MATERIALS AND METHODS
3. MATERIALS AND METHODS:

3.1 Pharmacognostical and Phytochemical studies:

Plant Materials:

The root of *C. arundinaceum* was obtained during the month of March 2003, from Sagar region (M.P.) and dried. The root was identified by the department of Pharmacognosy, L.M. College of Pharmacy, Ahmedabad. The dried roots were then crushed, finely powdered (40# sieve) and were used for present work.

Macroscopical Characters:

Size : 3-5 cm. in length.
Shape : Cylindrical.
Surface : Hard and uneven.
Fracture : Short, granular.
Color : White to creamish buff.
Odor : None.
Taste : Bland.

Drug swells considerably when soaked in water and becomes soft and flaccid.

Microscopical Observations:

Free hand sections were taken of roots and were stained with number of reagents for histochemical examination\(^{197}\).

Physico-Chemical Parameters:

**Determination of Ash Value\(^{198}\):**

**Determination of Total Ash:**

2g of accurately weighed root powder was incinerated in crucible at a temperature not exceeding 450° C on a hot plate, till carbon free ash
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was obtained. It was then cooled, weighed and percentage of ash was calculated with reference to the air-dried drug.

**Determination of Acid Insoluble Ash:**
The ash obtained from above procedure was boiled for 5 minutes with 25ml of dilute hydrochloric acid and filtered using an ash less filter paper to collect insoluble matter. The ash obtained was washed with hot water and filter paper was burnt to a constant weight. The percentage of acid-insoluble ash was calculated with reference to the air-dried drug.

**Determination of Water Soluble Ash:**
Ash was boiled for 5 minutes with 25ml of water, and insoluble matter was collected on an ash less filter paper, washed with hot water and ignited for 15 minutes at a temperature not exceeding 450° C. Difference in weight of ash and weight of water insoluble matter gave the weight of water-soluble ash. The percentage of water-soluble ash was calculated with reference to the air-dried drug.

**Determination of Extractive Value**

**Determination of Alcohol Soluble Extractive (Maceration Method):**
5g of accurately weighed root powder was macerated with 100ml of alcohol in a closed flask for 24 hours, shaking frequently at an interval of six hours. It was then allowed to stand for 18 hours and filtered rapidly. To prevent any loss during evaporation, 25ml of the filtrate was evaporated to dryness in a porcelain dish and dried at 105° C to a constant weight. The percentage of alcohol soluble extractive was calculated with reference to air-dried drug.

**Determination of Alcohol Soluble Extractive (Reflux Method):**
5g of accurately weighed root powder was mixed with 100ml of alcohol in a round bottom flask (RBF) and reflux it at 80-90° C for 1 hour. The content was filtered and the filtrate was collected. To prevent any loss
during evaporation, 25ml of the filtrate was evaporated to dryness in a porcelain dish and dried at 105° C to a constant weight and weighed. The percentage of alcohol soluble extractive was calculated with reference to air-dried drug.

**Determination of Water Soluble Extractive (Maceration Method):**
5g of accurately weighed root powder was macerated with 100ml of distilled water in a closed flask for 24 hours, shaking frequently at an interval of six hours. It was then allowed to stand for 18 hours and filtered rapidly. To prevent any loss during evaporation, 25ml of the filtrate was evaporated to dryness in a porcelain dish and dried at 105° C to a constant weight and weighed. The percentage of water-soluble extractive was calculated with reference to air-dried drug.

**Determination of Water Soluble Extractive (Reflux Method):**
5g of accurately weighed root powder was mixed with 80ml of distilled water in a RBF and reflux at 80-90° C for 1 hour. The content was filtered and the filtrate was collected. To prevent any loss during evaporation, 25ml of the filtrate was evaporated to dryness in a porcelain dish and dried at 105° C to a constant weight. The percentage of alcohol soluble extractive was calculated with reference to air-dried drug.

**Determination of Ether Soluble Extractive:**
5g of accurately weighed root powder was extracted with petroleum ether (40° to 60° C) in a soxhlet extractor for 6 hours and filtered. The solvent was evaporated off on a water bath and the residue was dried at 105° C to a constant weight. The percentage of ether-soluble extractive was calculated with reference to air-dried drug.
Phytochemical Screening:

Tests for Saponins¹⁹⁹,²⁰⁰:

(a) Froth Test:

0.1g of root powder was vigorously shaken with 5 ml of distilled water in a test tube for 30 seconds and was left undisturbed for 20 minutes. Persistent froth indicated presence of saponins.

