

Healing property of the *Piper betel* phenol, allylpyrocatechol against indomethacin-induced stomach ulceration and mechanism of action

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the mucin content of the gastric tissues. Compared to the ulcerated untreated rats, those treated with APC and misoprostol showed near normal MDA levels, while the protein levels were 86% and 78% of the normal value respectively ($P < 0.05$). Likewise, both APC and misoprostol increased the SOD, catalase, and mucin levels significantly ($P < 0.05$), the effect of APC being better.

CONCLUSION: APC can protect indomethacin-induced gastric ulceration due to its antioxidative and mucin protecting properties.

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Key words: Allylpyrocatechol; Antioxidant; Histopathology; Indomethacin; Mucin; *Piper betel*; Stomach ulcer

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Abstract

AIM: To evaluate the protective activity of allylpyrocatechol (APC), the major antioxidant constituent of *Piper betel*, against the indomethacin-induced stomach ulceration in the rat model and correlates with its antioxidative and mucin protecting properties.

METHODS: Male Sprague-Dawley rats were divided into five groups. Normal control rats (group I) were given the vehicle oral dose of gum acacia in distilled water (1 mL per rat); ulcerated control and treated rats (groups II-V) were given a single dose of indomethacin (30 mg/kg body wt.); group II rats were sacrificed 4 h after indomethacin administration; groups III-V rats were given the vehicle (1 mL per rat) or APC (2 mg/kg body wt.) or misoprostol (1.43 µg/kg body wt.) once daily by oral intubation for 7 d starting from 4 h after the indomethacin administration. After 7 d, the stomach tissues were excised for histological examination and biochemical analysis.

RESULTS: Treatment with APC (2 mg/kg body wt per day) and misoprostol (1.43 µg/kg body wt per day) for 7 d could effectively heal the stomach ulceration as revealed from the ulcer index and histopathological studies. Compared to the zero day ulcerated group, treatment with APC and misoprostol reduced the ulcer index by 93.4% and 85.4% respectively ($P < 0.05$). Both APC and misoprostol accelerated ulcer healing observed in natural recovery ($P < 0.05$), their respective healing capacities not being significantly different. The healing capacities of APC and misoprostol could be attributed to their antioxidant activity as well as the ability to enhance

INTRODUCTION

Gastrointestinal toxicity associated with nonsteroidal anti-inflammatory drugs (NSAIDs) is an important medical problem despite recent pharmaceutical advances^[1]. Besides being used as pain-killers, the NSAIDs are being increasingly used for prevention of malignancies, stroke, pre-eclampsia, Alzheimer's disease, and many other illnesses^[2-4]. The percentage of gastric ulcer cases induced by NSAIDs is emerging day by day, accounting for approximately 25% of gastric ulcers^[5]. In addition, various factors such as stress, hunger, *H. pylori* invasion etc are also known to cause gastric ulcer. Consequently, prevention of gastrointestinal disorder continues to be of concern for both medical professionals and researchers. Various synthetic anti-ulcer drugs are presently available, and some of these like misoprostol are specifically used to prevent or treat the NSAID induced gastric ulcer. However, each of these drugs confers simpler to severe side effects such as diarrhea, itching, skin rash, dizziness, and inactivation of

some antifungal drugs (proton pump inhibitors), confusion in elderly patients, headache and antiandrogenic effect (H-2 receptor blockers), constipation, vomiting, indigestion, back pain, and dizziness (sucralfate), bleeding diathesis and abortion for pregnant women (misoprostol)^[6]. Thus, there is a growing interest on non-toxic, antiulcer formulations from medicinal plants, and many taxa of medicinal plants have been assessed worldwide for their antiulcerogenic effects^[7,8]. In the developing nations, this turn of events has also been prompted, in part, by the high cost of the modern antiulcer medication.

The *Piper betel* plant is found widely growing in the tropical humid climate of South East Asia, and its leaves, with a strong pungent and aromatic flavor, are widely consumed as a mouth freshener. The Indian traditional system of medicine has identified the *P. betel* leaves with digestive and pancreatic lipase stimulant activities^[9,10]. Earlier, we also reported gastrocytoprotective properties of the leaf extract on experimentally induced gastric lesions^[11].

The major problem with the herbal drugs is the lack of their quality control and improper authentication. Identifying the major active principle(s), and ensuring their presence in optimum quantity in the herbal preparations can minimize problems associated with their questionable quality. Very recently we have found that the extraordinary antioxidant activity of the *P. betel* leaf extract is due to its major phenolic constituent, allylpyrocatechol (APC, Figure 1)^[12]. Consequently, the aim of the present study was to evaluate the healing effect of APC on indomethacin-induced acute gastric ulceration of rats and compare the activity with that of the drug, misoprostol.

