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Evaluation of anti-inflammatory activity of ethanolic extract of *Derris brevipes* (benth.) Baker leaves

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ABSTRACT

Background: Ethnopharmacological relevance: *Derris brevipes* (benth.) *Baker* is widely used as an anti-arthritic agent in traditional medicine. **Aims and Objectives:** This study evaluated the anti-inflammatory activity of ethanolic extract of *D. brevipes* (EEDB) leaves, a member of the *Leguminosae* family *in vitro* and *ex vivo.* **Materials and Methods:** The EEDB leaves of was screened for anti-inflammatory activities by protein denaturation analysis and proteinase inhibition analysis. Prednisolone was used as the standard. EEDB (at doses of 300 mg/kg and 600 mg/kg) was administered through the oral route. *Ex vivo* analysis was performed by estimating the expression of tumor necrosis factor-alpha (TNF-α) in collagen (chicken sternal collagen)-induced arthritis (CIA) in Sprague–Dawley rats using the reverse transcription polymerase chain reaction method. The data were analyzed using one-way analysis of variance, followed by Dunnet's multiple comparison tests. **Results**: Prednisolone (100 mcg/mL) and EEDB (600 mcg/mL) showed percentage inhibition of 80.32% and 80.04%, respectively, in protein denaturation assay. Similarly, prednisolone (600 mcg/mL) and EEDB (600 mcg/mL) showed 66.03% and 56.69% inhibition in the proteinase activity. Synovial tissue TNF-α levels showed a significant decrease in EEDB-treated groups and prednisolone-treated group compared to CIA control rats. **Conclusion**: From this study, it was concluded that the *D. brevipes* leaf extract possesses anti-inflammatory activity, which explains its beneficial role in rheumatoid arthritis.

Key words: Musculoskeletal and joint diseases; Arthritis; Rheumatoid arthritis; Collagen induced arthritis

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INTRODUCTION

Rheumatoid arthritis (RA) is a chronic autoimmune disease that leads to joint inflammation, damage, and disability over time. While the exact cause of RA is unknown, it is believed to involve a combination of genetic and environmental factors. Medications such as nonsteroidal anti-inflammatory drugs, disease-modifying antirheumatic drugs, and biological agents are frequently used to treat RA. Since traditional anti-inflammatory medications used for RA often come with side effects, and the search for natural alternatives has become important.

Many phytochemicals such as curcumin,[1] resveratrol,[2] quercetin,[3] and green tea catechins[4] have demonstrated anti-inflammatory and analgesic properties, which can help alleviate the symptoms of RA.

Oxidative stress and immune system dysfunction are mainly implicated in the development and progression of RA. Phytochemicals with antioxidant and immunomodulatory properties can help counteract oxidative damage and regulate immune responses, potentially reducing joint inflammation and damage. Phytochemicals may also possess disease-modifying effects by targeting specific

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pathways involved in the progression of RA, such as immune dysregulation and cartilage degradation. Hence, identifying and investigating various phytochemicals with disease-modifying potential could lead to the discovery of new compounds with therapeutic potential for RA.

In our study, we investigated *Derris brevipes*, which has been used as antifertility and antiarthritic agent in traditional medicine. Ethanolic extracts of *D. brevipes* and *Derris indica* on carbon tetrachloride-induced oxidative stress in Wistar rats were shown to activate antioxidant enzymes.[5] ß-sitosterols, one of the active components of *D. brevipes*,[6,7] were shown to have anti-inflammatory properties, as it inhibited pro-inflammatory mediators in the mouse air pouch model.

While *D. brevipes* has been conventionally used in folk medicine, there is limited scientific research specifically focused on this plant species. With this background, this study aimed to investigate *D. brevipes*'s potential as an anti-arthritis drug using *in vitro* and *ex vivo* methods.

MATERIALS AND METHODS

Plant collection and authentication

D. brevipes (Benth.) Baker leaves were collected in February from the Ernakulum district of Kerala, India and were certified by the Department of Botany, CMS College, Kottayam, Kerala, India. The Herbarium has a voucher specimen with the voucher number, 260 (Figure 1).

