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Effect of ethanol extract of *Piper betle* Linn leaf on healing of NSAID - induced experimental ulcer — A novel role of free radical scavenging action

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Treatment with ethanol extract of leaf of *P. betle* at a dose of 150 mg/kg body weight daily for 10 days, after induction of peptic ulcer by NSAID in albino rats, produced significant healing effect. During healing process, on treatment with the extractive, antioxidative factor, e.g. superoxide dismutase and catalase activity, mucus and total gastric tissue sulfhydryl group were increased. In contrast, oxidised lipid and oxidatively modified proteins were reduced to near normalcy, within 7 to 10 days, however, change in the untreated group was not significant. The extract also showed significant *in vitro* free radical scavenging action. The results suggest that the antioxidant or free radical scavenging activity of the plant extract, may be responsible for its healing action.

Piper betle Linn commonly known as tambula (Sanskrit); pan (Hindi and Bengla) is a widely growing plant in the tropical humid climate of South East Asia. In the traditional medicinal it has been reported to possess wound healing activity and enhancement of digestion[1,2]. Extracts of *P. betle* leaves also possess antimicrobial, antifungal and antiinflammatory activities[3]. Piperbetol, piperol A and piperol B are some constituents, isolated from *Piper betle*, that show highly specific Platelet activiting factor (PAF) receptor antagonism[4]. Triterpenes and beta sitosterol, isolated from the plant, possess antiplatelet and antiinflammatory activities[5]. Ethanol extract of leaf of *P. betle* exhibited an gastrocytopropective properties on experimently induced gastric lesions by antioxidative mechanism[6]. The significant stimulatory action on intestinal amylase and lipase activity has also been reported[7]. It is now known that antioxidative imbalance and disruption in the aggressive and defensive mucosal factors one the underlying causes of peptic ulcer[8,9]. Among the various factors that lead to initiation of peptic ulcer, alcohol abuse, acute and chronic stress and prolonged use of NSAID are the major causative ones, apart from the much controversial *Helicobactor pylori* infection. The conventional drugs used in the treatment include H-2 receptor antagonists, proton pump inhibitors, antacids and anticholinergics. However, most of these drugs contribute various undesirable side effects and or drug intractions[10]. These observation justify the investigation of the healing action of drugs from natural sources. Thus, in continuation with the recently reported antioxidant activity of *P. betle* leaf extract[11], the present investigation has been undertaken to study its curative action on NSAID's induced ulcers, and a possible correlation with it's antioxidant activity.

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Materials and Methods

Plant material — Leaves of *P. betle* were collected locally during March-May and identified by Botanical Survey of India as *Piper betle* Linn of family Piperaceae. The leaves were chopped into fine pieces and made into a paste in 95% ethanol forming a slime. The slime was then percolated at room temperature with 95% ethanol for 7 days using an aspirator, and the solvent was filtered through a nylon mesh. The process was repeated thrice. The alcohol soluble portions were taken together and evaporated under vacuum condition (~0 cm of Hg) in a rotary evaporator. The finally concentrated solution, devoid of alcohol, was dried in a lyophilizer, to obtain a solid, amorphous yellowish brown mass, that was stored in vaccum dessicator.

Animals and treatment — Healthy, pathogen free, colony breed, male Charles Foster rats (150-170g) were maintained in laboratory condition with 12 : 12 hr

L : D cycle and average room temperature, maintained at 25° ± 1°C for at least 10 consecutive days and fed with pellet food (Hindustan lever, India) and water *ad libitum*. They were housed in standard metal cages. Analytical grade of chemicals from Sigma Chemical Co, MO, St Louis USA, E Merck and SRL, India were used.

Rats were divided into following 3 groups of 6 animals each :

Group I : Ulcerated control [rats with NSAID induced ulcer but no treatment with ethanol extract]; Group II : Experimental [rats with induction of ulcer with NSAID (indomethacin) and subsequent treatment with ethanol extract]; and Group III : Normal control [normal healthy rats].

In Groups I and II, induction of ulcer was done using indomethacin, an NSAID, at a dose, standardised in this laboratory (5 mg/kg body weight for 5 days) with fasting on 4th day. During this period Group III was kept on normal diet. Thereafter, Group I was treated with 2% gum acacia for 10 consecutive days and Group II treated with ethanol extract of the above leaf at a dose of 150 mg/kg body weight in 2% gum acacia as binder. Rats were sacrificed on 0th, 3rd, 7th and 10th day with 0th day being considered as the day of the last dose of indomethacin. The rats were sacrificed using anaesthetic ether.

For the assessment of ulcer index, the stomachs were opened along the greater curvature and observed under microscope with ×40 resolution and ulcer spots if any, were examined and counted. The area of mucosal damage was calculated in square millimeter and expressed as percentage of total area of glandular stomach[12]. The antral portion of the stomach was taken for biochemical investigation.