(b) Haemolytic Zone:

0.5 ml of blood was mixed with gelatin solution (3g gelatin powder dissolved in 100 ml of 0.85% NaCl solution at 60° C and taken on a glass slide).

A thick section of root was placed on it. A clear haemolytic zone was formed around the section indicating the presence of saponins.

Test for Sterols²⁰¹,²⁰²:

Liberman Burchadt Test:

To 1g powder drug moistened with 1.0 ml of acetic anhydride on a clean tile, were added 2 drops of sulphuric acid. The powder was mixed well and the color gained by the powder was observed. Formation of green- blue- purple-red color indicated presence of sterols.

Estimation of Saponins¹⁹⁹,²⁰³:

(a) Froth Number¹⁹⁹,²⁰³:

It is the dilution corresponding to 1g of the drug which gives one centimeter high froth, when 10 ml of such diluted solution is shaken for 30 seconds and the froth height is measured after 15 minutes in a test tube having internal diameter 16 mm.

Preparation of Aqueous Extract:

1g of the root powder was macerated with 100 ml of distilled water in a conical flask for 12 hours. The content was refluxed for 10 minutes.
using boiling water bath. The extract was filtered while hot and after cooling to room temperature, the volume was adjusted to 100 ml using distilled water.

**Method:**

The extract was used separately for estimation of froth number. Twenty test tubes having uniform internal diameter of 16 mm were selected and were divided into 2 groups (10 in each group).

To each of these test tubes extracts were added in increasing order of 1 ml, 2 ml, 3 ml, ...... 10 ml and final volume in each of them was made to 10 ml using distilled water. Each test tube was shaken vigorously for 30 seconds and left undisturbed. The height of the froth formed was measured after 15 minutes.

**(b) Haemolytic Index:**

Haemolytic Index is the concentration of the drug corresponding to 1g, which causes complete haemolysis of R.B.C. in the blood. Fresh citrated human blood was obtained from the local pathological laboratory and was stored in the refrigerator at 5°C and used within 14 days.

**Preparation of Extract:**

1g of root powdered was refluxed for 1 hour with 100 ml of isotonic NaCl solution (0.9% w/v), filtered while hot and cooled. The final volume of the filtrate was adjusted to 100 ml using isotonic NaCl solution.

**Method:**

A10% suspension of erythrocytes (9 ml) in physiological normal saline (0.9% w/v NaCl) was incubated with different concentrations of sample solutions (1 ml) in 10% methanol for 30 min at 30°C. After incubation, 1 ml of each mixture was withdrawn and centrifuged at 2000 rev min⁻¹ for 1 min. The absorbance of each supernatant at 540
nm was measured. The value % haemolysis was calculated from the ratio of the absorptions. The tolerance of erythrocytes towards 10% methanol was also tested and it was found that no haemolysis occurred.

**ISOLATION AND FRACTIONATION:**

**Preparation of 50% Alcoholic Extract (Extract A)**

The dried roots (250 gm) were crushed and finally powdered and shifted from 40# sieve and defatted with petroleum ether (60°-80° C, 500ml X 4 times). Then extracted with 50% ethanol (500ml X 6 times). The pooled extract was concentrated under reduced pressure to yield 11.25% dry solid. It was stored in a refrigerator at 4° C in a glass container throughout the study.
SCHEME FOR EXTRACTION

Root powder

Extraction with absolute alcohol

Dry powder

Extracted with distilled water

Ethanolic extract (4.96% yield)
(Concentrated to semisolid mass and taken in Distilled Water)

Petroleum ether

Concentrated till soluble (0.064% yield)

Aqueous fraction in (MeOH : H₂O) (20 : 80)

EtOAC

EtOAC Soluble (0.5% yield)

EtOAC insoluble

n-Butanol

Precipitate of POLYSACCHARIDES
(Extract P, 16.43% yield)

n-Butanol fraction

Taken in Absolute alcohol

Add excess of Ether or Acetone

Precipitate of SAPONINS
(Extract S, 1.56% yield)
3.2 Experimental Gastro-duodenal Ulcers:

Animals:
Wistar albino rats of either sex 200± 20 g were divided into groups of 8-10 animals. The distribution of animals’ in-groups, the sequence of trials and the treatment allotted to each group were randomized. The animals received standard rat chaw diet and water ad libitum under standard conditions of 12h dark-light period, humidity and temperature. The animals were fasted for 36 h with free access to water prior to the ulcerogenic assay because standard drugs and the extract were administered orally.

