ANTI-ARTHRTIC EFFECTS OF ADHATODA VASICA, EMBLICA OFFICINALIS AND CLITORIA TERNATEA VIA DOWN REGULATION OF SYNOVIAL MMP-2 ACTIVITY: A COMPARATIVE STUDY

Rana Adhikary¹ and Biswadev Bishayi¹*¹

Department of Physiology, Immunology and Microbiology Laboratory. University of Calcutta, University Colleges of Science and Technology; 92, A.P.C. Road, Calcutta-700 009, West Bengal, India.

*Corresponding Author: Biswadev Bishayi
Department of Physiology, Immunology and Microbiology Laboratory. University of Calcutta, University Colleges of Science and Technology; 92, A.P.C. Road, Calcutta-700 009, West Bengal, India.

ABSTRACT
Antiarthritic activity of methanolic extracts of Adhatoda vasica leaves (AVE), Emblica officinalis fruits (EOE) and Clitoria ternatea flower petals (CTE) on arthritic mice. Plant materials were tested for total phenolic contents (TPC), total flavonoid contents (TFC), and DPPH radical scavenging activities by simple biochemical analysis. Arthritis was induced by injecting type II collagen in CFA and IFA; and the arthritic mice were administered with 100, 200 and 50 mg.kg body weight doses of AVE, EOE and CTE respectively at alternative days upto day 24. Antioxidant status, generation of superoxide anions and NO, as well as expression of TLR-2, iNOS, COX-2 and MMP-2 were assessed from synovial tissue. Levels of different cytokines and CRP were measured from both synovial tissue as well as serum. Among the plant materials, EOE was found to have highest TPC, and CTE was found to have highest TFC and DPPH radical scavenging activity. Highest antiarthritic effect was shown by CTE, in comparison to AVE and EOE in terms of synovial radical scavenging, cytokine production and MMP-2 activity. This can also be positively correlated to highest flavonoid content of CTE in comparison to AVE and EOE in the present study. It can be concluded that C. ternatea flower petals possess potential antiarthritic activity over that of other plant materials in this study which is directly correlated to its highest flavonoid content. Therefore further studies on flavonoids present in flower petals of C. ternatea are warranted for development of herbal antiarthritic drugs.

KEYWORDS: Rheumatoid arthritis, Flavonoids, Cytokines, Antioxidants.

INTRODUCTION
Rheumatoid arthritis (RA) is a very common form of arthritis that affects 1-2% of the world’s total population. The anti-inflammatory drugs introduced in last few decades during arthritis such as naproxen, oxaprin, piroxicam, salazate, tolmetin, few blockers of COX-2, were reported to possess potential side effects (Hinson, 2010). Medicinal plants are being used for treating arthritis for several thousands of years in eastern countries and are now getting high privilege from the aspects of biosafety. Among different medicinal plants Emblica officinalis (Family: Euphorbiaceae, commonly known as emblica in English) is well known for its antiarthritic effect both in contemporary as well as in traditional medicinal systems (Bhandari and Kamdod, 2012). Adhatoda vasica (Family: Acanthaceae, commonly known as Malabar nut in English) is well known to possess potential antiarthritic effect; while comparatively less reports on antiarthritic effect from Clitoria ternatea Linn (Family: Fabaceae, commonly known as butterfly pea plant in English) exists. The antioxidant potential from these plants in turn relies on the many biologically active components i.e., polyphenols, mainly flavonoids, from these plants.

Oxidative stress into the joint synovium during RA is chiefly contributed by the polymorphonuclear (PMN) cells that infiltrate to induce superoxide, nitric oxides production (Filippin, 2008). Synovial macrophages are contributors of local production of inflammatory cytokines and chemokines that shows pleiotropic effects to mount the symptoms of the disease (Bondeson, 2008). Synovial fibroblast has been considered as chief source of MMPs, collagenases and cathepsins in the degrading joints (Bartok and Firestein, 2010).

Free radicals in conjunction with pro-inflammatory cytokines degrade components of synovial fluid and also depolymerize hyaluronic acid, which leads to a loss of viscosity in the synovial fluid and inactivation of anti-proteinase enzymes resulting in bone resorption. Bone resorption induced by O2- is amplified in the presence of IL-1β (Garrett et al., 1990). It has been verified experimentally that over production of ROS accelerates
damage to cartilage and osteoclast activation (Tak et al., 2000). Activation of NF-κB by the RNS intermediates generate peroxynitrites (•ONOO−) that may interfere redox balance of glutathione (Mclmnes, 1996). TNFα also plays important role in phosphorylation of kinase kappa inhibitor (IκBK), allowing nuclear translocation of NF-κB dimers to stimulate NADPH oxidase, there by increasing •O2− generation (Moynagh PN, 2005).

In this study anti-arthritis effect of methanolic extracts of A. vasica leaves (AVE), E. officinalis fruits (EOE) and C. ternatea flower petal (CTE) were compared using multiple oral doses in the clinical regimen at every alternative day following induction of arthritis (CIA) in mice; and the clinical score of arthritis, as well as joint swelling were evaluated, followed by estimation of synovial and systemic levels of tumor necrosis factor (TNF)-α, interferon (IFN)-γ, interleukins (IL)-6, -10; as well as synovial chemokines like IL-8 and monocyte chemoattractant protein (MCP)-1, and C-reactive protein (CRP), along with measures of synovial free radical generation (•O2− and NO production), redox balance (reduced glutathione [GSH]; lipid peroxides [LPO]), and activities of superoxide dismutase (SOD) and catalase (CAT) enzymes. Synovial expression of TLR2, iNOS, and COX-2 expressions by immunoblot assay as well as zymographic analysis of MMP-2 activity were also carried out.

