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FLAVONOID FRACTION OF *ARGYREIA NERVOSA* LEAVES WITH ANTIULCER POTENTIAL IN DIFFERENT EXPERIMENTAL RAT MODELS

JYOTI THAKUR[1], SAURABH SHARMA[2], MINKY MUKHIJA[1], A.N.KALIA[1]

1. Department of Pharmacognosy, ISF College of Pharmacy, Moga.
2. Department of Pharmacology, ISF College of Pharmacy, Moga.

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Abstract: *Argyreia nervosa* has been used as an antiulcer in ethano medicine. So the aim of the present study was to evaluate the leaves of the plant for its antiulcer potential and to isolate the bioactive compounds. On the basis of the result of the preliminary screening for antiulcer potential of different fractions of methanolic extract of *Argyreia nervosa* leaves; n-butanol fraction was charged into silica gel column. The two major subfractions (A and B) obtained were screened for their antiulcerogenic activity by using indomethacin induced gastric ulcer model. Subfraction B (4.59 mg/kg, p.o.) has shown more significant antiulcer as well as antioxidant potential than Subfraction A (6.34mg/kg, *p.o.*). Subfraction B significantly increased superoxide dismutase (SOD), catalase and glutathione (GSH) levels whereas tissue lipid peroxidation and nitrite levels were found to be decreased as compared to the control group. Quercetin and Kaemferol were isolated from the subfraction B. Both are known antiulcer and antioxidant. Hence, the antiulcer potential of n butanol fraction of methanolic extract of *A. nervosa* may be due to the synergestic effect of Quercetin and Kaemferol.

Keywords: *Argyreia nervosa,* n-butanol fraction, Indomethacin induced gastric ulcer model, Pylorus ligation induced gastric ulcer model

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Corresponding Author: DR. A.N.KALIA

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INTRODUCTION

Peptic ulcers are sores in the lining of the upper gastrointestinal tract. They include duodenal ulcers (those that are located in the top of the small intestine or duodenum) and gastric ulcers (those found in the stomach). It is a complex pluricausal disease and is known to develop due to imbalance between aggressive (acid, pepsin) and protective factors (bicarbonate, prostaglandin, nitric oxide, peptides and growth factors)[1, 2].

The treatment of peptic ulcers is usually based on inhibition of gastric acid secretion using proton-pump inhibitors, H2-receptor antagonists, and antimuscarinics, as well as acid-independent therapy provided by antimicrobials against Helicobacter pylori, sucralfate and bismuth[3]. However, these drugs are expensive and generate numerous adverse effects (such as hypersensitivity, gynecomastia, impotence, arrhythmia and hematopoietic changes), thereby limiting their usefulness[4]. It is thus important that studies be carried out to investigate potential antiulcerogenic herbs which may lead to the discovery of new potential antiulcer drugs.

A. nervosa, a member of Convolvulaceae family, is a climbing shrub with woody tomentose stem, found mainly in Deccan, Karnataka and eastern slopes of the West Ghats at an altitude of 900 m[5]. It is commonly known as elephant creeper in English, Samudra-sok in Hindi and in Sanskrit, it is called as Vridhadaraka meaning 'anti-aging'[6].

It has been used as 'Rasayan' drug in the ayurvedic system of medicine to cure diseases of nervous system[7]. In the indigenous system of medicine, the plant is prescribed in gleets, gonorrhoea, strangury and chronic ulcer. The leaves are emollient, vesicant and are used externally in the treatment of ringworm, eczema, itch and other skin diseases and internally to cure boils swellings, etc. The leaves are also used as local stimulant and rubefacient. Traditionally leaves are used by Rajasthani tribes to prevent conception. A paste of the roots made with rice water is applied over rheumatic swelling and rubbed over the body to reduce obesity. The whole plant is reported to have analgesic property[8;9].

The roots of this plant have been regarded as anticonvulsant[10], analgesic, anti-inflammatory and anti-arthritic[11;12], adaptogenic and antioxidant[13], Antimicrobial[14], immunomodulatory[15], Hepatoprotective[16], tonic, diuretic and aphrodisiac[17], hypoglycemic[18] and central nervous depressant activity[7]. The roots are also used in obesity[19].

