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PHARMACOLOGICAL EVALUATION OF ANTI ULCER ACTIVITY OF *Lilium candidum* LINN ON RODENT MODELS

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ABSTRACT

Ulcers are an open sore of the skin or mucus membrane characterized by sloughing of inflamed dead tissue. Ulcers are lesions on the surface of the skin or a mucous membrane characterized by a superficial loss of tissue. Ulcers are most common on the skin of the lower extremities and in the gastrointestinal tract, although they may be encountered at almost any site. There are many types of ulcer such as mouth ulcer, esophagus ulcer, peptic ulcer, and genital ulcer .Of this peptic ulcer is seen among many people. Pylorus ligation has been reported to causes severe hemorrhagic necrosis .Results of Histopathological studies have shown that pre-treatment with the AEEA (100 mg/kg/p.o. and 200 mg/kg/p.o.) reduced pylorus ligation-induced hemorrhagic necrosis in rats and also reduced sub mucosal oedema and leukocyte infiltration. Histopathology of reserpine administered stomach showed severe mucosal injury along with oedema in sub mucosal layer. Pre-treatment with AEEA (100 mg/kg/p.o. and 200 mg/kg/ p.o.) reduced the effects and maintained the mucosal integrity in a dose dependent manner. It may be concluded that AEEA (50 mg/kg/p.o. and 100 mg/kg/ p.o.) exerts gastro protective and antioxidant effect as it reduces the oxidative stress and consequently improves the integrity of gastric mucosa and enhances the generation of nitric oxide and mucus in experimentally-induced gastric ulcers. It was also concluded that AEEA at a dose of 200 mg/kg was more potent than 100 mg/kg.

Key words: Excoecaria agallocha, Antioxidant, Antiulcer activity.

INTRODUCTION

Ulcers are an open sore of the skin or mucus membrane characterized by sloughing of inflamed dead tissue [1]. Ulcers are lesions on the surface of the skin or a mucous membrane characterized by a superficial loss of tissue. Ulcers are most common on the skin of the lower extremities and in the gastrointestinal tract, although they may be encountered at almost any site. There are many types of ulcer such as mouth ulcer, esophagus ulcer, peptic ulcer, and genital ulcer .Of this peptic ulcer is seen among many people. The peptic ulcers are erosion of lining of stomach or the duodenum [2].

The two most common types of peptic ulcer are called “gastric ulcer” and “duodenal ulcer.” The name refers to the site of ulceration. A person may have both gastric and duodenal ulcers at the same time. Gastric ulcers are located in the stomach, characterized by pain; ulcers are common in older age group. Eating may increase pain rather than relieve pain. Other symptoms may include nausea, vomiting, and weight loss. Although patients with gastric ulcers have normal or diminished acid production, yet ulcers may occur even in complete absence of acid [3].

Duodenal ulcers are found at the beginning of small intestine and are characterized by severe pain with burning sensation in upper abdomen that awakens patients from sleep. Generally, pain occurs when the stomach is empty and relieves after eating. A duodenal ulcer is more common in younger individuals and predominantly affects males. In the duodenum, ulcers may appear on both the anterior and posterior walls [4]. In some cases, peptic ulcer can be life threatening with symptoms like bloody stool, severe abdominal pain, and cramps along with vomiting blood [5].

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The term “Mangroves”, plants which exist in muddy, wet soil in tropical or subtropical tidal waters. Lilium candidum. (Euphorbiaceae) is an ancient mangrove species specified in “Thillai Lord Nataraja” temple, Chidambaram as “Tala virucham” in tamil. Common name of Excoecaria agallocha: Agallocha, blinding tree (General name); Thillai, Kampetti (in Tamil); Tilla, Tella and Chilla (in Telugu); Thelakiriya, Thalia (in Singhalese) It is widely distributed abundant in Pichavaram mangrove forest, Indian coastal regions, Australia from northern New South Wales, along the northern coastline around to Western Australia. According to Red list criteria it is a least concern position [6]

It is used traditionally in the treatment of various diseases such as epilepsy, ulcers, leprosy, rheumatism, and paralysis. The latex obtained from the bark is poisonous in nature and may cause temporary blindness, thus it is also known as the blind-your-eye mangrove plant. The aim of this research work is to evaluate the anti ulcer activity of the selected Indian medicinal plan by pylorus ligation method using standard methods.

MATERIAL AND METHODS

Collection, Authentication Plant Drugs

Organoleptic characters, morphological characters, and microscopically examination would help in identifying crude drug. For identification of unknown drugs herbariums and leading botanical gardens are of great help. The leaves of selected plant namely *Lilium candidum* was purchased from Moolchand Phoolchand herbal store, Bhopal, Madhya Pradesh. The entire plant drug was authenticated by expert botanist of Department of Botany Safiya College Bhopal. All collected plant drug were cleaned, shade dried, pulverized into moderately coarse powder and stored in airtight container for further use.

