MATERIAL AND METHODS

Toxicity Study

Experimental animals

Adult *Charles foster albino* rats (150 to 200 *g* in weight) of either sex were obtained from the Central Animal House, Institute of Medical Sciences, Banaras Hindu University, Varanasi, India. The animals were housed in polypropylene cages up to a maximum of 6 per cage and maintained under standard conditions (12 h light and dark cycle at an ambient temperature of 22 ± 3°C and 45-55% Relative humidity) and were fed with commercially available rat feed (*Hindustan Lever Ltd*., Mumbai, India) and water *ad libitum*. The animals were allowed to adapt the environment for 7 days before the commencement of experiments. The pharmacological experiments were performed after the approval obtained from the Central Animal Ethical Committee, Institute of Medical Sciences, Banaras Hindu University (Approval no.: Dean/2015/CAEC/983) and were conducted in accordance with accepted standard guidelines of National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985).

Acute oral toxicity study

Acute oral toxicity study of ethanolic root extract (EAD) was performed as per Organization for Economic Co-operation and Development (OECD) guidelines 425 (2008). The extract was administered as 0.5% carboxy-methyl cellulose (CMC) and Tween 80, suspension at doses of 1, 2.5 and 5 *g*/kg, *p.o*. body weight and control group received 0.5%CMC suspension only. The toxicity signs and symptoms or any abnormalities associated with EAD administration was observed at 0, 30, 60, 120, 180 and 240 min and then up to 48h. The behavioral and

neurological changes such as hyperactivity, ataxia, tremors, convulsions, salivation, diarrhea, lethargy, sleep and coma were closely monitored. Total observation period for eventual mortality rate was done up to 14 days and at the end of study survival rate of rats was recorded.

Sub-acute toxicity study

Adult *Charles Foster albino* rats (150±10 g) of either sex were distributed into 4 groups (A1, A2, A3, and control) of 10 animals each and OECD guideline 407 was followed. The three treated groups received the following doses of EAD 250, 500 and 1000 mg/kg, *p.o*. (A1, A2, and A3) respectively for 28 consecutive days. The animals were then weighed every five days from the start of the treatment, to note any weight variation, food, and water consumption were also monitored daily throughout the study period. At the end of experiment, rats fasted overnight and blood was collected on 29th day by *retro-orbital plexus* under light anesthesia. The blood samples were analyzed for hematological (hemoglobin, total leukocyte count (TLC), differential leukocyte count (DLC), packed cell volume (PCV), Mean corpuscular volume (MCV), Mean corpuscular hemoglobin (MCH) and Mean corpuscular hemoglobin conc.) (MCHC) and biochemical (glucose, cholesterol, Triglyceride, HDL, LDL, alkaline phosphatase, aspartate transaminase (AST), alanine aminotransferase (ALT), SGPT, SGOT, blood urea nitrogen, creatinine, total protein and albumin) parameters. After blood collection, animals were sacrificed for isolation of kidney and liver to observe histopathological changes, if any. The liver, kidney, heart and spleen were dissected out and were fixed in 10% formalin solution. Paraffin sections were made and stained with hematoxylin and eosin for detailed histopathology study (Singh & Kumar, 2011).

RESULTS

Acute toxicity study

Rats did not show any abnormal behavior except for mild sedation after EAD administration at doses of 1, 2.5 and 5 g/kg, *p.o.* for initial 4 h. After 14 days of treatment with EAD no mortality was observed. Hence, the drug was found to be safe up to 5000 mg/kg, *p.o.* and three doses of EAD 100, 200 and 400 mg/kg, *p.o.* were selected from the pilot study.

Sub-acute toxicity study

The body weight of EAD treated rats was normal in comparison to vehicle treated rats. EAD treatments did not significantly change the hematological and biochemical parameters as summarized in table 12 and 13. Histopathological examination of control and EAD treated rats exposed the absence of any gross pathological lesion in liver, kidney, heart and spleen (Figure 26).

Table 12: Hematological Parameters of Ethanolic root extract of *A. dichotoma*

Parameter	Control	250mg/kg	500mg/kg	1g/kg
Hemoglobin	11.21± 0.08	12.20±0.17	11.56±0.20	11.14±0.04
$R.B.C_s$	7.23±0.11	7.27±0.07	6.30±0.17	7.24±0.09
$W.B.C_s$	10.28±0.07	9.80±0.04	11.04±0.04	10.88±0.10
Hematocrit	41.88±0.09	41.66±0.25	43.05±0.26	42.95±0.14
Platelet count	891.33±1.45	868.31±1.76	899.66±2.33	882.12±2.89
TLC	6.82±0.03	6.68±0.07	6.84±0.04	6.82±0.03
Neutrophil	57.22±0.22	57.97±0.09	58.38±0.13	57.96±0.19
Lymphocyte	45.08±0.09	45.56±0.13	43.91±0.34	43.26±0.19
Eiosinophil	1.24±0.02	1.19±0.02	1.25±0.02	1.13±0.03
Monocyte	3.02±0.02	2.99±0.03	2.96±0.02	2.54±0.04
Basophil	0	0	0	0
PCV	44.27±0.03	44.17±0.07	44.08±0.06	41.98±0.01
MCV	55.14±0.03	54.62±0.06	55.36±0.02	54.93±0.04
MCH	18.65±0.03	18.86±0.05	18.06±0.20	18.21±0.08
MCHC	32.27±0.05	32.72±0.11	32.31±0.12	32.23±0.11

All statistical data was expressed in mean ± SEM (n=10) and determined by one way ANOVA followed by Tukey's multiple comparison test and no significant effect was observed.

Table 13: Biochemical Parameters of EAD

Parameter	Control	250mg/kg	500mg/kg	1g/kg
Glucose	86.18±0.08	86.56±0.13	85.76±0.20	86.54±0.03
Cholesterol	109.83±0.03	111.02±0.12	110.02±0.16	110.35±0.07
TG	90.48±0.03	90.30±0.07	90.81±0.07	90.04±0.11
HDL	78.56±0.20	78.55±0.25	78.62±0.12	78.53±0.15
LDL	27.31±0.10	27.13±0.04	27.33±0.09	27.42±0.13
Urea	39.25±0.24	39.29±0.09	39.99±0.10	39.91±0.22
Creatinine	0.85±0.02	0.89±0.03	0.93±0.01	0.91±0.01
Total Protein	6.05±0.02	6.11±0.05	6.11±0.01	6.34±0.01
Albumin	2.60±0.05	2.63±0.04	2.59±0.01	2.64±0.01
Globulin	35.73±0.01	35.84±0.02	36.08±0.03	36.32±0.04
ALT	62.47±0.02	62.83±0.02	63.08±0.02	62.10±0.10
AST	246.22±0.57	242.66±0.87	242.36±1.57	240.33±0.87
SGOT	57.47±0.08	57.94±0.09	56.67±0.22	56.50±0.48
SGPT	46.42±0.01	45.64±0.24	45.71±0.05	45.14±0.16

All statistical data was expressed in mean ± SEM (n=10) and determined by one way ANOVA followed by Tukey's multiple comparison test and no significant effect was observed.

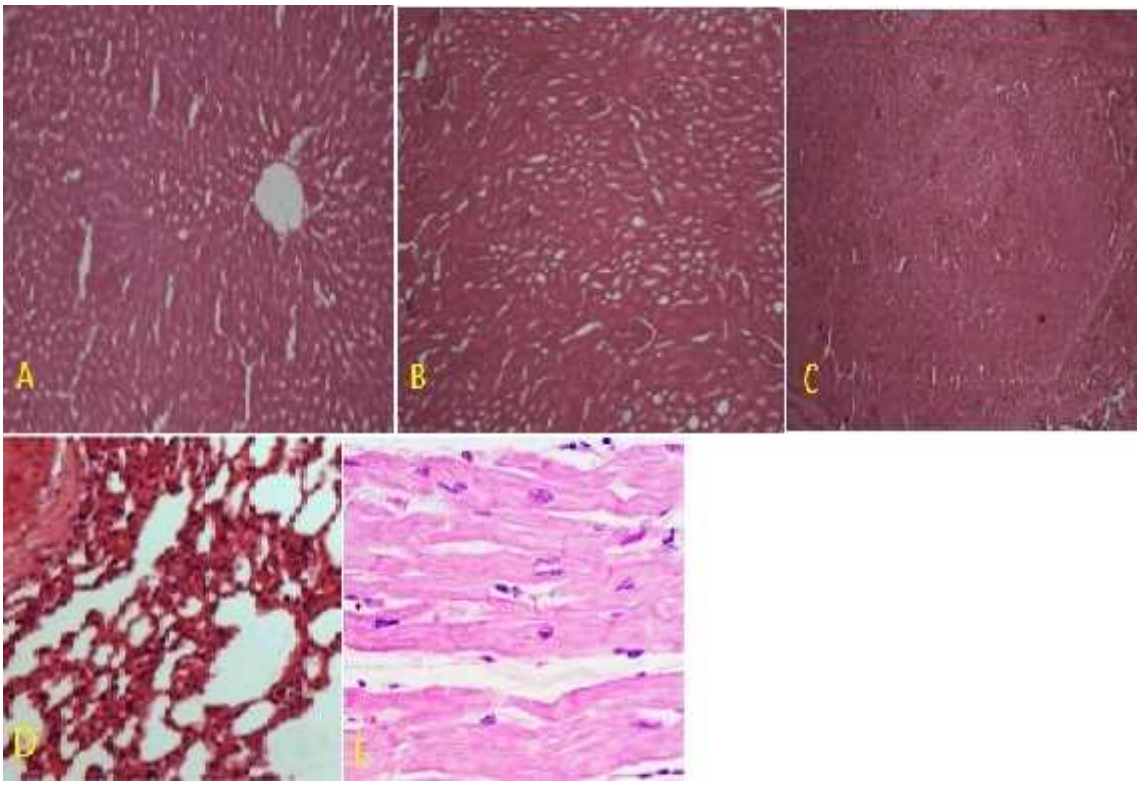

Figure 26: Histology of Visceral organs (A: Liver, B: Kidney, C: Spleen, D: Lung, E: Heart)

EVALUATION OF ANTI-ULCER ACTIVITY

MATERIAL AND METHODS

Pylorus ligation-induced ulcer (PL)

The animals were divided into seven groups of six animals each (n=6). The control group (1) received distilled water as the vehicle. The gastric ulcer control group (2) received 0.5% CMC suspended in distilled water, standard drug treated groups (3) received Omeprazole 20mg/kg, *p.o*. (*Cadila Pharmaceuticals Ltd*.), group 4-6 was EAD treated received 100, 200 and 400 mg/kg, *p.o*., and group 7 was ursolic acid (50 mg/kg, *p.o*.) treated. All the standard and tested drugs were administered orally for a period of seven days. Fasting of the rats was done overnight for 18h with free access to water *ad libitum* and on the seventh day, after the last dose of drug administration, pylorus ligation on the rat stomach was done under anesthetized condition according to the method of Shay *et al*, (1945). The abdomen was cut opened and the pyloric end was subjected to the ligation without detrimental effect to the blood supply. The stomach was cautiously replaced and with help of interrupted sutures the abdomen was closed. After this post-operative period, the rats were destitute of water up to 4h after that stomach was dissected out from greater curvature for ulcer scoring (Oliveira *et al*., 2004).

Ulcer score and ulcer index were calculated by adding the total number of ulcers per stomach and the total severity of ulcers per stomach. The total severity of the ulcers was determined by recording the severity of each ulcer after histological confirmation as follows:

[0]: no ulcer.

[+]: pin point ulcer and histological changes limited to superficial layers of mucosa and no congestion.

[++]: ulcer size less than 1 mm and half of the mucosal thickness showed necrotic changes.

[+++]: ulcer size 1–2 mm with more than two-thirds of the mucosal thickness destroyed with marked necrosis and congestion, muscularis remaining unaffected.

[++++]: ulcer either more than 2 mm in size or perforated with the complete destruction of the mucosa with necrosis and hemorrhage, muscularis still remaining unaffected. The pooled group ulcer score was calculated according to the method of Sanyal *et al*, (1983).

Evaluation of gastric secretion studies in PL

Determination of gastric juice volume

After the induction of pyloric ligation in rats, stomach was removed and its gastric juice was drained into a graduated centrifuge tube (centrifuged at 3000 rpm for 10 min at 25ºC). The volume of the supernatant was expressed as mL/100*g* body weight (Hawk *et al*., 1947).

Determination of gastric juice pH

To a 100 mL volumetric flask, 1 mL of gastric juice was pipetted and 10 mL of distilled water was added to the flash and mixed properly. The pH of this solution was noted with the help of pH meter (Hawk *et al*., 1947).

Determination of free and total acid output

After the pH was monitored, 2 to 3 drops of Topfer's reagent was added into the above solution and triturated with 0.01N NaOH until all traces of the red color disappeared and the color of solution turns to yellowish orange. The volume of alkali added was noted, whereby this volume indicates the free acidity. Finally, to the same solution 2 to 3 drops of 1% phenolphthalein solution was added and titration was continued until a definite red tinge reappeared. The total volume of

alkali added was noted and this corresponds to the volume of total acidity (Hawk *et al*., 1947).

Determination of pepsin output (peptic activity)

Peptic activity was illustrated by using hemoglobin powder as the substrate (Debnath *et al*., 1974). The assay was performed by diluting the gastric juice to 1:250 times with distilled water and 2% hemoglobin was prepared in 0.06N HCl. In this method, 0.4 mL of the diluted gastric juice and 1 mL of hemoglobin solution were together incubated at 37ºC for 10 min. The pre–incubated hemoglobin solution was then added to the diluted gastric juice and this mixture was incubated at 37ºC for exactly 20 min. Digestion was stopped by adding a 1.4 mL of ice-cold trichloroacetic acid and kept in ice bath for 15 min. The precipitated undigested protein was separated by filtration of a mixture. 0.4 mL of the digested filtrate was taken to determine the concentration of the liberated amino acid (tyrosine) by first adding 4 mL of alkaline reagent followed after 10 min by addition of 0.4 mL of diluted phenol reagent as per the method of Lowry and associates (1951). The optical density was estimated with the help of spectrophotometer at 610 nm against the blank prepared similarly using 0.01N HCl instead of diluted gastric juice after 10 min of adding phenol reagent. The peptic activity was intended in terms of mmol of tyrosine liberated per liter of gastric juice and the peptic output in gastric juice was expressed as µmol / mL and µmol / 4h of peptic concentration and output respectively.

Determination of gastric juice mucoprotein and mucosal scrap glycoprotein

The gastric juice or scrapings of gastric mucosal homogenate obtained from the 4h PL rat stomach was used to estimate the dissolved mucosubstances. This

parameter was estimated in the alcoholic precipitate obtained by adding 90% alcohol in 9:1 ratio (Goel *et al*., 1986).

Method:

0.5 mL of gastric juice or gastric mucosal homogenate was added into 4.5 mL of 90% alcohol. The mixture was shaken and kept for 10 min before it was centrifuged. The supernatant was redundant and the precipitate was dissolved in 0.5 mL of 0.1 N NaOH. 0.05 mL of this solution was taken for the estimation of protein by Lowry's method (1951) and the remaining solution was added into 1.8 mL of 6N HCl. This mixture was hydrolysed in a boiling water bath for 2h and hydrolysates were neutralized with 4N NaOH by using phenolphthalein as indicator. The volume was made up to 4.5 mL with distilled water and used for the estimations of total carbohydrate content.

Total protein estimation

The dissolved protein in gastric juice was estimated in the alcoholic precipitate by using the method of Lowry *et al*. (1951). 0.95 mL water was added into 0.05 mL solution of alcoholic precipitate of gastric juice in 0.1N NaOH. Out of this 1 mL solution, 0.4 mL was taken into another test tube and 4 mL of alkaline reagent (as prepared above in peptic activity) was then added and kept for 10 min. The color development was done by adding 0.4 mL of the phenol reagent (Folin-ciocalteu reagent) which was again allowed to stand for 10 min. The optical density of the solution was finally estimated at 610 nm against the blank prepared with water. The protein content was calculated from the standard curve prepared with bovine albumin and was expressed as µg/mL in gastric juice or µg/mL of gastric mucosa.

Total carbohydrate estimation

The total carbohydrate content was determined as per the calorimetric method described by Yemm (1954).

Preparation of anthrone reagent

Anthrone reagent was prepared by dissolving 0.2 *g* of anthrone in 100 mL of water. The reagent was allowed to stand for 30 to 40 min with occasional shaking until it was perfectly clear. The reagent was prepared freshly and should be used within 12h from the time of preparation.

Methodology

The 5 mL anthrone reagent was pipette out into a thick walled pyrex tube (150 x 25mm) and chilled in ice-cold water. Then 1 mL of alcoholic precipitate of gastric juice or gastric mucosal homogenate was added into a tube and then tubes were loosely fitted with corks, heated in a boiling water bath and later cooled for 5 min. Finally, absorption of mixture was analyzed by using spectrophotometer at 600 nm. Mucosubstance or mucin activity was expressed as the ratio of total carbohydrate and protein (TC: TP).

Determination of cell shedding and cell proliferation

Gastric juice cell shedding

The gastric juice cell shedding (DNA) was estimated according to method described by Mukhopadhyay *et al*., 1987.

Extraction of DNA: Stomach tissues were taken, defrosted in a shaking water bath at 37ºC for 30 min and centrifuged for 30 min at 180 rpm in a cooling centrifuge (4ºC). Then the supernatant was redundant and 5 mL of 5% trichloroacetic acid (TCA) was added to the precipitate and mixed thoroughly and the mixture was

centrifuged at 1800 rpm for 30 min at 4ºC and the supernatant was again discarded.

Hydrolysis: 2 mL of 5% TCA was added to the sample deposits in the centrifuge tubes with constant stirring and transferred into a glass-stoppard tubes for heating in a water bath at 90 ± 1ºC for 15 min and then centrifuged at 2500 rpm at room temperature for 30 min. The supernatant was collected in separate test tubes. A second hydrolysis was done on the deposits by adding 1.5 mL of 5% TCA and heating for 15 min at 90 ± 1ºC. It was then centrifuged at 2500 rpm for 30 min at room temperature and the supernatant was collected and added to the first hydrolysate in the test tubes.