Measurement of the red chromophore (TBARS)-thiobarbituric acid-peroxidised lipid complex, malonyl dialdehyde (MDA) was done according to Esterbaner & Cheeseman[13]. The activity of superoxide dismutase (SOD), one of the principle antioxidant enzymes in the eukaryotes's *in vitro* defence system, was determined as per by McCord and Fridovich[14]. The assay procedure involved the inhibition of epinephrine autooxidation in an alkaline medium (*p*H 10.2). The enzyme activity was expressed in arbritary units considering 50% inhibition in the reaction mixture under the experimental condition as one unit of SOD. Catalase activity was determined according to the method followed by Lück *et al*[15].

Oxidatively damaged protein was determined in terms of carbonyl content, according to Rodney *et al*[16]. Hexosamine content[17] in gastric tissue was assayed. The mucus content was estimated[18] by measuring the amount of Alcian Blue (1% in 3% acetic acid) bound to mucus, thus, indicating the rate of regeneration of gastroprotective barrier. The detemination of total sulfhydryl group was carried out according to the method followed by Ellman[19] with minor modification.

Both hydroxyl free radical scavenging action and superoxide anion deactivating capacity of the plant extract estimated *in vitro*, as damage by ROS has been found to be a major marker of occurrence of NSAID induced ulceration. Superoxide anion scavenging activity of EtOH extract of leaf of *P. betle* was measured after Maulik *et al* [20]. Superoxide anion generation in absence of, and inhibition of generation in presence of, the plant extract was measured following cytochrome C reduction at 550 nm. The ralative antioxidant potential was expressed in terms of m*M* of thiourea. The hydroxyl radical scavenging action of the plant extract was studied by its ability to inhibit oxidation of deoxyribose after Maulik *et al*[21] with mannitol as standard.

Statistical Analysis was carried using Analysis of Variance (ANOVA) test followed by using Students *t* tests, to estimate the level of significance among the mean ± SE values in different groups of animals.

Results and Discussion

The ethanolic extract of leaf *P. betle* exhibited significant healing effect on NSAID induced peptic ulcer, as evident from various biochemical parameters. Before the onset of treatment with the extract, as is evident from microscopic observation on the 0th day, a number of deep ulcers were observed throughout the glandular stomach. Continuous treatment with the extract resulted in gradual healing of NSAID induced gastric lesion on and from 3rd day. On 10th day, virtually no ulcer spot were found, while that of the control group, showing natural healing, still exhibited prominant ulcer spots (Table 1). Tissue peroxidised lipid levels, malonyl dialdehyde, MDA showed gradual lowering, with that of the experimental groups on the 7th and 10th day, nearing that of the normal controls, while the experimental control values were reduced by much lower amounts. The SOD activity showed gradual increase during the process of ulcer healing, the activity on the 7th and 10th day of the experimental groups were comparable to that of the normal control, however, the SOD level of the experimental control groups showed much less

Table 1 — Healing effect of ethanol extract of *P. betle* leaf on experimentally induced gastric lesions

[Values are mean ± SE of 6 animals in each group]

Treatment	Area of damaged glandular stomach Ulcer index (mm^2) No of days after induction of ulcer			
	0	3	7	10
Untreated control Group I	10.40 ± 2.42	9.80 ± 2.62	10.10 ± 2.51	9.20 ± 1.16
Treated with EtOH extract Group II	10.60 ± 2.54^{NS}	6.20 ± 1.51^{NS}	3.20 ± 1.12*	2.41 ± 1.72**

P values : *< 0.01 ; **< 0.005; ^{NS}- not significant

Table 2 — Effect of leaf extractives on lipid peroxidation, superoxide dismutase, catalase and damage protein

[Values are mean ± SE of 6 animals in each group]

No of days after induction of ulcer	Parameters (units)	Untreated control Group I	Treated with EtOH extract Group II	Normal healthy control Group III
0	Malondialdehyde [μM × 10]	25.09 ± 1.42	$26.90 \pm 2.32^{***,**}$	9.96 ± 0.10
3		26.84 ± 2.36	$18.90 \pm 4.30^{**,NS}$	
7		27.86 ± 3.26	$12.68 \pm 2.32^{*}$	
10		18.64 ± 3.42	$11.32 \pm 2.12^{NS, NS}$	
0	Superoxide dismutase (SOD) specific activity [U/min/mg protein]	5.76 ± 2.32	$5.78 \pm 1.20^{***,**}$	22.24 ± 2.34
3		12.23 ± 2.12	$14.64 \pm 2.32^{**,**}$	
7		12.12 ± 1.20	$17.20 \pm 2.32^{*,NS}$	
10		11.58 ± 1.32	21.10 ± 1.24^{NS}	
0	Catalase (CAT) specific activity [U/min/mg protein]	9.02 ± 2.54	$10.11 \pm 2.40^{**}$	21.21 ± 1.12
3		8.76 ± 1.12	$12.82 \pm 0.72^{**,NS}$	
7		10.26 ± 2.24	$16.72 \pm 2.54^{*,NS}$	
10		14.20 ± 2.84	$18.04 \pm 2.68^{NS, NS}$	
0	Carbonyl content [μM/mg protein]	17.29 ± 1.28	$16.04 \pm 1.72^{***,**}$	5.26 ± 1.34
3		21.80 ± 2.42	$12.59 \pm 1.14^{**,NS}$	
7		13.00 ± 2.12	$6.8 \pm 2.18^{NS,NS}$	
10		9.46 ± 2.15	$6.03 \pm 1.14^{NS,NS}$	

P values : *< 0.05; **< 0.01; ***< 0.001; ^{NS}- Not significant of Group II compared with Group III and with Group I respectively.

increase in the enzyme specific activity. The catalase, another *in vivo* antioxidant enzyme in the eukaryotic defense mechansim against free radicals, showed an increase level in the experimental group, but the increase in the experimental control being much lower. The carbonyl content showed significant reduction in the experimental group, in contrast to that of the ulcerated control group showing almost no reduction (Table 2).

