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Anti-ulcer and anti- secretory properties of the *Pongamia Pinnata* root extract with relation to anti -oxidant studies.

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ABSTRACT

The present study was undertaken to determine the anti-ulcer potential and antisecretory properties of the Methanol extract of *Pongamia Pinnata* root Extract with relation to *in vitro* anti-oxidant studies. Methanol extracts of the root P. *Pinnata* were tested orally at the doses of 15, 20 and 25 mg/kg, on gastric ulcerations experimentally induced by aspirin, alcohol and pylorus ligation models. The extract at the dose of 25 mg/kg showed 79.30 and 82.20 % inhibition when gastric ulcerations were induced by aspirin and ethanol and 66.38 % inhibition showed in pylorus ligation at a dose of 20 mg/kg respectively. The methanol extract at 20 and 25 mg/kg was showed significantly ($P<0.001$) inhibited ulcer formation. Methanol extract which contains flavonoids, triterpenes, carotinoids and saponins, which may exhibited an anti-ulcer properties. To understand the pharmacological actions, *in vitro* anti-oxidant activity of Methanol extract of the root P. *Pinnata* was investigated for activity of scavenging lipid peroxidation, reducing power, superoxide anion radicals and hydroxyl radical. In all the testing, a significant correlation existed between concentration of the extract and percentage of inhibition of free radicals. The extract inhibited 72.47, 75.86, 68.11 and 77.46 % on lipid per oxidation, reducing power, superoxide anion and hydroxyl radical scavenging activity at a 50 µg/ml concentration respectively. The anti-oxidant property may be related to the flavonoids and polyphenol present in the extract. These results clearly indicated that Methanol extract of the root P. *Pinnata* is effective against free radical mediated ulcer disease.

Keywords: *Pongamia Pinnata,* anti-ulcer, anti-secretory, *in- vitro* anti-oxidant

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INTRODUCTION

Peptic ulcer is the most common gastrointestinary disorder in clinical practice. Considering the several side effects (arrhythmia's, impotence, funaecomastia 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 inflammations and gastric ulcer [2]. In the anti-oxidant status following ulceration, indicating that free radicals seem to be associated with the pylorus ligation [3] and ethanol induced [4, 5] ulceration in rats. Drugs with multiple mechanisms of protective actions, including anti-oxidant properties, may be one way of minimizing tissue injury in human disease [6]. Molecular oxygen is essential for life. Add electrons to O_2 (reduction) and generate reactive oxygen species like superoxide (O_2^-), peroxide (O^{2-}_2) and its fragmentation product, hydroxyl radical (OH^-), all of which are damaging to biological systems because of their reactivity. Cells have developed enzymatic mechanism to handle reactive oxygen species, with superoxide dismutase, peroxidase and catalase being the most important 'deactivators'. Reactive oxygen species have also been implicated in drug induced gastric hyperacidity and stress induced gastric ulceration [7]. According to traditional system of medicine one of such plant, possessing anti-ulcer activity is P. *Pinnata*. The P. *Pinnata*(linn) Pierre (Syn P. glabra vent) is a medium sized glabrous tree, found through out India and further distributed East wards mainly in the littoral regions of south eastern Asia and Australia. [8]. The seeds and seed oil of this plant have been used for treating various inflammatory and infectious diseases such as leucoderma, leprosy, lumbago, muscular and rheumatism. [9]. Recently, we have reported the ulcer protective effect of extract of P. Pinnata(PP) and the ethnolic extract of the root of this plant tended to decrease acid-pepsin and increase mucin secretion. Although several factors are reported to be involved in ulcerogensis, the causative factor is basically the imbalance between offensive and defensive mucosal factors[10]. although many chemical constituent such as, kino-tannic acid, gallic acid and proanthocyanidins have been isolated from root [11]. Root of this plant has been used traditionally for treating gastric ulcers. Moreover in our initial studies, we found that Methanol extract of the Root P. *Pinnata* contain substantial amount of polyphenol. The presence of natural anti-oxidants has been confirmed in Soya been, garlic, red wine, green tea, in flowers and fruits of hawthorn. These plants are found to posses polyphenolic constituents like flavonoids. Hence in the present study, root of the plant has been selected for investigate the anti-ulcer activity along with correlation of *in vitro* anti-oxidant study.