MATERIALS AND METHODS

Chemicals and reagents

The *P. betel* leaves were collected from the local market and identified (collection no. 2610) by taxonomy by the Botanical Survey of India, Indian Botanical Garden, West Bengal. 2-Thiobarbituric acid (TBA), Tris, ethylenediaminetetraacetic acid (EDTA), acetic acid, methanol and ethanol were procured from E Merck (India), while trichloroacetic acid (TCA) was from Thomas Baker, India. Alcian blue, indomethacin, dimethylaminobenzaldehyde, epinephrine, Cu (II) sulphate, bovine serum albumin (BSA), hematoxylin, alum, mercuric oxide, eosin, Schiff's reagent and periodic acid were procured from Sigma Chemicals, St. Louis, MO (USA). Other reagents used were H₂O₂ (35%, Lancaster, England), and perchloric acid, 2,4-dinitrophenyl hydrazine (DNPH), K₂HPO₄, KH₂PO₄, KOH and HCl (all from SRL, India). Stock solutions of EDTA and H₂O₂ were prepared in triply distilled deaerated water just prior to use. Stock solutions (1% w/v) of TBA were prepared in 50 mmol/L NaOH solution and used within a week.

Isolation of APC from *P. betel*

APC was isolated from *P. betel* ethanol extract as in the reported procedure^[12]. The air-dried leaves of the *P. betel* (250 g) were chopped into fine pieces, soaked in 95% ethanol (1 L) for two days and the supernatant

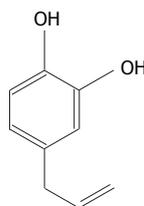


Figure 1 Chemical structure of allylpyrocatechol.

decanted. The entire process was repeated three times, the combined extracts were filtered through a nylon mesh, and evaporated in vacuo. The extract (8.0 g) was dissolved in methanol (50 mL), treated with activated charcoal (0.2 g), and the mixture warmed at -60°C. After filtration, the extract was concentrated under vacuum, and finally lyophilized to obtain a chlorophyll-free amorphous yellowish brown solid (yield: 1.23% w/w) that was stored in a vacuum desiccator.

The extract (4.7 g) was subjected to column chromatography over silica gel using gradient elution. The column was eluted with 0, 2%, 5% and 10% ethyl acetate/hexane followed by 0, 5%, 10%, 15%, 50% and 100% methanol/chloroform (500 mL each), and 50 mL fractions were collected. The fraction eluting with 10% ethyl acetate/hexane furnished APC (yield: 0.9% w/w of the extract) as a light yellow oil, which was characterized using IR and ¹H NMR spectroscopic data. The chemical structure of APC is shown in Figure 1.

3,4-Dihydroxyallylbenzene (Allylpyrocatechol, APC): ¹H NMR (CDCl₃): δ 3.28 (d, *J* = 6.5 Hz, 2H, ArCH₂), 4.99-5.08 (m, 2H, olefin), 5.36 (broad, 2H, 2 × Ar-OH), 5.80-6.01 (m, 1H, olefin), 6.59-6.79 (m, 3H, ArH).

Preparation of the drug

A specific dry weight of APC or misoprostol was macerated with a mortar and pestle in double distilled water containing gum acacia 2% (w/w) to provide the drugs, which were administered po.

Animals

The rats were bred at Dr. B. C. Roy Post Graduate Institute of Basic Medical Sciences, Kolkata, India and BARC Laboratory Animal House Facility, Mumbai, India. These were procured after obtaining clearance from the respective Animal Ethics Committee of the two centers and were handled following international Animal Ethics Committee guidelines. Male Sprague-Dawley rats (weighing 180-200 g) were reared on a standard laboratory diet (Ralston Purina, Chicago, Illinois, USA) and given tap water. They were kept in a room where temperature (20 ± 2°C), humidity (65%-70%), and day/night cycle (12/12 h) were controlled.

Experimental protocol for indomethacin-induced ulceration and healing

Ulceration in the rats was induced with a single dose of indomethacin (30 mg/kg body wt., oral intubation) dissolved in distilled water. The rats were deprived of food but had free access to tap water 24 h before ulcer induction.

For the standardization of drug dose, APC (0.5, 1, 2,

5, and 10 mg/kg body wt.) was given once daily by oral intubation for 7 d starting from 4 h after the indomethacin administration. Five rats were taken for each dose and each experiment was repeated three times. The extent of healing was assessed from the macroscopic damage scores. The effect of the treatment with APC (2 mg/kg body wt.) for 10 d was also studied.

Based on the results of dose optimization, the histopathological, and biochemical parameters were assessed in separate experiments. For this, a total of 25 rats were randomly divided into 5 groups in each set of experiment, which was replicated three times. Group I rats serving as the normal control received only the vehicle oral dose of gum acacia in distilled water (1 mL per rat). Ulceration was induced in the groups II-V rats. Group II rats serving as the experimental control received only indomethacin and were sacrificed 4 h after indomethacin administration. Group III-V rats were given the vehicle (1 mL per rat) or APC (2 mg/kg body wt.) or misoprostol (1.43 µg/kg body wt.) once daily by oral intubation for 7 d starting from 4 h after the indomethacin administration. After 7 d, the rats of group I and groups III-V were sacrificed under ether anesthesia, followed by cutting off the aorta abdominalis.