Preparation of suspensions

A total of 100 g of shade dried leaf powder of *D. brevipes* in a permeable muslin bag was placed in a round bottom flask under reflux at 600°C. Ethanol (rectified spirit) was used for extraction and extraction was done for 24 h. The marc obtained was extracted twice using the same method.

Selection of animals

Twenty-four 150–170 g female Sprague–Dawley rats were used. Animals were obtained from the College of Veterinary and Animal Sciences in Mannuthy, Thrissur, and were housed in the Animal House at the University College of Pharmacy on the Cheruvandoor campus in Ettumanoor, Kottayam. The animals were confined in polypropylene cages in a room with ideal conditions of 27±1°C temperature, 30–60% relative humidity, and 12-h light/dark cycles. The animals were given 14 days to acclimatize to their surroundings in four separate groups. They were fed a standard pellet diet obtained from Hindustan Lever Limited, Bangalore and were provided with water ad libitum. According to Indian National Science Academy requirements, all procedures and investigations were conducted during the day. After receiving approval from the Institutional Animal Ethical Committee, University College of Pharmacy, Cheruvandoor (IAEC number: 008/MPH/UCP/CVR/13), the experiment was conducted.

Preparation of emulsion

Type II chicken sternal collagen was dissolved in 0.1 M acetic acid at a concentration of 2 mg/mL and stored at 40°C for one night. Before use, this solution was added drop by drop to an equal volume of chilled Incomplete Freund's adjuvant and stored on ice to make the inducing agent. Prechilled glassware was used for this process.[8]

Induction of arthritis[9,10]

On day 1, each rat was administered 0.5 mg of collagen in 0.5 mL at five locations (the base of the tail and the region above each limb). It was followed by a booster injection on day 7 with the same emulsion concentration as the primary immunization. 0.1 mL of emulsion was administered at the base of the tail, a location distinct from the initial injection site. All injections were administered intradermally.[8]

Grouping

The animals were separated into four groups of six animals. Using an intragastric tube, the vehicle and the drug or extract were administered orally from day 20 to day 40 after the primary immunization with emulsion. On day 41, animals were euthanized by cervical dislocation, and the synovial tissues of each rat were collected for biochemical examination.

In-vitro studies

Protein denaturation assay

Standard drug prednisolone was prepared in various concentrations of 3, 6, 12, 25, 50, and 100 mcg/mL in distilled water. A total of 10 g of ethanolic extract of

Figure 1: *Derris brevipes* (Benth.) Baker

D. brevipes (EEDB) extract was dissolved in distilled water to prepare concentrations of 100, 200, 300, 400, 500, and 600 mcg/mL.[11] The reaction mixtures (0.5 mL) consisted of 0.45 mL of bovine serum albumin (5% aqueous solution) and 0.05 mL of *D. brevipes* extract in various concentrations against standard prednisolone. pH was adjusted at 6.3 using a small amount of 1 N HCI. The samples were incubated at 37°C for 20 min and then heated at 57°C for 3 min. After cooling the samples, 2.5 mL phosphate buffer saline (pH 6.3) was added to each tube. Turbidity was measured using spectrophotometer at 660 nm. For control, 0.05 mL distilled water was used.[11]

Proteinase inhibition assay

The reaction mixtures (2.0 mL) contained 0.06 mg trypsin, 1.0 mL. 25 mM tris HCI buffer (pH 7.4). 1.0 mL EEDB leaves (100–600 mcg/mL of final volume) was used as a test and prednisolone (100–600 mcg/mL of final volume) as standard; the mixtures were incubated at 37°C for 5 min. Then, 1.0 mL of 0.8% (w/v) casein was added. The mixtures were incubated for an additional 20 min. 2.0 mL of 70% perchloric acid was added to terminate the reaction. The cloudy suspension was centrifuged. The absorbance of the supernatant was read at 280 nm against the buffer as blank.

