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Study of Therapeutic Potential of *Dodonaea viscosa* against Rheumatoid Arthritis in Collagen Induced Arthritic Mouse Model

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Abstract

Rheumatoid arthritis (RA), is a complex autoimmune disease associated with high rates of mortality and extreme disability. It is characterized by gradual joint destruction and a chronic inflammatory response. As current medications are not economical and provide complications, plant-derived therapeutic practices are now being explored. *Dodonaea viscosa*, a flowering plant, is well-known for its antioxidant, anti-inflammatory, anti-diabetic, wound healing, and analgesic activities but no in vivo anti-arthritic study have been conducted so far. Hence, in this study, we aimed to evaluate the toxicity and therapeutic potential of *Dodonaea viscosa* in a collagen-induced arthritic mouse model. Chemical analysis exhibited that *Dodonaea viscosa* has high levels of beneficial bioactive compounds, including phenols, flavonoids, and other phytochemicals. In ex vivo and in vivo studies, *Dodonaea viscosa* showed significant antioxidant, anti-inflammatory, and anti-arthritic potential, while no toxic effects were found. Arthritic mice treated with *Dodonaea viscosa* showed reduced levels of rheumatoid factor and paw edema, while no significant effects were found on spleen indices and radiological examination of paws, compared to control untreated arthritic mice. Our study showed that treatment with *Dodonaea viscosa* resulted in improvements in arthritis and could therefore be a viable therapeutic source for treating patients with rheumatoid arthritis. However, further studies on humans are required for validation.

Keywords: Rheumatoid Arthritis; Dodonaea viscosa; Phytochemicals; Antioxidant; Antiinflammatory; Anti-rheumatic

Introduction

Rheumatoid arthritis (RA), a complex autoimmune illness characterized by gradual joint destruction and a chronic inflammatory response, resulting in severe disability. is The disease affects nearly 1% of the world's population, with a higher incidence in females compared to males (3:1) and is associated with high rates of mortality [1]. Genetic, environmental, and epigenetic factors play a major role in the etiology of RA. Genetic markers, such as *HLA class II histocompatibility antigen, DR beta chain 1* (*HLA-DRB-1), protein tyrosine phosphatase non-receptor type 22 (PTPN22)* and *cytotoxic T-lymphocyte associated protein 4* (*CTLA4*), and environmental factors including obesity and smoking, have been correlated with RA pathogenesis [2].

In addition, the pathophysiology of RA is influenced by a variety of immune modulators such as effector cells and cytokines as well as signaling pathways. The joint injury occurs at the synovial membrane and extends to most IA components due to the complicated interplay of immune modulators [3], [4], Mononuclear cells (B cells, T cells, dendritic cells, plasma cells, macrophages, and mast cells) influx, their local activation, or both can induce synovitis, as can angiogenesis. The synovial membrane swells and produces villi, and the synovial lining becomes hyperplastic [5].Oxidative stress also plays an important role in the pathophysiology of RA by initiating the inflammation cycle [6], [7], [8].

Early diagnosis of RA, before the appearance of symptoms, is important for the effective treatment of RA. An antibody test to detect levels of rheumatoid factor (RF) or anti-citrullinated peptide antibodies (ACPA) in the blood is often used in the diagnosis of RA [9].

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In case of its treatment, till date, numerous targeted therapeutic approaches, such as *P13K* inhibitors, *HDAC* inhibitor, nanoparticles, and targeted protein degradation, are being investigated in pre-clinical trials [10], [11]. Yet, due to a lack of existing and effective therapeutic targeting strategy against RA, the focus has been to manage pain and slow damage to joints through the use of non-steroidal anti-inflammatory drugs (NSAIDs), steroidal drugs, disease-modifying anti-rheumatic drugs (DMARDs) and biologics [12]. However many individuals get symptom remission when utilizing various combinations of DMARDs, such as csDMARDS, bDMARDS, and tsDMARDS, in conjunction with NSAIDs. Although DMARDs and biologics provide drastic control of disease activity, patients suffer from side effects, persuading scientists to now move towards natural treatment options [13].

Dodonaea viscosa (Linnaeus) Jacquin, (family Sapindaceae) originally from Australia and endemic to Western America, is now available in most countries. Various compounds like volatile oils, flavonoids, saponins, cardiac glycosides, alkaloids, tannins, phenols, terpenoids, steroids, carbohydrates, and proteins are present in *D. viscosa* [14]. Many species of *Dodonaea* have been used frequently and extensively by traditional healers and society for a variety of diseases, making use of the leaves, stems, and even fruits of the plant. *D. angustifolia* and *D. viscosa* are the most commonly studied *Dodonaea* species [15]. *D. viscosa* has been used in folk medicine for a variety of ailments and pathological conditions, including anti-inflammatory, analgesic, spasmolytic, antiviral, anti-microbial, laxative, rheumatism, hypotensive, gout, fractures, wound healing, hemorrhoids, and snake bites [16], [17].

The present study aimed to screen the qualitative and quantitative amounts of beneficial phenols and flavonoids in *D. viscosa* and to evaluate the antioxidant, anti-inflammatory, and anti-arthritic activity of its leaves extract in arthritic mice. While *D. viscosa* has previously been explored for its anti-inflammatory, antioxidative, wound healing properties, specifically focusing on small molecule inhibitor to elevate the symptoms [18], however no in vivo model based studies of *D. viscosa* extract for treatment of RA has been performed up till now. Hence, this study mainly focuses on this approach to potentiate an effective therapeutic strategy towards treating RA.

Materials and Methods

2.1. Plant collection and extract preparation

Leaves of the D. viscosa plant (Figure 1) were collected from Margalla Hills, Islamabad, Pakistan.

Its dried specimen was submitted to Pakistan Museum of Natural History (PMNH), and the accession number 00046453 was retrieved. Subsequently, its leaves were dried and powdered. Extract of leaves was prepared in a 1:10 ethanol mixture (15 g in 150 mL) in a Soxhlet apparatus for 2 days. The extract was then filtered and dried to powder form for further use.

