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Research Note

Histological Evidences of the Ulcer Healing Properties of *Perna viridis* Extracts

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Present study evaluates the ulcer healing properties of methanol and aqueous/ethanol (7:3) (Aq/EtOH) extracts of the Indian green mussel *Perna viridis,* against ethanol induced gastric ulcer model in rats. A preliminary chemical analysis for alkaloids, flavonoids, phenolics, terpenoids, saponins, anthraquinones and sterols of both extracts were carried out using general detection reagents. *In vitro* antioxidant activities were evaluated by Phosphomolybdenum method and ABTS radical scavenging assay. For ulcer induction, the rats were administered with 80% ethanol after 36 hours of food deprivation. Methanol and aqueous/ethanol extracts of *P. viridis* were given orally once, (100, 500 and 1000 mg/kg) 1h prior to the ethanol challenge and Ranitidine (50mg/kg body weight) was given as the reference drug. After sacrificing the animals, stomach was cut open and the tissue of each group was processed for histopathological studies. Pretreatment with both the extracts reduced the depth and severity of ethanol-induced gastric mucosal lesions effectively as evidenced by the histopathological evaluation. The healing property of *P. viridis* extracts on ethanol-induced gastric ulcer, could be attributed to their antioxidant properties and the presence of polyphenols and saponins.

Keywords: *Perna viridis*, antioxidant, anti-ulcer, ethanol, histopathology

Gastric ulcer is one of the most prevalent gastrointestinal (GI) disorders, which affects approximately 5-10% of people during their lives (Debashis et al., 2002). It is a benign lesion of gastric or duodenal mucosa occurring at sites where the mucosal epithelium is exposed to acid, alcohol and pepsin (Goel & Sairam, 2002). Gastric ulceration results from an imbalance between damaging factors within the lumen and protective mechanisms within the gastrointestinal mucosa (Rao et al., 2004).

Gastric ulcer therapy faces a major drawback in modern days due to the unpredictable side effects of commercially available drugs. The drugs used in the treatment of ulcer include receptor blockers, proton pump inhibitors, drugs affecting the mucosal barrier and drugs acting on the central nervous system. Even though a range of drugs are available for the treatment of ulcer, many of these do not ameliorate the whole symptoms and also leads to side effects. 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. Consequently, many drugs are being researched offering newer and better options for the treatment of gastric ulcers and the search extends to the systematic development of natural products.

Bivalves are considered vital in terms of nutritive point of view next to fish and prawns. They were reported to contain bioactive compounds which can influence human health in alleviating the symptoms of many disease conditions with a wide range of activities such as antimicrobial, antiangiogenic, antioxidant, anticancer activity and antiinflammatory, Annamalai et al., 2007; Sreejamole & Radhakrishnan, 2016; Praveena & Kaneez, 2017). Marine natural product research programmes have seldom attempted to extract compounds with gastroprotective/anti-ulcer properties, except for limited attempts on mussel (Rainsford & Whitehouse,

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1980) oyster shell (Nie et al., 1994) and fish oils (Khare et al., 2008). The present study investigates the ulcer healing property of *P. viridis* extracts, through histological evaluation of the ulcer induced stomach mucosa in rats.

The mussel, *Perna viridis* was collected from Anthakaranazhi, Alappuzha Dist (Kerala, India). The whole mussel tissue (300g) was macerated in a blender and extracted twice with 600ml of methanol (MeOH) by mechanical stirring overnight. The suspension was centrifuged at 8,000rpm for 20min at 4°C. The resultant residue was extracted with 600ml 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.

Identification of the chemical constituents (alkaloids, flavonoids, phenolics, terpenoids, saponins, anthraquinones and sterols) present in the extracts (MeOH and Aq/EtOH) of *P. viridis* were carried out using various general detection reagents described by Cannell (1998). For testing alkaloids; Mayer's test, Dragendorff reagent and Wagner reagent were used. Sesquiterpene lactones and cardiac glycosides were evaluated using Baljet reagent and Legal reagent. For flavonoids Shinoda's test and Sulfuric acid test were employed. Other polyphenols were evaluated using 5% Ferric chloride. Sterols in the extracts were estimated by Liebermann- Buchard test and Salkowski reaction. Saponins in the mussel extract was tested by persistent froth formation when treated with distilled water.