While the leaves of the plant are reported to have wound healing[20], anti-fungal[21], anti-microbial and anti-inflammatory[5] and to some extent aphrodisiac properties[17]. The flowers of the plant reported to have antidiarrhoeal activity[22]. Cardiovascular dysregulation and psychosis has been reported after consumption of *Argyreia nervosa* seeds[23; 24].

The major constituents isolated from the plant are friedelin, ergine, agroclavine, penniclavine, chanclavine, ergometrine, quercetin, kaempferol, scopoletin, and hexadecanyl p-hydroxycinnamate[25].

Seeds reported to have Ergoline alkaloidal constituents - clavine type[26;27], Hallucinogen (serotonin receptor agonist)-Lysergic acid amide[28], lysergacidamide and lysergacidethylamide (and their isomers)[29]. A new steroidal glycoside, (24*R*)-ergost-5-en-11-oxo-3β-ol-α-D-glucopyranoside, designated as argyroside was also isolated from the seeds of the *A. nervosa*[30]. The seeds yield fatty oil, which was found to contain the glycosides of palmitic, oleic, stearic, behenic, linoleic and linolenic acid. The free amino acids reported in the seeds were glutamic acid, glycine, isoleucine, leucine, lysine, phenylalanine, tyrosine, praline and α-aminobutyric acid[31].

The leaves were found to contain flavonoids - quercetin, kaempferol and kaempferol 3-O-L-rhamnopyranoside[31;8]. Two new flavone glycosides characterized as 7,8,3',4',5'-pentahydroxyflavone5-o-α-lrhamnopyranoside and 7,8,3',4',5'-pentahydroxyflavone5-o-α-l-glucopyranoside were also reported from leaves[32]. 1-tricontanol, epifriedelinol acetate, epifriedelinol and β-sitosterol were also present in the leaves[33]. Two new steroidal compounds 28-pentyl-3-galloyl-betulinate and 11-hydroxy friedelane have also been isolated from the leaves[34].

Investigations on *A. nervosa* roots revealed the presence of Aryl esters, coumarin glucoside, p-hydroxycinnamate and scopoletin as important phytoconstituents[16]. A lipid ester and a disubstituted tetrahydrofuran were isolated from the roots and characterized as tetradecanyl palmitate and 5, 8- oxidotetracosan-10-one[35]. Two new compounds isolated from the roots have been characterized as tetradecanyl palmitate 1 and 5, 8-oxidotetracosan-10-one[36] and two novel aryl esters characterized as stigmasteryl p-hydroxycinnamate and hexadecanyl p-hydroxycinnmate along with scopoletin[37].

MATERIALS AND METHODS

Collection and authentication of plant material

The fresh leaves of *A. nervosa* were collected in the month of September, 2011, from Punjab Agriculture University (PAU), Ludhiana (Pb.) and authentication was done by Dr. Cheema, PAU, Ludhiana (Pb.)

Preparation and fractionation of extract

The leaves were shade dried at room temperature, coarsely powdered and stored in air tight container till further use. The ground powdered leaves (503.7g) were exhaustively defatted

with Pet. ether (60-80°C) by maceration for 2 days at room temperature with occasional shaking. This process of extraction was repeated for four times, filtered and concentrated on rotary evaporator (Equitron Roteva). The marc left behind was extracted with 80% methanol by maceration. The methanolic extract (46.04g) was suspended in water and partitioned with toluene, dichloromethane, ethyl-acetate and n-butanol to yield toluene (16.63% w/w), dichloromethane (19.46% w/w), ethyl-acetate (13.57% w/w), n-butanol (21.28% w/w) and aqueous (27.75% w/w) soluble fractions.

Phytochemical studies

Qualitative testing of the ethyl-acetate and n-butanol fractions for alkaloids, steroids, terpenoids, anthraquinone glycosides, flavonoids, tannins, phenolic compounds, saponins, carbohydrates, proteins and amino acids was carried out according to the method described by Harborne et al., 1998[38].