Successive Solvent Extraction of Plant Drugs

The Collected plant drug (Leaves) was cleaned properly and washed with distilled water to remove any kind of dust particles. Cleaned and dried plant drug was converted into moderately coarse powder in hand grinder. Powdered plant drug was weighed (100 gm) and packed in Soxhlet apparatus. [7]

The plant Material (Leaves) was extracted with water (40°-60°C) for about 12 hrs. The Aqueous extracts were collected in a tarred conical flask. The solvent removed by drying on hot plate. The extracts obtained with each solvent were weighed to a constant weight and percentage w/w basis was calculated.

Preliminary Phytochemical Screening

Preliminary phytochemical screening means to investigate the plant material in terms of its active constituents. In order to detect the various constituents present in the different extracts of *Excoecaria agallocha,* was subjected to the phytochemical tests as per standard methods. Phytochemical screening was revealed for the presence of alkaloids, glycosides, carbohydrates, tannins, resins, flavonoids, steroids, proteins and amino acids [8].

Test Compound Formulations

The aqueous suspension of extract of leaves of *Lilium candidum* (AEEA) was prepared in 0.5 % carboxymethyl cellulose (CMC) solution in distilled water prior to oral administration to animals. Only freshly prepared solution was used. The vehicle alone served as control

Acute Toxicity study

The LD_{50} of the *Lilium candidum* was reported to be safe till 1000 mg/kg/i.p. Thus the studies were carried out by using two selected doses of aqueous extract of *Lilium candidum* 100 mg/kg and 200 mg/kg. The dose of plant was selected by hit and trial method. No death and side effects were found at both selected doses of plant.

Assessment of Anti-Ulcer Activity

Albino rats of either sex were divided into five groups of six animals each. Animals were fasted for 24 h before the study, but had free access to water. Animals in the disease control group received only distilled water before pylorus ligation. AEEA at 100 and 200 mg/kg, (p. o.) were given to the animals in the treatment group. Ranitidine (50 mg/kg) was used as a standard. After 1h of drugs treatment, they were anaesthetized with the help of anesthetic ether; the abdomen was opened by a small midline incision below the xiphoid process.

Pyloric portion of the stomach was slightly lifted out and ligated according to method of Shay et al. [9] avoiding traction to the pylorus or damage to its blood supply. The stomach was replaced carefully and the abdominal wall was closed by interrupted sutures. Rats were sacrificed by an over dose of anaesthetic ether after four hours of pyloric ligation. The abdomen was opened, cardiac end of the stomach was dissected out and the contents were drained into a glass tube. The volume of the gastric juice was measured and centrifuged at 2000 rpm for 10 min. From the supernatant, aliquots (1 ml of each) were taken for the determination of pH, total and free acidity. Each stomach was examined form lesions in the fore stomach portion and indexed according to severity.

Macroscopic evaluation of stomach

The stomachs were opened along the greater curvature, rinsed with saline to remove gastric contents and blood clots and examined by a 10X magnifier lens to assess the formation of ulcers. The numbers of ulcers were counted by the scoring method [10].

Scoring of ulcer will be made as follows

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
Percent inhibition of ulceration was calculated as below:

$$\% \text{ Inhibition of ulceration} = \frac{(\text{Ulcer Indexcontrol} - \text{Ulcer Indextest})}{(\text{Ulcer Indexcontrol})} \text{x100}$$

Determination of pH

An aliquot of 1ml gastric juice was diluted with 1ml of distilled water and pH of the solution was measured using pH meter [11].

Determination of total acidity

An aliquot of 1ml gastric juice diluted with 1ml of distilled water was taken into a 50 ml conical flask and two drops of phenolphthalein indicator was added to it and titrated with 0.01N NaOH until a permanent pink colour was observed. The volume of 0.01N NaOH consumed was noted [11].
The total acidity is expressed as mEq/L by the following formula:

$$\text{Acidity} = \text{Vol. of NaOH x} N \text{x} 100 \frac{\text{mEq}}{\text{L}} / 0.1$$

Assessment of Anti-oxidant activity

The stomach of rats was then weighed and homogenized in chilled phosphate buffer (10 mM, pH 7.4) at a concentration of 10% (w/v). The homogenate was then centrifuged at 10,000 x for 20 min. The clear supernatant was used for the assays of Lipid peroxidation (MDA content), MPO, endogenous antioxidant enzymes (nitrate/nitrite, Superoxide dismutase and reduced glutathione) and for the determination of gastric adhesion mucus content.

Biochemical Estimations

Superoxide dismutase was determined by the method [12]. Reduced glutathione was estimated by the method [13]. Lipid peroxidation or malondialdehyde formation was estimated by the method [14]. Nitrate level was determined by the method [15]. Gastric adhesion mucus content was determined by the method [16]. Assay of Gastric mucosal myeloperoxidase activity was estimated [17]. Total proteins were determined by the method of Biuret by using protein estimation kit.