METHODS AND MATERIALS
Preparation of plant extracts
Taxonomical identification of the A. vasica, E. officinalis and C. ternatea were done by the Central National Herbarium (CNH), Botanical Survey of India. Plant materials were dried under shade, and ten grams of finely ground A. vasica leaves, E. officinalis fruit pulp, and C. ternatea flower petals were separately soaked into 30 ml methanol at 30°C for 12h with shaking. The methanol was then allowed to evaporate completely under sterile conditions; it was repeated thrice. The final residues were then dissolved into 10 ml methanol and filtered through Whatman No.1 filter paper. Each filtrate was centrifuged at 2000 rpm for 10 minutes; the supernatant was collected and air-dried under sterile conditions. The final yields were 7.1%, 12.15% and 6.5% for A. vasica leaves, E. officinalis fruits and C. ternatea flower petals.

Initially the extracts were suspended into sterile saline were administered at a dose regimen of 250, 500, 1000, 2000, 4000, and 8000 mg/kg body weight for AVE, EOE as well as CTE for acute oral toxicity study. The results showed that dose upto 2000, 4000 and 1000 mg/kg for AVE, EOE and CTE did not cause death or morbidity for up to 72 hr, and therefore 1/20th of these doses, i.e., 100 mg AVE/kg, 200 mg EOE/kg and 50 mg CTE/kg body weight doses were considered safe for oral administration in this study (Oliveira et al., 2008).

Animals
Male Swiss-Albino mice (20-22 g, 3-4 weeks of age) were procured from Chittaranjan National Cancer Institute (CNCI), Kolkata, India for use in this study. All animals were housed in separate polystyrene cages in pathogen-free facilities maintained at 25 ±2 °C, with 50-60% relative humidity, and 12 h light: dark cycle. All mice had ad libitum access to normal laboratory diet as recommended for mice, by the National Center for Laboratory Animal Sciences and filtered tap water. All experiments involving animals were conducted according to the protocols approved by Department of Animal Ethical Committee, Department of Physiology, University of Calcutta (as it is affiliated to the University of Calcutta is called Institutional Animal Ethics Committee), under supervision of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines (Registration No. IAEC/IV/Proposal/BB-2/2014, dated August 26, 2014), Ministry of Environment and Forest, Government of India.

Quantitation of total phenolic content, total flavonoid content, and DPPH radical scavenging activity of the extracts
The total phenolic contents in methanolic extracts of A. vasica leaves, E. officinalis fruit pulp, and C. ternatea flower petals were determined by using the Folin-Ciocalteu assay (Singleton and Rossi, 1965). An aliquot (1 ml) of extracts or standard solution of Gallic acid (20, 40, 60, 80 and 100 μg/ml) was added to 25 ml of volumetric flask, containing 9 ml of distilled water. Reagent blank using distilled water was prepared. One ml of Folin-Ciocalteu phenol reagent was added to the mixture and shaken. After 5 minutes 10 ml of 7% Na2CO3 solution was added to the mixture. After incubation for 90 minutes at room temperature, the absorbance against the reagent blank was determined at 765 nm with an UV-Visible spectrophotometer. The amount of flavonoid was calculated from linear regression equation obtained from the quercetin calibration curve. The phenolic content was calculated as mean±SD (n=3) and expressed as mg/g of Gallic acid equivalents (GAE) of the extracts.

Total flavonoid contents in methanolic extracts of A. vasica leaves, E. officinalis fruit pulp, and C. ternatea flower petals were estimated by aluminium chloride colorimetric method, using quercetin-3β-D-glucoside (purchased from Sigma, MO) as standard to constitute the calibration curve (Madaan et al., 2011). Briefly, quercetin-3β-D-glucoside was dissolved in 80% ethanol and then diluted to 20, 40, 60, 80 and 100 μg/ml. The diluted standard solutions of quercetin or plant extracts (500 μl) of different concentrations were separately mixed with 1.5 ml of 95% ethanol, 100 μl of 10% aluminium chloride, 100 μl of 1 M potassium acetate and 2.8 ml of distilled water in a test tube. The test tubes were incubated for 30 min at room temperature to complete the reaction. The absorbance of the reaction mixture was measured at 415 nm with double beam UV-Vis spectrophotometer against blank (containing all the
The amount of flavonoid was calculated from linear regression equation obtained from the quercetin calibration curve. The flavonoid content was calculated as mean±SD (n=3) and expressed as mg/g of QG equivalent (QGE) of the extracts.

To evaluate the antioxidant property of methanolic extracts of *A. vasica* leaves, *E. officinalis* fruit pulps, and *C. ternatea* flower petals DPPH free radical scavenging method developed by Blois was used (Zheleva-Dimitrova et al., 2010). For this purpose, the antioxidant activity of the extracts was compared with the natural antioxidant, ascorbic acid. Stock solution of DPPH assay was prepared by dissolving 24 mg DPPH with 100 ml methanol and then stored at -20°C for further use. The working solution was prepared by adding 10 ml stock solution to 45 ml methanol to obtain an absorbance of 1.10±0.02 units at 515 nm. A 150 μl (1 mg/ml) volume of the extracts were added to react with 2850 μl of the DPPH solution for 30 minutes in the dark and absorbance was measured at 515 nm. The solution without any extract with DPPH and methanol was used as control. Ascorbic acid was used as standard. Inhibition of DPPH free radical in percentage was calculated by the formula:

\[
\text{Inhibition} \% = \left( \frac{A_{\text{control}} - A_{\text{test}}}{A_{\text{control}}} \right) \times 100
\]

Where, Abs\text{control} is the absorbance of DPPH radical in methanol; Abs\text{sample} is the absorbance of DPPH radical solution mixed with sample extract/standard.

