

# ***In vitro* and *in vivo* anti-arthritic evaluation of *Polystichum braunii* to validate its folkloric claim**

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**Abstract:** Medicinal plants are playing an imperative role in the therapy for treating various chronic ailments including arthritis. The present study was focused on finding *in vitro* and *in vivo* anti-arthritic potential of *P. braunii* roots. *In vitro* protein denaturation, membrane stabilization and anti-trypsinase assays were carried out to demonstrate anti-arthritic activity of the extracts. Furthermore, the extracts exerting promising *in vitro* anti-arthritic potential were tested orally at 150, 300 and 600mg/kg/day against formaldehyde induced arthritis in Wistar rats. The methanolic, aqueous and ethyl acetate extracts of the plant revealed noteworthy *in vitro* anti-arthritic activities while mitigating formaldehyde induced paw edema in dose dependent manner. Methanolic and aqueous extracts showed the highest inhibition ( $p < 0.05$ ) of paw edema, arthritic indices, reduced elevated level of platelets and leukocytes while increasing hemoglobin and body weight of arthritic rats. Anti-arthritic activity of the plant extracts may be due to inhibition of protein denaturation and lysosomal membrane stabilization. The plant exhibited good anti-arthritic potential.

**Keywords:** *Polystichum braunii*, arthritis, anti-trypsinase, protein denaturation.

## **INTRODUCTION**

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by the pain and inflammation of joints especially of hands and feet that proliferates to physical disability. Throughout the world, one-fifth of the elderly population mostly women suffer from arthritis (Mubashir *et al.*, 2014). Being a systemic disease, it affects the physiology of heart, liver and lungs, and thus accompanies various comorbidities (Gutiérrez-Rebolledo *et al.*, 2018).

Rheumatoid joints are surrounded by inflamed synovium filled with pro-inflammatory and bone resorbing cytokines including matrix metalloproteinases (MMPs), prostaglandin E2 (PGE2), tumor necrosis factor (TNF- $\alpha$ ) and interleukin IL-1 $\beta$  (Pan *et al.*, 2017; Kshirsagar *et al.*, 2014).

Generally, RA is treated symptomatically though lifetime treatment with Non-steroidal antiinflammatory drugs (NSAIDs), steroids, Disease-modifying anti-rheumatic drugs (DMARDs), immunosuppressants and cytotoxic drugs (Gutiérrez-Rebolledo *et al.*, 2018).

Adverse effects of these therapies are numerous that necessitate the development of safer and cost-effective anti-arthritic agents for lifelong use. Herbal drugs are promising alternatives for the development of effective

and safe drugs against arthritis is due to their ease of availability and cost-effectiveness in comparison to allopathic (Hasan, 2018).

*Polystichum braunii* (Spenn.) Fee is locally called as Holy fern and belongs to Dryopteridaceae family. It is traditionally used for treating rheumatism (Ch *et al.*, 2011). Moreover, it is also applied locally over rheumatic joints admixed with olive oil. It has a large habitats including Alps, Norway, Germany, Czech Republic, Pakistan, China, Nepal, India, and United States (Schwerbrock and Leuschner, 2016).

