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Anti-ulcer and antioxidant activity of Normacid, a herbomineral formulation

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Effect of various doses (125, 250, 500 and 1000 mg/kg, po) of Normacid was studied on gastric secretion and gastric ulcers in pylorus-ligation and on ethanol-induced gastric mucosal injury in rats. The reduction in ulcer index in both the models along with the reduction in total acidity and an increase in the *p*H of gastric fluid in pylorus-ligated rats proved the anti-ulcer activity of Normacid. The increase in the levels of superoxide dismutase, catalase, reduced glutathione and membrane bound enzymes like Ca^{2+}ATPase, Mg^{2+}ATPase and $Na^{+}K^{+}$ATPase and decrease in lipid peroxidation in both the models showed the antioxidant activity of the formulation. Thus it can be concluded that the anti-ulcer activity shown by Normacid may be due to the modulation of defensive factors by improvenent in gastric cytoprotection and partly due to antioxidant property.

Keywords: Anti-ulcer, Antioxidant, Lipid peroxidation, Superoxide dismutase, Catalase, Reduced glutathione
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Peptic ulcer is the most common gastrointestinal disorder in clinical practice. Considering the several side-effects (arrythmias, impotence, gynaecomastia and haematopoeitic changes) of modern medicine[1], indigenous drugs possessing fewer side-effects should be looked for as a better alternative for the treatment of peptic ulcer.

There is evidence concerning the participation of reactive oxygen species in the etiology and pathophysiology of human diseases, such as neurodegenerative disorders, inflammation, viral infections, autoimmune pathologies and digestive system disorders such as gastrointestinal inflammation and gastric ulcer[2]. Studies have shown alterations in the antioxidant status following ulceration, indicating that free radicals seem to be associated with the pylorus ligation-induced[3] and ethanol-induced[4,5] ulceration in rats. Drugs with multiple mechanisms of protective action, including antioxidant properties, may be one way of minimizing tissue injury in human disease[6].

Many indigenous drugs are known to possess anti-ulcer activity. The anti-ulcer property of *Solanum nigrum*[7] and Shankh bhasma[8] has been mentioned. Bhunimbadi Kwath, Mouktika bhasma and Kapardi bhasma are well-known for their antacid property and used in acid peptic disorders[9]. The antioxidant properties of *Solanum nigrum*[10], Swarnabhasma[11,12] and Shankh bhasma[8] were earlier investigated and were found to possess free radical scavenging properties. Some of the ingredients were also found to produce significant increase in the levels of various endogenous antioxidant enzymes.

The present study has been aimed to investigate the anti-ulcer effects of Normacid along with its effect on the anti-oxidant enzymes to justify whether the formulation exerts an anti-ulcer action by means of its antioxidant activity.

Materials and Methods

Composition—Each gram of Normacid (manufactured by Ayur. Herbals Pvt. Ltd., Baroda, India) contains Bhunimbadi Kwath (500 mg), *Solanum nigrum* (200 mg), Mouktika bhasma (60 mg), Shuddha Gairika bhasma (60 mg), Kapardi bhasma (120 mg), Swarnabhasma (20 mg), Praval bhasma (30 mg) and Shankh bhasma (10 mg).

Animals—Female albino rats of Wistar strain weighing between 150-225 g were used in the study. The animals were housed in an air-conditioned room at 23°± 1°C. They were fed *ad libitum* with standard pellet diet and had free access to water. Animal experiments were approved by the Social Justice and Empowerment Committee for the purpose of control and supervision of experiments on animals, Ministry of Government of India, New Delhi.

Experimental procedure—The animals were divided into five groups of six rats each. Group 1

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represented the control group, which received 5 ml/kg body weight of vehicle (1% gum acacia, po). Groups 2 to 5 received Normacid orally at the doses of 125, 250, 500 and 1000 mg/kg body weight, respectively. Group 6 received the standard drug, cimetidine orally at the dose of 50 mg/kg body weight.