The total sulfhydryl group content of experimental group (group II) increased, reaching almost that of normal control value, while that of experimental control value (group I) was much lower. The hexosamine level of the experimental group abruptly increased on the 3rd day of the extract administration, and reached almost normal value on the 10th day, however, in the experimental control group it was less than 50% of the normal value (Table 3).

In vitro superoxide scavenging action of the plant extract showed 73% inhibitory effect on hypoxanthine/xanthine oxidase system, as compared to 91% inhibition by a standard antioxidant, thiourea. Inhibition of Fe^{3+} as corbated mediated oxadative demage of deoxyribose, was found to achieve upto 51.11% as compared to the inhibitory effect of mannitol, a known hydroxyl radical scavenging agent (Table 4).

Table 3 — Effect of leaf extractive on thiol group (Total reduced sulfhydryl group) mucin and hexosamine content during healing process

[Values are mean ± SE of 6 animals in each group]

No of days after induction of ulcer	Parameters/Units	Untreated control Group I	Treated with EtOH extract Group II	Normal healthy control Group III
0		3.6 ± 1.26	3.4 ± 1.20^{**,NS}	
3	Reduced sulfhydryl group	8.0 ± 1.72	25.3 ± 8.62*,***	
7	(mM/gm protein)	12.0 ± 0.32	36.3 ± 82.62^{NS,***}	38.2 ± 0.32
10		8.8 ± 1.12	36.4 ± 2.54^{NS,***}	
0		0.94 ± 0.34	1.41 ± 0.32^{**,NS}	
3	Gastric mucin content	1.64 ± 0.36	4.43 ± 1.12^{NS,***}	
7	[mg of Alcian blue	1.22 ± 0.32	3.9 ± 1.12*,***	6.2 ± 1.14
10	binding/gm of tissue x 100]	1.3 ± 0.72	5.4 ± 1.24^{NS,***}	
0		1.28 ± 0.72	1.12 ± 0.36 $^{** NS}$	
3	Hexosamine concentration	6.14 ± 0.92	14.37 ± 2.32^{NS,***}	
7	[μg × 10/gm protein]	6.96 ± 1.76	14.36 ± 1.15^{NS,***}	16.54 ± 2.52
10		7.16 ± 0.82	15.24 ± 3.04^{NS,**}	

P values : *< 0.02; **<0.01; ***<0.001; ^{NS}- Not significant of Group II compared with Group III and with Group I respectively.

Table 4 — *In vitro* antioxidant/free radical scavenging action of ethanol extract of leaf of *P. betle* in superoxide anion and hydroxyl free radical scavenging pathway

Treatment	Concentration	Superoxide scavenging Potential (% of inhibition of XO mediated Oxidation)	Relative hydroxyl free Radical scavenging effect
EtOH extract of leaf of *P. betle*	1 mg/ml	73.00	51.11
Standard (known)	10 m*M*	91.00 (thiouresa)	100.00 (mannitol)

In the present study NSAID induced experimental model, the gastric mucosal barrier, primarily the hexosamine and mucus content gradually increased, reaching a maximum on the 10th day, indicating significant protective as well as healing action of the drug.

The antioxidant enzymes level also reached almost at normal values between 7th to 10th day while that in the experimental group was much lower. Thus, the free radical scavenging action, during the process of ulcer healing, may significantly influence the healing action of the plant extract.

Reduction of thiols is essential for recycling of other antioxidants in the biological system. Since perturbation of glutathione status causes oxidative stress and excessive peroxidation can cause increased thiol consumption, attainment of near normalcy of thiol group could be due to decreased lipid peroxidation. The high turnover rate of mucin a high molecular weight glycoprotein, by its viscoelastic properties and adherence to the epithelial surface, forms a protective barrier to epithelial digestion. The action can also be explained in light of the hydroxyl free radical scavenging action of mucin[22]. NSAID induces ulcer by prostaglandin synthetase inhibition, through cyclooxygenase pathway and overproduction of leukotrienes and other 5-lipo-oxygenase activity[23]. Peptic ulcer formation has also been attributed to xanthine oxidase and neutrophils. The scavenging of superoxide anion, in *in vitro* XO system, by the plant extract, strongly suggest that the cause of the healing action is due to antioxidative mechanism. This is further confirmed by *in vitro* studies. Thus, it can be concluded that a possible healing action of the leaf of *P. betle* could be due to the free radical scavenging activity of the plant extract.

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