The antioxidant activity of fractions was evaluated by phosphomolybdenum method according to the procedure of Prieto et al. (2005). An aliquot of 0.1ml of each concentration of extracts (0.2, 0.4, 0.8. 1.2, 2.4 and 4.8 mg ml^{-1}) was combined in a vial with 1ml of reagent solution (0.6M sulphuric acid, 28mM sodium phosphate and 4mM ammonium molybdate). The vial was capped and incubated in a water bath at 95°C for 90min. After the incubation, samples were cooled to room temperature, and the absorbance of the mixture was measured at 765nm against a blank. Percent inhibition was calculated by the following formula

% Inhibition = (1- absorbance of sample / absorbance of control) x 100

ABTS (2, 2-Azinobis (3-ethylbenzothiazoline)-6-sulphonic acid) (Sigma Aldrich USA) assay was carried out according to Gill et al. (1999). The assay is based on the capacity of antioxidants to scavenge ABTS radical cation causing a reduction in absorbance at 734 nm. The ABTS solution was prepared by mixing 7 mM ABTS and 2.45 mM potassium persulphate and incubated in the dark at room temperature for 16h. Different concentrations mussel extracts (0.2, 0.4, 0.8. 1.2, 2.4 and 4.8 mg ml^{-1}) were prepared in methanol. To each concentration, 3ml of ABTS solution was added and the absorbance was measured. All experiments were carried out in triplicate. Percentage inhibition was calculated using the formula:

ABTS+ % scavenging effect = $((A_B–A_A)/ A_B)\times100$, where, A_B is absorbance of ABTS radical A_A is absorbance of ABTS radical + mussel extracts.

Male Wistar rats (180-200g) were acclimatized in ventilated cages at 22-28°C temperature, 60-70% relative humidity 1 week prior to the experiments. They were fed with standard rat feed (Lipton, India) and sterile water *ad libitum.* For the experiment, rats were grouped into nine containing six animals in each. After 36h of food deprivation, they were administered with 1ml of 80% ethanol (control). Prophylactic treatment of MeOH and Aq/EtOH extracts orally (100, 500 and 1000mg/kg) was given to other groups, 1h prior to the ethanol challenge. A 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., 1990). Animal experiments were conducted following the guidelines and prior approval from the Institutional Animal Ethics Committee (Registered Number 149/1999/CPCSEA India/28/2/2004), constituted by the Animal Welfare Division, Government of India.

The excised stomachs were cut open through the ventral suture and a small portion was fixed in 10% formalin solution. The specimens were passed through ascending grades of alcohol, cleared in xylene and impregnated and embedded in paraffin. Tissue sections were prepared from each stomach tissue and stained with hematoxylin-eosin. The sections were mounted in DPX and observed under light microscope.

The results of the preliminary chemical analysis were shown in Table 1. MeOH extract was positive for alkaloids, polyphenols, sterols, anthraquinones

and saponins, whereas Aq/EtOH extract showed positive results only for alkaloids and saponins. The secondary metabolites isolated from molluscs make up a vast repository of compounds with a wide range of biological activities. But it is imperative that the dietary sources contribute significantly to the chemical diversity found in molluscs. Nevertheless, evidence for de novo biosynthesis has been reported in several molluscan taxa (Garson, 1993; Cimino & Ghiselin, 2001; Moore, 2006).