Figure 1. *Dodonaea viscosa*. The image shows the plant collected from Margalla Hills, Islamabad, Pakistan

2.2. Qualitative phytochemical screening

The ethanolic extract of *D. viscosa* was subjected to phytochemical analysis. The presence of alkaloids, phenols, flavonoids, tannins, terpenoids, glycosides, cardiac glycosides, saponins, steroids, sterols, anthraquinones, and anthocyanins was examined in the plant extract using previously published methods [19].

2.3. Quantitative phytochemical assays

2.3.1. Total phenolic content

Folin Ciocalteu (FC) reagent was used to determine total phenolic content (TPC), with few modifications in the preparation of chemicals [20]. Different dilutions (25, 50, 75, 100 µg/ml) of 1% stock solution of gallic acid and 0.5 mL of 1% stock solution of plant extract (not diluted) were prepared, to which 0.4 mL of FC reagent was added. The solution was vortexed and left to stand for 5 min. To this, 4 mL of 7.5% sodium carbonate was added and topped up with distilled water for a total volume of 10 mL. The mixture was then incubated in the dark for 2 hours. Absorbance was measured at 700 nm against a blank. A calibration curve was plotted of gallic acid and TPC was expressed in terms of gallic acid equivalent (mg of gallic acid/ g of extract).

2.3.2. Total flavonoid content

To determine total flavonoid content (TFC), a previously published method [21] was followed, with few changes. Different dilutions (25, 50, 75, 100, 125 µg/mL) of the standard rutin were prepared from stock solution (1 mg/ml). 100 µL of 1M

potassium acetate, 100 µl aluminum chloride and 2.8 mL distilled water were added to 100 µL of stock plant solution (1 mg/ml) and the prepared dilutions of standard rutin. The mixture was thoroughly shaken and then incubated at 25C for 30 min. Absorbance was measured at 415 nm against a blank. TFC was determined using a rutin calibration curve and the result was represented in mg rutin equivalent to per g dry weight of the extract.

2.4. in Vitro antioxidant activity measurement by DPPH assay

To determine the antioxidant activity of *D. viscosa* extract, a previously published protocol was followed [22], with few modifications in the chemical preparation. The ability to quench free radicals was determined by the purple staining of the DPPH (1-diphenyl- 2-picrylhydrazyl) solution. A stock solution of 0.2 mM DPPH (Sigma-Aldrich), standard ascorbic acid (5mg/5m-Lin methanol), and plant extract (1 mg/ml in methanol) were prepared and kept on ice. Ascorbic acid and plant extract solutions were prepared in different dilutions of methanol (10 µg, 20 µg, 30 µg, 40 µg). Following this, 50 µL of DPPH was added to 150 µL of prepared dilutions, in the dark. The plate was then also incubated in the dark, at room temperature for 30 min. Absorbance was measured at 515 nm using a plate reader. The percentage of inhibition was calculated by the formula:

$$\%\ Inhibition = \left(\frac{Control - Sample}{Control}\right) \times 100$$

2.5. in Vitro anti-inflammatory activity measurement by membrane stabilization assay

The red blood cell (RBC) membrane is comparable to the lysosomal membrane, and its stabilization is crucial for controlling inflammatory responses. A previously published protocol for the membrane stabilization assay [23] was followed, with few modifications. Fresh human blood was collected in ethylenediamine tetraacetic acid (EDTA) tubes and was centrifuged for 5 min at 3000 rpm, serum was discarded, and packed cells were washed 3 times with an isosaline solution. Then a 10% volume/volume suspension of packed cells was prepared with saline solution. The test solution contained 2 mL hypotonic saline solution, 1 mL sodium phosphate buffer, 0.5 mL of human red blood cell (HRBC) suspension, and different dilutions of 0.5 mL of 1% stock solution of the standard diclofenac potassium and plant extract. The mixture was incubated at 37°C for 30 min and then centrifuged at 3000 rpm for 5 min. Absorbance was measured at 560/620 nm. The % protection was found by the following formula:

$$\%\ Protection = \left(\frac{Control - Sample}{Control}\right) \times 100$$

2.6. in Vitro anti-arthritic determination by inhibition of albumin denaturation assay

Denaturation is a well-known biological cause of inflammation. It involves the loss of tertiary structures of proteins, and can occur under stress. In this assay, the potential of *D. viscosa* to prevent denaturation was assessed. The inhibition of protein denaturation was carried out using a previously published protocol [24], with few modifications. A reaction mixture of 5 mL was prepared. Different dilutions (200, 300, 400, 500, 600 µg/mL) of the standard ascorbic acid and plant extract were prepared. To this, 0.2 mL of 1% BSA and 2.8 mL of 0.2 M phosphate buffer saline (PBS) were added. The mixture was incubated in a 37°C water bath for 5 min and then transferred to a 70°C dry incubator for another 5 min. The mixture was cooled at room temperature and absorbance was measured at 620 nm. The percentage inhibition of albumin denaturation was calculated by the formula:

$$\%\ Inhibition\ of\ albumin\ denaturation = \left[\left(\frac{Control}{Sample}\right) - 1\right] \times 100$$

2.7. ex Vivo toxicity analysis by hemolytic assay

In the hemolytic assay, test reagents and RBCs are incubated at a specific pH. A spectrophotometer is used to quantify how much hemoglobin is released in the solution. Besides a few modifications, the protocol was followed as previously published [25]. Whole human blood was centrifuged in EDTA tubes at 500 x g at 4°C for 5 min. The supernatant (plasma) phase was discarded, and an equal volume of normal saline was added to the tubes. To obtain packed RBCs, the washing step was repeated three to four times. A 2 % erythrocyte solution (7.4 pH) was prepared by mixing the packed RBCs with PBS. The plant extract was serially diluted (1000, 100, 10, 1, 0.1 µL) in PBS. In a tube, 30 µL of already diluted plant extract and 550 µL of blood solution were combined. The mixture was incubated for 1 hour at 37°C. PBS in erythrocyte suspension was used as a negative control, while 20% triton-X in erythrocyte suspension was used as a positive control. After incubation, mixture tubes were centrifuged for 5 min at 500 x g to create blood pallets, and absorbance of supernatant was measured at 405-541 nm.