Total phenolic content

Total phenolic contents were determined spectrophotometrically according to the method of Singleton and Rossi (1965) but with some modifications[39]. Gallic acid was taken as standard. The content of total phenolics in n-butanol fraction and most active sub-fraction from n-butanol fraction was calculated using a calibration curve of gallic acid (the linearity range: 10–50 µg/ml, R2 = 0.998). All determinations were carried out in triplicate.

Isolation of the compound(s)

On the basis of pharmacological screening, the most potent n- butanol fraction (5.02 g) was subjected to column chromatography (CC) with silica gel (520 g, 0.120-0.250 mm particle size) as the stationary phase. It was eluted with a stepwise gradient of pure toluene followed by increasing amounts of dichloromethane (DCM) (9:1, 8:2, 7:3, 6:4, 1:1, 4:6, 3:7, 2:8, 1:9) to pure DCM, and then DCM-Ethyl Acetate (EA) to pure EA, followed by increasing amount of methanol in the same above manner. Total 990 fractions were collected. Homogeneous fractions profiles, using thin layer chromatography (TLC) were pooled to give major fractions. Therefore, 28 pooled out sub-fractions were collected from the column.

Experimental animals

Wistar rats of either sex weighing 150-200 g were procured from the Animal house, ISF College of Pharmacy, Moga. The animals were kept in metabolic cages (3 rats in each cage) at an ambient temperature 25±2°C and relative humidity 55-65%. A 12-12 h light and dark schedule was maintained in the animal house. The rats were fed over commercially available feed (Aashirwad Industries, Ropar, Punjab) and water *ad libitum*. Experimental protocol was

approved by Institutional Animal Ethical Committee (IAEC) with approval no. IAEC/M2/CPCSEA/P18/2011 and was conducted as per the international guidelines for animal experimentation and care.

Indomethacin induced gastric ulcer model[40]

The animals were divided into different groups (n=6): control received 0.5% CMC; misoprostol (100 µg/kg) as positive control; ethyl acetate fraction (36.48 mg/kg) and n-butanol fraction (46.16 mg/kg) of methanolic extract of *A. nervosa*. All the rats except control rats were pretreated with test drugs for 3 days. On day third, indomethacin (100 mg/kg, *p.o.*) suspended in 0.5% CMC was given to all the groups to induce acute gastric ulcers, after 30 min of test drug treatments. After 5 hrs the animals were sacrificed; each stomach was removed, washed with saline and kept in 10% formalin solution for 24 hrs. After 24 hrs the stomach were opened along the greater curvature and were examined for ulcer index[4].

Ulcer index (UI) - the lesions score for each rat was calculated as the number of lesions in the rat multiplied by their respective severity factor. The U.I. for each group was taken as the mean lesion score of all the rats in that group.

SEVERITY FACTOR/ SCORING OF ULCER[41]: 0 - no lesion; 1 - lesions < 1 mm length; 2 - lesions 1-4 mm of length; 3 - lesions > 4 mm length

The protection percentage was calculated from the following formula:

$$\% \text{ Protection} = [(\text{UIcontrol} - \text{UI treated}) / \text{UI control}] \times 100$$

The fraction showing maximum efficacy in this model had been subjected to column chromatography and on the basis of %age extractive value the sub-fractions A and B obtained from column were subjected to Indomethacin induced ulcer model in rat to estimate their anti-ulcer potential. The efficacy of sub-fraction showing maximum potential in this model had been further confirmed by pylorus ligation induced ulcer model in rat.