The antioxidant capacity of extracts of *P. viridis* are shown in Fig. 1. Among the two, methanolic extract exhibited slightly higher antioxidant activity. At higher dose, MeOH and aqueous/EtOH extract (4.8mg ml^{-1}), showed a percentage of inhibition of 75.3 and 87.8% respectively. The

Table 1. Chemical analysis of *P. viridis* extracts using general detection reagents

Test	Extracts of *P. viridis*	
	Methanol	Aqueous/EtOH
Alkaloids		
Mayer's reagent	-ve	+ve
Dragendorff's reagent	+ve	+ve
Wagner's reagent	-ve	-ve
Flavonoids		
Shinoda's test	-ve	-ve
Sulfuric acid	-ve	-ve
Poly phenols		
5% $FeCl_3$	+ve	-ve
Sesquiterpene lactones/Cardiac glycosides		
Baljet reagent	-ve	-ve
Legal reagent	-ve	-ve
Sterols		
Liebermann-Buchard test	+ve	-ve
Salkowski reaction	+ve	-ve
Anthraquinones		
Borntrager test	+ve	-ve
Saponins		
Presence of persistent froth	+ve	+ve

phosphomolybdenum method is based on the reduction of Mo (VI) to Mo (V) by the antioxidant compounds and the formation of green phosphate/ Mo (V) complex with the maximal absorption at 695nm. This may be explained by the fact that the transfer of electrons/hydrogen from antioxidants depends on the structure of the antioxidants (Loo et al., 2008).

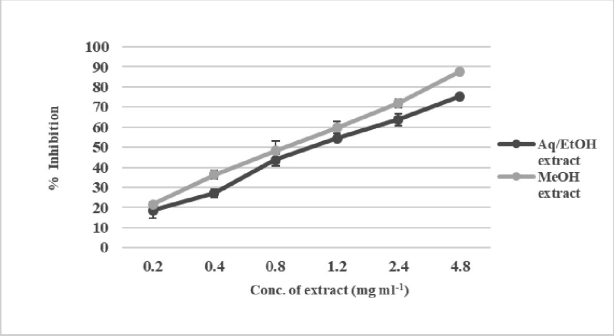

Fig. 1. Effects of *P. viridis* extracts on phosphomolybdinum

The results of ABTS+ radical scavenging activity of *P. viridis* extracts are shown in Fig 2. The percentage inhibitions were directly proportional to the concentrations for both MeOH and aqueous/EtOH extracts, and comparatively better radical scavenging activity was displayed by MeOH extract than aqueous/EtOH extract. The ABTS radical cation scavenging activity of a compound attributes to its hydrogen-donating capacity (Hagerman et al., 1998) and reported that the high molecular weight phenolic (tannins) have more ability to quench free radicals (ABTS+). The presence of polyphenols in MeOH extract can be the reason for it's radical scavenging property.

Gastric lesions in control group showed clear depth of penetration into the gastric mucosal surface, forming deep erosions and bleeding in rats receiving 80% ethanol (Plate 1 A) Pretreatment with MeOH and Aq/EtOH extracts reduced the depth and severity of ethanol induced gastric mucosal lesions effectively. Mild epithelial atrophy was noted, but no severe necrotic changes in mucus ridges were observed in the higher concentrations for both extracts.

Ethanol induced gastric lesion formation is found to be due to the stasis in gastric blood flow, hence contributing to the development of the hemorrhage and necrosis of tissue injury (Guth et al., 1984). Histological analysis of the stomach of rats showed severe acute gastric ulcerations (Plate 2). The lesions