2.8. Animal procurement, acclimatization, and ethical approval

Bagg albino (BALB/c) mice (n=4), 6-7 weeks old and weighing 25-30 g, were procured from Atta-ur-Rahman School of Applied Biosciences (ASAB) Animal House, National University of Sciences and Technology (NUST), Islamabad. All mice were acclimatized for one week at the Animal House and provided with distilled water and normal feed. Approval for studies using the mouse model was obtained from the institutional review board committee with Performa number 32-IRB-ASAB-2016 at NUST, Islamabad, Pakistan.

2.9. Acute toxicity test

A procedure established previously by Chinedu et al. in 2013 [26], was followed to check the toxicity of our plant extract. This procedure involved three stages in which each stage contained three groups of BALB/c mice, age 5-6 weeks. Mice were acclimatized for 2-3 days and fasted for 24 hours before the experiment. In the first stage, groups 1, 2, and 3 of mice were given 50, 200, and 400 mg/kg of *D. viscosa* extract, respectively. Behavioral changes, dullness, and mortality were examined. In the second stage, the same three groups of mice were given 1000, 1500, 2000 mg/kg of *D. viscosa* extract and again behavioral changes and mortality were assessed. In the last stage, mice were given 3000, 4000, 5000 mg/kg of body weight of *D. viscosa* extract and again the same changes were assessed. This process was used to determine the right dosing of the *D. viscosa* extract.

2.10. Collagen-induced arthritic (CIA) mouse model

For induction of rheumatoid arthritis in mice, bovine collagen type ll (Worthington biochemical cooperation, Lakewood, NJ, USA) and Complete Freund's Adjuvant (CFA; Sigma-Aldrich, St. Louis, MO, USA) were used [27]. 0.1 M glacial acetic acid was prepared in Hartmann Solution and to this, collagen type ll was added in a ratio of 2:1 and kept on ice. The solution was mixed with CFA in a ratio of 1:1 and vortexed for 2-3 min. On days 0, 7, and 14, 0.2 mL/200 µL of this mixture was injected in the sub-dermal region of BALB/c mice. On day 21, a booster injection of CFA was given to the hind paws of the mice. Each week before injection, the paw was measured for inflammation with vernier caliper (GmBH). One week after the booster injection, the level of inflammation was assessed, Figure 2.

An arthritic score was given to each mouse as a marker for arthritis induction (Table 1).

2.11. in Vivo therapeutic evaluation of D. viscosa

After arthritis induction and the scoring method discussed above, the mice with grade scores three and four were selected for further analysis. Four groupings of mice were created: Group 1 (normal untreated), Group 2 (arthritic untreated), Group 3 (standard drug treated orally; leflunomide 10 mg/kg), and Group 4 (*D.viscosa* treated orally, with a dose of 300 mg/kg). The doses were administered for 14 consecutive days and paw measurements were recorded before every immunization injection and after treatment on weekly basis with a vernier caliper on days 0, 7, and 14. The weight of the mice was also measured on days 0, 7, and 14 of treatment to check whether it is affected or not before and after treatment. After 14 days, mice were sacrificed and paws, blood, and spleen were collected for downstream X-ray analysis and blood tests.

The spleen was weighed to calculate spleen indices. It is

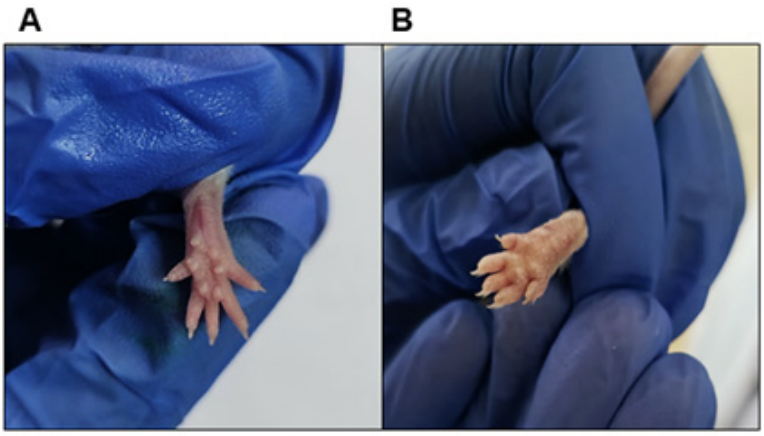

Figure 2. Representative images of arthritis induction. A- Normal paw with score 0; B- Arthritic paw with a score 4.

Table 1. Scoring table for arthritis induction. Scoring scheme ranges from 0 to 4 and associated conditions are described.

Grade Score	Condition
0	No swelling
1	Slight swelling of toe joint
2	Slight swelling of ankle, toe, wrist, and other small joints
3	Severe swelling of paw and abscess formation
4	Joint deformation, severe abscission

thought that the weight of the spleen is increased during induction. Spleen indices were calculated by the following formula:

$$Spleen\ Indices = \frac{Weight\ of\ Spleen\ in\ (mg)}{Total\ Body\ Weight\ of\ mice}$$

As RF is used as the diagnostic marker for rheumatoid arthritis in almost 80% of individuals, post-treatment with *D. viscosa*, RF levels were estimated in mice serum. The normal range of RF is between 14-20 IU/mL.

Paws were collected from sacrificed mice and examined by X-ray for joint structure destruction, spaces between joints, swollen joints, and any soft tissue inflammation.

2.12. Statistical analysis

Statistical analysis was performed with Prism 9.0 software (GraphPad Software). Variance analysis was performed by one-way and two-way ANOVA. Variations were considered significant at $p \leq 0.05$.