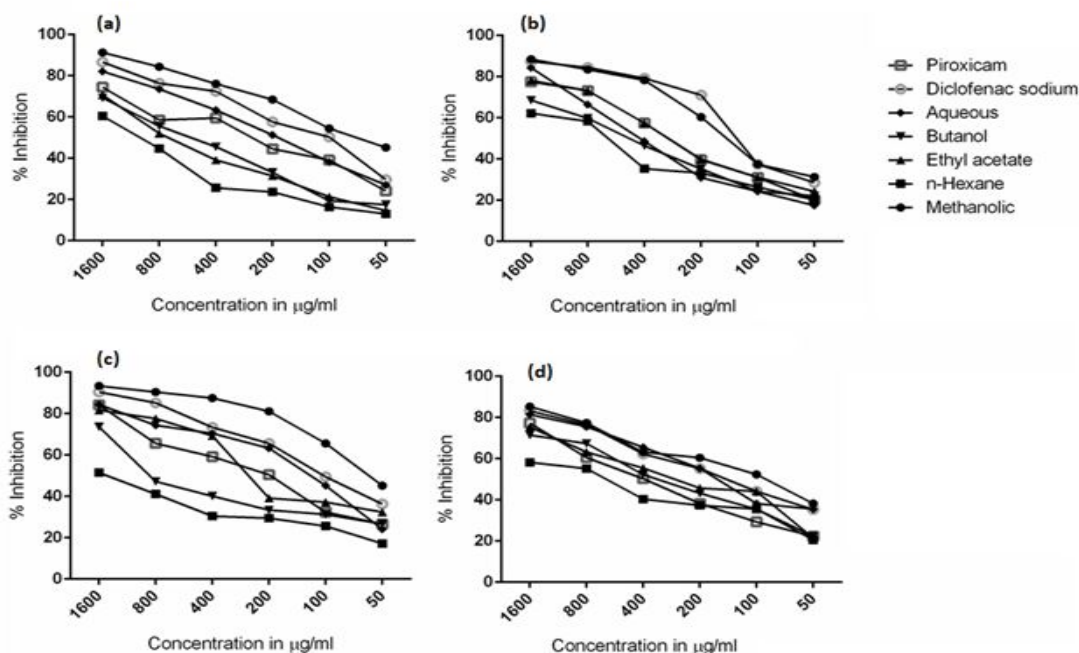
Literature survey reveals that no scientific authentication of anti-arthritic and anti-inflammatory activity of *P. braunii* has been carried out. Therefore, the present study was planned to demonstrate the anti-arthritic potential of the plant by employing various *in vitro* and *in vivo* tests.

## **MATERIALS AND METHODS**

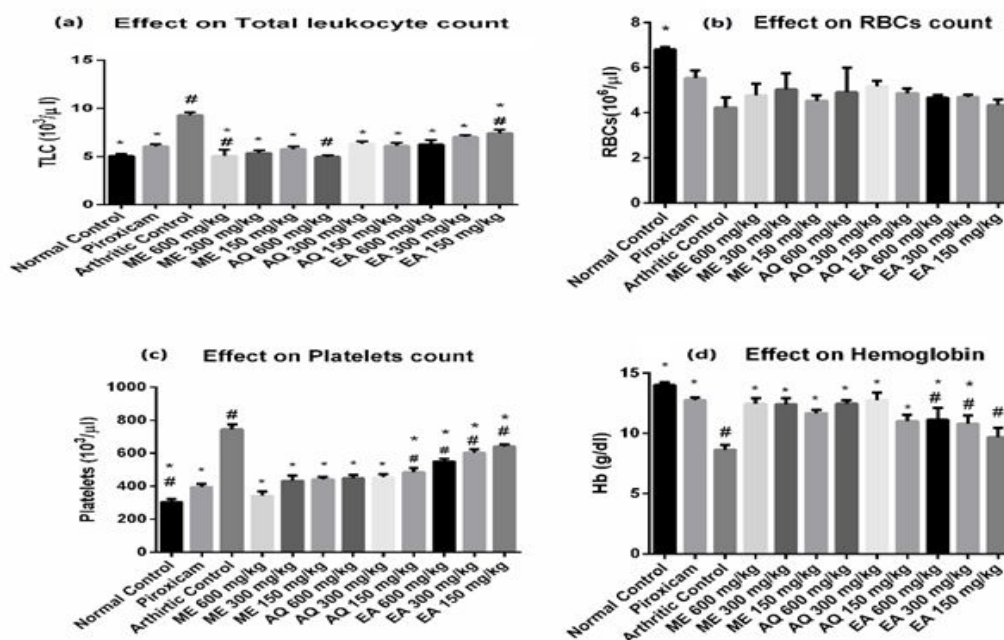
### ***Collection, extraction and fractionation***

Roots of *P. braunii* (5kg) were collected from Mirpur, Azad Jammu and Kashmir in June 2016 and identified by a taxonomist of the University of Agriculture, Faisalabad, Pakistan who assigned the Herbarium No.103-1-16. The roots were washed thoroughly. Shade dried and coarsely ground roots of the plant were subjected to extraction with methanol (1:10) by cold maceration technique for 7 days.

