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Original Article

Comparative Efficacy of Different Solvent Extracts of *Equisetum diffusum* Whole Plant in FCA-induced Arthritic Rat Model

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ABSTRACT

Background: *Equisetum diffusum* D. Don (Family *Equisetaceae,* common name 'Himalayan horsetail') is one such medicinally important herb that has a long history of traditional use among different tribal populations of the sub-Himalayan regions in India for treating back discomfort, bone fractures, and dislocations, as well as Rheumatoid Arthritis (RA). **Objectives:** This study aims to assess the *in vivo* anti-inflammatory and anti-arthritic characteristics of whole-plant of *E. diffusum* ethyl acetate (EDEAE) and n-hexane (EDHE) extracts. **Materials and Methods:** The adjuvant-induced arthritic rat model was utilized for assessing different arthritic parameters. The GC-MS studies was conducted for screening the bioactive phytocomponents in both solvent extracts. **Results:** In our study, both the EDEAE and EDHE significantly reduced the arthritic scores, normalized body weight, and restored paw-edema and hematological, biochemical, radiological, and histological states. From our GC-MS analysis, we found that EDHE and EDEAE consist of 75 and 133 unique bioactive phytocompounds, respectively. Out of which, eight (8) and seventeen (17) phytocompounds from the EDHE and EDEAE extracts showed the presence of anti-arthritic and/or anti-inflammatory characteristics, respectively. **Conclusion:** The findings of our study provide solid evidence for the anti-arthritic characteristics of different solvent extracts derived from *E. diffusum* in a chronic adjuvant-induced arthritic model. However, the EDEAE extract exhibited a more favorable outcome in comparison to the EDHE extract.

Keywords: *Equisetum diffusum*, FCA, GC-MS, Inflammation, Paw-edema, Rheumatoid arthritis.

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INTRODUCTION

Inflammation is the immediate defense response of the immune system to foreign substances at and after their entrance to the body.[1] Based on its persistence in the body, inflammation is divided into acute inflammation and chronic inflammation.[2] The unresolved acute phase causes a protracted inflammatory response, which is associated with many chronic inflammatory conditions, including cancer, diabetes, Rheumatoid Arthritis (RA), atherosclerosis, and inflammatory bowel disease.[3] RA, at its 1% prevalence rate, is prevalent in one out of three males.[4] Current medical approaches like Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) can treat prolonged inflammation. However, long-term exposure to these drugs can severely affect the kidneys and gastrointestinal tract through the inhibition of Cyclooxygenase (COX) and prostaglandin production.[5] As a result, herbal medicines are gradually becoming a popular alternative among the scientific community due to their fewer side effects.

The genus *Equisetum*, a pteridophyte with high silica and phytol content, is widely used as a medicinal plant due to its therapeutic potential.[6] The whole plant of *Equisetum diffusum* D. Don (*Equisetaceae*) is a highly valued medicinal herb in the sub-Himalayan belt of India. Due to its 'horsetail'-like appearance, the plant finds its common name as 'Himalayan horsetail'. The plant is also locally known as 'Kurkure Jhar', 'Chasmaa Jhar', and 'Aankhle Jhar'.[7] The plant is found mainly in the hilly regions of tropical and subtropical areas, especially in southeast Asian countries like India, Nepal, Bangladesh, Bhutan, China, Tibet, Myanmar, Vietnam, Thailand, and Pakistan.[6] The whole plant extract is also used for the healing of bone fractures by the Adi, Galo, Tagin, and Nyshi tribes in Arunachal Pradesh, India.[8] The Munda, Ho, Kharia, Oraoans, Bedia, Purans, Lohra, and Santhals communities use the whole plant powder with mustard to treat bone fractures, back pain, muscular pain, osteoporosis, and arthritis.[9] The herb also finds use among the ethnic tribes of Jammu-Kashmir, Sikkim, and Madhya Pradesh for curing arthritis, gonorrhoea, bone fractures, and bone dislocation.[10] The Raute community in Nepal uses the stem and whole plant

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paste to treat rheumatoid arthritis, inflammation, and joint pain.[7] The Mulam people in Guangxi, China, use the plant for its anti-inflammatory properties in wound healing.[11] The native communities in Pakistan often use the whole plant to alleviate arthritis.[12]

Despite the strong ethnobotanical evidence of the plant, there is limited experimental evidence published to date. The essential oil isolated from the *Equisetum diffusum* aerial parts showed the presence of 52 important bioactive phytochemicals, with phytol as a major component using the GC-FID and GC-MS study.[6] In our prior studies, we have confirmed the presence of satisfactory total phenols, flavonoids, tannins, and saponins content in the EDME.[13] The methanol extract of the whole plant also showed substantial antioxidant properties against ABTS, DPPH, the FRAP technique, and superoxide radicals.[13] The anti-inflammatory characteristics of the plant were also confirmed by *in vitro* protein-denaturation inhibition, membrane-stabilization hemolysis tests, and *in vivo* paw-edema tests induced by carrageenan.[13] The efficacy of the methanolic extract of the whole plant in treating arthritis was also validated from the *in vivo* FCA-induced arthritic model studies, where the methanol extract was found to have significantly improved the RA symptoms like arthritic score, paw edema, loss of body weight, hematological, biochemical, radiological, and histopathological parameters.[14]

Despite these findings, the main aim of the current work is to examine the anti-arthritic characteristics of two solvent extracts (mid-polar, ethyl acetate, and non-polar, n-hexane) of the whole plant. Our present study was also aimed at identifying the bioactive phytocompounds possessing anti-arthritic and anti-inflammatory characteristics from the different solvent extracts using GC-MS analysis.

MATERIALS AND METHODS

Chemicals and Reagents

HPLC grade ethyl acetate and n-hexane were purchased from Sisco Research Laboratories (SRL), India. Carboxymethylcellulose (CMC) was procured from HiMedia, India. Freund's Complete Adjuvant (FCA) was purchased from Sigma-Aldrich, USA. All the chemicals used were of molecular biology grade.

Collection of plant specimen and identification

We collected the fresh part of the whole plant from Naya Basti, Lebong, located in the Darjeeling district of West Bengal's sub-Himalayan Terai area. The specific coordinates of the collection site are Latitude 27.053171^{0}N and Longitude 88.265506^{0}E. An herbarium of our plant specimen with accession number 10971 was submitted to the Department of Botany, University of North Bengal and it was authenticated by Dr. M. Chowdhury. We have also verified our plant specimen from the Botanical Survey of India, Howrah, India. A voucher specimen with the identification number NBU/SS-002 was submitted to the Central National Herbarium, BSI, India.

