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RESEARCH ARTICLE

## Anti-rheumatic activity of *Ananas comosus* fruit peel extract in a complete Freund's adjuvant rat model

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### ABSTRACT

**Context:** Rheumatoid arthritis is a chronic, autoimmune and systemic inflammatory disease, which targets synovial joints leading to joint destruction mediated in part by migration of inflammatory cells into the synovial tissue.

**Objective:** The present study evaluates the anti-rheumatic effect of a methanol extract of *Ananas comosus* (L.) Merr. (Bromeliaceae) peel in rats.

**Materials and methods:** Anti-rheumatic activity of crude extract of peels of *A. comosus* in complete Freund's induced arthritis model in rats was studied at doses of 50, 100, 250 and 500 mg/kg b.w. for 21 days. Parameters such as paw size, levels of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), C-reactive proteins (CRP) and prostaglandins (PGE<sub>2</sub>) were analysed.

**Results:** Oral administration of the extract significantly reduced the swelling in the paw of rats (EC<sub>50</sub> 65.1 ± 2.95 mg/kg b.w.) with a maximal inhibition of 77.01 ± 10.53% on 21st day at 500 mg/kg b.w. The extract also significantly reduced the levels of SOD, CAT and GPx in liver (EC<sub>50</sub> 26.84 ± 16.37, 68.37 ± 19.22, 106.54 ± 34.81 mg/kg b.w., respectively), kidney (EC<sub>50</sub> 261.75 ± 81.5, 176.38 ± 8.08, 14.32 ± 6.64, mg/kg b.w., respectively) and spleen (EC<sub>50</sub> 152.14 ± 39.57, 83.97 ± 14.6, 47.1 ± 10.45 mg/kg b.w., respectively); and CRP (EC<sub>50</sub> 36.37 ± 12.4 mg/kg b.w.) and PGE<sub>2</sub> (EC<sub>50</sub> 191.06 ± 71.54 mg/kg b.w.) in tissue homogenate and serum, respectively, at 500 mg/kg b.w. as compared to arthritic control group.

**Discussion and conclusion:** These results suggest that *A. comosus* fruit peel extract exerts anti-rheumatic activity.

### ARTICLE HISTORY

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Catalase; c-reactive proteins; glutathione peroxidase; prostaglandins; rheumatoid arthritis; superoxide dismutase

### Introduction

Rheumatoid arthritis (RA) is a systemic, chronic and autoimmune inflammatory disease that leads to joint destruction, caused partly by the migration of inflammatory cells into the synovial tissue. It affects approximately 1% of the adult population globally each year (Wei et al. 2014). The common indications of RA are pain, stiffness, fatigue, sleep disturbances and fever. Pathogenesis of RA is complicated with pathological changes at multiple targets. Progression of RA is associated with release of proinflammatory cytokines such as tumour necrosis factor (TNF)- $\alpha$ , interleukin (IL)-1 $\beta$  and IL-6 from monocytes, synovial fibroblasts and macrophages. Inflammatory mediators such as superoxide and hydrogen peroxide are also produced by activated polymorphonuclear leucocytes and macrophages. Superoxide radicals cause cellular disruption through peroxidation of membrane lipids (Devi et al. 2007). However, activated oxygen intermediates together with secondarily formed hydroxyl radicals (OH) and antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx), cause damage to the membrane lipids, proteins, DNA, hyaluronic acid and cartilage. Additionally, the levels of inflammatory markers such as C-reactive proteins (CRP) and specifically, prostaglandins (PGE<sub>2</sub>) produced from arachidonic acid by cyclic oxidase isozymes (COX<sub>2</sub>), also rise during the inflammatory process causing tissue damage (Ricciotti & Fitzgerald 2011). Currently, the development of natural anti-inflammatory drugs for prevention or treatment of inflammatory diseases is receiving increasing attention. Medicinal plants are the main sources of chemical substances with potential therapeutic

effects and many of compounds from plants have already been characterized.

*Ananas comosus* (L.) Merr. (pineapple) is a tropical plant native to South America. *A. comosus* is by far the most economically important plant in the Bromeliaceae family with an edible fruit (Coppens d'Eeckenbrugge & Leal 2003). *Ananas comosus* is reported to possess several medicinal properties such as antidiabetic (Weidong et al. 2005), antitumour (Kalra et al. 2008), antioxidant, hepatoprotective (Doughnon et al. 2009), platelet aggregation (Bhattacharyya 2008), anti-helminthic (Gillian et al. 2004), anti-inflammatory (Eric et al. 2005) and immunomodulatory (Engwerda et al. 2001) activities. Various pharmacologically active phytochemicals present in this plant are ananasate,  $\beta$ -sitosterol, chlorogenic acid, rutin, naringenin (Makoto et al. 2007), bromelain (Eric et al. 2005) glycosides, flavonoids (Chao et al. 2007) and neurotransmitters, such as serotonin (Feldman & Lee 1985), dopamine, adrenaline and non-adrenaline (Ođjakova & Hadjiivanova 1997). Bromelain extracted from *A. comosus* stem belongs to a family of sulphhydryl proteolytic enzymes and is reported to possess anti-inflammatory properties. In a clinical study, bromelain showed decrease in swelling and pain in 72% of rheumatoid arthritis (RA) patients (Cohen & Goldman 1964).

