Original Article

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Pithecellobium dulce Fruit Extract exerts Antiulcerogenic effect by Influencing the Gastric expression of H^+, K^+-ATPase and Mucosal Glycoproteins

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ABSTRACT

Objective: *Pithecellobium dulce* (*P. dulce*) fruits are evidenced by its traditional use for gastrointestinal disorders. The aim of the study is to elucidate the molecular mechanisms associated with the antiulcerogenic effects of *P. dulce* fruits. **Methods:** Gastric and duodenal ulcers were induced by administering ethanol and cysteamine in rats pretreated with hydroalcoholic fruit extract of *P. dulce* (200 mg/kg bwt) for 30 days. The antiulcerogenic effect of *P. dulce* extracts was screened by quantitative analysis of H^+, K^+-ATPase β subunit, MUC6 and MUC2 expression in stomach and duodenal tissue by real time PCR. **Results:** The *P. dulce* fruit extracts exerted its antiulcerogenic effect by down regulation of H^+, K^+-ATPase β subunit expression ($p<0.01$) and up regulation of gastroprotective mucins, MUC6 and MUC2 ($p<0.05$) in stomach and duodenum respectively. **Conclusion:** Our findings suggest that *P. dulce* exerts gastroprotective effect by down regulating gastric H^+, K^+-ATPase synthesis and up regulation of mucin secretion in stomach and duodenum. Thus the findings strongly suggests that *P. dulce* may be included in antiulcer drug formulations either singly or with other known herbal medicines for the prevention and treatment of peptic ulcer.

Key words: H^+, K^+-ATPase β subunit, MUC6, MUC2, *P. dulce* (*Fabaceae*), Peptic Ulcer.

INTRODUCTION

Peptic ulcer disease (PUD) is a major chronic gastrointestinal disorder, affecting millions of people worldwide. The disease is characterized by shallow breaches in the mucosal lining of stomach and duodenum. The loss of mucosal integrity was due to the imbalance between mucoprotective components such as prostaglandins, bicarbonate, mucosal glycoproteins[1] and aggressive ulcerogenic factors such as hypersecretion of gastric acid and pepsin. The hypersecretion of ulcerogenic factors were reported to be stimulated by caffeine, alcohol, *Helicobacter pylori* (*H. pylori*) infection and use of Non steroidal anti-inflammatory drugs (NSAID's).[2] Also, changes in traditional lifestyle and stress were reported to be a major precipitating factor for the higher incidence of PUD. Hydrochloric acid (HCl) produced by gastric H^+, K^+-ATPase plays an important role in acidification process and hence the inhibition of enzyme reduces the excess production of HCl in stomach.[3] Mucins were found to involve in protecting the gastrointestinal tract from acid, proteases, pathogenic microorganisms as well as from mechanical trauma. Therefore, H^+, K^+-ATPase

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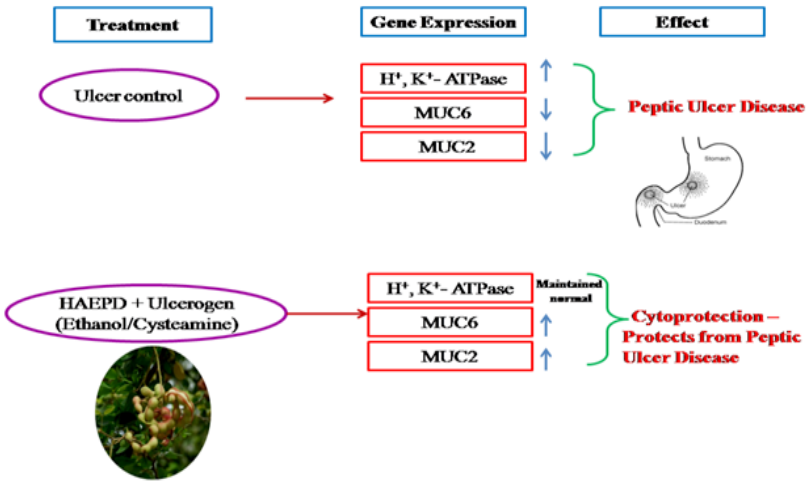