Preparation of whole plant extract

We properly cleaned the collected whole plant with the rhizome and sun-dried it for 3-4 days. After complete drying, the whole plant was pulverized into a fine powder with the help of an electric grinder. The powdered sample (10 g) was then taken into Whatman filter paper and wrapped with a piece of muslin cloth. The HPLC-grade ethyl acetate and n-hexane solvents were used for extraction for 6-7 hr with the help of a Soxhlet apparatus. After that, both extracts underwent concentration at 50°C to 60°C temperature and 600 psi of pressure with the help of a rotary evaporator. The yield percentages for the *Equisetum diffusum* ethyl acetate (EDEAE) and n-hexane (EDHE) extracts were found to be around 1.6% (w/w) and 2.9% (w/w), respectively. Both the extracts were then stored separately in a sealed vial at 4°C for future use. Both the EDEAE and EDHE extracts were dissolved into 0.5% CMC for the purpose of animal feeding.

Animal maintenance

In this *in vivo* experiment, we used healthy rats of Wistar albino strain aged between 8 and 10 weeks and weighed between 120 and 140 g. These rats were acquired from a licensed vendor and were accommodated in the Departmental animal house. The rats were kept up at a standard temperature of 25±3°C with a normal day-night cycle (12 hr of light/12 hr of darkness). All the animals could get access to standard pellets and normal saline water (0.9% NaCl). The animals had a 2-week acclimatization period before the start of the studies.

Acute oral toxicity test

The OECD guideline 423 was followed for conducting the oral acute toxicity assay.[15] In this assay, the experimental groups consisted of both sexes of Wistar albino rats weighing 120±10 g. The rats were categorized into 5 groups for each extract, each consisting of 6 rats: 3 males and 3 females. Group I for both extracts were considered a 'normal group' where rats received a normal diet and 0.9% saline. The remaining four groups were designated as experimental dose-groups, and they were administered individual dosages of 250, 500, 1000, and 2000 mg/kg of respective plant extracts (EDHE and EDEAE) orally. After the administration of the respective doses, behavioral changes of all the rats were thoroughly observed individually for the first 30 min and then every 1 hr for the next 24 hr. All the animals were then observed individually for 14 days from the day of the experiment to assess whether there was any appearance of late toxicological effects. After the experiment, the Lethality value (LD_{50}) of both the extracts was determined, based on which the dose for the chronic arthritic model was selected.

FCA-induced chronic arthritic model

A total of 24 male rats weighing 140±10 g was categorized into four groups, with each group consisting of 6 rats for each extract (EDHE and EDEAE) (n=6). The animal groupings for the chronic arthritic model are as follows:

Group I (Arthritic Control group): All the rats received 0.9% normal saline throughout the experimental tenure.

Group II (High-dose group of EDHE and EDEAE): Rats received single-time oral administration of 500 mg/kg b.w. of EDHE and EDEAE from the beginning of the study (day 0) till the end of the study (day 28).

Group III (Low-dose group of EDHE and EDEAE): Rats received single-time oral administration of 250 mg/kg b.w. of EDHE and EDEAE from the beginning of the study (day 0) till the end of the study (day 28).

Group IV (Vehicle Control group): The rats in this group only received 0.5% CMC from day 0 to day 28.

The EDHE and EDEAE doses were administered once daily from day 0 till the end of experimental tenure (day 28).

Induction of arthritis

The induction of arthritis was conducted following standard established methodology.[16] For this, 0.1 mL of FCA was intradermally administered into the right hind paws (sub-plantar area) of all the experimental rats, except the group IV vehicle control rats. The trial commenced on day 0 with the administration of FCA, followed by a booster dosage of 0.1 mL on the day 14 of the study.[16]

Biometric studies

The body weight of individual rats in the experimental groups was measured weekly, starting from day 1 until the end of the experimental tenure. After the experimental tenure at day 28, all the rats were sacrificed following previous standard protocols.[17] After the sacrifice, the major internal organs, like the kidney and liver, were collected, and their weight was recorded. The absolute organ weight was used to calculate relative weights, following the standard formula.[18]

Paw diameter of each rat was measured with the help of the vernier caliper at two different planes following the standard formula.[19,17] The measurements were recorded starting from day 0 and continued every 2 days until the completion of the experiment.[14]

The arthritis progression was assessed by conducting arthritic scoring using standard published protocols, which involved assessing paw-inflammation, swelling, and redness.[20,21] The scoring system was used to evaluate the extent of swelling, which ranges between 0 and 4. A score of '0' indicates absence of swelling, swelling in one toe joint indicates as '1', swelling in toes and toe joints indicates as '2', ankle joints swelling indicates as '3', and swelling in the whole paw indicates as '4'. We recorded these scores on days 1, 7, 14, 21, and 28, respectively.

Hematological parameters

After sacrifice, the blood samples from each rat in the respective groups were collected in an EDTA-coated vial following standard methodology.[17] The collected blood was then used for assessing the following hematological parameters: Red Blood Cell (RBC) count, Hemoglobin (Hb) content, White Blood Cell (WBC) count, and platelet count.

Biochemical assay

For the biochemical analysis, the following parameters, like total protein, albumin, ceruloplasmin, creatinine, and urea concentration were measured from the blood serum samples collected on the day of sacrifice using standard methodology.[17] For the serum collection, the blood from each rat in all experimental groups was taken in a non-EDTA vial and allowed to clot for 30 min. Next, the vials underwent centrifugation at 5000 rpm for a duration of 10 min at 4°C and the collected serum was used to measure the parameters. The Coral Kit (Coral Clinical Systems, India) was used to evaluate the levels of total protein, albumin, creatinine, and urea. The level of serum ceruloplasmin was measured by assessing the activity of p-phenylenediamine oxidase.[14]

Radiological analysis

Allengers 325/625 X-ray machine (Mumbai, India) was used for the radiological analysis of the right hind-paw of the FCA-induced arthritic rats to evaluate the degree of arthritis progression. The imaging technique used is a high-resolution digital X-ray machine with the following settings for conducting the study: a peak of 50 kV, 50 mA, and an exposure period of 3-seconds. After that, the original X-ray images were cropped and analyzed carefully.[22]

Histopathological parameters

On the day of sacrifice, the ankle joints of the right hind paw were surgically taken out and preserved in 10% formalin, followed by decalcification in 3% HCl. Moreover, the soft tissues like the liver and kidneys were preserved in 4% formalin. Subsequently, all the specimens were subjected to dehydration using a serial dilution of ethanol concentration and properly embedded with paraffin. Then, thin (5 µm) sections for paw ankle-joints (longitudinal) and organ tissues (transverse) were cut using a microtome machine, followed by a double staining procedure with eosin and haematoxylin stains. The tissue specimens were then subsequently examined with the help of Nikon Eclipse E200 microscope (Tokyo, Japan) at magnifications of 10X and 40X.[17] The scale bars for 10X magnification were assigned to 100 µm and for 40X magnification were assigned to 25 µm.