Pylorus ligation induced ulcer model

A simple and reliable method for production of gastric ulceration in the rats based on ligature of the pylorus has been published by[42]. The most potential sub-fraction was subjected to pylorus ligation induced ulcer model. Rats were divided into three groups (n=6) as control received 0.5% CMC; omeprazole (50mg/kg) as standard and sub-fraction B (4.59mg/kg, p.o.) of the n-butanol fraction as a test. After 48 hrs fasting of rats, all the drugs and vehicle were administered (p.o.). After 45min. rats were anesthetized with ketamine (70 mg/kg, *i.p.*). Then abdomen was incised and pylorus ligated. Four hours later, the animals were sacrificed; each

stomach was removed, washed with saline and kept in 10% formalin solution for 24 hrs. After 24 hrs the stomach were opened along the greater curvature and were examined for ulcer index**.**

Analysis of gastric juice

Gastric juice collected from each animal was centrifuged at 3000 rpm for 10 min to remove any solid debris and the volume of the supernatant was measured. The pH of the gastric juice was measured using the pH meter. The total acid secretion in the gastric juice in the supernatant volume was determined by titration using a 0.01M NaOH solution, and phenolphthalein as an indicator. Acidity was calculated by using the formula:

Total Acidity = (Vol of NaOH X normality of NaOH)/ 0.1 X 100 mEq/L

Tissue biochemical estimation

Stomachs were cut into small pieces and then homogenized in ice-cold PBS (phosphate buffer solution) to give 10% homogenate. The homogenate was then made into aliquots and used for the assessment of biochemical parameters.

The protein was measured in all tissue samples by the Biuret method using bovine serum albumin (BSA) as a standard[43].

The quantitative measurement of lipid peroxidation (LPO/MDA) in the stomach tissue was performed according to the method of Wills, 1965. Nitric oxide (NO) content was determined as total nitrites/nitrates, the stable degradation products of NO[44;45].

Catalase activity,]. Reduced glutathione (GSH) and SOD was also estimated[46;47;48].

Statistical analysis:

Results were expressed mean ± SD; analyzed by one way ANOVA followed by Tukey's multiple comparison test as post hoc analysis. $p < 0.05$ statistically significant.

RESULTS

Phytochemical screening: The preliminary phytochemical screening carried out on ethyl-acetate and n-butanol fraction of methanolic extract of *A.nervosa* leaves extract revealed the presence of phytoconstituents such as flavonoids, steroids, triterpenoids and phenolic compounds.

Total phenolic content: The total phenolic content of most active sub-fraction B was found to be 39.45 µg/ml (% w/w), which was higher than n-butanol fraction (26.63 µg/ml, % w/w). The calibration curve of gallic acid is shown in **Figure 1.**

Isolation, purification and characterisation of flavonoidal compounds

Two Yellow crystalline powder (Compound A and B) has been obtained from Sub-fraction B from coloumn. Crystalline powders were separated from mobile phase and purification was done by recrystallization using solvents acetone and methanol.

The Melting point of the compound A and B was found to be 310-313°C and 278-281°C respectively. Due to less amount obtained, confirmatory analysis was carried out only through NMR (^{1}H) spectral analysis and compared with the literature. For Compound A ^{1}H NMR (DMSO, 400 MHz, δ with TMS=0) showed signals at δ 7.71 (d, *J*=2.2 Hz, 1H, 2'), 7.54 (dd, *J*=2.2, 8.4 Hz, 1H, 6'), 6.87 (d, *J*=8.5 Hz, 1H, 5'), 6.36 (d, *J*=1.96 Hz, 1H, 6), 6.16 (d, *J*=2.12 Hz, 1H, 7). For Compound B ^{1}H NMR (DMSO, 400 MHz, δ with TMS=0) showed signals at δ 8.06 (dd, *J*=7.04, 1.84 Hz, 2H, 2', 6'), 6.91 (dd, *J*=1.8, 8.84, 2H, 3', 5'), 6.38 (d, *J*=1.92 Hz, 1H, 6), 6.19 (d, *J*=2.12 Hz, 1H, 8). By ^{1}H NMR spectral analysis and literature comparison reveals that Compound A has total 5 hydroxy groups present at the molecule, out of which 3 at chromenone ring and 2 at phenyl ring, possibly similar to Quercetin **(Figure 2a)** while the Compound B has one hydroxy group less on the phenyl ring, which is of Kaempferol structure **(Figure 2b)**.