Graphical Abstract

inhibition and enhancement of cytoprotective action by mucins were the pharmacological target to develop drugs against PUD. In 2003, WHO reported that 80% of population affected by PUD in developing countries resort to ayurvedic drugs, as they could not afford much towards commercially available anti-ulcer drugs. Hence, a demand for the development of new drug preferably from natural sources with gastroprotective components is on the rise and also these natural compounds should be less toxic and cost effective in nature.

India is a land of origin for Ayurveda and Siddha forms of medicine, which uses the naturally occurring plants and herbs as medicines. *P. dulce* belongs to *Fabaceae* is commonly called as 'Vilayati babul' in Hindi and 'Kodukkapuli' in Tamil. The arils of the plant have been consumed for its high nutritive and medicinal value in various parts of India. Besides this, the plant has been used for treating various complications since ages and to name a few, the aqueous extract of the bark was used to treat dysentery, febrifuge, dermatitis and inflammation of eyes,[4] leaves of the plants were used to relieve indigestion and intestinal disorder and also as a folk remedy to treat leprosy and peptic ulcer.[5] The plant has been reported to possess numerous pharmacological effects including antivenomous activity,[6] anti-inflammatory activity[7] and antidiabetic and antihyperlipidemic activity in streptozotocin induced diabetic rats.[8]

The current approach for PUD therapy involves various mechanisms in reducing gastric acid secretion and mechanical damage. Recently, several pharmacological products such as Histamine blocker, gastro protectors and proton-pump inhibitors are being used to manage hyperacidity and mucosal damage. However, these drugs also have limitation because of their adverse side effects on human health. Thus a modest approach to treat PUD has to down regulate H^+, K^+-ATPase in gastric parietal cells and enhance the mucin secretion in the gastric and duodenal mucosa. Hence this study was carried out to elucidate the molecular mechanisms by which the *P. dulce* fruits exert its antiulcerogenic property.

MATERIALS AND METHODS

Plant material and extract preparation

P. dulce fruits were collected from Thiruvallur district, Chennai. The plant material was authenticated by Dr. P. Jayaraman, Director, Plant Anatomy Research Center, Chennai, and allotted the Voucher no: PARC/2008/208. The hydroalcoholic fruit extract of *Pithecellobium dulce* (HAEPD) was prepared by macerating about 300 g of air dried ground homogenous powder of the fruits in 3l of 70% ethanol for a week. The evaporated filtrate under low pressure yielded 90 g of HAEPD (30% from the dry weight). The residue was reconstituted with water and used for the experimental studies.

Experimental Animals

Male albino Wistar strain rats (150-200 g) were obtained from the Centre for Animal Health studies, Madhavaram, Chennai. Animals were maintained under standard conditions of temperature and relative humidity with 12:12 hour light: dark cycle and were given standard pellet diet and water *ad libitum*. The institute ethics committee for Supervision and Control of Experiments on Animals clearance had approved the study (290/CPCSEA/12/12/08-02).

Table 1: Forward and Reverse primer sequences used for gene expression studies

Gene Accession. No	Primer	Sequence (5'-3')	Product size
β actin NM_0311442	Forward Reverse	GACAGGATGCAGAAGGAGATTACT TGATCCACATCTGCTGGAAGGT	142 bp
H^+,K^+-ATPase β subunit M55655.1	Forward Reverse	TGACCCCCAAAAGCCCCGGA GTGGACAGCCACGCCCACTC	288 bp
MUC 6 XM_215127.6	Forward Reverse	ACACCTGTGGGCCCCTCCTACTTG TGTGGGCCTTGTGGGTGTTGACTT	207 bp
MUC2 U07615.1	Forward Reverse	GCCAGATCCCGAAACCA TATAGGAGTCTCGGCAGTCA	127 bp