GC-MS study

We subjected both solvent extracts, EDEAE and EDHE, to GC-MS analysis using the GCMS-QP2010 Ultra spectrometer (Shimadzu, Japan) in accordance with standard methodology.[17] The study was conducted from the AIRF center in JNU, New Delhi. The phytocomponents in both the solvent extracts were identified by comparing their retention time and mass spectra to the data available in the Willey 8 and NIST11 libraries connected with the GC-MS equipment.[17]

Statistical analysis

The statistical analyses of our study were obtained using the GraphPad Prism Version 7.00 statistical software. The measurement of arthritic score, paw circumference, and body and organ weight data were expressed in mean±standard error mean (mean±S.E.M). The results for the other hematological and biochemical tests are given as mean±standard deviation (mean±S.D). Two-way/one-way analyses of variance (ANOVA) were applied for comparisons involving more than two groups, and Dunnett's multiple comparisons test was conducted following the post hoc analysis. Statistically significant values were set at $p \leq 0.05$.

RESULTS

Acute oral toxicity test

Both extracts showed no mortality or toxicity, or behavioral changes within 24 hr of the experiment's commencement. There was also no emergence of any late toxicology effects during the 14-day observation period after the treatment schedule. The amount of water and food intake was normal for all the rats. Therefore, it can be concluded that both extracts were 'non-toxic' at 2000 mg/kg b.w. dose. As a result, the LD_{50} for both the extracts was found to be more than that of 2000 mg/kg b.w. Therefore, for the chronic arthritic model study, the low dose was selected to be $1/8^{th}$ of the LD_{50} value, i.e., 250 mg/kg b.w., and the high dose was selected to be $1/4^{th}$ of the LD_{50} value, i.e., 500 mg/kg b.w.

Chronic anti-arthritic properties of EDEAE and EDHE

Effect of both extracts on paw diameter analysis

The paw-circumference data for all the experimental groups of both extracts were depicted in Figure 1. In the rats treated with EDHE, there was no notable reduction in paw diameter until the 14^{th} day in both the group II and group III rats in comparison to the group I control arthritic rats. After booster dose administration on day 14^{th}, a significant ($p \leq 0.05$) amelioration in paw edema was seen in the group II rats (62.662±4.190) in comparison to the rats of arthritic control group (71.620±1.336) (Figure 1). On day 18, significant reduction in paw-edema was seen in both the group II (59.729±4.195; $p \leq 0.05$) and group III (61.069±2.632; $p \leq 0.01$) rats than the group I control arthritic rats (71.008±1.287) (Figure 1). From the 21^{st} to the 27^{th} day, the reduction of paw-edema in group II rats were found to be significant ($p \leq 0.01$) than the group I arthritic control rats.

In the case of ethyl acetate (EDEAE)-fed rats, no such significant amelioration of paw-edema was seen from the 3^{rd} day until the 12^{th} day (Figure 1). However, after the booster dose administration, there was a notable ($p \leq 0.05$) amelioration in paw-edema seen in both extract-treated dose groups in comparison to the group I control arthritic rats. Similarly, on days 18 and 21, both the extract-fed group II (61.328±1.109; $p \leq 0.01$) and group III (61.743±0.378; $p \leq 0.01$) rats showed a notable amelioration of paw-swelling than the group I control arthritic rats (71.008±1.287) (Figure 1). On day 24, the paw-swelling showed a notable ($p \leq 0.05$) reduction in both dose groups in

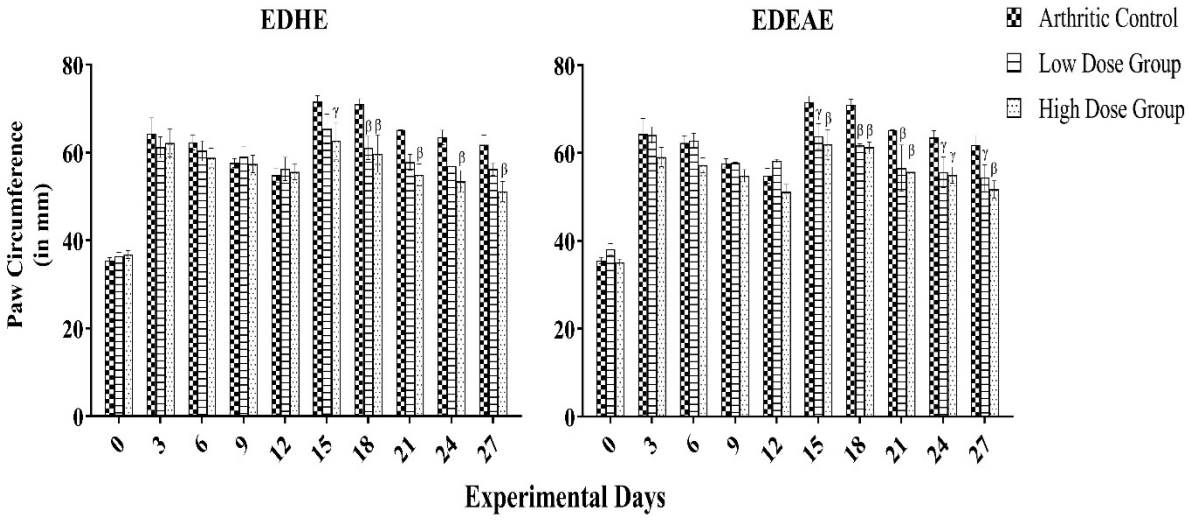

Figure 1: Effect of EDHE and EDEAE on rat paw-edema of different experimental groups of chronic arthritic experiment. The data is presented as the mean±S.E.M. of six rats each group (n=6). Two-way ANOVA was performed following Dunnett's post hoc comparison test (β denotes $p \leq 0.01$; $^{\gamma}$denotes $p \leq 0.05$).

comparison to the group I control arthritic rats. On day 27, a notable amelioration of paw-swelling was found in the group II rats (51.724±1.960; $p \leq 0.01$) than the group I control arthritic rats (61.765±2.228) (Figure 1).

Effect of both extracts on arthritic scoring

The results of arthritic scoring for all the experimental groups of both extracts were depicted in Supplementary 1. In the case of EDHE-fed rats, both the high- and low-dose groups rats did not exhibit any significant changes in the arthritic index in comparison to the group I control arthritic rats. From day 21 and day 28, a notable ($p \leq 0.05$) reduction was shown in the high-dose group in comparison to the rats of control arthritic group [see Supplementary 1].

In the case of ethyl acetate (EDEAE)-treated rats, both the group II high-dose rats (2.5±0.204) and the group III low-dose rats (2.5±0.204) showed a notable ($p \leq 0.05$) reduction of the arthritic scoring in comparison to the group I control arthritic rats on day 21 [see Supplementary 1]. Similarly, the high-dose group (2.125±0.125) on day 28 resulted in a significant ($p \leq 0.01$) reduction of arthritic scoring than the group I control arthritic rats [see Supplementary 1]. The images of the paw among all the animal groups for both extracts are provided in Supplementary 2.

Effect of both extracts on body weight study

All the experimental groups showed no notable alterations in the final body weight in both the EDHE and EDEAE-fed rats. The body weights of all experimental groups remained within a range of 140±10 g at the completion of the treatment regimen. The body weight data of all the experimental groups for both extracts is represented in Supplementary 3.

