https://doi.org/10.48047/AFJBS.6.15.2024.14665-14681

African Journal of Biological Sciences

Journal homepage: http://www.afjbs.com

Research Paper Open Access

Evaluation of anti-arthritic activity of *Pluchea wallichiana* leaves in complete freund's adjuvant induced arthritis in rats

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Volume 6, Issue 15, Oct 2024

Received: 15 Aug 2024

Accepted: 25 Sep 2024

Published: 21 Oct 2024

doi: 10.48047/AFJBS.6.15.2024.14665-14681

Abstract

Rheumatoid arthritis (RA) is a chronic autoimmune disease marked by joint inflammation and damage, affecting 0.5 to 1% of the global population and leading to irreversible cartilage and bone changes. *Pluchea wallichiana* is known for its anti-inflammatory potential. The plant was subjected to phytochemical quantitative analysis using the aluminum chloride method. The flavonoid content was found in the leaf, stem, and root of *P. wallichiana* as 27.81%, 18.85%, and 13.81%, respectively. The IC_{50} values for antioxidant activity were 20 µg/ml for the leaf, 21.5 µg/ml for the root, and 40 µg/ml for the stem, compared to 32.3 µg/ml for ascorbic acid, indicating superior antioxidant potential of leaf. In an *in vitro* anti-inflammatory study using the protein denaturation method, the ethanolic extract of *P. wallichiana* leaves showed significant inhibition, reducing protein denaturation by 98.40%. This study explores the anti-rheumatic effects of *Pluchea wallichiana* in rats with arthritis induced by complete freund's adjuvant (CFA). The ethanolic extracts of *P. wallichiana* leaves at doses of 100, 250, and 500 mg/kg was given as a treatment from 14th day. By day 28, histological analysis showed significant reductions in bone degradation, joint inflammation, and pannus formation. Blood tests revealed that *P. wallichiana* lowered C- reactive protein, rheumatoid factor, and total lipid levels, while improving RBC counts and hemoglobin. Higher doses of the leaves extract were more effective in alleviating RA symptoms. The findings indicate that *P. wallichiana* has substantial antiarthritic potential.

INTRODUCTION

The herbal medicine remained integral to the medical practices of certain communities. The gradual development and transmission of knowledge about plant-based remedies formed the basis of numerous traditional medical systems worldwide. [Kunle et al., 2012] Several prevalent autoimmune diseases such as myasthenia gravis, serum sickness, pernicious anemia, and arthritis. Among them rheumatoid arthritis (RA) is a chronic, inflammatory and systemic autoimmune disease.[Mehta et al., 2012] The primary symptoms of RA include pain, swelling, and destruction of cartilage and bone as a result of which permanent disability occur.[Lin, B et al. (2014)] The significant challenges to the medical and pharmaceutical communities due to the unclear pathophysiological mechanisms of RA, but it is suggested that the release of specific free radicals, including nitric oxide and superoxide radicals generated during cellular metabolism, may play a pivotal role. These free radicals could potentially trigger the synthesis of interleukins (IL) and tumor necrosis factor (TNF-α) by T cells which in turn, have the capability to modulate the production of growth factors, cytokines, and adhesive molecules on immune cells. Such molecular events are believed to contribute to tissue inflammation and destruction. Traditional herbal medicines used in various tribal cultures. Many herbal medicinal plants are being increasingly recognized for their potential therapeutic roles in RA. This is particularly significant because currently available synthetic drugs like NSAIDs (Non-Steroidal Anti-Inflammatory Drugs) and DMARDs (Disease-Modifying Anti-Rheumatic Drugs) not only pose economic burdens but also come with a range of adverse effects. such as ulcers, gastrointestinal bleeding, kidney as well as liver damage, and hypertension.[Nimesh, S. (2018)] *Pluchea wallichiana* DC, a species within the genus *Pluchea*, is widely distributed across America, Africa, Asia, India, Pakistan, and Australia.[Hussain, H et al., (2013)] The phytoconstituents reported in *Pluchea wallichiana* include β-amyrin and β-sitosterol, syringaresinol, Pluviatilol, and flavanoids including Apigenin 7-O-β-D-glucoside,[Kazmi et al., (2012)] Kaempferol 3-O-β-D-galactoside, and Kaempferol 3-(2’’, 3’’-diacetyl-4’’-p coumaroyl rhamnoside). [Bera, K. and Patel, M. (2023)] The genus *Pluchea* exhibits diverse biological properties including anticancer,[Bauer S, et al. (2011)] immunosuppressive,[Bhagwat, D., et al. (2010)] antioxidant,[Sen, T., et al. (2002)] anti-acetylcholinesterase,[Noridayu, et al. (2011)] antimicrobial,[Cordova, W., et al (2006)]

hepatoprotective,[Sen, T., et al. (1993)] anti-ulcer,[Sen, T., et al. (1993)] anti-inflammatory,[Chawla, et al. (1991)] and antinociceptive activities.[Figueredo, S., et al. (2011)] Given its broad therapeutic potential, establishing the *Pluchea wallichiana* pharmacological profile is essential to provide scientific evidence for future reference to ensuring its safety and effectiveness.

