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Original Article

Exploring the Ameliorative Role of *Equisetum diffusum* D. Don Whole-Plant Methanolic-extract in Acute Inflammation and Molecular Docking Analysis of GC-MS-identified Phytocompounds with Few Prominent Inflammatory Markers/Cytokines for Inspecting the Potent Drug Targets

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ABSTRACT

Background: *Equisetum diffusum* D. Don (Fam. *Equisetaceae*), an important medicinal pteridophyte species has been traditionally used in the treatment of bone fracture, bone dislocation, and arthritis by various tribal communities of India. **Objectives:** To validate the potent anti-inflammatory properties of the whole plant methanolic-extract (EDME) using *in silico*, *in vitro*, and *in vivo* strategies. **Materials and Methods:** The EDME was characterized through total phenolic, flavonoid, tannin, saponin content and five antioxidant assays. The GC-MS analysis was done to screen the active phyto-constituents followed by *in silico* molecular docking studies with inflammatory markers. The *in vitro* anti-inflammatory activity was assessed by measuring protein denaturation-inhibition test, heat-induced and hypotonicity-induced haemolysis test. Carrageenan-induced paw-edema test was used to determine the *in vivo* anti-inflammatory activity of EDME. **Results:** Our results showed quite higher tannin content in EDME compared to saponin content, and showed satisfactory phenol and flavonoid contents. From the antioxidant survey, it was found that EDME has the highest activity against ABTS radical and the least against superoxide radical. Furthermore, GC-MS analysis of EDME reported the presence of 47 phyto-compounds, out of which few compounds showed good inhibition against Cyclooxygenase-2 (Cox-2) and IL-6 compared to standard NSAIDs, in molecular docking analysis. *In vitro* anti-inflammatory studies indicated that EDME inhibits protein denaturation, heat-induced, and hypotonicity-induced haemolysis significantly ($p<0.001$) compared to standard drugs. The *in vivo* anti-inflammatory study of the plant showed 52.26% and 73.36% reduction in paw-edema in both protective-dose groups respectively, when compared to the carrageenan control group. **Conclusion:** Our findings established the anti-inflammatory roles of the whole plant methanolic extract of *Equisetum diffusum* on strong ground which may lead to drug development for the treatment of inflammation-related complications.

Keywords: Carrageenan, *Equisetum diffusum*, GC-MS, Inflammation, Molecular docking, Paw edema.

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INTRODUCTION

Inflammation is the protective immunological response of the body against any harmful aggressive stimuli and plays a pivotal role in maintaining the tissue homeostasis.[1] Based on the time course, inflammation can be categorized into: acute and chronic types.[2] Acute inflammation involves the initial response of the body against injurious stimuli and is achieved by the movement of immune cells from the blood into the injurious tissues.[1,3] When this acute phase remains unresolved it leads to prolonged inflammatory response which is linked to several chronic inflammatory disorders, such as atherosclerosis, diabetes, inflammatory bowel disease, cancer, and rheumatoid arthritis.[4,5] To treat the consequences of these chronic inflammatory disorders, various commercially available Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) or steroids are commonly practised. However, long-term use of these drugs

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elicit several adverse side-effects, including gastrointestinal and renal toxicity.[6,7] In order to overcome these issues, an alternative medication with less side-effect is required. In this regard, the field of Complementary and Alternative Medicines (CAMs) can play a major role owing to their easy availability, low cost, and low-risk side-effects.[8] According to the World Health Organization (WHO), 80% of the world population rely directly or indirectly on 'CAMs' for their primary healthcare.[8]

Equisetum diffusum D. Don is one of the major medicinally important pteridophyte species belonging to the *Equisetaceae* family of Pteridophyta. It is widely distributed in tropical and subtropical countries like India, Bangladesh, Pakistan, Vietnam, Myanmar, China, Tibet, and Japan.[9,10] It is commonly known as 'Himalayan horsetail' and popularly called as 'Kurkure Jhar' in Nepali.[11,12] The main stem of the plant is annual, hollow, monomorphic and is differentiated into nodes and internodes. These are found along the hilly road-side in thickets or semi-shaded places.[9] Due to the presence of different minerals, mainly the silicic acid, several species of *Equisetum* have been used in traditional medicine worldwide.[11] The stem of *Equisetum arvense* has been reported to have anti-nociceptive and anti-inflammatory property;[13] bone tissue regeneration property.[14] The stem and leaves of *E. hyemale* are reported to possess antioxidant, anti-inflammatory activity.[15] The aerial parts of *E. giganteum* possess anti-inflammatory, antioxidant and antitumor property.[16]

The whole plant of *Equisetum diffusum* has also been reported to have various ethnobotanical properties and are used by various tribal communities of Eastern-Himalayan Global Biodiversity hotspot of India.[17] The macerated leaf extract of *Equisetum diffusum* D. Don along with *Acacia nilotica* (L.) Del. is used in the treatment of back pain and abrasives among the ethnic groups of Manipur, India like Meiteis, Nagas, Kukis, and Meitei Pangals.[17] The Tagin, Nyshi, Galo tribes, and Adi community of Arunachal Pradesh, India use the whole plant extract in treating bone fracture.[18,19] The ethnic groups of Sikkim, Madhya Pradesh, Jammu and Kashmir also use the plant in the treatment of kidney ailments, bone fracture, and bone dislocation;[10,20,21] gonorrhea and arthritis.[22,23] The whole plant powder along with mustard oil is used in the treatment of bone fractures, backache, and muscular pain by the tribal people of Ranchi of Jharkhand, India.[24] The Mulam people of Guangxi, China apply the grounded fresh plant parts over the affected area for their anti-inflammatory properties.[25] The aerial parts of the plant has been used as a Vietnamese folk medicine for its hypertensive and anti-inflammatory properties.[26] Phytochemical screening of different extracts of *Equisetum diffusum* showed the presence of carbohydrate, phenols, alkaloids, saponin, and proteins.[27,28] Previously conducted pharmacological studies also confirmed the anti-microbial and anti-fungal activities of the plant.[28]

Despite the diverse folklore uses of *Equisetum diffusum*, there is no detailed work validating its ethnobotanical uses reported in animal models to date. So far, very limited studies have been published on *Equisetum diffusum* related to its phytochemical composition and antioxidant activity. In this present context, the prime objective of our study was to evaluate the anti-inflammatory activities of the whole plant methanolic extract of *Equisetum diffusum* D. Don (EDME) through appropriate *in vitro* assays and *in vivo* animal model study. The present study also designed to assess the antioxidant activity and to screen the active phyto-constituents of methanolic extract using GC-MS analysis and to identify potential anti-inflammatory bioactives against potent pro-inflammatory mediators through *in silico* molecular docking studies.

MATERIALS AND METHODS

Chemicals and Reagents

All the chemicals used were of molecular biology grade. Ethanol, HPLC grade methanol were purchased from Merck, India. Carrageenan was purchased from Sigma Aldrich, USA. Carboxymethylcellulose (CMC) was purchased from HiMedia, India. Nitroblue tetrazolium (NBT), NADH solution, Phenazine Methosulfate (PMS), potassium ferricyanide was procured from SRL, India. Diclofenac sodium were purchased from Novartis, India. Indomethacin was purchased from Cipla, India.

Collection and Identification of plant material

The whole plant of *Equisetum diffusum* D. Don was collected from the Darjeeling foothills adjacent to the sub-Himalayan Terai regions of the northern part of West Bengal. Collection of the plant material were done during November-December, 2020-21. The collected plant specimen was identified and authenticated by the Scientist-E and Head of Office (Dr. R. K. Gupta) of Central National Herbarium, Botanical Survey of India, Howrah (BSI, Govt. of India) with an official deposition of herbarium specimen with specimen number (NBU/SS-002) [see Additional file 1]. The detailed methodology of the workflow has been provided as Additional file 2.

Preparation of whole plant methanolic extract

Collected fresh plants with small rhizomes (3 kg) were properly cleaned and washed with tap water to remove dust particles. Plants were then shade-dried at room temperature for 1 week to obtain the dry sample. The dried plants were then crushed to powder using an electrical grinder. The coarsely powdered plants were extracted with 100 mL of HPLC grade methanol using the Soxhlet apparatus (Borosil, India) for 48 hr. Then, the supernatant liquid was collected and filtered by the Whatman No. 1 filter paper. The obtained *Equisetum diffusum* whole plant methanolic extract (EDME) was then concentrated using a Buchi-type rotary evaporator (KNF, RC600, Germany) under reduced pressure and

temperature (45°C) and the percentage yield of the extract was 4.8% (w/w). The final yielded products were stored in an airtight glass container at 4°C for further experimental uses. For animal feeding, the extract (EDME) was reconstituted in 0.5% CMC.