Effect of both extracts on organ weight

In EDHE-treated rats, the relative weight of the kidney in the group II high-dose (0.88±0.02 g), group III low-dose (0.72±0.01 g), and group IV vehicle control rats (0.77±0.02 g) showed no significant differences compared to the group I arthritic rats (0.76±0.13) (Table 1). The relative weight of the liver also showed a similar trend in both the extract-treated groups (Table 1).

In case of EDEAE, no significant alterations were found in the relative weight of the kidney among the extract-treated group II high-dose (0.75±0.03), group III low-dose (0.81±0.03), and group IV vehicle control rats (0.77±0.02) compared to the group I arthritic rats (0.76±0.13) (Table 1). The low-dose (3.48±0.18), high-dose (3.76±0.14 g), and vehicle control (3.68±0.11 g) groups also expressed a non-significant ($p \geq 0.05$) alteration in the relative liver weight of EDEAE rats than the arthritic control group (3.19±0.31 g) (Table 1).

Effect of both extracts on hematological parameters

For the hematological analysis, the following parameters were measured: total count of RBC, Hb content, WBC, and platelet counts, which were represented in Figure 2. In the case of EDHE-fed groups, no notable ($p \geq 0.05$) alterations were observed in the RBC count and Hb content in both the extract-treated group II and group III rats than the group I control arthritic rats (Figures 2A and 2B). However, a notable ($p \leq 0.05$) reduction in the WBC count was observed in both the extract-treated group II (6.79±0.0700 $\times 10^{\wedge 3}$/uL) and group III rats (6.32±1.57 $\times 10^{\wedge 3}$/uL) in comparison to the group I arthritic control rats (9.32±0.963 $\times 10^{\wedge 3}$/uL). The group IV vehicle control rat (4.25±0.800 $\times 10^{\wedge 3}$/uL; $p \leq 0.001$) also showed the maximum reduction of WBC count among the experimental groups (Figure 2C). The platelet count, however, exhibited a non-significant ($p \geq 0.05$) decline in both the group II (684±41.5 $\times 10^{\wedge 3}$/uL) and group III rats (712±63.1 $\times 10^{\wedge 3}$/uL), whereas the control vehicle group (514±50.1 $\times 10^{\wedge 3}$/uL) exhibited a significant ($p \leq 0.01$) reduction in the platelet count in comparison to the group I control arthritic rats (740±73.3 $\times 10^{\wedge 3}$/uL) (Figure 2D).

In the case of EDEAE-treated rats, the total RBC count exhibited a non-significant ($p \geq 0.05$) change among the extract-treated group II (7.45±0.480 $\times 10^{\wedge 6}$/uL) and group III (7.99±0.298 $\times 10^{\wedge 6}$/uL) rats, in comparison to the group I control arthritic rats (Figure 2E). Nevertheless, the WBC count resulted in a notable reduction in the group II high-dose rats (4.65±0.575 $\times 10^{\wedge 3}$/uL; $p \leq 0.001$), group III low-dose rats (5.91±1.15 $\times 10^{\wedge 3}$/uL; $p \leq 0.01$), and group IV vehicle control rats (4.25±0.800 $\times 10^{\wedge 3}$/uL; $p \leq 0.001$) in comparison to the group I control arthritic rats (9.32±0.963 $\times 10^{\wedge 3}$/uL) (Figure 2G). The group II high-dose rats (612±61.7 $\times 10^{\wedge 3}$/uL) and group III low-dose rats (744±19.9 $\times 10^{\wedge 3}$/uL) revealed a non-significant ($p \geq 0.05$) reduction, whereas the group IV control vehicle rats (514±50.1 $\times 10^{\wedge 3}$/uL) revealed a notable ($p \leq 0.01$) reduction in platelets in comparison to the group I control arthritic rats (740±73.3 $\times 10^{\wedge 3}$/uL) (Figure 2H).

Effect of both extracts on biochemical parameters

The results of the biochemical parameter analysis for the chronic arthritic model for both extracts were represented in Table 2. For the EDHE-treated rats, we found a non-significant increase of total protein in the low-dose group (7.04±0.373 g/dL) and high-dose group (7.18±0.105 g/dL), whereas the group IV vehicle control rats (7.88±0.659 g/dL; $p \leq 0.05$) showed a significant increment when compared to the group I control arthritic rats (6.37±0.608 g/dL) (Table 2). We also observed no notable ($p \geq 0.05$) alterations in the albumin concentration in comparison to the group I control arthritic rats (2.45±0.184 g/dL) among all the EDHE-treated experimental groups (Table 2). However, a significant reduction in ceruloplasmin concentration was found in the EDHE-treated low-dose (104±16.3 mg/dL; $p \leq 0.01$), high-dose (116±10.5 mg/dL; $p \leq 0.05$), and vehicle control

group (100±19.1 mg/dL; $p \leq 0.01$), in comparison to the group I control arthritic rats (155±13.8 mg/dL) (Table 2). The creatinine concentration exhibited non-significant ($p \geq 0.05$) alterations in the extract-treated low-dose (1.06±0.0644 mg/dL) and high-dose (1.07±0.106 mg/dL) groups compared with the control arthritic group (1.24±0.210 mg/dL) (Table 2). The serum urea level of EDHE-treated rats revealed a non-significant ($p \geq 0.05$) decline in comparison to the group I control arthritic rats (27.5±3.88 mg/dL) (Table 2).

In the EDEAE-treated rats, the serum protein concentration revealed a non-significant ($p \geq 0.05$) alteration in both the extract-fed group II high-dose rat groups (6.98±0.520 g/dL) and group III low-dose (6.79±0.467 g/dL) rats, when compared with the control arthritic rats (6.37±0.608 g/dL) (Table 2). The group IV vehicle control rats, however, revealed a notable ($p \leq 0.05$) increment in total protein concentration (7.88±0.659 g/dL) in comparison to the group I control arthritic rats. The serum albumin level also showed a similar trend in comparison to the group I control arthritic rats (2.45±0.184 g/dL) (Table 2). No notable ($p \geq 0.05$) alterations were seen in the ceruloplasmin level of the low-dose (153±6.85 mg/dL) and the high-dose rats (144±12.9 mg/dL) in comparison with the control arthritic rats (155±13.8 mg/dL) (Table 2). The administration of EDEAE also revealed a non-significant ($p \geq 0.05$) decline in the creatinine concentration in both the extract-treated group II rats (1.00±0.059 mg/dL) and group III rats (1.01±0.025 mg/dL) in comparison to the group I control arthritic rats (1.24±0.210 mg/dL) (Table 2). However, the vehicle control rats (100±19.1 mg/dL;

Table 1: Absolute (in g) and relative organ weights of the chronic arthritic model experimental rats.

Group	Arthritic control	EDHE Low dose	EDHE High dose	EDEAE Low dose	EDEAE High dose	Vehicle control
Absolute kidney weight	1.01±0.08	1.03±0.02	1.12±0.01	1.11±0.05	1.01±0.16	0.93±0.09
Relative kidney weight	0.76±0.13	0.72±0.01	0.88±0.02	0.81±0.03	0.75±0.03	0.77±0.02
Absolute liver weight	4.20±0.27	5.01±0.24	4.63±0.41	4.65±0.35	4.92±0.31	4.44±0.47
Relative liver weight	3.19±0.31	3.53±0.19	3.63±0.316	3.48±0.18	3.76±0.14	3.68±0.11

The data is presented as the mean±S.E.M. of six rats each group (n=6). One-way ANOVA was performed following Dunnett's post hoc comparison test. $p \leq 0.05$ indicates significance level.