REAGENTS AND APPARATUS

Quercetin standard was sourced from Yucca Enterprise (Mumbai). Methanol, aluminum chloride, and sodium nitrite were obtained from Fisher Scientific (USA). Complete Freund's Adjuvant (CFA) and Diclofenac Sodium was purchased from Sigma Chemical Co. (USA), and Ascorbic acid from Merck (Germany). All other chemicals utilized were of analytical grade. The digital plethysmometer used was from orchid scientific (Nashik).

MATERIAL AND METHODS

Collection of plant material

In the month of September 2022, the entire *Pluchea wallichiana* plant was harvested from the herbal garden of Parul Institute of Ayurved, Parul University Limda, Vadodara, Gujarat. The plant material was identified and authenticated by Blatter Herbarium, Parul Institute of Ayurved, Parul University, Vadodara. The leaves, stem and root part of the plant were cleaned and shade dried for 14 days and made a fine powder. The plant materials were stored separately in airtight containers for further use.

Preparation of extract

The prepared coursed powder of leaves, stems, and roots of *Pluchea wallichiana* was extracted with ethanol (95%) using soxhlet extractor up to 4-6 hrs. Then the extract was concentrated in rotary vaccum evaporator. The percentage yield of extract was calculated in reference to material taken initially.

Preliminary phytochemical screening [Bera, K. and Patel, M. (2023)]

The Preliminary phytochemical screening of the *Pluchea wallichiana* ethanolic extract of root, stem and leaf was conducted to detect for the presence of several chemical components such as tannins, steroids, proteins, starches, flavonoids, saponins, terpenoids, and alkaloids.

Total Flavonoid Content

The extract was evaluated using an aluminium-chloride colorimetric assay. In a 10 ml volumetric flask, 1 ml of the extract (leaf, root and stem) solution (1 mg/ml) was mixed with 4 ml of distilled

water. To this, 0.30 m of 5% sodium nitrite solution was added. After 5 minutes, 0.30 ml of 10% aluminium chloride ($AlCl_3$) solution was introduced. Following another 5 minute interval, 2 ml of 1.0 M sodium hydroxide (NaOH) was added, and the mixture was diluted to the 10 ml mark with distilled water. Standard solutions at concentrations of 100, 80, 60, 40, and 20 µg/ml were prepared in the same way. The absorbance of the root, leaf, and stem extracts, along with the standard solutions, was measured at 420 nm using a UV/Visible spectrophotometer [Bera & Patel, 2023; Saeed et al., 2012].

2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay

Plant extracts (leaf, root, and stem) and standard ascorbic acid solutions (0.1 ml) at varying concentrations (10, 20, 40, 60, 80, and 100 µg/ml) were added to 3 ml of a 0.004% w/w ethanol solution of DPPH. Ethanol and DPPH in equal propotion served as the control. After 30 minutes of incubation in the dark, absorbance was measured at 517 nm, and the percentage inhibition activity was calculated using the formula [(A0 - A1) / A0] × 100, where A0 represents the absorbance of the control and A1 represents the absorbance of the extract or standard. The antioxidant activity was expressed as IC_{50}, which is the concentration of the extract (in µg/ml) required to inhibit 50% of DPPH radicals. All tests were conducted in triplicate, and the results were averaged to plot the graph. [Bera, K. and Patel, M. (2023), Srisook, K., et al. (2012)]

***In-vitro* anti-inflammatory activity**

Protein denaturation assay

The test solution (5 ml) was consisted 0.2 ml of bovine serum albumin (BSA**,** 5% w/v aqueous solution), 2.8 ml of phosphate buffer solution and 2 ml of sample solution(1mg/ml root, stem ad leaves extract) of different concentrations (50 µg/ml, 100 µg/ml, 200 µg/ml, 400 µg/ml, 800 µg/ml, 1600 µg/ml). Control solution was prepared by replacing sample solution with 2 ml of distilled water. Standard solution (5 ml) consisted of 0.2 ml of bovine serum albumin (5% w/v aqueous solution) add 2.8 ml of phosphate buffer solution and 2 ml of diclofenac sodium of different concentrations (50 µg/ml-1600 µg/ml). Following adjustment of pH to 6.3 using 1 N HCl, the samples were incubated at 37°C for 20 minutes and subsequently heated at 70°C for 5 minutes. After cooling to room temperature, absorbance (Abs) was measured at 660 nm using a UV-visible spectrophotometer. The control group represented 100% protein denaturation. The results were compared against diclofenac sodium, used as the standard. [Volluri, et al. (2011), Alamgeer, Uttra,