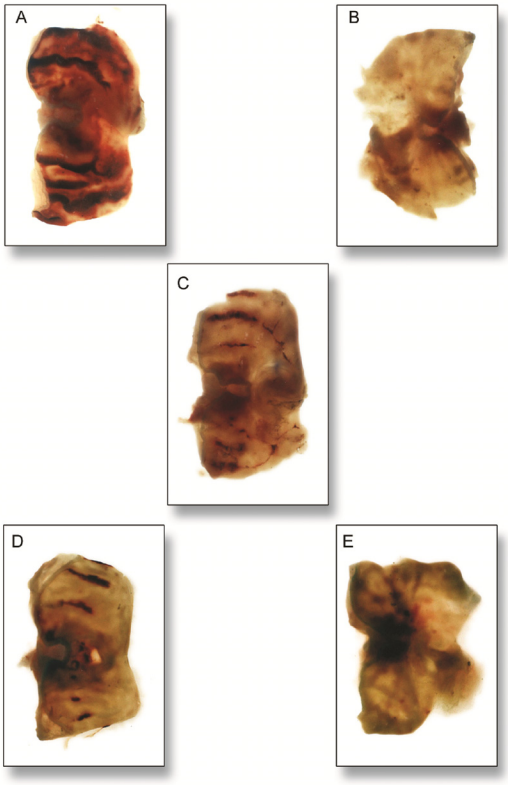

Plate 1. Opened Stomach of ulcer induced rats showing lesions with/without treatment with *P. viridis* extracts. A) Control B) Normal C) MeOH extract 100mg/kg D) MeOH extract 500mg/kg E) MeOH extract 1000mg/kg

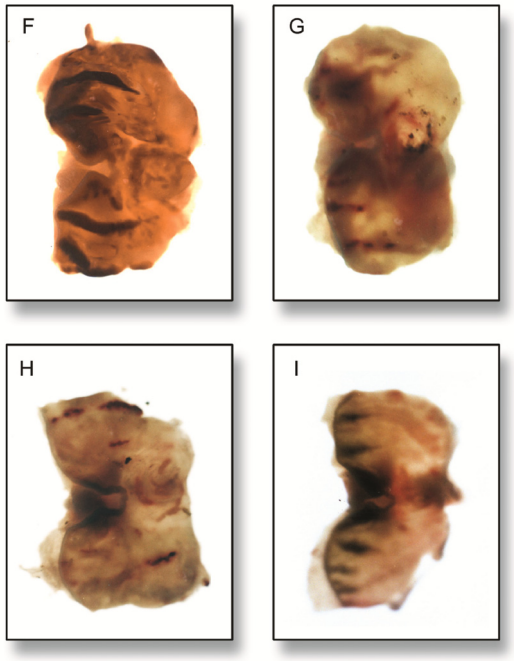

Plate 2. Opened Stomach of ulcer induced rats showing lesions with/without treatment with *P. viridis* extracts. F) Aqueous/ EtOH extract 100 mg/kg G) Aqueous/ EtOH extract 500 mg/kg H) Aqueous/ EtOH extract 1000 mg/kg

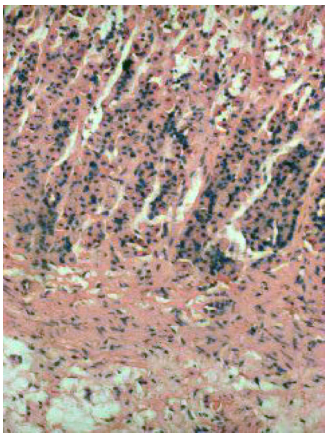

Plate 2 a. Photomicrograph of stomach of control group showing predominant disintegration and sloughing off of superficial and underlying epithelial cells with complete necrosis

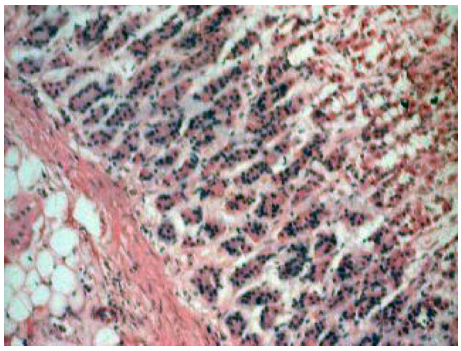

Plate 2 b. Photomicrograph of MeOH extract (100mg kg^{-1}) treated group showing surface mucous cells with necrosis and epithelial necrosis not extended to deeper layers.

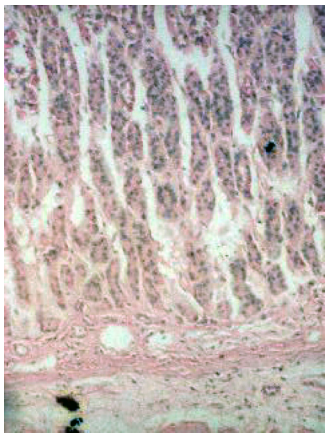

Plate 2 c. Photomicrograph of MeOH extract (500mg kg^{-1}) showing inflammatory exudates in the mucosa and submucosa mild atrophy of glands