Bromelain, to the best of our knowledge, is the only component studied for its anti-rheumatic activity with specific inhibitory activities against Cox-2 and PGE<sub>2</sub> expression (Gaspani et al. 2002). Considering the commercial use of *A. comosus* as a cash crop and the fact that bromelain is extracted from the stem of the plant, we evaluated the anti-rheumatic activity of the fruit peel, an

inedible part that is discarded as waste. We studied the effect of methanol extract of fruit peel (MEFP) of *A. comosus* on complete Freund's adjuvant (CFA) induced arthritis in a rat model to understand its possible mechanism of action.

## Materials and methods

### Plant material and extraction

The whole plant of *A. comosus* was collected in September 2011 from Ratnagiri district, Maharashtra, India. The plant material was authenticated by Blatter Herbarium, St. Xavier's College, Mumbai and a voucher specimen has been deposited with accession number OS-1a. The fruit peel of *A. comosus* was air-dried in shade and ground into fine powder using a grinder. Powdered fruit peel (50 g) was extracted with 300 mL of methanol for 48 h in a Soxhlet apparatus. The MEFP obtained was then concentrated to dryness under vacuum at 60 °C using a rotary evaporator and the yield was calculated. The methanol content in the extract was determined by gas chromatography at Geo-Chem Laboratories Pvt Ltd., Mumbai, India. The extract was suspended in water for *in vivo* studies.

### Phytochemical component analysis of MEFP

#### Qualitative phytochemical analysis

The MEFP of *A. comosus* was analysed for the presence of various phytoconstituents such as carbohydrates, proteins, triterpenes, phytosterol, cardiac glycosides, anthroquinone glycosides, flavonoids, alkaloids and tannins using standard methods (Chandana & Prabhakaran 2014).

#### Quantitative analysis of flavonoids

The total flavonoid content in the MEFP was estimated using the  $AlCl_3$  method with quercetin as a standard (Chang et al. 2002). Briefly, to 0.5 mL of the extract, 1.5 mL methanol, 0.1 mL aluminium chloride, 0.1 mL potassium acetate solution and 2.8 mL distilled water were added and mixed well. The absorbance was measured at 415 nm.

#### Quantitative analysis of tannins

The total tannin content in MEFP was estimated by modified Prussian Blue method with gallic acid as a standard (Graham 1992). Briefly, 0.1 mL of the extract was added to 6.9 mL of distilled water, 1 mL of 0.008 M potassium ferric cyanide, 1 mL of 0.2 M ferric chloride in 0.1 M HCl and mixed well. The absorbance was measured at 700 nm.

#### Thin layer chromatography (TLC) analysis of MEFP

Thin layer chromatography (TLC) of MEFP of *A. Comosus* was carried out on the G60 F254 silica plates (Merck KGaA, Darmstadt, Germany). Briefly, 5  $\mu$ L of the sample was spotted on the plate and the chromatogram was developed using the solvent system toluene:ethylacetate:methanol:formic acid (4:6:4:0.2). The chromatograms were derivatized with 2-aminoethyl diphenylborinate (NP reagent) and vanillin sulphuric acid

reagent (1% vanillin in 95% ethanolic sulphuric acid) for detecting flavonoids and tannins, respectively.

### Experimental animals

Male Wistar rats weighing 100–150 g were purchased from Bharat Serum and Vaccines, Mumbai, India. The animals were kept in cages and maintained under standard housing conditions (24–27 °C with 12:12 light:dark cycles). Food was provided in the form of dry pellets (Nutrivet Life Science, Pune, India) and water *ad libitum*. The animals were acclimatized for a week under laboratory conditions before commencement of experiments.

The use of animals in the study was approved (CPCSEA/IAEC/SOS/P-36/2012) by the Institutional Animal Ethics Committee (IAEC) and all the experiments were carried out according to the guidelines of the Committee for the Purpose of Control on Supervision and Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India.

### Acute toxicity studies

The acute toxicity studies with MEFP of *A. comosus* were conducted as per the OECD Guideline 423. The rats were fasted for 12 h prior to initiation of the experiment. Briefly, MEFP was administered orally to the rats as a single dose of 2000 mg/kg body weight. The rats were observed continuously for behavioural changes for the first 4 h then observed daily for 14 days for any toxic symptoms and/or mortality. Parameters such as breathing (palpitation), eye movement, eye colour, writhing effect, vomiting, diarrhea, abnormal body movements and texture and colour of fur were monitored.