A, and Hasan, U. (2017), Jayaprakasam, R. and Ravi, T. (2012)] The percentage inhibition of protein denaturation was determined using the following formula.

$$\% \text{ inhibition} = \frac{\text{Abs of control - Abs of test}}{\text{Abs of control}} \times 100$$

Experimental animals

Healthy female rats, weighing between 150 and 280 g, were housed in polypropylene cages under standard conditions. They were kept on a 12-hour light/dark cycle at an ambient temperature of 25 ± 2°C with a relative humidity of 45–55%. The animals had ad libitum access to commercially available feed and water. The experimental protocol (PIPH 03/22) was approved by the Institutional Animal Ethics Committee.

Evaluation of anti-arthritic potential [Lin, B et al. (2014)**,** Alamgeer, Uttra, A, and Hasan, U. (2017)**]**

The study spanned from day 0 to day 28, segmented into two intervals: 0–14 days (developing phase) and 14–28 days (developed phase). Arthritis was induced in all rats, except those in the control group, by sub-plantar administration of 0.1 ml of Freund's complete adjuvant (CFA) into the left hind paw. Group 1 (Normal control, NC) received a 0.5% saline solution orally. Group 2 (Disease control, DC) was administered CFA via sub-plantar injection. Group 3 (Positive control, STD) received diclofenac orally at 10 mg/kg. Groups 4, 5, and 6 were orally treated with ethanolic extract of *Pluchea wallichiana* leaves at doses of 100 mg/kg (T1), 250 mg/kg (T2), and 500 mg/kg (T3) respectively. Treatment began on the 14th day post-CFA injection and continued daily until the 28th day. Following CFA injection, the volume of the left hind paw of each rat was measured using a plethysmometer on days 0, 7, 14, 21, and 28, while joint diameters were measured with a Vernier caliper on the same days. On the 28th day, blood was collected from all groups via retro orbital vein puncture after anesthesia with diethyl ether to assess biochemical parameters including Hemoglobin (Hb), White Blood Cell (WBC) count, Red Blood Cell (RBC) count, Erythrocyte Sedimentation Rate (ESR), C - reactive protein (CRP), Lipid profile, and Rheumatoid Factor (RF). Subsequently, the animals were euthanized using a high dose of diethyl ether. Ankle joints from the right hind paws were excised and preserved in 10% formalin solution. These joints underwent hematoxylin and eosin staining and were examined under a light microscope to evaluate bone erosion, inflammation, pannus formation, and destruction of joint space.

Statistical analysis

The results are presented as Mean ± SEM for six animals. Statistical comparisons between drug-treated and disease control groups were performed using two-way ANOVA, followed by the Bonferroni test. Biochemical data were analyzed using one-way ANOVA, followed by Dunnett's multiple range test. All analyses were conducted with GraphPad Prism 5.0 software, with a significance level set at $P < 0.05$. The values are Means ± S.E.M. (n=6).

RESULT

Extraction yield and Preliminary phytochemical screening

The percentage yield of the ethanolic extracts of leaf, stem and root of *P. wallichiana* were calculated as 10%, 10% and 5.5% w/w respectively. The Preliminary phytochemical screening results depicted in table 1.

Table 1. Phytochemical constituents present in ethanolic extract of *P. wallichiana*.

Sr. No.	Chemical Test	Phytochemicals	Leaf	Stem	Root
1.	Dragendorff's test	Alkaloids	-	-	-
2.	Shinoda and lead acetate test	Flavonoids	+++	++	++
3.	Borntrager's and foam test	Glycosides	-	-	-
4.	$Fecl_3$ and lead acetate test	Tannins	-	+++	++
5.	Salkowski's & Liebermann burchard test	Steroids	+++	++	+
6.	Biuret test	Proteins	-	+	-
7.	Iodine test	Starch	+	+	+

[(+) = Present and (-) = Absent]

Determination of total flavonoid content

The result mention in table 2 suggested that the leaf consisted highest amount of flavonoids as compared to root and stem. The amount of flavanoids found in leaf stem and root was 27.81%, 18.85% and 13.81%, respectively.