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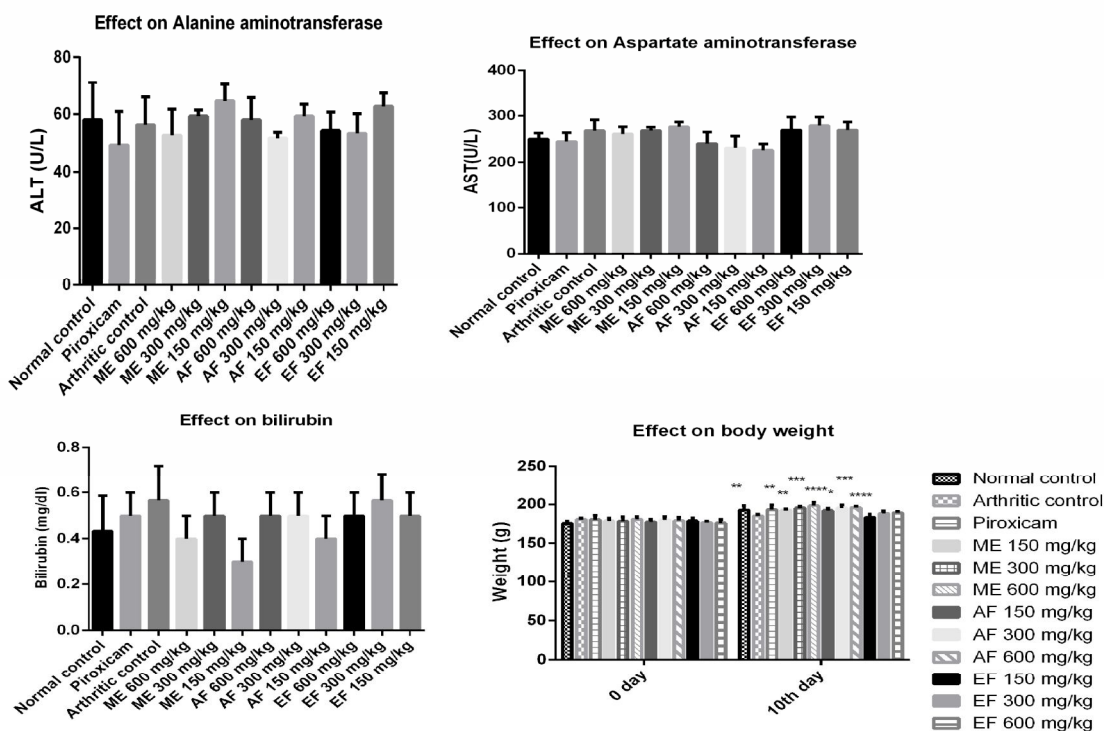


**Fig. 1:** *In vitro* anti-arthritic effects of different extracts of *Polystichum braunii* (a) Inhibition of egg albumin denaturation (b) Inhibition of bovine serum albumin (c) Red blood stabilization assay (d) Anti-trypsinase activity. Results are presented as mean  $\pm$  S.D. The values were statistically analyzed by two-way analysis of variance (ANOVA) followed by Tukey's test. Results considered significant ( $p < 0.05$ ) as compared to piroxicam and DS.