Macroscopic and histopathological studies

The stomach from the normal and treated groups were removed rapidly, opened along the greater curvature, and thoroughly rinsed with normal saline. After recording the ulcers produced in the stomach, a longitudinal section of the gastric tissue was taken from the anterior part of the stomach and fixed in a 10% formalin solution. After 24 h of fixation followed by embedding in a paraffin block, it was cut into sections of 5 micron onto a glass slide and stained with hematoxylin-eosin for histological assessment of the gastric mucosa. For biochemical studies, the stomach was opened along the greater curvature, and the gastric antral portion was used. The wet weight of the portion was also recorded.

Quantification of ulceration

Area of glandular portion comprising of the fundic and corpus region of each stomach was measured in square millimeters. The total eroded gastric mucosal areas (lesions) were also measured (mm²) with a dissecting microscope under × 20 magnification. The percentages of the whole glandular area contributing to the damaged part are referred to as the ulcer indices according to a reported procedure^[13]. The ulcer indices for the cold stress rats were also calculated in the same manner.

Preparation of tissue homogenate

The stomach tissue was homogenized in a 50 mmol/L phosphate saline buffer (PBS) pH 7.2 under cold condition, using a glass-teflon homogenizing tube. The homogenate was centrifuged at 2500 r/min for 10 min and the supernatant was carefully removed from the pellet and used for biochemical analyses.

Quantification of lipid, protein and DNA damages

The lipid peroxidation products were estimated^[14] in terms

of malondialdehyde (MDA) formation. The amounts of protein carbonyls and DNA contents were estimated following reported methods^[15,16].

Assessment of enzymatic activities

The specific activity of catalase (CAT) in the tissue was estimated according to a reported method^[17]. The tissue homogenate (20 µL) was added to a H₂O₂-phosphate buffer mixture (3 mL), maintaining the optical density at 240 nm to 0.500 ± 0.010 ($d = 1$ cm) against buffer. The rate of change of optical density at 240 nm with time was recorded for the calculation of the catalase activity.

Following a reported method^[18], the tissue superoxide dismutase (SOD) activity was measured. This involves assaying the SOD-mediated inhibition of epinephrine autooxidation at an alkaline medium. For this, the absorbances of the samples at 480 nm were noted at an interval of 30 s. The enzyme activity was measured in arbitrary units, considering 50% inhibition as 1 unit of enzyme activity.

Mucin assay

Following a reported method^[19], the free mucin in the gastric tissues was estimated. Briefly, the gastric tissues were incubated with a 1% buffered solution of Ab in 3% aqueous acetic acid (0.5 mL) at 37°C for 30 min. After centrifuging, the concentration of Ab in the solution was measured from the absorbance at 615 nm.

Hexosamine assay

The hexosamine concentrations in gastric tissues were assayed according to a reported method^[20] with a minor modification. The gastric tissues were hydrolyzed in acidic medium, the hydrolysate neutralized with 3 mol/L NaOH (litmus) and diluted to 10 mL with double distilled water. Acetyl acetone solution (1 mL, prepared by dissolving 1 mL of acetyl acetone in 50 mL 0.5 mol/L sodium carbonate) was added to the above solution (1 mL), which was mixed well, and heated on a boiling water bath for 15 min avoiding evaporation. After cooling, ethanol (5 mL, 95%) was added to the mixture followed by Ehrlich's reagent (1 mL, prepared by dissolving 0.8 g para-dimethylaminobenzaldehyde in 30 mL methanol and 30 mL conc. HCl). The mixture was diluted to 10 mL with 95% ethanol, allowed to stand for 30 min, and its absorbance at 530 nm was read.

Statistical analyses

All the results were expressed as mean ± SE. Student's paired *t*-test for comparison between groups and one way analysis of variance (ANOVA) for multi-sample groups at $P < 0.05$ were used to assess statistical significance in various groups of animals.

RESULTS

Effect of APC on healing indomethacin-induced gastric ulceration in rats

Oral administration of APC at different doses accelerated the rate of healing of gastric lesion induced by indomethacin. As shown in Table 1, for a 7-d treatment,

Table 1 Comparative healing capacity of different doses of APC against indomethacin-induced ulceration in rats¹

Samples	Treatment		Ulcer index ¹
	Dose (mg/kg body wt.)	d	
Unulcerated control			0 ± 0
Ulcerated control (4 h) ²	-	-	26.71 ± 1.62
APC treated	0.5	7	20.22 ± 1.17
APC treated	1	7	11.21 ± 0.91
APC treated	2	7	1.78 ± 0.34
APC treated	5	7	1.67 ± 0.28
APC treated	10	7	1.83 ± 0.51
APC treated	2	10	1.65 ± 0.31

¹Stomach ulceration in rats was induced by oral administration of indomethacin (30 mg/kg body wt.). The ulcer indices values are mean ± SE (*n* = 15). ²The ulcer indices were measured 4 h after indomethacin administration. For other samples the measurement was carried out after 7 d.

increasing the dose of APC from 1 to 2 mg/kg body wt. led to a significantly improved ulcer healing. Increasing the doses of APC further did not provide any additional benefit. Even when the treatment with APC (2 mg/kg body wt.) was continued for 10 d, the extent of ulcer healing remained almost the same. Thus, administration of APC at a dose of 2 mg/kg body wt. for 7 d after ulcer induction was found to be optimal for effective ulcer healing. Hence all subsequent experiments were carried out with the same protocol. The dose of the positive control, misoprostol (1.43 µg/kg body wt.) was decided based on its recommended therapeutic dose for humans. Earlier misoprostol has been used at significantly higher doses with the rat models^[21]. However, our studies were primarily aimed at developing anti-ulcer drugs for humans. For this it was essential to compare the ulcer-healing efficacy of APC with that of the established drug, misoprostol at the dose recommended for humans.