Study of anti-ulcer activity using pylorus ligation — The method of Shay rat ulcer[13] was adopted. Rats were fasted for 48 hr. The drug, Normacid or cimetidine was administered to the animals. During the course of the experiment food was withdrawn. After the pretreatment period of 1 hr, the animals were anaesthetised with anaesthetic ether. The abdomen was opened by a small midline incision below the xiphoid process; pylorus portion of stomach was slightly lifted out and ligated. Precaution was taken to avoid traction to the pylorus or damage to its blood supply. The stomach was placed carefully in the abdomen and the wound was sutured by interrupted sutures. After 19 hr of pylorus ligation the rats were sacrificed and the stomach was removed. The gastric content was collected and centrifuged. The volume, *p*H and total acidity of gastric fluid was determined. The stomach was then incised along the greater curvature and observed for ulcers. The number of ulcers was counted using a magnifying glass and the diameter of the ulcers was measured using a vernier caliper. Ulcer index was determined by following the scoring method of Suzuki *et al.*[14]

Score 1: maximal diameter of 1mm
Score 2: maximal diameter of 1-2mm
Score 3: maximal diameter of 2-3mm
Score 4: maximal diameter of 3-4mm
Score 5: maximal diameter of 4-5mm
Score 10: an ulcer over 5mm in diameter
Score 25: a perforated ulcer

Study of anti-ulcer activity using ethanol-induced ulcer method—The method described by Dhuley[15] was adopted. Normacid or cimetidine was administered orally to the rats for 10 days. On the 10th day, 1 hr after the final dose of the drug, 96% ethanol (5 ml/kg, po) was administered to the overnight fasted rats of all groups. The animals were then sacrificed 1 hr after the dose of ulcerogen. The stomach was removed, incised along the greater curvature and its mucosal erosion was determined randomly by measuring the area of the lesions. The sum of the areas was expressed as ulcer index (mm^2).

Study of anti-oxidant activity of Normacid—The stomach of rats of Group 1 (control) and Groups 2 to 5 (Normacid-treated groups) was then weighed and homogenized in chilled Tris buffer (10 m*M*, *p*H 7.4) at a concentration of 10% (w/v). The homogenates were centrifuged at 10,000×g at 0°C for 20 minutes using Remi C-24 high speed cooling centrifuge. The clear supernatant was used for the assays of lipid peroxidation (MDA content), endogenous antioxidant enzymes (superoxide dismutase and catalase) and reduced glutathione (GSH). The sediment was resuspended in ice cold Tris buffer (10 m*M*, *p*H 7.4) to get a final concentration of 10% and was used for the estimation of different membrane bound enzymes (Na^+K^+ATPase, Ca^{2+}ATPase and Mg^{2+}ATPase) and proteins.

Superoxide dismutase (SOD)[16], catalase[17], reduced glutathione[18], lipid peroxidation or malondialdehyde formation[19], inorganic phosphorus[20] and total proteins[21] were determined. Membrane bound enzymes namely, Na^+K^+ATPase[22], Ca^{2+}ATPase[23] and Mg^{2+}ATPase[24] were assayed.

Statistical analysis—Results were presented as mean ± SE. Difference between the groups (Normacid and control groups) was statistically determined by analysis of variance followed by Tukey-Kramer Multiple Comparisons test, with the level of significance set at $P<0.05$. Ranitidine group was compared with the control group by using unpaired Student's *t*-test.

Results

Study of anti-ulcer activity using pylorus ligation method—In the vehicle treated control group the ulcer index was 92.75 ± 10.40 and the maximum number of ulcers were of the ulcer score 4 and 5. In the rats of this group a number of perforated ulcers (score 25) were also observed.

Normacid was found to produce a significant ($P<0.001$) decrease in ulcer index at the doses of 500 and 1000 mg/kg; the percentage reduction being 66.31 and 80.59%, respectively. All the ulcers were of score 1 and 2 and no perforated ulcers were observed. The formulation at the same two doses significantly ($P<0.001$) increased the *p*H of the gastric fluid from 1.20±0.07 to 2.78±0.17 and 2.83±0.18, respectively. It also significantly reduced the total acidity at all the four doses, but however did not affect the volume of gastric fluid (Table 1).