Assessment of phytochemical contents of EDME

Estimation of total phenol content

The total phenolic content of EDME was assessed following previously published standard protocol.[29] Briefly, the reaction mixture was prepared by adding 1 mL of EDME, 95% ethanol, 5 mL distilled water, 50% Folin–ciocalteau reagent, and 5% sodium carbonate. After 1 hr of incubation, the absorbance of the reaction mixture was measured at 725 nm (UV-1900i VIS-spectrophotometer, Shimadzu, Japan) and the phenolic content was calculated using gallic acid as a standard.

Estimation of flavonoid content

The flavonoid content of EDME was done following the standard protocol.[30] For that, 4 mL of double distilled water, 5% sodium nitrite, 10% aluminium chloride, and 2 mL of sodium hydroxide (1 M) was added to 0.5 mL of EDME and the absorbance of the resultant mixture was measured at 510 nm (UV-1900i VIS-spectrophotometer, Shimadzu, Japan). Rutin standard was used to determine the flavonoid content.

Estimation of saponin content

The vanillin-sulphuric acid method was followed for the estimation of saponin content of EDME.[31] For that, 500 µl of 8% vanillin and 5 mL of 72% H_2SO_4 were added to 500 µL of EDME and were mixed well in an ice-bath. After that, the mixture solution was heated at 60°C in the water bath. Absorbance was measured at 535 nm after cooling the mixture in ice-cold water (UV-1900i VIS-spectrophotometer, Shimadzu, Japan).

Estimation of tannin content

The amount of tannin in EDME was estimated using a standard protocol.[32] For this estimation, the reaction mixture consisting of 100 µL of EDME, 7.5 mL of double distilled water, 500 µL of Folin-Denis reagent, and 1 mL of 35% sodium carbonate was added and the mixture was incubated for 30 min. Absorbance was measured spectrophotometrically at 700 nm and tannin content was calculated using the tannic acid standard (UV-1900i VIS-spectrophotometer, Shimadzu, Japan).

Assessment of antioxidant activity of EDME

The antioxidant activity of the whole plant methanolic extract of *Equisetum diffusum* was evaluated using the following antioxidant assays.

DPPH radical scavenging assay

DPPH (2, 2-diphenyl-1-picrylhydrazyl) assay was performed according to standard protocol.[33] Briefly, the reaction mixture containing 1800 µL of 100 mM DPPH and 200 µL of EDME was incubated for 30 min at room temperature in dark and the absorbance was measured at 517 nm (UV-1900i VIS-spectrophotometer, Shimadzu, Japan). Another reaction mixture was prepared without sample extract and was considered as a control. The inhibition percentage was calculated using the following equation:

$$\text{Percent inhibition} = [(Ac - As)/Ac) \times 100\%$$

where 'Ac' is the absorbance of the control and 'As' is the absorbance of the sample extract.

$ABTS^+$ radical scavenging assay

The spectrophotometric analysis of $ABTS^+$ (2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) radical cation(s) scavenging activity was carried out following standard methodology,[34] with some minor modifications. ABTS (2 mL) solution was added to 1 mL of EDME and the absorbance of the mixture solution was measured at 734 nm after 30 min (UV-1900i VIS-spectrophotometer, Shimadzu, Japan). The percent inhibition was calculated using the same formula as stated above.

Superoxide scavenging assay

Superoxide scavenging activity was analyzed according to standard protocol.[35] For this, the reaction mixture contained 1 mL of EDME, 1 mL of NBT solution (312 µmol/L), 1 mL of NADH solution (936 µmol/L), and 10 µL of PMS (120 µmol/L). The photo-induced reactions were performed under fluorescent lamps and the decrease in absorbance, which signifies the radical scavenging activity, was measured at 560 nm (UV-1900i VIS-spectrophotometer, Shimadzu, Japan).

FRAP assay

Ferric reducing antioxidant power of the plant extract was determined using a standard protocol.[36] EDME (1 mL) was mixed with 2.5 mL of potassium phosphate buffer (200 mM, pH-6.6) and 2.5 mL of potassium ferricyanide (1%) and was incubated for 20 min at 50°C. After that, 2.5 mL of trichloroacetic acid (600 mM) was added to the mixture and was centrifuged at 3000 rpm for 10 min. After the centrifugation, 2.5 mL of supernatant (upper layer) was mixed with an equal volume of distilled water and ferric chloride (0.5 mL; 6 mM). The absorbance of the resultant solution was measured at 700 nm using a UV-visible spectrophotometer (UV-1900i VIS-spectrophotometer, Shimadzu, Japan). The FRAP activity of the plant was expressed in µg Ascorbic Acid Equivalent (AAE) per mg plant sample.

Metal chelating activity

The chelating activity of the sample extract for Ferrous ions (Fe^{+2}) was spectrophotometrically measured as per standard protocol.[37] To 400 µL of EDME, 1600 µL of methanol, and 40 µL of $FeCl_2$ (2 mM) were added and mixed well. Simultaneously, 800 µL ferrozine (5 mM) was added and after 10 min of incubation, the absorbance of the Fe^{+2}-Ferrozine complex was measured at 562 nm (UV-1900i VIS-spectrophotometer, Shimadzu, Japan).

Anti-lipid peroxidation capability of EDME

Anti-lipid peroxidation potentiality of EDME was estimated following the standard TBARS method.[38] For estimation, 2.8 mL of freshly prepared liver homogenate, 100 µL of 15 mM ferrous sulphate, and 100 µL of EDME (different grades of dilution) were added and the mixture was incubated at 37°C for 30 min. After that, 1 mL of the reaction mixture was taken and to that 1.5 mL of 10% TCA solution was added. After 10 min of incubation, 1.5 mL of 0.67% TBA solution was added to the existing reaction mixture, and test tubes were placed in a hot water bath (at 80°C) for 30 min followed by centrifugation to obtain clear supernatant solution. The absorbance of this supernatant was recorded at 535 nm using a spectrophotometer (UV-1900i VIS-spectrophotometer, Shimadzu, Japan). A control set was prepared using the same protocol without adding the plant sample. The anti-lipid peroxidation capability of the plant sample was expressed in terms of inhibition percentage and IC_{50} value was calculated using the following formula:

$$\text{Inhibition percentage} = [(\text{Absorbance of control} - \text{Absorbance of the sample})/\text{Absorbance of control}] \times 100\%.$$

GC-MS study

The GC-MS analysis of EDME was carried out using GCMS-QP2010 Ultra (Shimadzu, Japan) following standard protocol[39] with slight modification and performed at Advanced Instrumentation Research Facility (AIRF) centre, Jawaharlal Nehru University (JNU), New Delhi. The chromatographic column was a fused silica capillary column Rtx-5MS having a column length 30.0 m and a column diameter 0.25 mm. The 1 µL sample was injected with a split ratio of 10.0. The ultra-high purity helium gas (99.9%) served as the carrier gas at a flow rate of 1.21 mL/min. The injector temperature was set at 260°C and the ion source temperature was operated at 220°C. The non-polar components were separated using a temperature program of 60°C for 2 min, then ramped at 10°C/min to 300°C and held for 16 min. The mass spectra were taken at a scan-interval of 0.2 sec and fragment ranges were scanned from 40 to 650 m/z. The total run time of the program was 42 min. The relative quantity of the chemical components found in EDME was determined using Total Ion Count (TIC) and expressed as a percentage based on the peak area produced in the chromatogram. The spectrums of the component were identified via their mass spectra available in the computer library (NIST11 and Willey 8) attached to the GC-MS instrument.[39]

Molecular Docking study

The key inflammatory markers/cytokines like Cyclooxygenase-2 (COX-2), IL-6, and TNF-α were selected for molecular docking analysis based on their major role in prolonging the chronic inflammatory diseases.[39-41] The 3D structures of those marker proteins were retrieved in PDB format from Protein Data Bank. The proteins were then prepared using Schrödinger (Maestro Version 12.5.139, Schrödinger, LLC, New York), utilizing the force field OPLS3. The information regarding the PDB of selected proteins are provided as an Additional File 3. The total number of phytochemicals (47) identified in GC-MS analysis were used as ligands for the docking studies. The SDF format of each of these ligands were retrieved from NCBI PubChem database.[42] The ligands were then prepared using the force field OPLS3 in LigPrep (Maestro Version 12.5.139, Schrödinger, LLC, New York). For molecular docking analysis, the receptor grids for each protein were generated first in Glide (Maestro Version 12.5.139, Schrödinger, LLC, New York) followed by grids generation with specific coordinates for the ligands [see Additional File 3]. The binding affinities for each ligand were recorded using Glide's Extra-Precision (XP) docking models.

