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POTENT ANTIULCEROGENIC ACTIVITY OF ETHANOL EXTRACT OF LEAF OF *Piper betle Linn* BY ANTIOXIDATIVE MECHANISM

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ABSTRACT :

Pretreatment of an ethanolic extract of leaf of *Piper betle linn* at a dose of 200mg/kg body weight, orally administered to rats for ten consecutive days, was found to possess a significant protective action against gastric lesions induced by indomethacin. The extract pretreatment resulted in significant increase in superoxide dismutase (SOD) and catalase (CAT) activity, increase in mucus, hexosamine and total thiol group content, but marked reduction in oxidatively damaged protein and peroxidised lipid levels as compared to untreated ulcerated control. The extract was also found to possess both superoxide and hydroxyl free radical scavenging action. The present observations establish the efficacy of the extract in prevention of experimentally induced peptic ulcer by indomethacin and antioxidant property appears to be predominantly responsible for such cytoprotective activity in the experimental model.

KEY WORDS :

Antioxidant, Antiulcer, Free radical scavenger, *Piper betle Linn*,

RUNNING TITLE :

Piper betle linn as antioxidant and antiulcerogenic agent

INTRODUCTION

Piper betle Linn (Piperaceae), popularily known as pan (Bengali), pan (Hindi), tambuli (Sanskrit), is a widely growing plant in India and some tropical countries .In the traditional medicinal systems, the leaf of this plant has been advocated for the treatment of wounds, boils, bites of insects and for enhancing digestion (1,2). A literature survey also reveals its significant stimulatory influence on intestinal lipase and amylase activity(3). Triterpenes and β-sitosterol, isolated from *Piper betle Linn* , have been shown to possess antiplatelet and anti-inflammatory activity(4).

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Induction of peptic ulcer is a common side effect of most non-steroidal anti-inflammatory drugs and the role of oxygen free radicals(5,6) in the development of pathogenesis in acute experimental gastric lesions by NSAID is well known(7,8,9,10). Moreover 75%-85% of chronic ulcers in human is due to *Helicobacter pylori* infection in stomach wall, which potentiates the polymorphic nuclear leucocyte oxidative burst, leading to considerable production of reactive oxygen metabolites (11), which degenerate the tissue causing ulcer (12). In view of the undesireable side effects and/or drug interaction, in conventional therapy of peptic ulcer that includes antibacterial agents (13), H2 receptor antagonist (14), proton pump inhibitors, prostaglandins (15), and considering the mentioned antioxidative effect of *Piper betle Linn,* the anti parasitic and antimicrobial efficiency of the leaf against human pathogenic bacteria (16), phytopathogenic fungi and anti -inflammatory effect , investigations were undertaken to ascertain

any possible effect of the leaf as antiulcerogenic and antioxidant and any plausible link between these two properties.

MATERIALS AND METHODS

Plant Materials

Commonly edible fresh leaves of *Piper betle Linn* (pungent variety) , collected during the months of October -December from the local market of Kolkata , and identified by Botanical Survey of India , as Piper betle Linn (piperaceae family) were cut into small pieces and pasted with 95 % ethanol , forming a slime . The slime was then cold percolated with ethanol for seven days in an aspirator and the solvent was separated . The process was repeated thrice . The ethanol soluble fraction was evaporated under vacuum in a rotary evaporator and the residue (yield 1.2% w/w in terms of starting material -a yellowish brown mass) was kept in a vacuum desiccator until use. The material was macerated with double distilled water and gum acacia (final concentration of gum acacia in solution is 2%) for experimental use. This was considered as "Drug" in subsequent experiments .

Experimental Animals

Charles-Foster rats (120-150g), maintained under uniform laboratory conditions, maintaining a light and dark cycle 12:12 hours, and uniformly controlled temperature of 25 $\pm 1^{0}$ C for at least 10 days, housed in standard metal cages and fed with pellet diet (Hindustan Lever, India) and water *ad libitum,* were used for experiments. The analytical grade chemicals used, were either from Sigma or E. Merck.

Animal Experiments

Rats were divided into three groups of six each, as follows:-

Group A : Normal healthy rats (normal control)

Group B: Ulcerated, with no use of "Drug" (Experimental control)

Group C : Ulcerated, with pretreatment with "Drug"(Experimental)

While the animals of Group A and B were gavaged with gum acacia solution (2%) , those in group C were fed the "Drug" with 2% gum acacia as vehicle for ten consecutive days. Thereafter, all the groups were kept fasting for 48hrs. Indomethacin was given to Groups B and C (15 mg /kg body weight x two days = total 30mg/ kg body weight) for two consecutive days during fasting condition, and the animals were sacrificed under ether anaesthesia 3 hours after the last dose of indomethacin.