### Experimental groups
Mice were randomly allocated into five different groups (n = 6/group): control (Con) group, collagen-induced arthritis (CIA) group, collagen-induced arthritis+AVE(100 mg/kg) (CIA+AVE) group, collagen-induced arthritis+EOE(200mg/kg) (CIA+EOE) group and collagen-induced arthritis +CTE(50mg/kg) (CIA+CTE) group.

### Preparation of type II collagen emulsion
Lyophilized bovine type II collagen (Sigma, St. Louis, MO) was dissolved in 0.05 M acetic acid solution at 2 mg/ml concentration. An equal volume of Freund's complete adjuvant (CFA) was then added and the solution was emulsified slowly. The booster dose was prepared by emulsifying bovine type II collagen with equal volume of Freund's incomplete adjuvant (IFA) (Brand et al., 2007).

### Immunization and induction of collagen-induced arthritis
Mice from all the six groups excepting control group were injected with 100 µl of type II collagen-CFA emulsion, and all the mice were maintained and provided normal laboratory diet for 20 days. At day 21 after the primary immunization, the mice were again immunized with 100 µl of type II collagen in IFA (Brand et al., 2007). The mice were then carefully monitored for onset of early signs of arthritis, i.e., redness/deformities/swelling in the joints and/or toes etc. (Fig. 1).

### Fig. 1: Study design.

The experimental schedule of total 45 days was divided into pre-treatment regimen (0-21 days after primary immunization with CII-CFA) and a therapeutic regimen (0-24 days after secondary immunization with CII-IFA). Mice from all the five experimental groups were treated with PBS, AVE, EOE or CTE respectively as shown at different time-points. Arthritic scorings and joint swellings were measured from the date of onset of first signs of arthritis. Blood and tissues for experimental use were collected from all the groups at day 24 at therapeutic regimen after euthanasia.

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Treatment of animals
Mice were randomly allocated into five groups (n = 6/group): control (CON), collagen induced arthritis group (CIA) only, AVE+collagen induced arthritis group (AVE+CIA), EOE+collagen induced arthritis group (EOE+CIA), and CTE+collagen induced arthritis group (CTE+CIA). After secondary immunization, AVE (100 mg/kg), EOE (200 mg/kg) or CTE (50 mg/kg) at a volume of 200 µl, dissolved into sterile PBS were administered per orum at every alternative days beginning from the date of onset of first signs of arthritis (i.e., day 23 after primary immunization or day 2 after secondary immunization) routinely up to day 45 of the experiment (i.e., 24 days post-secondary immunization). On day 45 post primary immunization, mice from all the groups were euthanized after ether anaesthesia.

Assessment of arthritic scores and joint swelling
Mice were monitored for signs of arthritis for which severity scores were derived as: 0 = no signs of arthritis; 1 = swelling/redness in only one joint; 2 = swelling/redness in more than one joint; 3 = swelling/redness in entire paw; 4 = severe swelling of entire paw with deformity and/or ankylosis. The macroscopic arthritic score of each mouse was presented as the sum of each score of the four limbs, with the maximum score being 16 for all four limbs. Swelling of the knee joint was evaluated with a dial calliper every other day beginning on day 21 after primary immunization (i.e. day 4 after secondary booster immunization). On day 24 post-secondary immunization (i.e. day 45 after primary immunization) mice were euthanized for analysis.

Collection of blood and synovial joint samples
Immediately after being euthanized, blood samples were obtained from each mouse by cardiac puncture. The blood samples were placed at 4°C for 45 min and then centrifuged at 3000 rpm for 5 min at 4°C. The serum was collected and aliquots were assessed for total protein using the Lowry protein estimation method; all remaining aliquots were stored at -20°C for later analysis (Lowry et al., 1951). Synovial joint isolated from each mouse were stored at -20°C for later use as well.

Assessment of cytokine and chemokine concentrations into the synovial joint and serum
Isolated synovial joint tissues (≈ 1 g/mouse) were lysed in 1 ml lysis buffer (containing 300 mM NaCl, 15 mM Tris [pH 7.4], 2 mM MgCl2, 2 mM Triton X-100, 20 ng pepstatin A/ml, 20 ng leupeptin/ml, and 20 ng aprotinin/ml) and homogenized as described earlier (Bergeron et al., 1998). Thereafter, the sample was centrifuged at 2900 rpm for 15 min at 4°C; the resulting supernatant was isolated, 100 µl was analyzed for protein content, and another 100 µl aliquot was placed at -20°C until use (Lowry et al., 1951). Concentrations of pro-inflammatory cytokines including TNFα, IFNγ IL-6, and IL-10, in serum as well as in synovial homogenates were then determined using commercial kits (RayBiotech, Norcross, GA), according to manufacturer instructions. Concentrations of chemokines like IL-8 and MCP-1 were determined in the synovial homogenates only. Levels for each cytokine in a sample were extrapolated from standard curves prepared in parallel. All samples were evaluated using a microplate reader (BioRad, Hercules, CA). All values were reported as pg of cytokine/mg synovial joint tissue protein in homogenate samples per ml serum. The level of sensitivity (per ml materials placed in kit wells) of the kits was 60 pg TNFα, 2 pg IL-6, 2 pg IL-8, 45 pg IL-10, 5 pg IFNγ, and 3 pg MCP-1.