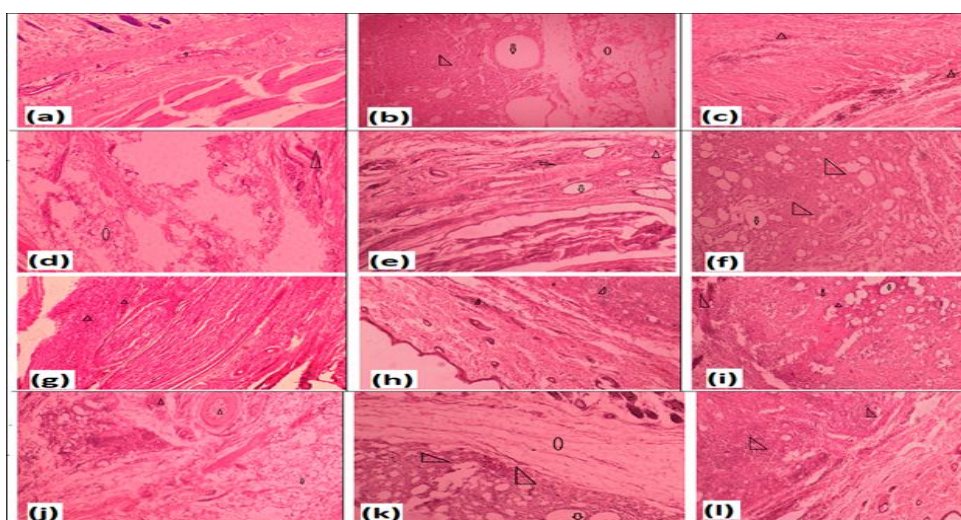
**Fig. 2:** Effect of *Polystichum braunii* on blood parameters of formaldehyde induced arthritic rats

Results are presented as mean  $\pm$  S.D. Results of blood tests were analysed by one-way analysis of variance (ANOVA) followed by Tukey's test. Significant level for body weight was  $p < 0.05$ . Where \* and # showed statistically significant in comparison to arthritic control and piroxicam treated groups respectively. ME: methanolic extract, AQ: aqueous extract, EA: Ethyl acetate extract



**Fig. 3:** Effect of *Polystichum braunii* on liver function parameters and body weight of formaldehyde induced arthritic rats

Results were analyzed by one-way analysis of variance (ANOVA) followed by Tukey’s test. Where \* showed statistically significant in comparison to arthritic control group. ME: methanolic extract, AQ: Aqueous extract, EA: Ethyl acetate



**Fig. 4:** Effect of *Polystichum braunii* on joint histology of formaldehyde induced arthritis at 40X magnification

Where (a) Normal control (b) Arthritic control (c) Piroxicam (d) Methanolic extract 600 mg/kg (e) Methanolic extract 300 mg/kg (f) Methanolic extract 150 mg/kg (g) Aqueous extract 600 mg/kg (h) Aqueous extract 300 mg/kg (i) Aqueous extract 150 mg/kg (j) Ethyl acetate 600 mg/kg (k) Ethyl acetate 300 mg/kg (l) Ethyl acetate 150 mg/kg treated groups

The extraction procedure was repeated twice and the filtrate was pooled. The filtrate was concentrated using rotary evaporator (Model: RE300, Stuart®, UK) at 40°C under reduced pressure. The dried crude methanolic extract was dissolved in distilled water (DW) and

fractionated with n-hexane, ethyl acetate and n-butanol by liquid-liquid extraction technique. After fractionation, each filtrate was dried with rotary evaporator and finally stored in a refrigerator at 8°C till further use.

**Table 1:** Values of percentage inhibition in different in vitro anti-arthritic tests by *Polystichum braunii* extracts expressed as IC<sub>50</sub> (µg/ml)

Extract	Egg albumin denaturation assay	Bovine serum albumin denaturation assay	Anti-trypsinase activity	HRBC Membrane assay
Methanolic	105.6	190.2	295.0	123.8
n-Hexane	1386	516.9	332.9	545
Ethyl acetate	508.1	326.2	255.6	318.5
Butanol	468.4	407.9	280.6	887.2
Aqueous	168.7	657.2	262.3	150.2
Diclofenac Sodium	200.7	148.1	313.2	233.9
Piroxicam	263.7	289.7	399.5	253.4

**Table 2:** Percentage inhibition of paw volume by *Polystichum braunii* extracts against formaldehyde induced arthritis