Evaluation of *in vitro* anti-inflammatory potential

The following standard assays were used for evaluating the *in vitro* anti-inflammatory property of whole plant methanolic extract of *Equisetum diffusum*.

Protein denaturation-inhibition test

The test was performed following standard protocols with minor modifications.[41,43] Hen's egg albumin was used as a protein source and the experiment was carried out in triplicate sets (per dose). The reaction mixture (5 mL) consisted of 0.2 mL of egg albumin with saline of 2.8 mL phosphate buffer (PBS, pH 6.4) and 2 mL of varying concentrations of EDME so that final concentrations become 600 µg/mL, 800 µg/mL, and 1000 µg/mL. The similar volume of distilled water was used as control set. Incubation was done for 15 min at 37°C and then the mixture was heated for 15 min at 70°C. After cooling, the turbidity of the solution was measured at 660 nm using a spectrophotometer (UV-1900i VIS-spectrophotometer, Shimadzu, Japan). For reference Diclofenac sodium was used at a concentration of 100 µg/mL. The Percentage Inhibition (PI) of protein denaturation was calculated using the formula:

$$PI = [(OD_2 - OD_1)/ OD_2)] \times 100\%$$

where 'OD_2' is the absorbance of the heated control sample and 'OD_1' is the absorbance of the heated test sample.

Membrane stabilization assay

Preparation of erythrocyte suspension

Erythrocyte suspension was prepared according to standard protocol with slight modification.[44] Briefly, 3 mL of whole rat blood was collected in an EDTA-coated vial and centrifuged at 3000 rpm for 10 min. Sterile saline solution (0.9% NaCl) was used for washing followed by centrifugation at 3000 rpm for 5 min. The process of washing was done three times and the volume of the dissolved red blood pellets was measured. For cellular component reconstitution, a 40% (v/v) suspension were prepared with an isotonic phosphate-buffered buffer solution (10 mM sodium phosphate buffer, pH 7.4).

Heat-induced haemolysis

Heat-induced haemolysis test were carried out following the standard methodology with minor modifications.[41,44] For this test, the isotonic buffer solution (5 mL) containing 600, 800, and 1000 µg/mL of EDME were taken into 3 duplicate sets of centrifuge tubes. The same amount of distilled water (5 mL) was taken as control tubes in another set of centrifuge tubes. 100 µL of erythrocyte suspension was added to each tube and gently mixed by inverting the tubes. Then the tubes were incubated for 20 min at 54°C in a regulated water bath. After that, the reaction mixtures were centrifuged at 1300 g for 3 min at room temperature. The absorbance (OD) of the supernatant was then measured at 540 nm using a spectrophotometer (UV-1900i VIS-spectrophotometer, Shimadzu, Japan). Standard NSAID drug, indomethacin (200 µg/mL) was used as reference standard. The Percent Inhibition (PI) of haemolysis was calculated using the equation:

$$PI = [(OD_2 - OD_1)/ OD_2)] \times 100\%$$

where 'OD_2' is the absorbance of the heated control sample (distilled water) and 'OD_1' is the absorbance of the heated test sample (isotonic buffer).

Hypotonic solution-induced haemolysis

This test was performed by following the standard protocol with minor modifications.[41,44] In this test, the hypotonic solution was prepared using distilled water (5 mL) containing 600, 800, and 1000 µg/mL of EDME into 3 duplicate sets of centrifuge tubes. In other sets of centrifuge tubes, the isotonic buffer solution (5 mL) was prepared containing the similar graded doses of EDME. Distilled water (5 mL) and indomethacin (200 µg/mL) were served as control and standard reference drug, respectively. 100 µl of stock erythrocyte suspension was added to each tube and gently mixed. Then, all the tubes were incubated at 37°C for 1 hr followed by centrifugation at 1300 g for 3 min at room temperature. The absorbance (OD) of the supernatant was measured at 540 nm using a spectrophotometer (UV-1900i VIS-spectrophotometer, Shimadzu, Japan). The Percentage Inhibition (PI) of haemolysis was calculated using the following equation:

$$PI = [1-(OD_2 - OD_1)/(OD_3-OD_1)] \times 100\%$$

where 'OD_3' is the absorbance of the control sample in hypotonic solution (distilled water); 'OD_2' is the absorbance of the test sample in hypotonic solution (distilled water) and 'OD_1' is the absorbance of the test sample in isotonic solution.

Experimental animals

For the *in vivo* studies, Wistar albino rats (8-12 weeks old; 120 ± 10 g) were purchased from authorized animal dealers (M/s Chakraborty Enterprise, Kolkata, India; Regd. No. 1443/PO/Bt/s/11/CPCSEA). All the animals were kept in polypropylene cages (max. 4 rats per cage) and were maintained at a room temperature of 25±3°C. Rats were supplemented with standard feed and water *ad libitum*. The animals were kept in the animal house of the Department of Zoology, University of North Bengal, where they were acclimatized to laboratory conditions for 7 days before the commencement of experiments. The experimental procedures were carried out from 2020 to 2022 in strict compliance with the ethical guidelines approved by the Institutional Animal Ethical Committee [Approval number: IAEC/NBU/2018/03] (IAEC) of CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals) of the University of North Bengal, West Bengal, India.

Acute toxicity studies

Acute toxicity test was performed as per the Organization for Economic Cooperation and Development guidelines (OECD) 423.[45] In this test, Wistar albino rats (120 ± 10 g) of both sexes were categorized into 5 groups; each contained 6 rats; 3 males and 3 females. The first group contained normal animals receiving only normal water; the other four groups were considered as experimental groups. For the experimental groups, the *Equisetum diffusum* methanolic whole plant extract (EDME) was administrated orally by using gavage in single doses of 250 mg/kg, 500 mg/kg, 1000 mg/kg, and 2000 mg/kg of body weight on day one. All animals were then observed individually at 30 min, 1 hr, 2 hr, 4 hr, 6 hr, 12 hr, and 24 hr and, thereafter, for 14 days for any delated toxicological effects. Based on acute toxicity results we choose the doses for *in vivo* anti-inflammatory study.

Evaluation of *in vivo* anti-inflammatory potential

Carrageenan-induced paw edema

For evaluating the *in vivo* anti-inflammatory property of EDME, the carrageenan-induced paw edema was established following previously established protocol.[46,47] Inflammation was induced by injecting 0.1 mL of 1% (w/v) carrageenan suspended in 0.9% normal saline subcutaneously into the sub-plantar tissues of the right hind paw of each rat. Rats were randomly placed into seven groups, each group consisting of 6 animals (n=6). Group 1 served as vehicle control group, receiving 0.5% CMC orally for single

dose and paw edema was induced by 0.9% NaCl. Group 2 served as negative control group which received 0.9% NaCl orally for single dose and paw edema induced by 1% carrageenan. Group 3 and 4 served as Low dose group where rats were treated with 250 mg/kg b.w. dose of EDME for single day and for 7 days, respectively and then paw edema induced by 1% carrageenan. Group 5 and 6 served as high dose group where rats received 500 mg/kg b.w. dose of EDME for single day and for 7 days and then paw edema induced by 1% carrageenan. Group 7 served as positive control group where rats received standard Non-Steroidal Anti-Inflammatory Drug (NSAID) diclofenac sodium (10 mg/kg of b.w.) followed by 1% carrageenan injection. The paw thickness was measured using vernier calliper before injecting the carrageenan and after selected intervals at 0.5 min, 1, 2, 3, 4, and 5 hr. The percentage-inhibition of oedema was used to assess the anti-inflammatory activity of the extract treated group in comparison to the carrageenan control group. The following formula was used to calculate the percentage inhibition:

$$\% \text{ Inhibition} = [(C_t - C_0)\ NC - (C_t - C_0)\ T] / (C_t - C_0)\ NC \times 100$$

where '$(C_t - C_0)$ NC' is the difference in the paw circumference in 5 hr in negative control rats and '$(C_t - C_0)$ T' is the difference in the size of paw circumference at 5 hr, either treated with EDME or Standard drug.

Statistical analyses

The paw diameter measurement data was expressed as mean ± Standard Error Mean (SEM). For the remaining assays, all data were expressed as mean ± Standard Deviation (SD). One-way Analysis of Variance (ANOVA) or two-way ANOVA, following the post hoc analysis with Dunnett's multiple comparisons test, was performed. Values of $p \leq 0.05$ were taken to indicate a statistical difference. All the statistical analyses were performed using GraphPad Prism Version 7.00 (San Diego, United States of America).