Antiulcerogenic studies

Indomethacin induced gastric ulcer model

The percentages protection produced by ethyl acetate fraction (36.48 mg/kg) and n-butanolic fraction (46.16 mg/kg) were 47.94% and 58.17% respectively. The standard drug misoprostol showed 72.44% protection against control group. **(Figure3)**

The percentages protection produced by sub-fraction A (6.34mg/kg, *p.o.*) and sub-fraction B (4.59mg/kg, *p.o.*) were 53.15% and 74.58% respectively. The standard drug Misoprostol showed 72.44% protection against control **(Figure 4)**.

In the Pylorus ligation induced gastric ulcer model, ligation of pylorus end of stomach caused significant increase in gastric volume, acid concentration and decrease in pH. Oral administration of subfraction B (4.59 mg/kg) produced significant ($p < 0.05$) reduction in gastric secretion volume. Considering the pH values, are also increased, when compared with the control. The acid concentration is also reduced significantly ($p < 0.05$) as compared to control. The ulceration area has been significantly decreased in Subfraction B treated group as compared to control group of wistar rats. **(Table 1)**

Figure 5 shows that the Standard drug omeprazole (50 mg/kg) and subfraction B significantly decreased the ulcer index as compared to vehicle control group. Effect of The effect of Sub-fraction B on levels of free radicals and anti-oxidants in rat gastric mucosa in pylorus ligated induced gastric ulceration is shown in **Table 2.**

DISCUSSION

Phytochemical screening of ethyl acetate fraction and n-butanol fraction revealed the presence of phenolic compounds, flavonoids, steroids and triterpenoids by positive reaction with the respective test reagent. n-butanol fraction and its sub-fraction B revealed that sub-fraction B has more anti-ulcer potential as compared to n-butanol fraction due to higher amount of phenolic content, which was confirmed by Indomethacin and Pylorus ligated ulcerative models in Wistar rats.

The present study has been designed to isolate and characterize the bioactive component(s) from the n-butanol fraction of methanolic extract of *A. nervosa* (leaves) for anti-ulcer activity. The study confirms the pathological manifestations of indomethacin induced gastric ulcers *in-vivo.* Pretreatment with ethyl acetate and n-butanol fractions of methanolic extract of *A. nervosa* leaves and standard misoprostol showed significant prevention of gastric ulceration. The n-butanol fraction is rich in flavanoids and triterpenoids and thus, showed a significant reduction in ulcer index, which may be due to the synergystic effect of these phytoconstituents.

Overuse of NSAIDs in the population is commonly seen. NSAID like indomethacin inhibits COX1 thereby inhibits the prostaglandin synthesis, consequently lipooxygenase pathway is enhanced liberating leukotrienes and these leukotrienes are reported to have a role in ulcerogenesis. In addition there is some evidence that NSAIDs may induce ulcer by causing the back diffusion of H^+ ion in to mucosal cells[49;50]. Therefore, the gastroprotective effect of the test extract may be due to its ability to inhibit the synthesis of prostaglandins/leukotrienes.

The two known flavonoidal compounds, Quercetin and Kaempferol, have been isolated, purified and characterized from the sub-fraction of the n-butanol fraction. They have well reported anti-inflammatory and anti-oxidant properties[51;52].

On the basis of yield, sub-fractions- A and B were further subjected to indomethacin induced gastric ulcer model. Sub-fraction B has shown better antiulcer activity, so therefore, further confirmed by using pylorus ligation induced ulcer model.

Pylorus ligation is an important procedure that shows the possible changes of the parameters for gastric content, e.g., volume of gastric juice, acid concentration and pH.

Sub-fraction B reverses the pylorus ligation induced gastric ulceration in Wistar rats. The anti-ulcer property of Sub-fraction B in pylorus ligation model is evident from its significant reduction in total acidity and ulcer index. Because Sub-fraction B treated animals significantly inhibited the formation of ulcers in the pylorus ligated rats and also decreased both the concentration and increased the pH, it is suggested that Sub-fraction B can suppress gastric damage induced by aggressive factors and has anti-secretory activity.

