant parameter used by several investigators as a marker of ulcer severity (Khayyal et al., 2001; Albiero et al., 2002). A significant decrease in ulcer index values compared to control suggests the cytoprotective effect of *P. viridis*. This holds good as

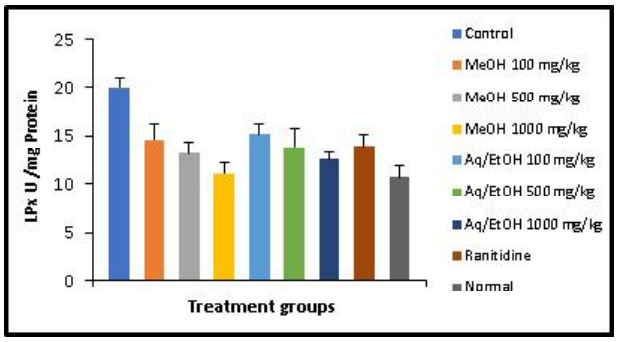

Fig. 3. Effect of *Perna viridis* extracts on the gastric mucosal lipid peroxidation in ethanol induced ulcer in rats

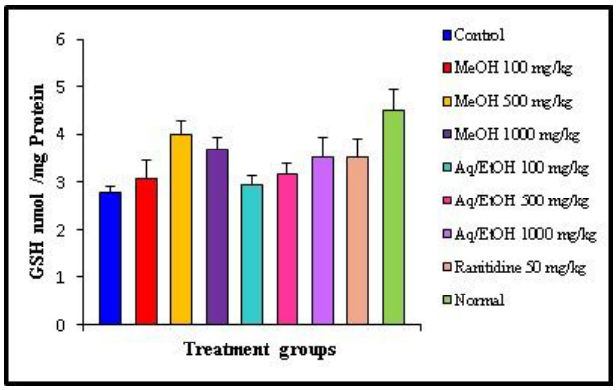

Fig. 4. Effect of *P. viridis* extracts on the gastric mucosal glutathione reductase activity in ethanol induced ulcer in rats

cytoprotection of anti-ulcer drug is assessed in terms of absence or reduction in macroscopically and microscopically visible lesions. The lowered gastric volume in the treated groups against the control suggests the possible anti-secretory nature of the *P. viridis* extracts.

Gastric lesions induced by ethanol are thought to arise as a result of direct damage of gastric mucosal cells, resulting in the development of free radicals (Pihan et al., 1987) and peroxidation of lipids (Puurunen et al., 1980), which are the key mediators for the pathogenesis of ethanol-induced gastric mucosal injury *in vivo* (Pihan et al., 1987) causing damage to the cell and cell membranes (Fridovich, 1978). ROS also decreases the levels of endogenous antioxidants, such as GSH, á-tocopherol and ascorbate and make the mucosa more prone to oxidative damage (Phull et al., 1995). Moreover, they have also been shown to have a significant effect on the cellular system, damaging its structure, and inducing alteration, especially in its high molecular weight components. Administration of *P. viridis* extracts proved effective in augmenting the antioxidant enzyme levels (SOD, CAT and GSH) as a whole, indicating the cytoprotection by free radical scavenging.

The higher SOD and CAT activities in *P. viridis* treated rats and ranitidine (50 mg/kg) ($p<0.01$) reveal its potent activity to scavenge the ROS formation. Preventive antioxidants like superoxide dismutase (SOD) and catalase (CAT) are the first line of defense against reactive oxygen species (Halliwell, 1885) and they protect the cells against their toxic effects. This protective action may be, via the increase and maintenance of near normalcy in the activity of SOD, which is said to prevent neutrophil induced damage (Terano et al., 1986; Wallace et al., 1988). It can be suggested that free radical scavenging and antioxidant activities of the *P. viridis* extracts play an important role in preventing the ethanol induced damage of the stomach mucosa.

Lipid peroxidation is a process which occurs naturally as part of the development of free radicals. It brought about many changes in cells, such as, the production of free radicals and the rearrangement of double bonds in unsaturated lipids, ultimately leading to the destruction of membrane lipids (Sathish et al., 2011). Biological membranes are often rich in unsaturated fatty acids and bathed in oxygen-rich metal containing fluid. Therefore, it is unsurprising that membrane lipids are susceptible to prooxidative attack. Ethanol is known to induce rapid lipid peroxidation which is the critical event responsible for mucosal haemorrhage and oedema in vascular smooth muscle cells and endothelial cells. The significant decrease in the level of lipid peroxidation and increase in activities of antioxidant enzymes in the treated animals, suggests the ability of *P. viridis* extracts to protect gastric mucosa against free radical-mediated tissue injury. Given the above findings, the mussel would prove to be a good ingredient in functional food formulations and as antioxidant leads.

Reduced glutathione (GSH) plays a central role in co-ordinating the body's antioxidant defense processes. It is a major low molecular weight scavenger of free radicals in the cytoplasm. GSH is also thought to be important in maintaining the integrity of the gastric mucosa. It was noted that ethanol significantly reduced gastric glutathione concentration in control group. This reduction may be due to the oxidation of GSH because of ethanol induced generation of toxic oxygen metabolities (Pihan *et al.*, 1987).

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