### Evaluation of anti-rheumatic activity of MEFP

#### CFA induced arthritis in rats

Experiments were carried out as per the method previously described by Pearson and Wood (1959). Briefly, 0.1 mL of CFA (Sigma-Aldrich, MO) containing 10 mg/mL of heat killed *Mycobacterium tuberculosis* was injected into the left hind paw of the rats. The MEFP at doses of 50, 100, 250 and 500 mg/kg b.w. was administered orally to each rat; the control group received distilled water (0.1 mL/kg b.w.); and reference group received prednisolone (10 mg/kg b.w.) daily for 21 days. Treatment was initiated from day 1 after FCA injection and the oedema on the left hind paw was evaluated daily using a digital plethysmometer. After 21 days, the animals were sacrificed by cervical dislocation and the blood was collected by cardiac puncture. The liver, kidney and spleen were immediately removed and washed with ice cold 10 mM phosphate buffered saline (PBS). The tissues were cut into pieces and homogenized using ice cold 10 mM PBS for further analysis.

#### Evaluation of scavenging enzymes

Tissue homogenates of liver, kidney and spleen were assessed for levels of SOD, CAT and GPx. SOD was estimated using a modification of the method described by Marklund and Marklund (1974). Briefly, the reaction mixture contained 100  $\mu$ g tissue homogenate in 2 mL of 50 mM Tris EDTA buffer (pH 8.2) and 1 mL of 4 mM pyrogallol. Absorbance was measured every minute for 3 min at 420 nm.

CAT was estimated using a modification of the method described by Chance and Herbert (1950). Briefly, the reaction mixture contained 200 µg of tissue homogenate and 19.6 mM H<sub>2</sub>O<sub>2</sub> in 2 mL of 10 mM potassium phosphate buffer (pH 7.4). Absorbance was measured every minute for 2 min at 240 nm.

GPx was estimated using a modification of the method described by Awasthi et al. (1979). Briefly, the reaction mixture contained, 100 µg tissue homogenate, 10 mM sodium azide, 1 mM reduced glutathione and 2 mM H<sub>2</sub>O<sub>2</sub> in 0.2 mL of 0.2 M phosphate buffer (pH 7.6). Following incubation at 37 °C for 10 min, 0.4 mL of 5% TCA was added and centrifuged at 3200g for 20 min. One micromolar Ellman's reagent (1 mL) was added to 0.2 mL supernatant and incubated for 5 min. The absorbance was measured at 412 nm.

### Evaluation of inflammatory markers

The serum samples were assessed for levels of CRP and PGE<sub>2</sub>. The CRP was estimated using CRP latex kit (Aspen-Rapid Diagnostic Co. Kit, Delhi, India). The PGE<sub>2</sub> was estimated using Prostaglandin E<sub>2</sub> assay kit as per manufacturer's instructions (Catalog No- KGE004B, R&D Systems, McKinley Place NE, Minneapolis, MN). The absorbance was measured at 450 and 570 nm on an ELISA plate reader (BIO-RAD, CA).

### Histological analysis of paw

Histological studies of the left hind paw of the animals were performed. The tissues were fixed in formalin, decalcified and embedded in paraffin blocks. The tissue sections were stained with haematoxylin and eosin and examined under microscope.

### Statistical analysis

The values obtained are expressed as mean ± SEM. The data were analysed by a one-way ANOVA, followed by Dunnett's post-test using Prism 5.0 (GraphPad Software, Inc., CA). *p*-Values less than 0.05 (*p* < 0.05) were considered as significant.