Methodology

2 mL of hydrolysate was taken in a glass-stoppard test tube and then 0.2 mL of 60% (w/v) perchloric acid was added and blank taken consisted of 2 mL of 5% TCA. 2 mL of 2% diphenylamine reagent was added to the mixture and kept in a refrigerator for 48h. The concentration of DNA was evaluated at 600 nm and the DNA content was expressed as µg/ mL gastric juice/100 *g* weight of rats.

Mucosal scrap cell proliferation

DNA and protein were estimated in the rat gastric mucosal scrapping following the method of Goel (1986). The homogenisation of mucosal scrapping was done in 2.5 mL of ice cooled 0.6N perchloric acid. The precipitate thus obtained was allowed to flocculate at 0ºC for 10 min and then centrifuged at 2000 rpm for 5 min at 2ºC and precipitate was washed twice with 5 mL of 0.2N ice-cold perchloric acid. Then 4 mL of 0.3N KCl was added to the precipitate and incubated at 37°C for 1h, for the alkaline hydrolysis of RNA. After incubation, all the test tubes were ice-cooled and to each test tube 2.5 mL of ice-cooled and 1.2N

perchloric acid was added. After centrifugation, the supernatant was discarded and the precipitate was hydrolysed by adding 2 mL of 0.5N perchloric acid at 80ºC for 30 min and the supernatant was used for DNA estimation (Schneider, 1957; Goel *et al*., 1986). The concentration of DNA was expressed as µg DNA / mg protein.

Determination of inhibition of H^+K^+-ATPase enzymatic activity

H^+K^+-ATPase inhibitory activity was assessed by using the method described by Reyes-Chilpa *et al*. (2006). H^+K^+-ATPase enzymatic inhibition was evaluated on gastric microsomes from 4h PL rats. The mucosa was scraped from gastric fundus region and suspended in EGTA-sucrose buffer solution (250 mM sucrose, 2mM MgCl2, 1mM EGTA and 2mM Tris buffer, maintained at pH 7.4) followed by 3 min homogenization. Preparation of gastric microsomes was processed by successive centrifugation at 3000 rpm for 10 min and 20,000 rpm for 30 min at 4ºC two times, from which the nuclei, mitochondria, and microsomes were obtained. The supernatant of the resulting solution was discarded and the microsome pellet was obtained, which was dissolved and homogenized in mannitol buffer solution (250mM mannitol, 2 mM magnesium chloride and 2mM Tris buffer maintained at pH 7.4). The obtained homogenate (0.1 mL) was further processed by adding 0.2 mL Tris buffer-HCl (20mM, pH 7.4), 0.2mL 2mM $MgCl_2$ and 0.2mL 2mM KCl. After that 0.2mL 2mM ATP was added to start the reaction and incubated at 37ºC for 30 min and later ended by adding 10% trichloro acetic acid followed by centrifugation at 2000 rpm for 10min. The inorganic phosphate (Pi) generated was evaluated by spectrophotometer at 640nm and result was expressed in terms of µM Pi liberated/mg protein/min (Griswold *et al*., 1951).

Cold restrain stress induced ulcer (CRS)

The animals were subjected to cold restrain stress after 1h treatment with EAD at a dose of 100, 200 & 400 mg/kg, *p.o*., ursolic acid (50 mg/kg, *p.o*.) and omeprazole (20mg/kg, *p.o*.). The fore and hind limbs were tied up on the wooden slab of overnight fasted rats and kept at a temperature of 4-6ºC for 2h to induce cold restrain stress (Sairam *et al*., 2003). Thereafter, the animals were sacrificed 3h after CRS and ulcers were evaluated on the dissected stomach. Ulcer index was scored as per methods described under the PL-induced ulcer model.

Ethanol-induced ulcer (EtOH)

To induce ulcer with ethanol, animals were fasted for 24h and rats were given ursolic acid (50 mg/kg, p.o), EAD at the doses of 100, 200 and 400 mg/kg; *p.o*., positive control received omeprazole at the dose of 20 mg/kg, *p.o*., while negative control received distilled water. After 1.5h, absolute ethanol (95-99%), (1mL/200*g*, *p.o*.) was administered. After 1h administration of ethanol, animals were sacrificed by euthanasia and the stomach was incised along the greater curvature for scoring of ulcer (Adinortey *et al*., 2013). Ulcer score (in mm^2) was carefully monitored and the ulcer index and percentage protection were calculated following the standard method of Hollander *et al*. (1985). The percentage protection was calculated following the equation as represented above. Determination of mucosal microvascular permeability, mucus content, levels of antioxidant enzymes, microscopy and histology studies were performed in EtOH induced ulcer model.

Determination of mucosal microvascular permeability

The microvascular permeability test was performed on 1h absolute ethanol-induced gastric ulcer rats by using Evans blue dye (EBD). EBD (10mg/kg, i.v.)

was administered 30 minutes prior to animal sacrifice. The mucosal tissue was scraped from the stomach and soaked in 1mL 1N KOH at 37ºC overnight. 9mL of a mixed solution of 0.6N phosphoric acid and acetone (5:13) was prepared and added to the tube, followed by vigorous shaking for a few seconds after which was centrifuged at 3000 rpm for 15min. The supernatant was subjected under spectrophotometer at 620 nm to obtain absorbance and the results were indicated as µg Evans blue/*g* of tissue (Katayama *et al*., 1978).

Estimation of mucus content in the gastric wall

According to Corne *et al*. (1974), gastric mucus content was determined from the 1h absolute ethanol-induced ulcer rats by reacting with alcian blue dye. Briefly, 0.5*g* of the glandular segment of the stomach was put in 10 mL alcian blue solution (0.1%, w/v in 0.16M sucrose solution) buffered with 0.05M sodium acetate adjusted to pH 5.5 with HCl and left to stain for 2h. Uncomplexed dye was detached by two washes of 15 and 45 min in sucrose 0.25M. Complexed dye was extracted from 5 mL of 0.5M $MgCl_2$ solution for 2h with proper shaking. The extracted dye was then shaken vigorously with equal volumes of diethyl ether and finally centrifuged at 3600 rpm for 20 min. The concentration of the dye in aqueous layer was estimated by using spectrophotometer at 580 nm from the alcian blue standard calibration curve. The mucus content was intended and articulated in terms of µg alcian blue/*g* wet tissue.

Antioxidant activity and free radical determination

Antioxidant enzymes like superoxide dismutase (SOD), catalase (CAT), glutathione (GSH) and lipid peroxides (LPO) were determined on mucosal tissue scrap accessed from the rat stomach of 1h absolute ethanol-induced gastric ulcer. Followed by homogenization of mucosal scrap in 0.9% ice-cold saline for 30 sec.

successive centrifugation was done initially at 800 xg for 10 min and later at 12,000 xg for 15 min. The clear supernatant was used for the following estimations (Goel *et al*., 2001).

Estimation of lipid peroxidase (LPO) levels:

LPO level was estimated and articulated in terms of malondialdehyde (MDA). 0.4 mL of the gastric mucosal homogenate was taken and 0.2 mL of 8.1% sodium dodecyl sulfate (SDS), 1.5 mL of 20% acetic acid solution was added and the pH was attuned to 3.5 with NaOH. Then 1.5 mL of 0.8% aqueous solution of thiobarbituric acid (TBA) was added to the above mixture. The mixture volume was made up to 4 mL with distilled water and then heated in an oil bath at 95°C for 60 min. After cooling with tap water, 1 mL of distilled water and 5 mL mixture of n–butanol, and pyridine (15:1, v/v) were added and shaken vigorously. Then centrifugation was done at 4000 rpm for 10 min and the organic layer was separated and absorbance was measured at 532 nm against the blank containing 0.4 mL distilled water in place of sample. 1, 1, 3, 3–tetra–methoxypropane was used as external standard and the LPO was expressed as nmol MDA/*g* wet tissue (Okhawa *et al*., 1979).

Estimation of superoxide dismutase (SOD) levels

SOD was estimated by a method described by Kakkar (1984). The inhibition of reduction of nitroblue tetrazolium (NBT) to blue colored formazan in presence of phenazine methosulphate (PMS) and NADH was measured at 560 nm using n–butanol as blank. 0.4 mL of the homogenate was taken and 1.2 mL of sodium pyrophosphate buffer (pH 8.3, 0.052M), 0.1 mL of 186 µM of phenazine methosulphate and 0.3 mL 300 µM NBT was added. The volume of the mixture was made up to 3 mL with 0.8 mL of distilled water. The reaction was initiated by

the addition of 2 mL NADH (780 µM) and incubate the mixture at 30°C for 60 sec. After incubation reaction was stopped by the addition of 1 mL of glacial acetic acid. The reaction mixture was then stirred vigorously and shaken with 4 mL of n–butanol and was allowed to stand for 10 min, centrifuged and the butanol layer was taken out. The color intensity of the chromogen in n–butanol layer was measured at 560 nm in the spectrophotometer against n–butanol. A system devoid of enzyme served as control. 1 unit of enzyme activity is defined as enzyme concentration required for inhibiting the optical density at 560 nm of chromagen protection by 50% in one min under the assay conditions, and the results have been expressed as units (U) of SOD activity/*g* wet tissue.

Estimation of Catalase (CAT) levels

The decomposition of hydrogen peroxide (H_2O_2) in the presence of catalase was determined by the methods of Beers and Sizer (1952). The assay was started by addition of tissue homogenate to 20 mM hydrogen peroxide in 20 mM Tris buffer, pH 8. The initial absorbance change (typically the first 30 sec) was used to calculate the rate of hydrogen peroxide decomposition. The molar absorption coefficient for hydrogen peroxide at 240 nm was assumed to be 43.6 M^{-1} cm^{-1} and one unit (U) of catalase activity was defined as the amount of enzyme required to degrade 1 µmol of hydrogen peroxide per minute at 25°C. Results obtained were expressed as units (U) of CAT activity/*g* of tissue.

Estimation of glutathione (GSH) levels

400 mg sample of gastric mucosal scrap was homogenized with 8 mL of 0.02M EDTA and homogenate was kept in an ice-bath until use. Aliquots of 5 mL homogenates were taken in a 15 mL test tube and then add the 4 mL of distilled water, 1 mL of 50% TCA. The tubes were shaken intermittently for 10-15 min

and centrifuged for 15 min at 3000 xg. The resultant supernatant (3 mL) was collected and mixed with 4 mL of 0.4M tris buffer and 0.1 mL of 5,5′–dithio–bis–2–nitrobenzoic acid (DTNB) then sample was shaken. The absorbance was measured within 5 min after the addition of DTNB at 412 nm against a reagent blank (Sedlak and Linsday, 1968). The results were expressed as µmol/*g* wet tissue and were calculated from the standard curve prepared by using glutathione.

Histopathological studies

Stomach sample from 1h absolute EtOH-induced ulcerated rats of all the groups were fixed using 10% formalin. The tissue was then embedded in paraffin blocks for preparing sections (1-3 µm) which were then stained using hematoxylin and eosin dye and photographed by using a Nikon digital microscope (Eclipse 200) at 10x magnification.

Aspirin induced ulcer

Pretreatment of animals was done with EAD at a dose of 100, 200 and 400 mg/kg, *p.o*., ursolic acid (50 mg/kg, *p.o*.) and with omeprazole 20 mg/kg, *p.o*. for 7 days. On the 7^{th} day of the experiment, aspirin (ASP) in the dose of 200 mg/kg, *p.o*. was administered to the overnight fasted animals. The animals were sacrificed after 4h of aspirin administration and the ulcer was scored as per the method described in PL-induced ulcer model (Rao *et al*., 2004).

Statistical analysis

Results are expressed as mean ± S.E.M. with n=6 per group. Statistical significance was determined using one-way analysis of variance (ANOVA) followed by Tukey's post-test for multiple groups comparison. Graph Pad Prism

(version 5) software was used for all statistical analysis. The difference was considered to be significant when $p < 0.05$.

RESULTS

Gastric ulcer studies

Effect of EAD at doses of 100, 200 & 400 mg/kg, *p.o*. for 7 days showed a protective effect against ulcer in dose dependant manner. EAD at higher doses (200 & 400 mg/kg, *p.o*.) showed significant effect in pyloric ligation, ethanol induced, CRS models and ASP- induced gastric ulcer model while ursolic acid protect the ulcer only in PL- induced and ethanol induced gastric ulcer model. Standard drug omeprazole (20 mg/kg, *p.o*.) showed significant protection against all the tested gastric ulcer models. The percentage protection and score of ulcer index were determined in Table 14.

Gastric secretion study

In 4h pylorus-ligated rats, ursolic acid (50 mg/kg, *p.o*.) and EAD (200 and 400 mg/kg, *p.o*.) in dose dependent manner decreased the gastric juice volume, increase the gastric pH when compared to ulcer control group. Acid-pepsin output was significantly reduced with omeprazole (20 mg/kg, *p.o*.), ursolic acid (50 mg/kg, *p.o*.) and EAD (200 and 400 mg/kg, *p.o*.) groups; however, EAD at all doses, ursolic acid and omeprazole did not show any significant effect on DNA content of gastric mucosa, thus demonstrating the absence of effect on cell proliferation, while EAD (200 & 400 mg/kg, *p.o*.), ursolic acid (50 mg/kg, *p.o*.) and omeprazole (20 mg/kg, *p.o*.) showed significant effect on cell shedding (Table 15). Furthermore, EAD, ursolic acid and standard drug did not produce any significant effect on mucin activity as compared to the ulcer control group (Table 16).

Table 14: Effect of graded dose ethanolic extract of root of *A. dichotoma* (EAD) and ursolic acid on pylorus ligated (PL, 4h), absolute ethanol (EtOH, 1h), cold restrain stress (CRS, 2h) and aspirin (ASP, 4h) induced gastric ulcers in rats.

Groups	**Ulcer index and Percentage protection**							
	PL	**% protection**	**Etoh mm^2/rat**	**% protection**	**CRS**	**% protection**	**ASP**	**% protection**
Ulcer control (0.5% CMC)	24.16±1.05	-	32.83±0.79	-	17.50±0.56	-	22.83±1.01	-
Omeprazole (20 mg/kg)	6.33±0.88[a]	73.80	5.66±0.66[a]	82.74	9.16±0.79[a]	47.66	6.83±0.70[a]	70.08
EAD (100 mg/kg)	21.5±1.06[b]	11.01	30.16±0.75[b]	8.12	14.00±1.06[ab]	20.00	20.16±1.17[b]	11.70
EAD (200 mg/kg)	14.60±1.05[abc]	39.32	15.33±0.88[abc]	53.30	12.66±0.88[ab]	27.60	14.30±0.76[abc]	37.23
EAD (400 mg/kg)	7.50±0.76[acd]	68.96	7.33±0.49[acd]	77.66	10.00±0.68[ac]	42.86	10.00±0.85[acd]	56.20
Ursolic Acid (50 mg/kg)	18.61±0.71[abe]	22.97	22.75±0.67[abcde]	30.70	14.76±0.81[be]	19.31	18.92±0.54[abde]	15.41

All statistical data was expressed in mean ± SEM and determined by one way ANOVA followed by Tukey's multiple comparison test.

[a] $p < 0.05$ statistically significant as compared to ulcer control

[b] $p < 0.05$ statistically significant as compared to omeprazole

[c] $p < 0.05$ statistically significant as compared to EAD 100

[d] $p < 0.05$ statistically significant as compared to EAD 200

[e] $p < 0.05$ statistically significant as compared to EAD 400

Table 15: Effects of extract and ursolic acid on gastric juice volume, pH, free, total acid output and DNA content of gastric juice (cell shedding) and stomach mucosa (cell proliferation) in 4h pylorus ligated rats for 7 days.

Groups	Gastric juice volume (ml/100g)	Gastric pH	Acidity (µEq/ml)		Total Acid output (µEq/4h)	Pepsin		Gastric juice cell shedding (µg DNA/mL)	Mucosal cell proliferation (µg DNA/100mg wet tissue)
			Free Acid	Total Acid		Conc. (µmol tyrosine/mL)	Output (µmol tyrosine/4h)		
Ulcer control	1.63±0.04	3.74±0.11	42.80±1.50	112.20±4.51	279.56±15.44	289.21±12.91	721.34±41.10	95.81±1.87	134.30±6.76
Omeprazole 20	0.89±0.05[a]	5.33±0.11[a]	23.21±0.90[a]	45.46±3.50[a]	59.32±6.12[a]	163.18±9.30[a]	213.70±19.81[a]	41.40±1.01[a]	141.97±7.02
EAD 100	1.48±0.02[b]	3.89±0.18[b]	41.06±1.52[b]	110.13±5.26[b]	242.68±10.09[ab]	285.02±7.00[b]	630.12±22.90[b]	92.31±2.20[b]	139.96±3.83
EAD 200	1.06±0.05[ac]	4.89±0.12[ac]	28.26±0.73[ab c]	65.93±2.50[ab c]	106.75±6.03[ab c]	253.50±12.11[b]	413.74±34.10[a bc]	76.15±2.32[abc]	146.13±2.44
EAD 400	0.92±0.03[ac]	5.05±0.21[ac]	26.33±0.54[ac]	52.86±3.00[ab c]	69.27±2.87[acd]	198.90±7.30[acd]	261.88±11.00[a cd]	49.31±1.36[abc d]	153.46±4.41
Ursolic acid 50	1.07±0.04[ac]	4.78±0.23[ac]	32.71±0.88[ab ce]	74.23±2.86[ab ce]	87.23±4.21[ac]	211.06±6.14[abc d]	344.51±16.10[a bc]	68.43±1.78[abc e]	141.22±2.12

All statistical data was expressed in mean ± SEM and determined by one way ANOVA followed by Tukey's multiple comparison test.
[a] $p < 0.05$ statistically significant as compared to ulcer control
[b] $p < 0.05$ statistically significant as compared to omeprazole
[c] $p < 0.05$ statistically significant as compared to EAD100
[d] $p < 0.05$ statistically significant as compared to EAD200
[e] $p < 0.05$ statistically significant as compared to EAD 400

Table 16: Effect of EAD and ursolic acid on content of gastric juice mucoprotein and mucosal scrap glycoprotein in 4h PL rats.