Results

3.1. Visual appearance

The prepared *D. viscosa* extract was dark greenish in color, it was filtered in a reagent bottle, covered with aluminum foil, and stored at 4°C for further use in experiments. Some of the filtered extract was also poured into a petri dish and air dried to solidify it, and after that, it was scratched and stored in a falcon at 4°C. It was found to be crystalline in appearance and sticky in texture if left outside the refrigerator at room temperature for a long period of time.

3.2 Qualitative phytochemical screening

To assess phytochemicals present in the plant, screening tests were performed as discussed in section 2. Alkaloids, anthraquinones, and anthocyanins were found to be absent in the ethanolic extract of *D. viscosa,* while all other phytochemicals tested were present (Table 2). The phytochemicals present show that this plant has antioxidant and anti-inflammatory activity.

3.3. Quantitative phytochemical screening (TPC and TFC in D. viscosa)

The Folin-Ciocalteu reagent was used to quantitatively determine the total phenolic content in terms of gallic acid equivalent. A linear regression graph was plotted of gallic acid and TPC was articulated as gallic acid equivalent (mg of gallic acid/g of extract). Total Phenolic Content was calculated from a linear regression equation (which in the conducted experiment was (y = 0.0003x - 0.002). A significant quantity of phenols was found in the ethanolic extract (291.22 ± 0.025).

Total Flavonoid Content was calculated by the aluminum chloride method. The total flavonoid concentration was determined using a rutin calibration curve, and the result was represented in mg rutin equivalent per g dry weight of the extract).

Table 2. Qualitative Phytochemical screening of *D. viscosa*.

Phytochemical Test	Phytochemical detection in *D. viscosa*	Appearance of Extract
Alkaloid	-	Yellow precipitate
Phenols	+	Blue/black colour solution
Anthraquinones	-	Pink/red/violet colour solution
Flavonoids	+	yellow precipitates
Anthocyanins	-	Pinkish red to bluish violet colour solution
Tannins	+	Transient green to black colour solution
Coumarins	-	Yellow colour solution
Terpenoids	+	Deep red colour solution
Steroids	+	Reddish brown colour solution
Sterols	+	Red colour at interface
Saponins	+	Formation of foam
Cardiac glycosides	+	Green/blue colour or brown ring

The + sign shows a positive result and the – sign shows a negative result.

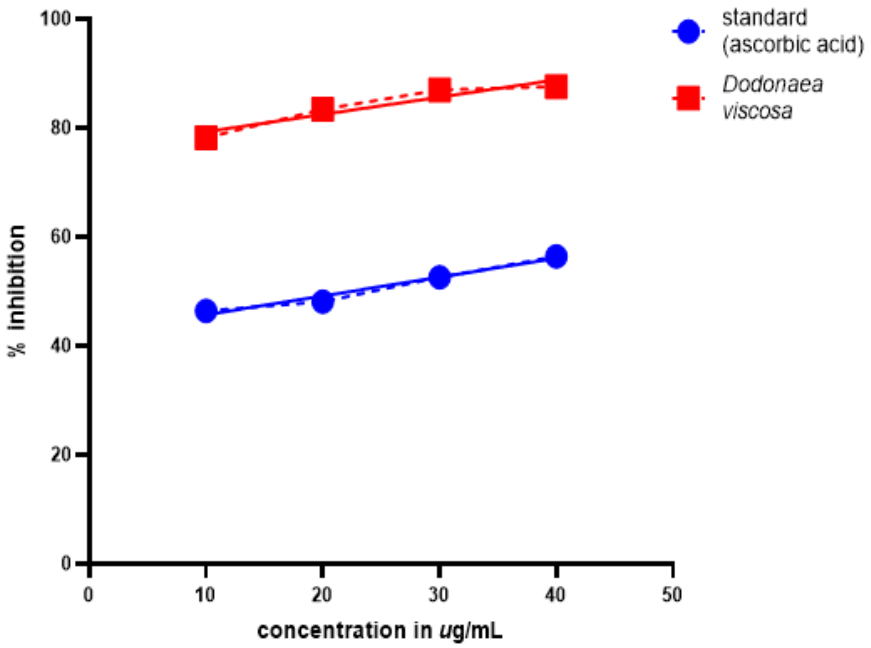

Figure 3. Percent radical scavenging activity of *D. viscosa* by DPPH assay. Red line indicates standard and blue line for *D. viscosa*.

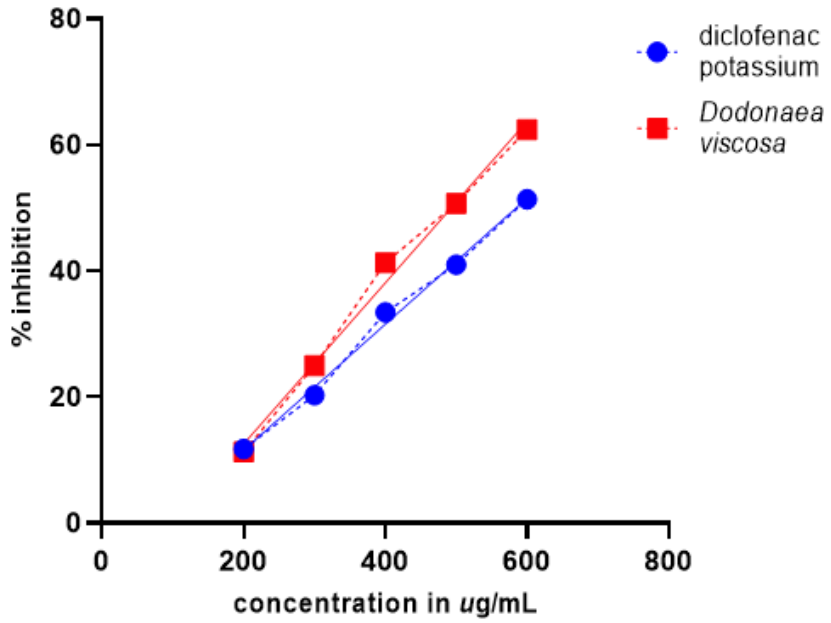

Figure 4. Percent protection/ inhibition of membrane stabilization assay of *D. viscosa*. Red line indicates standard and blue line for *D. viscosa*.