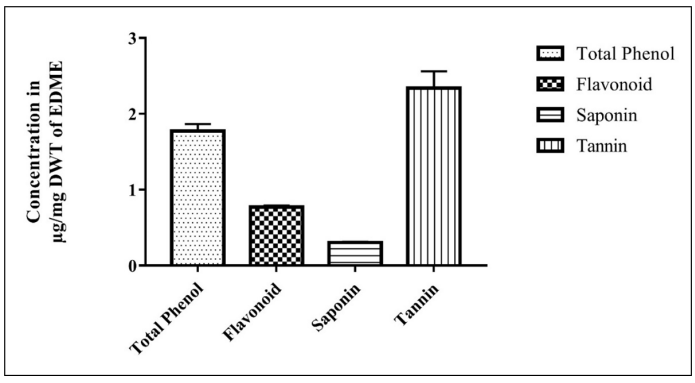

Figure 1: Total phytochemical contents of *E. diffusum* whole plant methanolic extract. Data presented as Mean ± SD of triplicate determination.

RESULTS

Phytochemical content of *E. diffusum* whole plant methanolic extract

Phenolics and flavonoids are important antioxidants responsible for the deactivation of free radicals by donating hydrogen atoms to free radicals. Total phenol and flavonoid contents of whole plant methanolic extract (EDME) of *E. diffusum* were expressed as gallic acid and rutin equivalent respectively. The present study showed almost 130% higher phenol content (1.773 ± 0.093 µg gallic acid equivalent/mg dry weight tissue) in comparison to flavonoid (0.770 ± 0.022 µg rutin equivalent/mg DWT) [Figure 1]. Saponins are glycosidic compounds having the capability to haemolyze blood cells and form insoluble complexes with cholesterol. Our plant sample showed a satisfactory amount of saponin content (0.304 ± 0.010 µg diosgenin equivalent/mg DWT). However, the EDME showed quite higher tannin content (2.340 ± 0.222 µg tannin equivalent/mg DWT) in comparison to saponin content [Figure 1].

Antioxidant activity of *E. diffusum* whole plant methanolic extract

In vitro antioxidant activity of EDME was evaluated by DPPH, ABTS, superoxide, metal chelation, and FRAP method. In the present study, the antioxidant activity of EDME was expressed as both IC_{50} and percent inhibition and is presented in Table 1 and Figure 2A. The lower IC_{50} value corresponds to the higher antioxidant activity of any tested sample. From the obtained results it can be summarized that EDME has the highest activity against ABTS radical (having an IC_{50} of 8.946 ± 0.105 mg/mL) and the least against superoxide radical (with an IC_{50} of 48.496 ± 1.280 mg/mL) [Table 1]. Furthermore, EDME demonstrated a dose-dependent inhibition pattern, in which the increase in plant extract concentration corroborates with the increase in its inhibition percentage against any radical. EDME at 25 mg/mL concentration showed 36.84, 90.21, 29.19, and 74.27% inhibition against DPPH, ABTS, superoxide radical, and metal chelating activity respectively [Figure 2A]. The EDME also exhibited

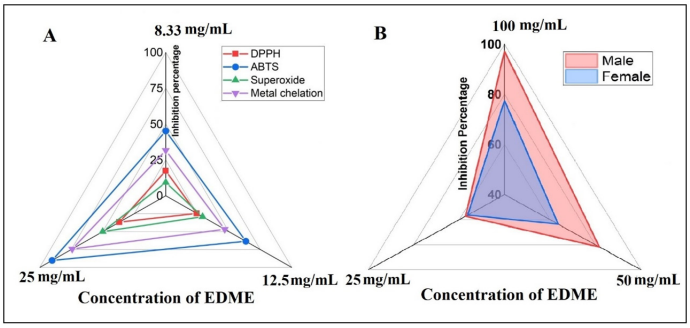

Figure 2: Antioxidant activity (A) of EDME at different concentration showing inhibition percentage (%) against different free radical; and anti-lipid peroxidation activity (B) of EDME at different concentration showing percentage inhibition (%) through anti-lipid peroxidation using male and female rat liver.

Table 1: Antioxidant activity of *E. diffusum* whole plant methanolic extract evaluated through different radical scavenging assay.

Inhibition Percentage 50 (IC_{50}) in mg/mL				FRAP activity (µg AAE/mg DWT)
DPPH	ABTS	Superoxide	Metal Chelation	
36.466 ± 0.404	8.946 ± 0.105	48.496 ± 1.280	14.933 ± 0.266	17.683 ± 0.236

Data presented as Mean ± SD of triplicate determination. AAE: Ascorbic acid equivalent.

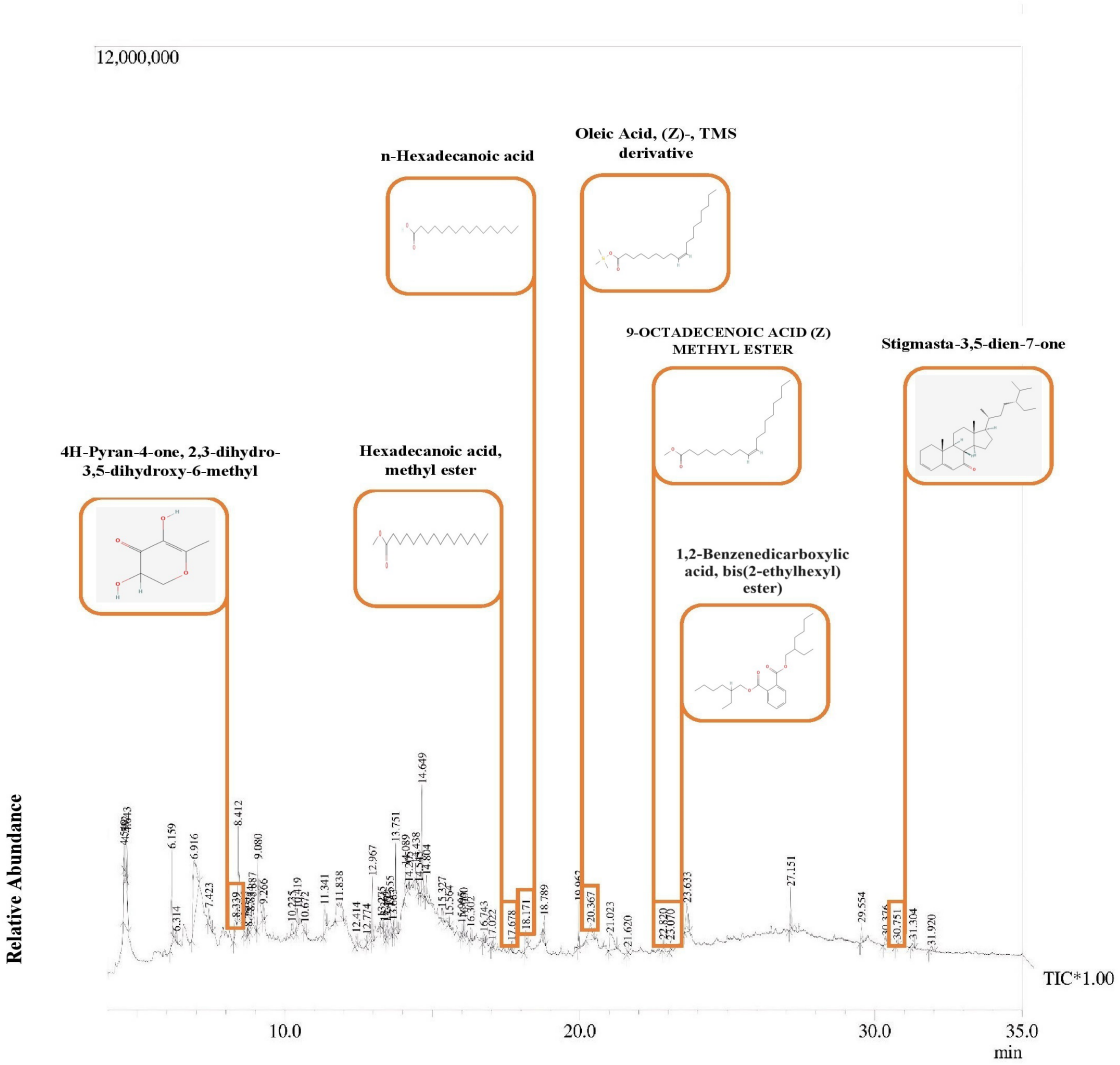

Figure 3: Gas-chromatogram of *Equisetum diffusum* D. Don whole plant methanolic extract. Out of 47 phyto-compounds, seven (7) were found to possess anti-inflammatory and the anti-arthritic properties.

substantial capability to resist the tested free radical even at low concentration (8.33 mg/mL) as evident from our experimental results; in which the inhibition percentage against DPPH, ABTS, superoxide radical, and for metal chelation capability was found to be around 17.40, 45.13, 9.26, and 31.64% respectively [Figure 2A]. In Ferric Reducing Antioxidant Power (FRAP) assay, the transformation of ferric cyanide to the ferrous form by contributing an electron indicates the existence of reductants in the plant samples. FRAP assay revealed that plant extract showed satisfactory (17.683 ± 0.236 µg ascorbic acid equivalent/mg DWT) iron-reducing (Fe^{3+} to Fe^{2+}) capability [Table 1].