The stomach was examined under light microscope and ulcer spots counted were for scoring ulcer index, according to Vogel and Vogel (17) , to evaluate the optimal dose of the "Drug". A graduation of doses of the extract was administered orally, the different doses being used were, 100mg/kg body weight , 150 mg/kg body weight, 200mg/kg body weight and 250 mg/kg body weight . From the highest percentage decrease of ulcer index , i.e. maximum protection (Table I), the optimal dose, found to be 200mg/kg body weight, was used throughout the experiment. The extent of protection of the "Drug " treated group was compared with the normal control and experimental control. For biochemical analysis, gastric tissues were taken from the antral portion of the stomach. After scrapping off the outer fibrous layer , the inner layer of the stomach was homogenized in 4mM phosphate saline buffer and the supernatant was used for comparison of subsequent parameters .

Assay of Malondialdehyde (MDA)

Lipid peroxide was estimated by thiobarbituric acid method using malondialdehyde (MDA) as described by Das *et al* (18).

Assay of Oxidatively Damaged Protein

Oxidatively damaged protein was determined in terms of carbonyl content according to Rodney *et al* (19).

Assay of Superoxide dismutase (SOD) activity

The method of McCord and Fridovich (20) was followed, involving inhibition of epinephrine auto-oxidation in an alkaline medium at 480nm. The readings were noted at 30secs interval and the enzyme activity was expressed in arbitrary units, considering 50% inhibition of autooxidation of epinephrine, as 1 unit of enzyme activity.

Assay of Catalase (CAT)

The catalase activity was determined according to Lück *et al* (21).100 (lml of tissue supernatant was added to a solution containing 3 ml of H_2O_2 -phosphate buffer mixture. The change in optical density (OD) at 240nm was aeasure of catalase activity. The concentration of the H_2O_2-buffer was standardized to get the OD against buffer at 240 nm to 0.500 ± 0.01 (d=1cm).

Assay of Hexosamine

Hexosamine content in gastric tissue was assayed according to the method followed by Glick *et al* (22). The tissue was hydrolysed with 6N HCl overnight . The hydrolysate was neutralized with 6 N NaOH and treated with acetylacetone on a waterbath for 15 mins. The product coupled with Ehrlich's Reagent, containing p-dimethylaminobenzaldehyde, to form a coloured derivative, which was measured spectrophotometrically at 530nm.

Assay of Mucus

The mucus content in gastric tissue was estimated as described by Corne *et al* (23) by measuring the amount of Alcian Blue(AB) (1% in 3% acetic acid) bound to mucous, spectrophotometrically at 615nm.

Assay of tissue sulfhydryl group

Tissue homogenate was taken up with 10mM phosphate buffer (pH =8.0) and 0.2 ml of 10mM DTNB (5,5'-dithiobis -2-nitrobenzoic acid) of pH 8.0 , prepared in 10 mM phosphate buffer, pH=7.0. The reading was taken spectrophotometrically after 20 mins of incubation at 37(C , at 412nm (24).

Assay of in vitro hydroxyl free radical scavenging potential of ethanol extract of leaf of *Piper betle* Linn

The hydroxyl scavenging action of the plant extractive was examined by its ability to inhibit OH. catalysed oxidation. The final concentration of the assay, in a total volume of 1ml, includes , 28mM deoxyribose , 20mM Tris-HCl, pH=7.4, 100μM EDTA ,100μM $FeCl_3$ and 100μM ascorbate . The reaction mixture was incubated at 37°C for 1 hour, after which 1 ml of 30% ice cold HCl and 0.75% thiobarbituric acid (TBA) in 0.5% sodium acetate were added . The reaction mixture was boiled for 20 mins and cooled to room temperature, centrifuged at 1000rpm for 5 mins , and reading of coloured chromophore was taken spectrophotometrically at 535nm (25). The relative antioxidant activity is expressed in terms of mM equivalent of thiourea.

Assay of in vitro superoxide radical scavenging action of ethanol extract of leaf of *Piper betle* Linn

Superoxide generation was measured in presence of the plant extract following cytochrome C reduction at 550 nm. The final concentration of the assay mixture in a total volume of 1 ml were 100 μM cytochrome C , 100 μM hypoxanthine, 10mM Tris-HCl . The reaction was initiated with the addition of 8mU of xanthine oxidase (XO). The relative antioxidant activity is expressed in terms of mM equivalent of thiourea.