Total Flavonoid Content was calculated from a linear regression equation (which in the conducted experiment was y = 0.0004x - 0.0037). A significant quantity of flavonoid was found in the ethanolic extract of Dodonaea viscosa (400.75 ± 0.0026).

3.4. Antioxidant activity

An *in vitro* DPPH assay was used to assess the antioxidant capacity of *D. viscosa* extract to a standard value. Ascorbic acid served as the standard. The result was plotted as a linear regression curve (Figure 3). The radical scavenging potential or % inhibition of *D. viscosa* increased with higher concentrations and was significantly greater than that of the standard -ascorbic acid, $p \leq 0.05$. This suggests that the plant is a good source of antioxidant activity.

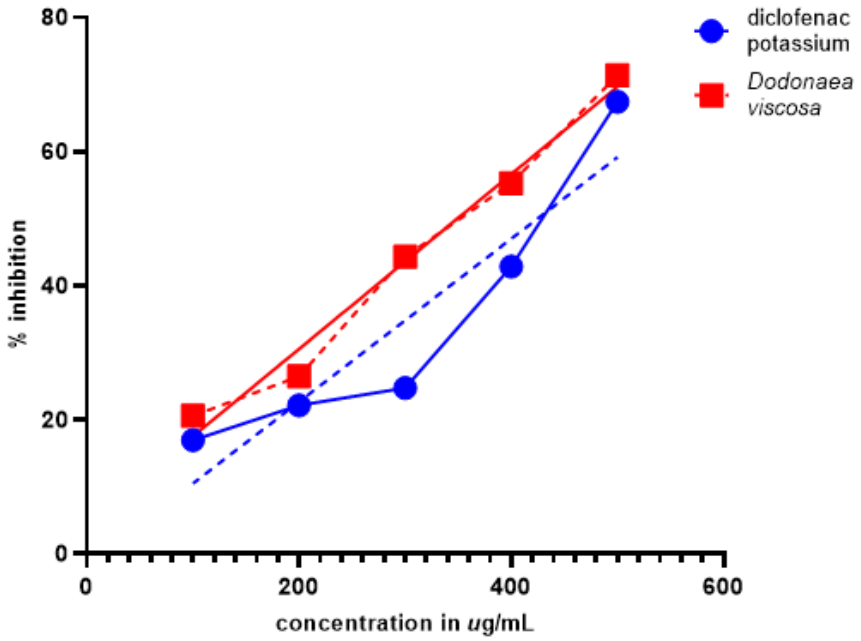

Figure 5. Percent inhibition of albumin denaturation activity of *D. viscosa*. Red line indicates standard and blue line for *D. viscosa*.

3.5. Anti-inflammatory activity

A membrane stabilization assay was used to determine the ability of *D. viscosa* plant extract to provide membrane stabilization to lysosomes, as the stabilization of lysosomes is critical in inflammatory responses. Diclofenac potassium was used as a standard in this experiment and a graph of linear regression was plotted (Figure 4). Percent protection or percent inhibition of *D. viscosa* extract and standard increased with higher concentrations, but in case of D.viscosa this was significantly greater than the standard indicating a direct relationship between the two variables (concentration and % inhibition), $p = 0.0003$. This suggests that with the increase in concentration, anti-inflammatory activity of plant extract also increases and can be a valuable source to treat inflammation.

3.6. Anti-arthritic activity

An inhibition of albumin denaturation assay was used to calculate the plant extract's capacity to inhibit protein denaturation. Diclofenac potassium was utilized as a standard in this experiment. A graph of linear regression was plotted (Figure 5). Results indicate that the percent inhibition of *D. viscosa* increased significantly higher with concentration ($p \leq 0.001$), in comparison to the standard (diclofenac potassium). This result suggests our plant extract has good anti-inflammatory activity and can be used as a treatment option to treat inflammation which is a symptom of arthritis.

3.7. ex vivo toxicity analysis by hemolytic assay

An *ex vivo* toxicity analysis revealed no hemolytic activity of *D. viscosa* within the range of 0.1-1000 µg/mL, indicating that our plant extract is safe to use within this range. It indicates that it is biocompatible against RBCs.

Table 3. Acute toxicity analysis observations. Mortality and other observations corresponding to stages and doses are detailed.

Stage	D. viscosa Dosing	Mortality	Other Observations
1	50 mg/kg, 200 mg/kg, 400 mg/kg	0	Calmness and fur improvement
2	1000 mg/kg, 1500 mg/kg, 2000 mg/kg	0	Calmness and fur improvement
3	3000 mg/kg, 4000 mg/kg, 5000 mg/kg	0	Calmness and fur improvement