MATERIALS AND METHODS

Plant Material

P. Pinnata root was collected from the rural area of north Karnataka. The plant was identified and authenticated by Prof. K. Prabhu, Department of Pharmacognosy, S.C.S. College of Pharmacy, Harapanahalli, Karnataka, India. A voucher specimen (021/2008) has been deposited at the museum of our college. The root was collected in the month of May 2008 and shade dried at room temperature.

Preparation of extract

The *Pongamia Pinnata* root was powdered in electrical grinders. This powder was packed into Soxhlet column and extracted successively with petroleum ether, chloroform and methanol. The Chloroform extract was concentrated under reduced pressure (yield: pet ether: 3.06%, chloroform: 3.96%, methanol: 8.29%). The dried extracts were stored in air tight container in refrigerator below 10° C. The Methanol extract was dissolved in water before use the following studies. The chemical constituents of the Methanol extract were identified by qualitative analysis [12].

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Experimental Animals

Albino rats (150-200 g) and albino mice (20-30 g) were procured from National Institute of Mental Health and Neuro Sciences, Bangalore India. After procuring the animals were acclimatized for 10 day's under standard husbandry conditions, room temperature (27 ± 3° C), relative humidity (65 ± 10 %) and 12 hours light / dark cycle. They were allowed free access to standard dry pellet diet (Gold mohr, Lipton India Ltd., Bangalore, India) and water *ad libitum* under strict hygienic conditions. All the described procedure were reviewed and approved by the Institutional Animal Ethical Committee (Reg No: 157/1999/CPCSEA).

Toxicity study

Acute toxicity study of Methanol extract of the *P. Pinnata* was carried out for determination of LD_{50} by adapting fixed dose method of CPCSEA, OECD guidelines no 420. The female albino mice weighing between 20-30 g were used for the study. The animals were continuously observed 12 h to detect changes in autonomic or behavioral responses. Mortality was observed for 24 h. The doses of 15, 20 and 25 mg/kg, p.o. were selected based on the results of preliminary toxicity testing.

Aspirin-induced gastric ulcer

The male rats were randomly divided into five groups and fasted for 24 h. To the first group it was given of vehicle (normal saline, 1ml/100 g, p.o.), and the Group II, III IV received orally 15, 20, 25 mg/kg of Methanol extract of the *P. Pinnata* The remaining Group V was treated with Omeprozoal (8 mg/kg) orally. An hour later aspirin (250 mg/kg) was administered orally to all the animals and 6 h later the animals were sacrificed by cervical dislocation, [15]. The stomachs were removed, opened along the greater curvature and examined under microscope. Scoring of ulcer was done by the following method: 1= erosions 1 mm or less, 2 = 1-2 mm, 3 = < 2 mm. The overall score was divided by a factor of 10 which was designated as the ulcer index [16]. The percentage of ulcer inhibition was calculated as follows;

Percentage of ulcer inhibition = Mean ulcer index of control - Mean ulcer index of test / Mean ulcer index of control x 100.

Alcohol-induced gastric ulcer

The male rats were randomly divided into five groups and fasted for 24 h with free access to water. Animals were given vehicle or Methanol extract of the *P. Pinnata* at dose of 15, 20 and 25 mg/kg or Omeprozole (8 mg/kg) orally. One hour later, 1 ml of 80% ethanol was administered orally to each animal [17]. Animals were sacrificed by cervical dislocation, one hour after ethanol administration, stomachs were isolated and cut open along the greater curvature and pinned on a soft board. The length of each gastric lesion was measured and the lesion index was expressed as sum of the length of the entire lesion in mm.

Pylorus- ligation induced gastric ulcer

In this method male albino rats were fasted in individual cages for 24 h. The vehicle or Methanol extract of the *P. Pinnata* (15, 20, 25 mg/kg) or reference drug (Omeprozale, 8 mg/kg) was administered 30 min prior to pyloric legation orally. Under light ether anesthesia, the abdomen was opened and the pylorus was legated. The abdomen was then sutured. At the end of 4 h after legation, the animals were sacrificed with excess of anesthetic ether, and the stomach was dissected out. Gastric juice was collected and its volume, pH, free acidity and total acidity, was determined. The glandular portion was then exposed and examined for ulceration. Ulcer index was determined [18]. Determination of total acid and free acid were estimated from gastric juice collected from the 4 h pyloric ligated rats. Total acid output of the gastric juice was estimated by titration of 0.1 ml of gastric juice with 0.01N sodium hydroxide using phenolphthalein as indicator. Total acid output was expressed as mEq/L per 100 gm of body weight [19].