Anti-oxidant potential

The study was revealed the IC_{50} value of leaf (20 µg/ml), root (21.5µg/ml) and stem (40 µg/ml) while the IC_{50} value of ascorbic acid was found 32.3 µg/ml. The result indicated the higher

antioxidant potential of leaf (Figure 1)

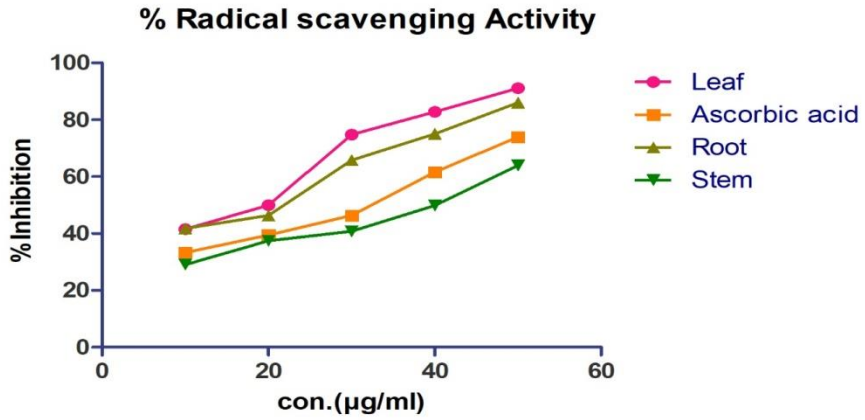

Figure 1. DPPH radical scavenging activity of ethanol extract of *Pluchea wallichiana*

Effect on protein denaturation

The inhibition of protein denaturation caused by various concentrations of ethanolic extract of *P. wallichiana*. Ethanolic extract of *P. wallichiana* leaves, stems, and roots at a concentration of 1600 g/ml produces considerable inhibition ($p < 0.001$) of 98.40%, 93.86%, and 29.70%, respectively, whereas diclofenac sodium produced 77.03% inhibition. Based on the findings from our study (Figure 2), it can be concluded that the ethanolic extract of *P. wallichiana* leaves exhibited significant inhibition of protein denaturation.

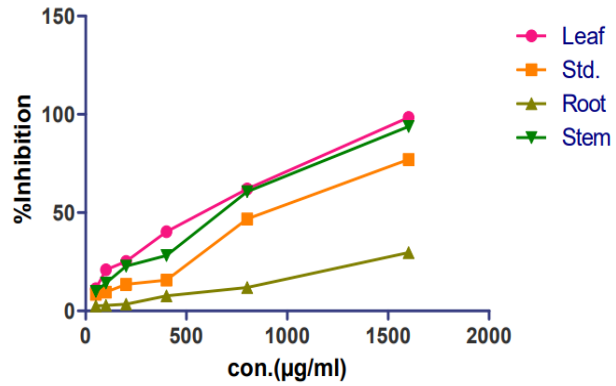

Figure 2. Effect on protein denaturation by *Pluchea wallichiana* root, stem and leaf

Effect of *P. wallichiana* leaves extract on paw volume

During the primary phase of arthritis (Day 7 to 14), a non-significant reduction in paw volume was observed in the extract-treated rats. However, following this phase, a significant decrease in paw volume was noted in the extract-treated group compared to the arthritic control rats (Figure 3).

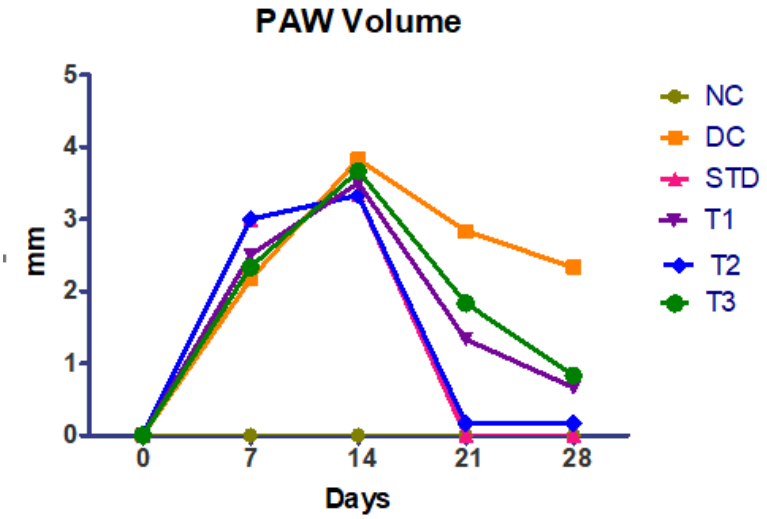

Figure 3. Effect of *P. wallichiana* leaf extract o paw volume

Effect of *P. wallichiana* on Paw diameter

Rats treated with CFA showed a significant increase in joint diameter compared to the normal control group. However, treatment with the ethanolic extract of *P. wallichiana* led to a significant reduction in joint diameter between Day 14 and Day 28, compared to the CFA-treated rats (Figure 4).