Assessment of CRP concentration into the synovial joint and serum
Concentrations of C-reactive protein (CRP) in synovial joint tissue homogenate and serum were determined using a commercial ELISA kit (MyBiosource, San Diego, CA) according to manufacturer instructions. Levels of CRP in the same samples analyzed above were extrapolated from a standard curve prepared in parallel. All values were reported as ng CRP/ml serum or ng CRP/mg synovial joint tissue protein in homogenate. The level of sensitivity of the kit was 2000 ng CRP/ml.

Assessment of local •O2- and NO production in synovial joint
Portions of the isolated synovial joint (≈ 1 g/mouse) were homogenized in 1 ml ice- cold PBS and homogenized as above. The resulting mixture was centrifuged (12,000 rpm, 30 min, 4°C), and an aliquot of the resulting supernatant was collected. To determine •O2- release, an assay was employed that measured the change in color of cytochrome C (CytC) when reduced by •O2-. Equal volumes (100 µl) of each aliquot of supernatant were combined with 100 µl of a solution of cytochrome C (2 mg/ml in PBS) and incubated at 37°C for 30 min. The reaction was then terminated by placing each tube on ice for 5 min. Total •O2- was monitored at 550 nm in the UV-1800 spectrophotometer against a reference blank that contained the same components except for distilled water in place of supernatant. The amount (µM) of •O2- production/sample was calculated as mean absorbance at 550 nm x 15.87 (Absolom 1986).

Nitrates concentrations in synovial joints were measured to reflect local NO levels. The same supernatants as above were analyzed for NO production using a modified Griess spectrophotometric method as described in (Sun et al., 2003). In brief, 50 µl supernatant was combined with an equal volume of a solution of 40 µM Tris (pH 7.9) containing 40 µM NADPH, 40 µM flavin adenine dinucleotide (FAD), and 0.05 U nitrate reductase/ml (all Sigma) and incubated at 37°C for 15 min. The reduced sample was then incubated with an equal volume of Griess reagent containing 0.25% sulphanilamide (w/v) and 0.025% N-1- naphthyl-ethylene-diamine (w/v); Sigma for 10 min at 37°C, and
the absorbance was measured at 550 nm in the spectrophotometer. The amount (µM) of NO produced was determined by extrapolation from a standard curve prepared in parallel using sodium nitrite.

Local GSH, lipid peroxide, and SOD/CAT enzyme activity status in synovial joint

Synovial joint tissues isolated from mice (= 1 g/mouse) were homogenized into ice-cold 50 mM potassium phosphate buffer (pH =7.4). Homogenates were then centrifuged (9000 rpm, 20 min, 4°C). Supernatants were collected, and aliquots assessed for protein content. To assess anti-oxidant status, levels of reduced GSH were estimated using a spectrophotometric method that employs DTNB (5,5'-dithio-bis-[2-nitrobenzoic acid]; Ellman’s reagent; Sigma) (Sedlak and Lindsay, 1968). A 300 µl aliquot of sample supernatant was mixed with an equal volume of 10% trichloroacetic acid (TCA) solution and then centrifuged at 5000 rpm for 10 min. The supernatant was isolated and a 250 µl aliquot was mixed with 500 µl Tris–HCl buffer (pH 6.5) and 25 µl 10 mM DTNB and incubated for 5 min at 37°C in the dark. Thereafter, the sample absorbance was measured in the spectrophotometer at 412 nm (vs. solution containing all components, with saline in place of homogenate). All outcomes were expressed as µM GSH/mg tissue protein in homogenate sample by taking the mean absorbance and dividing by 14,150 M⁻¹cm⁻¹ (extinction coefficient value).

The tissue contents of lipid peroxidation products were determined from the generation of thiobarbituric acid reactive substances (TBARS) from the isolated homogenates (Buege and Aust, 1978). In brief, 1 ml sample supernatant was mixed with an equal volume of TCA-TBA-HCl mixture and heated in a boiling water bath for 15 min. The mixture was then cooled to room temperature and centrifuged at 10000 rpm (10 min, 4°C). The resultant supernatants were collected and the absorbance of each was measured at 532 nm in the spectrophotometer. All outcomes were expressed in nM TBARS/mg synovial joint tissue protein in homogenate sample by taking the mean absorbance and dividing by 1.56 x 10⁵ M⁻¹cm⁻¹ (extinction coefficient value).

SOD enzyme activity in tissue homogenate was measured as the amount of enzyme capable inhibiting the auto-oxidation of pyrogallol (Dutta et al., 2009). Specifically, a solution (A) containing 100 µl sample supernatant and 1.5 ml Tris–EDTA–HCl buffer (pH 8.5) was prepared. In parallel, a solution (B) containing all components (but with saline in place of homogenate material) was prepared. 100 µl of 7.2 mM pyrogallol was added to each tube, and all samples were incubated at 25°C for 10 min. The reaction was terminated by addition of 50 µl 1 M HCl to each tube and the absorbance at 420 nm was measured in the spectrophotometer. Based on the equation % inhibition = 100 x (1 – [Abs(A)/Abs(B)]), one unit of SOD activity was defined as the amount of enzyme that reduced the rate of auto-oxidation of pyrogallol by 50% under these experimental conditions and it was expressed as U/mg tissue protein in homogenate sample. Catalase activity in supernatants was determined spectrophotometrically as decrease in hydrogen peroxide (H₂O₂) production (Lee et al., 2003). In a cuvette 200 µl supernatant was mixed with 2 ml phosphate buffer (pH 6.5) and 10 mM H₂O₂ was then added. The absorbance in the sample was then immediately monitored at 240 nm in the spectrophotometer against a blank that contained all materials except for homogenate. Measures of absorbance were taken at 15 sec interval after addition of the H₂O₂-buffer. The rate of change in absorbance was calculated and the activity expressed in terms of µM H₂O₂ consumed/min/mg tissue protein in the homogenate (supernatant) sample.