Assesment of integrity of stomach using Histopathological studies

The stomach was excised and immediately immersed in 10% buffered formalin. They were then dehydrated in the graded concentrations of ethanol, immersed in xylene, and then embedded in paraffin. From the paraffin blocks, 4-mm thin sections were cut, and staining is done using with haematoxylin (0.6% w/v) for 15 min followed by counterstaining with eosin (1% w/v) for 2 min. They were then examined using light microscopy to analyze integrity of stomach, using an image analysis program (NIH Scion image analyzer).

Statistical analysis

The results were analysed statistically using one way analysis of variance (ANOVA) followed by tukey test. A probability value of $p < 0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

Extraction of Plant Drugs

The yields were found to be 9.12g (9.12% w/w of crude drug) of Aqueous extract with Bark brown colour semisolid mass, for *Lilium candidum* Leaves.

Evaluation of in Vitro Antioxidant Activity
Quantitative antioxidant activity
Estimation of total phenolic content

The total phenolic content of the ***Lilium candidum*** leaf extracts was compared with standard curve of catechol (y=0.004x-0.001, R2=0.990) and the results were expressed as the number of equivalents of catechol (µg/mg of extract). Among the Four solvents used, aqueous extract showed prominent total phenolic activity (76µg of catechol/mg of extract).

Estimation of total flavonoids content

The standard curve of catechol (y=0.027x-0.075, R2=0.987) was used to express the total flavonoid content of the ***Lilium candidum*** leaf extracts in terms of µg/mg of extract (Fig.). Aqueous extract of ***c*** found to be 25µg of catechol/mg.

Determination of total antioxidant capacity

The results of total antioxidant capacity revealed that ***Lilium candidum*** leaf under study has prominent potentials for the parameter. The activity is expressed in terms of the equivalence of ascorbic acid (µg/mg of extract) using the standard curve of ascorbic acid (y=0.006x-0.04, R2=0.991). The extract, Aqueous extract has shown higher activity (76 µg/mg of extract).

Qualitative antioxidant activity
i. DPPH radical scavenging

The results of DPPH radical scavenging activity was found to be highest in terms of EC_{50} values in Aqueous extracts (66.70µg/ml) .The results were compared with the standard, ascorbic acid with an EC_{50} value of 14.37µg/ml .
Nitric oxide radical scavenging

The results of nitric oxide radical scavenging activity revealed that ***Lilium candidum*** possessed scavenging activity. The aqueous extract (320 µg/ml) has good scavenging ability .Ascorbic acid recorded 194.44 µg/ml of EC_{50}.

Hydroxyl radical scavenging

The selected plant showed good hydroxyl radical scavenging activity. The hydroxyl radical scavenging activity of ascorbic acid was found to be 6.58µg/ml and that of extracts was (32µg/ml) .The results were presented in Fig.

iv. Fe2+ chelating

The selected plant under study, ***Lilium candidum*** aqueous extract (754µg/ml), The results were compared with standard disodium EDTA (57 µg/ml) and expressed in terms of EC_{50} values (Fig. 4.19).

Reducing power assay

The plant have shown prominent reducing power effect in a dose dependent manner. ***Lilium candidum*** aqueous extract has shown maximum activity with an effective concentration (EC_{50}) of 191.47µg/ml .Standard ascorbic acid recorded an EC50 value of 78.43µg/ml (Fig).

Animal Study (Anti ulcer activity)

Study of antiulcer and antioxidant activity using pylorus ligation model. It was observed that in disease control, the ulcer index was 92.50 ± 5.93 and the maximum numbers of ulcers were of the score 4 and 5, and a number of perforated ulcers (score 25) were also observed. AEEA (100mg/kg/p.o. and 200 mg/kg/p.o.) was found to produce significant decrease in ulcer index. All the ulcers were of scores 1 and 2 and no perforated ulcers were observed.

In control rats, pylorus ligation for 4 h resulted in accumulation of 3.1 ± 0.3 ml of gastric secretion with pH 3.11 ± 0.354 and a total acid output of 104.2 ± 9.34 mEq/L/100 gm. The volume of gastric secretion in the rats treated with 100 mg/kg/p.o. and 200 mg/kg/p.o. of AEEA significantly reduced to 2.67 ± 0.24 ml and 0.97 ± 0.08 ml respectively.

A marked increase in the pH of gastric content was observed with AEEA at a dose of 200 mg/kg (5.25 ± 0.405).

However, AEEA at lower dose (100 mg/kg/p.o.) produced a non significant effect on pH of gastric content. A significant decrease in total acid output was observed in the rats treated with AEEA (100 mg/kg/p.o. and 200 mg/kg/p.o.)

As compared to normal rats, pylorus-ligation was found to increase lipid peroxidation and MPO activity. A significant decrease in SOD, reduced glutathione, gastric adhesion mucus content and nitrate/nitrite levels in the disease control has observed.