Cimetidine (50 mg/kg) was found to produce a significant reduction in ulcer index, the percentage reduction being 58.13%. It also reduced the total acidity and increased the volume of gastric fluid significantly (Table 2) and increased the *p*H to 3.73 ± 0.26.

Study of anti-ulcer activity using ethanol-induced ulcer method—Administration of ethanol produced significant ulcers (287.98 ± 17.79) in the control group. There was a significant reduction in ulcer index at all the four doses of Normacid (Table 3). Cimetidine (50 mg/kg) produced a percentage reduction of 78.75% in ulcer index (Table 2).

Study of anti-oxidant activity of Normacid—Pylorus-ligation was found to increase lipid peroxidation and decrease SOD, catalase and reduced glutathione in the control group, thus leading to oxidative stress. Administration of Normacid brought about a significant reduction in lipid peroxidation and an increase in the level of reduced glutathione as compared to control. An increase in the activities of antioxidant enzymes namely, SOD and catalase along with the enhancement in the membrane bound ATPases was also observed in the drug treated groups (Table 4).

Table 1—Effect of Normacid on various gastric parameters of pylorus-ligated rats

[Values are mean ± SE. Figures in parentheses are % reduction in ulcer index]

Groups	Ulcer Index	Volume of gastric fluid (ml/100g)	Total acidity (mEq/l/100g)
Control	92.75±10.40	9.22±0.63	127.0±3.63
Normacid (125mg/kg)	86.50±5.14^{NS} (6.74)	8.56±0.36^{NS}	103.75±7.17^{a}
Normacid (250mg/kg)	77.75±3.66^{NS} (16.17)	9.81±0.13^{NS}	89.00±6.19^{b}
Normacid (500mg/kg)	31.25±4.25^{b} (66.31)	8.72±1.11^{NS}	75.75±4.15^{b}
Normacid (1000mg/kg)	18.00±2.94^{b} (80.59)	6.95±0.84^{NS}	47.5±2.78^{b}
F value	62.399	2.169	34.786
P value	<0.0001	<0.001	<0.0001

Normacid treated groups were compared with control group.
P values: ^{a}<0.05; ^{b}<0.001; ^{NS}non-significant

Table 2—Effect of cimetidine on various gastric parameters of pylorus-ligated and ethanol-treated rats.

[Values are mean ± SE. Figures in parentheses are % reduction in ulcer index]

Gastric Parameters	Control	Cimetidine (50mg/kg)
Ulcer Index		
Pylorus-ligated rats	92.75±5.20	38.83±5.36^{a} (58.13)
Ethanol-treated rats	287.98±17.79	61.19±21.22^{a} (78.75)
Volume of gastric fluid (ml/100g)	9.22±0.63	7.05±3.53^{a}
Total acidity (mEq/l/100g)	127.01±3.63	63.56±8.56^{a}

Cimetidine treated groups were compared with control group.
^{a}*P* <0.001

Ethanol administration was found to increase lipid peroxidation and decrease SOD, catalase and reduced glutathione in the control group when compared to normal rats. Administration of Normacid significantly decreased lipid peroxidation and increased the levels of SOD, catalase, reduced glutathione and all membrane bound ATPases (Table 3).

Discussion

Although in most of the cases the aetiology of ulcer is unknown, it is generally accepted that it results from an imbalance between aggressive factors and the maintenance of the mucosal integrity through the endogenous defense mechanism[25]. To regain the balance, different therapeutic agents including herbal preparations are used to inhibit the gastric acid secretion or to boost the mucosal defense mechanism by increasing mucus production. The anti-ulcer effect of Normacid was tested against gastric lesions induced by pylorus-ligation and ethanol, the experimental models related to lesion pathogenesis with production of reactive species. Normacid prevented the mucosal lesions induced by pylorus-ligation and ethanol. It also increased the *p*H and decreased the total acidity of gastric fluid. These effects of Normacid treatment on the parameters that influence the initiation and induction of ulceration may be considered as highly desirable property of anti-ulcerogenic agent. Normacid was found to be 10 and 5 times less potent than the standard drug, cimetidine in protecting against pylorus-ligation and ethanol-induced ulcers, respectively.