Groups	Total Carbohydrate	Total Protein	TC:TP
Gastric juice mucoprotein estimation (µg/mL)			
Ulcer control	422.09±24.60	307.06±16.71	1.41±0.15
Omeprazole20	495.72±11.50[a]	286.92±4.30	1.72±0.04
EAD 100	427.54±10.00[b]	270.95±5.21	1.58±0.05
EAD 200	460.12±11.10	266.09±7.10[a]	1.73±0.06
EAD 400	463.91±7.30[a]	264.27±9.11[a]	1.75±0.08
Ursolic acid50	441.64±7.28[b]	276.41±3.41	1.59±0.03
Mucosal scrap glycoprotein estimation (µg/100 mg tissue)			
Ulcer control	1512.36±18.70	1043.99±27.20	1.45±0.05
Omeprazole20	1593.57±24.92	990.51±26.00	1.61±0.05
EAD 100	1504.48±17.00	1015.51±23.22	1.48±0.03
EAD 200	1562.97±23.12	988.43±26.41	1.58±0.05
EAD 400	1590.24±29.00	983.57±9.42	1.61±0.04
Ursolic acid50	1538.12±14.10	996.48±18.21	1.54±0.04

All statistical data was expressed in mean ± SEM and determined by one way ANOVA followed by Tukey's multiple comparison test.
[a] $p < 0.05$ statistically significant as compared to ulcer control
[b] $p < 0.05$ statistically significant as compared to omeprazole

Effect of EAD and ursolic acid on H^+K^+-ATPase enzymatic activity

The antisecretory effect of EAD (200 and 400mg/kg, *p.o.*) and omeprazole (20mg/kg, *p.o.*) was confirmed from H^+K^+-ATPase activity as observed in Pyloric ligated rat model, while ursolic acid did not show any significant action on H^+K^+-ATPase activity. The percentage inhibition in descending order was omeprazole (76.63%), EAD 400 (58.47%), and EAD 200 (16.44%) as shown in figure 27.

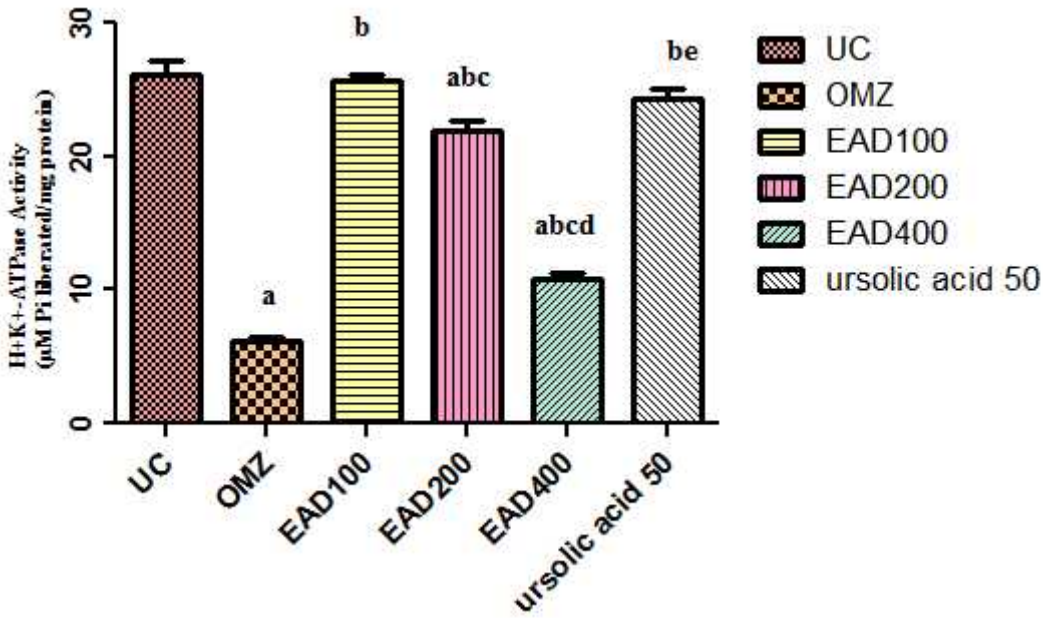

Figure 27: Effect of EAD (100, 200 and 400 mg/kg, *p.o.*) and ursolic acid on H^+K^+-ATPase enzymatic activity activity in 4h PL rats.

Statistical comparison was determined by one way ANOVA followed by the Tukey's multiple comparison tests.
(a) $P < 0.05$, statistically significant as compared to ulcer control.
(b) $P < 0.05$, statistically significant as compared to omeprazole.
(c) $P < 0.05$, statistically significant as compared to EAD 100.
(d) $P < 0.05$, statistically significant as compared to EAD200.
(e) $p < 0.05$ statistically significant as compared to EAD 400.

Effect of EAD on mucosal microvascular permeability

EAD 400 mg/kg, *p.o.*, ursolic acid (50 mg/kg, *p.o.*) and omeprazole (20mg/kg., *p.o.*) were showed significant action on microvascular permeability in gastric mucosa induced by absolute ethanol as shown in figure 28. All were considerably decreased the extrasavated amount of Evans blue dye in the gastric mucosa by 54.83%, 23.49 % and 59.35% respectively as compared to the ulcerated control group.

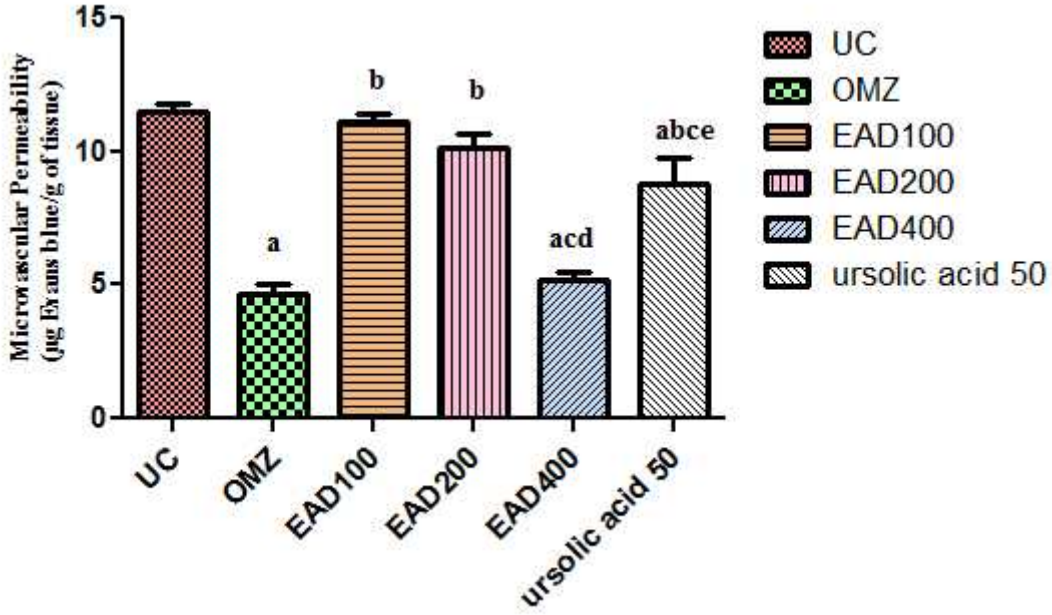

Figure 28: Effects of EAD, ursolic acid and omeprazole on microvascular permeability in gastric mucosa induced by absolute EtOH.

Statistical comparison was determined by one way ANOVA followed by the Tukey's multiple comparison tests.
(a) $P < 0.05$, statistically significant as compared to ulcer control.
(b) $P < 0.05$, statistically significant as compared to omeprazole.
(c) $P < 0.05$, statistically significant as compared to EAD 100.
(d) $P < 0.05$, statistically significant as compared to EAD200.
(e) $p < 0.05$ statistically significant as compared to EAD400.

Effect of EAD on Mucus content

Graded doses of EAD, ursolic acid and omeprazole did not show any significant effect on the mucus content when compared to the ulcer control group (Figure 29).

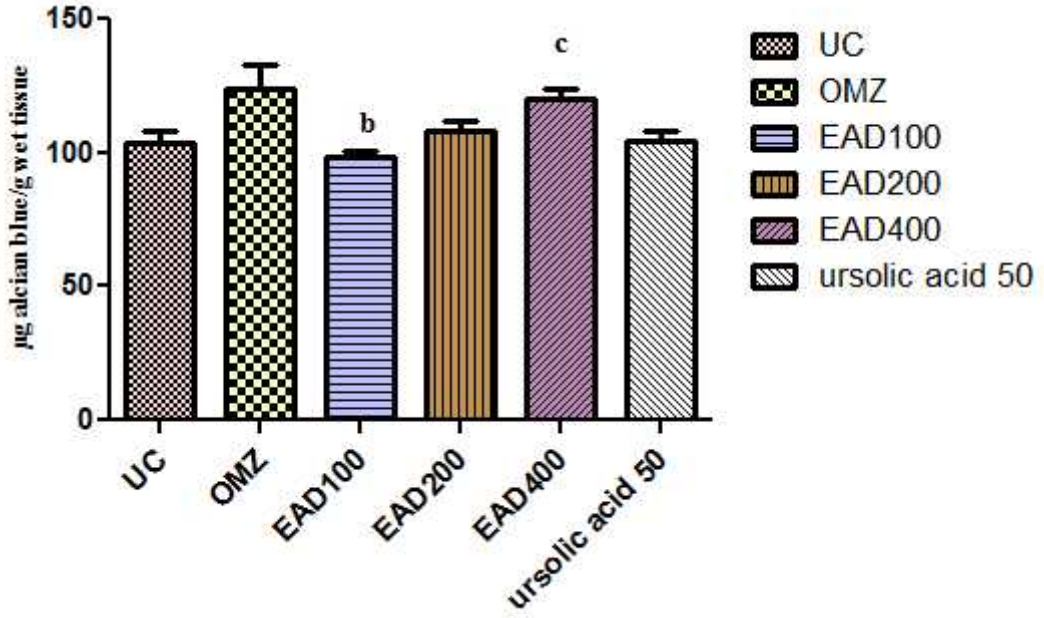

Figure 29: Effects of mucus content by EAD (100, 200 and 400mg/kg, *p.o.*), ursolic acid (50 mg/kg, *p.o.*) and omeprazole (20mg/kg, *p.o.*) in 1h absolute EtOH induced gastric ulcer in rats.
All statistical comparison was determined by one way ANOVA followed by the Tukey's multiple comparison tests.
(b) $P < 0.05$, statistically significant as compared to omeprazole.
(c) $P < 0.05$, statistically significant as compared to EAD 100.

Effect of EAD on antioxidant enzymes and free radical generation

Administration of absolute ethanol in the ulcerated control group increased level of LPO while decreased the level of SOD, GSH & CAT as compared to the normal control group. EAD (200 & 400 mg/kg, *p.o.*), ursolic acid (50 mg/kg, *p.o.*) and standard drug omeprazole (20 mg/kg, *p.o.*) pretreatment significantly leveled the antioxidant enzyme as a result leading to decrease in LPO level while SOD, GSH and CAT values showed significant increase (Table 17).

Table 17: Effect of EAD and ursolic acid on the levels of LPO, SOD, CAT and glutathione (GSH) in rats with gastric ulcers induced by absolute EtOH.

Groups	LPO (MDA, nmol/g tissue)	Antioxidant Enzymes		
		SOD (units/ g tissue)	CAT (units/ g tissue)	GSH (µg GSH/g tissue)
Normal control	70.78±1.44	37.01±0.33	32.29±0.75	248.84±4.83
Ulcer control	93.05±0.93[a]	18.79±0.41[a]	13.96±1.88[a]	108.51±7.17[a]
Omeprazole 20	44.24±1.20[ab]	32.41±0.32[ab]	29.19±0.70[b]	234.77±4.36[b]
EAD 100	75.93±1.51[bc]	19.41±0.38[ac]	17.33±1.72[ac]	148.19±4.71[abc]
EAD 200	63.09±1.17[abcd]	24.19±0.56[abcd]	24.71±0.44[abd]	197.61±5.04[abcd]
EAD 400	43.73±1.60[abde]	31.22±0.42[abde]	27.12±0.85[abd]	234.05±2.89[bde]
Ursolic acid 50	71.47±1.98[bcef]	22.16±1.38[abcf]	21.41±0.93[abcf]	156.73±3.17[abcef]

All statistical data was expressed in mean ± SEM and determined by one way ANOVA followed by Tukey's multiple comparison test.
[a] $p < 0.05$ statistically significant as compared to normal control
[b] $p < 0.05$ statistically significant as compared to ulcer control
[c] $p < 0.05$ statistically significant as compared to omeprazole
[d] $p < 0.05$ statistically significant as compared to EAD100
[e] $p < 0.05$ statistically significant as compared to EAD200
[f] $p < 0.05$ statistically significant as compared to EAD 400

Histopathological studies

In the microscopic observation of ethanol induced ulcer causes gastric lesions, leading to disruption of surface epithelium, erosion, necrosis and hemorrhage, thus changing the normal architecture of the stomach mucosa. Protection against these histopathological changes was observed in pretreatment with EAD graded doses (100, 200 & 400mg/kg, *p.o.*), ursolic acid (50 mg/kg, *p.o.*) and omeprazole (20 mg/kg, *p.o.*) offered significant protection to the mucosa and reduced size of ulcer caused by ethanol (Figure 30 and 31).

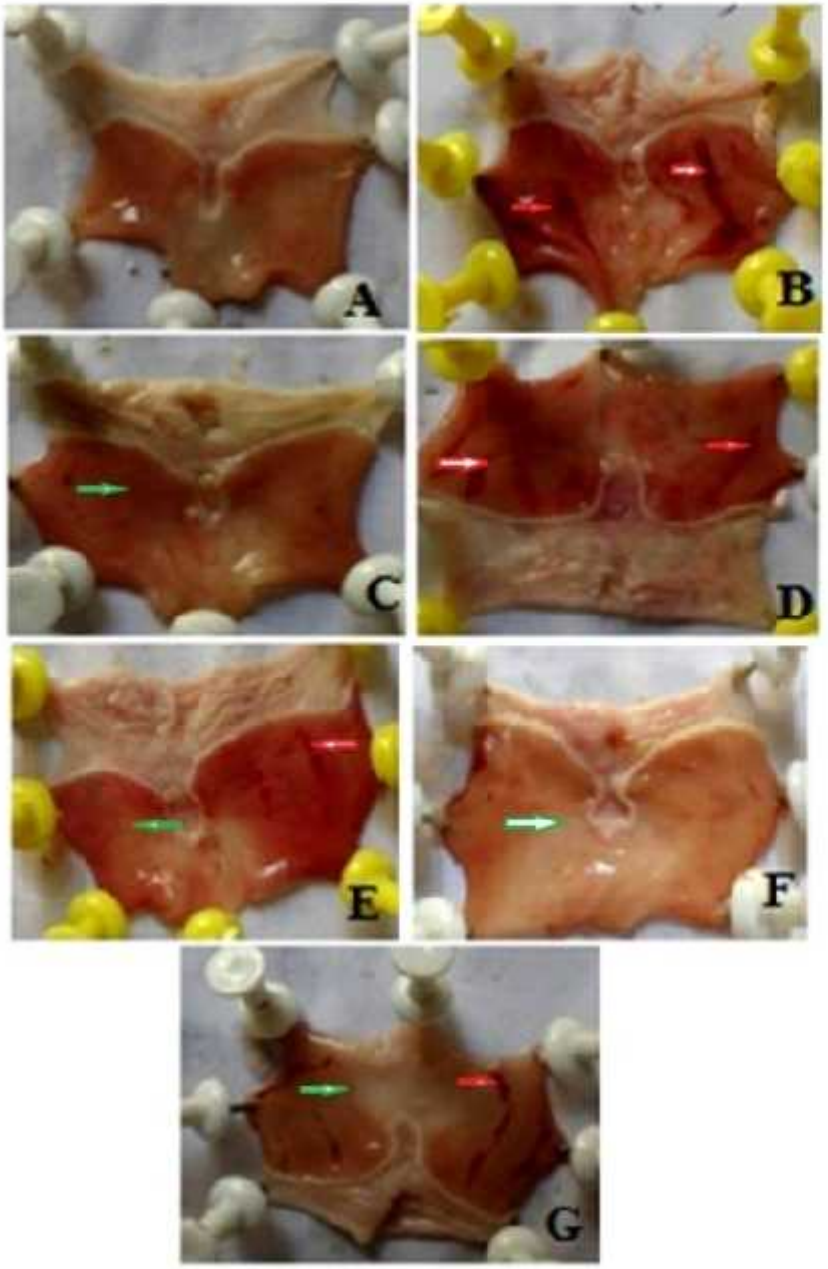

Figure 30: Macroscopic observation of gastric mucosal lesions in ethanol induced ulcer model. (A) Normal control (B) ulcer control (C) Omeprazole (20 mg/kg) pretreated group (D-F) Pretreated EAD doses at 100, 200 & 400 mg/kg, *p.o.* (G) Ursolic acid (50 mg/kg, *p.o.*). ((Indications of arrow marks: Red: Gastric pit lesions formation; Green: stomach normal architecture with less or no ulcer formation).