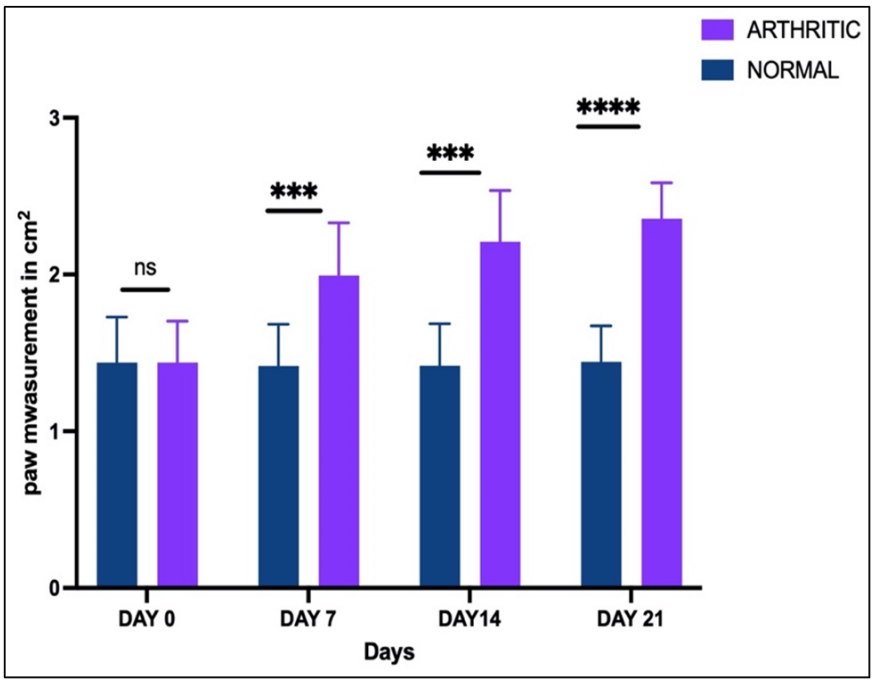

Figure 6. Paw measurement of mice over the course of Arthritis induction. Paw measurement is indicative of level of inflammation.

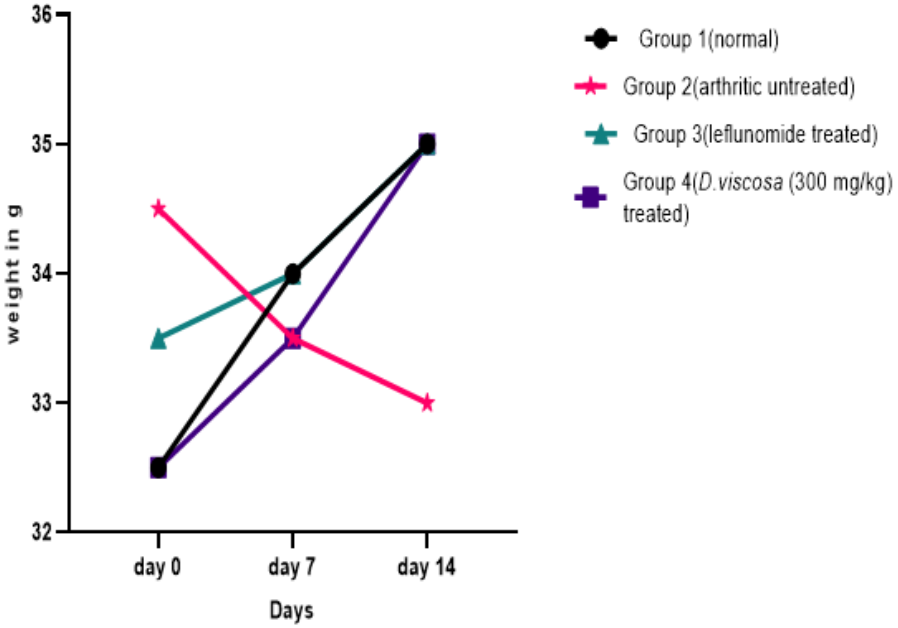

Figure 7. Body weight of arthritic mice following *D. viscosa* treatment. Line colors are representative of groups as described.

3.8. in Vivo evaluation of *D. viscosa*

3.8.1. Acute toxicity evaluation of *D.viscosa*

In BALB/c mouse the safety profile of *D.viscosa* was evaluated. The median lethal dose (LD_{50}) of *D. viscosa* was found to be greater than 5000 mg/kg as no significant behavioral change and/or mortality was observed in mice treated up to this dose in acute toxicity testing (**Table 3**).

Other observations noted include fur improvement and calmness in mice. These findings suggest that *D. viscosa* has a high safety profile and does not cause acute illness of other acute symptoms.

3.8.2. Therapeutic evaluation of *D. viscosa* in mice model

To evaluate the therapeutic efficacy of *D. viscosa* in mice, rheumatoid factor, spleen indices, and body weights were calculated. Mice with grade scores three and four were selected as discussed before in methodology section. After selection, mice were divided into 4 groups as discussed earlier, Group 1 (nor-

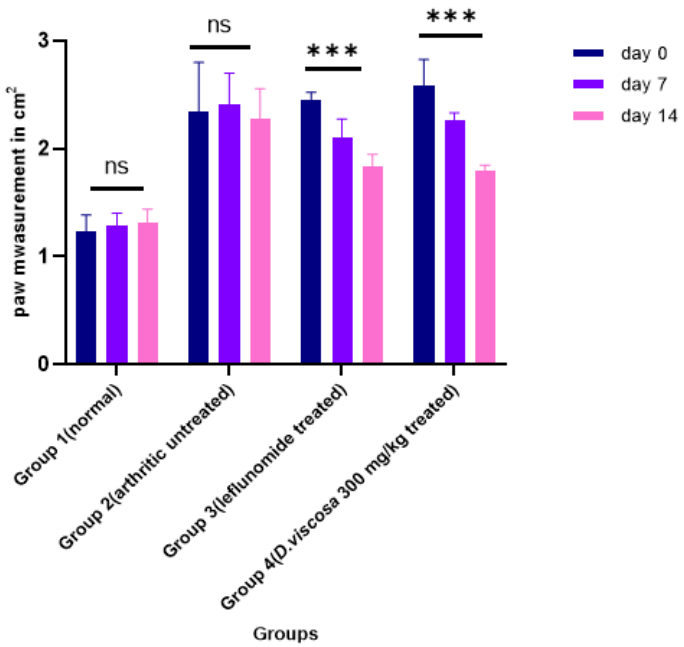

Figure 8. Paw measurement of mice following treatment. Group 1-4 were studied at day 0, 7 and 14.