Immunoblot analysis for local TLR2, COX-2 and iNOS expression in synovial joints

Isolated synovial joint tissues (= 1 g/mouse) were placed into RIPA lysis buffer (0.5 M EDTA, 1 M Tris buffer, 5 M NaCl, 10% sodium deoxycholate, 10% SDS supplemented with 1% Triton X-100) and homogenized as above. After isolating supernatant and equivocating protein content by Lowry protein estimation method, 60 µg tissue lysate/mouse was denatured at 100°C for 5 min and then resolved over separate 10% SDS-PAGE gels to permit subsequent separate analyses of TLR2, COX-2, and iNOS (Lowry et al., 1951). Thereafter, all materials were electrotransferred to dedicated nitrocellulose membranes. In each case, after blocking for 2 hr at 4°C in TBST (20 mM Tris-HCl [pH 7.4], 150 mM NaCl, 0.1% [v/v] Tween-20) containing 5% bovine serum albumin (BSA), the membrane was washed three times with TBST and then incubated overnight at 4°C in TBS containing appropriate rabbit anti-mouse- primary antibody, i.e., anti-TNFRF1 (Abcam, USA), anti-TLR2, anti-COX-2, or anti-iNOS antibody (Biorbyt Ltd., Cambridge, UK), each at 1:100 dilution. In each case, antibody against β-tubulin was used to correct for lane loading. Blots were then washed three times in TBST before then being incubated for 2 h at 4°C in TBS containing horseradish peroxidase-conjugated secondary antibody (1:5000 dilution; Biorbyt). Final signals were developed using Super Signal-chemiluminescent-substrate (Thermo Scientific, Pittsburgh, PA). Each blot was then exposed to X-Omat BT films (Kodak, Windsor, CO) and bands were quantified using a BioRad GS-900 densitometer and associated Quantity One software.

Extraction and analysis of MMP-2 by activity gel assay

Analysis of MMP-2 activity was done in synovial joint extracts collected on day 1 post booster immunization, and were analyzed using a gelatin substrate gel in sodium dodecyl sulfate-polyacrylamide gel electrophoresis zymography (Momi et al., 2009). The synovial joints were removed and then homogenized, in 1 ml/100 mg joint weight of RPMI 1640 medium, and were then
centrifuged at 2000g (3450 rpm) for 10 min. The supernatants were sterilized by passing through a millipore filter (0.45 mm) and stored at -80°C until analyzed. The total concentration of soluble protein was determined using Lowry protein estimation method. Twenty micrograms of soluble proteins from tissue sample was diluted with non-reducing sample buffer, and the samples were separated in a 7.5% polyacrylamide gel containing 1 mg/ml of gelatin. Gels were subjected to electrophoresis at 20 mili Ampere (mA) for 90 min, and then incubated in 2.5% Triton X-100 Tris-buffered saline for 60 min, followed by 2 h at -37°C in buffer containing 100 mM CaCl₂. Bands were visualized with 0.2% coomassie blue, and the intensity of each band was determined. Gels were scanned into Adobe Photoshop version 7.0 and visualized as black and white images, and results were analyzed.

RESULTS
Total phenolic content (TPC), total flavonoid content (TFC) and antioxidant (DPPH) effects of AVE, EOE and CTE
The TPC and TFC were found in various concentrations in methanolic extracts of A. vasica leaf, E. officinalis fruit pulp and C. ternatea flower petal. Phenolic content was found to be highest in methanolic extract of E. officinalis fruit pulps (90.5±1.5 mg GAE/g dry wt.) in comparison to methanolic extract A. vasica leaf (76.23±1.23 mg GAE/g dry wt.) and C. ternatea flower petals (61.4±2.0 mg GAE/g dry wt.) which was significant at p<0.05 (Fig. 2A). Flavonoid content was found maximum in methanolic extract of C. ternatea flower petals (67.2±0.72 mg QE/g dry wt.) in comparison to A. vasica leaf and E. officinalis fruit pulps (significant at p<0.05), followed by total flavonoid content in methanolic extract E. officinalis fruit pulps (62±0.83 mg QE/g dry wt.), and then methanolic extract of A. vasica leaf (29.76±0.54 mg QE/g dry wt.) (Fig. 2B). DPPH radical is a stable free radical (absorption band at 517 nm) which loses absorbance following acceptance of electron or free radical species, and results in a visually noticeable discoloration from purple to yellow. Our study revealed that DPPH scavenging activities of the extracts were different (Fig. 2C). The methanolic extract of C. ternatea flower petals (CTE) yielded highest DPPH radical scavenging activity (86.24% inhibition) in comparison to methanolic extracts of A. vasica leaves (54.67% inhibition) and E. officinalis fruit pulps (78.87% inhibition).

![Fig. 2: Total phenolic content, total flavonoid content and radical scavenging potential of AVE, EOE and CTE.](image-url)
methanolic extract of \textit{A. vasica} leaf; EOE - methanolic extract of \textit{E. officinalis} fruit pulps; and CTE - methanolic extract of \textit{C. ternatea} flower petal.

**Effect on macroscopic arthritic scoring and joint swelling**

Arthritis was induced in mice ≈3 weeks after primary immunization with collagen II-CFA, i.e. 2 days after booster immunization with collagen II-IFA. It was observed that AVE (100 mg/kg), EOE (200 mg/kg) and CTE (50 mg/kg) treatment following induction of CIA inhibited arthritic progression in terms of macroscopic arthritic scores and joint swelling from day 20 for AVE and EOE, and day 14 for CTE respectively (Fig. 3).