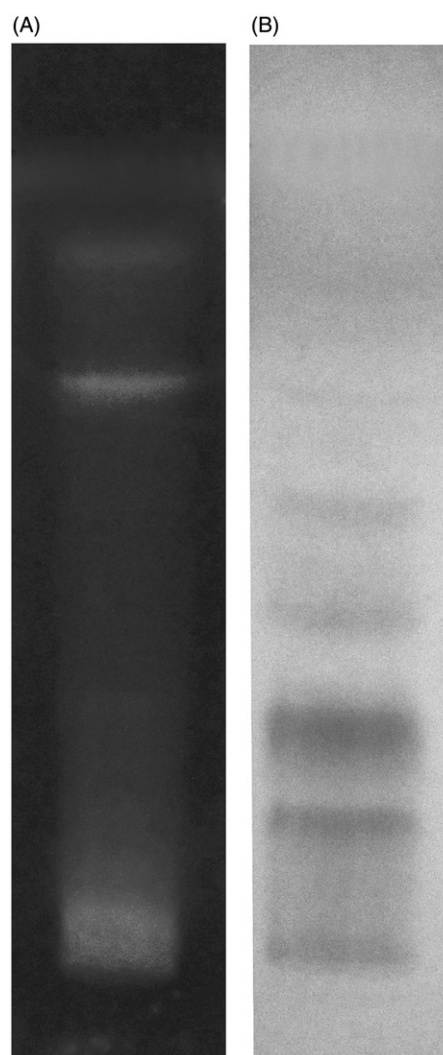
## Results

### Extraction of plant material

The yield of the extract from powdered fruit peel was 28.14%. The gas chromatography analysis of the dried extract showed the presence of approximately 7.63% w/w of methanol content. For all the assays conducted in the study, the extract suspended in distilled water at concentrations of 50, 100, 250 and 500 mg/kg b.w. was administered orally. The methanol content received by the different groups of animals was estimated to be 3.815, 7.63, 19.08, 38.15 mg/kg b.w., respectively. The minimum lethal dose of methanol via oral administration has been reported to be approximately 5628 mg/kg b.w. (Sax & Lewis 1989), indicating that the extract was not toxic to the animals. This was also confirmed by histopathological analysis where no difference was observed between control and test groups (data not shown).

### Phytochemical component analysis of MEFP

Qualitative phytochemical analysis of MEFP of *A. comosus* showed the presence of phytoconstituents, namely flavonoids, tannins, triterpenoids and phytosterols. The TLC analysis also



**Figure 1.** Chromatogram showing bands of (A) flavonoids derivatized with NP reagent and (B) tannins derivatized with vanillin sulphuric acid reagent.

showed presence of flavonoids (Figure 1A) and tannins (Figure 1B) in MEFP when derivatized with NP reagent and vanillin sulphuric acid reagent, respectively. The contents of flavonoids and tannins in MEFP were estimated to be  $1.76 \pm 0.067$  mg/g and  $4.52 \pm 0.45$  mg/g, respectively.

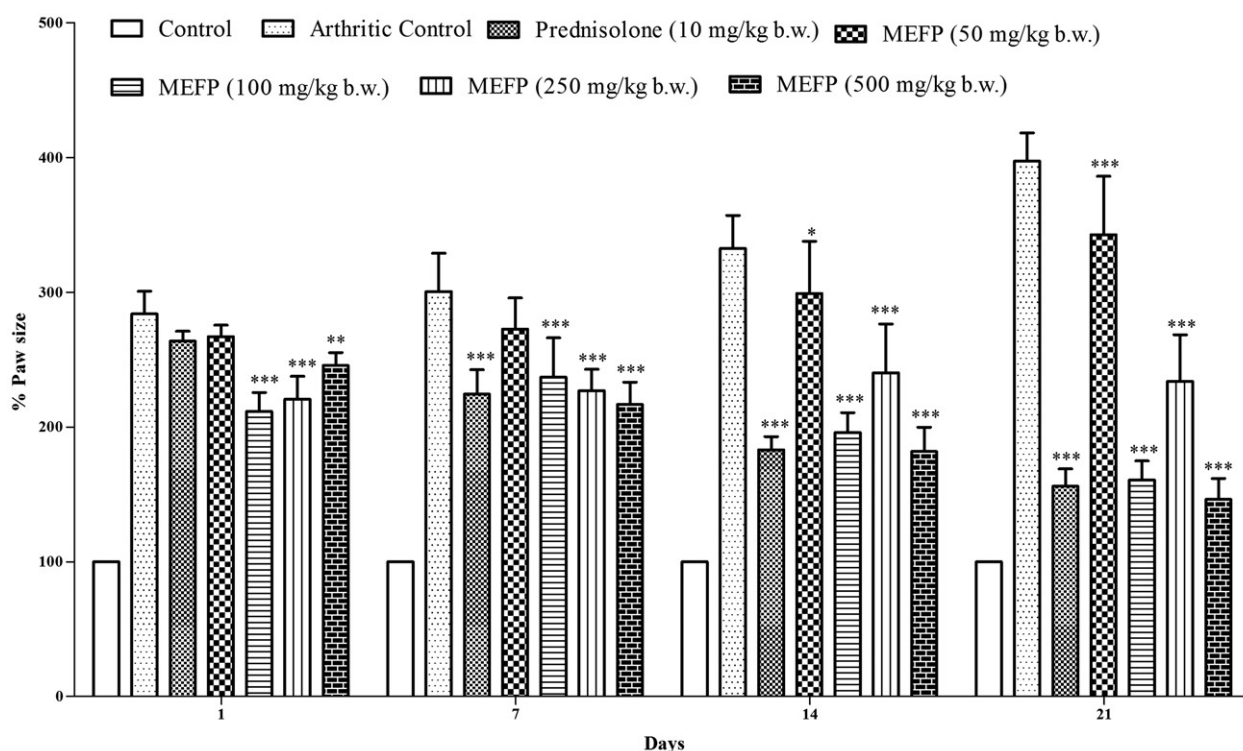
### Acute toxicity studies

No deaths were observed in the group of animals administered with 2000 mg/kg b.w. of MEFP. No effect on behavioural or any other toxicological parameters was observed during the 14-day observation period. Hence, MEFP was considered to be safe.