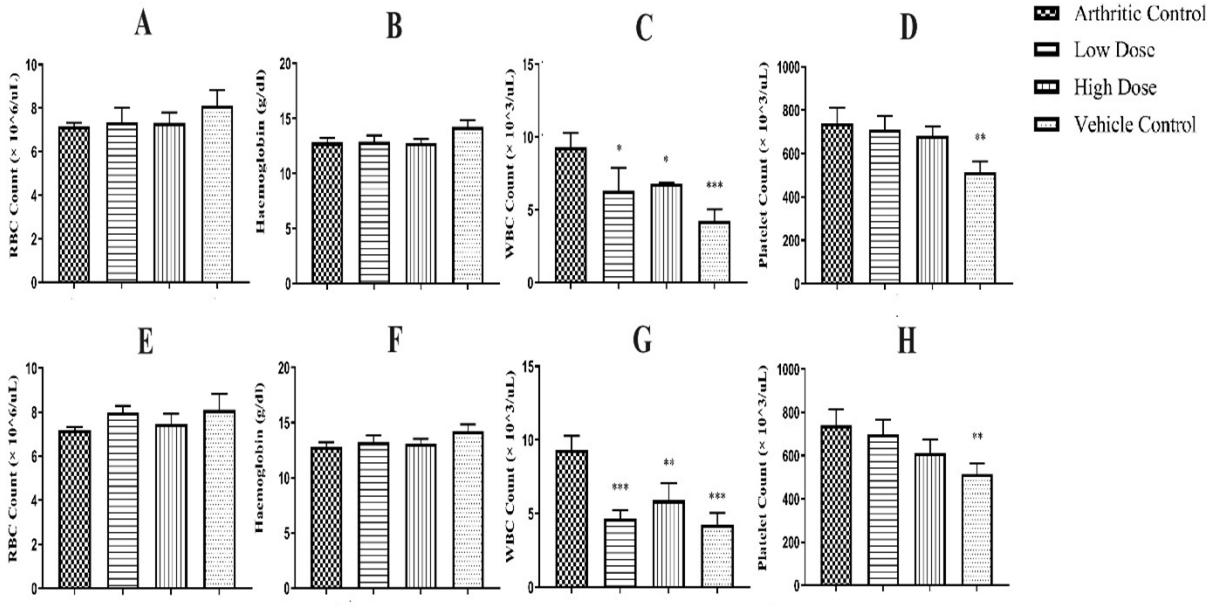

Figure 2: Effect of EDHE (upper panel) and EDEAE (lower panel) on hematological parameters of different experimental animal groups of chronic arthritic experiment. (A, E) Total count of RBC; (B, F) Hb content; (C, G) WBC content; and (D, H) Platelet counts. The data is presented as the mean±S.D. of six rats each group (n=6). One-way ANOVA was performed following Dunnett's post hoc comparison test (***denotes $p \leq 0.001$, ** denotes $p \leq 0.01$, and *denotes $p \leq 0.05$).

Table 2: Effect of EDHE (upper panel) and EDEAE (lower panel) on biochemical characteristics of various experimental rat groups of chronic arthritic experiment.

EDHE					
Group	**Total protein (g/dL)**	**Albumin (g/dL)**	**Urea (mg/dL)**	**Creatinine (mg/dL)**	**Ceruloplasmin (mg/dL)**
Arthritic control	6.37±0.608	2.45±0.184	23.1±1.16	1.24±0.210	155±13.8
Low dose	7.04±0.373	2.74±0.286	23.9±2.41	1.06±0.064	104±16.3**
High dose	7.18±0.105	2.83±0.301	25.8±1.10	1.07±0.106	116±10.5*
Vehicle control	7.88±0.659*	2.95±0.200	27.5±3.88	0.988±0.033	100±19.1**
EDEAE					
Arthritic control	6.37±0.608	2.45±0.184	23.1±1.16	1.24±0.210	155±13.8
Low dose	6.79±0.467	2.81±0.235	23.9±3.49	1.01±0.025	153±6.85
High dose	6.98±0.520	2.92±0.386	27.2±3.95	1.00±0.059	144±12.9
Vehicle control	7.88±0.659*	2.95±0.200	27.5±3.88	0.988±0.033	100±19.1**

The data is presented as mean±S.D. of six rats each group (n=6). One-way ANOVA was performed following Dunnett's post hoc comparison test (**denotes $p \leq 0.01$, and * denotes $p \leq 0.05$).

$p \leq 0.01$) showed a significantly lower level of ceruloplasmin than the group I control arthritic rats. The serum urea level exhibited a non-significant decline among all experimental groups than the group I control arthritic rats (27.5±3.88 mg/dL; $p \geq 0.05$) (Table 2).

Effect of both extracts on radiological analysis

After the completion of the experimental schedule, the radiographs obtained from the right hind-paws were visually assessed using the standard radiographic imaging. It was clear from the radiological analysis that the arthritic control rats experienced noticeable bone disintegration with uneven joint space and growing soft-tissue edema (Figure 3A). Compared to the arthritic control rats, the joint space looked normal, and the amount of soft tissue edema was reduced in both the high- and low-dose groups of EDHE and EDEAE-treated rats (Figures 3B-3E). Among all the extract-treated groups, the paw-joint spaces of the EDEAE high-dose group showed the highest degree of restoration without any amount of soft tissue edema (Figure 3E). However, it was noted that the vehicle control rats had a normal bone structure compared to the control arthritic rats (Figure 3F).

Effect of both extracts on histopathological analyses of paw ankle joint

The histological images of the rat paw-ankle joint of all the experimental groups of both the EDHE and EDEAE-treated rats are represented in Figure 4. In the Figure 4, the sections of the group IV control vehicle rats showed normal joint cartilage, without any immune cell's infiltration in between the cartilage lining (Figure 4D). On the other hand, abnormal cartilage lining and a high level of immune cell infiltration were observed throughout the arthritic control joint section due to severe inflammatory arthritis (Figure 4A). However, administration of both EDHE and EDEAE extracts in both the group II and group III rats showed reformation of cartilage lining and reduction of infiltrating cells compared to the group I control arthritic rats (Figures 4B, 4C, 4E, and 4F).

Effect of both extracts on histopathological analyses of liver and kidney

The histoarchitecture of liver and kidney sections among all the experimental groups showed no appreciable structural alterations. The liver sections revealed a well-organized Hepatocyte (H), prominent Central Vein (CV), portal vein, prominent sinusoids with Kupffer cells, and no anomalies among all the groups [see Supplementary 4]. Similarly, the renal sections showed well vascularized glomeruli and well-organized Bowman's capsule cells among all the experimental groups without any abnormalities [see Supplementary 5].