Treatment of rats with indomethacin (30 mg/kg body wt.) by oral intubation produced typical time-dependent acute lesions in the gastric mucosa, while the rats receiving vehicle only showed no visible mucosal lesions. Compared to the zero day ulcerated group (group II) for which an ulcer index was taken as 100%, treatment with APC and misoprostol reduced the ulcer index by 93.4% and 85.4% respectively. The ulcer healing data of the treated groups (APC and misoprostol) were significantly different from those of both 0 and 7 d untreated experimental control rats (groups II and III) (*P* < 0.05). However, the difference in healing between the two treated groups (groups IV and V) was insignificant (Table 2).

The visible morphological features of the ulcerated rat stomachs of the treated and untreated groups were compared to those with normal rats. This revealed that the zero-day ulcerated stomach had a number of blood clots in the ulcer spots and perforations. The stomach of the untreated experimental control rats (group III) showed lesser spots, but the tissues were hyaline in nature. The result with misoprostol treatment was marginally better than that of the group III rats. In comparison, stomachs of the APC treated rats (groups IV) were healthy, and equivalent to those of the normal control rats (group I).

Table 2 Comparative healing capacity of APC and misoprostol on ulcerated rats¹

Samples	Ulcer index ¹	Protection(%) ²
Unulcerated control	0 ± 0	-
Ulcerated control (4 h) ³	26.71 ± 1.62	0
Ulcerated untreated control	17.64 ± 1.24	36.08 ± 1.38
Ulcerated APC treated	1.78 ± 0.34	93.26 ± 6.77
Ulcerated misoprostol treated	3.91 ± 0.30	85.40 ± 3.51

¹Stomach ulceration in rats was induced by oral administration of indomethacin (30 mg/kg body wt.). APC (2 mg/kg body wt.) and misoprostol (1.43 µg/kg body wt.) were used for these experiments. The ulcer indices values are mean ± SE (*n* = 15). ²Considering an ulcer index of 100 for the ulcerated, untreated rats. ³The ulcer indices were measured 4 h after indomethacin administration. For other samples the measurement was carried out after 7 d.

Histopathological investigation of the healing capacity of APC

The photomicrographs of rat stomachs belonging to groups I-V shown in Figure 2A-E gives a better comparison of the gastric lesion and its healing. Significant acute ulceration in rats was developed within 4 h of indomethacin (30 mg/kg body weight) administration as revealed from Figure 2A (normal rats) and Figure 2B (rats after 4 h of indomethacin administration). Indomethacin intake induced severe and extensive macroscopic gastric mucosal damage in the irrigated starved rats, characterized by injury in the epithelial layer of the mucosa. The lamina propria also were greatly damaged along with elongated hemorrhagic lesions confined mainly to the gastric corpus and running parallel to the long axis of the stomach that had the highest ulcer scoring rate. The disruption in the gastric mucosa was partially restored after 7 d even without any treatment, revealing autohealing. These observations matched well with the ulcer index parameter. Histology of the rat stomach of the untreated ulcerated group showed some damage in the mucosal layer with moderate infiltration in the mucous membrane (Figure 2C). However, the gastric mucosal tissues of the APC treated group (Figure 2D) showed almost normal and continuous mucosal layer and formation of the epithelial layer. The efficacy of APC was better than that of misoprostol as revealed from the Figures 2D and E.

Effect of APC on lipid peroxidation and DNA and protein contents in gastric tissues of ulcerated rats

The effects of indomethacin intake alone, and following administration of APC on the extent of lipid peroxidation (measured in terms of MDA), protein oxidation (measured in terms of total proteins and protein carbonyls), and DNA damage in the gastric tissues of rats are shown in Table 3. Indomethacin administration markedly stimulated lipid peroxidation in gastric tissues, and the MDA content was elevated by about 14 times compared to the normal rats. This was reduced by 11% after 7 d due to autohealing for the untreated control rats (group III), although the MDA content remained significantly high (13 times) compared to that in the normal rats. Administration of APC for 7 d significantly reduced the MDA level (2.02 nmol/mg prot.)