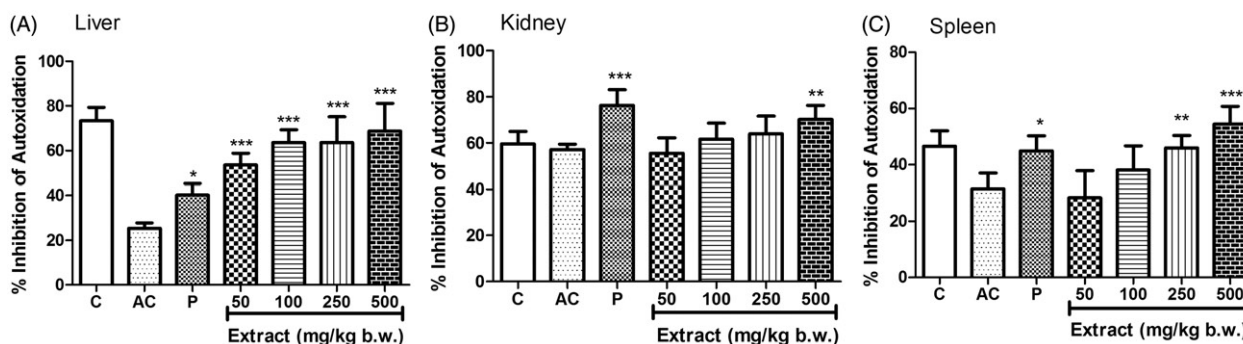
### Evaluation of anti-rheumatic activity of MEFP

#### CFA induced arthritis in rats

The paw size was measured on day 1 following injection with FCA in the left hind paw. The treatment was initiated on day 1 and the paw size was measured daily for 21 days. Figure 2 shows mean paw size for animals in each group at days 1, 7, 14 and 21. The CFA-induced arthritis control group showed continuous increase in the paw size until day 21, indicating no improvement in the arthritic lesions. The inflammation was significantly lower



**Figure 2.** Effect of MEFP on paw volume in CFA-induced arthritic rats. The values are expressed as mean  $\pm$  SD.  $p < 0.05$  was considered significant with respect to arthritic control group (\* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ).



**Figure 3.** Effect of MEFP on SOD levels in liver, kidney and spleen. The values are expressed as mean  $\pm$  SD.  $p < 0.05$  was considered significant with respect to arthritic control group (\* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ).

in groups treated with MEFP when compared to the arthritic control group. The reduction in inflammation with 500 mg/kg b.w. of MEFP was  $77.01 \pm 10.53\%$  and  $49.11 \pm 20.69\%$  with prednisolone.

### Evaluation of scavenging enzymes

The level of SOD activity decreased significantly in tissue homogenates of liver (Figure 3A) and spleen (Figure 3C) in the arthritic control group compared to the normal control; however, the decrease in tissue homogenate of kidney was not significant (Figure 3B). The SOD activity significantly increased in liver ( $EC_{50} 26.84 \pm 16.37$ ), kidney ( $EC_{50} 261.75 \pm 81.5$ ) and spleen ( $EC_{50} 152.14 \pm 39.57$ ) when treated with MEFP with maximum increase at 500 mg/kg b.w.

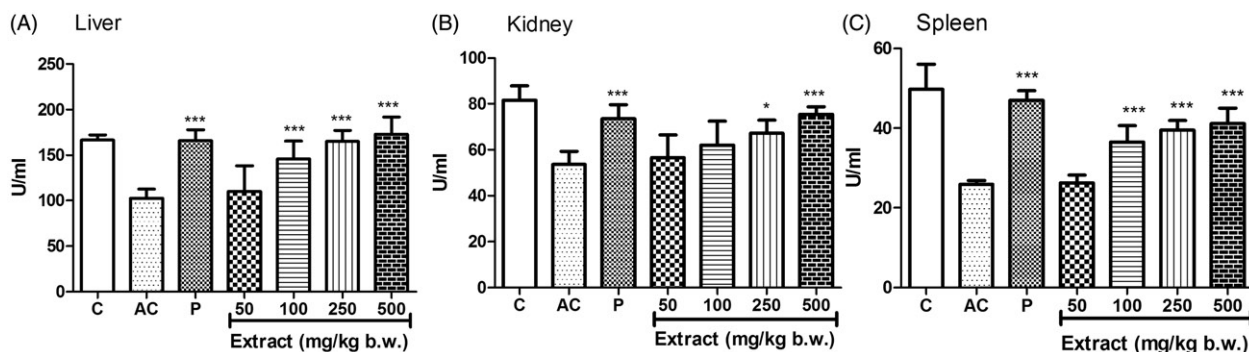
The level of CAT activity decreased significantly in the tissue homogenates of liver (Figure 4A), kidney (Figure 4B) and spleen (Figure 4C) in the arthritic control group compared to the normal control. The CAT activity increased in a dose-dependent manner in liver ( $EC_{50} 68.37 \pm 19.22$ ), kidney ( $EC_{50} 176.38 \pm 8.08$ ) and spleen ( $EC_{50} 83.97 \pm 14.6$ ) when treated with MEFP with a

significant increase at 250 and 500 mg/kg b.w. compared to the arthritic control group in all three tissue homogenates.