GC-MS Study of EDHE and EDEAE

The chemical fingerprinting for EDHE and EDEAE was determined using GC-MS study. The phytocomponents identified from EDHE and EDEAE are listed in Supplementary 2 and Supplementary 3, respectively. The Molecular Formula (M.F.) and Weight (M.W.), Retention Time (RT), area percentage, and Similarity Index (SI) of all the identified phytocomponents were provided in the Supplementary 6 and Supplementary 7. The chromatogram of both the solvent extracts were depicted in Supplementary 8. From our in-depth survey, we found that the GC-MS metabolomics study of the whole plant in n-hexane and ethyl acetate solvents was done for the first time. From our analysis, we found that EDHE and EDEAE consisted of 75 and 133 unique bioactive phytocompounds, respectively (see Supplementary 6 and 7). Out of the 75 phytocompounds identified from EDHE, eight (8) bioactives were determined to possess anti-arthritic and/or anti-inflammatory characteristics (Table

3). Similarly, 17 compounds out of 133 from EDEAE possess the anti-inflammatory and/or anti-arthritic characteristics (Table 3).

DISCUSSION

RA is a common chronic inflammatory illness, more prevalent among adults and seniors alike. The prevalence of RA ranges from 0.5% to 1%, among which the number of affected women is three times higher than that of men worldwide.[4] NSAIDs, DMARDs, and glucocorticoids are the mainstays of treatment for RA prevention and control, although these medicines are not without side effects. That is why herbal medicinal research has come to the limelight as an alternative nowadays. The practice of herbal medicines has been carried out for many years by the tribal communities without knowing their scientific background. As a result, these ethnobotanical supports provide the main basis of the research to validate the scientific background of herbal medicines. For scientific validation, the toxicity test and *in vivo* efficacy of the medicines need to be conducted along with the *in silico* analysis of the plant extracts. For this, the anti-inflammatory and anti-arthritic efficacy of EDHE and EDEAE have been examined through detailed *in vivo* experimentation on the FCA-induced arthritic rat model. Alongside this, the GC-MS study of both extracts was analyzed to identify the presence of valuable anti-inflammatory and anti-arthritic bioactives.

From our oral acute toxicity test, we found that both the extracts, EDHE and EDEAE, were safe up to 2000 mg/kg b.w. dose throughout the 14-day observation period. These results also corroborated the oral acute toxicity results of the methanol extract of the *Equisetum diffusum* whole plant reported earlier.[13]

The adjuvant-induced arthritic rat model is a significant and reliable animal model to evaluate the anti-arthritic characteristics of any drug because it provides similar features to human RA.[16] Several parameters were analyzed to determine the efficacy of plant extracts for the treatment of arthritis. Among such parameters, we performed biometric parameters (including paw edema, arthritic index, body weight, and relative organ weight measurements); hematological parameters (including total count of RBC, Hb content, WBC count, and platelet counts); and biochemical tests (like albumin, total protein, ceruloplasmin, urea, and creatinine concentration). We have also performed radiological and histopathological analyses to determine the severity of the disease.

The measurement of paw-edema is a very important parameter to determine the inflammation level, and the efficacy of the extracts depends upon their ability to reduce paw-edema. Our study clearly showed that the paw-swelling of the extract-fed rats for both extracts was reduced at the end of the treatment schedule (Figure 1). However, the EDEAE high-dose showed a higher reduction of paw-swelling than the EDHE high-dose group than the control arthritic rat group (Figure 1). Arthritic scoring in arthritis-induced rats showed a similar trend in paw-edema measurement. After administration of the booster dose, both the extracts significantly decreased the arthritic scoring comparison to control arthritic rats, indicating their efficacy in reducing paw edema [Supplementary 2]. In this case too, the EDEAE high-dose group showed higher efficacy compared to the EDHE high-dose group (Figure 2). A similar result for the arthritic index was found in a previous study after the treatment of n-hexane and methanol extracts of the leaf of *Tragia involucrata*.[53]

No significant difference has been found in the body and relative organ weights of the experimental animals after treatment with both the extracts (Supplementary 3 and Table 1). From this, it can be stated that neither of the extracts affects the growth or metabolic rates of the rats. A similar study conducted by Porwal *et al.*, (2017) on experimental rats also showed no such changes in body and relative organ weight when treated with an ethanolic extract of *Marsdenia tenacissimental* leaves.[18]

In arthritic conditions, the total count of RBC usually decreases due to the reduced activity of bone marrow, thereby failing to produce RBC.[54] Our study revealed that the total count of RBC was normalized after treatment with both the extracts in comparison to the group I control arthritic rats (Figure 2). The

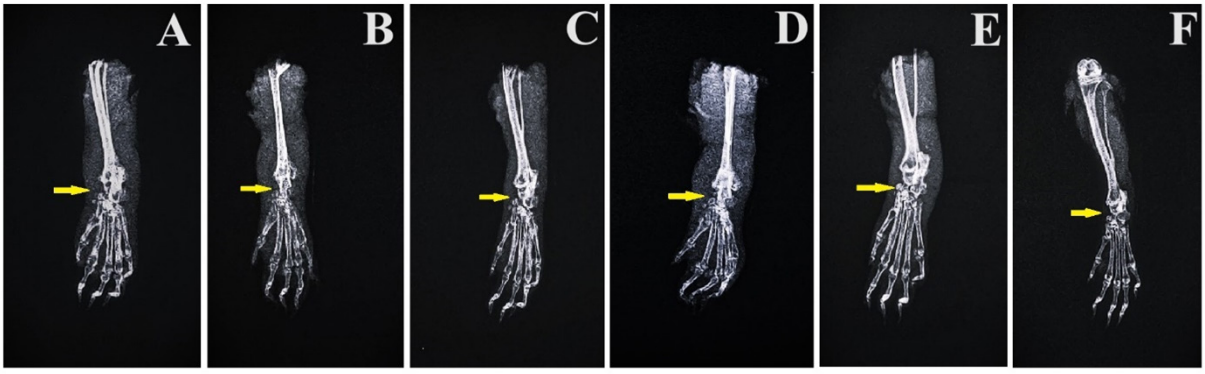

Figure 3: Radiographic examination of the ankle joints in the right hind paws of the experimental rat groups. Arrow symbols are used to depict the enlargement and deterioration of cartilage in experimental animals. A-Arthritic control group; B-EDHE low; C-EDHE high; D-EDEAE low; E-EDEAE high; F-Vehicle control group.

Hemoglobin (Hb) content in the blood also increases with the increase in the RBC count, and based on our results, we also found a similar trend of Hb normalization to that of the RBC count. Inflammation generally leads to an increase in total WBC count, indicating immune system activation.[17] After the treatment with EDHE and EDEAE, the WBC count showed a significant reduction in both solvent extract-treated rats in comparison to the group I control arthritic rats (Figure 3). The Platelets (Pt) count also increases in arthritic conditions.[53] Our study also showed a normalization of Pt counts in both the high- and low-dose groups for both extracts in comparison to the group I control arthritic rats. The hematological parameters in

Table 3: Biological properties of the identified phytocompounds from the GC-MS analysis of both solvent extracts, EDHE and EDEAE, respectively.