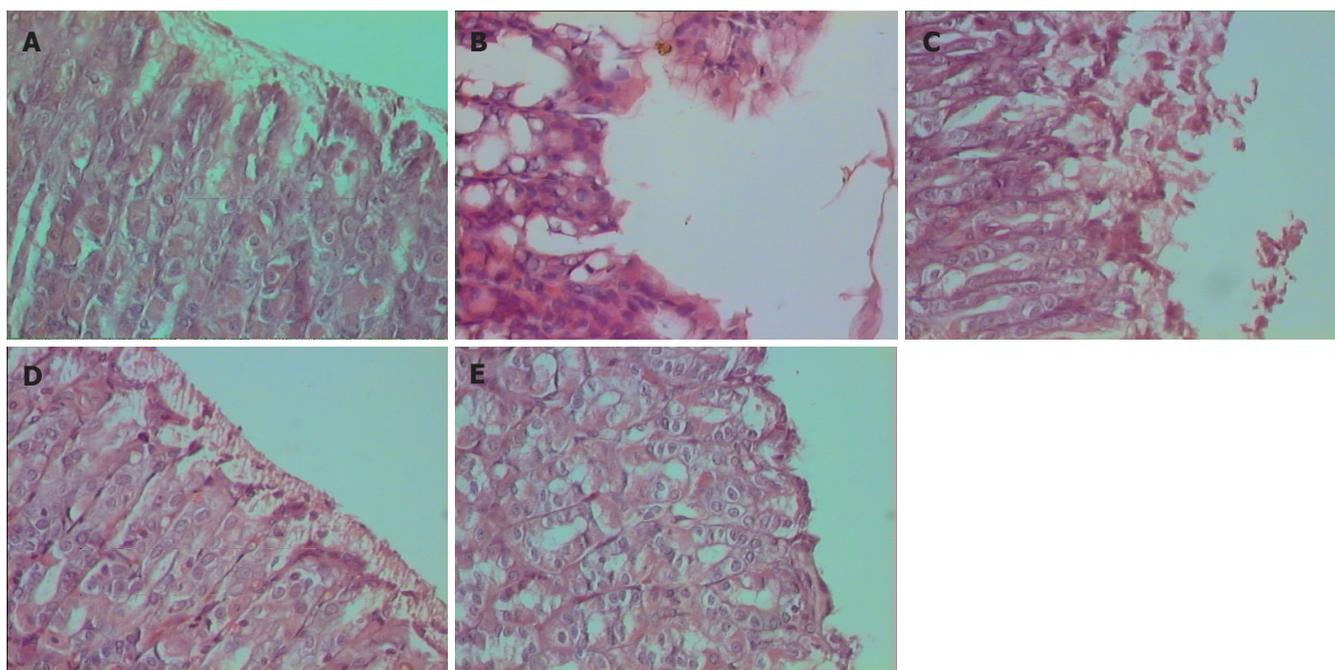


Figure 2 Histological assessment of acute gastric mucosal injury induced by indomethacin in rats and its prevention by APC (2 mg/kg body wt.) and misoprostol (1.43 µg/kg body wt.). Section of rat stomachs obtained from **A:** normal control rats; **B:** ulcerated untreated control rats 4 h after indomethacin administration; **C:** ulcerated untreated control rats 7 d after indomethacin administration; **D:** ulcerated rats treated with APC for 7 d; **E:** ulcerated rats treated with misoprostol for 7 d.

Table 3 The effect of APC and misoprostol on lipid, protein, DNA, SOD and CAT levels of ulcerated gastric tissue of rats¹

Parameters	Group I	Group II ²	Group III	Group IV	Group V
MDA (nmol/mg protein)	1.89 ± 0.01	27.04 ± 0.84	24.28 ± 1.12	2.02 ± 0.03	2.62 ± 0.02
Proteins (mg/mL)	16.52 ± 6.05	9.03 ± 1.28	12.55 ± 1.18	14.21 ± 1.8	12.89 ± 0.87
CO (mg/mg protein)	5.26 ± 1.34	17.29 ± 1.83	9.46 ± 2.15	6.03 ± 1.51	6.54 ± 1.12
DNA (mg/g tissue)	1.82 ± 0.22	0.81 ± 0.06	1.12 ± 0.21	3.56 ± 0.05	1.68 ± 0.23
SOD (U/min/mg protein)	22.2 ± 2.34	5.76 ± 2.32	11.58 ± 1.32	21.14 ± 1.24	16.71 ± 2.57
CAT (U/min/mg protein)	21.2 ± 1.12	9.02 ± 2.54	14.20 ± 2.84	19.04 ± 2.15	15.84 ± 2.09

¹Stomach ulceration in rats was induced by oral administration of indomethacin (30 mg/kg body wt.). APC (2 mg/kg body wt.) and misoprostol (1.43 µg/kg body wt.) were used for these experiments. The values are mean ± SE (*n* = 15). ²The assays were carried out 4 h after indomethacin administration. For other samples these were carried out after 7 d.

in gastric tissues almost to that of the normal rats (1.89 nmol/mg prot.). The effect of misoprostol was marginally less (MDA level 2.62 nmol/mg prot.) to that of APC. The data for the APC and misoprostol treatment are significant compared to that of the indomethacin induced untreated ulcerated control rats of groups II and III (*P* < 0.05).