Ex-vivo analysis

Reverse transcription-polymerase chain reaction (RT-PCR) analysis of tumor necrosis factor-alpha (TNF-α) expression in arthritic rats

Isolation of total RNA

One ml of trizol reagent was added to the synovial tissue sample from rats with collagen-induced arthritis (CIA) until it formed a fine paste. A volume of 200 µL of chloroform was added to the sample and incubated for 2–3 min at room temperature. Sample was centrifuged at 14,000 rpm for 15 min at 4°C. Then, aqueous layer was collected and 100% 500 µL of isopropanol was added. It was incubated for 10 min at room temperature. Supernatant was then removed, and the pellet deposited at the side of the tube was washed with 1 mL of 75% of ethanol. Tube was then centrifuged at 1000 rpm for 5 min at 4°C. The RNA pellet obtained was then dried and dissolved in TE buffer.[12]

Reverse transcriptase PCR analysis

About 5 µL of leaf extract-treated RNA, 1 µL of forward primer and reverse primer were added to an RNAse-free tube. The sequence of the forward primer TNF α is (5'CCCAGGCAGTCAGATCATCCTTC3') and the sequence of the reverse primer TNF α is (5'AGCTGCCCCTCAGCTTGA3'). To this mixture, 10 µL of prime RT PCR premix was added. Then, total reaction volume was made up to 20 µL with the addition of distilled water. The solution was gently mixed by pipetting up and down. The PCR machine was then programmed to undergo complementary DNA synthesis and amplification. Initial DNA synthesis was done at 42°C for 30 min, denaturation at 94°C for 10 min, denaturation repeated at 94°C for 1 min, annealing at 58°C for 1 min, and extension at 72°C for 1 min. The steps of denaturation, annealing, and extension were repeated for 35 cycles and the final extension at 72°C for 5 min. After the amplification, the PCR product was separated by agarose gel electrophoresis.

Agarose gel electrophoresis

Agarose gel electrophoresis was performed to check the purity of isolated mRNA·1% agarose gel was prepared in 1X TE buffer and melted in the hot water bath at 90°C. Then, the melted agarose was cooled down to 45°C. 3 µL of 0.5 mg/mL of ethidium bromide was added and poured into the gel casting apparatus with the gel comb. After setting, the comb was removed from the gel. The electrophoretic buffer was poured in the gel tank and the platform with the gel was placed in it so as to immerse the gel. The amplified RNA sample was carefully pipetted into the wells in the agarose gel. RNA bands started migrating toward the anode once the electric field was switched on. The stained gel was visualized using an UV transilluminator. The intensity of the bands was analyzed using the IMAGE J analysis software.

Statistical analysis

Results were analyzed using one-way analysis of variance, followed by Dunnet's multiple comparison tests using Graph Pad Prism (version 5).

RESULTS

Protein denaturation assay

Prednisolone (100 mcg/mL) and EEDB (600 mcg/mL) showed 80.32% and 80.04% inhibition, respectively, in the protein denaturation assay. The mean percentage inhibitions (n=3) obtained with different concentrations of sample extract were comparable to that of a standard concentration (Figure 2).

From the graph (Figures 2 and 3), IC_{50} of prednisolone and EEDB were found to be 20.76 mcg/mL and 137.05 mcg/mL, respectively.

Proteinase inhibition assay

EEDB leaves have shown a significant inhibition in proteinase activity (n=3). Prednisolone (600 mcg/mL) and EEDB (600 mcg/mL) showed 66.03% and 56.69% inhibition in the proteinase activity, respectively. From the graph (Figure 4), IC_{50} of prednisolone and EEDB