STATISTICAL ANALYSIS

Analysis of variance test was followed using Students' "t" test, for determination of significance among the mean ± SEM values in different groups of animals .

RESULTS

Cytoprotective activity of the drug

Indomethacin induced ulcerated group showed a number of perforations with blood spots. A thin flaccid mascular layer was also observed in each case. Pretreatment with ethanol extract of the leaf at doses of 100 mg /kg body weight, 150 mg/kg body weight, 200 mg/kg body weight and 250 mg/kg body weight inhibited the formation of indomethacin induced gastric ulcers, as is exhibited by reduction of ulcer index by 51.47 %, 59.81%, 75.90% and 45.59%, with virtually no ulceration in the third case particularly(Table I).

Effect of drug on lipid peroxidation

Tissue peroxidised lipid levels in case of group, pretreated with "Drug" at the dose of 200mg/kg body weight, showed a significant low value, as compared to ulcerated control ($p< 0.001$) (Table II) .

Effect of the drug on damaged protein content

The carbonyl content (due to oxidative damage) of the group, pretreated with the "Drug", at the dose of 200mg/kg body weight, showed a significant reduction ($p<0.001$) as compared to ulcerated control (Table II).

Effect of the drug on Superoxide dismutase (SOD) activity of the gastric mucosa

The SOD activity of the group, pretreated with the "Drug", at the dose of 200mg/kg body weight, was significantly high and attained normal value compared to untreated group (Table III).

Effect of the drug on catalase activity and toal sulfhdryl (-SH) group of gastric mucosa

The catalase activity and the total sulfhydryl content of the group pretreated with the "Drug", at the dose of 200mg/kg body weight, remained almost near the normal values, while the values of untreated ulcerated group were very low ($p<0.001$) (Table III).

Effect of drug on hexosamine and mucous content of gastric mucosa

A significant increase in mucous and hexosamine contents of the gastric mucosa were observed in the group, pretreated with the "Drug", at the dose of 200mg/kg body weight, as compared to the untreated ulcerated group ($p<0.001$) (Table II).

Effect of ethanol extract of leaf of Piper betle Linn in scavenging hydroxyl free radicals in in vitro system

Figure 1 shows that, after an initial relaxation upto a concentration of 2.5 mg/ml, there is almost a linear dose dependent nature of the extract as hydroxyl free radical scavenger.

Effect of ethanol extract of leaf of Piper betle Linn in scavenging of superoxide anion in invitro system

Figure 2 shows an almost dose dependent antioxidant, superoxide anion scavenging action of the above extract with concentration.

DISCUSSION

Ulcer formation, induced by indomethacin, a non -steroidal anti inflammatory drug (NASID) is related to the inhibition of cyclooxygenase , that prevents prostaglandin biosynthesis and in turn inhibits the release of mucus (a defensive factor against gastrointestinal damage). It is evident from the results, that the hexosamine and mucus contents of gastric tissue of rats pretreated with the "Drug" increases (Table III), indicating its cytoprotective action on indomethacin induced gastric lesions, also supported by a decrease of ulcer index (Table I). The overriding evidences strongly indicate the involvement of free radical scavenging action of the drug. The significant decrease in the level of peroxidised lipid, a product of reactive oxygen species(ROS) injury on proteins, also supports the above presumption. High tissue SOD (Table III) and catalase (CAT) activity (Table III), two major biological antioxidant agents (27), may be taken to be an interpretation, which envisages interplay of protective action of antioxidant enzymes within the system in counteracting the formation of ulcers in gastric mucosa. The observed protective action of the extract is possibly due to scavenging of ROS by both superoxide and hydroxyl free radical pathway. This is supported by the in vitro, direct free radical scavenging action of the extract. Thus it is tempting to interpret, that the preventive potency of the extract is through antioxidative pathway. Glutathione, a major non -protein thiol in the biological systems, play a central role in co-ordinating the antioxidant status. The increase in the total -SH group content (Table III) could be due to decreased consumption of glutathione, via decreased level of lipid peroxidation and thereby protecting the gastric mucosa. Superoxide is inactivated by SOD, the activity is effective only when it is followed up by the increase in the activity of catalase(CAT) or glutathione peroxidase , since SOD generates hydrogen peroxide as a metabolite, which is more tissue -toxic than oxygen radical and has to be scavenged by catalase or reduced glutathione. Thus a concomitant increase in catalase or glutathione peroxidase activity is essential, if the beneficial effect from increased SOD activity is to be expected.