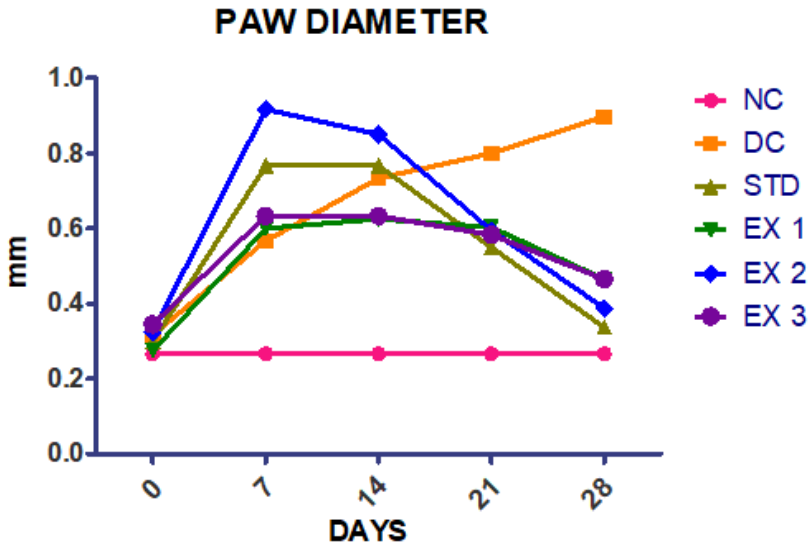

Figure 4. Effect of ethanolic

extract of *P. wallichiana* on paw volume

Hematological parameters

Hematological parameters including Hb, RBC, WBC, ESR, RF and CRP were measured for all animal groups including normal control group (Figure 5). All the haematological parameters assessed in the CFA-control group were abnormal in comparison to normal rats. There was a significant increase in WBC, ESR, CRP, RF and a significant decrease in RBC together with Hb in disease control group. Rats treated with *P. wallichiana* ethanol extract (100, 250 and 500 mg/kg) showed significant decrease in WBC when compared to disease control groups whereas treatment with *P. wallichiana* extract (500 mg/kg) significantly increase in the RBCs and Hb level as compared to disease control groups.