Induction of Gastric and duodenal ulcers

A colony of rats (n=6) abstained from any experimental procedures were used as controls. A colony of rats (n=6) were given 60% ethanol (1 ml/200 g/1h) and were used as gastric ulcer disease controls. Rats (n=6) treated with HAEPD (200 mg/kg bwt) for 30 days were used as drug controls. A group of rats (n=6) were pretreated with HAEPD (200 mg/kg bwt) for 30 days and on the last day, gastric ulcers in them were induced by administering a single oral dose of 60% ethanol (1 ml/200 g/1h). The animals were sacrificed after 24 hours and the gastrum was examined for focal ulcers[9] and were used to study the gastroprotective properties of *P. dulce*.

The animal groupings mentioned in the induction of gastric ulcers were followed here, however the duodenal ulcers in the experimental animals (n=6) were induced by administering a single oral dose administration of cysteamine hydrochloride (420 mg/kg body weight). The animals were sacrificed after 24 h of cysteamine administration and the duodenum was screened for ulcer lesions.[10]

Isolation of Gastric and Duodenal mucosa

The gastric and the duodenal tissues obtained from the respective study groups were collected separately and homogenized in TRIZOL for RNA extraction. Total RNA from the samples was extracted using Qiagen RNeasy Mini Kit, Germany. The quality and quantity of the isolated RNA was tested using Eppendorf Biophotometer Plus, Germany. Further, the integrity of the RNA was verified using 1.4% agarose-formaldehyde gel electrophoresis. cDNA from the isolated RNA was synthesized using High capacity cDNA reverse transcription kit (Applied Biosystems Inc., CA) following the manufacturer's instructions.

Quantitative Real-time (qRT-PCR) analysis of H^+, K^+-ATPase subunit, MUC6 and MUC2 mRNA Expression in experimental rats

The expression of H^+, K^+-ATPase β subunit and MUC6 and MUC2 genes in gastric and duodenal mucosal tissues in all the animal groups were analyzed by quantitative RT–PCR using SYBR green chemistry (the primer sequences are provided in the Table 1). The expression of the genes was compared with expression of β-actin gene (endogenous control). All C_t values were the mean of duplicate samples tested. The ΔC_t values indicate the difference in the C_t values between the target gene and the endogenous gene.

Western blot analysis of expressed proteins

The protein expression level of H^+, K^+-ATPase β subunit in the gastric mucosal preparations were determined by Western Blot and densitometry analysis. The total protein was extracted from the tissues and was resolved in 12% SDS-PAGE gel. The gel was then transferred to the nitrocellulose membrane for immunodetection. The membrane was initially blocked with 5% W/V of Bovine serum albumin in Tris Buffered Saline (TBS) at 4°C for 2 h and was incubated with primary and secondary antibody coupled with alkaline phosphatase for 1 hr. The nitrocellulose strips are then incubated in the substrate mixture for 10-30 min for colour development.

Statistical analysis

The ΔC_t values were compared across the groups by one way ANOVA followed by Bonferroni multiple comparison test. Statistical analysis was carried out in Graph pad prism 5 software (Santiago,USA). The p value of <0.05 was considered significant.

RESULTS

Gene expression status in gastric and duodenal ulcer models

The gene expression of H^+, K^+-ATPase β subunit, MUC6 and MUC2 in all the groups of animal models revealed that the H^+, K^+-ATPase β subunit mRNA expression was elevated significantly ($p<0.01$) in ethanol induced gastric ulcer group than in control group (Figure 1). MUC6 and MUC2 expression was observed to be considerably reduced in ulcer models in comparison with control animals (Figure 2 and Figure 3).