In Vitro Anti-oxidant activity

CCl_4-induced lipid peroxidation activity

The inhibition of *In vitro* lipid peroxidation was determined by using the method of Fairthurst et al. A solution of 30% (w/v) rate liver homogenate in ice cold KCl (0.15 M) were made in a homogenizer and 0.5 ml of the homogenate was transferred to small conical flasks. The flasks were then incubated at 37° C in a constant shaker bath (150 cycles/min) for 45 minutes with 1.5 ml of 0.15 M KCl and 0.5 ml of Methanol extract of the *P. Pinnata* in different concentration (10, 25 and 50 μg/ml) in different flasks respectively, 10 μl of CCl_4 were added to the control and the test samples prior to incubation. After incubation, the reaction was stopped by addition of 4.0 ml of 10% w/v trichloroacetic acid. The mixtures were centrifuged; 2 ml of thiobarbituric acid (0.68% w/v) was added to the tubes (containing 2 ml supernatant) prior to heating in a water bath for 15 minutes. The color was stabilized with KOH and the optical density was measured at 543 nm. Increased absorbance indicates greater MDH concentration. Conversely, reduction in absorbance indicates lesser concentration of MDA and indirectly, less extent of lipid peroxidation [20]. The percentage reduction in the absorbance was calculated which is proportional to the reduction in the lipid peroxidation. The experiment was performed in triplicate.

Reducing Power activity

The reducing power of Methanol extract of the *P. Pinnata* was determined according to the method of Oyaizu [21]. Chloroform extract or sodium metabisulphate (standard) dissolved in 1ml of distilled water so as to get 10, 25 and 50 μg concentration in the final volume of reaction. This was mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$; 2.5 ml, 1%]. The mixture was incubated at 50° C for 20 min. To the mixture 2.5 ml of 10% trichloroacetic acid was added. This was then centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5 ml) was mixed with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml, 0.1%), and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. The experiment was performed in triplicate. Percentage of increase in the absorbance was calculated.

Superoxide anion scavenging activity

Measurement of superoxide anion scavenging activity of Methanol extract was done based on the method described by Nishimiki [22] and slightly modified. About 1 ml of nitroblue tetrazolium (NBT) solution (156 μM NBT in 100 mM phosphate buffer, pH 7.4), 1 ml of nicotinamide adenine dinucleotide reductase (NADH) solution (468 μM NADH in 100 mM phosphate buffer, pH 7.4) and 0.1 ml of sample solution of Methanol extract of the *P. Pinnata* at different concentration (10, 25 and 50 μg concentration in the final volume of reaction) or sodium metabisulphate (10, 25 and 50 μgl, as standard) in water were mixed. The reaction started by adding 100 μl of phenazine methosulphate (PMS) solution (60 μM PMS in 100 mM phosphate buffer, pH 7.4) to the mixture. The reaction mixture was incubated at 25° C for 5 min, and the absorbance at 560 nm was measured against blank samples, Decreased absorbance of the reaction mixture indicated increased superoxide anion scavenging activity. The experiment was performed in triplicate. Percentage of decrease in the absorbance was calculated.

Hydroxyl radical scavenging activity

Hydroxyl radical generation by phenyl hydrazine was measured by the 2-deoxyribose degradation assay of Barry Hathwell and John Gutteridge method. Solution of 1 mM deoxyribose and 0.2 mM phenyl hydrazine hydrochloride were prepared in 50 mM phosphate buffer (pH 7.4). Deoxyribose 0.6 ml (1 mM) and 0.4 ml of *P. Pinnata* extract (10, 25 and 50 μg concentration in the final volume of reaction) or sodium metabisulphate (10, 25 and 50 μg, standard) were taken and phosphate buffer was added to make the volume

up to 1.6 ml. After 10 min of incubation, 0.4 ml of 0.2 mM phenyl hydrazine hydrochloride was added. Incubation was terminated after 1 and 4 h. To this mixture 1 ml each of 2.8% TCA and 1% w/v thiobarbituric acid were added and mixture was heated for 10 min in a boiling water bath. The tubes were cooled and absorbance was taken at 532 nm. Decreases in absorbance indicate the increase in hydroxyl free radical scavenging activity. The percentage of decrease in absorbance was calculated [23].