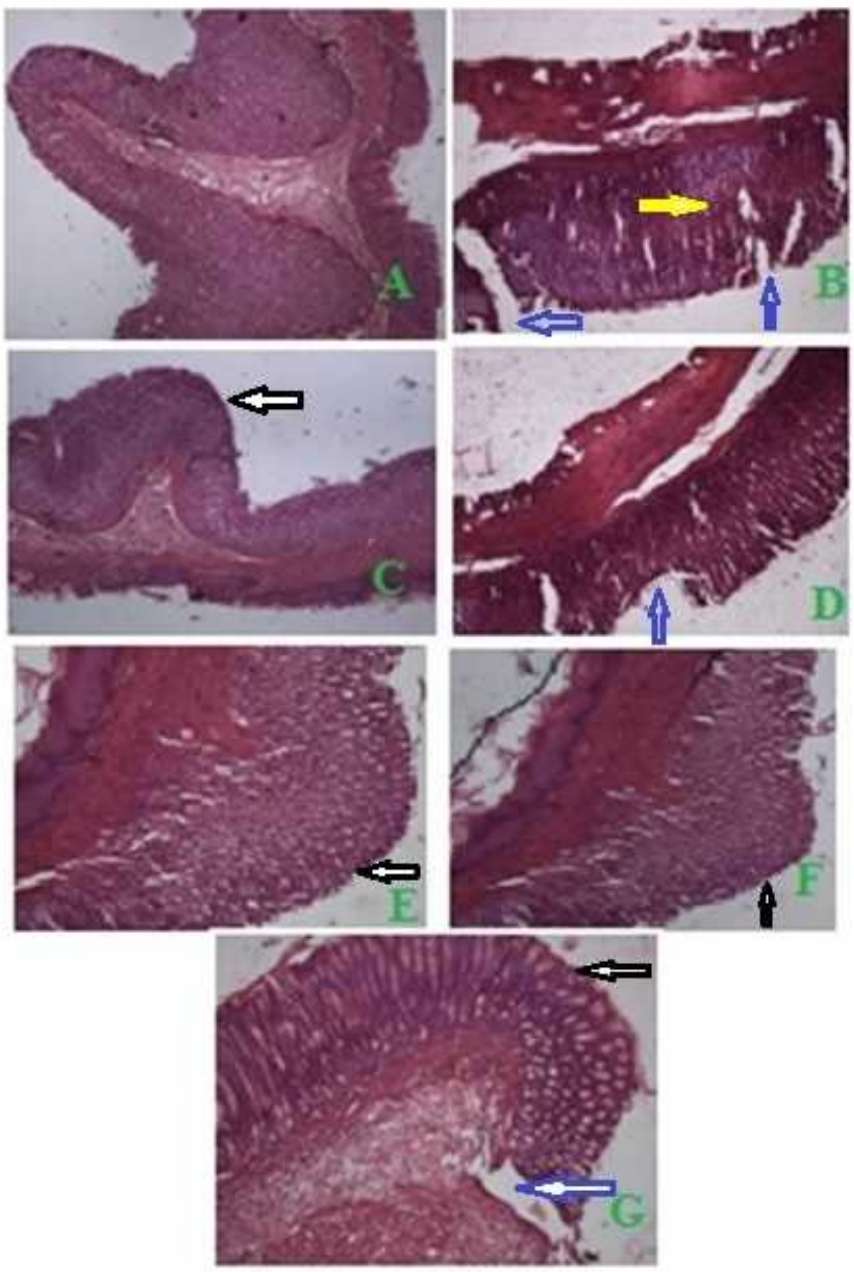

Figure 31: Histological evaluations for the protective effect of EAD and ursolic acid. (A) Normal control (B) ulcer control (C) Omeprazole (20 mg/kg, *p.o.*) pretreated group (D-F) Pretreated EAD doses at 100, 200 & 400 mg/kg, *p.o.* (G) Ursolic acid (50 mg/kg, *p.o.*) on ethanol-induced gastric damage in rat stomach tissues. (Indications of arrow marks: Blue: Severe surface epithelium detachment; Yellow: hemorrhage, Black: stomach normal architecture with less or no ulcer formation).

DISCUSSION

The oral acute toxicity study illustrates that EAD was safe up to 5000mg/kg, *p.o*. and result of sub-acute toxicity study revealed that there is no change in haematological and biochemical parameters, as well as absence of pathological lesion in visceral organs occurred up to the administration of 1000 mg/kg, *p.o*. of EAD.

Anti-ulcer activity of ethanolic root extract of *A. dichotoma* and isolated ursolic acid

The anti-ulcer effect of EAD and ursolic acid has been demonstrated and it efficiently protected the animals against acute gastric ulcer caused by various physical and chemical ulcerogens. Ethanol and pyloric ligation models lead to the determination of mechanism of action by the biochemical studies on various aggressive and defensive parameters. 200 and 400 mg/kg, *p.o*. dose of EAD and ursolic acid (50 mg/kg, *p.o*.) showed significant gastric ulcer protective effect on absolute ethanol and PL- induced gastric ulcers in rats.

Pyloric ligation induced ulcers model increased the accumulation of gastric acid and pepsin which is responsible for auto-digestion of gastric mucosa and rupture the gastric mucosal barrier (Goel *et al*., 1991). The increased gastric acid secretion stimulates the pressure receptors in the antral gastric mucosa, which activate the vagus-vagal reflux in the hypersecretion model of pylorus ligature (Baggio *et al*., 2007). The current data clearly demonstrated that EAD and ursolic acid treated rats showed a significant increase in gastric pH as compared to ulcer control while gastric juice volume and total acid-pepsin output was notably reduced in the 4h PL-rats. However defensive factors (total mucin activity) were not altered significantly. Effect of EAD (200 & 400 mg/kg, *p.o*.) and ursolic acid (50 mg/kg,

p.o.) on cell shedding in the 4h PL-rats depicted decrease in the gastric juice DNA content which was significant, but the level of mucosal DNA content which signifying the lack of effects on the mucosal cell proliferation were unaffected. Thus the possible mode of action of EAD and ursolic acid might be accredited mainly to the antisecretory effect (offensive factor) and not due to the mucin activity (defensive factor).

Antisecretory effect of EAD was further supported by the evaluation of H^+K^+-ATPase activity. H^+K^+-ATPase residing in parietal cells is a membrane bound enzyme which acts as vehicle of H^+, by utilizing energy released by hydrolysis of ATP which on reaction with luminal Cl^- generate HCl in the stomach. Thus H^+K^+-ATPase plays pivotal role in generation of gastric acid (Laloo *et al*., 2013). Hence, antisecretory effect may be result of H^+K^+-ATPase inhibition. The result showed that EAD (200 & 400mg/kg, *p.o.*) significantly inhibited the H^+K^+-ATPase enzyme. Therefore, decreased acid secretion as a result of proton pump inhibition might impart the gastroprotective activity to EAD while ursolic acid does not act on proton pump inhibition. Although, ursolic acid may play an important role in ulcer treatment but it shows lesser effect as compared to EAD 400 mg/kg, *p.o.*

Ethanol depletes the defensive factors of the mucosa, particularly the mucosal barrier, leading to the destruction of gastric wall mucus due to its potent necrotizing effect (Wallace, 2001). The result of ethanol induced ulcer showed that pretreatment with EAD (400mg/kg, *p.o.*) protected the mucosa from the erosive effect of ethanol with percentage inhibition of ulcer score of 77.6% which was as high as comparable to the standard drug omeprazole (82.74%). Pre-treatment with EAD at all dose levels showed a significant decrease in the LPO

level while increase in SOD, CAT, and GSH level. Reactive oxygen species is one of the endogenous aggressive factors in ulcer and can be excluded by augmenting articulation of antioxidant enzymes such as SOD, CAT, and GSH or by eliminating the free radicals like LPO (Ikasari *et al*., 2017; Joshi *et al*., 2016). Generation of free radicals including peroxynitrite and hydroxyl radicals are inhibited by elevated level of superoxide dismutase (SOD) as a result of dismutation of superoxide radicals. Moreover, antioxidant like glutathione (GSH) in turn eradicates deleterious free radicals by scavenging or by catalyzing reduction of H_2O_2 and lipid peroxides (Joshi *et al*., 2013).

Histopathological studies of ethanol-induced positive control rats suggested the extensive rupture of surface epithelium, development of gastric pit lesions, haemorrhage and loss of normal glandular architecture of the stomach. Rats pre-treated with standard drug omeprazole showed a restitution of normal control stomach in which the gastric mucosal cells, gastric pits and gastric glands were together, normal and no profusion and haemorrhage observed. Rats pre-treated with EAD (200-400 mg/kg; *p.o*.) and ursolic acid (50 mg/kg, *p.o*.) were close to normal architecture of the entire gastric mucosa as compared to normal rats.

Ursolic acid has been reported to possess hepatoprotective, anti-inflammatory, anti-tumor, anti- hyperlipidemic, and anti-ulcer activity (Liu, 1995). It is a triterpenoid compound having a free hydroxyl group at position C-3 of the triterpenoid structure which mainly attributes to its anti-ulcer activity (Navarrete *et al*., 2002). According to Lee et al. (2009), ursolic acid also diminished HCl-induced gastric lesions in mice due to antacid effects or cytoprotective properties in gastric mucus. Therefore, presence of ursolic acid in EAD may contribute to

the anti-ulcer activity because it also acts on the offensive mechanism which is similar to the EAD.

Quantification study revealed the presence of significant amount of polyphenolics and saponin in EAD. Anti-ulcer efficacy of flavonoids is primarily by the ability to defend the gastric mucosa against ulcer causing agents by diverse mechanism such as free-radical scavenging and antioxidant properties, marked increase in mucus production and antisecretory activity. Phenolics exhibit antioxidant properties by the virtue to scavenge free radicals by breaking radical chain reactions, attenuating peroxides level and triggering antioxidant defence enzyme system contributing to anti-ulcer effect. The astringent action of tannin stimulates protein precipitating and vasoconstriction resulting in the formation of impenetrable protective barrier preventing gastric ulcer by reducing the number of ulcer score. Moreover, saponin activates mucous membrane protective elements (Sumbul *et al*., 2011; Borrelli & Izzo, 2000). Therefore polyphenolics and saponin along with ursolic acid present in the EAD may contribute in anti-ulcer activity.

EVALUATION OF ANTI-NOCICEPTIVE, ANTI-INFLAMMATORY AND ANTI-ARTHRITIC ACTIVITY

MATERIAL AND METHODS

Anti nociceptive activity

Acetic acid induced writhing test

Rats were divided into 10 groups (n=6), group 1 was control, while group 2-3 animals were treated with standards indomethacin (5 mg/kg, *p.o.*) and curcumin (100 mg/kg, *p.o.*) respectively. Animals in group 4-6 received EAD 100, 200 & 400mg/kg, *p.o.* while group 7-8 received PF 100 & 200 mg/kg, *p.o.*, group 9-10 animals were treated with CF 100 & 200 mg/kg, *p.o.* After 45 minutes of drug administration, each rat was injected with acetic acid i.p. (0.8%, v/v, 10 mL/kg). The number of writhing response was recorded after 15 min of acetic acid administration for each rat during a subsequent 5 min. After that, the percentage inhibition of writhing was calculated (Nonato *et al*., 2011).

Formalin-induced pain

After thirty minutes of drug administration (grouping was similar to the previous model), pain was induced by injecting 0.05 mL of 2.5% formalin in distilled water in the sub-plantar of the right hind paw. Then individual rats were placed in a transparent plexiglass cage (25cm×15 cm×15 cm) observation chamber. The time spent in licking of injected paw was indicative of pain. The number of lickings from 0 to 5 min (first phase) and 15–30 min (second phase) were counted after injection of

formalin. These phases represented neurogenic and inflammatory pain responses, respectively (Young *et al*., 2005).

Tail Flick test

The tail flick test was conducted according to the method described by D' Amour and Smith (1941), where animal grouping was kept similar to the writhing test but morphine (5 mg/kg, *p.o*.) was used as a standard drug in this model. The rat's tail was placed at the window of the tail flick apparatus (UGO Basile, Italy) and reaction time was recorded before and at 30, 60 and 90 min in both treated and control groups.

Anti-inflammatory activity

Carrageenan-induced rat paw edema

Subcutaneous injection of carrageenan in rat paw produces inflammation resulting from plasma extravasation, increased tissue water, and plasma protein exudation, along with neutrophil extravasation, due to the metabolism of arachidonic acid. Grouping of animals was done as described in acetic acid induced writhing test. One hour after dosing, edema was induced by a subcutaneous injection of 0.1mL of 1% solution of carrageenan into the sub plantar side of the left hind paw. The paw volume was measured again at 1, 2, 3, 4, and 5h after injection. The increase in paw volume was calculated as a percentage compared with the basal volume. The difference of average values between treated animals and control group was calculated for each time interval and evaluated statistically. The percent inhibition was calculated using the formula as follows:

$$\% \text{ edema inhibition} = [1 - (Vt/Vc)] \times 100$$

Where, Vt and Vc are edema volume in the drug-treated and control groups, respectively (Eddouks *et al*., 2012).

Cotton pellet induced granuloma

According to Swingle and Shideman (1972), granulomatous lesions was induced by surgically implanting two cotton pellets subcutaneously in the dorsal region of the rats, one near each axilla (grouping was same as in carrageenan-induced rat paw edema). One hour after the first dosing, adsorbent cotton wool was cut into 20 ± 1 mg pieces and pellets were made. The abdomen was shaved cleanly, swabbed with 70% ethanol then rats were anesthetized under light ether and an incision was made in the lumbar region by blunted forceps, a subcutaneous tunnel was made and a sterilized cotton pellet (20 ± 1 mg) was inserted in the groin area. EAD, PF, and CF were administered once daily throughout the experimental period of 7 days. On the 8^{th} day, cotton pellets were removed surgically. Extraneous tissue was removed from the pellet and dried at 60ºC for 18h until weight become constant. The mean weight of the cotton pellets of the control group as well as of the treatment groups was calculated. The transudative weight, granuloma formation and percent granuloma inhibition of the treatment groups were calculated (Sengar *et al*., 2015).

Anti-arthritic activity

Freund's adjuvant-induced arthritis

Arthritis was induced by single 0.1 mL intra-dermal injection of complete Freund's adjuvant (FCA) (Sigma-Aldrich) in foot pad of the left hind paw of rats. FCA was prepared by suspending dry heat-killed *Mycobacterium tuberculosis* in liquid paraffin at 10mg/mL. A glass syringe (1mL) with the locking hubs and a 26G needle was used

for injection. Prior to adjuvant injection rats were anesthetized with ether due to viscous nature of adjuvant which causes difficulty during injection. Animals were divided into 11 groups with six no. of animal in each group.

Group 1- Normal control

Group 2- Freund's adjuvant induced arthritic rats (AIA)

Group 3- AIA + Methotrexate (MTX) (3mg/kg, *p.o.*)

Group 4- AIA + Curcumin (100 mg/kg, *p.o.*)

Group 5 to 7 – AIA + EAD (100, 200, 400 mg/kg, *p.o*)

Group 8 to 9 – AIA + PF (100 and 200 mg/kg, *p.o*)

Group 10 to 11 – AIA + CF (100 and 200 mg/kg, *p.o*)

Treatment of rats was started with EAD, PF, and CF from day 2. Paw volume was measured at 0, 7, 14, 21 and 28 days by using Plethysmometer while body weight and arthritic index score was also evaluated at every 7 days. On 28^{th} day x-ray radiography was done on left hind limb and blood samples were collected from all rats through retro-orbital plexus. Hematological parameters were evaluated from blood samples. Then serum was separated for the estimation of cytokine (IL-1β, IL-6, TNF-α) level thereafter rats were sacrificed by euthanasia and ankle tissue was used for the estimation of various biochemical parameters. At the end of experiments thymus, spleen and ankle joint was dissected out for the estimation of thymus and spleen index and ankle joints were decalcified and embedded in paraffin section and stained with hematoxylin and eosin to observe the histopathological changes.

Evaluation of body weight, paw volume, and arthritic score

Alteration in body weight is an essential factor in RA, thus, body weight of each rat was measured at every 7^{th} day up to 28^{th} day. The left hind paw volumes of all animals were estimated just before the administration of FCA injection and thereafter at every 7^{th} day time intervals up to day 28^{th} by using a Plethysmometer (UGO Basile, Italy). The change in paw volume was calculated as the difference between the final and initial paw volumes. The morphological aspect of arthritis like redness, swelling, erythema and use of joint was monitored by set visual criteria as follows: normal paw = 0, mild swelling and erythema = 1, low to moderate swelling and erythema = 2, severe swelling and erythema with limited joint use = 3, gross deformity and inability to use the limb = 4 on respective days (Cai *et al*., 2007).

Hematological parameters

The hematological parameters for instance hemoglobin, RBCs, WBCs, and ESR were determined by standardized laboratory method described by Mythilypriya *et al*. (2008).

Evaluation of thymus and spleen index

On 28^{th} day animals were sacrificed followed by prompt removal of thymus and spleen. The thymus index and spleen index were articulated as the ratio (mg/g) of thymus and spleen wet weight versus body weight, respectively (Zhang *et al*., 2004).

Antioxidant parameters

The ankle tissue of rats was isolated and washed in ice-cold saline. Tissue homogenates were prepared with 0.1M tris-HCl buffer (pH 7.4) and centrifugation was done initially at 800 xg for 10 min and later at 12,000 xg for 15 min. The

supernatant obtained was used to estimate superoxide dismutase (SOD), lipid peroxidation (LPO), catalase (CAT) and reduced glutathione (GSH) as described in anti-ulcer study.

Estimation of cytokines level

Rat blood was collected and centrifuged with 3000 rpm at 4ºC for 15 min. Serum was separated and frozen at −20ºC until assayed. The concentration of TNF-α, IL-1β, and IL-6 were assessed in serum by ELISA (Koma Biotech) as per manufacturer's protocol.

Radiological analysis of ankle joints

The adjuvant injected hind paws of anesthetized rats were radiographed on 28th day by digital X-ray (Brivo XR 115 unit, Wipro GE healthcare). Radiographic analysis of hind paws was performed at 200 kHz peak, 100mA and the exposure time was 5s.

Histopathology of ankle joints

On 28th day, ankle joints were alienated from the hind paw and immersed in 10% buffered formalin for 24h followed by decalcification in 5% formic acid, processed for paraffin embedding sectioned at 3µ thickness. The sections were stained with hematoxylin-eosin and evaluated for the presence of inflammatory cells, hyperplasia of synovium, pannus formation and destruction of joint space (Ohmachi *et al*., 2002).

Statistical Analysis

All data are expressed as mean ± SEM with n=6 per group. Statistical significance was determined using one-way analysis of variance (ANOVA) followed by Dunnett's post-test for multiple groups comparison. However, two-way ANOVA followed by Bonferroni post test was performed for determining the significance level in models

such as the tail flick method, carrageenan-induced rat paw edema and FCA induced change in paw volume. Graph Pad Prism (version 5) software was used for all statistical analysis. P value < 0.05 was considered significant.

RESULTS

Anti nociceptive activity

Acetic acid induced writhing test in rats

EAD (100, 200 and 400 mg/kg, *p.o*) showed a significant and dose dependent decrease in the number of writhes as compared to control. PF and CF treated groups (100 and 200 mg/kg, *p.o*) also demonstrated a significant effect. The inhibition rates of the number of writhes for the extract EAD (100, 200 and 400) were (21.90%, 57.64%, 82.87%) while for PF (100 and 200) were (18.37%, 54.18%) and for CF (100 and 200) were (16.82%, 40.93%). EAD at the dose of 400 mg/kg showed higher percentage inhibition of writhes than the standard drug indomethacin 5 mg/kg (80.69%) and curcumin 100 mg/kg (60.37%) (Figure 32).