Administration of AEEA (100 mg/kg/p.o. and 200 mg/kg/p.o.) brought about a significant reduction in lipid peroxidation and MPO activity and an increase in the activities of antioxidant enzymes namely, SOD and GSH. An increase in the level of tissue nitrate/nitrite has also observed. A similar observation was done with gastric wall mucus. The treatment of rats with AEEA (100 mg/kg/p.o. and 200 mg/kg/p.o.) significantly increased the alcian blue binding capacity of gastric wall mucus as compared to disease control (Table 6.2).

Histological evaluation of gastric lesions induced by Pylorus ligation

Histological observation of pyloric ligation-induced gastric lesions in disease control showed comparatively extensive damage to the gastric mucosa, oedema and leucocytes infiltration of the submucosal layer as compared with normal control (Figure 1 and 2). Rats that received pretreatment with AEEA (100 mg/kg/p.o. and 200 mg/kg/p.o.) had comparatively better protection of the gastric mucosa as seen by reduction in ulcer area, reduced or absence of submucosal oedema and leucocytes infiltration (Figures 3 and 4). The AEEA has been shown to exert the cytoprotective effects in a dose-dependent manner.

The anti-ulcer activity of the leaves of *Lilium candidum* was evaluated by employing pylorus ligation ulcer model. This model represents 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 pylorus ligation. Pylorus ligation -induced ulcers occur as a result of discernible increase in acid-pepsin accumulation due to pylorus obstruction and subsequent mucosal digestion. Further, literature review reveals that in pylorus ligation model, the interference of gastric blood circulation is too responsible for induction of ulcers.

Gastric acid and pepsin are important factors for the formation of ulcers in pylorus ligated rats . In addition to this, increase oxidative stress is also responsible in the progression of ulceration . This contention is supported by results obtained in our study that there is marked increase in lipid peroxidation along with significant attenuation in the level of various antioxidants like GSH and SOD. Pylorusligation also caused a significant depletion of gastric adhesion mucus content along with increase MPO activity characterized by leukocyte infilteration.

It is evident from the result obtained in our study that the aqueous extract of *Lilium candidum* leaves (100 mg/kg and 200 mg/kg) significantly inhibit gastric ulceration in pylorus ligated rats.

The anti-ulcer activity of *Lilium candidum* extract in pylorus ligation model is evident from its significant reduction in gastric volume, total acidity, free acidity and increase in pH of gastric juice. It is suggested that AEEA can suppress gastric damage induced by aggressive factors. The ulceration in pyloric-ligated rats is generally caused by increased acid and peptic activity.

Also AEEA (100 mg/kg and 200 mg/kg) produced a significant increase in the level of various antioxidants with subsequent decrease in the level of oxidative stress parameters, which provides its role as antioxidant. AEEA

significantly restore the level of NO and also restore the depletion of gastric adhesion mucus content.

Pylorus ligation has been reported to causes severe hemorrhagic necrosis .Results of Histopathological studies have shown that pre-treatment with the AEEA (100 mg/kg/p.o. and 200 mg/kg/p.o.) reduced pylorus ligation-induced hemorrhagic necrosis in rats and also reduced submucosal oedema and leukocyte infilteration. Histopathology of reserpine administered stomach showed severe mucosal injury along with oedema in submucosal layer. Pre-treatment with AEEA (100 mg/kg/p.o. and 200 mg/kg/ p.o.) reduced the effects and maintained the mucosal integrity in a dose dependent manner.