Days	Methanol			Ethyl acetate			Aqueous			Piroxicam 10 mg/kg
	150 mg/kg	300 mg/kg	600 mg/kg	150 mg/kg	300 mg/kg	600 mg/kg	150 mg/kg	300 mg/kg	600 mg/kg	
2	27.67 ±0.48 <sup>a</sup>	31.44±0.30 <sup>a</sup>	36.49±0.49 <sup>a</sup>	13.15±0.15 <sup>a</sup>	21.51±0.32 <sup>a</sup>	25.39±0.17 <sup>a</sup>	24.65±0.50 <sup>a</sup>	34.43±0.43 <sup>ns</sup>	37.37±0.35 <sup>a</sup>	35.02±0.64
4	45.51 ±0.29 <sup>a</sup>	46.46±0.42 <sup>a</sup>	56.62±0.87 <sup>a</sup>	34.51±0.28 <sup>a</sup>	41.47±0.08 <sup>a</sup>	45.17±0.20 <sup>a</sup>	38.45±0.29 <sup>a</sup>	49.05±0.176 <sup>a</sup>	50.56±0.22 <sup>a</sup>	55.64±0.46
6	56.28 ±0.36 <sup>a</sup>	62.62±0.24 <sup>a</sup>	68.48±0.38 <sup>a</sup>	45.66±0.31 <sup>a</sup>	49.82±0.67 <sup>a</sup>	55.34±0.28 <sup>a</sup>	53.36±0.38 <sup>a</sup>	58.47±0.180 <sup>a</sup>	63.48±0.26 <sup>a</sup>	64.82±0.49
8	65.77 ±0.23 <sup>a</sup>	70.85±0.22 <sup>ns</sup>	76.56±0.45 <sup>a</sup>	53.46±0.31 <sup>a</sup>	56.96±0.65 <sup>a</sup>	61.33±0.11 <sup>a</sup>	58.61±0.32 <sup>a</sup>	65.3±0.22 <sup>a</sup>	71.23±0.42 <sup>ns</sup>	71.4±0.38
10	72.45 ±0.47 <sup>a</sup>	76.31±0.21 <sup>a</sup>	81.43±0.41 <sup>a</sup>	56.69±0.09 <sup>a</sup>	61.44±0.23 <sup>a</sup>	64.63±0.39 <sup>a</sup>	67.51±0.340 <sup>a</sup>	71.52±0.34 <sup>a</sup>	77.61±0.45 <sup>ns</sup>	77.75±0.37

Results are presented as mean±S.D, n=6. The values were statistically analysed by two-way analysis of variance (ANOVA) followed by Tukey's test. Results considered significant (p<0.05) as compared to Piroxicam on respective day. a: statistically significant as compared with piroxicam, ns: Not significant.

***In vitro anti-arthritic potential***

***Protein denaturation assay using egg's albumin***

The assay was performed according to the method described earlier (Pavithra *et al.*, 2015). The 5ml reaction mixture consisted of 0.2ml egg albumin, 2.8ml PBS (pH 6.4) and 2ml of extract solution at 50, 100, 200, 400, 800 and 1600µg/ml concentrations. The standard solution contained piroxicam/ diclofenac sodium (DS) instead of extract. The control solution contained DW while 5% DMSO for n-hexane and butanol instead of the extract solutions (in all the *in vitro* anti-arthritic tests). These solutions were incubated at 37°C for 0.25 h followed by heating at 70°C for 5 min. Then the solutions were cooled to room temperature and their absorbance was measured at 660 nm. The test was performed in triplicate and percentage inhibition of protein denaturation was determined.

Percentage inhibition = (Absorbance of control – Absorbance of test sample) × 100 / Absorbance of control

***Protein denaturation assay using BSA***

The assay was performed by following the procedure described earlier (Naz *et al.*, 2017). In this assay, 0.5ml test control solution contained 0.45ml BSA and 0.05ml DW. Product control contained 0.5ml extract solution. Test solution (0.5ml) comprised of 0.45ml BSA and 0.05

ml of extract solution at 50, 100, 200, 400, 800 and 1600 µg/ml concentrations. The standard solution (0.5ml) comprised of 0.05ml piroxicam/DS in place of extract solution. pH of all these solutions was set at 6.3 and incubated at 37°C for 20 min followed by heating at 60°C for 3 min. After cooling to room temperature, 2.5ml PBS (pH 6.3) was added to each tube and absorbance was measured at 660 nm. The assay was carried out thrice and percentage inhibition of BSA denaturation was calculated.