Formalin induced pain

The result demonstrated that EAD at 400 mg/kg, *p.o*., PF (100 and 200 mg/kg, *p.o*.) and CF (100 and 200 mg/kg, *p.o*.) produced a significant dose dependent inhibition of neurogenic phase (0-5 min). While EAD, PF and CF at all dose levels showed significant action on inflammatory phase (15-30 min) of formalin induced licking (Table 18). However, anti nociceptive effect of both extract and fractions were more pronounced in the second phase of formalin induced pain. Where, indomethacin and curcumin reduced the formalin induced pain in both the phases.

Tail Flick test

Figure 33 represents analgesic activity of EAD, PF and morphine, which did not show any significant activity up to 30 min of treatment. However, EAD (100, 200 and 400 mg/kg, *p.o*.) and PF (100, 200 mg/kg, *p.o*.) exhibited significant effect after

60 min and increased latency in flicking tail. The observed effect was found to be more pronounced ($p < 0.001$) in rats treated with EAD at (100, 200, 400 mg/kg, *p.o.*), PF 100 and 200 mg/kg, *p.o.* ($p < 0.05$, $p < 0.01$) which was quite comparable with standard morphine while CF at any dose did not show significant effect on latency in flicking.

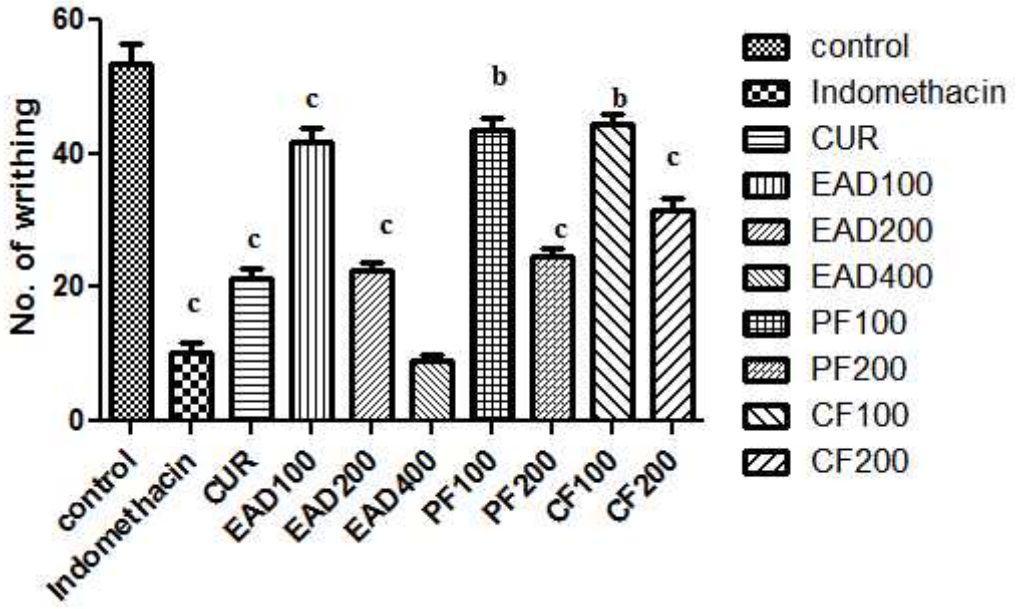

Figure 32: Effect of extract and fractions on acetic acid induced writhing test.

All statistical data was expressed in mean ± SEM and determined by one way ANOVA followed by Dunnett's post test.
[b] $p < 0.01$ statistically significant as compared to control.
[c] $p < 0.001$ statistically significant as compared to control.

Table 18: Effect of EAD, PF and CF on formalin induced pain

Group	First Phase	% Inhibition	Second Phase	% Inhibition
Control (0.5%CMC)	77.00±2.36	-	57.50±1.88	-
Indomethacin (5mg/kg)	22.33±1.28[c]	71.00	10.50±0.99[c]	81.73
Curcumin (100mg/kg)	26.16±1.62[c]	66.02	18.33±1.35[c]	68.12
EAD (100mg/kg)	72.33±1.33	6.06	49.61±1.22[b]	13.72
EAD (200mg/kg)	69.78±1.70[a]	9.37	41.52±1.81[c]	27.79
EAD (400mg/kg)	22.16±1.66[c]	71.22	9.43±1.11[c]	83.60
PF (100mg/kg)	61.80±1.86[c]	19.74	45.34±1.89[c]	21.14
PF (200mg/kg)	56.50±1.14[c]	26.62	28.61±1.67[c]	50.24
CF (100mg/kg)	68.23±1.71[b]	11.68	46.82±1.54[c]	18.57
CF (200mg/kg)	54.82±1.25[c]	28.80	37.71±1.91[c]	34.41

All statistical data was expressed in mean ± SEM and determined by one way ANOVA followed by Dunnett's post test.
[a] $p < 0.05$ statistically significant as compared to control.
[b] $p < 0.01$ statistically significant as compared to control.
[c] $p < 0.001$ statistically significant as compared to control.

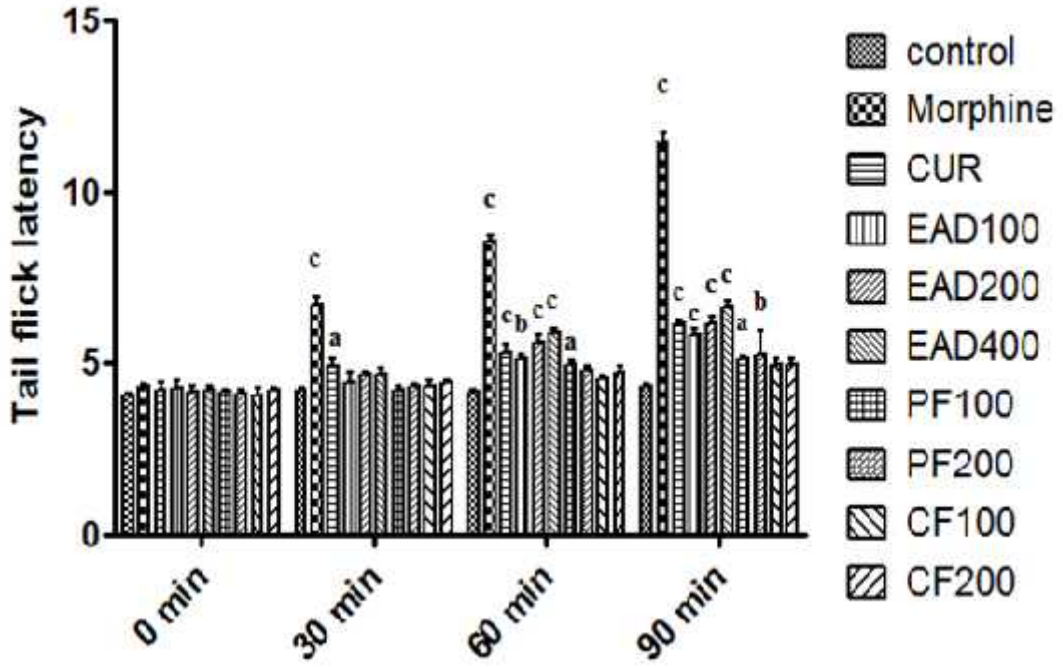

Figure 33: Central analgesic activity of EAD, PF and CF in tail flick method.

All statistical data was expressed in mean ± SEM and determined by Two way ANOVA followed by Bonferroni post test.
[a] $p < 0.05$ statistically significant as compared to control.
[b] $p < 0.01$ statistically significant as compared to control.
[c] $p < 0.001$ statistically significant as compared to control.

Anti-inflammatory activity

Carrageenan-induced rat paw edema

As shown in table 19, the EAD at 200 and 400 mg/kg., *p.o*. doses significantly ($p < 0.001$) inhibited the paw edema from 3 to 5 h, while EAD 100 mg/kg, *p.o*. produced significant action only at 5 h. Fraction PF and CF (100 and 200 mg/kg, *p.o*.) significantly ($p < 0.001$) inhibit the paw volume from 3-5 h. The maximum inhibitory effect at 3h and 5h of the extract was recorded at the 400 mg/kg dose

(35.82%, 46.86%) as compared to indomethacin (38.05%, 45.38%) and curcumin (21.64%, 38.37%) at 3 h and 5 h, respectively.

Cotton pellet induced granuloma

Table 20 shows that the EAD (200 and 400 mg/kg, *p.o.*), PF and CF (200 mg/kg, *p.o.*) exhibited significant ($p < 0.001$) and dose dependent inhibition of dry weight of the cotton pellet granuloma. EAD 400 mg/kg dose was displayed higher % inhibition in granuloma formation (42.49%) than the standard drugs indomethacin (40.69%) and curcumin (34.13%).

Table 19: Effect of different doses of EAD, PF and CF on carrageenan induced paw edema

Groups	**Dose (mg/kg, *p.o.*)**	**Left hind paw volume (ml)**					
		0 h	1 h	2 h	3 h	4 h	5 h
Control	0.5%CMC	1.42±0.08	1.84±0.08	2.23±0.14	2.68±0.10	2.65±0.10	2.71±0.04
Indomethacin	5	1.39±0.04	1.46±0.04[c]	1.61±0.04[c]	1.66±0.02[c]	1.65±0.03[c]	1.48±0.04[c]
Curcumin	100	1.44±0.04	1.61±0.03[a]	1.93±0.04[b]	2.10±0.05[c]	1.88±0.03[c]	1.67±0.03[c]
EAD	100	1.40±0.03	1.80±0.01	2.15±0.02	2.47±0.10	2.43±0.14	2.21±0.15[c]
EAD	200	1.39±0.03	1.75±0.06	2.06±0.05	2.28±0.10[c]	2.11±0.07[c]	1.86±0.05[c]
EAD	400	1.38±0.05	1.54±0.05[b]	1.68±0.04[c]	1.72±0.05[c]	1.69±0.02[c]	1.44±0.03[c]
PF	100	1.41±0.01	1.51±0.06[c]	1.81±0.02[c]	2.06±0.03[c]	1.96±0.02[c]	1.95±0.05[c]
PF	200	1.41±0.06	1.61±0.02[a]	1.99±0.04[a]	1.96±0.02[c]	1.91±0.03[c]	1.71±0.03[c]
CF	100	1.45±0.04	1.78±0.03	2.48±0.06[a]	2.24±0.05[c]	2.13±0.04[c]	1.98±0.04[c]
CF	200	1.39±0.04	1.73±0.06	1.84±0.08[c]	2.19±0.02[c]	1.99±0.06[c]	1.87±0.02[c]

All statistical data was expressed in mean ± SEM and determined by Two way ANOVA followed by Bonferroni post test.
[a] $p < 0.05$ statistically significant as compared to control.
[b] $p < 0.01$ statistically significant as compared to control.
[c] $p < 0.001$ statistically significant as compared to control

Table 20: Effect of EAD, PF and CF on cotton pellet granuloma in rats

Groups	Dose(mg/ kg, *p.o.*)	Granuloma dry weight (mg)	% Inhibition
Control	0.5%CMC	89.26±1.78	-
Indomethacin	5	52.94±1.94[c]	40.69
Curcumin	100	58.79±1.22[c]	34.13
EAD	100	81.53±2.78	8.66
EAD	200	68.45±2.56[c]	23.31
EAD	400	51.33±1.56[c]	42.49
PF	100	79.47±2.54[a]	10.96
PF	200	68.91±1.22[c]	22.79
CF	100	81.34±2.82	8.87
CF	200	74.56±1.66[c]	16.46

All statistical data was expressed in mean ± SEM and determined by one way ANOVA followed by Dunnett's post test.
[a] $p < 0.05$ statistically significant as compared to control.
[b] $p < 0.01$ statistically significant as compared to control.
[c] $p < 0.001$ statistically significant as compared to control.

Anti arthritic activity

Effect on body weight, paw swelling and arthritic score

A close relationship between the extent of joint inflammation and weight loss was observed in this study as it was found that rats of control group showed marked weight loss in second week after adjuvant injection, followed by normal weight gain in subsequent weeks. Whereas methotrexate, curcumin, EAD, PF and CF treated groups did not show significant weight loss (Figure 34). Joint swelling was observed in all arthritic rats 24h after the injection of FCA. Administration of EAD 100 and 200 mg/kg, *p.o*. significantly decreased the paw volume from 14 to 28 days as compared to control group while PF and CF at all dose levels inhibit the paw volume

from 21 to 28 days as shown in figure 35. Whereas, MTX (34.43%), curcumin (31.12%) and EAD 400 mg/kg, p.o. (33.19%) shows maximum % inhibition of paw volume on 28 days respectively. The reduction of clinical arthritis score was observed in the group treated with EAD, PF and CF at all doses and demonstrated significant effect through-out the treatment period from 21st day. MTX and curcumin treated group showed potent efficacy from day 14 to 28 as shown in figure 36.

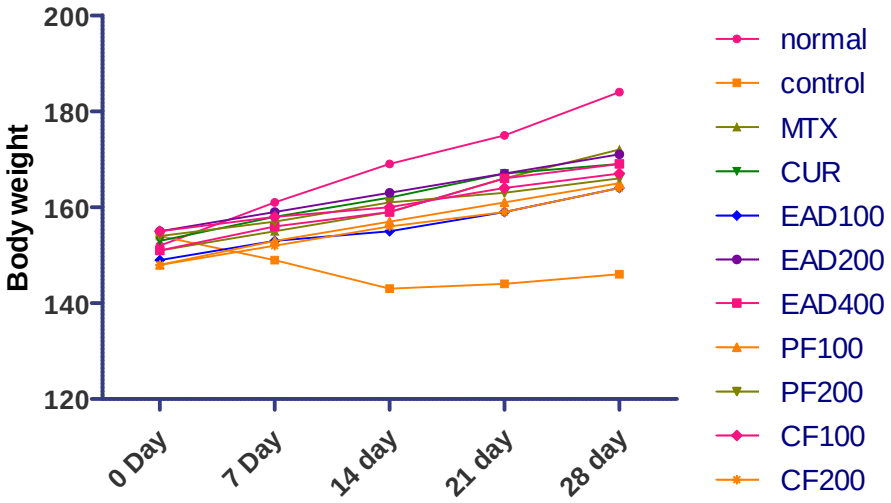

Figure 34: Effect of extract and fractions on body weight after FCA administration. All statistical data was expressed in mean ± SEM and determined by one way ANOVA followed by Dunnett's post test. Only control group shows significant weight loss ($p < 0.001$) on 14 days as compare to normal rats. MTX, Curcumin (CUR), EAD, PF and CF treated rat shows non significant action on body weight as compared to normal rats.

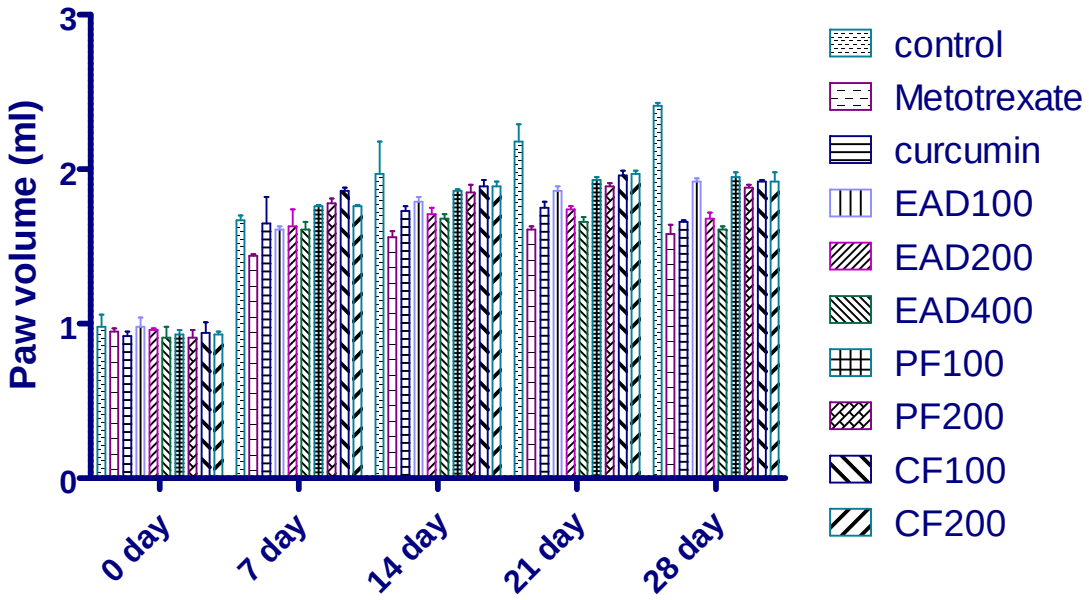

Figure 35: Effect of EAD, PF and CF on change in paw volume in FCA induced arthritis.

Values are expressed as mean ± SEM for six animals and analysed by two-way ANOVA followed by Bonferroni post test. MTX, Curcumin (CUR), EAD, PF and CF at all dose level shows significant change in paw swelling ($p < 0.001$) from 14 to 28 days. While only MTX ($p < 0.001$) and EAD 200 and 400 mg/kg, *p.o*. shows ($p < 0.05$) significant action on 7^{th} day.