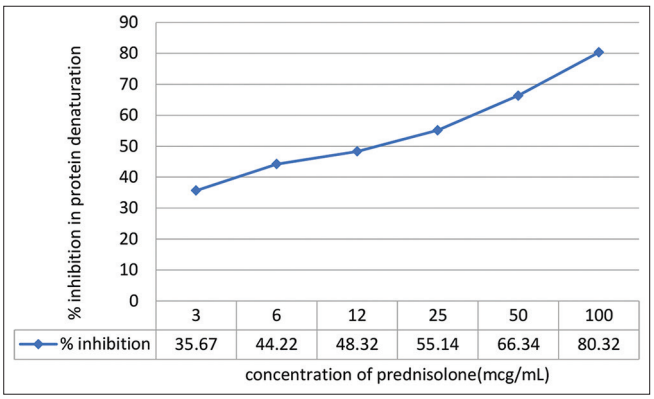

Figure 2: Effect of prednisolone in protein denaturation

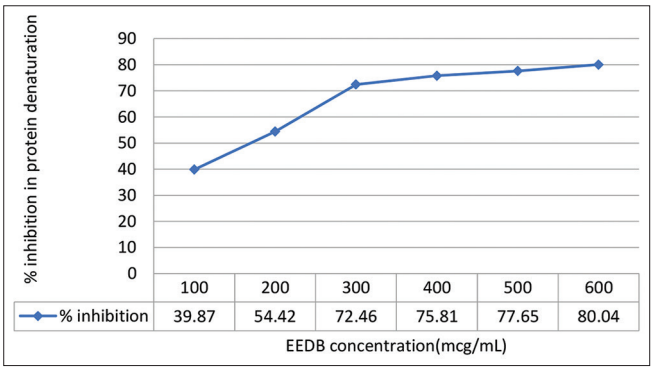

Figure 3: Effect of ethanolic extract of *Derris brevipes* on protein denaturation

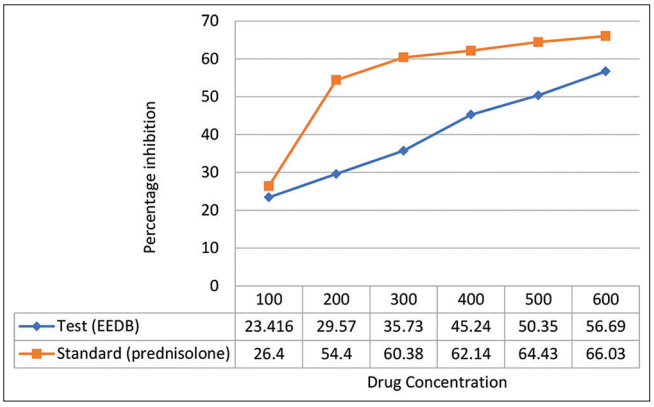

Figure 4: Proteinase inhibition assay of ethanolic extract of *Derris brevipes* leaves versus prednisolone

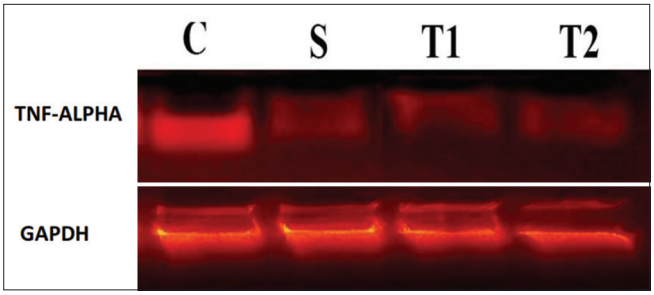

Figure 5: Expression of Tumour necrosis factor alpha and glyceraldehyde 3 phosphate dehydrogenase mRNA in collagen induced arthritis rats treated with ethanolic extract of *Derris brevipes*

were found to be 267.23 mcg/mL and 450 mcg/mL, respectively.

Ex-vivo analysis

The expression of TNF α was measured from the intensity of bands obtained from the RT-PCR analysis. The intensity of band produced by the control group is higher than that of the treatment groups. That means treatment groups inhibit TNF α expression (Figure 5). (C-control group, S-prednisolone treated group, T1-EEDB 300 mg/kg, T2-EEDB 600 mg/kg). Synovial tissue TNF α levels in CIA control rats was elevated; however, a significant decrease was seen in EEDB-treated groups and prednisolone-treated group (Figure 6). The percentage expression exhibited by both EEDB-treated groups was similar to that of the prednisolone-treated group.