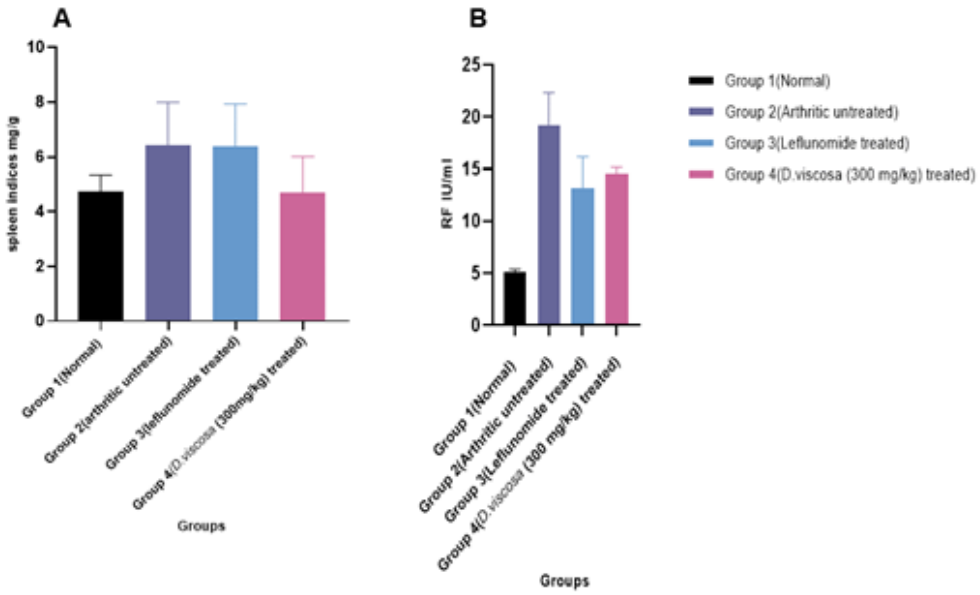

Figure 9. Arthritic mice post-treatment with *D. viscosa*. A- Spleen indices and B- Rheumatoid factor levels.

mal untreated group), Group 2 (Arthritic untreated group), Group 3 (standard drug treated; leflunomide 10mg/kg), and Group 4 (*D.viscosa* treated with a dose of 300 mg/kg). The paws of mice were measured weekly for 28 days to measure the level of inflammation (Figure 6).

Paw measurements were done in cm^2 of control and arthritic group following CIA injection. Data represented as mean ± standard deviation (SD). Statistical analysis was performed by two-way ANOVA. ***P < 0.001; ****P < 0.0001.

Mice with grade scores 3 and 4, as described earlier were selected for further processes. During the treatment phase, an increase in body weight was observed in the Group 4 (*D. viscosa* treated) and Group 3 (leflunomide-treated) groups as well as the normal untreated group, while the arthritic untreated group showed a decrease in body weight over the course, Figure 7.

D.visocsa treatment in mice resulted in reduced paw edema and inflammation as determined by paw measurements (Figure 8), which may be attributed to the anti-arthritic potential of *D. viscosa*.

No significant difference in spleen indices was seen in any group as shown in Figure 9A.

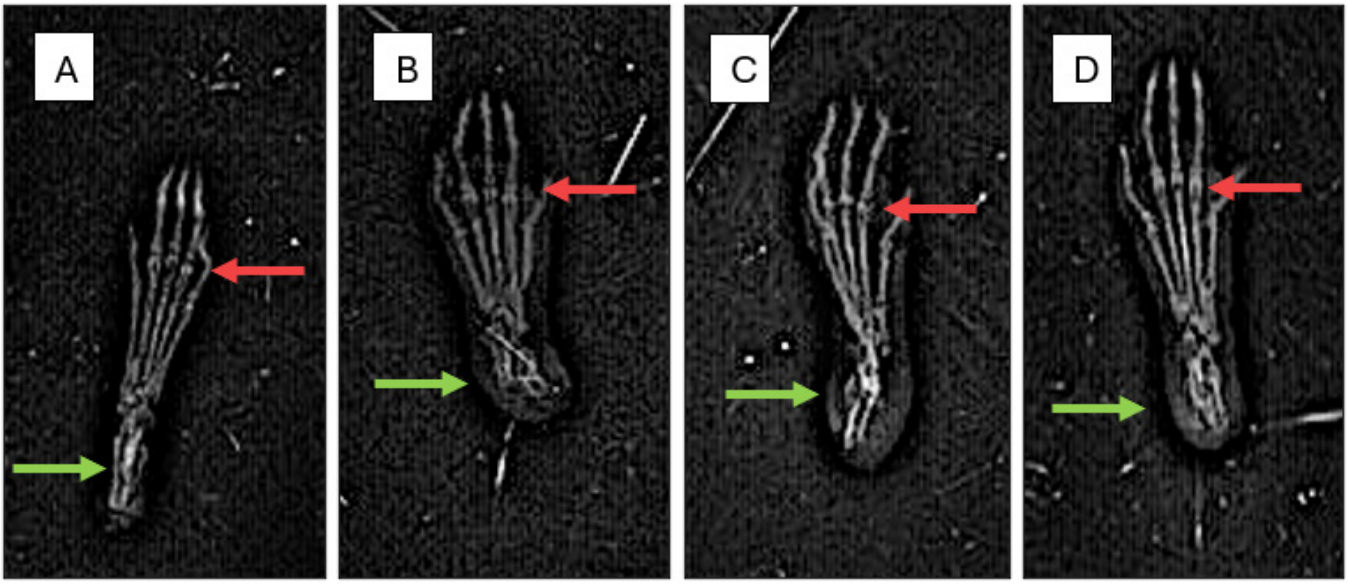

Figure 10. X-ray analysis of mice paws. A-Group 1(normal); B- Group 2 (arthritic untreated); C- Group 3 (leflunomide treated), and D- Group 4 (*D.viscosa* treated). Red arrows show joint spaces and joint deformation; Green arrows show ekinflammation of soft tissues.