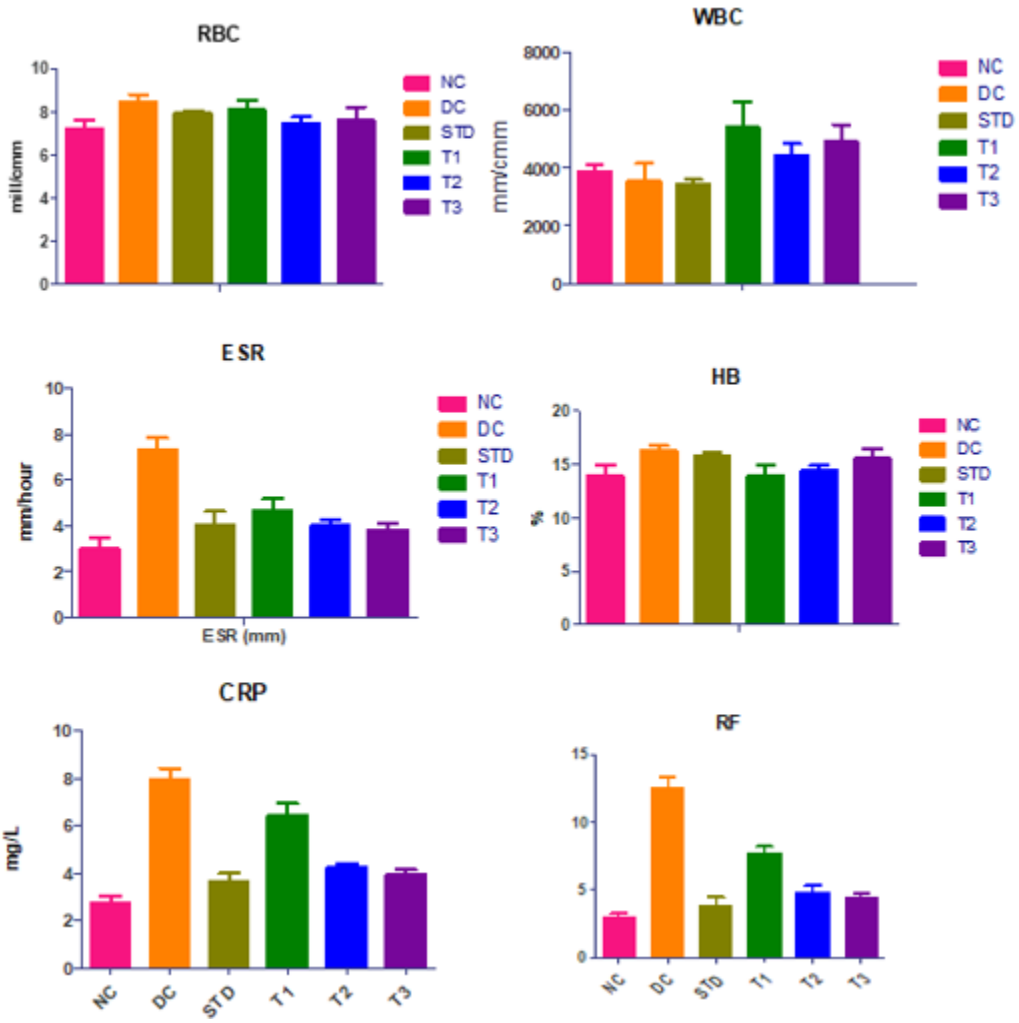

Figure 5. Effect of *P. wallichiana* leaves extract on hematological profile

The values are Means ± S.E.M. (n=6), * P < 0.05, ** P < 0.01, ***P < 0.001compared with control group. Statistical Analysis was carried out using One way ANOVA followed by Dunnett's test, indicating significant difference compared to arthritic group.

Lipid profile

Effect of *P. wallichiana* extract on the lipid profile of arthritic rats as seen in Figure 6, on administration of CFA, a marked increase was seen in the plasma triglyceride levels, plasma cholesterol, LDL, VLDL levels and decrease in HDL levels. On treatment with *P. wallichiana* and Diclofenac sodium, a marked decrease in plasma triglyceride levels, plasma cholesterol, LDL, VLDL levels was seen, along with an increase in HDL levels.