Compared to the protein contents in the normal animals, the ulcerated group showed poor protein content (54% reduction), which improved for the untreated group on the 7 d and became 76% of the normal value. Treatment with APC and misoprostol increased the protein levels respectively to 86% and 78% of the normal value. The increase of the protein contents by the APC was significantly different (*P* < 0.05) compared to both ulcerated and untreated ulcerated controls (groups II and III). However, the data of misoprostol treatment group (group V) was not significantly different from that due to autohealing (group III).

The oxidative damage to tissue proteins was also

evaluated by assessing the contents of protein carbonyls. The amount of protein carbonyls that increased significantly (329% compared to normal rats) due to indomethacin administration, was reduced by 45.3% due to autohealing. APC treatment restored the level of protein carbonyls to normalcy (6.03 µg/mg *vs* 5.26 µg/mg protein in normal rats), while the effect of misoprostol was less (6.54 µg/mg protein). The reduction of protein carbonyls by APC was significant (*P* < 0.05) as compared to untreated ulcerated controls (group III).

The tissue DNA concentration was significantly (56%) reduced by indomethacin administration. During autohealing, the DNA level increased to 61.5% of the normal value. Surprisingly the APC treatment increased the DNA level by 196% compared to the normal rats, while treatment with misoprostol restored the level of DNA to normalcy (1.68 mg/g tissue for treated *vs* 1.82 mg/g tissue for normal rats). The augmentation of DNA by APC was significant (*P* < 0.05) compared to the ulcerated control (group II) as well as the misoprostol group.

Effect of APC on the SOD and CAT levels in gastric tissues of ulcerated rats

The effect of indomethacin intake alone, and following administration of APC on the tissue levels of SOD and CAT in gastric tissues of rats are also presented in Table 3. Following indomethacin administration, the levels of these enzymes in gastric tissue were also depleted. The SOD activity decreased by 74%, while that of CAT was reduced by about 57% compared to those in normal control rats. Treatment with APC increased the SOD activity ($P < 0.05$) to near normalcy (21.1 U/min *vs* 22.2 U/min per mg protein in normal rats), while the CAT level showed 90% recovery ($P < 0.05$). The effect of misoprostol was significantly less than that of APC ($P < 0.05$) restoring the SOD and CAT levels to 75% and 74% of the normal values. In comparison, the autohealing restored the SOD and CAT levels to 52% and 67% of the normal values only. The data for the APC-treated rats were significantly different ($P < 0.05$) from those of the untreated 0 and 7 d groups.

Effect of APC on hexosamine and mucin contents of ulcerated gastric mucosa

Alcian blue assay: Indomethacin administration to rats significantly decreased the secretion of mucin (43.5%, $P < 0.05$) and mucosal glycoproteins (76.4% decrease, $P < 0.05$) in the ulcerated rats of group II compared to those in un ulcerated rats (group I). Treatment with APC enhanced the tissue mucin level to that in normal un ulcerated rats, while the mucosal glycoprotein content was also increased to 65.4% of the normal value. The augmentation of mucin level by APC was significant ($P < 0.05$) as compared to ulcerated controls (group II). Misoprostol also increased the mucin secretion and mucosal glycoproteins in the ulcerated rats to 94.2% and 89.9% of the respective normal values. The results are summarized in Table 4.

PAS staining: The photomicrographs of groups I-V rat stomachs after PAS staining are shown in Figure 3A-E. The indomethacin-induced stomach ulceration led to a drastic reduction in the mucin content that was partially restored due to autohealing. However, treatment with APC improved the mucin content in the stomach tissues (Figure 3D) further, bringing to a normal level (Figure 3A) along with considerable restoration of stomach morphology and epithelium lining. In comparison, the effect of misoprostol was not significantly different from that due to autohealing.

DISCUSSION

Oxygen free radicals are known to play a role in the induction and pathogenesis of gastroduodenal injury^[22]. Extensive research has proven that antioxidants might be effective not only in protecting against gastric mucosal injury, but also inhibiting progression of a gastric ulcer. Ulcer progression is caused by free radical-induced chain processes. Consequently, its arrest by radical scavengers helps in faster healing. Indomethacin is known to induce the reactive oxygen metabolites in animal models, which

Table 4 The effect of APC and misoprostol on mucin and hexosamine levels of ulcerated rats¹

Samples	Hexosamine (mg/mg protein)	Mucin ²
Unulcerated control	61.27 ± 2.28	4.62 ± 2.24
Ulcerated control ³	14.48 ± 1.62	2.60 ± 4.14
Ulcerated APC treated	40.05 ± 3.78	4.53 ± 1.38
Ulcerated misoprostol treated	55.10 ± 1.21	4.35 ± 0.68

¹Stomach ulceration in rats was induced by oral administration of indomethacin (30 mg/kg body wt.). APC (2 mg/kg body wt.) and misoprostol (1.43 µg/kg body wt.) were used for these experiments. The values are mean ± SE ($n = 15$). ²Ab binding proteins. ³The measurement was carried out 4 h after indomethacin administration. For other samples the measurement was done after 7 d.

may contribute to mucosal injury^[23]. The cytoprotective role of antioxidants in the prevention and healing of gastric lesions has been widely investigated in a number of studies^[24,25].