Sl. No.	Phytocomponents with M.F. and M.W.	RT (min)	Biological properties	References
		EDHE		
1.	Tetradecane [$C_{14}H_{30}$, 198]	10.359	Anti-inflammatory	[23,24]
2.	Eicosane [$C_{20}H_{42}$, 282]	13.680	Anti-inflammatory	[25,26]
3.	Tetracosane [$C_{24}H_{50}$, 338]	16.717	Anti-inflammatory	[23]
4.	Neophytadiene [$C_{20}H_{38}$, 278]	17.677	Anti-inflammatory	[27-29]
5.	Phytol [$C_{20}H_{40}O$, 296]	20.394	Anti-inflammatory; anti-arthritic property	[29-31]
6.	Heptacosyl heptafluorobutyrate [$C_{31}H_{55}F_7O_2$, 592]	28.101	Anti-inflammatory	[32]
7.	gamma-Sitosterol [$C_{29}H_{50}O$, 414]	30.380	Anti-inflammatory	[33]
8.	Tris(2,4-di-tert-butylphenyl) phosphate [$C_{42}H_{63}O_4P$, 662]	33.702	Anti-inflammatory	[34,35]
		EDEAE		
9.	Pentadecane [$C_{15}H_{32}$, 212]	9.660	Anti-inflammatory; anti-arthritic property	[26,36]
10.	Nonadecane [$C_{19}H_{40}$, 268]	11.156	Anti-inflammatory	[37-39]
11.	2Z,6E-Farnesol [$C_{15}H_{26}O$, 222]	12.275	Anti-inflammatory	[40]
12.	Tetradecane [$C_{14}H_3O$, 198]	12.540	Anti-inflammatory	[23,24]
13.	Eicosane [$C_{20}H_{42}$, 282]	13.675	Anti-inflammatory	[25,26]
14.	Octane, 2-methyl- [C_9H_{20}, 128]	13.867	Anti-inflammatory	[41]
15.	1-Decanol [$C_{10}H_{22}O$, 158]	14.963	Anti-inflammatory	[42]
16.	Pluchidiol [$C_{13}H_{20}O_2$, 208]	17.394	Anti-inflammatory	[43]
17.	Neophytadiene [$C_{20}H_{38}$, 278]	17.676	Anti-inflammatory	[27-29]
18.	Tetracosane [$C_{24}H_{50}$, 338]	18.847	Anti-inflammatory; anti-arthritic property	[23]
19.	Phytol [$C_{20}H_{40}O$, 296]	20.397	Anti-inflammatory	[29-31]
20.	2-Methylhexacosane [$C_{27}H_{56}$, 380]	20.509	Anti-inflammatory	[44]
21.	9-Octadecenoic acid (Z)-2,3-dihydroxypropyl ester [$C_{21}H_{40}O_4$, 356]	23.122	Anti-inflammatory	[45]
22.	13-Docosenamide, (Z) [$C_{22}H_{43}NO$, 337]	25.850	Anti-inflammatory	[46-48]
23.	2,2,4-Trimethyl-3-(3,8,12,16-tetramethyl-heptadeca- 7,11,15-tetraenyl)-cyclohexanol [$C_{30}H_{52}O$, 428]	26.014	Anti-inflammatory; anti-arthritic property	[49]
24.	Stigmast-5-en-3-ol, (3. beta.) [$C_{29}H_{50}O$, 414]	27.506	Anti-inflammatory	[50-52]
25.	Tris(2,4-di-tert-butylphenyl) phosphate [$C_{42}H_{63}O_4P$, 662]	33.675	Anti-inflammatory	[34,35]

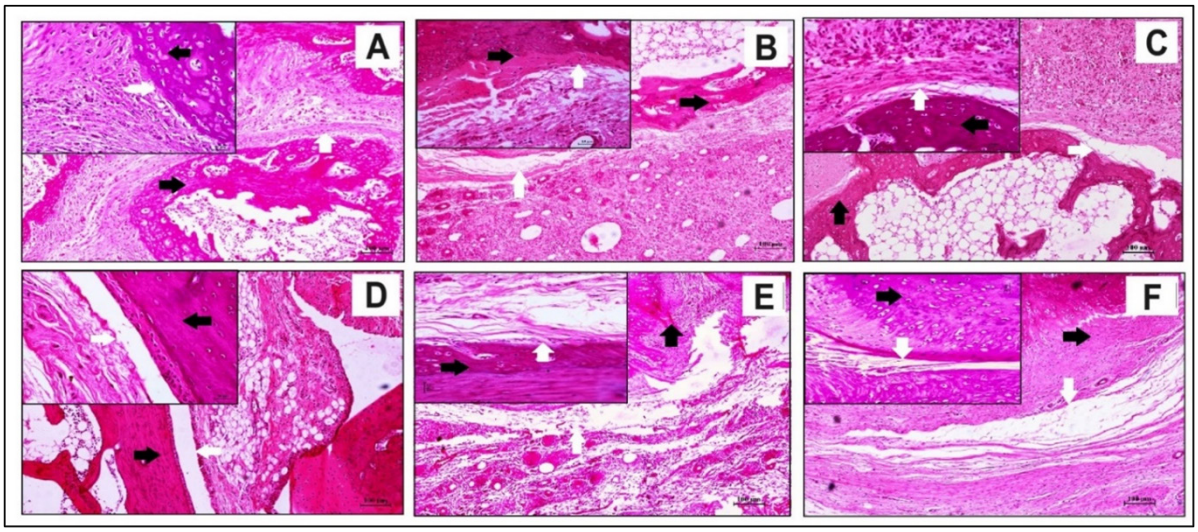

Figure 4: Histopathological sections (longitudinal) of the paw ankle joint among all the various experimental rat groups of chronic arthritic experiment. The white arrows indicate deterioration of the lining of the cartilage, whereas the black arrows indicate invasion of immune cells among the experimental rat groups. A-Arthritic control group; B-EDHE low; C-EDHE high; D-Vehicle control group; E-EDEAE low; F-EDEAE high. The main pictures are parts seen at a 10X magnification, and the smaller pictures in the inset are parts seen as 40X magnified images. In 10X, the scale bars are assigned to 100 µm, and in 40X, they are 25 µm.

both extracts showed promising results but the EDEAE seems to be more effective in keeping the parameters within the normal range in comparison to EDHE (Figure 3).