Further, the antiulcer potential of the Sub-fraction B has also confirmed by tissue biochemical estimations - MDA/LPO, SOD, Catalase, GSH and Nitrite levels.

The level of anti-oxidant enzymes (SOD, GSH and catalase) increases significantly, while LPO and nitrite level decreases in the stomach tissue of drug treatment group.

Due to their antioxidant capacity proved above, their capacity to preserve the integrity of mucosal tissue near normal and their ability to prevent the depletion of antioxidant enzymes, it is clear that Sub-fraction have a great anti-ulcerogenic activity.

The sub-fraction B has much higher antiulcer activity than n-butanolic fraction due to the presence of higher amount of total phenolic content.

The estimation of sub-fraction B phenolics content was made using Folin–Ciocalteu method[53]. Much of literature refers to the plant phenolics compounds a direct antioxidant effect, which acts as ROS scavengers via hydrogen donating ability from their hydroxyl group[54;55;56]. Such aptness could breakdown lipid chain peroxidation reactions and contributes to the sub-fraction B an antioxidant and antiulcerogenic effects.

Therefore, it is suggested that Sub-fraction B of n-butanol fraction of methanolic extract of *A. nervosa* leaves due to their richness in the total phenolic content, can suppress gastric damage induced by aggressive factors, which is generally accepted that gastric ulcers result from an imbalance between aggressive factors and defensive factors.

CONCLUSION

n-butanol fraction of methanolic extract of *A. nervosa* was found to have anti ulcer potential. This fraction on purification by column chromatography found to have two major compounds Quercetin and Kaemferol. The subfraction from which these compounds are seperated are found to have significant antiulcer potential in Indomethacin induced and Pylorus ligation models. Thus these two compounds are responsible for antiulcer potential of n-butanol fraction as well as it confirms the traditional claim of the plant for its anti-ulcer activity.

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Figure 1. Calibration curve of Gallic acid for estimation of total phenolic compounds