Fig. 3: Effect of AVE, EOE and CTE on arthritic scores and joint swelling.

Macroscopic arthritic scores (A); and joint swelling in mm (B) were measured at every alternative days from day of secondary immunization upto day 45 was measured. The data were represented as mean±SD; (n=6/group).

**Effect on synovial TNF\(\alpha\), IFN\(\gamma\), IL-6, IL-10, IL-8 and MCP-1 concentrations**

TNF\(\alpha\), IFN\(\gamma\), IL-6, IL-8 and MCP-1 in synovial joints were found to decrease significantly (\(p<0.05\)) in AVE, EOE and CTE treated mice with concomitant restoration in IL-10 levels at day 45 post-secondary immunization (Fig. 4). However, CTE also exhibited comparatively better (significant at \(p<0.05\)) inhibitory effect on synovial TNF\(\alpha\), IL-6, and MCP-1 levels in respect to AVE and EOE.
Fig. 4: Effect of AVE, EOE and CTE on synovial TNFα, IFNγ, IL-6, IL-10, IL-8 and MCP-1 concentrations.

Synovial TNFα (A), IFNγ (B), IL-6 (C), IL-10 (D), IL-8 (E), and MCP-1 (F) production in different experimental groups were measured as pg/mg of tissue protein at day 45 post-secondary immunization. The data were represented as mean±SD; (n=6/group). *indicates significantly (p<0.05) different compared to CON group; †indicates significantly (p<0.05) different compared to CIA group; ‡indicates significantly (p<0.05) different compared to AVE+CIA group; and • indicates significantly (p<0.05) different compared to EOE+CIA group.

Effect on serum TNFα, IFNγ, IL-6, and IL-10 concentrations

TNFα, IFNγ, and IL-6 in serum were found to decrease significantly (p<0.05) in AVE, EOE and CTE treated mice with significant (p<0.05) restoration in IL-10 levels at day 45 post-secondary immunization (Fig. 5). However, CTE also exhibited comparatively better (significant at p<0.05) inhibitory effect on serum TNFα, IFNγ and IL-6 in respect to AVE and EOE. This suggests CTE (50 mg/kg) to be potential regulator of synovial and serum pro-inflammatory cytokines in arthritic mice in comparison to AVE or EOE.
TNFα (A), IFNγ (B), IL-6 (C), and IL-10 (D) production in different experimental groups were measured as pg/ml of serum at day 45 post-secondary immunization. The data were represented as mean±SD; (n=6/group). *indicates significantly (p<0.05) different compared to CON group; †indicates significantly (p<0.05) different compared to CIA group; ‡indicates significantly (p<0.05) different compared to AVE+CIA group; and •indicates significantly (p<0.05) different compared to EOE+CIA group.

Effect on CRP concentrations in synovial joints and serum
CRP concentrations in synovial joints and in serum were found to decrease significantly (p<0.05) in AVE, EOE and CTE treated mice at day 45 post-secondary immunization (Table 1). However, CTE exhibited comparatively better (significant at p<0.05) effect on decreasing CRP concentration in synovial joints and serum of arthritic mice at day 45 post-secondary immunization with respect to AVE or EOE.

Table 1. Effect of AVE, EOE and CTE on synovial and serum CRP concentrations.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Synovial CRP in ng/mg tissue protein (Mean ± SD)</th>
<th>Serum CRP in ng/ml (Mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CON</td>
<td>15.26 ± 4.12</td>
<td>102.16 ± 4.12</td>
</tr>
<tr>
<td>CIA</td>
<td>97.05 ± 9.12*</td>
<td>176.05 ± 8.12*</td>
</tr>
<tr>
<td>CIA+AVE (100 mg/kg)</td>
<td>76.72 ± 3.59*†</td>
<td>159.12 ± 4.12*†</td>
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<tr>
<td>CIA+EOE (200 mg/kg)</td>
<td>71.71 ± 3.78*†</td>
<td>156.71 ± 4.56*†</td>
</tr>
<tr>
<td>CIA+CTE (50 mg/kg)</td>
<td>60.72 ± 4.12*†‡•</td>
<td>143.12 ± 6.75*†‡•</td>
</tr>
</tbody>
</table>

CRP concentration in synovial joints, and serum from different experimental groups were measured as pg/mg tissue protein and pg/ml respectively at day 45 post-secondary immunization. The data were represented as mean±SD; (n=6/group). *indicates significantly (p<0.05) different compared to CON group; †indicates significantly (p<0.05) different compared to CIA group; ‡indicates significantly (p<0.05) different compared to AVE+CIA group; and •indicates significantly (p<0.05) different compared to EOE+CIA group.

Effect on synovial free radical generation in terms of •O2 - and NO production
It was observed that AVE, EOE and CTE pre-treatment significantly (p<0.05) decreased •O2 - and NO production in synovial joints of mice day 45 post-secondary immunization (Fig. 6). EOE was comparatively more effective (p<0.05) in reducing synovial •O2 - and NO production in comparison to AVE, and CTE was more effective in this context in comparison to AVE as well as EOE to reduce synovial free radical generation.
Synovial $\cdot O_2^-$ (A) and NO production (B) in different experimental groups were measured as μg/mg of tissue protein at 45 days post-secondary immunization. The data were represented as mean±SD; (n=6/group). *indicates significantly ($p<0.05$) different compared to CON group; †indicates significantly ($p<0.05$) different compared to CIA group; ‡indicates significantly ($p<0.05$) different compared to AVE+CIA group; and •indicates significantly ($p<0.05$) different compared to EOE+CIA group.