PRELIMINARY ANTI ULCER STUDY:
Preliminary anti-ulcer study was carried out with 50% ethanolic extract (Extract A, 300 mg kg⁻¹) of roots, on ethanol-induced gastric ulcers in rats47. Based on these results, extraction was performed to get two major fractions, Polysaccharides (Extract P) and Saponins (Extract S), which were screened for their anti-ulcer effects on ethanol-induced gastric ulcers in rats47. Saponins, the active fraction giving better protection, were then further evaluated in other experimental models.

Ethanol-induced Gastric Mucosal Ulcers in Rats207:
The ethanol induced lesion assay was carried according to the method of Robert et al207. Animals were given 1.0 ml of absolute alcohol, one hour after the administration of the Extracts A-300 mg kg⁻¹, P-300 mg kg⁻¹, S (100 mg kg⁻¹, 200 mg kg⁻¹ and 300 mg kg⁻¹) orally. Pentoprazole (20 mg kg⁻¹) was given as positive control. Two hours after ethanol treatment the animals were killed, the stomachs were opened along the greater curvature and the ulcer index calculated208. The stomach glandular tissue was weighed and subjected to estimation of gastric wall mucus content209, lipid peroxidation assay210, superoxide dismutase211, catalase212 and reduced glutathione213.
HCl-Ethanol-induced Gastric Mucosal Ulcers in Mice\textsuperscript{214}:
Gastric mucosal lesions were induced by the method of Yamada et al\textsuperscript{214}, based on the modifications of Mizui and Doteuchi\textsuperscript{215}. Animals were given 0.2ml of 0.3 M HCl / 60% ethanol orally fifty minutes after the administration of Extract S 100 mg kg\textsuperscript{-1} (p.o). Cimetidine (100 mg kg\textsuperscript{-1}) was given as positive control. The animals were sacrificed after one hour. The stomachs were excised, opened along the greater curvature and examined for lesions in glandular portion. Gastric lesions were measured and expressed in terms of ulcer index. Extent of lipid peroxidation was determined by estimating thiobarbituric acid reactive substance by method of Kiso et al\textsuperscript{210}.

Ethanol-induced Pylorus Ligated Gastric Mucosal Ulcers in Rats\textsuperscript{216}:
One hour before the administration of ethanol, Extract S (100 mg kg\textsuperscript{-1}) and pantoprazole (20 mg kg\textsuperscript{-1}, positive control) were administered orally. Pylorus of anesthetized rats was ligated one hour after the administration of 1ml absolute ethanol. Two hours after pylorus ligation, rats were killed by cervical dislocation and the esophagi were clamped. The stomach was removed and inspected externally and its content drained into graduated centrifuge tube and centrifuged at 2500 rev minutes\textsuperscript{-1} for 10 minutes. The volume of supernatant and pH were recorded. The juice was further subjected to analysis of various biochemical parameters. Each stomach was examined for lesions in the glandular portion, which were measured and expressed in terms of the ulcer index.

Ethanol-induced Gastric Mucosal Ulcers in N-ethylmaleimide (NEM) pretreated Rats\textsuperscript{217}:
N-ethylmaleimide was injected subcutaneously at the dose of 10 mg kg\textsuperscript{-1}, 30 minutes before the treatment of Extract S 100 mg kg\textsuperscript{-1} orally. Animals were given 1.0 ml of absolute ethanol, one hour after the administration of the extracts. Two hours after ethanol treatment the
animals were killed, the stomachs were opened along the greater curvature and the ulcer index calculated\textsuperscript{208}.