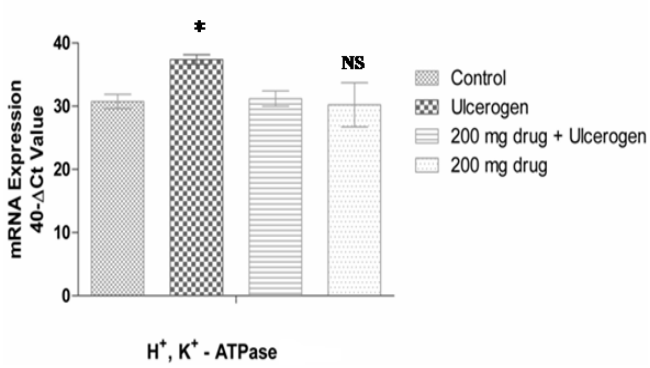


Figure 1: Expression of H^+, K^+-ATPase β subunit gene in the gastric mucosa of control and gastric ulcer model and drug pretreated animal groups

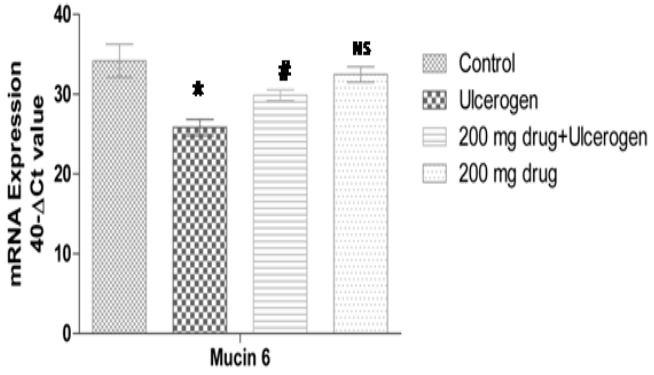


Figure 2: Expression of MUC6 gene in the gastric mucosa of control and gastric ulcer model and drug pre treated animal groups

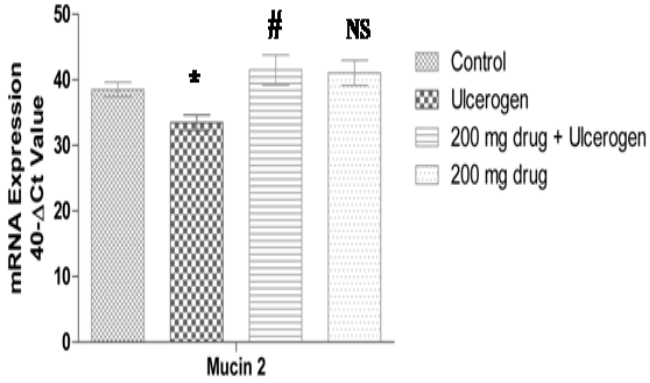


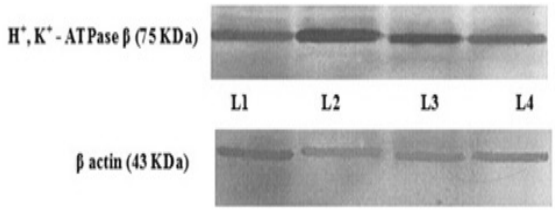
Figure 3: Expression of MUC2 gene in the duodenal mucosa of control and duodenal ulcer model and drug pre treated animal groups