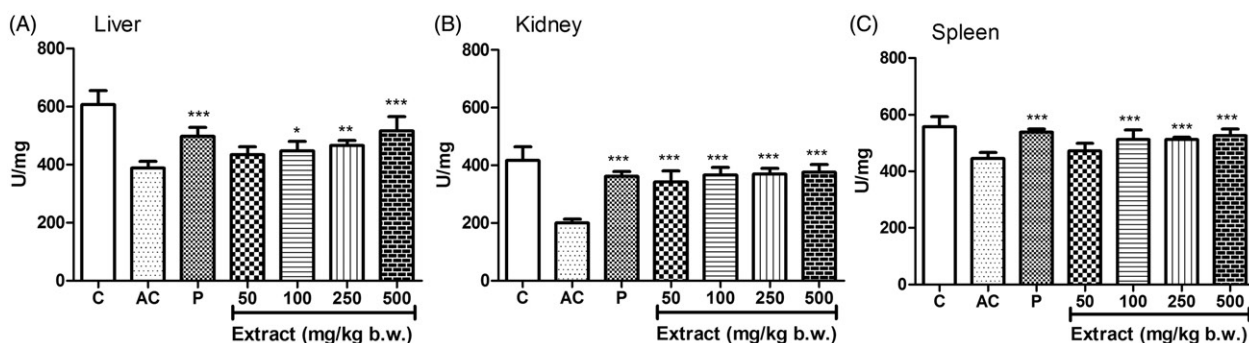
The level of GPx activity decreased significantly in the tissue homogenates of liver (Figure 5A), kidney (Figure 5B) and spleen (Figure 5C) in the arthritic control group compared to the normal control. The GPx activity increased in a dose-dependent manner in liver ( $EC_{50} 106.54 \pm 34.81$ ), kidney ( $EC_{50} 14.32 \pm 6.64$ ), spleen ( $EC_{50} 47.1 \pm 10.45$ ) when treated with MEFP with a significant increase at 100, 250 and 500 mg/kg b.w. compared to the arthritic control group in all three tissue homogenates.

### Effect on inflammatory markers

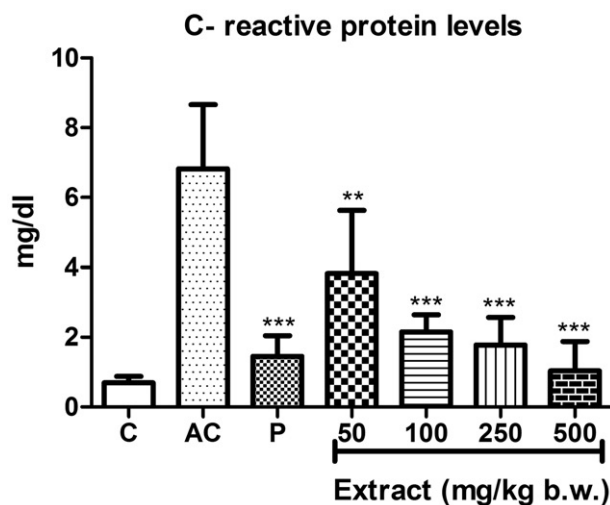
The levels of CRP increased significantly in serum samples of rats in the arthritic control group compared to that of the normal control (Figure 6). When treated with different concentrations of MEFP, the levels of CRP decreased in a dose-dependent manner with all the concentrations ( $EC_{50} 36.37 \pm 12.4$ ), showing significant decrease compared to the arthritic control group.



**Figure 4.** Effect of MEFP on CAT levels in liver, kidney and spleen. The values are expressed as mean  $\pm$  SD.  $p < 0.05$  was considered significant with respect to arthritic control group (\* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ).



**Figure 5.** Effect of MEFP on GPx levels in liver, kidney and spleen. The values are expressed as mean  $\pm$  SD.  $p < 0.05$  was considered significant with respect to arthritic control group (\* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ).

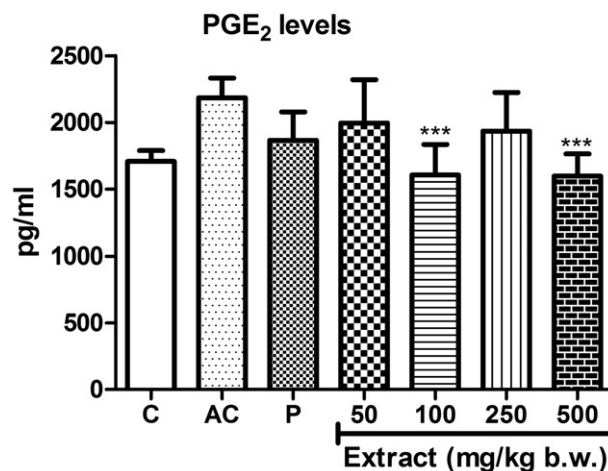


**Figure 6.** Effect of MEFP on CRP levels. The values are expressed as mean  $\pm$  SD.  $p < 0.05$  was considered significant with respect to arthritic control group. (\*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ).