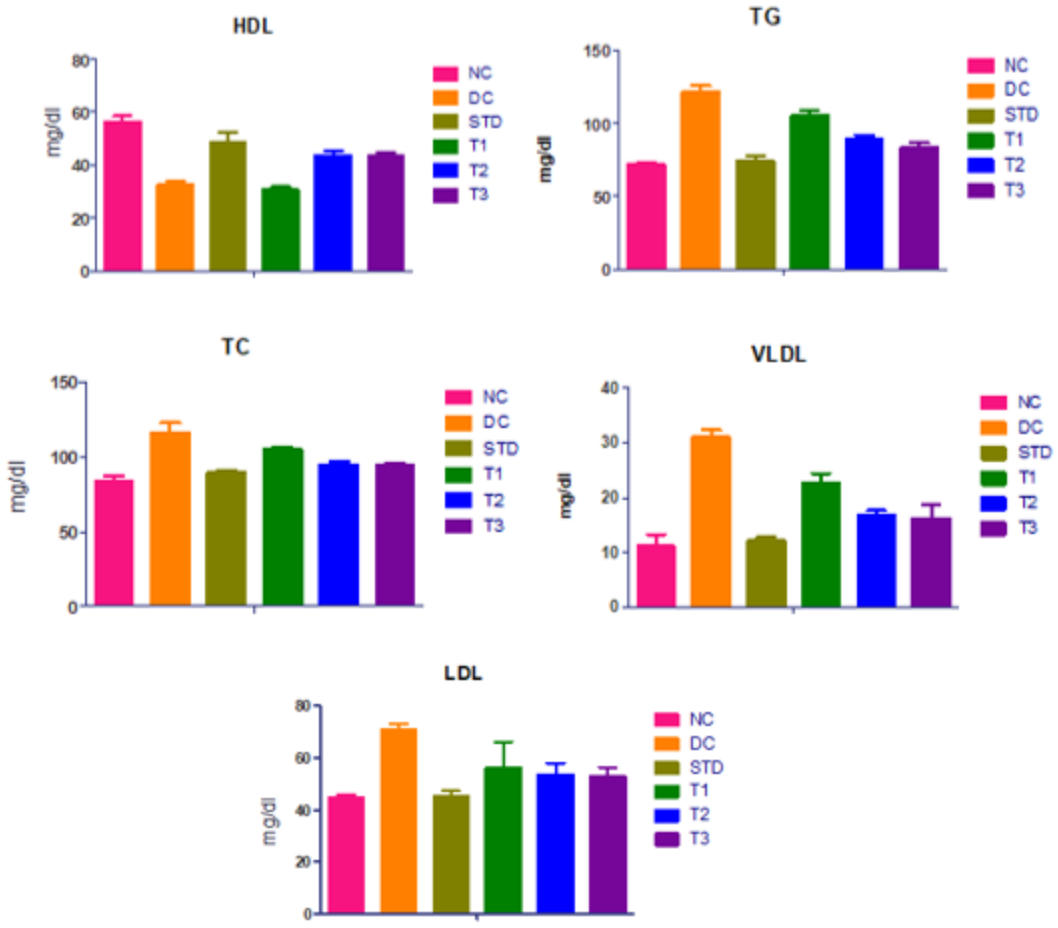

Figure 6. Effect of *P. wallichiana* leaves extract on lipid profile

Histopathological Changes in Ankle Joints

The section of collected hind paws, stained with Hematoxylin & Eosin, and examined under a light microscope. Figure 7 showed the corresponding results. There was no evidence of inflammation in the synovial tissue in the normal group. The synovial space retained its original structure, and the cartilage surface remained smooth (Figure 7(A)). However, in CFA treated group (Figure 7(B)), rats showed rough cartilage surfaces, and increased synovial spaces. The paw tissue of arthritic rats showed intense inflammatory infiltration in the synovial and pericapsular tissue, and pannus formation. Additionally, rats treated with an ethanolic leaf extract of *P. wallichiana* was capable to recover from RA. These histopathological alterations were greatly reduced by treatment with an ethanolic extract of *P. wallichiana* (Figures 7 (D)). There was no visible inflammation in the synovial and perisynovial soft tissue, pannus formation and the cartilage surfaces remained smooth (Figures 7 (E), and (F)).

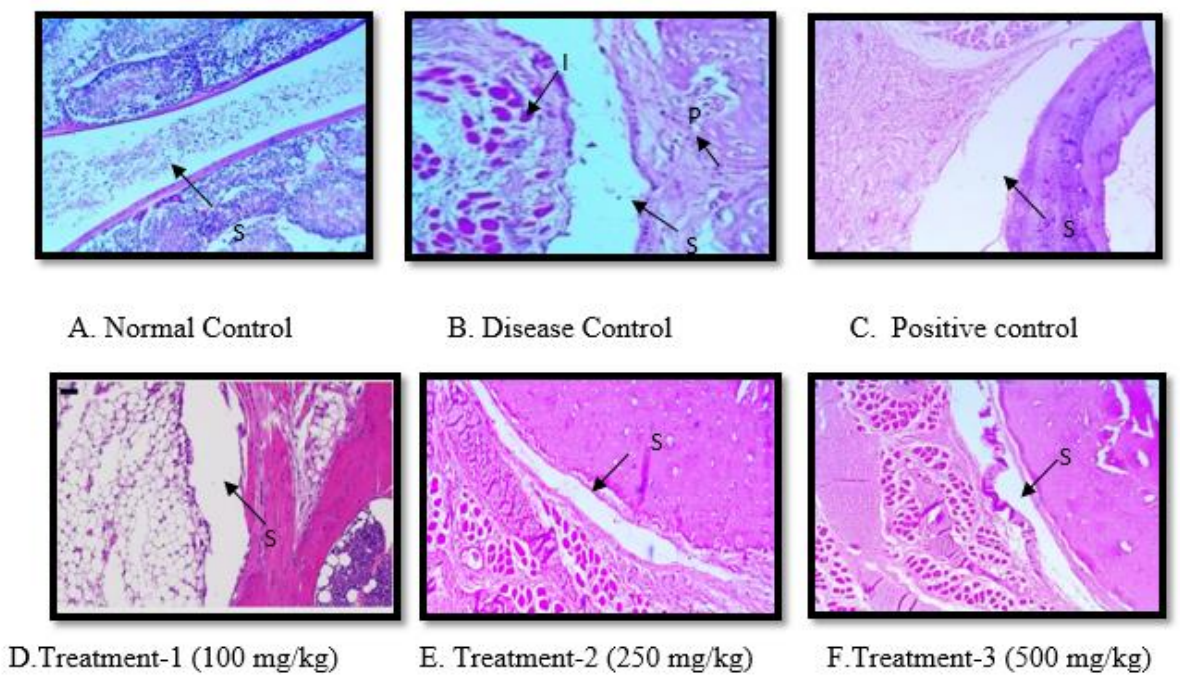

Figure 7. Histopathological changes in ankle joints. A. Normal synovial space(S) B. Presence of pannus (P), and infiltration of inflammatory cells (I) C. Reduction of inflammation, and pannus D,E. normal synovial structure and absence of inflammation and pannus .