Though it is not possible to altogether exclude the cytoprotective effect, exerted by the drug itself, development of ulcer 48 hours after administration of the last dose under fasting condition as also increase in mucin and hexosamine contents and consequent increase in gastric barrier of the stomach wall could involve the inductive effect of the drug in triggering other gastric mechanism in action including antioxidant activity (28), which might as well scavenge the hydroxyl free radical. From the above study it is not illogical to project that the ethanol extract of leaf of *Piper betle Linn* at a dose of 200mg/kg body weight possesses cytoprotective action against experimentally induced peptic ulcer by indomethacin and the underlying cause may be due to its potent antioxidant action.

Altough, the infection of *Helicobater pylori*, that has been detected as one of the prime causes in human gastric and duodenal ulcers, is related to the oxidative burst and production of free radicals, the efficiency of the above leaf extract indirectly inhibiting and / or eradicating the growth of *H. pylori* has not yet been known and is still to be evaluated and explored. Further studies, concerning any co-relation with the mechanism of inhibition of growth of *H. pylori* by the extract of the leaf are currently in progress in our laboratory.

Table I

Effect of *Piper betle Linn* extract on gastric lesions of mucosa

Status	Mean Ulcer Index (mm^2)
Ulcerated Untreated	10.55 ± 0.36
Ethanol extract pretreated (100mg/kg body weight)	5.12 ± 0.94 *
Ethanol extract pretreated (150mg/kg body weight)	4.24 ± 0.88*
Ethanol extract pretreated (200mg/kg body weight)	2.55 ± 1.15*
Ethanol extract pretreated (250mg/kg body weight)	5.74 ± 2.02**
Normal healthy control	0.00 ± 0.00

Values represent mean ± SEM

*Significant (p<0.001) compared to ulcerated control

**Significant (p<0.01) compared to ulcerated control

N /group =6, number of animals in each group.

Table II

Effect of *Piper betle Linn* extract on peroxidised lipid , oxidatively damaged protein(Carbonyl content), hexosamine and mucus content of gastric tissue

Status	Malondialdehyde (MDA) concentration of gastric tissue (μM x 10 /mg protein)	Carbonyl content (μM /mg protein)	Hexosamine (μg x 10)	Mucus (mg AB* binding)
Untreated ulcerated	13.20 ± 0.80	22.70 ± 1.50	5.38 ± 0.15	3.90± 0.25
Ethanol extract pretreated (200mg/kg body weight)	4.70 ± 0.35 *	4.46 ± 1.65*	26.42 ± 2.85*	6.22±0.11*
Normal healthy control	2.47 ± 0.78	2.83 ± 0.35	36.45 ± 3.24	7.54±0.05

Values represent mean±SEM

*Significant (p<0.001) compared to ulcerated control

**Significant (p<0.01) compared to ulcerated control

N /group =6, number of animals in each group.

AB* alcian blue

Table III

Effect of *Piper betle Linn* extract on Superoxide Dismutase (SOD) activity, catalase (CAT) activity and total sulfhydryl (-SH) group content

Status	Superoxide Dismutase of gastric tissue [specific activity] (U)	Catalase of gastric tissue [specific activity]	Total sulfhydryl (-SH) group (mM /mg protein)
Untreated ulcerated	2.65 ± 0.33	8.11 ± 0.96	25.00 ± 0.72
Ethanol extract pretreated (200 mg/kg body weight)	6.26 ± 0.65*	12.61 ± 1.36**	52.00 ± 0.98*
Normal healthy control	6.91 ± 0.45	14.11 ± 0.09	61.20 ± 1.05

Values represent mean ± SEM

*Significant ($p<0.001$) compared to ulcerated control

**Significant ($p<0.01$) compared to ulcerated control

N /group=6, number of animals in each group.