Treatment with MEFP showed decrease in levels of PGE<sub>2</sub> in the serum samples of rats (EC<sub>50</sub> 191.06  $\pm$  71.54) compared to the arthritic control when treated with significant decrease at concentrations of 100 and 500 mg/kg b.w. (Figure 7).

#### Histopathological analysis of paw

The histopathological analysis of hind paw of the arthritic control group rats injected with FCA showed infiltration of mononuclear cells, inflammatory reaction around the bone and under the subcutaneous tissue; necrosis and degeneration in the inner regions

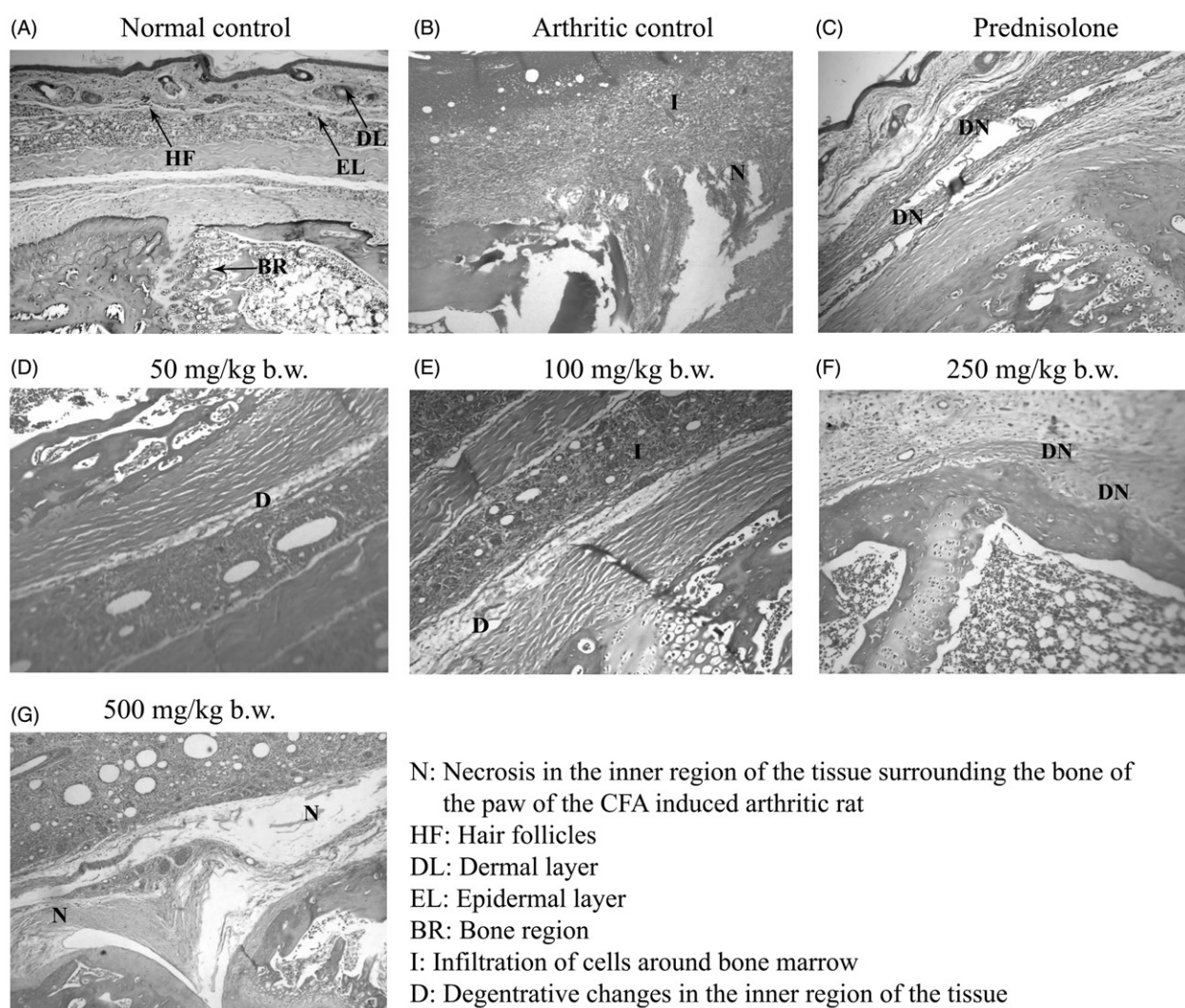


**Figure 7.** Effect of MEFP on PGE<sub>2</sub> levels. The values are expressed as mean  $\pm$  SD.  $p < 0.05$  was considered significant with respect to arthritic control group (\*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ).

of the tissue surrounding the bone (Figure 8). Treatment with MEFP showed reduction in necrosis at all the concentrations tested.