**Effect on synovial anti-oxidant status**

Synovial GSH content and CAT activities were significantly ($p<0.05$) restored with AVE, EOE and CTE treatment; and also LPO content and SOD activities were decreased in synovial joints of arthritic mice at day 45 post-secondary immunization (Table 2). CTE also exerted potential effect on restoration of all these parameters significantly ($p<0.05$) in comparison to AVE and EOE.

**Table 2: Effect of AVE, EOE and CTE on synovial anti-oxidant status.**

<table>
<thead>
<tr>
<th>Groups</th>
<th>GSH concentration in μM/mg of tissue protein (Mean ± SD)</th>
<th>TBARS generation in nM/Mg of tissue protein (Mean ± SD)</th>
<th>CAT activity in μM of H2O2 consumed/min/mg of tissue protein (Mean ± SD)</th>
<th>SOD activity in U/mg of tissue protein (Mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CON</td>
<td>1.474 ± 0.04</td>
<td>15.26 ± 1.98</td>
<td>36.31 ± 2.34</td>
<td>5.021 ± 0.45</td>
</tr>
<tr>
<td>CIA</td>
<td>1.069 ± 0.10 *</td>
<td>47.56 ± 3.65 *</td>
<td>21.55 ± 5.34 *</td>
<td>14.919 ± 1.55 *</td>
</tr>
<tr>
<td>CIA+AVE (100 mg/kg)</td>
<td>1.652 ± 0.07 *</td>
<td>21.23 ± 1.23 †</td>
<td>50.16 ± 2.87 *</td>
<td>6.164 ± 0.60 †</td>
</tr>
<tr>
<td>CIA+EOE (200 mg/kg)</td>
<td>1.695 ± 0.08 †</td>
<td>20.12 ± 1.09 †</td>
<td>56.78 ± 1.52 †</td>
<td>5.745 ± 0.67 †</td>
</tr>
<tr>
<td>CIA+CTE (50 mg/kg)</td>
<td>1.732 ± 0.06 †</td>
<td>17.25 ± 1.45 †</td>
<td>61.23 ± 1.76 †</td>
<td>4.534 ± 0.31 †</td>
</tr>
</tbody>
</table>

Plantar tissue GSH content in μM/mg tissue protein (A), LPO content in nM/mg tissue protein (B), enzymatic activities of SOD in U/mg tissue protein (C) and CAT in μM H2O2 consumed/min/mg tissue protein (D) at day 45 post-secondary immunization in all experimental groups. The data were represented as mean±SD; (n=6/group). *indicates significantly ($p<0.05$) different compared to CON group; †indicates significantly ($p<0.05$) different compared to CIA group; ‡indicates significantly ($p<0.05$) different compared to AVE+CIA group; and •indicates significantly ($p<0.05$) different compared to EOE+CIA group.

**Effect on synovial TLR2, COX-2 and iNOS expressions**

Synovial TLR2, COX-2, and iNOS expressions were significantly ($p<0.05$) increased (Fig. 7; Lane 2) in CIA mice at day 45 post-secondary immunization. AVE (100 mg/kg), EOE (200 mg/kg) and CTE (50 mg/kg) doses significantly decreased synovial TLR2, COX-2, and iNOS CTE (50 mg/kg) pre-treatment significantly ($p<0.05$) decreased synovial TLR2 expressions (Fig. 7; Lane 3), although CTE (Fig. 7; Lane 5) exerted better effect in decreasing synovial TLR2 in comparison to AVE (Fig. 7; Lane 3) as well as EOE (Fig. 7; Lane 4) treated groups. CTE was found to be more effective in decreasing (significantly at $p<0.05$) synovial COX-2 and iNOS expressions in comparison to AVE and EOE at day 45 post-secondary immunization in arthritic mice.
Synovial joint homogenates were prepared for the analysis of TLR2, COX-2, and iNOS expression (A) by immunoblot analysis. All the samples were probed with β-tubulin to show equal protein loading. Expression of TLR2 (B), COX-2 (C), and iNOS (D) was measured in terms of fold changes (in arbitrary unit) over control. The data were expressed as mean±SD; (n=3/group). *indicates significantly (p<0.05) different compared to CON group; †indicates significantly (p<0.05) different compared to CIA group; ‡indicates significantly (p<0.05) different compared to AVE+CIA group; and •indicates significantly (p<0.05) different compared to EOE+CIA group.

**Effect on synovial MMP-2 activity**

Synovial MMP-2 activity was significantly (p<0.05) increased (Fig. 8; Lane 2) in CIA mice at day 25 post-secondary immunization. CTE (Fig. 8; Lane 5) exerted better effect in decreasing synovial MMP-2 activity in comparison to AVE (Fig. 8; Lane 3) as well as EOE (Fig. 8; Lane 4) treated groups.