Reactive oxygen species are involved in the pathogenesis of pylorus ligation-induced[3] and ethanol-induced[4] gastric mucosal injury *in vivo*. Results of the present study also indicate similar alterations in the antioxidant status after pylorus ligation and ethanol induced ulcers. Preventive antioxidants, such as superoxide dismutase (SOD) and catalase (CAT) enzymes are the first line of defense against reactive oxygen species. Reduced glutathione (GSH) is a major low molecular weight scavenger of free radicals in the cytoplasm and an important inhibitor of free radical mediated lipid peroxidation[26]. Administration of Normacid resulted in a significant increase in the SOD, catalase and reduced glutathione levels as compared to control animals, which suggests its efficacy in preventing free radical induced damage.

Table 3—Effect of Normacid on the ulcer index and biochemical parameters in stomach of ethanol-treated rats
[Values are mean ± SE. Figures in parenthesis are % reduction in ulcer index]

Groups	Ulcer Index	Lipid Peroxidation (nmoles of MDA/ mg protein)	Reduced Glutathione (μg of GSH/ mg protein)	Superoxide Dismutase (Units/mg protein)	Catalase (μmoles of H_2O_2 consumed/ min/ mg protein)	Na^+K^+ATPase (μmoles of inorganic phosphorus liberated/min/ mg protein)	Ca^{2+}ATPase (μmoles of inorganic phosphorus liberated/min/ mg protein)	Mg^{2+}ATPase (μmoles of inorganic phosphorus liberated/min/ mg protein)
Normal	-	3.45 ± 0.24	3.31 ± 0.13	5.64 ± 0.32	8.27 ± 0.22	5.29 ± 0.18	3.65 ± 0.30	3.52 ± 0.20
Control	287.98 ± 17.79	6.75 ± 0.72^c	0.79 ± 0.17^c	2.23 ± 0.14^c	5.12 ± 0.21^c	2.03 ± 0.11^c	1.50 ± 0.21^c	1.49 ± 0.13^c
Normacid (125 mg/kg)	85.20 ± 4.93^c (70.41)	6.40 ± 0.44^{NS}	1.14 ± 0.08^{NS}	2.24 ± 0.12^{NS}	5.02 ± 0.17^{NS}	2.13 ± 0.05^{NS}	1.75 ± 0.09^{NS}	1.91 ± 0.11^{NS}
Normacid (250 mg/kg)	56.83 ± 2.37^c (80.27)	5.79 ± 0.22^{NS}	2.72 ± 0.18^c	3.13 ± 0.25^{NS}	5.72 ± 0.27^{NS}	2.57 ± 0.08^a	2.18 ± 0.08^{NS}	2.39 ± 0.09^b
Normacid (500 mg/kg)	37.59 ± 3.61^c (86.95)	3.97 ± 0.29^c	3.21 ± 0.08^c	3.47 ± 0.13^b	6.94 ± 0.08^c	4.21 ± 0.10^c	2.46 ± 0.17^a	3.04 ± 0.11^c
Normacid (1000 mg/kg)	5.75 ± 1.11^c (98.00)	3.97 ± 0.08^c	3.96 ± 0.32^c	4.32 ± 0.16^c	7.23 ± 0.13^c	5.12 ± 0.11^c	3.01 ± 0.08^c	3.23 ± 0.12^c
F *value*	173.49	13.524	49.985	42.458	45.802	174.16	21.341	37.669
P value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

Control group was compared with normal group. Normacid groups were compared with control group.
P values: ^{a}<0.05; ^{b}<0.01; ^{c}<0.001; ^{NS}non-significant

Table 4—Effect of Normacid on the biochemical parameters in stomach of pylorus ligated rats
(Values are mean ± SE)