Figure 1

Superoxide Radical scavenging profile of leaf of *Piper betle Linn*

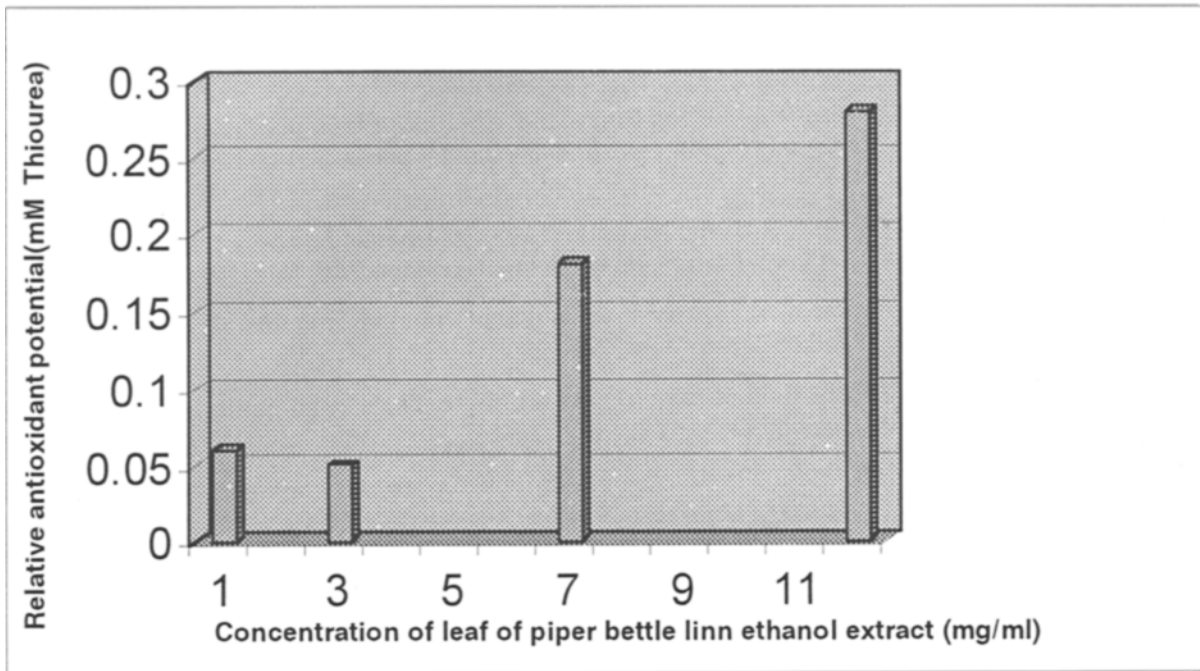
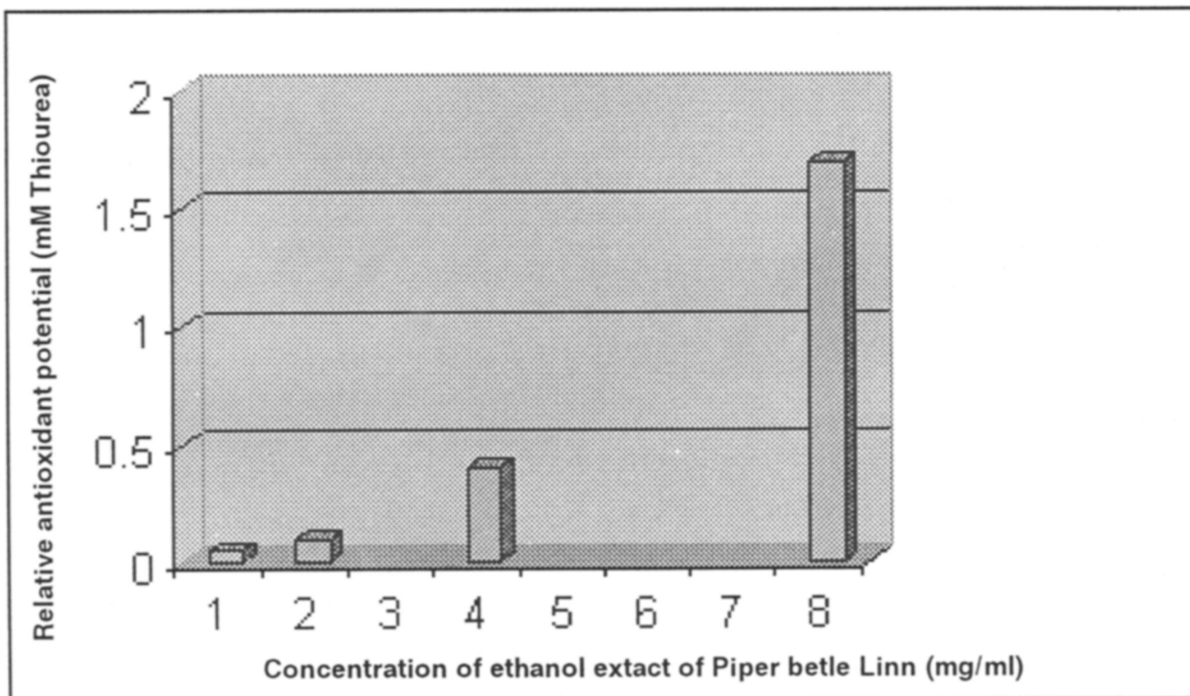


Figure 2

Hydroxyl Free Radical scavenging Profile of leaf *Piper Betle Linn*



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