Figure 1: Total phenolic estimation of Excoecaria agallocha

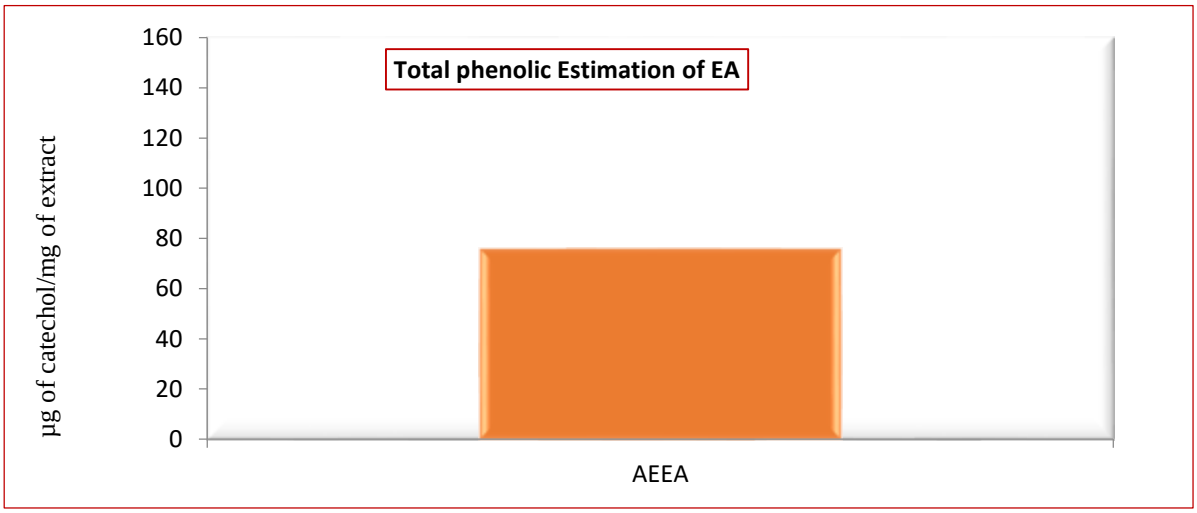


Figure 2: Total Flavonoid estimation of Excoecaria agallocha.

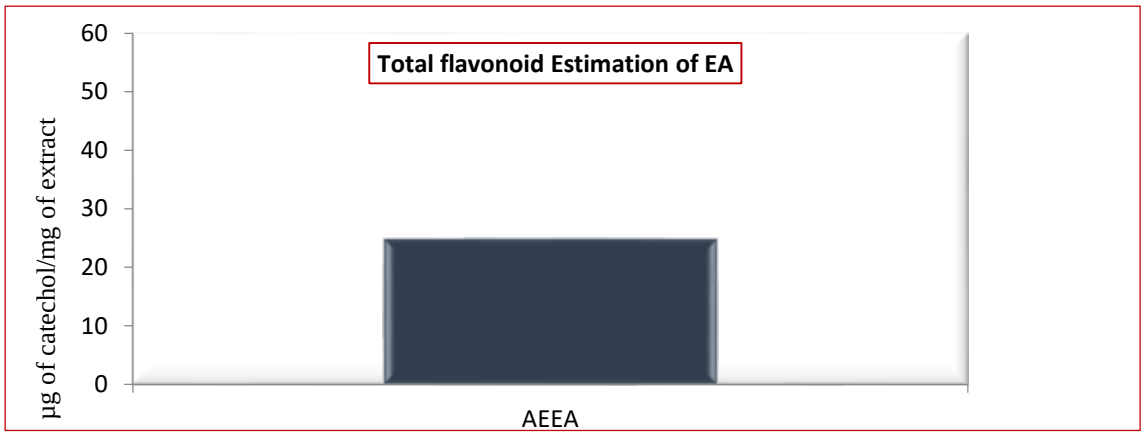


Figure 3: Total antioxidant capacity of Excoecaria agallocha.