***Human red blood cell membrane (HRBC) stabilization assay***

For this assay, 3 ml blood from a healthy volunteer was drawn who had not taken any NSAID for the last two weeks. Blood was mixed with Alsever's solution and centrifuged at 3000 rpm for 15 min to separate packed cells. Packed cells were washed with isosaline solution (0.85% w/v NaCl, pH 7.2) thrice. Packed cell suspension (10% v/v) was made using isosaline solution and used immediately (Ghavi pour *et al.*, 2017).

Test solution contained 1ml PBS (pH 7.2), 2ml hypotonic saline solution (0.36% w/v NaCl), 0.5ml extract solution of different concentrations (similar to protein denaturation assay) and 0.5ml blood suspension. The standard solution contained piroxicam/DS while control solution contained

2ml DW or DMSO in place of extract solution. All solutions were incubated at 37°C for 0.5h followed by centrifugation at 3000 rpm. The absorbance of supernatant from each tube was measured at 560 nm. The test was performed in triplicate and mean value were calculated. The percentage stabilization of HRBC was calculated.

#### **Proteinase inhibition assay**

The assay was performed by following the method described in a previous method (Naz *et al.*, 2017). In this assay, 2ml sample solution was comprised of 60µl trypsin (0.6µg), 1ml test extract (in concentrations similar to protein denaturation assay) and 1ml tris HCl buffer (25mM pH 7.4). The blank solution contained tris HCl buffer while the standard solution contained piroxicam /DS instead of the extract solution. All solutions were incubated at 37°C for 5 min. afterwards 1ml casein (0.8% w/v) added. Again, solutions were incubated for 20 min. Perchloric acid (2ml, 70%v/v) was added to stop the reaction followed by centrifugation at 5000 rpm for 5 min. The absorbance of the supernatant was determined at 280 nm. The test was carried out thrice and the percentage inhibition of proteinase enzyme was calculated.

#### **In-vivo anti-arthritis activity**

##### *Formaldehyde induced arthritis in Wistar rats*

Wistar rats of both sexes, weighing 150-200g were housed in the Animal house of the Faculty of Pharmaceutical Sciences, GC University Faisalabad under standard laboratory conditions (25±2°C and 60-70% humidity). These rats were acclimatized for two weeks in steel cages with 5 animals in each cage having free access to standard pellet diet and water. The animal study protocol was approved by the Institutional Review Board, GC University, Faisalabad, Reference No. GCUF/ERC/19574.

The experiment was carried out according to the previous method (Hasan, 2018). Wistar rats were divided into 12 groups with 6 animals in each group. Normal and arthritic control groups received DW while standard group received piroxicam (10mg/kg). Treatment groups received methanolic, aqueous and ethyl acetate extracts at 150, 300 and 600mg/kg/day orally for 10 days respectively. Arthritis was induced on day 1 by subplantar injection of 0.1ml 5% v/v formalin in left hind paw 1 h post administration of treatment. Injection was repeated on the third day.

#### **Assessment of the severity of arthritis**

Paw volume was measured with plethysmometer (Panlab, Spain) starting from day 1 before induction. Percentage inhibition of paw edema was calculated. Body weight of each animal was also measured at the start and end of the study. At the end of study, the rats were anesthetized with chloroform and blood was withdrawn by cardiac puncture for appraising the effect of treatment on blood and biochemical parameters.

#### **Histopathological evaluation**

Animals were killed by cervical dislocation after anesthetising with ether. The ankle joints from left hind paws were excised and stored in 10% formalin solution. Ankle joints were decalcified by placing in 10% EDTA for 30 days. Afterwards, ankle joints were embedded in paraffin wax and sectioned into 5µm thickness. The tissues were stained with H&E dye and observed under a photomicroscope (Meiji Techno, Co. Japan) for pathological changes (Akhtar *et al.*, 2018).

#### **STATISTICAL ANALYSIS**

The results were expressed as mean±S.D. The data were analyzed by Graphpad Prism software version 6 by one or two way analysis of variance (ANOVA) followed by Tukey's multiple comparison test.