Statistical analysis

Values are expressed as mean ± S.E.M. The data were statistically evaluated by analysis of variance (ANOVA) coupled with student't' test. $P<0.05$ were consider statistically significant.

RESULTS

Preliminary phytochemical screening revealed the presence of flavonoids, triterpenes, saponins, carotinoids, alkaloids, glycosides and carbohydrates. Acute toxicity studies of the Methanol extract of the *P. Pinnata* did not exhibit any signs of toxicity up to 2 g/kg body weight. Since there was no mortality of the animals found at high dose. Hence 15, 20 and 25 mg/kg dose of the extract selected for evaluation of anti-ulcer activity.

Aspirin induced ulcer

Table 1 summarizes the results obtained in the experimental model of aspirin-induced gastric ulceration in rats. The Methanol extract was found to possess remarkable ulcer-protective properties at 15, 20 and 25 mg/kg. The maximum effect of ulcer protection (79.30%) was produced at 500 mg/kg and the standard drug (Omeprozole) gave 88.89% of ulcer protection (Table 1).

Alcohol induced ulcer

Pretreatment of rats with *P. Pinnata* extract produced a dose dependent protection in the ethanol induced ulceration model as compared to control group. However the protection was statistically significant reduced the severity of ulcer and caused a significant reduction of ulcer index in this model. Omeprozole produced significant gastric ulcer protection as compared to control group (Table 1).

Pylorus ligation induced ulcer

The Methanol extract of the *P.Pinnata* in the doses of 15 and 20 mg/kg produced a reduction in the ulcer index, gastric volume, free acidity, total acidity and raised gastric pH significantly in comparison with control group. Omeprozole, reference drug produced significant reduction gastric ulcer and total acid output as compared to control group (Table 2).

In- Vitro Anti-oxidant activity

The different concentration of Methanol extract (10, 25 and 50 µg/ml) was evaluated the anti-oxidant activity by employing various *in vitro* anti-oxidant models. It was observed that free radicals were scavenged by the test compound in a concentration dependent manner. The maximum percentage inhibition in all the models viz, CCl_4-induced lipid peroxidation, reducing power, superoxide anion and hydroxyl radical scavenging activity were found to be 72.47, 75.86, 68.11 and 77.46% respectively at 50 µg/ml concentration. (Fig: 1-4). Sodium metabisulphate was used for the reference standard for comparison of all the models.

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Table 1: Effect of Methanol extract of the root *P. Pinnata* against aspirin and alcohol induced gastric ulcer in rats

Treatment	Dose (mg/kg, p.o.)	Aspirin		Alcohol	
		Ulcer index Mean ± SEM	% of Ulcer protection	Ulcer index Mean ± SEM	% of Ulcer protection
Control group	--	7.50 ± 0.50	--	4.67 ± 0.56	--
P. Pinnata	15	5.00 ± 1.00*	33.00	2.67 ± 0.54*	35.80
P. Pinnata	20	2.58 ± 0.27***	65.70	1.75 ±0 .46***	62.50
P. Pinnata	25	1.50 ± 0.39***	79.30	0.83 ± 0.21***	82.20
Omeprozole	8	0.83 ± 0.21***	88.89	0.67 ± 0.16***	89.27

Results are mean ± S.E.M.(n = 6). Statistical comparison was performed by using ANOVA coupled with student't' test.* P<0.05, ** P<0.01, *** P<0.001 were consider statistically significant when compared to control group.

Table 2: Effect of Methanol extract of the root *P. Pinnata* against pylorus ligated rats.

Treatment (Dose)	Gastic juce volume (ml/4h)	pH	Free acidity (mEq/L/ 100 g)	Total acidity (mEq/L/ 100 g)	Ulcer index	% Inhibition of Ulcer
Control group	5.02 ± 0.11	2.02 ±0.08	87.53 ± 0.99	1.71 ± 1.77	5.83 ± 0.54	--
P. Pinnata (15 mg/kg,)	4.22 ± 0.12***	3.33 ± 0.16***	66.33 ± 3.81***	136.00 ± 3.43***	3.83 ± 0.38**	34.30
P. Pinnata (20 mg/kg)	2.68 ± 0.15***	4.72 ± 0.17***	65.00 ± 2.67***	87.83 ± 1.17***	1.92 ± 0.40***	66.38
Omeprozole (8 mg/kg)	1.95 ± 0.08***	5.12 ± 0.12***	50.00 ± 2.12***	67.67 ± 3.32***	0.67 ± 0.17***	88.57

Results are mean ± S.E.M.(n = 6). Statistical comparison was performed by using ANOVA coupled with student't' test.* P<0.05, ** P<0.01, *** P<0.001 were consider statistically significant when compared to control group.