**Ethanol-induced Gastric Mucosal Ulcers in \textit{N^G}-nitro-L-arginine methylester (l-NAME) pretreated Rats}\textsuperscript{217}:

\textit{N^G}-nitro-L-arginine methylester was injected intraperitoneally at the dose of 70 mg kg\textsuperscript{-1}, 30 minutes before the treatment of Extract S 100 mg kg\textsuperscript{-1} orally. Animals were given 1.0 ml of absolute ethanol, one hour after the administration of the extracts. Two hours after ethanol treatment the animals were killed, the stomachs were opened along the greater curvature and the ulcer index calculated\textsuperscript{208}.

**Indomethacin-induced Gastric Mucosal Ulcers in Rats\textsuperscript{218}:

Indomethacin was used to block the formation of prostaglandin by inhibition of cyclooxygenase\textsuperscript{219}. Indomethacin was suspended in 1% carboxymethylcellulose in water and administered 30 mg kg\textsuperscript{-1} orally to 36 hours fasted rats. Extracts S (50 mg kg\textsuperscript{-1}, 100 mg kg\textsuperscript{-1} and 200 mg kg\textsuperscript{-1}) and pentoprazole (20 mg kg\textsuperscript{-1}) orally as positive control were administered thirty minutes before the treatment of Indomethacin. The rats were killed 6 hours after the treatment of Indomethacin. Ulcerated area of gastric lesions and score for intensity was measured\textsuperscript{220}.

**Cysteamine-induced Duodenal Ulcers in Rats\textsuperscript{220}:

The method described by Szabo\textsuperscript{220}, was followed. Wistar albino rats of either sex weighing 200-220g were used; food and water were freely allowed throughout the study. Duodenal ulcers were induced by the administration of 2 doses of cysteamine hydrochloride (400 mg kg\textsuperscript{-1}, p.o. each) in 10% aqueous solution at intervals of 4 hours. Animals were given p.o. Extract S (100 mg kg\textsuperscript{-1}) and cimetidine (100 mg kg\textsuperscript{-1}) as positive control, 30 minutes before each dose of cysteamine. Animals were sacrificed 24 hours after the first dose of cysteamine and duodenum were excised carefully and opened along the antimesentric
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side. The duodenal ulcers were scored for intensity using a scale of 0 to 3, where,

- No Ulcer = 0
- Superficial mucosal erosion = 1
- Deep or transdermal necrosis = 2
- Perforated ulcer = 3

The ulcer index is the sum of the arithmetic means of the intensity in a group and the ratio of positive / total multiplied by 2.

**Parameters under Investigation:**

**Physical Parameters:**

Ulcer index and total lesion area\(^{208}\).

Volume of gastric secretion.

pH of gastric juice

Score of intensity of ulcer\(^{209}\)

**Biochemical Parameters:**

**Acid Secretory Parameters:**

Total acidity\(^{221}\)

Total acid output\(^{221}\)

Pepsin activity\(^{222}\)

**Mucoprotective Parameters:**

Total carbohydrates (TC)\(^{223}\)

Protein content (PR)\(^{224}\)

Gastric wall mucus barrier\(^{209}\)

Mucin activity (TC / PR ratio)
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*Lipid Peroxidation:*
Estimation of malondialdehyde content

*Preventive Antioxidants:*
Superoxide dismutase (SOD)
Catalase

*Chain Breaking Antioxidants:*
Reduced Glutathione (GSH)

*Physical Parameters:*

**Ulcer Index**: Each linear lesion was measured along its greater curvature. For circular lesion, diameter (d) was measured and finally area was calculated. 5 petechies were considered to be equivalent to 1 mm² of ulcerated area. The ratio (X) of total area of stomach mucosa and that of ulcerated mucosa was calculated.

\[
X = \frac{\text{Total area of stomach mucosa}}{\text{Total area of ulcerated mucosa}}
\]

Ulceter index = \(10 / X\)

Area of circular lesion = \(\pi d^2 / 4\) (\(d = \) diameter of circular lesion)
Area of linear lesion = \(L \times B\)
Area of stomach mucosa = \(\pi D^2 / 8\) (\(D = \) diameter of stomach mucosa)

**Volume of Gastric Secretion:**
The contents of stomach were drained into a graduated tube, which was centrifuged at 2000 rev min⁻¹ for 10 minutes. The volume of gastric juice was measured and calculated for 100g body weight of animals.
Score of Intensity of Ulcer:
- No ulcer = 0
- Superficial mucosal erosion = 1
- Deep ulcer = 2
- Perforated ulcer = 3

Biochemical Parameters:

Acid Secretory Parameters:

pH of Gastric Juice:
pH was recorded with a digital pH meter.