#### **RESULTS**

The percentage yield of all extract of *P. braunii* was calculated. The percentage yield of plant methanolic (18.64%) extract was the highest followed by aqueous (15.78%), butanol (8.42%), ethyl acetate (6.66%) and n-hexane (4.02%) respectively.

#### **In vitro anti-arthritis potential**

##### *Activity against egg albumin denaturation*

The percentage inhibition of egg albumin denaturation was maximum with methanolic extract (91.27±0.28%) at 1600µg/ml in disparity to aqueous (82.14±0.12%), ethyl acetate (70.67±0.58%), butanol (69.2±0.18%) and n-hexane (60.48±0.41%) extracts. All plant extracts impeded protein denaturation dose dependently with remarkable effect observed at the highest concentration as mentioned in (fig. 1a). The value of IC<sub>50</sub> of percentage inhibition of egg albumin denaturation was demonstrated in table 1.

##### *Activity against BSA denaturation*

The protection of BSA against denaturation by the plant extracts was tested. The maximum percentage denaturation of BSA was shown by methanolic extract (88.42±0.36%) followed by aqueous (84.28±0.24%), ethyl acetate (78.14±0.12%), butanol (68.56±0.48%) and n-hexane (62.18±0.17%) at 1600µg/ml as depicted in (fig. 1b). Percentage denaturation by methanolic extract was statistically insignificant from standard DS at all equivalent concentrations. The value of IC<sub>50</sub> (µg/ml) in BSA denaturation assay was mentioned in table 1.

##### *HRBC membrane stabilization activity*

The plant extracts stabilized the RBC membrane in dose dependent manner with the highest stabilization at 1600 µg/ml. The percentage inhibition of RBC membrane lysis was the maximum with methanol (93.35±0.31%) and aqueous (84.40±0.34%) extracts as compared with DS

(92.01±0.02%) and piroxicam (81.34±0.29%) at 1600 µg/ml as shown in (fig. 1c). The result of IC<sub>50</sub> of percentage stabilization of the plant extract and fractions was mentioned in the table 1.

#### **Effect on anti-trypsinase activity**

All tested extracts showed the maximum hindrance to trypsinase activity as noticed at 1600µg/ml and the minimum at 50µg/ml. The percentage inhibition of trypsinase activity was maximum with methanolic extract (85.28±0.24) in contrast to aqueous (81.42±0.37%), ethyl acetate (75.35±0.30%), butanol (71.42±0.37%) and n-hexane (58.21±0.18%) fractions. The results were mentioned in the (fig. 1d). The IC<sub>50</sub> value of percentage inhibition of trypsinase activity was expressed in table 1.

Results are presented as mean ± S.D. The values were statistically analyzed by two-way analysis of variance (ANOVA) followed by Tukey's test. Results considered significant (p<0.05) as compared to piroxicam and DS.

#### **In vivo anti-arthritic activity**

##### **Effect on paw volume in formaldehyde induced arthritis**

The paw volume increased in all groups after arthritis induction with formaldehyde. The volume reduced steadily as treatment with the plant extracts continued and in dose reliant with pronounced effect exhibited by the highest dose (600mg/kg) of each extract of the plant. The percentage inhibition of paw volume was more with the methanolic extract than aqueous and ethyl acetate extract at 600mg/kg at day 10 as shown in table 2. The percentage inhibition by methanolic extract was statistically significant (p<0.05) at all doses from the piroxicam (10mg/kg) at respective day exclusive of 300 mg/kg dose of the extract at day 8.

##### **Effect on blood parameters**

The level of RBCs, hemoglobin (Hb), total leukocyte count (TLC) and platelets fluctuated at varying degrees in different groups in contrast to the arthritic group, normal control and piroxicam group as shown in (fig. 2).

##### **Effect on liver function tests and body weight**

The level of total bilirubin, ALT and AST was not statistically altered in all groups in contrast to the normal, standard and arthritic control group as depicted in (fig. 3a, b &c). Arthritic control rats failed to achieve significant gain in body weight 10 days post arthritis induction in contrast to rats treated with piroxicam therapy or methanolic and aqueous extracts at 150, 300 and 600 mg/kg. The effect of *P. braunii* extracts on body weight of arthritic rats is expressed in (fig. 3d).