Effect of P. dulce treatment on H^+, K^+-ATPase , MUC6 and MUC2 gene expression

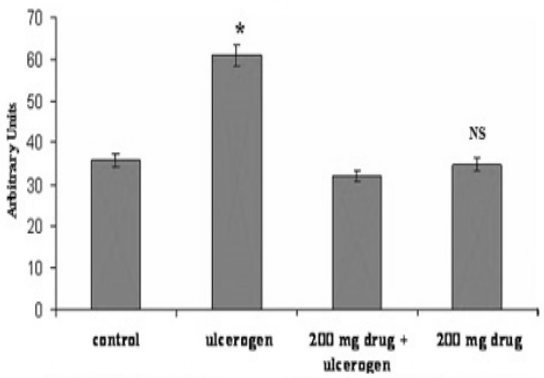
Pre-treatment with HAEPD resulted in decrease expression of H^+, K^+-ATPase β subunit in gastric ulcer models (Figure 1). This in turn would protect the gastric mucosa by reducing the hydrochloric acid secretion. The finding is suggestive that the extract from *P. dulce* exerts a proton pump inhibitor like activity. In addition, it was observed that the expression of MUC6 and MUC2 genes in the gastric and duodenal mucosa of the *P.dulce* pre-treated rats were significantly higher ($p<0.05$) in comparison with the disease models (Figure 2 and Figure 3). It was also observed that the expression of these gastroprotective proteins is up regulated in the *P. dulce* pretreated animals and the effect is similar to that of the control animals. The western blot and densiometric analysis of the expression of H^+, K^+-ATPase β subunit in the gastric mucosa of the control, gastric ulcer model, drug control groups and drug pretreated animal groups of gastric ulcer model is given in Figure 4a and Figure 4b.

DISCUSSION

Naturopathy is a traditional Indian system of medicine which utilizes the naturally occurring plants and plant products to cure the common diseases. The use of this form of medicine is on the rise around the globe in view



4a



4b

Figure 4a and 4b: Western blotting for gastric H^+, K^+-ATPase β subunit protein expression in gastric ulcer animal models. Densitometry analysis of H^+, K^+-ATPase β subunit protein expression in gastric ulcer animal models

of its minimal or no side effects. This study was carried out to elucidate the mechanism of gastro-protective property of a novel and non-toxic medicinal plant *P. dulce*. The gastroprotective and mucoprotective effects were tested by analyzing the influence of the hydroalcoholic extracts of *P. dulce* on expression of gastric H^+, K^+-ATPase β subunit and MUC6, MUC2 genes in stomach and duodenum in animal models of gastric ulcer.

The loss of mucosal integrity due to hypersecretion of HCl is the basis for all the acid base disorders such as GERD, gastric ulcer, duodenal ulcer, Zollinger-Ellison syndrome and Barrett's oesophagus. From this study, it was observed that the pretreatment of animals with *P. dulce* extracts down regulated the expression of H^+, K^+-ATPase β subunit, which was further confirmed by the western blot analysis. These findings suggest that the gastroprotective effect of *P. dulce* might be due to the down regulation of H^+, K^+-ATPase β subunit and eventual reduction of gastric acid secretion in the stomach. Inhibition of gastric acid secretion by *P. dulce* extract observed in this study is similar to the pharmacodynamics of the proton pump inhibitors.[11]

From the previous findings, the HPLC-UV chromatogram of the *P. dulce* fruit extracts were found to have various phenolic acids such as ellagic acid, gallic acid, mandelic acid, ferulic acid, vanillic acid and p-coumaric acid and flavonoids such as quercitrin, rutin, kaempferol, naringin and daidzein.[12] It was reported that the phenolic compounds were a potent blockers of H^+, K^+-ATPase enzyme. Hence it may be inferred from the findings that the phenolic compounds present in the *P. dulce* extracts might have exerted the gastroprotective effect by inhibiting the H^+, K^+-ATPase enzyme and thereby reducing the secretion of gastric acid.[13] Thus the findings of this study offers a future research scope to identify individual components in the *P. dulce* extract and to test its efficacy in the prevention of gastric ulcer with a well elucidated pharmacodynamics at molecular level.