Anti-lipid peroxidation capability of EDME

The lipid peroxidation protection ability of the EDME was evaluated using the rat liver of both sexes (i.e., male and female) separately. From the experimental results, it was found that the plant extract provides better inhibition potentiality in the case of male rats when compared to female rats. At 100 mg/mL concentration, EDME demonstrated 97.20% inhibition against lipid peroxidation for male rats which is 25.33% higher than the female rats exposed to liver peroxidation in *in vitro* medium [Figure 2B]. The studied plant extract also showed a

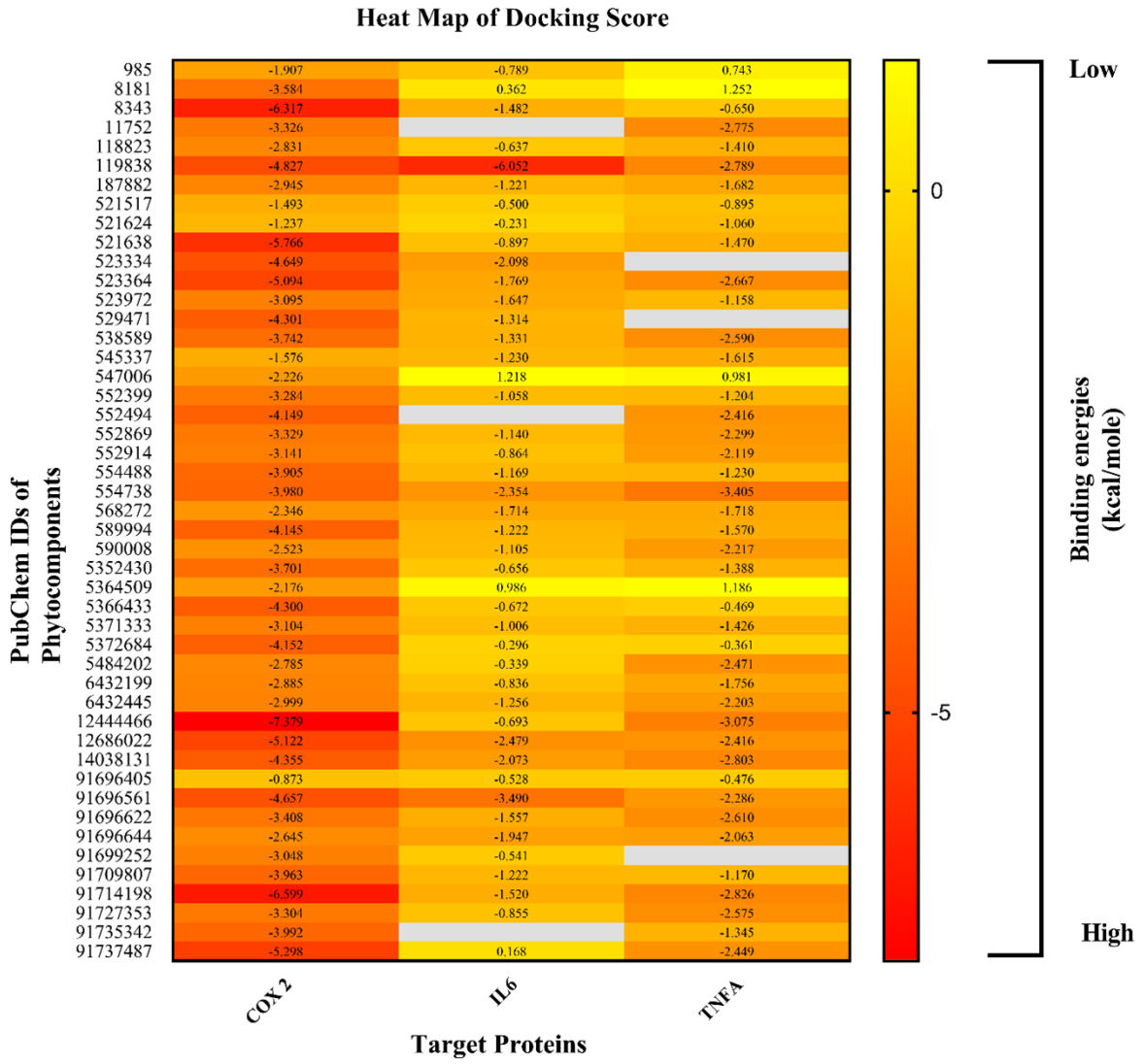

Figure 4: Heat map representing the docking score of 47 phytocomponents with potent anti-inflammatory markers: COX-2; IL-6 and TNFα. The red colour represents higher binding interactions, and the yellow represents lower binding energies.

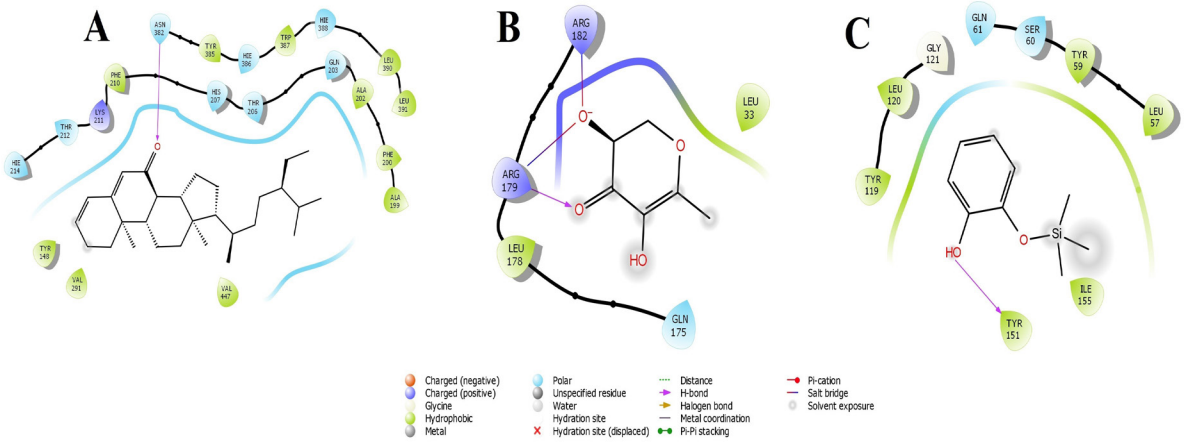

Figure 5: Molecular docking 2D interaction between best docked phyto-compounds and key inflammatory targets proteins. A: Stigmasta-3,5-dien-7-one binds with COX-2; B: 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl binds with IL-6; C: Catechol, TMS derivative binds with TNFα.

Table 2: List of compounds identified from GC-MS analysis of whole plant methanolic extract of *E. diffusum*.