The concentration of total protein and albumin usually decreases in arthritic conditions because of alterations in concentration of plasma-derived protein and the presence of certain inflammatory markers such as bradykinin and prostaglandins that enhance vascular permeability.[55] The treatment with EDHE and EDEAE showed an increase in total protein along with albumin concentration in both the extract-fed group II high-dose and group III low-dose rats compared to the control arthritic group rats (Table 2). In case of total protein, the low dose of both solvent extracts showed higher efficacy, whereas the EDHE high dose showed a more significant effect on the serum albumin level. The urea and creatinine levels for both extracts decreased in both dose groups in comparison to the group I control arthritic rats, thereby suggesting normal kidney functions after extract treatment. Serum ceruloplasmin is one of the significant acute-phase protein usually increases its concentration during RA progression, causing severe injury to the liver tissue.[56] In our investigation, the level of the serum ceruloplasmin significantly decreased when treated with EDHE. However, treatment with EDEAE also decreased the ceruloplasmin level to some extent, indicating the efficacy of both extracts in the treatment of arthritis (Table 2).

Radiographs are the primary diagnostic tool used in clinical practice for the diagnosis and monitoring of RA because they provide valuable information about the disease severity. Edema in soft tissue is typically the first symptom of RA, although in more advanced stages, joint gaps constrict and bone degradation happens. In our investigation, the radiographic patterns of arthritic rats exhibited improvement after EDEAE and EDHE treatments, showing restoration of bone degradation (Figure 3). The histoarchitectures of the right ankle joints corroborated these radiographic changes in arthritic rats (Figure 4). Arthritic control rats had abnormal cartilage linings and increased immune cell infiltration compared to the EDEAE and EDHE-treated experimental groups (Figure 4). The liver or kidney histological sections of the experimental groups also showed no significant structural alterations, thereby eliminating the harmful side effects of the plant extracts. A comparable result of radiography and histopathology was conducted by Sarkar *et al.*, (2025), which showed restoration of bone erosion, an increased influx of immune cells inside the articular cartilage linings, and an abnormal lining of cartilage of the control arthritic rats, and no discernible structural changes of the histopathological sections of the major organs (liver and kidney) across the experimental groups.[14]

In summary, it can be said that both the solvent extracts (EDEAE and EDHE) seem to exhibit the anti-inflammatory and anti-arthritic characteristics. Both the extracts showed ameliorative properties in the FCA-induced arthritic model. The treatment regime of the extracts dose ameliorated the inflammatory paw-edema and arthritis scoring, restored the hematological parameters to the normal range, and improved the concentration of biochemical parameters without causing any toxic effects. However, the EDEAE showed a better effect on the biometric and hematological parameters compared to the EDHE extract. In the case of biochemical parameters, although both the extracts have promising effects, the n-hexane extract (EDHE) showed comparatively higher efficacy in improving the concentration of the parameters compared to EDEAE.

Researchers commonly conduct the GC-MS metabolomics study to identify the presence of bioactive volatile substances in a plant-derived extract.[57] The two solvent extracts, EDHE and EDEAE, yielded a total of 75 and 133 unique bioactive phytocompounds, respectively. However, the literature survey revealed that 8 and 17 phytocompounds from the respective EDHE and EDEAE extracts have anti-inflammatory and anti-arthritic characteristics (Table 3). The presence of phytocompounds such as phytol, stigmast-5-en-3-ol-(3. beta.), and pentadecane in both extracts confirm the plant's anti-inflammatory and anti-arthritic efficacy.[26,29,31,51] Prior research on the GC-MS study of the methanol extract of the whole *Equisetum diffusum* plant identified a total of seven (7) phytocompounds with anti-inflammatory and anti-arthritic attributes, including phytol derivative.[13] Further studies on the role of phytochemical compounds on gene and protein-level expressions would provide deeper evidence of the plant's anti-inflammatory and anti-arthritic characteristics.

CONCLUSION

Our study assesses the anti-arthritic and anti-inflammatory characteristics of two solvent extracts of the *Equisetum diffusum* whole plant. All the parameters, including arthritic index, paw-circumference measurement, body and organ weight, hematological parameters, biochemical parameters, radiological analysis, and histological examinations, indicate that both plant extracts have valuable medicinal properties for treating rheumatoid arthritis and other inflammatory diseases. The GC-MS studies also screened for the presence of many bioactive phytocompounds with proven anti-inflammatory and anti-arthritic properties. The identified anti-inflammatory phytocomponents additionally required separation, purification, and bioavailability tests. So, the outcome of our study indicates that both plant extracts exhibit a unique and significant medicinal value with no such side effects. However, the mid-polar solvent extract (ethyl acetate) seems to have a more promising effect compared to the non-polar solvent extract (n-hexane). Thus, *Equisetum diffusum* presents a new avenue to investigate an alternative approach for treating inflammation-related diseases, including arthritis.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

ANOVA: Analysis of variance; **CMC:** Carboxymethyl cellulose; **COX:** Cyclooxygenase; **CCSEA:** Committee for Control and Supervision of Experiments on Animals; **EDEAE:** *Equisetum diffusum* ethyl acetate extract; **EDHE:** *Equisetum diffusum* n-hexane extract; **FCA:** Freund's Complete Adjuvant; **GC-MS:** Gas chromatography-Mass spectrometry; **IAEC:** International Animal Ethical Committee; **LD_{50}:** Lethal dose 50; **NSAID:** Non-steroidal anti-inflammatory drugs; **OECD:** Organization for Economic and Development; **RA:** Rheumatoid arthritis; **SD:** Standard Deviation; **SEM:** Standard Error of Mean.

AUTHORS CONTRIBUTIONS

SB: Conceptualization, Supervision, Visualization, Writing-review & editing; SS: Methodology, Software, Investigation, Validation, Writing - original draft; DM: Methodology, Investigation, Validation, BC: Methodology, Investigation, Writing - original draft; RD: Methodology, Investigation. SB: Soumen Bhattacharjee; SS: Sourav Sarkar; DM: Debabrata Modak; BC: Barnana Chakrabarti, RD: Roopsa Datta.

ETHICAL STATEMENT

These rats were accommodated in the Department of Zoology's Animal House, University of North Bengal. The rats were acquired from a licensed vendor (Chakraborty Enterprise, Kolkata, India; Registration No. 1443/PO/Bt/s/11/CPCSEA). The experimental procedures were conducted in strict adherence to the ethical norms authorized by the International Animal Ethical Committee (IAEC) of the Committee for Control and Supervision of Experiments on Animals (CCSEA) of the University of North Bengal, West Bengal, India. This study was granted approval under the reference number IAEC/NBU/2022/24.

SUMMARY

The manuscript explores the anti-arthritic and anti-inflammatory properties of different solvent extracts of *Equisetum diffusum* (Himalayan horsetail) in an adjuvant-induced arthritic rat model. The study compares ethyl acetate (EDEAE) and n-hexane (EDHE) extracts, analyzing their effects on arthritis-related parameters, including paw edema, hematological and biochemical markers, radiological and histological findings, and GC-MS-based phytochemical profiling. The GC-MS analysis identified 75 and 133 bioactive compounds in EDHE and EDEAE, respectively, with several possessing anti-inflammatory and anti-arthritic properties. Both extracts significantly reduced

arthritis symptoms, with EDEAE showing superior effects. The study therefore supports the traditional use of *E. diffusum* for inflammatory conditions and suggests as a treatment measure for arthritic conditions.

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