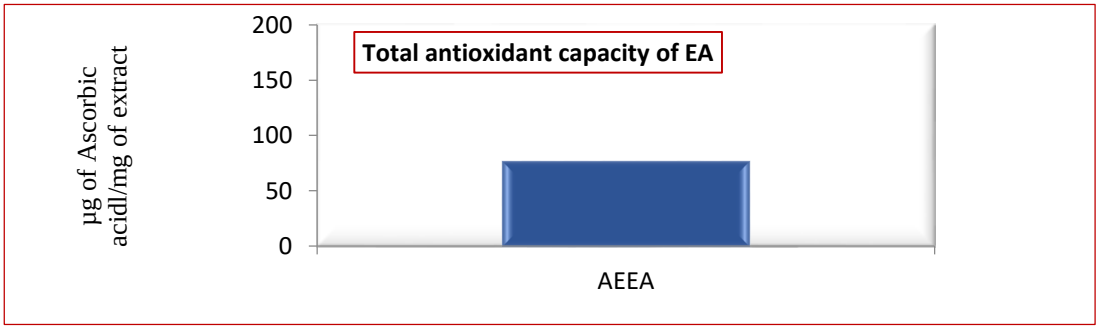


Figure 4: DDPH radical Scavenging activity of Excoecaria agallocha

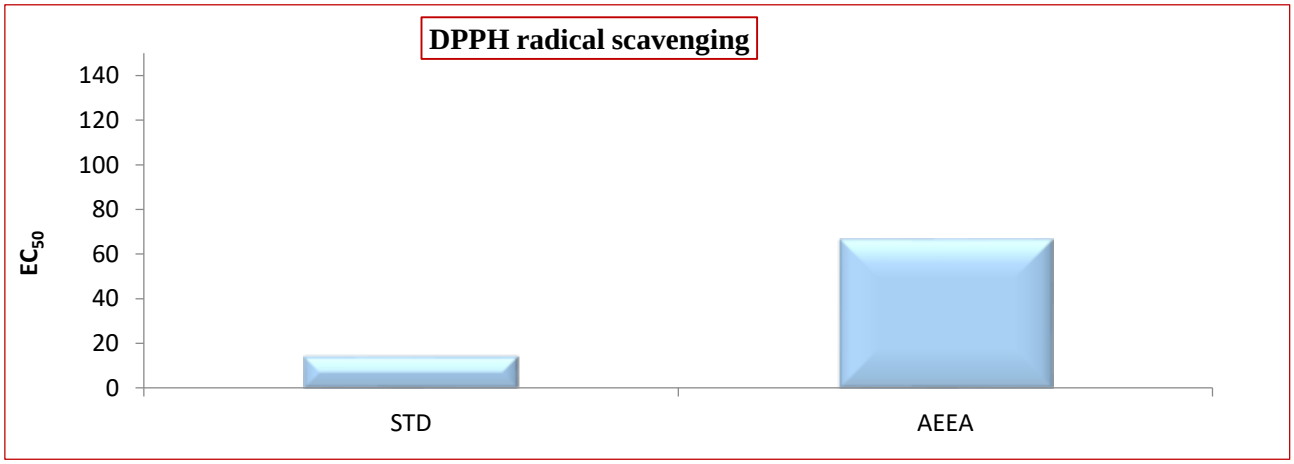


Figure 5: Nitric oxide radical scavenging of Excoecaria agallocha

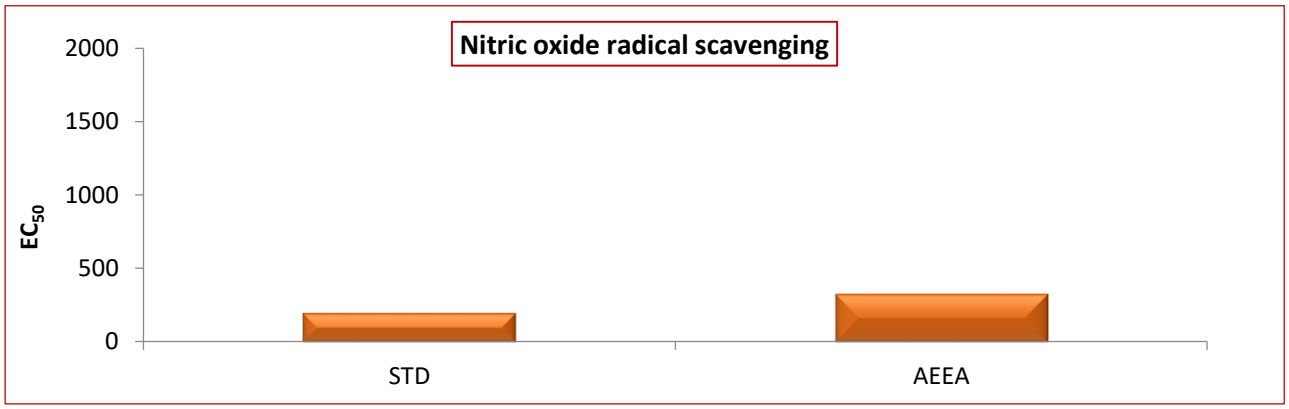