Total Acidity:
The total acidity of gastric juice was estimated by titration of 0.1 ml of gastric juice with 0.01N NaOH to pH 7.0 using phenolphthalein as an indicator. The total amount of acid was calculated and expressed as mEq L⁻¹. These results may also be expressed in terms of 0.01N NaOH required for 100 ml gastric juice.

\[
\text{Total acidity} = \frac{\text{ml of NaOH} \times N \times 100 \text{ mEq L}^{-1}}{0.1}
\]

Total Acid Output:

Total acid output = \frac{\text{Total acidity} \times \text{volume of gastric content}}{100 \text{ g of body weight}}

It is expressed as \(\mu\text{Eq L}^{-1} / 100 \text{ g body weight}\).

Pepsin Activity:
The pepsin activity was estimated as described by Debnath et al.

Reagents:

0.01N & 0.06N HCl: These solutions were prepared by dilution with distilled water from stock solution prepared of 1N HCl, at the time of use.
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**Hemoglobin solution**: 2% Hemoglobin solution in 0.06 N HCl was freshly prepared filtered and used.

**Trichloroacetic acid**: To 10% TCA solution in distilled water charcoal was added, mixed thoroughly, kept for 10 minutes and filtered.

**Folin Ciocalteau reagent**: 1N Folin ciocalteau reagent was prepared by 2 times dilution with water.

**Alkaline mixture**: Freshly prepared 2% solution of Na₂CO₃ in 0.1N NaOH.

**Alkaline Reagent**: 100 ml of alkaline mixture was mixed with 1 ml of % aqueous solution of potassium tartarate and 1 ml of 2% aqueous copper sulphate.

**L. Tyrosine**: Standard in estimation of digested substrate to indicate pepsin activity.

**Procedure:**
0.4 ml of the diluted gastric juice (1:250 dilutions) and 1 ml of hemoglobin solution in 0.06N HCl were taken in separate test tubes and incubated at 37°C for 10 minutes. The hemoglobin solution was then added to the diluted gastric juice and the mixture was again incubated at 37°C for 20 minutes. Protein digestion was stopped by adding equal volume (1.4 ml) of 10% ice-cold trichloroacetic acid, kept in an ice bath for 15 minutes and filtered to separate the precipitated undigested protein. To determine the concentration of liberated amino acid tyrosine 0.4 ml of filtrate was first mixed with 4 ml of alkaline reagent, followed after 10 minutes by 0.4 ml of diluted phenol reagent as per method of Lowry et al (1951)²²⁴. The optical density was measured by using UV-VIS SCHIMADZU spectrophotometer at 610 nm against similarly prepared blank using 0.01N HCl instead of diluted gastric juice after 10 minutes of adding phenol reagent. The pepsin activity was calculated in terms of μg ml⁻¹ of tyrosine liberated per ml of gastric juice.
**Mucoprotective Parameters:**

**Total Carbohydrates**:

**Reagents:**

**Phenol 5%**: 5% phenol solution was prepared in distilled water and filtered.

**H₂SO₄**: 96%

**Glucose Standard**: Glucose solution of different concentration was prepared for standard curve.

**Procedure:**

To 0.15 ml of gastric juice were added 1.0 ml of 5% phenol and 5 ml of 96% H₂SO₄ and mixed. After 10 minutes, the test tubes were shaken and placed in a water bath at 28-30° for 20 minutes. The optical density was measured at 482 nm against similarly prepared blank using 0.15 ml of distilled water. The standard curve was prepared using different concentration of glucose.

**Total Proteins**:

**Reagents:**

**Alkaline Reagent**: (a) Freshly prepared 2% solution of Na₂CO₃ in 0.1N NaOH

(b) 100 ml of (a) mixed with 1 ml of 4% aqueous solution of potassium tartarate and 1ml of 2% of aqueous copper sulphate.