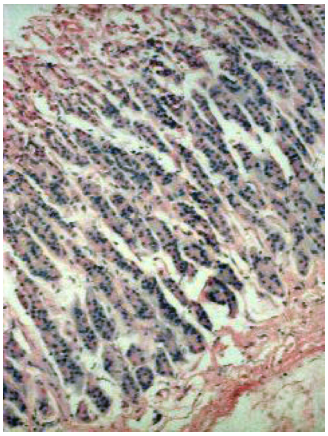

Plate 2 d. Photomicrograph of MeOH extract (1000mg kg^{-1}) showing intact glands, mild oedema in the mucosa, sub mucosal layer shows no oedema

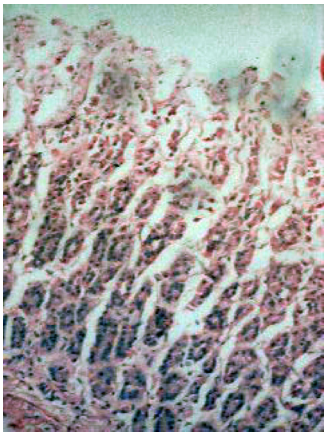

Plate 2 e. Photomicrograph Aq/EtOH extract (100mg kg^{-1}) showing necrosis in the epithelial layers, moderate atrophy of glands, oedema in the mucosa

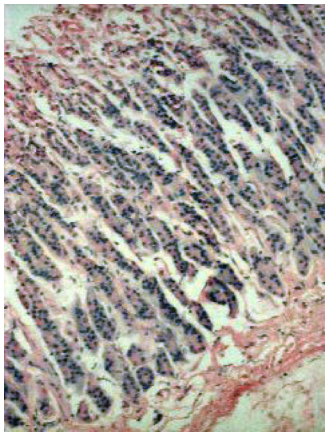

Plate 2 f. Photomicrograph of Aq/Et-OH (500mg kg^{-1}): surface epithelium shows mild necrosis, inflammatory cell infiltration, oedematous sub mucosa

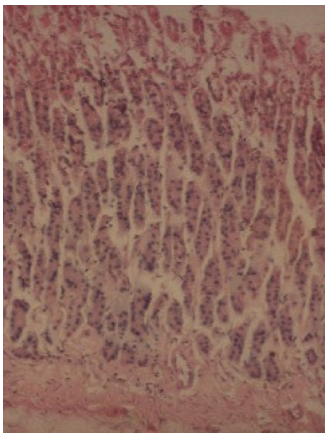

Plate 2 g. Photomicrograph of Aq/Et-OH 1000mg kg-1: oedema in the mucosa, necrosis in the epithelial layer, non-oedematous sub mucosa

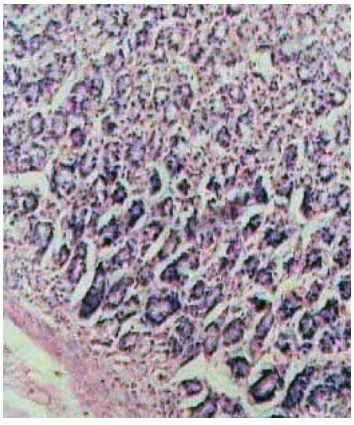

Plate 2 h. Photomicrograph normal group: glands intact, no edema in the mucosal and sub mucosal layers

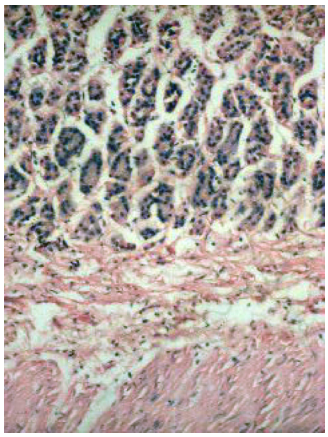

Plate 2 i. Photomicrograph of Ranitidine 50mg kg^{-1}: mild atrophy of glands, mild oedema in mucosa and submucosa

Plates 2 a-i: Effect of P. viridis on the gastric mucosal damage induced by absolute ethanol in rats (200X)

consisted of extensive hemorrhagic necrotic patches involving the full mucosal layer, hyperemia, prominent deep submucosal oedema as well as leucocyte infiltration. Histologically, the stomach of *P. viridis* extract pretreated groups (500 and 1000mg/kg) showed superficial erosion in the surface epithelium and moderate degree of submucosal oedema with neutrophilic infiltration (Plates 2 c, d, e & g). Reduction in gastric damage, glandular necrosis and submucosal leucocyte infiltration was also evidenced compared to the control group.