Figure 6: Hydroxyl radical scavenging of Excoecaria agallocha

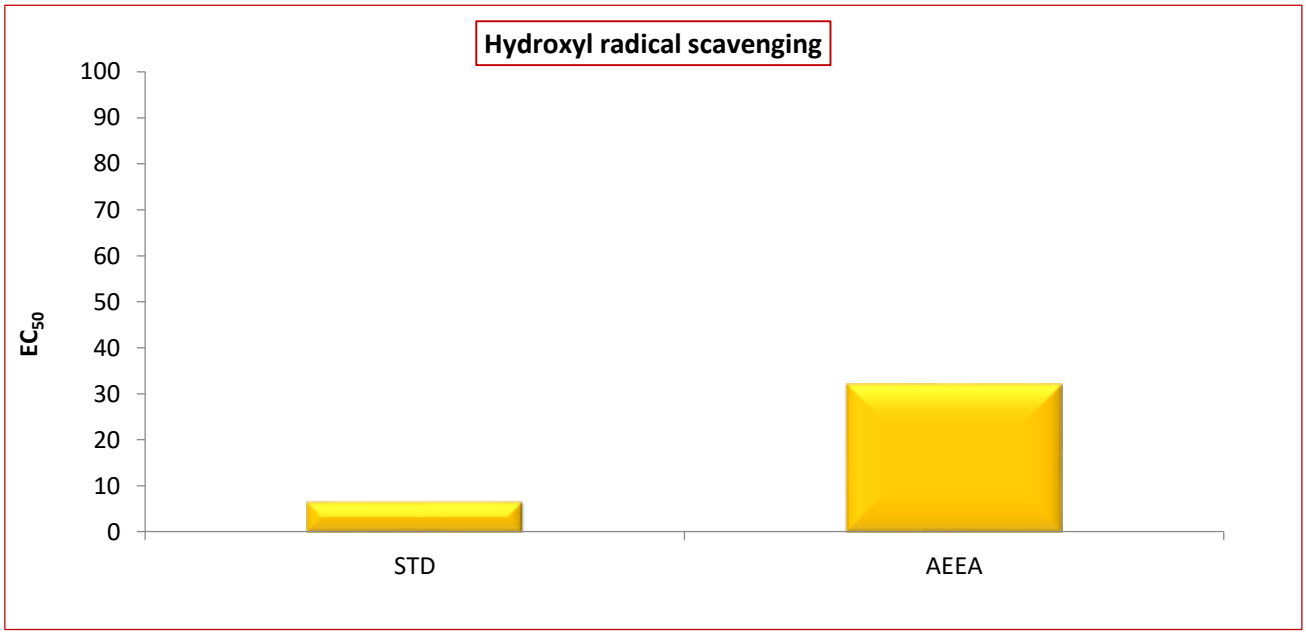


Figure 7: Fe2+ chelating capacity of Excoecaria agallocha.

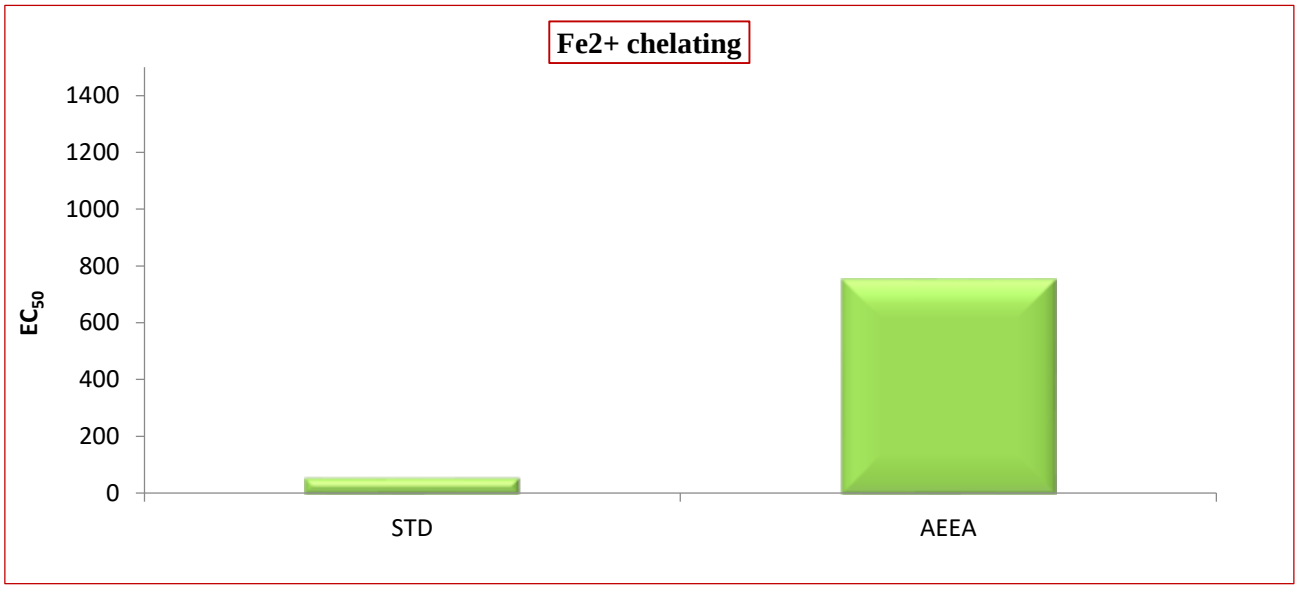


Figure 8: Reducing power assay of Excoecaria agallocha.