**Folin's Ciocalteau-reagent**: 1 ml of reagent was diluted with 2 ml of distilled water.

**Bovine albumin**: Standard
Procedure:

Alcoholic Precipitation:
1 ml of gastric juice was mixed with 9 ml of 95% alcohol and centrifuged at 3000 rev min$^{-1}$ for 15 minutes. The precipitate formed was dissolved in 1 ml of 0.1N NaOH from which 0.1 ml was used to estimate protein. To 0.1 ml of NaOH solution (precipitate in 0.1N NaOH), was added 0.9 ml of distilled water, 0.4 ml of this reconstituted solution was mixed with 4 ml of alkaline reagent. After 10 minutes, 0.4 ml of Folin's ciocalteau reagent was added and the optical density was measured after 10 minutes at 610 nm against similarly prepared blank using distilled water. The protein content was calculated with the help of standard curve prepared by using bovine albumin and was expressed in terms of $\mu$g mL$^{-1}$ of gastric juice.

Gastric Wall Mucus Content$^{209}$:

Reagents:

01 % w/v Alcian Blue dye Solution: 100 mg of Alcian Blue 8GX (Sigma) dissolved in 0.16 M sucrose, buffered with 0.05 M sodium acetate, pH 5.8 with 1M HCl.

0.5M Magnesium chloride

0.25 M Sucrose Solution

Procedure:

Gastric wall mucus was determined according to the method of Corne et al.$^{209}$ The glandular segments from stomach, which previously had been opened along the large curvature, were removed and weighed. Each segment was immersed for 2 hours in 10 ml of 0.1% w/v alcian blue dissolved in 0.16M sucrose solution buffered with 0.05M sodium acetate and finally adjusted to pH 5.8 with 1M HCl. Excess of dye was removed by washing the segments twice with 0.25M sucrose solution for a period of 15 and 45 minutes, respectively. Mucus-dye complex formed was extracted by placing the glandular tissue in 10 ml of 0.5M
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Magnesium chloride and shaking intermittently for 1 minute at 30 minutes intervals for 2 hours. 4 ml of blue extract was mixed with an equal volume of diethyl ether and shaken vigorously for 2 minutes. The emulsion obtained was centrifuged for 10 minute at 3600 rev minutes⁻¹ and the absorbance of the aqueous layer was recorded using UV-VIS SCHIMADZU spectrophotometer at 598 nm. Alcian blue extracted per gram of wet glandular tissue was calculated

\[
\text{Absorbance} \times 10^5 \quad \frac{\text{Mucus barrier}}{E_{1\text{cm}}^\text{10\%}} \times \text{weight of glandular tissue}
\]

(mg of alcian blue dye per gram of wet stomach glandular tissue)

\[E_{1\text{cm}}^\text{10\%} \text{ of Alcian blue} = 189.\]

**Mucin Activity (TC/PR):**

The ratio between the total carbohydrate and protein was determined and was used as an index of dissolved mucosubstances in gastric juice.

**Lipid Peroxidation:**

**Thiobarbituric Acid Reactive Substances Assay:**

**Reagents:**

- \( \text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O} \): 0.2 M
- \( \text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O} \): 0.2 M
- \( \text{NaCl} \): 0.9 %
- \( \text{Sodium dodecyl sulphate (SDS)} \): 8 %w/v
- \( \text{Thiobarbituric acid (TBA)} \): 1% w/v in hot water (freshly prepared)
- \( \text{Trichloroacetic acid} \): 10%
Procedure:
The glandular tissue from stomach, which previously had been opened along the greater curvature, were removed and weighed after thoroughly washing with phosphate buffer solution. Reaction mixture containing homogenate (0.2 ml, 10% w/v), sodium dodecyl sulfate (0.2 ml, 8% w/v), freshly prepared thiobarbituric acid (1.5 ml, 1% w/v), acetic acid (1.5 ml, 20%) and distilled water (0.6 ml) was mixed well, made up to 4 ml with distilled water and heated at 95° for 45 minutes on a boiling water bath. After cooling, to 2 ml of this reaction mixture was added equal volume of 10% trichloroacetic acid and was centrifuged at 1000 rev min⁻¹ for 5 minutes. The absorbance was measured at 532 nm against similarly prepared blank using phosphate buffer solution and was expressed as nmol malondialdehyde.