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Fig 1. Effect of Methanol extract of the root *P. Pinnata* on CCl_4-induced lipid peroxidation activity. Each value represents mean ± S.E.M. (n = 3).

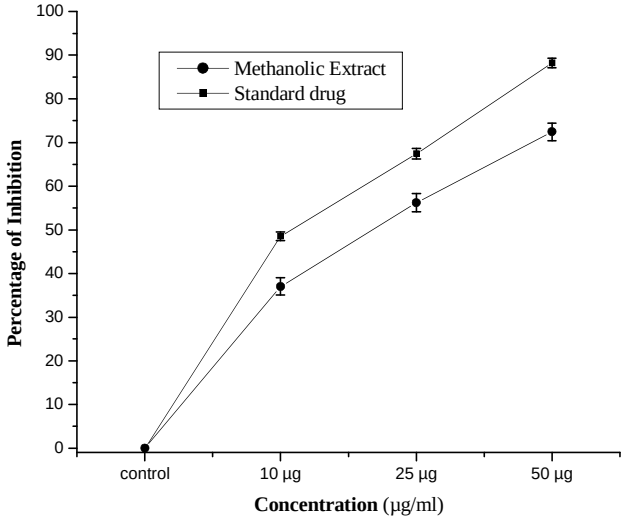

Fig 2. Effect of Methanol extract of the root *P. Pinnata* on Reducing power activity. Each value represents mean ± S.E.M. (n = 3).

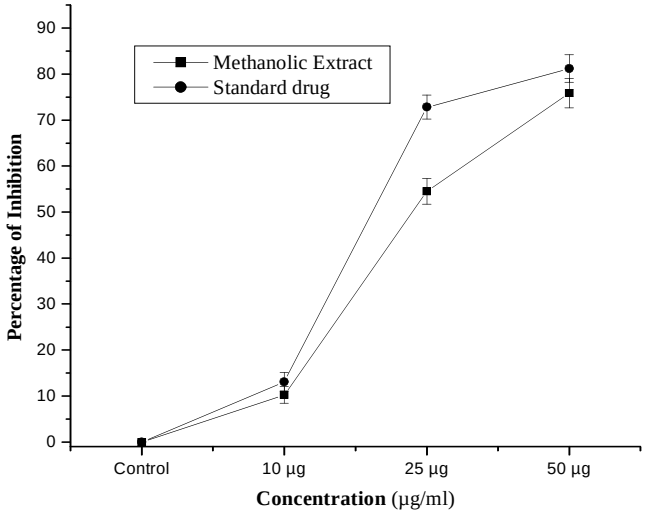

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Fig 3. Effect of Methanol extract of the root *P. Pinnata* on Superoxide anion scavenging activity. Each value represents mean ± S.E.M. (n = 3).

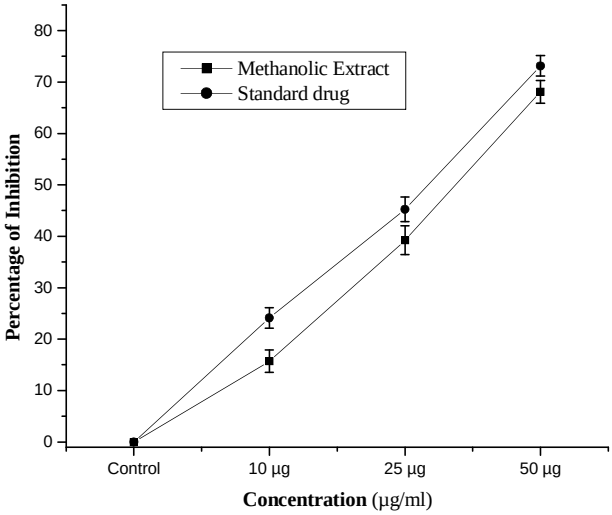
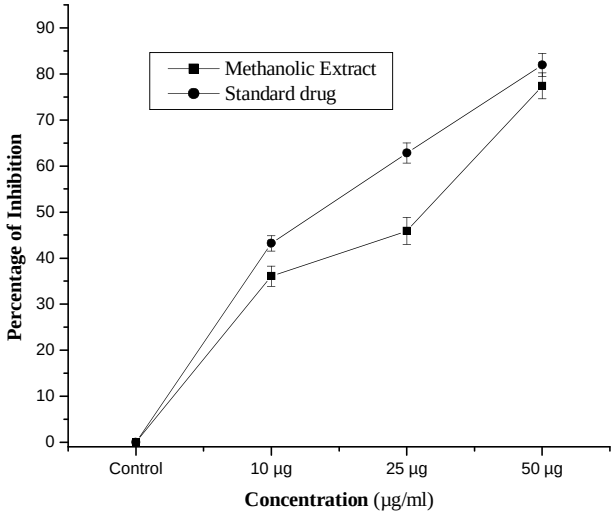