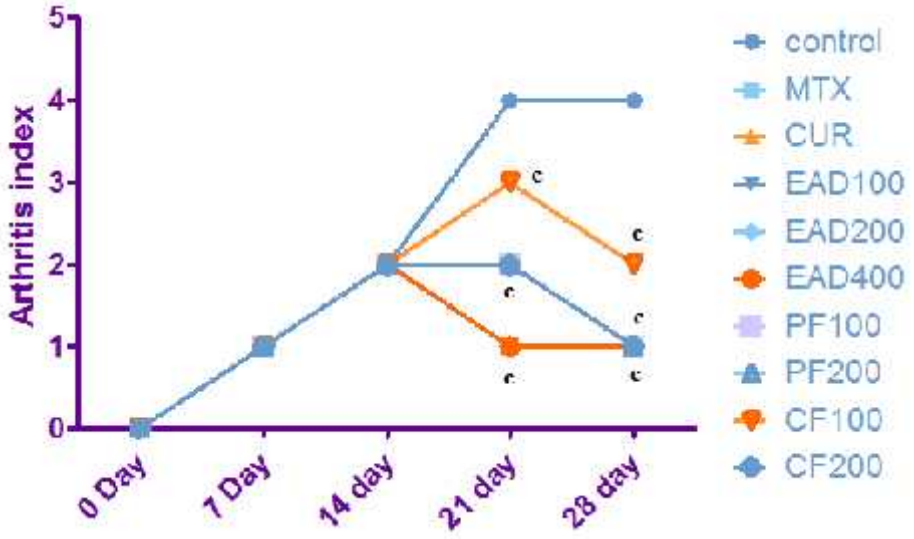

Figure 36: Effect of EAD, PF and CF on arthritic index in FCA induced arthritic rats. Values are expressed as mean ± SEM (n=6) and analysed by two-way ANOVA followed by Bonferroni post test. MTX, Curcumin (CUR), EAD, PF and CF at all dose level shows significant action (c = $p < 0.001$) on arthritis index as compared to control group from 21st to 28th days.

Evaluation of Hematological parameters

Table 21 represents the changes in hematological parameters in FCA induced arthritic rats. Treatment groups of EAD, PF and CF demonstrated significant changes such as increase in RBCs and hemoglobin and decrease in total WBCs and ESR as compared to control group which indicated stimulation of immune response.

Table 21: Effect of EAD, PF and CF on Hematological parameters

Groups	Dose(mg/kg, *p.o.*)	RBC(millions/mm^3)	WBC(thousands /mm^3)	Hb(g/dl)	ESR(mm/hr)
Control	0.5%CMC	4.63±0.38	25.63±1.28	8.11±1.72	9.44±1.34
MTX	3	7.57±0.21[c]	14.21±0.89[c]	13.38±0.52[b]	4.87±0.48[b]
Curcumin	100	7.14±0.38[b]	16.16±0.63[c]	13.61±0.34[c]	4.23±0.41[c]
EAD	100	5.82±0.41	21.28±1.78[a]	11.26±1.12	6.66±0.93
EAD	200	6.69±0.27[a]	17.63±0.37[c]	13.17±0.37[b]	5.12±0.61[b]
EAD	400	7.13±0.46[b]	15.11±0.38[c]	14.61±0.28[c]	4.93±0.34[b]
PF	100	6.92±0.29[b]	17.67±0.93[c]	12.79±1.23[b]	5.83±0.78[a]
PF	200	7.22±0.34[b]	16.48±0.73[c]	13.46±0.78[b]	5.11±0.52[b]
CF	100	5.72±0.89	20.61±1.26[b]	12.24±0.97[a]	6.98±1.06
CF	200	6.39±0.61	19.72±1.12[b]	12.98±0.87[b]	5.87±0.88[a]

All statistical data was expressed in mean ± SEM and determined by one way ANOVA followed by Dunnett's post test.
[a] $p < 0.05$ statistically significant as compared to control.
[b] $p < 0.01$ statistically significant as compared to control.
[c] $p < 0.001$ statistically significant as compared to control.

Effect on Thymus and spleen index

The spleen and thymus index were determined at 28th day after sacrificing the rats, and the results showed that the index of spleen and thymus in FCA induced group were markedly increased as compared with the normal group. As shown in figure 37 only curcumin, EAD (200 and 400) and PF 200 mg/kg, *p.o.*, treated rats exhibited a significant ($p<0.01$) decrease in spleen index as compared to control group whereas, curcumin, EAD, PF and CF had no significant effect on thymus index. Metotrexate (3 mg/kg, *p.o.*) administered rat demonstrated decrease in both thymus and spleen index as compared to control group.

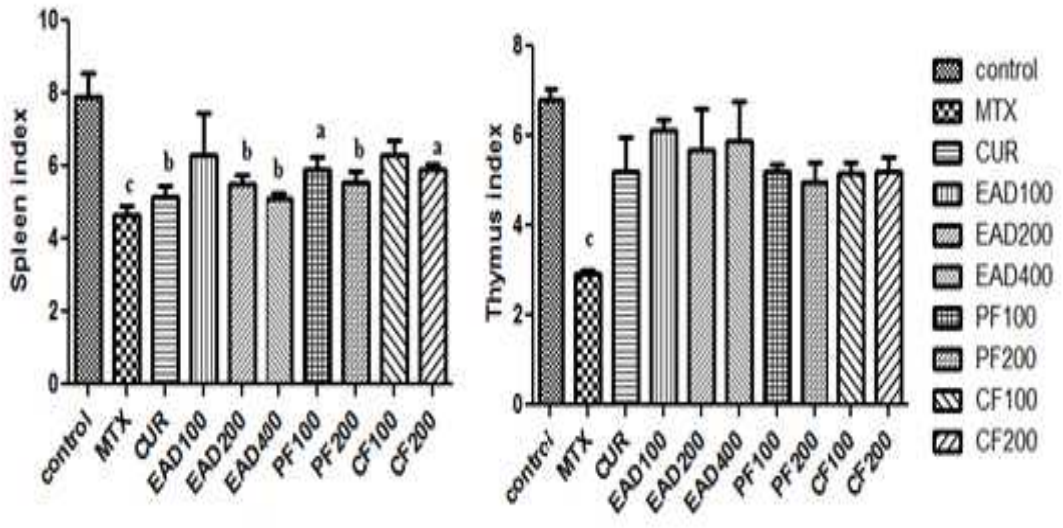

Figure 37: Effect of EAD, PF and CF on spleen and thymus index.

Values are expressed as mean ± SEM (n=6) and determined by one way ANOVA followed by Dunnett's post test.
[a] $P < 0.05$ statistically significant as compared to control.
[b] $p < 0.01$ statistically significant as compared to control.
[c] $p < 0.001$ statistically significant as compared to control.

Effects of extract and fractions on in-vivo antioxidant activity

Results of different biochemical parameters evaluated in the left hind paw tissue obtained from FCA induced model are represented in table 22. The results elicited a significant ($p < 0.05$) reduction in lipid peroxidation (LPO) while increase in SOD, GSH and CAT level in serum.

Determination of TNF-α, IL-1β and IL-6

A significant increase in TNF-α, IL-1β and IL-6 level was observed in FCA induced rats as compared to normal rats. MTX, curcumin, EAD, PF and CF showed a significant effects and decrease the production of TNF-α, IL-1β and IL-6 as compared to control group shown in table 23. Furthermore, MTX, curcumin and EAD 400

showed potent inhibition in TNF-α (61.14%, 44.09%, 57.32%), IL-1 β (56.29%, 45.65%, 51.66%) and IL-6 (73.19%, 68.27%, 64.42%) respectively.

Table 22: *In-vivo* antioxidant effect of EAD, PF and CF

Groups	Dose (mg/kg, *p.o.*)	LPO (MDA, nmol/*g* tissue)	SOD (units/ *g* tissue)	CAT (units/ *g* tissue)	GSH (µg GSH/*g* tissue)
Normal		74.46±1.67	62.32±0.97	38.43±1.46	234.78±3.11
Control	0.5%CMC	97.51±1.22[a]	20.37±0.48[a]	11.78±1.92[a]	104.61±4.23[a]
MTX	3	38.89±1.46[ab]	57.36±1.78[b]	36.41±0.97[b]	238.31±4.36[b]
Curcumin	100	45.61±1.21[ab]	48.39±1.27[abc]	31.78±1.67[b]	226.71±3.56[b]
EAD	100	86.93±1.53[abcd]	21.40±0.38[acd]	14.56±1.98[acd]	134.67±4.78[acd]
EAD	200	67.48±1.17[bcde]	37.53±0.47[abcde]	25.61±1.23[abce]	197.61±3.55[abce]
EAD	400	43.73±1.60[abef]	58.22±0.59[bdef]	34.51±1.78[bef]	218.09±2.67[be]
PF	100	72.34±1.76[bcdeg]	28.15±1.54[abcdefg]	21.76±1.41[abcdf]	198.46±3.18[be]
PF	200	54.65±1.34[abcdefgh]	46.38±1.72[abcefgh]	28.77±1.41[abce]	217.56±3.85[be]
CF	100	61.82±1.89[abcdeghi]	31.38±0.89[abcdefgi]	19.38±1.19[abcdfh]	187.20±3.24[abce]
CF	200	58.44±1.34[abcdefg]	39.78±0.75[abcdeghij]	26.75±1.67[abce]	215.23±3.81[be]

All statistical data was expressed in mean ± SEM and determined by one way ANOVA followed by Tukey's multiple comparison test.
[a] $p < 0.05$ statistically significant as compared to normal control.
[b] $p < 0.05$ statistically significant as compared to negative control.
[c] $p < 0.05$ statistically significant as compared to MTX.
[d] $p < 0.05$ statistically significant as compared to Curcumin.
[e] $p < 0.05$ statistically significant as compared to EAD 100.
[f] $p < 0.05$ statistically significant as compared to EAD 200.
[g] $p < 0.05$ statistically significant as compared to EAD 400.
[h] $p < 0.05$ statistically significant as compared to PF 100.
[i] $p < 0.05$ statistically significant as compared to PF 200.
[j] $p < 0.05$ statistically significant as compared to CF 100.

Table 23: Effect of extract and fractions on cytokine levels (TNF-α, IL-1β and IL-6) in serum.

Groups	Dose(mg/kg, *p.o.*)	IL-1β (pg/ml)	IL-6 (pg/ml)	TNF-α (pg/ml)
Control	0.5% CMC	128.51±3.26	294.51±39.27	68.22±4.12
MTX	3	56.17±2.54[c]	78.94±44.61[c]	26.51±1.79[c]
CUR	100	69.84±5.04[c]	93.44±38.44[b]	38.14±2.05[c]
EAD	100	117.12±7.32	164.88±31.25	66.59±2.14
EAD	200	98.34±5.72[b]	134.51±44.29[a]	42.27±1.56[c]
EAD	400	62.19±3.44[c]	104.78±29.16[b]	29.11±1.38[c]
PF	100	97.45±3.19[b]	143.72±34.55[a]	53.10±0.92[c]
PF	200	77.54±8.12[c]	119.79±28.92[b]	36.33±1.24[c]
CF	100	106.72±7.45[a]	136.53±22.45[a]	58.19±0.78[b]
CF	200	89.41±3.44[c]	124.57±34.08[b]	36.21±2.16[c]

All statistical data was expressed in mean ± SEM and determined by one way ANOVA followed by Dunnett's post test.
[a] $p < 0.05$ statistically significant as compared to control.
[b] $p < 0.01$ statistically significant as compared to control.
[c] $p < 0.001$ statistically significant as compared to control.

X-ray radiography

Arthritis (FCA) induced group produced an observable signs of inflammation represented as ankylosis (narrowing of joint space), osteophytes formation, bone erosion and subchondral cyst formation. MTX and curcumin treated group did not show any visible sign of bone and joint deformation but mild sign of inflamed tissue were observed. EAD, PF and CF showed the suppression of inflammation and subsequent arthritic joint development. Rat treated with EAD 400 mg/kg, *p.o*. dose

shows a marked increase in joint space and reduced the bone erosion with less distorted metatarsal joint (Figure 38).

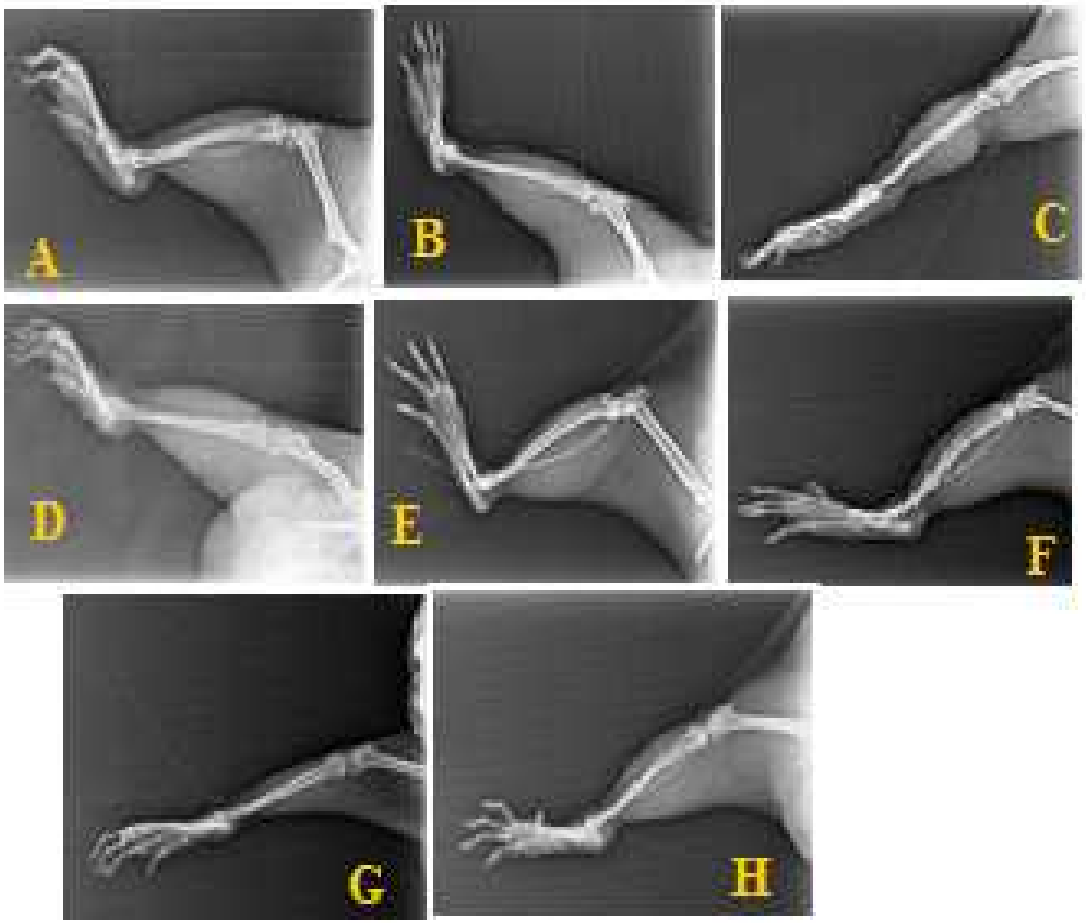

Figure 38: Radiological analysis of ankle joint. (A) Arthritic control. (B) MTX (3 mg/kg, *p.o.*) treated rat. (C) Curcumin (100 mg/kg, *p.o.*) treated rat. (D-F) EAD (100, 200 and 400 mg/kg, *p.o.*) treated. (G) PF 200 mg/kg treated. (H) CF 200 mg/kg treated rat.

Histopathology study of ankle joint

Histopathological evaluation of ankle joint in FCA induced group was shown in figure 39 which demonstrated mononuclear cell infiltration, damaged articular cartilage and subchondral bone, pannus formation and synovial membrane proliferation. MTX and curcumin treated rats showed significant attenuation of the synovial membrane with decreased infiltration of mononuclear cells and reduced pannus growth. The rats treated with EAD showed significant protection and reduced pathological changes in a dose dependent manner as compared to control group. Reduced pannus growth and synovial erosion was also observed in PF 200 mg/kg, *p.o*. dose treated rats while CF 200 mg/kg, *p.o*. treated rat shows influx of inflammatory cells with disturbed synovial lining and bone necrosis.

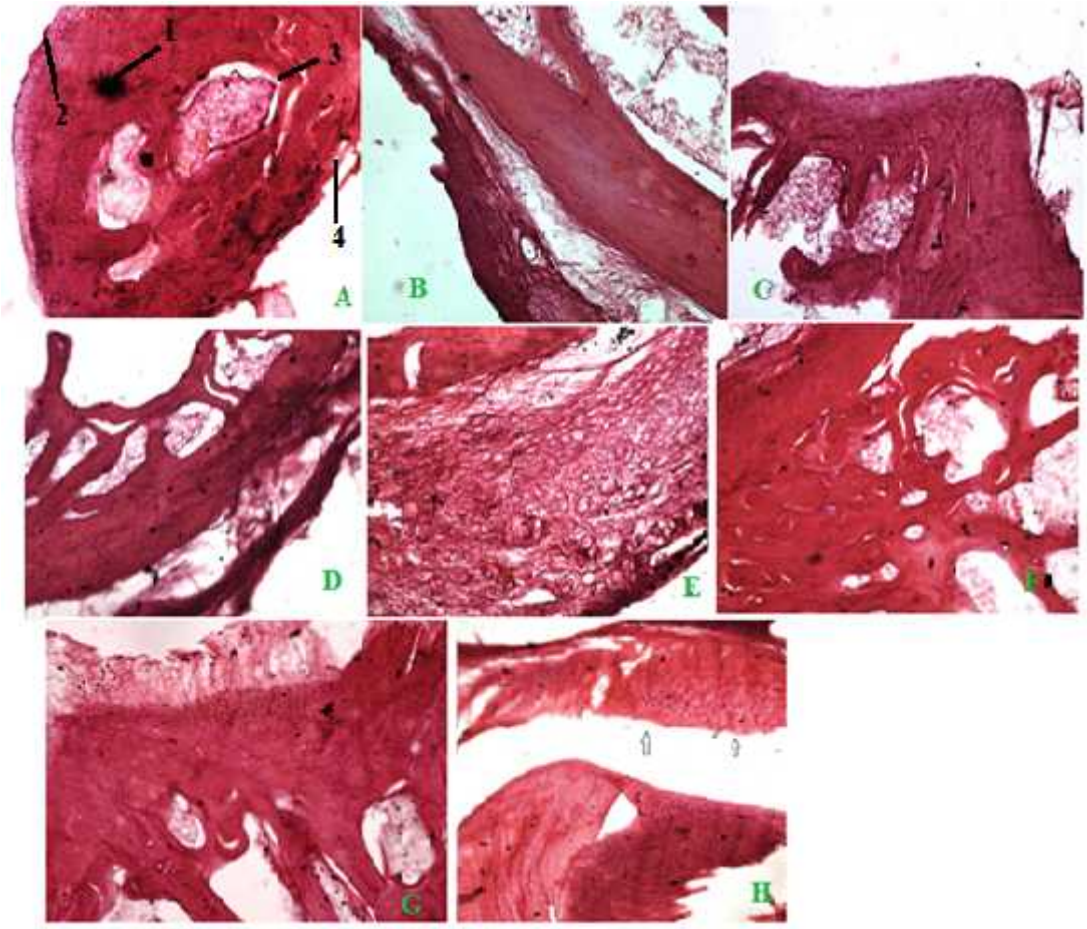

Figure 39: Histology of ankle joint of adjuvant induced arthritic rats. (A) Arthritic control. (B) MTX treated group. (C) Curcumin treated group. (D-F) EAD (100, 200, and 400 mg/kg, p.o.) treated rats. (G) PF 200 mg/kg, p.o. treated group. (H) CF 200 mg/kg, p.o. treated rat. (Indication of arrow) **1**: Dense mononuclear cell infilteration, **2**: Damaged articular cartilage, **3**: Pannus formation, **4:** Synovial membrane proliferation.