Preventive Antioxidants:
Superoxide dismutase (SOD)²¹¹
Reagents:
EDTA : (1 x 10⁻⁴ M)
Epinephrine : (3 x 10⁻³ M)
Carbonate buffer : (pH=9.7)

Procedure:
Superoxide dismutase level was measured as per the method described by Misra et al²¹¹. In this method 0.1 ml of sample was mixed with 0.1 ml of EDTA (1 x 10⁻⁴ M), 0.5 ml of Carbonate Buffer (pH=9.7) and 1 ml of epinephrine (3 x 10⁻³ M). The optical density of formed adrenochrome was measured at 480 nm for 3 minutes at an interval of 30 seconds. Results were expressed as mUnits/mg protein. One unit of enzyme activity defined as the enzyme concentration required to inhibit the chromogen production by 50% in one hour under the defined assay conditions.
Catalase (CAT)\textsuperscript{212}

**Reagents:**
- **Potassium phosphate (A):** (pH=7.0, 0.05 M)
- **Hydrogen peroxide:** (0.059 M in A)
- **Enzyme:** Dilute the enzyme in A to obtain a rate of 0.03 – 0.07
- **Reagent grade water**

**Procedure:**
Decomposition of \( \text{H}_2\text{O}_2 \) in presence of catalase was studied at 240 nm. A 0.1 ml of diluted enzyme was added to the cuvette containing reagent grade water (1.9 ml) and buffered substrate (1.0 ml) and made total volume 3.0 ml. The decrease in absorbance was recorded at 37°C for 2-3 minutes at an interval of 15 sec. The activity was calculated using extinction coefficient of \( \text{H}_2\text{O}_2 \), 0.041 μmoles/cm\(^2\) at 240 nm and results have been expressed as μmoles of \( \text{H}_2\text{O}_2 \) unites/min/mg protein.

**Chain Breaking Antioxidants:**

**Reduced glutathione (GSH)\textsuperscript{213}

**Reagents:**
- **Trichloroacetic acid**: 10% w/v
- **Disodium hydrogen phosphate**: 0.03M
- **5,5’-dithiobis-2-nitrobenzoic acid**: 40 mg/100ml in 1% w/v sodium citrate

**Procedure:**
The GSH level was measured as per method described by Beutler et al\textsuperscript{213}. GSH contents in tissue homogenates were measured after precipitation of protein with 10% w/v chilled trichloroacetic acid. Samples were kept in ice bath and were centrifuged after 30 minutes at 1000 g for 10 minutes at 4°C. GSH levels were measured in the supernatant. Supernatant (0.5 ml) was mixed with 2.0 ml 0.3 M
disodium hydrogen phosphate solution and 0.25 ml 5,5'-dithiobis-2-nitrobenzoic acid (40 mg/100 ml in 1% w/v sodium citrate) was added just before measuring the absorbance at 412 nm. Simultaneously different concentrations of GSH were also processed similarly to prepare a standard curve. Results were expressed as μmole of GSH/mg protein.

3.3 In-Vitro Antioxidant Activity:

Antiradical Activity (Free radical scavenger activity)\textsuperscript{226}:
Antiradical activity was measured by a method based on the reduction of a methanolic solution of the colored free radical DPPH (1, 1-diphenyl – 2 picrylhydrazyl)\textsuperscript{226}. A stock solution of DPPH (4.3 mg/3.3ml methanol) was prepared so that 75 μl of it in 3 ml of methanol gave an initial absorbance of 0.9 at 516 nm wavelength. The activity was expressed as an effective concentration at 50% (EC\textsubscript{50}) i.e. the concentration of the test solution required to give a 50% decrease in absorbance compared to that of a blank solution. For results linear regression was applied to the data values between 10 and 80%. Ascorbic acid was used as a standard.

Reagents:
- DPPH free radical
- Ascorbic acid

Superoxide Radical Scavenging Activity\textsuperscript{227}:
The assay was based on the capacity of the plant extracts to inhibit Nitro blue tetrazolium (NBT) up to 50% in presence of riboflavin-light-NBT system\textsuperscript{227}. The reaction medium contained 200 μl, 12 mM EDTA, 300 μl NBT (1 mg/ml), 200 μl riboflavin (1 mg/ml), and phosphate buffer solution pH 7.6, to make the final volume of test solutions 3 ml. The reaction was initiated by illuminating the sample cuvettes at regular intervals of 30 sec and increase in absorbance was
measured at 390 nm up to 2.5 min and also at 4.0 min. The superoxide radical scavenging activity was calculated using the formula:

\[
\text{OD (control) - OD (extract)} \\
\text{OD (control)}
\]

\[
\% \text{ Inhibition of superoxide radical} = \frac{\text{OD (control) - OD (extract)}}{\text{OD (control)}} \times 100
\]