##### **Effect on histology**

Normal control rats were devoid of any inflammation, pannus formation and bone erosion. The histopathology of ankle joints after formaldehyde induced arthritis

displayed substantial inflammation in the arthritic joints in comparison to normal control and treatment groups as shown in fig. 4a and 4b. Significant reduction in inflammation, pannus formation and bone erosion were reported with methanolic extract (fig. 4d, e, f) than aqueous and ethyl acetate extracts treated rats with the most prominent reduction in inflammation noticed at 600 mg/kg as shown in (fig. 4d-i). Moreover, piroxicam treated rats also showed reduction in arthritic indices (fig. 4c).

## **DISCUSSION**

Proteins denaturation, occurring under stressful conditions due to exposure to chemicals and heat, provokes the generation of autoantigens that adversely affects joint synovial membrane and cartilage. All extracts hindered the denaturation of BSA and egg albumin at 50, 100, 200, 400, 800 and 1600µg/ml in dose dependent manner. The highest activity explored by methanolic extract than other tested fractions and standards. The presence of secondary metabolites like phenol, flavonoids, alkaloids in *P. braunii* extracts might have contributed to the protection from protein denaturation that may be one of the mechanisms involved in anti-arthritic activity of the plant (Hasan, 2018).

Lysis of lysosomal membranes causes leakage of enzymes like proteases and phospholipase A2 in case of arthritis. The RBCs have membranes similar to lysosomes. Therefore, agents protecting RBC membrane under stress might be capable of stabilizing endogenous lysosomal membrane. This membrane stabilizing potential of *P. braunii* extracts might be due to the presence of various phyto-phenols and flavonoids that have been implicated for protection of RBC membranes, and protecting the activities of ATPase enzymes (Anosike *et al.*, 2012).

Serine proteinases present in lysosomes of neutrophils provoke tissue injury in arthritis. Thus proteinase inhibition may reinforce and contribute to the anti-arthritic activity of drugs (Naz *et al.*, 2017). The plant extract and fractions exhibit anti-trypsinase activity at all tested concentration with maximum inhibition exhibited by methanolic and aqueous extracts.

Formaldehyde induced arthritis is a simple chronic animal model that clinically and pathologically resembles human arthritis (Thite *et al.*, 2014). Drugs exhibiting anti-arthritic potential in formaldehyde induced arthritis model block both neurogenic and inflammatory phases (Hasan, 2018). In the present research, the plant extracts effectively prevented arthritis by blocking the production and release of prostaglandin and proinflammatory cytokines.

Anemia, weight loss, thrombocytopenia and elevated TLC are attributed to arthritis as extra-articular

manifestations. In the present study, a decrease in Hb and RBCs count were observed in disease controlled rats however, although these were insignificantly reinstated by the plant extracts in arthritic rats. Weight loss in arthritis may be linked to malabsorption of nutrients from small intestine as observed in the disease control group. Treatment with the methanolic and aqueous extracts restored the increase in body weight possibly by improving intestinal function or mitigating disease severity. Moreso, thrombocytopenia and leukocytosis were corrected by *P. braunii* extracts, although the effect of methanolic and aqueous extracts was more pronounced than the ethyl acetate counterpart (Perumal *et al.*, 2017).

In this study, liver function parameters were insignificantly altered in all groups as compared with normal and disease control groups, may be an indicative of the safety of the plant extracts.

The methanolic and aqueous extracts of the plant exhibited the highest anti-arthritic potential in both *in vitro* and *in vivo* models can be associated to their flavonoid and phenolic contents as well as radical scavenging potential as reported earlier (Shabbir *et al.*, 2018).

## CONCLUSION

*P. braunii* roots have anti-arthritic potential as obvious from aforementioned results. Further studies on the mechanistic analysis of anti-arthritic activity as well as isolation of pure compounds of *P. braunii* should be carried out.

## ACKNOWLEDGMENTS

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