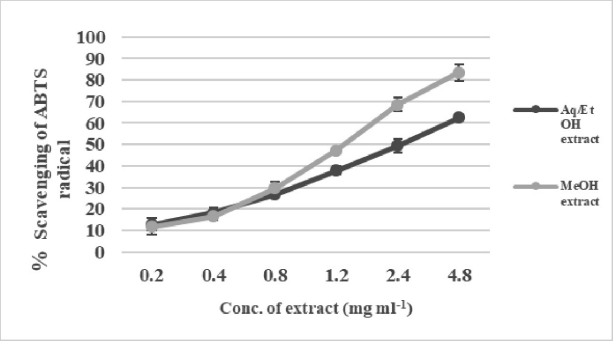

Fig. 2. Effects of *P. viridis* extracts ABTS+ radical scavenging activity

Moderately atrophic glands and submucosal oedema was observed for 100mg/kg treated groups of both extracts (Plates 2 b & e). On histopathological analysis, epithelial necrosis, mild atrophic glands, mild hyperplasia with slightly edematous mucosa and sub mucosa were observed for animals treated with ranitidine (50 mg/kg) (Plate 2 i). The group supplemented with saline without any treatment showed no signs of ulceration (Plate 2 h).

The study establishes significant inhibitory effect of *P. viridis* extracts in reducing inflammation and gastric lesion induced by 80% ethanol, owing to its significant free radical scavenging property which is an important factor causing mucosal damage. Moreover, its anti-inflammatory property has been proved earlier (Sreejamole et al., 2011). The possible mechanism of action of the extract in preventing gastric lesion produced by alcohol may be due to the formation of a protective layer of polyphenolic compounds present in the extract. Raphael & Kuttan (2003) has reported that polyphenolics react with the protein of the stomach lining by hydrophobic interaction. Saponins, especially triterpene type has been implicated in antiulcer activity by forming protective mucus on the gastric mucosa and protect the mucosa from the effects of acid by selectively inhibiting PGF2á (Agwu & Okunji, 1986; Lewis & Hanson, 1991). Moreover, several alkaloids are reported to have anti-ulcerogenic activities (Falcão et al., 2008).

Therefore, it can be concluded that pretreatment with the *P. viridis* extracts may either produced a protective lining on the stomach and reducing free radical production and its effect on the liver (Fig. 3). However, the active compounds responsible for the anti-ulcerogenic activity exhibited by these extracts are yet to be understood.

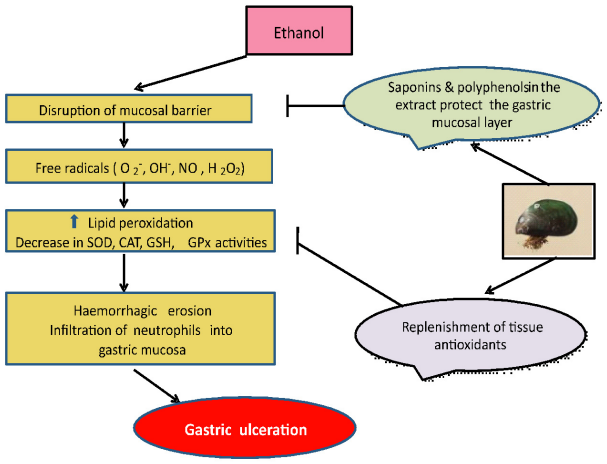

Fig. 3. Possible inhibitory mechanism of *P. viridis* extracts on ethanol induced ulcer in rats

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