**FeSO\textsubscript{4} stimulated lipid peroxidation in rat liver homogenate (in vitro).**

The effect of Extract S on rat liver homogenate with FeSO\textsubscript{4} and lipid peroxidation was determined by the MDA-TBA adduct according to the modified method\textsuperscript{228}. A mixture containing 0.5 ml of liver homogenate, 0.1 ml of Tris-HCl buffer (pH=7.5), 0.05 ml of 4 mm FeSO\textsubscript{4}, 0.05 ml of various concentration of extract S were incubated for 1 hour at 37° C. After incubation 9 ml of distilled water and 2ml of 0.6% TBA were added to 0.5 ml of the incubated mixture and the letter was shaken vigorously. The mixture was heated for 30 min in a boiling water bath. After cooling, 5 ml of n-butanol was added and the mixture was again shaken vigorously. The n-butanol layer was separated by centrifugation at 1000 g for 10 minutes, the MDA production was measured at 532 nm\textsuperscript{229}.
3.4 Effect on Central Nervous System:

Barbiturate Sleeping Time\textsuperscript{230}:

Pentobarbitone Sodium (50 mg kg\(^{-1}\), i.p.) was administered to male mice, which had been previously (30 minutes) treated with Extract S (i.p.). The time elapsed between loss and recovery of the righting reflex was recorded as the sleeping time, limited to a maximum of 2 hours.

Nociceptive Response:

Writhing Test\textsuperscript{231}:

Female mice were given 0.6\% acetic acid (1ml 100g\(^{-1}\), i.p.), which had been previously (90 minutes) treated with Extract S (100 mg kg\(^{-1}\), 200 mg kg\(^{-1}\) and 400 mg kg\(^{-1}\), p.o.) and with Aspirin (50 mg kg\(^{-1}\), p.o.) as positive control or with vehicle as negative control. The animals were isolated and the numbers of times the animal stretched or writhed during 5-15 minutes were counted. The percentage protection for each group was calculated using following equation:

\[
\% \text{ Protection} = \frac{W_c - W_t}{W_t} \times 100
\]

Where,

\(W_c\) = Mean writhing of the control group
\(W_t\) = Mean writhing of the treated group

Tail Flick Test\textsuperscript{232}:

Female mice were given Extract S (400 mg kg\(^{-1}\), p.o.) and Pethidine (5 mg kg\(^{-1}\), p.o.). The inhibition of the tail flick response was elicited by immersing the terminal 3 cm of tail in a water bath maintained at a constant temperature of 54 ± 1°C. The intensity of the heat stimulus in the test was adjusted so that the animal flicked its tail within 3 to 5 sec. The tail flick latencies were measured before \((T_0)\) and after giving
the dose of extract \( (T_1) \). The inhibition of the tail flick response was expressed as \% maximum possible effect \((\% \text{ MPE})\).

\[
\% \text{ MPE} = \left[ \frac{(T_1 - T_0)}{(T_2 - T_0)} \right] \times 100
\]

Where, cutoff time \( (T_2) \) was set at 6 sec.

**ED\textsubscript{50} Determination\textsuperscript{234}:**

ED\textsubscript{50} value of a drug in one particular parameter is defined as the dose of the drug at which 50\% of the maximum possible response is obtained in that particular parameter.

In present study, ED\textsubscript{50} of Extract S was determined in ethanol and indomethacin-induced gastric ulcer models. ED\textsubscript{50} was obtained by plotting log dose verses \% protection of drug. From this plot, the log dose of drug that gave 50\% of the maximal protective response \((\text{EC}_{50})\) was determined. Based up on this, ED\textsubscript{50} value was calculated as follows:

\[
\text{ED}_{50} = \text{antilog} \ (\text{EC}_{50})
\]

**Statistical analysis:**

Results were expressed as the mean ± SEM. Statistical significance was determined by Student's 't' test, one-way analysis of variance (ANOVA single factor) followed by Tukey's multiple range tests. The level of significance was set at \( p < 0.05 \).