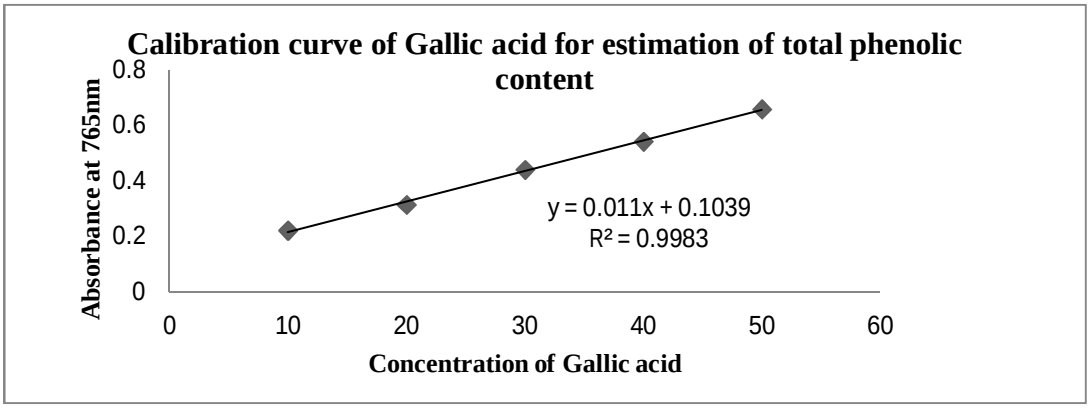


Figure 2: Chemical structure of (a) Quercetin and (b) Kaemferol

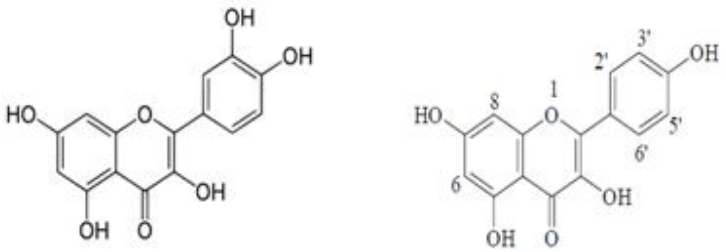
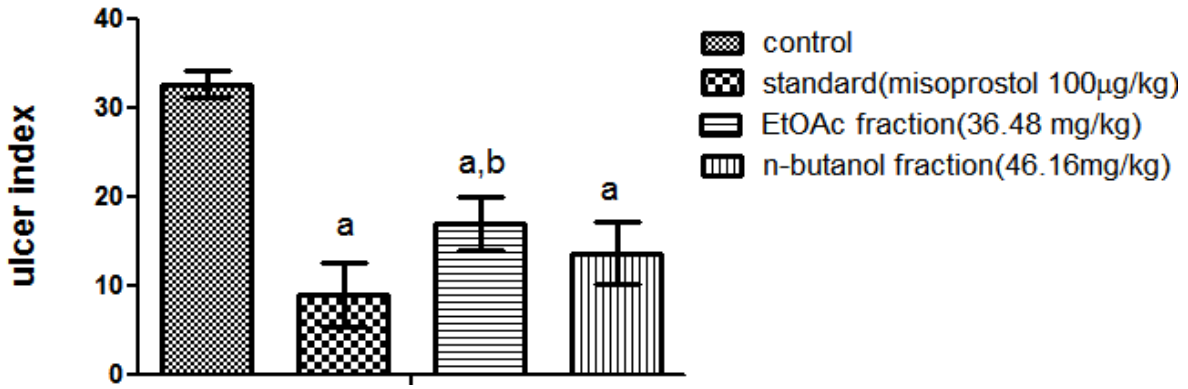
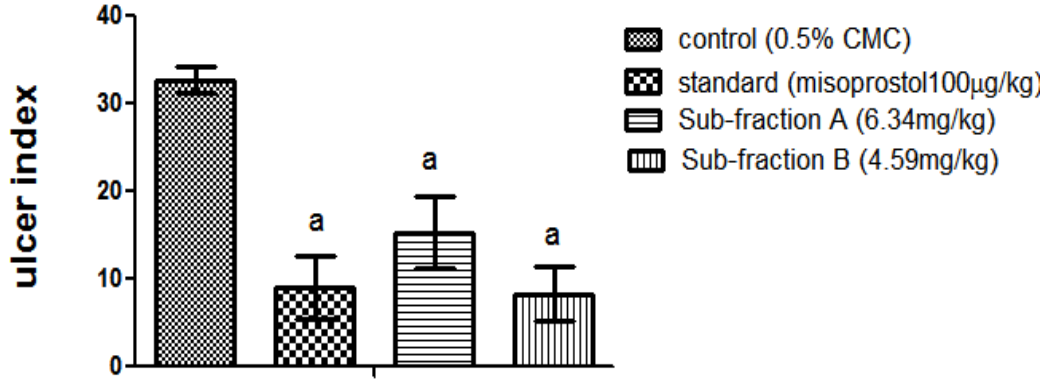


Figure 3: Effect of disease control (vehicle treated), standard, Et-OAc and n-butanol fraction on Indomethacin induce ulcer model