The notion that APC has shown a powerful antioxidant potential in various *in vitro* models^[26] warranted our attention to address its possible protective effects against indomethacin-induced gastric lesions in rats. Earlier, the cytoprotective and healing properties of *P. betel* crude extract against indomethacin as the gastric mucosal irritant was established by us^[11]. The aim of the present study was, therefore, to study the healing effect of its principal antioxidant constituent, APC on indomethacin-induced acute stomach ulceration of rats.

Administration of indomethacin to rats induced marked damage to the gastric mucosa as evident by macroscopic and histopathological examinations. This led to elongated hemorrhagic lesions, confined to the glandular portion, with the highest subjective ulcer-scoring rate.

In the ulcerated control animals that were given the vehicle only during the seven-day period of treatment, the ulcer craters also receded through the process of autohealing, reducing the ulcer index by 36.2% for the group III rats even without any drug treatment. The present study demonstrated that APC had a potent healing effect on indomethacin-induced gastric lesions in rats. The rate of healing in the APC-treated animals was significantly faster than that found in the case of autohealing. The histopathological observations revealed that the ulceration was acute explaining the autohealing observed in the untreated control rats.

Tissue damage is always associated with the loss/reduction of DNA content and loss or impairment of protein synthesis^[27] due to excess generation of free radicals. These free radicals also damage the cellular antioxidant enzymes such as CAT, SOD and others, acting as the first line of cellular defense against oxidative injury. This might lead to aggravated tissue damage during stomach ulceration^[28]. Our results revealed that the indomethacin-induced stomach ulceration was accompanied with a severe oxidative stress in gastric tissue causing damages to key biomolecules such as lipids, proteins and DNA. This was apparent from the stimulated lipid and protein oxidation leading to increased accumulation of MDA and

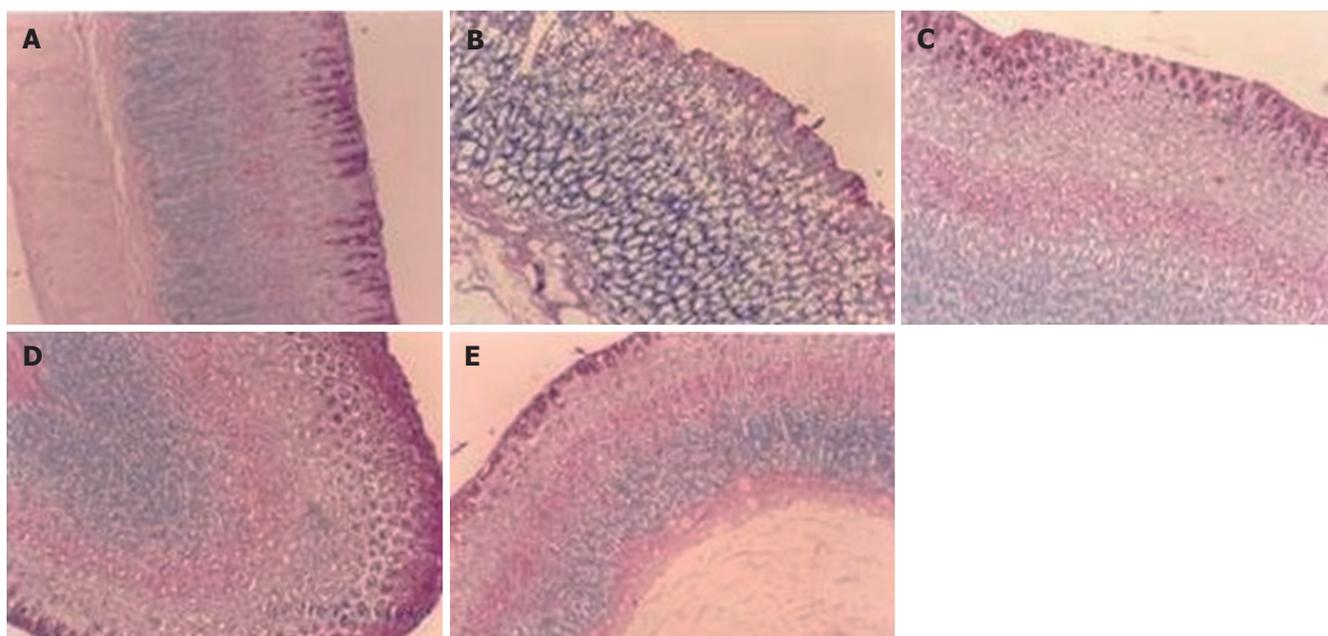


Figure 3 Depletion of mucin level in rat stomachs due to indomethacin-induced acute gastric ulceration and its prevention by APC (2 mg/kg body wt.) and misoprostol (1.43 μ g/kg body wt.) as revealed by PAS staining. Section of rat stomach obtained from **A**: normal control rats; **B**: ulcerated untreated control rats 4 h after indomethacin administration; **C**: ulcerated untreated control rats 7 d after indomethacin administration; **D**: ulcerated rats treated with APC for 7 d; **E**: ulcerated rats treated with misoprostol for 7 d.

protein carbonyls, as well as reduction in the tissue protein and DNA contents. These findings are in tune with many previous reports. APC provided a marked suppression of oxidative damage due to its excellent radical scavenging capacity and brought most of these parameters to normal levels, than observed in natural recovery.