Sl. No.	Name of compound with PubChem ID (PCID)	Chemical formula	M. W. (gm)	Retention time (min)	Area %	Similarity index
1.	N-(2-Methylbutylidene) isobutylami [545337]	$C_9H_{19}N$	141	4.540	1.59	83
2.	1-Butanamine, 2-methyl-N-(2-methylbutylidene)- [521517]	$C_{10}H_{21}N$	155	4.582	1.44	85
3.	3-Methylbutanoic acid, TMS derivative [187882]	$C_8H_{18}O_2Si$	174	4.643	2.30	95
4.	1-Butanamine, 3-methyl-N-(3-methylbutylidene)- [118823]	$C_{10}H_{21}N$	155	6.314	0.49	85
5.	2-Ethoxyethanol, TMS derivative [552399]	$C_7H_{18}O_2Si$	162	6.916	12.50	81
6.	Trimethylsilyl 2,2-dimethyl-3,6,9,12,15,18,21-heptaoxa-2-silatrico san-23-oate [91696561]	$C_{20}H_{44}O_9Si_2$	484	7.423	1.23	80
7.	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl [119838]	$C_6H_8O_4$	144	8.339	1.48	78
8.	Acetin, bis-1,3-trimethylsilyl ether [91696644]	$C_{11}H_{26}O_4Si_2$	278	8.412	6.88	86
9.	1,2,3-Butanetriol, 3TMS derivative [552869]	$C_{13}H_{34}O_3Si_3$	322	8.645	1.21	77
10.	2H-Pyran-2-one, tetrahydro-4-hydroxy-6-pentyl- [538589]	$C_{10}H_{18}O_3$	186	8.753	0.44	66
11.	Catechol, TMS derivative [554738]	$C_9H_{14}O_2Si$	182	8.834	0.61	83
12.	1-Ethyl-1-cyclohexyloxy-1-silacyclopentane [568272]	$C_{12}H_{24}OSi$	212	8.887	2.50	65
13.	Methyl trans-2-trimet hylsilyl-cyclopropane-1-carboxylate [91699252]	$C_8H_{16}O_2Si$	172	9.080	2.75	72
14.	Butanoic acid, 4-((trimethylsilyl)oxy)-, trimethylsilyl ester [589994]	$C_{10}H_{24}O_3Si_2$	248	9.266	0.69	87
15.	Tyramine, TMS derivative [91727353]	$C_{11}H_{19}NOSi$	209	10.235	0.79	65
16.	Octanoic acid, TMS derivative [521624]	$C_{11}H_{24}O_2Si$	216	10.419	1.10	76
17.	n-Octanoic acid, pentamethyldisilanyl ester [554488]	$C_{13}H_{30}O_2Si_2$	274	10.672	0.35	72
18.	Tetraethylene glycol, 2TMS derivative [12686022]	$C_{14}H_{34}O_5Si_2$	338	11.341	2.03	79
19.	Pyroglutamic acid, TMS derivative [14038131]	$C_8H_{15}NO_3Si$	201	11.838	5.67	76
20.	3-Pentanol, 2,4-dimethyl- [11752]	$C_7H_{16}O$	116	12.774	0.35	66
21.	4-Hydroxy-5-(hydroxymethyl) oxolan-2-one, 2TMS derivative [523334]	$C_{11}H_{24}O_4Si_2$	276	12.967	2.75	83
22.	3,8-Dioxa-2,9-disiladecan-5-one, 2,2,6,6,9,9-hexamethyl- [91696622]	$C_{12}H_{28}O_3Si_2$	276	13.275	0.45	70
23.	1-(2-Methoxy-1-methylethoxy)-2-propanol, TMS derivative [91709807]	$C_{10}H_{24}O_3Si$	220	13.462	0.69	80
24.	2-Ethyl-1,3-bis(trimethylsilyloxy)propane [590008]	$C_{11}H_{28}O_2Si_2$	248	13.751	4.51	81

Sl. No.	Name of compound with PubChem ID (PCID)	Chemical formula	M. W. (gm)	Retention time (min)	Area %	Similarity index
25.	D-(-)-Tagatofuranose, pentakis(trimethylsilyl) ether (isomer 1) [523972]	$C_{21}H_{52}O_6Si_5$	540	14.205	0.34	76
26.	2,2,9,9-Tetramethyl-5,6-bis[(trimethylsilyl) oxy]-3,8-dioxa-2,9-disiladecane [6432199]	$C_{16}H_{42}O_4Si_4$	410	14.545	0.41	72
27.	5,6,7,7-Tetramethyl-3,5-octadien-2-one [5371333]	$C_{12}H_{20}O$	180	15.564	0.30	65
28.	1,2,5,6-Hexanetetrol, tetrakis-O-(trimethylsilyl)- [552914]	$C_{18}H_{46}O_4Si_4$	438	15.995	0.46	78
29.	D-Ribo-Hexonic acid, 3-deoxy-2,5,6-tris-O-(trimethylsilyl)-, lactone [523364]	$C_{15}H_{34}O_5Si_3$	378	16.060	0.60	76
30.	5,5-Dimethyl-2-(trimethylsilyl)-1,2,3,4,4a,5,6,7-octahydro-2-naphthalenol [552494]	$C_{15}H_{28}OSi$	252	16.302	0.57	60
31.	9,12-Octadecadienoic acid (z, z)-, trimethylsilyl ester [5352430]	$C_{21}H_{40}O_2Si$	352	16.743	0.71	65
32.	1,5-Anhydrohexitol, 4TMS derivative [529471]	$C_{18}H_{44}O_5Si_4$	452	17.022	0.27	68
33.	Hexadecanoic acid, methyl ester [8181]	$C_{17}H_{34}O_2$	270	17.678	0.33	88
34.	n-Hexadecanoic acid [985]	$C_{16}H_{32}O_2$	256	18.171	1.97	79
35.	Palmitic Acid, TMS derivative [521638]	$C_{19}H_{40}O_2Si$	328	18.789	1.37	94
36.	Phytol, TMS derivative [5372684]	$C_{23}H_{48}OSi$	368	19.962	1.64	95
37.	Oleic Acid, (Z)-, TMS derivative [5366433]	$C_{21}H_{42}O_2Si$	354	20.367	3.26	83
38.	6-Methyl-2-tridecanone [547006]	$C_{14}H_{28}O$	212	21.023	3.89	79
39.	Fumaric acid, decyl 2,4-dimethylpent-3-yl ester [91737487]	$C_{21}H_{38}O_4$	354	21.620	0.22	64
40.	9-Octadecenoic acid (z)-, methyl ester [5364509]	$C_{19}H_{36}O_2$	296	22.820	0.32	82
41.	1,2-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester [8343]	$C_{24}H_{38}O_4$	390	23.070	0.41	92
42.	13-Docosenoic acid, (Z)-, TMS derivative [91696405]	$C_{25}H_{50}O_2Si$	410	23.633	3.54	90
43.	Stigmasta-5,22-dien-3-ol, acetate, (3.beta.)- [6432445]	$C_{31}H_{50}O_2$	454	27.151	2.13	73
44.	Stigmast-5-ene, 3.beta.-(trimethylsiloxy)-, (24S)- [91714198]	$C_{32}H_{58}OSi$	486	29.554	1.39	89
45.	24-Norursa-3,12-diene [91735342]	$C_{29}H_{46}$	394	30.376	0.80	81
46.	Stigmasta-3,5-dien-7-one [12444466]	$C_{29}H_{46}O$	410	30.751	0.46	63
47.	Stigmast-4-en-3-one [5484202]	$C_{29}H_{48}O$	412	31.304	0.67	77

dose-dependent inhibition pattern, in which the lowest dose of EDME (25 mg/mL) showed almost the same amount of inhibition capability against both male (57.26%) and female (56.28%) rats [Figure 2B]. IC_{50} value of the anti-lipid peroxidation against the male and female rat calculated was found to be around 0.84 mg/mL and 2.38 mg/mL respectively.

GC-MS analysis of EDME

The phyto-chemical compositions of the EDME were identified from the GC-MS study and the resultant gas-chromatogram is presented in Figure 3. As per our survey, the GC-MS analysis of whole plant methanolic extract of *E. diffusum* D. Don is reported for the first time. The GC-MS data showed the presence of 47 potent bioactive phyto-compounds in EDME. The IUPAC name of each compound identified by GC-MS analysis along with their Retention Time (RT), molecular formula, molecular weight, area (%), and similarity index are presented in Table 2. Out of 47 phyto-compounds, seven (7) compounds were found to possess anti-inflammatory and the anti-arthritic properties [see Additional File 4].

Molecular docking analysis

The 47 phyto-compounds identified from *E. diffusum* were docked with potent inflammatory markers/cytokines like COX-2, TNF-α, and IL-6 to monitor the binding affinity of phyto-compounds with the proteins. The binding energy of all the phyto-compounds with the inflammatory protein is summarized as a heat map representation in Figure 4. The best-docked 2D conformation between the phyto-compounds and inflammatory protein is represented in Figure 5. The highest binding energy and binding interactions in details between the phyto-compounds and standard NSAIDs with the key inflammatory marker proteins/cytokines was provided as an additional file [see Additional file 5].

From our binding study, we found that the phyto-compounds showed better binding affinity against COX-2 protein, compared to the other two markers (IL-6 and TNF-α). Of the 7 shortlisted bioactive compounds having anti-inflammatory and anti-arthritic activities, two were found to have highest binding affinity with the respective inflammatory protein, COX-2, and IL-6. Stigmasta-3,5-dien-7-one showed the highest binding affinity (-7.379 Kcal/Mol) with target inflammatory protein COX-2 (PDB ID-5IKQ) and formed one H-bond at ASN382 residue [Figure 5A]. Alongside the H-bond, it also forms several hydrophobic interactions (TYR148, ALA199, PHE200, ALA202, PHE210, VAL291, TYR385, TRP387, LEU390, LEU391, and VAL447 residues). Similarly, 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl also showed highest binding affinity (-6.142 Kcal/Mol) with IL-6 protein (PDB ID-1ALU) [see Additional file 5]. However, it forms one H-bond at ARG179 residue and two hydrophobic interactions at LEU33 and LEU 178 residues of the target protein [Figure 5B]. The ligand Catechol, TMS derivative showed good binding affinity (-3.405 Kcal/Mol) with large number of hydrophobic interactions at LEU57, TYR59, TYR119, LEU120, TYR151, and ILE155 residues of target protein TNF-α (PDB ID-2AZ5) [Figure 5C].