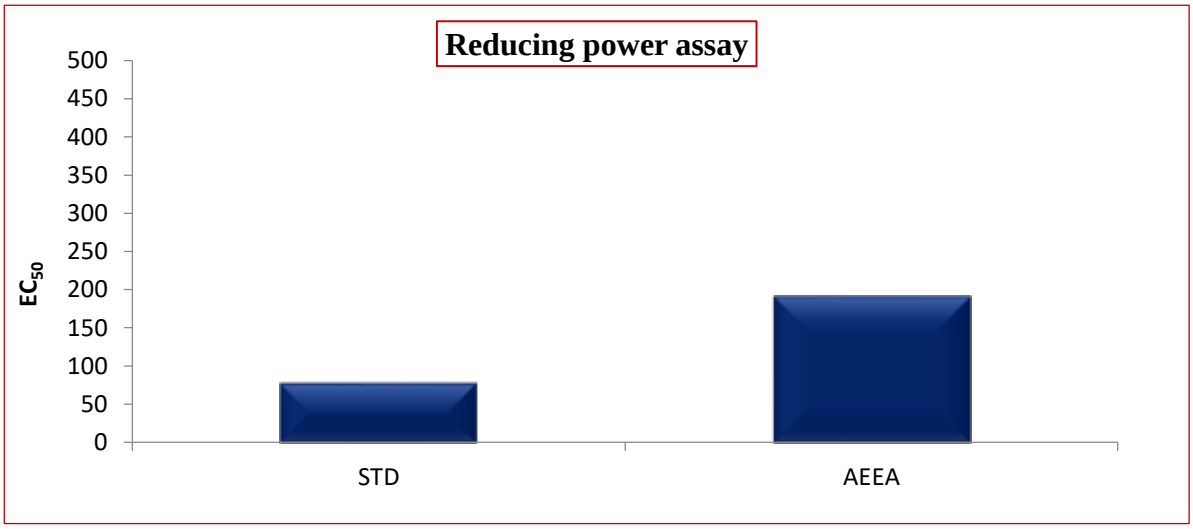


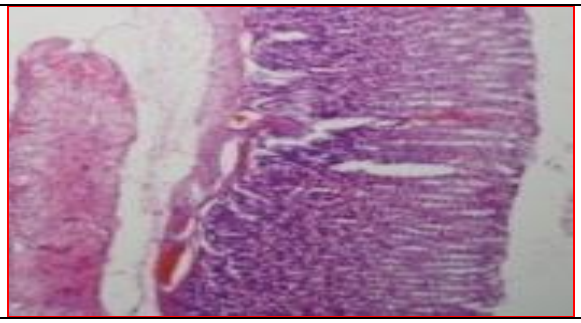
Figure 9: Histological section of gastric mucosa of normal control rat.	**Figure 10: Histological section of gastric mucosa in a pylorus-ligation control rat.**
	

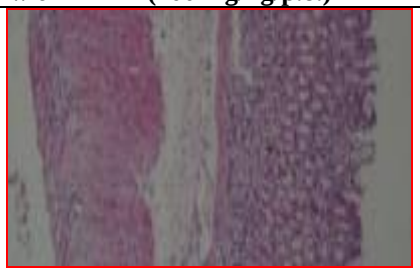
Figure 11: Histological section of gastric mucosa in a rat pre-treated with AEEA (100 mg/kg/p.o.)	Figure 12: Histological section of gastric mucosa in a rat pre-treated with AEEA (200 mg/kg/p.o.)
	

Table 1: Parameters of Anti ulcer activity.

Parameters	Pylorus Ligation Control	Ranitidine Treated	*Lilium candidum* Treated	
			100 mg/kg	200 mg/kg
Ulcer Index (mm2)	94.50 ± 7.93	8.5 ± 3.36*** (89.97%)	57.83 ± 8.85** (37.21%)	13.67 ± 2.81*** (85.33%)
Volume of Gastric Fluid (ml)	3.1 ± 0.3	0.8 ± 0.095***	1.67 ± 0.14**	0.87 ± 0.07***
pH of Gastric fluid	3.11 ± 0.254	5.32 ± 0.35***	2.63 ± 0.34	5.25 ± 0.305***
Total Acidity (m Eq/l/100g)	104.2 ± 9.24	17.5 ± 3.7***	71.33 ± 7.20*	26.67 ± 7.86***

Values are expressed as mean+SEM. Control group was compared with normal control. Treated groups were compared with disease control. *p>0.05 ; ** p>0.01 ; ***p>0.001 ; ns = non significant; Values in parenthesis indicate the % reduction in ulcer index in relation to the control group.

Table 2: Parameters of Anti ulcer activity.

Parameters	Normal Control	Disease Control	Ranitidine Treated	*Lilium candidum* Treated	
				100mg/kg	200 mg/kg
SOD (unit/mg protein)	9.52 ± 0.94	1.65 ± 0.28***	7.88 ± 0.64***	6.47 ± 0.33**	9.24 ± 0.84***
Reduced glutathione (µmol/mg protein)	0.85 ± 0.029	0.24 ± 0.033***	0.77 ± 0.039***	0.49 ± 0.034***	0.77 ± 0.047***
Nitrate (µmol/mg protein)	11.06 ± 0.39	3.65 ± 0.49***	8.86 ± 0.48***	8.05 ± 0.58***	9.72 ± 0.47***
Lipid peroxidation (nmoles/mg protein)	4.82 ± 0.61	18.91 ± 1.83***	4.84 ± 0.69***	11.82 ± 1.38**	5.87 ± 0.69***
MPO (µ/g of protein)	13.95 ± 0.70	22.38 ± 1.20***	14.32 ± 0.80***	18.97 ± 0.49*	14.17 ± 0.70***
Gastric adhesion mucus content (µg/g wet glandular tissue)	222 ± 8.29	160.2 ± 11.25***	211 ± 5.85***	193.8 ± 2.22*	216.2 ± 6.16***

CONCLUSION

It may be concluded that AEEA (50 mg/kg/p.o. and 100 mg/kg/ p.o.) exerts gastro protective and antioxidant effect as it reduces the oxidative stress and consequently improves the integrity of gastric mucosa and enhances the generation of nitric oxide and mucus in experimentally-induced gastric ulcers. It was also concluded that AEEA at a dose of 200 mg/kg was more potent than 100 mg/kg

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