DISCUSSION

Administration of EAD (100, 200 and 400 mg/kg, *p.o*), PF (100 and 200 mg/kg, *p.o*) and CF (100 and 200 mg/kg, *p.o*) produced consistent anti-nociceptive, anti-inflammatory and ant-arthritic effects in different models of pain and inflammation. Acetic acid induced writhing response is widely used as a visceral pain model. It causes release of pain mediators such as prostaglandins, kinins, etc. augmenting their level in the dorsal horn of the central nervous system via peripheral tissue fluid entering it which in turn induces excitation of primary afferent nociceptors (Gorzalczany *et al*., 2011). Oral administration of EAD, and its fraction PF and CF produced significant anti-nociceptive effect in acetic acid-induced writhing animals.

Formalin-induced pain model is inclusive of two distinctive phases indicating different types of pain (Hunskaar, 1987). The early phase being neurogenic phase is probably a direct result of stimulation in the paw and reflects centrally mediated pain with release of substance P while later phase (inflammatory phase) involves the release of histamine, serotonin, bradykinin and prostaglandins (Shibata *et al*., 1989). In the present study it was found that EAD (400mg/kg, *p.o*.), PF (100 and 200 mg/kg, *p.o*.) and CF (100 and 200 mg/kg, *p.o*.) were potent enough to block both phases of the formalin response but the effect was more prominent in the second phase. Considering the inhibitory property of EAD and its fractions on the second phase of formalin, we suggest that its anti-nociceptive activity is due to an anti-inflammatory action.

The tail flick response is a result of spinal reflex, which is inflected by supraspinal inhibitory mechanism which exhibits the central mechanism of anti-nociceptive

activity (Sengar *et al*., 2015). The study demonstrated that EAD and PF increase the tail flicking latency after the 60 and 90 min of treatment and produce the significant activity. Therefore, EAD and PF might possess centrally and peripherally mediated anti-nociceptive properties while CF act on peripheral mediated analgesic activity.

The anti-inflammatory activity of the extract and fractions were investigated in carrageenan-induced paw edema and cotton pellets induced granuloma in rats. Carrageenan-induced rat paw edema is a sensitive model for acute inflammation and useful to investigate the orally active anti-inflammatory agents (Sofidiya *et al*., 2014). Carrageenan induced edema constitutes of two phase, the first phase is mediated by release of histamine and serotonin while the late phase is associated with the neutrophil infiltration, eicosanoid release, production of free radicals and also release of other neutrophil derived mediators (Arawwawala *et al*., 2012). EAD, PF, and CF significantly inhibited the paw edema in late phase. Thus, the activity of extract and fractions may be accredited to its ability to inhibit the release of pro-inflammatory mediators mainly, prostaglandin. The cotton pellet induced granuloma model was used to investigate the proliferative phase of inflammation and can serve as a sub-chronic inflammatory model. The adsorbed fluid by the pellet acutely influences the wet weight of the granuloma, whereas amount of granulomatous tissue correlates with the dry weight of the pellets (Olajide *et al*., 1999). The EAD, PF, and CF produced a significant decrease in granuloma formation that reflected its ability to reduce elevated level of fibroblasts as well as to synthesize collagen with mucopolysaccharide, which are natural proliferative actions of granulation tissue formation (Panthong *et al*., 2003).

FCA induced arthritis model is characterized by infiltration of the synovial membrane and associated with destruction of the joints (Shinde *et al*., 1999). Paw swelling and arthritic scores are indicative measures to determine anti-arthritic activity of any drug. Here, EAD, PF, and CF significantly decrease both the indexes as compared to control group in a dose-dependent manner.

Haemoglobin and RBCs decrease in RA due to reduced bone marrow erythropoietin response and demolition of premature RBCs (Patil *et al*., 2011). Increased synthesis of endogenous protein such as fibrinogen, α and β globulin and IL-1β mediated increase in the respective colony stimulating factor accounts for increase in the level of ESR and WBCs count. Hence these parameters form key biomarkers that are regulated during inflammation, stress and cell necrosis (Hu *et al*., 2005). In this study, treatment with EAD, PF, and CF in arthritic rats significantly increased the level of Haemoglobin and RBCs whereas it decreased the level of ESR and WBCs which may support its anti-inflammatory potential.

Thymus and spleen are two vital organs involved in immune response and their relative weights are used as a primary indicator to evaluate the immune regulatory activity. Spleen plays a key role in preventing infection and acting as a first line of defence against invading pathogens by detecting the damaged blood cells and eradicates antigens by phagocytosis (Zheng *et al*., 2014; Huang *et al*., 2008). The primary role of thymus in an organism is processed immature precursor T-lymphocytes into the mature immune competent T-cells of the medulla (Vos *et al*., 1998). Curcumin, EAD, and PF at higher dose decrease the spleen index while no effect was observed on thymus index as compared to control group. Therefore, EAD,

and PF showed their beneficial effect on RA which confirms that they exhibit anti-arthritic activity by immune-suppressant mechanism.

TNF-α, IL-6, and IL-1 are pivotal mediators of cell migration and inflammation in RA in which TNF-α have potential to degrade cartilage and bone while IL-1β is abundantly found in the synovial membrane may be responsible for joint inflammation as well as systemic signs of inflammation and IL-6 have critical role in the pathogenesis of RA, which is responsible for bone resorption and also contributes to the synthesis of auto antibodies such as rheumatoid factor (Alvarez *et al*., 2009). EAD, PF, and CF treated rats markedly decrease the cytokines level in serum and control the progression of RA.

A change in RA condition was analyzed by radiography which specifies the severity of disease. In AIA group ankylosis, osteophytosis, bone erosion, and soft tissue swelling were observed in developed stage of arthritis. Moreover, protective effect of EAD, PF, and CF in progression of joint damage was confirmed by radiological study and they effectively reduced the disease progression in arthritic rats.

Histopathology study provides a perceptible morphological distinctiveness and identified the ability of the bones to re-form upon treatment with EAD, PF, and CF. The EAD 400 mg/kg dose exhibited potential pharmacological effect and have the ability to suppress inflammation, synovitis and protect the joint. HPTLC fingerprint analysis of EAD, PF, and CF confirmed the presence of ursolic acid and lupeol and both compounds possess anti-inflammatory and anti-arthritic activity which may impart anti-arthritic effect (Kang *et al*., 2008; Agarwal and Rangari, 2003).

EVALUATION OF ANTI-UROLITHIC ACTIVITY

MATERIAL AND METHODS

Ethylene glycol induced urolithiasis model

Ethylene glycol (EG) induced hyperoxaluria method was used to assess the anti-urolithic activity in male albino rats. Urolithiasis was induced by administration of 0.75% (v/v) of ethylene glycol (EG). Animals were divided into twelve groups, each containing six animals. Group I animals served as normal control and maintained on regular laboratory diet and water *ad libitum*. Group II to XII animals was treated with 0.75% ethylene glycol (EG) in drinking water to induce calcium oxalate crystals up to 28 days. All treatments were given once daily by oral route (Ramachandran *et al*., 2011).

Group II: Disease control.

Group III: animals received standard anti-lithiatic drug cystone (750 mg/kg, *p.o*.).

Group IV to VI: animals treated with EAD (100, 200 and 400 mg/kg, *p.o*.).

Group VII, VIII: animals treated with PF (50 and 100 mg/kg, *p.o*.).

Group IX, X: animals treated with CF (50 and 100 mg/kg, *p.o*.).

Group XI, XII: Animals treated with BF (50 and 100 mg/kg, *p.o*.).

Collection and analysis of urine

All animals were kept in metallic cage separately and urine samples of 24h were collected on 28^{th} day and a drop of concentrated hydrochloric acid was added to the urine sample before being stored at 4°C. Animals had free admittance to drinking water during the urine collection period. The collected urine sample was analyzed for urine volume, calcium, oxalate, phosphate, total protein, blood urea nitrogen (BUN),

uric acid and creatinine content by using the commercially available kit (Atmani *et al*., 2004).

Serum analysis

On 29th day blood was collected from retro-orbital plexus and allowed to cool centrifuge with 3000 rpm at 4°C for 15 min. Serum was recovered and frozen at -20ºC and analyzed for creatinine, BUN, total protein and uric acid.

Kidney homogenate analysis

Both kidneys of each animal were removed and cleaned off the extraneous tissue then preserved in 10 % formalin. Isolated kidneys were dried at 80°C in hot air oven. 100 mg of dried tissue was boiled in 1N hydrochloric acid and centrifuged at 2000 xg for 10 min to prepare homogenate. The calcium, oxalate, and phosphate content were analyzed in the supernatant (Selvam *et al*., 2001).

Polarized microscopy of urine

Urine samples were collected in the morning for microscopical study. A fresh urine sample (1 mL) from each group was collected in centrifuge tube then centrifuged at 2500 rpm for 5 minutes. After that 0.1 mL of sediment was taken on the glass slide covered with cover slip and observed using Nikon digital microscope (Eclipse 200) at 10x magnification (Vyas *et al*., 2012).

Histopathological studies

At the end of experiment, all rats were sacrificed by euthanasia and remove the kidney then fixed in 10 % formalin and tissue were then embedded in paraffin blocks for preparing sections (1-3 µm) which were then stained using hematoxylin and eosin

dye. Pathological changes were observed through photographed by using a Nikon digital microscope (Eclipse 200) at 10x magnification (Onyenmechi *et al*., 2002).

Statistical analysis

Results are expressed as mean ± S.E.M. with n = 6 per group. Statistical significance was determined using one-way analysis of variance (ANOVA) followed by Dunnett's post-test for multiple group comparison. The difference was considered to be significant when $p < 0.05$.

RESULTS

Estimation of urine volume

Increased urine volume indicates the diuretic property by diluting the urinary electrolyte concentration. Cystone (750 mg/kg, *p.o*), EAD 400 mg/kg., *p.o*. and all fractions at high doses were significantly ($p<0.001$) increases the urine output at 28 days while only PF and CF at 100 mg/kg., *p.o*. showed significant action at 14 days (Table 24).

Table 24: Effects of extract and fractions on urine output.

Groups	Urine output in mL at 14th day	Urine output (mL) at 28th day
Normal control	7.5 ± 0.4	10.8 ± 0.91
Disease control	14.38 ± 0.51	15.5 ± 0.34
Cystone (750 mg/kg)	14.08 ± 0.49	22.13 ± 0.76[c]
EAD (100 mg/kg)	13.83 ± 0.45	15.8 ± 0.67
EAD (200 mg/kg)	14.4 ± 0.38	18.71 ± 0.41[a]
EAD (400 mg/kg)	16.15 ± 0.5	19.93 ± 0.91[c]
PF (50 mg/kg)	13.98 ± 0.36	14.45 ± 0.49
PF (100 mg/kg)	18.06 ± 0.71[c]	22.85 ± 0.67[c]
CF (50 mg/kg)	14.21 ± 0.6	16.28 ± 0.5
CF (100 mg/kg)	16.98 ± 0.64[b]	22 ± 0.56[c]
BF (50 mg/kg)	12.7 ± 0.63	16.4 ± 0.83
BF (100 mg/kg)	16.45 ± 0.48	21.98 ± 0.66[c]

All statistical data were expressed in mean ± SEM and determined by one way ANOVA followed by Dunnett's post test.
[a] $p<0.05$ statistically significant as compared to disease control.
[b] $p<0.01$ statistically significant as compared to disease control.
[c] $p<0.001$ statistically significant as compared to disease control.

Estimation of calcium, oxalate and phosphate in urine and kidney homogenate

The oxalate is the main product of EG metabolism which is the important initiative factor for lithiasis, so animals of group II showed greater excretion of oxalate. However, Extract and PF in dose dependent manner lower the urinary oxalate level while CF and BF showed significant action ($p<0.01$) only at higher dose (100 mg/kg., *p.o.*). Level of oxalate in kidney homogenate sample was decreased by all the dose of EAD, PF and CF in dose dependent manner, while BF 50 mg/kg., *p.o.* did not produce any significant action on oxalate level as compared to disease control group. Urolithic rats showed increase in calcium output while both extract and fractions treated group showed significant action ($p<0.001$) in dose dependent manner on calcium level in urine and kidney homogenate samples while BF 100 mg/kg., *p.o.* in kidney homogenate showed lesser action ($p<0.05$) as compare to lithiasis induced rats. Similarly, phosphate excretion was also increased in stone forming animals that favours the nucleation and precipitation of calcium oxalate. Administration of EAD and PF significantly decreased the level of phosphate in kidney homogenate and urine samples while CF and BF at higher doses showed significant action on urine samples only (Table 25).

Table 25: Effect of EAD, PF, CF and BF on oxalate, calcium and phosphate level in both urine and kidney homogenate in rats.

Parameter	Disease control	Cystone	EAD 100	EAD 200	EAD 400	PF 50	PF 100	CF 50	CF100	BF 50	BF 100
Urine (mg/24 h)											
Oxalate	2.37±0.07	0.83±0.06[c]	1.73±0.07[c]	1.32±0.12[c]	0.91±0.06[c]	1.78±0.12[b]	1.22±0.11[c]	2.2±0.07	1.76±0.12[b]	2.06±0.2	1.82±0.11[b]
Calcium	4.35±0.1	1.64±0.08[c]	2.85±0.11[c]	2.19±0.1[c]	1.96±0.12[c]	2.85±0.1[c]	2.22±0.12[c]	2.84± 0.12[c]	2.44±0.22[c]	2.96± 0.08[c]	2.19±0.11[c]
Phosphate	3.26±0.11	1.21±0.11[c]	3.1±0.11	2.04±0.09[c]	1.32±0.12[c]	1.7±0.17[c]	1.4±0.11[c]	2.94±0.14	2.29±0.14[c]	2.83±0.12	2.32±0.17[c]
Kidney homogenate (mg/g)											
Oxalate	2.48±0.18	0.94±0.09[c]	2.01±0.14[a]	1.51±0.17[c]	0.99±0.09[c]	1.42±0.13[c]	1.13±0.05[c]	1.99± 0.08[a]	1.61±0.09[c]	2.04±0.14	1.79±0.14[c]
Calcium	4.96±0.08	3.55±0.13[c]	4.02±0.09[c]	3.83±0.12[c]	3.7±0.19[c]	4.12±0.15[c]	3.97±0.18[c]	4.08± 0.17[c]	4.04±0.14[c]	4.13± 0.09[c]	4.4±0.08[a]
Phosphate	1.83±0.15	0.84±0.09[c]	1.35±0.08[a]	0.99±0.17[c]	0.86±0.08[c]	1.14±0.14[c]	1.07±0.09[c]	1.51±0.08	1.47±0.13	1.55±0.11	1.38±0.09[a]

All statistical data were expressed in mean ± SEM and determined by one way ANOVA followed by Dunnett's post test.
[a] $p < 0.05$ statistically significant as compared to disease control.
[b] $p < 0.01$ statistically significant as compared to disease control.
[c] $p < 0.001$ statistically significant as compared to disease control.

Estimation of creatinine, BUN, uric acid and protein in urine and serum samples

Crystal formation in renal tissue leads to obstruction in outflow of urine that reduces the glomerular filtration and causes an accumulation of waste product in blood like creatinine, BUN and uric acid. Marked renal damage was also seen in lithogenic rats by the elevated level of these waste products in serum. However, administration of EAD, PF, CF and BF in dose dependent manner significantly ($p<0.001$) decreased the level of BUN and creatinine in both urine and serum samples as compared to untreated EG group except CF 50 mg/kg., *p.o.*, which showed significant action ($p<0.05$) on serum creatinine level. Similarly, level of uric acid in serum was decreased by both extract and fractions in dose dependent manner while in urine sample only EAD 400 mg/kg., *p.o*. ($p<0.01$), EAD 200 and PF 100 mg/kg., *p.o*. ($p<0.05$) showed significant action on level of uric acid (Table 26).

Estimation of Protein in urine and serum samples

As shown in figure 40, both extract and fractions at all doses inhibits the loss of protein in serum of rats while in urine only extract and PF showed significant action while in CF and BF only CF 100 mg/kg., *p.o*. showed action ($p<0.05$) on total protein loss.

Table 26: Effect of extract and fractions on creatinine, BUN and uric acid level in serum and urine samples.

Parameter	Disease control	Cystone	EAD 100	EAD 200	EAD 400	PF 50	PF 100	CF 50	CF100	BF 50	BF 100
Urine (mg/24 h)											
Creatinine	4.06±0.15	1.87±0.08[c]	3±0.11[c]	2.47±0.06[c]	1.85±0.25[c]	2.96±0.09[c]	2.4±0.13[c]	2.91±0.07[c]	2.7±0.1[c]	2.76±0.16[c]	2.77±0.2[c]
BUN	23.34±0.68	10.41±0.57[c]	16.83±0.61[c]	14.21±0.77[c]	13.27±0.43[c]	17.57±0.8[c]	14.2±0.63[c]	16.02±0.84[c]	13.54±0.41[c]	16.81±0.6[c]	15.31±0.82[c]
Uric acid	2.1±0.08	1.41±0.04[c]	1.89±0.08	1.72±0.11[a]	1.66±0.06[b]	1.77±0.07	1.72±0.11[a]	1.99±0.07	1.88±0.1	1.9±0.09	1.82±0.13
Serum (mg/dL)											
Creatinine	3.05±0.19	0.37±0.05[c]	1.87±0.11[c]	1.37±0.12[c]	0.83±0.06[c]	2.26±0.16[b]	1.76±0.2[c]	2.46±0.12[a]	2.15±0.1[c]	2.17±0.19[c]	2.16±0.18[c]
BUN	25.66±0.79	11.67±0.59[c]	17.81±1.04[c]	16.04±1.23[c]	13.81±0.64[c]	16.02±0.64[c]	16.18±0.98[c]	15.34±0.63[c]	14.75±0.82[c]	15.2±0.62[c]	13.15±0.71[c]
Uric acid	3.13±0.17	1.52±0.18[c]	2.15±0.14[c]	2.08±0.16[c]	1.99±0.09[c]	1.95±0.2[c]	1.76±0.09[c]	2.27±0.16[b]	2.11±0.15[c]	2.24±0.16[b]	2.25±0.19[c]

All statistical data were expressed in mean ± SEM and determined by one way ANOVA followed by Dunnett's post test.
[a] $p < 0.05$ statistically significant as compared to disease control.
[b] $p < 0.01$ statistically significant as compared to disease control.
[c] $p < 0.001$ statistically significant as compared to disease control.