In vitro anti-inflammatory activity of EDME

Effect of EDME on protein denaturation-inhibition test

The whole plant methanolic extract of *E. diffusum* were able to inhibit denaturation of protein in a concentration-dependent manner [Figure 6A]. EDME showed inhibitory effect of 37.4 ± 1.79%, 43.4 ± 2.94% and 48.2 ± 3.74% for different dose concentrations of 600 µg/mL, 800 µg/mL, and 1000 µg/mL respectively [see Additional file 6]. The inhibition of protein denaturation by the extract was found to be statistically significant ($p \leq 0.001$) when compared to the control untreated group (distilled water). Diclofenac sodium, the standard reference drug, however, showed the maximum inhibition of 57.6 ± 3.70% at a concentration of 100 µg/mL [see Additional file 6].

Effect of EDME on heat-induced haemolysis

The EDME have effectively inhibited the heat-induced haemolysis of RBCs in a dose-dependent manner [Figure 6B]. The maximum inhibition (33.6 ± 2.55%) was observed in the 1000 µg/mL dose group. All the experimental dose groups showed statistically significant ($p \leq 0.001$) percentage inhibition of haemolysis when compared to the control untreated group (distilled water). However, the standard drug, indomethacin at a concentration of 200 µg/mL showed the highest inhibition of haemolysis of 45.6 ± 2.36% [see Additional file 6].

Effect of EDME on hypotonicity-induced haemolysis

In this test, the EDME of different concentrations significantly ($p \leq 0.001$) protected the erythrocyte membrane against hypotonic-induced haemolysis [Figure 6C]. All the experimental dose groups 1000 µg/mL (58.1 ± 3.39%), 800 µg/mL (32.6 ± 6.3%), and 600 µg/mL (19.5 ± 3.26%) dose group, showed a dose-dependent of inhibition of haemolysis, in hypotonic conditions [Figure 6C]. However, the percentage of inhibition obtained from the standard drug, Indomethacin seems to be maximum (67.2 ± 1.45%) [see Additional file 6].

Acute toxicity studies

All the animals were alive from the start of the experiment till the next 14 days and no death or emergence of toxicological signs were observed among the animals. The lethality value (LD_{50}) of EDME was found to be more than 2000 mg/kg body weight which was considered "non-toxic" up to the feeding range according to the OECD guidelines. Based on this result, the feeding dose of low dose group (250 mg/kg body weight or $1/8^{th}$ of LD_{50} value) and high dose group (500 mg/kg body weight or $1/4^{th}$ of LD_{50} value) were selected for the *in vivo* anti-inflammatory assay.

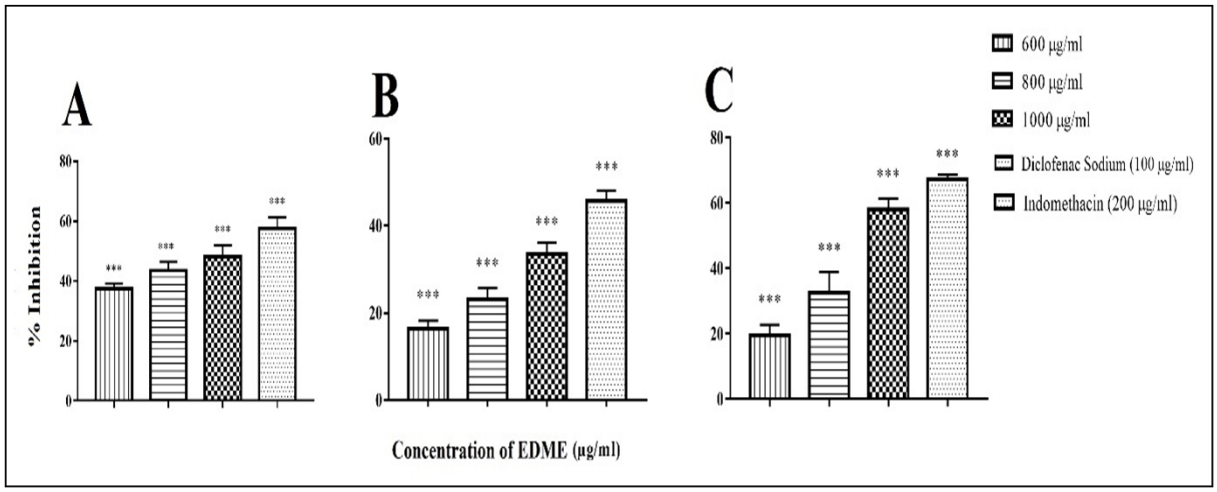

Figure 6: Effects of EDME on protein denaturation (A), heat-induced haemolysis (B) and hypotonicity-induced haemolysis (C). Statistical analysis was done against control untreated group (distilled water) and is considered to have 100% protein denaturation or heat-induced or hypotonicity-induced haemolysis (bar not shown in the graphs). ***indicates $p \leq 0.001$. Data are presented as the mean ± SD of triplicate determinations.

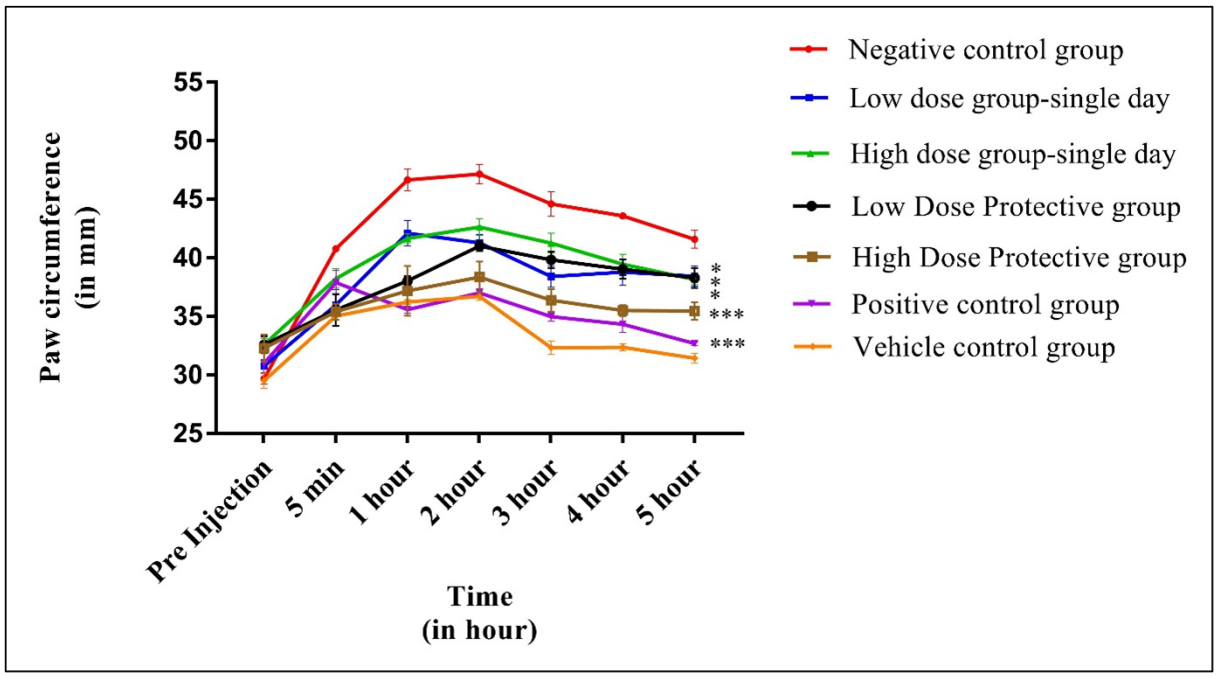

Figure 7: Effect of EDME in the amelioration of paw edema induced by carrageenan. Measurement of paw circumference (in mm) in rats of different experimental groups (n=6). ***indicates $p \leq 0.001$, *indicates $p \leq 0.05$, when compared with negative control group; Comparison between experimental groups were done using two-way Analysis of Variance (ANOVA) following the post hoc analysis with a Dunnett's multiple comparisons test.