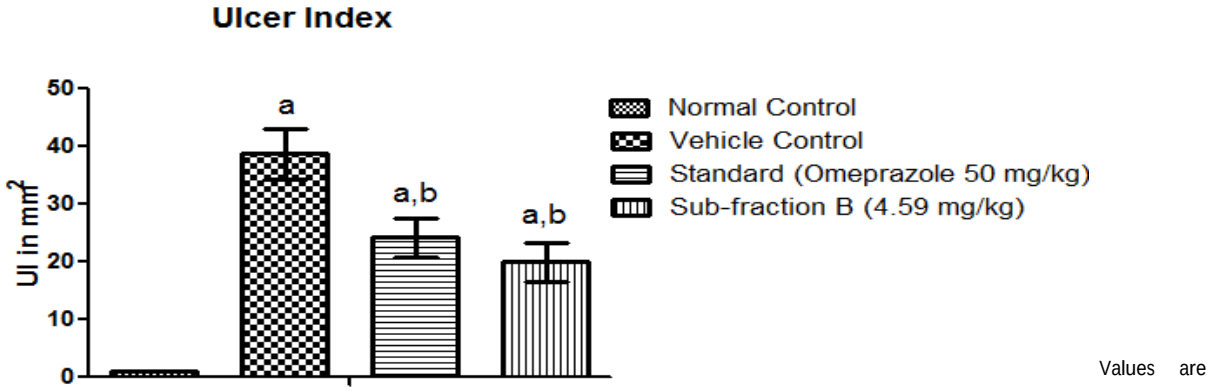
Values are expressed as mean ± S.D; n=6; a denotes for P<0.05 vs. vehicle control, b denotes for P<0.05 vs. standard control drug (misoprostol 100 µg/kg)

Figure 4: Effect of disease control (vehicle treated), standard, sub-fractions A and B from n-butanol fraction on Indomethacin induce ulcer model.



Values are expressed as mean ± S.D; n=6; a denotes for P<0.05 vs. vehicle control

Figure 5: Effect of Sub-fraction B on ulcer index of pylorus ligation induced gastric ulcer model



Values are expressed as mean ± S.D; n=6; a denotes for p<0.05 vs. normal control, b denotes for p<0.05 vs. vehicle control.

Table 1: Effect of sub-fraction B and omeprazole on the biochemical parameters of gastric juice obtained from pylorus ligature in Wistar rats.

Treatment	Dose	Gastric volume (ml)	pH	Acid concentration (meq/ml)
Normal Control	-	2.03 ± 0.25	3.7 ± 0.34	23.95 ± 1.29
Ind. Vehicle Control	-	1.80 ± 0.06	2.5 ± 0.26[a]	35.53 ± 3.08[a]
Omeprazole	50 mg/kg	0.85 ± 0.10[a,b]	3.8 ± 0.17[b]	28.05 ± 2.58[b]
Sub-fraction B	4.59 mg/kg	0.60 ± 0.18[a,b]	3.8 ± 0.40[b]	25.25 ± 3.14[b]

Values are expressed as mean ± s.d; n=6; a denotes for p<0.05 vs. normal control, b denotes for p<0.05 vs. vehicle control. Abbreviations- ind: indomethacin (50 mg/kg).

Table 2: Effect of Sub-fraction B on levels of free radicals and anti-oxidants in rat gastric mucosa in pylorus ligated induced gastric ulceration

Treatment	MDA (µmoles/mg pr.)	Nitrite/nitrate (µmoles/mg pr.)	SOD (U/mg protein)	Catalase (µM of H_2O_2/mg pr.)	Glutathione (µM of GSH/mg of protein)
Normal control	10.18 ± 0.94	0.32 ± 0.04	25.65 ± 1.5	18.48 ± 1.58	4.04 ± 0.5
Ind. Vehicle control	16.09 ± 1.9[a]	0.41 ± 0.03[a]	14.98 ± 1.1[a]	12.4 ± 1.35[a]	2.64 ± 0.6[a]
Sub-fraction B (4.59mg/kg)	10.45 ± 0.34[b,c]	0.31 ± 0.01[b]	24.05 ± 1.3[b,c]	14.98 ± 1.33[a]	3.99 ± 0.3[b,c]
Omeprazole (50mg/kg)	14.43 ± 1.41[a]	0.35 ± 0.02[b]	20.79 ± 1.9[a,b]	15.95 ± 2.1[b]	3.06 ± 0.4[a]

Values are expressed as mean ± s.d; n=6; a denotes for p< 0.05 vs. normal control, b denotes for p < 0.05 vs. vehicle control; c denotes for p<0.05 vs. standard drug (omeprazole 50mg/kg), d denotes for p< 0.05 vs. sub-fraction b (4.59 mg/kg). Abbreviations- ind: indomethacin (50 mg/kg).

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