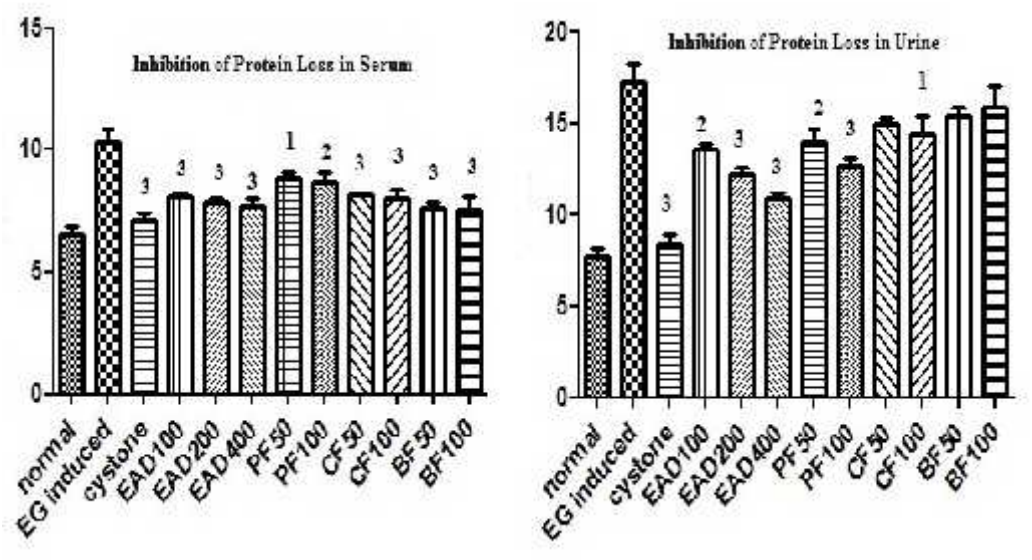

Figure 40: Effects of extract and fractions on inhibition of protein loss in serum and urine.

All statistical data was expressed in mean ± SEM and determined by one way ANOVA followed by Dunnett's post test.
1- $p < 0.05$ statistically significant as compared to disease control.
2- $p < 0.01$ statistically significant as compared to disease control.
3- $p < 0.001$ statistically significant as compared to disease control.

Polarized microscopy of urine

Calcium oxalate crystals were present as monohydrate (COM) and dehydrate (COD) forms. COM crystals were generally in the form of biconcave ovals while COD were bipyramidal shape. Rat urine microscopy revealed that COM was more abundant in urine samples and also the size of crystals was observed comparatively larger in untreated urolithiatic animals. Whereas, number and size of calcium oxalate crystals were observed lesser after treatment with the different doses of extract, fractions and cystone (750 mg/kg., *p.o.*) as shown in figure 41.

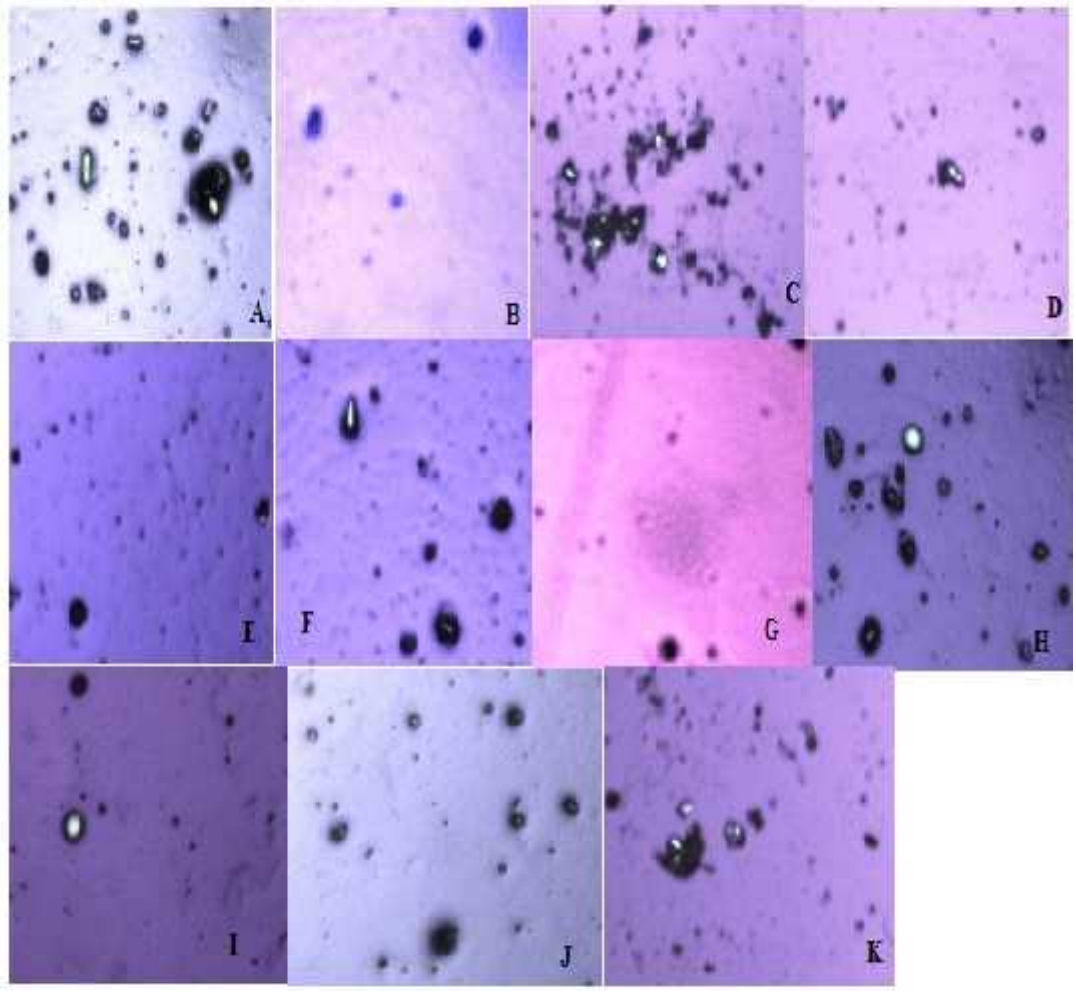

Figure 41: Polarized microscopy of rat urine. (A) Lithiasis induced. (B) Cystone (750 mg/kg, *p.o.*) treated rat. (C-E) EAD (100, 200 and 400 mg/kg, *p.o.*) treated. (F-G) PF 50 and 100 mg/kg treated. (H-I) CF 50 and 100 mg/kg treated. (J-K) BF 50 and 100 mg/kg treated rats.

Histopathological study

Histopathological evaluation revealed the deposition of calcium oxalate crystals in different groups as well as some other histopathological finding was observed such as dilation of proximal tubules, tubular epithelial necrosis and interstitial inflammation. As shown in figure 42, normal control group (fig. 3L) possessed intact nephron structure with normal glomerular capsule and juxtaglomerular cells while in disease control group (fig. 3B) many calcium oxalate deposits and

dilation of proximal tubule was observed. Cystone (750 mg/kg., *p.o.*), EAD (400 mg/kg., *p.o.*) and PF (100 mg/kg., *p.o.*) treated rats significantly prevented the rupture of kidney cells as compared to disease control group and close to normal architecture of kidney section while CF and BF showed less protection even at higher dose.

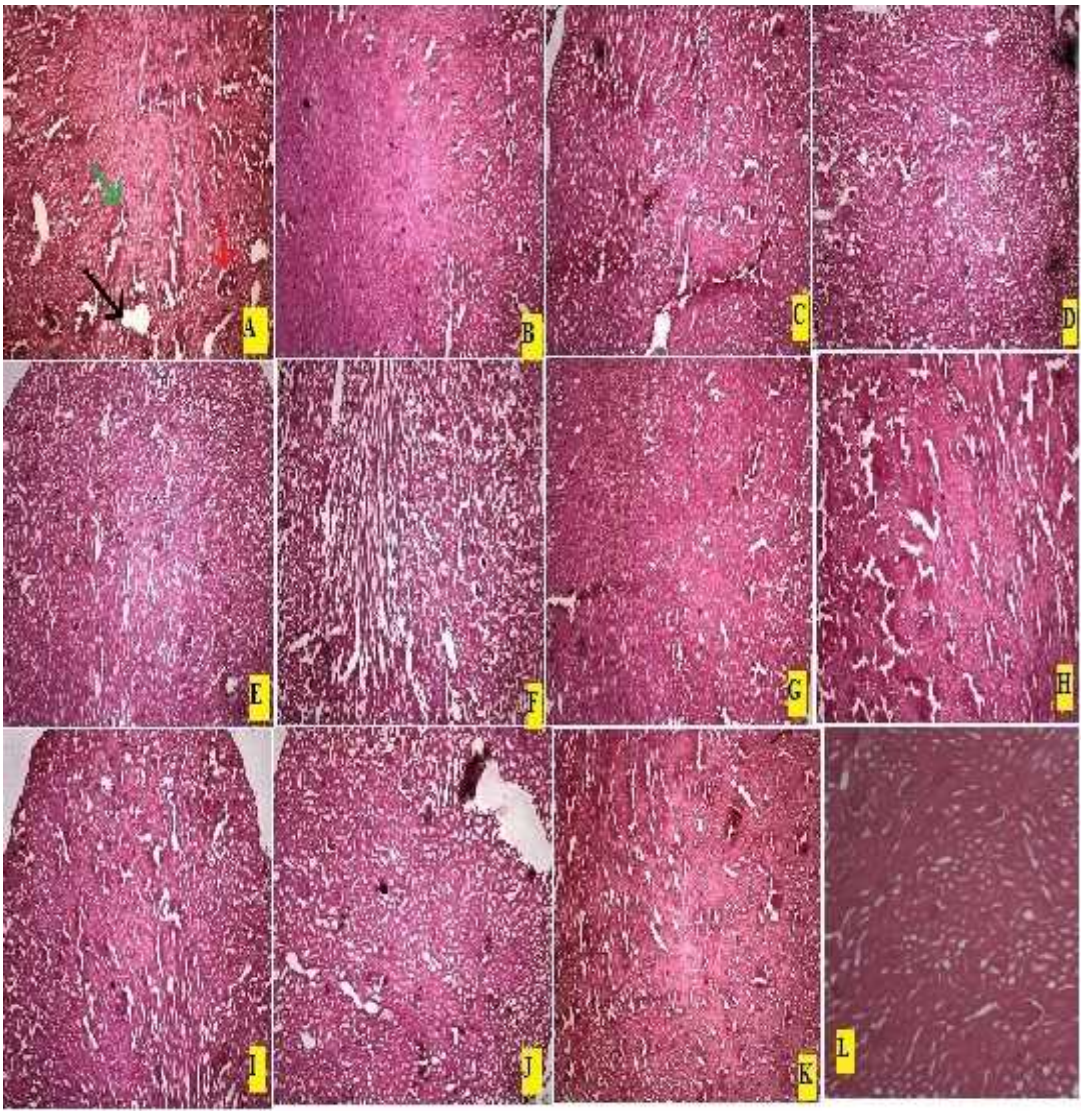

Figure 42: Histology of rat kidney. (A) Lithiasis induced. (B) Cystone (750 mg/kg, *p.o.*) treated rat. (C-E) EAD (100, 200 and 400 mg/kg, *p.o.*) treated. (F-G) PF 50 and 100 mg/kg, *p.o.* treated. (H-I) CF 50 and 100 mg/kg, *p.o.* treated. (J-K) BF 50 and 100 mg/kg, *p.o.* treated rats. (Indication of arrow) Black arrow indicates the dilation of tubule, Red arrow indicates the ruptured glomeruli and Green arrow indicates the hemorrhage.

DISCUSSION

Anti-urolithic activity of root extract and bioactive fractions

According to Khan (1997), urolithiasis can be induced in rats by using a variety of agents such as ethylene glycol, sodium oxalate, ammonium oxalate, hydroxyl-L-proline and glycolic acid but Kidney being the principal target for EG-induced toxicity. Thus, the present study involved the induction of hyperoxaluria in male albino rats with the oral administration of ethylene glycol which stimulates the nidus formation, calcium oxalate saturation, crystal aggregation and retention within kidney tubules (Vyas and Argal, 2012). Male rats were selected for the study due to the resemblance of urinary system with the human and also earlier studies have shown that frequency of stone deposition was significantly high in male as compared to female rats (Aggarwal *et al*., 2012). This study demonstrates that ethanolic root extract and petroleum ether fraction showed significant anti-urolithic activity in dose-dependent manner as compared to standard drug cystone. EG is rapidly absorbed and metabolized in the liver into glycolic acid via alcohol dehydrogenase/ aldehyde dehydrogenase then glycolic acid is further oxidized to glyoxylic acid. Glyoxylic acid is again oxidized into oxalic acid by glycolate oxidase. High doses of EG (>2,500 mg/kg, body weight) through an oral bolus, cause the saturation dependent accumulation of glycolic acid in the plasma so glycolate oxidase is one of the rate-limiting enzymes in the metabolism of EG (Green *et al*., 2005). Thus oxalate is the main product of EG metabolism which is the important initiative factor for urolithiasis.

Calcium and phosphate play a vital role in renal calculogenesis and increased level of calcium and phosphate in urine could be due to defective tubular re-absorption in the kidney (Varalakshmi *et al*., 1990). Urinary supersaturation is

also considered to be one of the causative factors in calculogenesis. Similarly, phosphate excretion was also increased in stone forming animals that favour the nucleation and precipitation of calcium oxalate (Bahuguna *et al*., 2009). In the present study, increased urinary phosphate excretion along with the oxalate stress seems to provide an environment appropriate for stone formation by forming calcium phosphate crystal, which epitaxially induces calcium oxalate depositions (Low and Stoller, 1997).

However, treatments with ethanolic root extract and their fractions lower the levels of calcium as well as oxalate in urine; this is possibly by increasing/ restoring the tubular re-absorption in the renal tubules.

Administration of EG causes the development of persistent crystalluria in all rats, due to agglomeration of particles. It is a crucial step in urinary stone formation because agglomerates getting trapped in renal tubules and retain in the kidney (Atmani *et al*., 2003). Crystal formation in renal tissue leads to obstruction in the outflow of urine that reduces the glomerular filtration and causes an accumulation of waste product in blood like creatinine, BUN, and uric acid (Khatib *et al*., 2010). However, extract and fractions at all doses decreased the concentration of these waste products and inhibits the loss of protein in serum and urine of rats. This data clearly support that the ethanolic extract of root of *A. dichotoma* and their bioactive fractions can reduce supersaturation of urine with calculogenic ions such as calcium and oxalate while decreased urinary creatinine reflects improved renal function. According to Selvam *et al*. (2001), uric acid interfere the solubility of calcium oxalate by binding with it and decreased the inhibitory activity of glycosaminoglycans. The predominance of uric acid crystals in calcium oxalate stones improve the capability of calcium oxalate binding with uric acid binding

protein and modulates its crystallization. Treatment of EAD and PF reduced the excretion of uric acid and thereby reduces the risk of stone formation.

From various reports, it was also observed that calcium oxalate crystals cause direct oxidative stress which damages the glomerulus and tubules due to the generation of reactive oxygen species. From the histopathological study of kidney samples, it was also confirmed that EG damage the kidney. Lipid peroxidation represents oxidative tissue damage due to hydrogen peroxide (H_2O_2) and superoxide anion (O_2) and hydroxyl radicals (OH), which causes the structural alteration of the membrane due to loss of essential fatty acid with the formation of cytosolic aldehydes and peroxide products (Balakrishnan *et al*., 2011). Catalase enzyme also regulates the H_2O_2 level which can lead to hydroxyl radical surplus through the metal catalyzed fenton (Fe/Cu) and Habber- Weiss reactions. The release of xanthine oxidase during uric acid formation is the main factor for release of hydrogen peroxide. Catalase is the only enzyme that regulates the hydroxyl radical formation and its decreased activity in urolithiasis may cause excessive accumulation of H_2O_2 in the kidney. So, elevated antioxidant enzymes in the kidney can remunerate the oxidative stress (Parmar *et al*., 2012). Thus *in-vitro* and *in-vivo* antioxidant activity have been also studied in our previous study which further act as a supporting evidence that extract would also be producing the protective effect on urolithiasis due to its antioxidant potential (Pandey *et al*., 2015; Pandey *et al*., 2017).

Various phytoconstituents like lupeol, ursolic acid, quercetin, and β-sitosterol have been identified in HPTLC study of extract and fraction in which quantity of lupeol was high in the ethanolic extract as compared to other bioactive fractions. According to Anand *et al*., (1994) lupeol was acting as anti-urolithic agent and

helps in the removal of lithogenic substances and prevent their deposition in the urinary bladder. Thus, lupeol may contribute in anti-urolithic activity. The present study demonstrates that mainly EAD and PF showed significant anti-urolithic activity in dose-dependent manner while CF and BF possessed lesser action as compared the standard drug cystone. The mechanism underlying this effect is still unknown but the possible mode of action includes increased diuresis, lowering the urinary concentration of stone forming salts and prevent the super-saturation of the crystallizing salts, decreasing the crystal size and restore normal kidney architecture by reducing the renal tissue injury. Further, studies are required to illuminate the chemical constituents and mechanism liable for pharmacological activities.