**DISCUSSION**

In the present study we have shown that oral administration of the test extracts in mice have provided significant protection against the progression of
experimentally induced arthritis (CIA) as indicated from the results of arthritic scoring, i.e., day 20 with 100 mg AVE/kg, 200 mg EOE/kg and day 14 with 50 mg CTE/kg body weight doses in mice after booster immunization with collagen-IFA. However CTE also produced comparatively better effect in down regulating oxidative stress parameters as well as inflammatory markers, as the results were in line effects of CTE>EOE>AVE. This pattern was found to be maintained throughout the result section. Persistence of activated and long-surviving neutrophils in synovial proinflammatory milieu is considered to be responsible for arthritic progression in existing reports (Ottonello et al., 2002). Generation of •O₂⁻ and NO as by-products of oxidative stress into inflammatory milieu of joint synovium destructed redox balance via increasing generation of hydroxyl radical (OH⁻), lipid peroxides, and depleted the reduced glutathione stores of cells (Leonard et al., 2000). Since lipid peroxide as a result of oxidative stress activates NF-κB, it might lead to over expression of inflammatory genes, e.g. iNOS and COX-2 into joint synovium (Ayala et al., 2014). In the present study scavenging of free radical generation (•O₂⁻ and NO production) by AVE, EOE and CTE was observable at day 25 post-secondary immunization with collagen-IFA also reflected as restoration in synovial GSH and lipid peroxide content, along with modulated enzymatic activities of superoxide dismutase (SOD) and catalase (CAT) in arthritic joints (Fig. 9).

In few reports the antioxidant capacity of plant extracts was correlated to total flavonoid contents (Chayaratanasin et al., 2015). Present study also have shown that the total phenolic content was lowest in CTE in comparison to EOE and AVE; however the highest antioxidant activity shown by CTE over EOE and AVE might be directly correlated to its total flavonoid content. Therefore most of the biological effects shown by CTE in providing protection against arthritis progression might be claimed as resultant of its flavonoid components (Bharathe et al., 2014). Existing reports on anti-arthritic effects of plant derived flavonoids stated that dietary supplementation of flavonoids could decrease pathogenesis of arthritis therefore the antiarthritic potential of the plant extracts in the present study might be positively correlated to flavonoids (Leyva-López et al., 2016). Therefore the beneficial effect of CTE over EOE and AVE in context to protection against RA might be directly correlated to its flavonoid content and antioxidant capacity.

Elimination of ROS/RNS by flavonoids present in dietary plants has been shown to attenuate synovial expression of TLR2 (Lucas and Michael, 2013). Therefore decrease in TLR2 expression in arthritic joints from CIA mice in this study following treatment with AVE, EOE and CTE might be correlated to the antioxidant capacities. C-reactive proteins were considered as endogenous ligand of TLR2 in few studies (He et al., 2009).

Generation of antibody mediated response after type II collagen injection in emulsion with IFA and CFA as well as formation and deposition of immune complexes into the joint synovium stimulates local macrophages as well as fibroblasts. Increase in pro-inflammatory cytokines and chemokines leads to generation of inflammation by inducing the fibroblast proliferation and PMN accumulation into joint synovium, as well as local oxidative stress. Activation of MMP-2, collagenases as well as cathepsins by synovial fibroblasts appears as

Fig. 9: Scheme.
final determinant of pathogenesis by increasing bone matrix and cartilage degradation. Treatment with AVE, EOE and CTE interfered production of the pro-inflammatory cytokines and chemokines, to decrease the oxidative stress and local inflammation, which is reflected as final regulation of synovial iNOS, COX-2 and MMP-2 to decrease the degradation of bone matrix as well as cartilages.

Since regulation of TLR2 expression has been correlated to protection in many auto-immune diseases, treatment of arthritic mice with AVE, EOE and CTE that affect CRP and IL-6 could reasonably be expected to translate into therapeutic efficacy against RA (Pepsys and Hirschfield, 2003). Decreased synovial TLR2 expression in AVE, EOE and CTE treated mice was also reflected as decrease in TNFα, IFNγ and IL-6 levels as well as oxidative stress parameters in synovial tissues of CIA mice (Gierut et al., 2010). Decrease in synovial as well as circulatory levels of TNFα, IFNγ and IL-6 is also reflected as decreased signaling of TNFα/TNFR1 mediated expression of synovial MMPs (Burrage et al., 2006). Therefore the decreased MMP-2 activity as measured in this study in synovial joints of arthritic mice after treatment with the plant extracts might be correlated with decreased TLR2 expression (Stanczyk et al., 2008).

Since bioactivities of many flavonoids e.g. quercetins were reported to impart long lasting anti-inflammatory action via inhibition of iNOS, COX and LOX enzyme activities, decreased NF-κB-DNA binding, the decreased expression of iNOS and COX2 in arthritic synovial joints from CTE treated mice might be correlated to its high flavonoid content in comparison to AVE and EOE (Williams et al., 2004; Chen et al., 2005). However AVE failed to reproduce any significant decrease in synovial iNOS, COX-2 and MMP-2; EOE as well as CTE mediated decrease in synovial iNOS, COX-2 and MMP-2 did not reach basal levels. This might indicate existence of other signals besides TLR2 mediated responses which have increased synovial iNOS, COX-2 and MMP-2 in CTE treated mice. Contribution of TNFα/TNFRI mediated signal in maintaining iNOS, COX-2 and MMP-2 levels cannot be overruled. TNFα/TNFRI plays important role in O2 generation and inflammatory responses via inducing NF-κB mediated transcription of inflammatory genes, i.e. iNOS, COX-2, TNF, IL-1, MMP-2, 9 etc. TNFR1 signaling dependent NO production into inflamed synovium has been described as a major cause of synovial hyperplasia in RA through onset of hypoxia mediated angiogenesis.

CONCLUSION
To summarize all the results in the present study it can be concluded that methanolic extracts of C. ternatea flower petal possess highest antioxidant activity in comparison to methanolic extracts of A. vaska leaves and E. officinalis fruit, which might be translated into comparatively better protection against damage of bone matrix and degradation of cartilages in arthritic joints from mice, which might be greatly contributed by flavonoid content of C. ternatea flower petal.

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R.A. undertook all the statistical analysis. R.A. wrote the manuscript and B.B. approved the final manuscript. None of the authors declare any conflicts of interest.

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