Loss of integrity in gastric mucosa triggers the over expression of ulcer healing Epithelial growth factor (EGF) and its receptor (ErK1 and ErK2) in the gastric epithelial cells, which further activates EGF-R–MAPK signal transduction pathway and results in the secretion of mucoprotective glycoproteins and down regulation of gastric HCl synthesis. Blocking this pathway was reported to delay ulcer healing. The gastroprotective effect of EGR-R signaling pathway was found to involve many adaptor proteins such as Grb2, Shc, Sos, Ras, Raf1 kinase and MAP Kinase, whose main function is to restore the epithelial components and favour the healing of gastric ulcers.[14-16] Though the effects of *P. dulce* extract on the EGF-R–MAPK signal transduction pathway were not analyzed, the findings of this study have widened the scope to further explore the molecular mechanisms of antiulcerogenic property of *P. dulce* extracts by its interactions with the EGF-R-MAPK signaling pathway.

Gastric mucin, a protective covering of gastrointestinal mucosa consists of water and glycoproteins.[17] Mucin also acts as an antioxidant and reduces free radicals induced mucosal injury. Oxygen derived free radicals such as superoxide, hydrogen peroxide, hydroxyl radical have been well established in pathogenesis of gastrointestinal mucosal damage induced by ethanol and anti-inflammatory drugs.[18,19] MUC2 and MUC6, the natural gastric mucins, play a major role in maintaining the integrity of gastrointestinal lining by protecting them against the action of HCl secretion, proteases, pathogenic microorganisms and mechanical trauma.[20] The loss of mucosal integrity due to excessive degradation of mucosal glycoprotein is a prominent feature in the pathogenesis of gastric diseases.[21] The drug pretreated rats, in comparison with the untreated rats, upon exposure to the ulcerogens were observed to have an up regulated MUC6 and MUC2 expression and exerted enhanced mucosal resistance by augmenting the mucin production and offered a mucoprotective effect. This is an another mechanism of mucoprotection exerted by *P. dulce* fruit extracts in addition to the previous reports of ulcer healing effect by increasing hexosamine and carbohydrate/protein ratio and adherent mucus content in ulcerogen treated rats.[22,23]

CONCLUSION

In conclusion, the results suggest that *P. dulce* fruits exert gastroprotective effect by down regulating gastric H^+, K^+-ATPase synthesis and up regulation of mucin secretion in stomach and duodenum. Thus the findings strongly suggests that *P. dulce* may be included in antiulcer drug formulations either singly or with other known herbal medicines for the prevention and treatment of peptic ulcer. In addition, its interaction with molecules associated with gastroprotection needs to be explored.

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CONFLICT OF INTEREST

The author has no conflict of interest to disclose.

ABBREVIATION

HAEPD:	Hydroalcoholic fruit extract of Pithecellobium dulce
MUC 6:	Mucin 6,
MUC 2:	Mucin 2
qRT-PCR:	Quantitative real time Polymerase Chain Reaction
PUD:	Peptic Ulcer Disease

Highlights of Paper

- Peptic Ulcer is a chronic inflammatory disease of gastrointestinal tract.
- *Pithecellobium dulce* was traditionally used to treat gastric complications.
- The fruit extract exerts the mucoprotective and gastric acid antisecretory effect.
- Enhanced mucin production and decreased HCl secretion has lowered ulceration.
- *Pithecellobium dulce* may be included for anti-ulcer drug formulations.

Author Profile

- **Dr. Megala Jayaraman:** Is serving as Assistant Professor in the Department of Genetic Engineering, SRM University, Chennai. She has completed her doctorate in the field of clinical Biochemistry at Bharathiar University, Coimbatore. Her research is focused in the field of experimental gastroenterology which involves isolation and characterization of novel active principles from medicinal plants and molecular characterization of its anti-ulcerogenic property in experimental animals. In addition, she is engaged with the study of genetic factors influencing the susceptibility of Helicobacter pylori infection in Peptic ulcer disease in humans.

- **Dr. Panneer Devaraju:** Has obtained his masters in Veterinary Microbiology from Pondicherry University and currently pursuing his PhD in Clinical Immunology at Jawaharlal Institute of Post Graduate Medical Education and Research (JIPMER), Pondicherry. His research mainly focuses on immunogenetics of autoimmune diseases and establishing the molecular mechanism of autoimmune pathogenesis.

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