DISCUSSION

Protein denaturation is a process in which proteins lose their tertiary structure and secondary structure by the application of external stress or compound such as strong acid or base, a concentrated inorganic salt, an organic solvent, or heat. Most biological protein loses their function when denatured.[13] Agents that can prevent protein denaturation, therefore, would be worthwhile for anti-arthritic drug development.[11] Denaturation of protein is the well-documented cause of RA. The production of auto-antigens in certain rheumatic diseases may be due to *in vivo* denaturation of protein.[11] Here, the extract showed dose-dependent ability to inhibit thermally induced protein denaturation. In thermal induced protein denaturation assay the IC_{50} of EEDB was found to be 137.05 mcg/mL and the prednisolone exhibit IC_{50} of 20.76 mcg/mL. The ability of EEDB to bring down the thermal denaturation of protein is possibly a contributing factor for its anti-inflammatory activity.

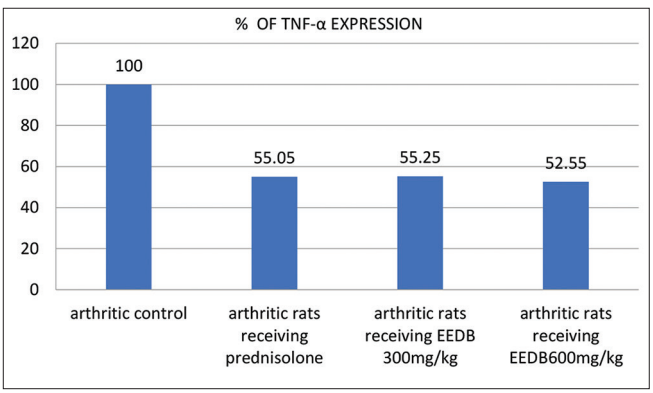

Figure 6: Effect of ethanolic extract of *Derris brevipes* leaves on tumor necrosis factor - alpha expression in collagen induced arthritis rats

Serine proteases, a subcategory of the protease family, are involved in different arms of the immune system and play an important role in inflammation. They have been evaluated as therapeutic targets in several inflammatory diseases. Neutrophils carry proteinases in their lysosomal granules. Leukocyte proteinases play an important role in the development of tissue damage during inflammatory reactions and significant level of protection was provided by proteinase inhibitors.[14] In the proteinase inhibition assay also, EEDB showed 56.69% inhibition in the proteinase activity and the IC_{50} was found to be 450 mcg/mL and 267.23 mcg/mL for EEBB and prednisolone, respectively.

TNF-α plays an important role in the pathogenesis of inflammatory joint diseases, including RA. The inflamed synovium, and the invasive pannus, are major sites of TNF-α production in RA. It was suggested that TNF-α is important in the pathogenesis of RA not only for its ability to directly promote connective tissue degradation but also for its involvement in the induction of the chronic inflammatory state.[15,16] TNF-α can stimulate the synoviocytes and cartilage cells to synthesize the PGE_2, IL-1, IL-6, IL-8, and collagenases, which are implicated in joint damage in RA.[17]

In the CIA rats treated with both doses of EEDB groups and prednisolone-treated group showed a significant reduction in the TNF-α expression. ß-sitosterol, a major phytosterol, had been isolated from the plant *D. brevipes* leaves.[6] In a study by Liz et al., ß-sitosterol exhibits potent TNF-α inhibition activity.[7] Preliminary phytochemical screening showed the presence of sterols, triterpenoids, and flavonoids in EEDB extract.[18] Hence, we assumed that the anti-arthritic effect of the EEDB leaves could be attributed to the inhibition of TNF alpha expression, which may be due to the presence of ß-sitosterol and it can be one of the underlying mechanism for anti-arthritic activity in CIA.

CONCLUSION

From the above findings, we concluded that the leaf extract of *D. brevipes* possess protective action against CIA. The TNF-α inhibition may be one of the underlying mechanisms of amelioration of inflammation in RA by *D. brevipes* leaf extract. Therefore, it seems to be a promising drug for the treatment of RA for ameliorating cytokine expression *in vivo*.

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Author's Contribution:
NMM- Definition of intellectual content, literature survey, prepared first draft of manuscript, implementation of study protocol, data collection, data analysis, manuscript preparation; **VR-** Concept, design, clinical protocol, manuscript preparation, editing, and manuscript revision; **SMS-** Review manuscript and editing; **JAJ-** Review manuscript; **SK-** Manuscript preparation, literature survey, preparation of figures, manuscript revision, coordination and submission of article.

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