DISCUSSION

The chemical evaluation revealed the presence of steroids, tannins, flavanoids, and phenolics in *P. wallichiana*. The various plant species have been reported to exhibit antioxidant activities, which

have been attributed to phenols and flavonols [Zovko Koncic, et al. (2010), Ruiz, et al. (2010)]. The ethanol extracts of root, leaf, and stem were evaluated for their antioxidant potential by measuring their ability to scavenge the stable diphenyl-2-picryl hydrazyl (DPPH) free radical. DPPH is a stable radical with an unpaired electron, commonly used to assess radical scavenging potential. The process involves observing the discoloration of the DPPH radical as it is neutralized by the plant extract, which indicates the extent of antioxidant potential. [Saeed, N, et al.(2012)] Denaturation occurs when a protein loses its natural structure due to external influences such as heat, extreme pH levels, or chemical agents. This structural alteration can result from modifications in electrostatic interactions, hydrogen bonds, hydrophobic interactions, and disulfide bonds within the protein molecule. [Grant, N, et al. (1970)] In this study, *P. wallichiana* was found to inhibit the denaturation of albumin. This suggests that compounds present in *P. wallichiana* may protect proteins from losing their secondary and tertiary structure under stressful conditions. [Prasad, S. and Yashwant, Aeri V. (2013)]. Among the different parts of the *P. wallichiana* plant studied, the leaf extract showed the highest inhibitory effects on protein denaturation compared to other parts of the plant. This suggests that the bioactive compounds responsible for inhibiting protein denaturation are more concentrated in the leaves. The increase in absorbance observed in the test samples compared to the control suggests that the extract has the ability to reduce thermal denaturation of proteins, specifically albumin. Edema refers to swelling caused by fluid accumulation.[Scallan, J., et al. (2010)] The study found that *P. wallichiana* inhibits edema in the paws. This anti-edematous effect indicates potential anti-inflammatory properties of *P. wallichiana*. In the current study, initial lesions appeared 3 to 5 days following CFA injection, while secondary lesions developed around days 11 to 12. Previous research has indicated that rheumatoid arthritis (RA) deformities are triggered by the release of certain inflammatory mediators such as , PDGF, IFN α, and cytokines like IL-1, IL-6, and TNF-α. [Patil, M. et al. (2012).]. Anemia, the most common extracellular manifestation in rheumatoid arthritis (RA), arises from lowered levels of hemoglobin (Hb) and red blood cells (RBCs). This condition is attributed to decreased erythropoietin [29] and reduced plasma iron, influenced by IL-1. [Hasan, U. H., et al. (2015).]. Furthermore, the release of IL-1 and TNF-α during arthritis leads to an increase in white blood cell (WBC) and platelet counts [Maria, M., et al. (1983)]. Additionally, proteins such as fibrinogen, α- and β-globulins, produced in response to inflammation, elevate the erythrocyte sedimentation rate (ESR) [Maria, M., et al. (1983)]. In

addition, rheumatoid factor (RF) serves as a primary serologic marker in arthritis [Martinez-Prat, L, et al. (2018).], acting as an autoantibody directed against the Fc segment of IgG. The findings indicated that extracts of *P. wallichiana* have positive influence on hematologic changes and histological damage. The decrease in rheumatoid factor (RF) levels due to the plant leaf extract point out a favorable indication of healing. The results depicted that *P. wallichiana* leaf extract had positively altered hematologic modifications and noticeable decrease in histological damage.

CONCLUSION

The present study investigated in *vitro* antiarthritic activity and anti-oxidant potential of ethanolic extract of *Pluchea wallichana* root, stem and leaf. It is concluded that *Pluchea wallichana* leaves has been observed to exert significant anti-arthritic and anti-oxidant effect. *P. wallichiana* shows promise as a medicinal plant with anti-arthritic effects, potentially mediated by its flavonoid content. These findings provide a basis for further research into its therapeutic applications and appraisal of exact mechanism of action.

ABBREVIATIONS

Abs: Absorbance; BSA: Bovine serum albumin; CFA: Complete freund's adjuvant; ESR: Erythrocyte sedimentation rate; Hb: Hemoglobin; RA: Rheumatoid arthritis; RBC: Red blood cell; RF: Rheumatoid factor; WBC: White blood cell; DPPH: diphenyl-2-picryl hydrazyl

Acknowledgements

We extend our sincere thanks to the management of Parul University for providing necessary facilities to carry out this research. The study was financially supported by Center for research and development, Parul University, Gujarat, India.

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