As shown in the figure, the treatment did not affect the spleen of mice. A normal RF value which is between 14-20 IU/mL was observed in the treated groups (groups 3 and 4) compared to the arthritic group (group 2) which is higher than the normal range (Figure 9B). This indicates that our plant extract has therapeutic effect. A normal RF value was observed in the treated groups (groups 3 and 4) compared to the arthritic group (group 2), Figure 9B. The X-ray analysis also revealed reduced soft tissue inflammation and bone deformation in groups 3 and 4, as depicted in Figure 10.

As depicted in (A) normal paw showed joint spaces with regular bone formation and no inflammation of soft tissues, (B) Arthritic paw showed 2-3 fused joints with joint deformation and severe inflammation of soft tissues, (C) Leflunomide-treated paw show less joint deformation and joint spaces with less inflammation and (D) *D. viscosa* treated paw showing reduced bone deformation and joint spaces with reduced soft tissue inflammation compared to arthritic paw.

Discussion

RA is a chronic polyarthritis condition that is highly inflammatory and results in joint deformity, damage, and impaired function. Fibroblast-like synovial cells, found in synovial tissue, contribute to creating vascular pannus, synovium hyperplasia, and inflammation, which further leads to bone erosion and cartilage destruction in RA [3], [4], [5]. Since current available treatment options for RA are less cost-effective and have several side effects, researchers are now moving towards herbal medications. In this study we carried out qualitative and quantitative phytochemical screening of *D. viscosa*. *In vitro* antioxidant, anti-inflammatory, and anti-arthritic potential of *D. viscosa* were examined and further *in vivo* anti-arthritic efficacy of the plant was also evaluated.

D. viscosa contained several important phytochemicals, confirming results from a previous study [17]. Important phytochemicals, such as flavonoid compounds, viscosol, and viscosine have been isolated previously from *D. viscosa,* which supports our finding that this plant can potentially be a beneficial source of phytochemicals [28], [29].

Previously, it was understood that the production of free radicals resulted in oxidative stress, which is a defining feature of many chronic inflammatory disorders, including RA [30]. Flavonoids and phenols play a role as antioxidants and in this way decrease oxidative stress [31]. Significant amounts of TPC and TFC were found in *D. viscosa*. In an antioxidant assay, significant levels of DPPH were found in *D. viscosa*, which may be attributed to the presence of high levels of TPC and TFC. Similar results were also found previously [32]. As oxidative stress can contribute to inflammatory diseases, *D. viscosa* can be a useful antioxidant source to reduce the risk of inflammatory diseases, such as RA.

The formation of autoantigens linked to RA is caused by the denaturation of proteins due to heat, stress, and exposure to chemicals. The stability of the albumin protein was indicated by a decrease in the absorbance of *D. viscosa* extract with an increase in concentration, which in turn increased the percent inhibition of albumin denaturation. The percent inhibition of *D. viscosa* was significantly greater than the standard (ascorbic acid) at the same concentration. These results are in agreement with a previous study [33]. This suggests that the *D. viscosa* extract may be a potent source of anti-arthritic and anti-inflammatory potential.

To evaluate the therapeutic effects of *D. viscosa* in CIA mice, Group 1 (normal untreated group), Group 2 (arthritic untreated group), Group 3 (standard drug treated orally; leflunomide 10mg/kg), and Group 4 (*D.viscosa* treated orally with a dose of 300 mg/kg). Both groups (groups 3 and 4) showed nearly the same results in reducing paw edema and inflammation and an overall increase in the body weight of mice over the course of the treatment. Moreover, when spleen indices were compared, no significant difference was found between the groups. In autoimmune diseases, the spleen size typically increases along with body weight due to malfunctioning, this was not observed in our mice. Here, CIA model was utilized as it is essential for understanding the autoimmune perspective of RA pathogenesis. Using other models in the future to assess *D. viscosa's* potential may provide in depth insights into how it may work across various types of arthritis.

Moreover, RF is used as a diagnostic test for RA in nearly 80% of people. In this test, RF values were abnormal in the arthritic group but in the leflunomide and *D. viscosa* treated groups RF values were in the normal range. This suggests that our treated groups were in a disease remission process.

Lastly, the radiological examination of mice paws concluded that in the standard drug (leflunomide) treated group, the joints of toes were deformed with 2-3 joints fused and severe soft tissue inflammation, while in the *D. viscosa* treated group there was reduced soft tissue inflammation and less joint deformation and joint spaces present.

In support of this, previously, the potential of *D. viscosa* bioactive compounds was assessed for targeting *tumor necrosis factor alpha (TNFA),* a key gene in RA pathogenesis where it proved to be a good source to decrease disease progression [18]. In addition to this, several other compounds are also being explored for targeted therapy. For instance, in RA patients, *JAK* inhibitors which are currently under study, may be able to precisely regulate inflammation and immunology [34].

Other therapeutic strategies for RA under trial include a combination of NSAIDs and DMARDS specifically focusing on disease remission. These approaches are also being used in individuals at a risk of developing RA. In line with these, present study's findings present potential therapeutic targets that may be pursued further to fully explore their role in treating RA.

Conclusion

Dodonaea viscosa is reported to contain several bioactive compounds of therapeutic relevance, Present study examined the therapeutic efficacy of *Dodonaea viscosa* ethanolic extract in a CIA BALB/c mouse model. This study's findings confirmed that *D. viscosa* possesses good antioxidant, anti-arthritic, and anti-inflammatory potential, with no acute toxicity. Moreover, significant therapeutic effects of *D. viscosa* were observed in CIA mice. Further research is still required to clarify the impact of *D. viscosa* on several enzymes and genes implicated in oxidative stress and inflammation in RA.

Authorship contribution statement

Zainab Ali: Conceptualization, Original draft, Investigation, Writing, Methodology, Editing, Reviewing. **Peter John**: Project administration, funding acquisition, Reviewing, Editing. **Attya Bhatti**: Project administration, Reviewing, Editing.

Conflict of Interest

The authors declare no conflicts of interest.

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