Groups	Lipid Peroxidation (nmoles of MDA/ mg protein)	Reduced Glutathione (µg of GSH/ mg protein)	Superoxide Dismutase (Units/mg protein)	Catalase (µmoles of H_2O_2 consumed/ min/mg protein)	Na^+K^+ATPase (µmoles of inorganic phosphorus liberated/min/ mg protein)	Ca^{2+}ATPase (µmoles of inorganic phosphorus liberated/min/ mg protein)	Mg^{2+}ATPase (µmoles of inorganic phosphorus liberated/min/ mg protein)
Normal	3.45 ± 0.24	3.31 ± 0.14	5.64 ± 0.32	8.27 ± 0.22	5.29 ± 0.18	3.65 ± 0.30	3.52 ± 0.20
Control	10.09 ± 0.44^c	0.58 ± 0.14^c	2.37 ± 0.26^c	5.93 ± 0.60^b	1.49 ± 0.10^c	1.62 ± 0.17^c	1.54 ± 0.13^c
Normacid (125mg/kg)	5.38 ± 0.48^c	1.43 ± 0.18^{NS}	2.34 ± 0.26^{NS}	5.73 ± 0.38^{NS}	1.53 ± 0.10^{NS}	2.59 ± 0.23^a	2.42 ± 0.18^a
Normacid (250mg/kg)	4.00 ± 0.17^c	2.44 ± 0.19^c	2.73 ± 0.10^{NS}	6.52 ± 0.36^{NS}	2.06 ± 0.12^{NS}	2.65 ± 0.15^a	2.75 ± 0.17^c
Normacid (500mg/kg)	3.35 ± 0.43^c	3.36 ± 0.20^c	3.17 ± 0.08^{NS}	6.93 ± 0.15^{NS}	2.50 ± 0.40^{NS}	3.06 ± 0.10^c	3.17 ± 0.17^c
Normacid (1000mg/kg)	2.89 ± 0.15^c	4.45 ± 0.34^c	4.55 ± 0.20^c	8.23 ± 0.14^b	3.64 ± 0.29^c	3.56 ± 0.05^c	3.52 ± 0.07^c
F value	61.058	45.725	36.698	9.991	42.265	16.318	22.819
P value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

Control group was compared with normal group. Normacid groups were compared with control group.
P values: ^{a}<0.05; ^{b}<0.01; ^{c}<0.001; ^{NS}non-significant

Lipid peroxidation is a free radical mediated process, which has been implicated in a variety of disease states. It involves the formation and propagation of lipid radicals, the uptake of oxygen and rearrangement of double bonds in unsaturated lipids which eventually results in destruction of membrane lipids. Biological membranes are often rich in unsaturated fatty acids and bathed in oxygen-rich metal containing fluid. Therefore, it is not surprising that membrane lipids are susceptible to peroxidative attack[27]. The study revealed a significant decrease in lipid peroxidation by Normacid in both the experimental models, which suggests its protective effect.

Na^+K^+ATPase, Ca^{2+}ATPase and Mg^{2+}ATPase are membrane bound enzymes. The drug significantly increased the activity of all the ATPases in both the models.

One of the constituents of Normacid, namely *Solanum nigrum*, has been reported to possess hepatoprotective effect due to its ability to suppress the oxidative degradation of DNA in the tissue debris[10]. Another constituent of Normacid, Swarnabhasma, has been used since ancient times in several clinical manifestations including loss of memory, defective eyesight, infertility, overall body weakness and incidence of early aging. According to modern concept, the scientific basis for its application in degenerative diseases may arise, atleast in part, through enhancement in free radical concentrations. It was found to induce enhanced activity of SOD and catalase. Swarnabhasma was found to produce no signs of toxicity indicating that the drug can be used safely in oral route for atleast a few months[11]. Shah and Vohora have also reported the antioxidant effects of calcined gold preparations used in Indian systems of medicine against global and focal models of ischemia[12]. Pandit *et al.*[8] have suggested that Shankh bhasma may act as gastric cytoprotective agent by modulating scavenging of free radicals. It is thus apparent that the antioxidant effect of Normacid may be due to the antioxidant property of its constituents mentioned above.

On the basis of the present results and available reports, it can be concluded that the anti-ulcer activity elucidated by Normacid could be mainly due to the modulation of defensive factors through an improvement of gastric cytoprotection and partly due to acid inhibition and free radical scavenging activity.

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