#### Discussion

CFA-induced arthritis in rats show clinical and pathological similarity to human RA and is thus the most widely used chronic test model (Pearson & Wood 1959). Most researchers have reported that inhibition of adjuvant-induced arthritis in rats is one of the most suitable methods to screen anti-arthritic agents. In this study, we investigated the effect of MEFP of *A. comosus* on paw



**Figure 8.** Parameters analysed in histopathology of paw region.

inflammation following injection of FCA in hind paw. The extract showed a significant reduction in inflammation measured in terms of paw-size at 100 and 500 mg/kg b.w., which was comparable to the effect shown by the standard drug prednisolone at a concentration of 10 mg/kg b.w. The histopathological analysis also showed reduction in necrosis when treated with MEFP.

The body possesses antioxidant defences, repair mechanisms, physical defences and preventive mechanisms against free radical oxidative stress. Enzymes such as SOD, CAT and GPx are necessary for protective mechanisms including quenching of singlet oxygen, potential complexing of pro-oxidant metals and free radical scavenging and reducing activity.

SOD acts as the first line of defence against oxygen derived free radicals and protects cells by converting superoxide anion to hydrogen peroxide (Afonso et al. 2007). In the present study, although there was significant decrease in level of SOD in liver and spleen, there was no significant decrease in level of SOD in kidney. This is because the body tries to overcome the oxidative stress by maintaining the levels of SOD in kidney unlike in liver and spleen (Chandankhede & Gupta 2013). The increase in SOD activity observed in liver, kidney and spleen when treated with MEFP exhibit the protective effect of the extract against the extra-cellular oxygen-derived free radicals.

CAT, localized in peroxisomes or microperoxisomes (Devi et al. 2007) cleaves hydrogen peroxide generated in the body to water and oxygen, and thus, protects the cell from oxidative damage. Increase in CAT activity in liver, kidney and spleen when administered with MEFP again exhibits its protective effect against oxygen-derived free radicals.

GPx, a detoxifying enzyme, forms the primary mitochondrial defence against peroxides (Devi et al. 2007). Selenium is related closely with GPx activities in tissues (Rister & Bancher 1976). In RA, the free radicals decrease levels of selenium thereby decreasing the activity of GPx. Similar to as observed with SOD and CAT, the increase in GPx activity in liver, kidney and spleen when administered with MEFP again exhibits its protective effect against oxygen-derived free radicals through restoration of activity of radical scavenging enzymes. The results were similar to as observed with the standard drug, prednisolone.

CRP is a member of the class of acute phase reactants that is produced only in liver (Bashir et al. 2014). CRP acts as a marker of systemic inflammation since during inflammatory processes, its levels rise dramatically in the body as was observed in case of arthritic control rats in the present study (Figure 6). Significant reduction in CRP levels in rats when administered with MEFP

compared to arthritic control rats indicates the potential of MEFP as an anti-inflammatory agent.

PGE<sub>2</sub>, the main products of COX pathways (Fattahi & Mirshafiey 2012), are important mediators of inflammation in RA (Ricciotti & Fitzgerald 2011). Production of PGE<sub>2</sub> by rheumatoid synovial tissue causes erosion of cartilage and juxta articular bone. The decrease in the levels of PGE<sub>2</sub> when administered with MEFP indicates the anti-inflammatory potential of *A. comosus*. It also suggests the possible use of MEFP for protection of cartilage from damage caused during RA.

The qualitative phytochemical analysis of MEFP showed the presence of flavonoids, tannins, triterpenoids and phytosterols. Of these, flavonoids and tannins can be attributed with the anti-rheumatic activity observed in the present study. Various studies have reported the anti-inflammatory properties of flavonoids and tannins from plants (Jiang et al. 2014). Reports have shown that triterpenoids and phytosterols do not possess anti-inflammatory properties (Saxena et al. 2013). There are no reports on the phytochemical composition of *A. comosus* fruit peel. Studies related to identification of active constituents through bioassay-guided fractionation is presently ongoing.

## Conclusion

The MEFP of *A. comosus* exhibited anti-rheumatic activity by increasing the levels of SOD, CAT and GPx in liver, kidney and spleen, and by decreasing the levels of CRP and PGE<sub>2</sub> prostaglandin in serum of arthritic rats. The histopathology of paw also exhibited reduction in necrosis when treated with the extract. We can conclude that flavonoids and tannins present in the crude extract may be responsible for the anti-arthritic activity as observed in this study.

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## Disclosure statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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