Fig 4. Effect of Methanol extract of the root *P. Pinnata* on Hydroxyl radical scavenging activity. Each value represents mean ± S.E.M. (n = 3).



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DISCUSSION

The anti-ulcer activity of the root *P. Pinnata* was evaluated by employing asprin, alcohol and pylorus ligation ulcer models. These models represent some of the most common causes of gastric ulcer in humans. Many factors and mechanisms are implicated in the ulcerogenesis and gastric mucosal damage induced by different models employed in the present study involving, depletion of gastric wall, mucin mucosal damage induced by non-steroidal anti-inflammatory drugs and free radical production [7]. NSAID's like aspirin causes gastric mucosal damage by decreasing prostaglandin levels through inhibition of PG synthesis [24]. Methanol extract of the root *P. Pinnata* was significantly effective in protecting gastric mucosa against aspirin induced ulcers at all the dose level studied. Ethanol induced gastric injury is associated with significant production of oxygen free radicals leading to increased lipid peroxidation, which causes damage to cell and cell membrane [25]. The extract of the root *P. Pinnata* has significantly protected the gastric mucosa against ethanol challenge as shown by reduced values of lesion index as compared to control group suggesting its potent cytoprotective effect. It has been proposed that in pyloric ligation, the digestive effect of accumulated gastric juice and interference of gastric blood circulation are responsible for induction of ulceration [26]. The anti-ulcer activity of *P. Pinnata* extract in pylorus ligation model is evident from its significant reduction in gastric volume, total acidity, free acidity, ulcer index and increase in pH of gastric juice. Because of animals treated with *P. Pinnata* extract significantly inhibited the formation of pylorus ulcer in the stomach and also decreased both acid concentration, gastric volume and increased the pH values. It is suggested that *P. Pinnata* root extract can suppress gastric damage induced by aggressive factors.

Lipid peroxidation is a free radical mediated process, which has been implicated in a variety of disease state. 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]. For the measurement of the reducing activity, the Fe^{3+}- Fe^{2+} transformation was investigated in the presence of *P. Pinnata*. The reducing capacity of a compound may serve as a significant indicator of its potential anti-oxidant activity. However, the activity of anti-oxidants has been assigned to various mechanisms such as prevention of chain initiation, binding of transition-metal ion catalyst, decomposition of peroxides, prevention of continued hydrogen abstraction, reductive capacity and radical scavenging [28]. Superoxide is biologically important since it can be decomposed to form stronger oxidative species such as singlet oxygen and hydroxyl radicals, is very harmful to the cellular components in biological system.The results suggest that extract had important superoxide radical scavenging effect. Hydroxyl radical is very reactive and can be generated in biological cell through the phenyl hydrazine. *P. Pinnata* extract exhibited concentration dependent scavenging activities against hydroxyl radical generated in a phenyl hydrazine system. The potential scavenging abilities of phenolic or flavonoids substance might be due to the active hydrogen donor ability of hydroxyl substitution. Similarly, high molecular weight and the proximity of many aromatic rings and hydroxyl groups are more important for the free radical scavenging by specific functional groups.

CONCLUSION

On the basis of the present results and available reports, it can be concluded that the anti-ulcer activity elucidated by *P. Pinnata* root could be mainly due to the modulation of defensive factors through an improvement of gastric cytoprotection and partly due to acid inhibition. The results also supported in the *in vitro* anti-oxidant studies, the Methanol extract of the root *P. Pinnata* involving in the scavenging process of free radical generation.

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