Accumulation of the reactive products is known to markedly alter the antioxidant enzymes leading to enhanced oxidative damage during stomach ulceration^[28]. In the present study, the gastric activities of SOD and CAT were found to be reduced notably following indomethacin intake. Treatment with APC reversed these oxidative changes with concomitant increase in SOD and CAT levels, thereby suppressing most of the biochemical adverse effects induced by indomethacin. This might decrease the ulcer progression and promote healing of gastric lesions induced by acute intake of indomethacin.

Ulcer-healing is a complex process involving a combination of wound retraction and re-epithelization^[29]. Release of preformed mucus also plays a role in promoting epithelial recovery after acute injury by forming a mucoid cap beneath which re-epithelization occurs^[30]. Besides providing significant buffering capacity for the neutralization of luminal acid, the mucus can offer protection against the endogenous aggressors like pepsin and oxidants produced in the gastric lumen, as well as against exogenous damaging agents, such as NSAIDs.

The macromolecular glycoprotein, hexosamine constitutes the major fraction of gastric mucin and is accepted to be an ideal index of gastric mucus production^[31]. The NSAID-produced mucosal hemorrhagic ulcer may be due to a decrease of gastric mucus production^[32]. Thus, drugs that arrest ulcer progression by antioxidant action, and also increase the synthesis and secretion of gastric mucus

would accelerate gastric ulcer healing.

In this study, the decreased mucin secretion in the indomethacin-administered rats indicated reduced ability of the mucosal membrane to protect the mucosa from physical damage and back diffusion of hydrogen ions. The decrease in the glycoprotein content of the gastric mucosa further proved the decreased ability of the gastric mucosa to withstand the offensive onslaught. Mucosal damage can be easily produced by the generation of exogenous and endogenous active oxygen and free radicals^[33]. An increase in mucus production usually assist the healing process by protecting the ulcer crater against irritant stomach secretions (HCl and pepsin) thereby enhancing the rate of the local healing process. Treatment with APC and misoprostol significantly accelerated the ulcer healing process, which is associated with an increase in the mucus layer in the gastric mucosa. Apparently, the free radicals scavenging property of APC might be contributing in protecting the oxidative damage to gastric mucosa.

It is well known that PAS stain is the best method to identify gastric metaplasia of duodenal mucosa. Metaplastic gastric type epithelium in the duodenum has been regarded as an adaptive defensive response to mucosal injury of any kind, including acid and/or pepsin^[34]. The presence and extent of gastric metaplasia have been correlated with acid secretory capacity in men^[35], and they have been induced in experimental animals by stimulation of excessive acid secretion. In contrast to the untreated controls in which the autohealing process can be attributed to other mechanisms, the healing action of APC is evidently related to its ability to improve mucus production. All these indicated that enhancement of the mucus modulation by APC play a significant role in its ulcer healing effect.

Given that some drugs can show mild-to-severe side

effects even after short-term intake, we also evaluated the possible toxic effects of APC up to a dose of 25 mg/kg body wt. with both mice and rats. There was no observable physical sign change and the animals had normal food and water as well as stool during the experimental period. These findings suggested that APC given at the current dose does not have any potential side effects in the animals. The non-toxicity of APC was expected considering that it is the major constituent of *P. betel* leaves that is freely consumed in India and South-East Asian countries.

In conclusion, the present study established that the APC, the major constituent of *P. betel* leaves can heal indomethacin-induced stomach ulceration in rats by its antioxidant action and ability to form mucus. Apparently, by scavenging free radicals, APC might be protecting the gastric mucosa, and in turn, the stomach epithelium from the oxidative damage. This accelerates healing of gastric ulcers. Earlier, our group has also reported the cytoprotective activity of the crude ethanol extract of *P. betel* leaves against indomethacin-induced stomach ulceration. These results, taken together along with the non-toxicity of APC indicate its potential as an anti-ulcerogenic drug for further investigations. Comparison of its efficacy with that of misoprostol further confirmed the findings.

Ulceration due to NSAID is also believed to occur because of non-selective inhibition of cyclooxygenases that hampers the release of mucus due to reduction in prostaglandin synthesis. The relationship between prostaglandins and leukotrienes, the products of prostaglandin H synthase (PGHS) and 5-lipoxygenase, respectively, seems to be an important factor in gastric ulcers. Many phenolic compounds stimulate prostaglandin synthesis by acting as reducing substrates for the oxidized intermediates of PGHS, thereby accelerating the peroxidase cycle and by functioning as electron-donating co-substrates for the peroxidase component of PGHS. In this way, they can modulate the PGHS and 5-lipoxygenase pathways of arachidonic acid^[36]. Thus, it would be of interest to study the effect of APC on the PG-dependent pathway of healing gastric ulcer. Investigation in this regard is currently in progress in our laboratory and the results will be reported later.

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