In vivo anti-inflammatory activity of EDME

Effect of EDME on carrageenan-induced paw-edema test

The *in vivo* anti-inflammatory activity of EDME was assessed by the carrageenan-induced rat paw-edema test and the result has been shown in Figure 7 and Additional file 7. From our study, we found that the treatment of EDME has significantly (p<0.05) ameliorates the paw edema in both the protective groups (Group 4 and 6) as well as the single-day fed extract treated groups (Group 3 and 5) when compared to the negative control group (Group 2) as shown in Figure 7. Group 3 fed with 250 mg/kg b.w. dose EDME for single-day has inhibited the paw swelling by 35.29% compared to that of the negative control group; whereas Group 4 fed with same dose of EDME for 7 days showed 52.26% inhibition of paw swelling [see Additional file 7]. However, the high dose group of both single-day fed (Group 5) as well as pre-treated (Group 6) has showed maximum inhibition of the paw swelling compared to the negative control group. The single-day fed EDME with 500 mg/kg b.w. dose (Group 5) has inhibited the paw swelling up to 53.10%, while the high dose protective group (Group 6) showed

the highest inhibition of 73.36% among the extract-treated group [see Additional file 7]. Group 7 served as the positive control group (treated with 10 mg/kg b.w of diclofenac sodium) showed maximum inhibition of paw swelling up to 85.88% compared to the negative control group. The paw images of the rats of the experimental groups are provided as an additional file [see Additional file 8]. So, from our results it was observed that both the protective groups showed better amelioration of paw swelling compared to the single day-fed dose groups.

DISCUSSION

The medicinal properties of any plant depend on the presence of several active secondary metabolites of the plant. The quantitative phytochemical analysis of the methanolic extract of our plant indicates the presence of phenol, flavonoid, saponin, and tannins. The presence of these phytochemicals often contribute to the medicinal properties of plants.[48] Flavonoids are polyphenols reported to have anti-oxidant, anti-diabetic, and anti-inflammatory properties due to the presence of hydroxyl phenol in their chemical structure.[49,50] Our experimental results reported satisfactory phenol and flavonoid contents as reported in other species of *Equisetum*.[51] Indeed, recent reports showed a close relationship between the anti-arthritic, anti-oxidant, and anti-inflammatory activity of total phenolics and total flavonoids of nine traditionally used South African plants.[52] Tannins are also polyphenols and act as anti-irritant having anti-phlogistic, anti-secretolytic, anti-microbial, and anti-parasitic properties.[53] Previous works also reported the use of crude saponin extracts in the treatment of inflammatory diseases like rheumatoid arthritis, hemorrhoids, and gout.[54] Natural antioxidants extracted from plants are in demand by the clinicians and biologists as they are consequently linked to anti-cancer, anti-inflammatory, hypo-lipidemic, and anti-aging activities.[55] From our study, we also found that EDME exhibited resistance against hydrogen peroxide, superoxide, and nitrous oxide radicals as revealed through a wide range of free radical scavenging assays. Our experimental results also depicted quite high IC_{50} values in comparison to *E. ramosissimum*.[56] Besides that, the *in vitro* anti-lipid peroxidation capability of EDME also showed similar potential compared to *E. hyemale*.[57] The anti-lipid peroxidation and antioxidant properties of EDME indicated its role in the medication of several inflammatory diseases, as anti-oxidants are reported to protect tissue from damage and prevents unwanted inflammatory responses.[58]

To determine the *in vitro* anti-inflammatory activity of any compound protein denaturation test is widely practiced.[43] In the protein denaturation-inhibition test, the egg albumin was denatured by heat treatment. This heat treatment denatures the three-dimensional configuration of proteins thereby altering their biological activity. Most of the chronic inflammatory diseases is linked with this protein denaturation.[39] The commercially available standard NSAID drugs (Diclofenac sodium) can inhibit the denaturation of protein.[41] In our study, it was found that the methanolic extract of *E. diffusum* can significantly inhibit the heat-induced denaturation of protein in a concentration-dependent manner [Figure 6]. Erythrocyte membrane stability test is another widely followed method used for determining the anti-inflammatory potency of any drug or herbal products. The lysis of the lysosomal membrane during inflammatory conditions prolonged the inflammatory responses by releasing the lysosomal contents.[39,59] Therefore, it is necessary to stabilize the lysosomal membrane in order to resolve the inflammatory responses. The use of non-steroidal anti-inflammatory drugs (NSAIDs) (Indomethacin) inhibits the release of lysosomal enzymes by stabilizing the lysosomal membranes.[59] The RBCs membrane shows structural similarity to that of the lysosomal membrane and exposure to high temperature and hypotonic medium lyse the RBC membranes resulting in its haemolysis and oxidation of haemoglobin.[39,59] In our study, the methanolic extract of *E. diffusum* showed a significant dose-dependent protective effect on membrane stabilization on both the heat-induced and hypotonicity-induced damage [Figure 6]. The results of *in vitro* anti-inflammatory study further encourage for validating the efficacy of the plant in *in vivo* inflammatory conditions.

For evaluating the efficacy of the plant in *in vivo* conditions, the toxic characteristic of the natural plant-product must be screened first. In this respect, the acute oral toxicity test was performed. In this test, EDME at a dose of 2000 mg/kg body weight had no adverse effect on the experimental rats up to 14 days of observation and considered as 'unclassified'. Based on these observations, the EDME is considered as 'low toxic product'.

Carrageenan-induced paw-edema is a reliable model used for determining the *in vivo* anti-inflammatory activity of any compound. This model can induce both local and acute inflammatory responses and the development of paw-edema is believed to be a triphasic event.[60] The initial phase of which begins within 1 hr upon carrageenan induction and involves with the release of serotonin and histamine, the second phase (2-3 hr) is mediated by kinins and the third phase occurs in after 5 hr of induction and is mediated by the prostaglandins, cyclooxygenases, and lipoxygenases.[60] From our study, we also found similar trend of increment of paw-edema in the first 2-3 hr. However, both the protective-group rats, pre-treated with EDME for 7 days, showed maximum paw-edema inhibition compared to single day fed rats [Figure 7]. Additionally, the positive control group rats those received diclofenac sodium, an established NSAID, also showed similar degree of paw edema inhibition like that of the EDME protective groups of rats. Our results also showed better inhibition of paw-edema compared to the results of hydroalcoholic extracts of *E. arvense* in carrageenan-induced mice model.[13] The anti-inflammatory activity of the plant may be

attributable to the presence of various phytochemical bioactives like flavonoids, terpenoids and saponins.[52,54] The GC-MS analysis of EDME also confirmed the presence of compounds like Hexadecanoic acid, methyl ester; Oleic Acid, (Z)-, TMS derivative; Stigmasta-3,5-dien-7-one; 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl; and 1,2-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester which have been reported to be have potent anti-inflammatory and anti-arthritic activity.[61-64]

We have also evaluated the molecular docking studies of all the phyto-compounds of *E. diffusum* extract with potent anti-inflammatory marker protein (COX-2, IL-6, and TNF-α) to demonstrate the collaboration between the phyto-compounds and protein at the molecular level, in order to corroborate the results of the *in vitro* and *in vivo* anti-inflammatory activity. From our results, it was found that Stigmasta-3,5-dien-7-one (-7.379 Kcal/Mol) and 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl (-6.142 Kcal/Mol) showed the highest binding affinity against target inflammatory protein COX-2 and IL-6, respectively [Figure 4]. Hydrogen bond formation is one of the major parameters in predicting docking score. The formation of hydrogen bond between ligand and protein structure reflects the firmness of their binding.[65] Our results also showed formation of single H-bond between Stigmasta-3,5-dien-7-one and COX-2 at ASN382 residue, and between 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl and IL-6 at ARG179 residue [Figure 5]. The docking score with these ligands was found to be better than that of the docking score of standard drugs, Indomethacin (-5.155 Kcal/Mol) and Diclofenac sodium (-5.148 Kcal/Mol) against COX-2 [see Additional file 5]. Most of these drugs used for combating inflammation mainly function by downregulating the prostaglandin pathway and thereby reduce the expression of pro-inflammatory markers/cytokines like COX-2, TNF-α, and IL-6.[66] The presence of several anti-inflammatory compounds and their binding score with these pro-inflammatory markers/cytokines validate the *in vitro* and *in vivo* anti-inflammatory activity of the plant. All our findings indicate that the bioactive phyto-components in EDME could possibly inhibit the inflammatory response by downregulating the prostaglandin pathway.

CONCLUSION

Our study experimentally explores the activity of *Equisetum diffusum* D. Don whole plant Methanolic Extract (EDME) in acute inflammation through *in vitro* and *in vivo* tests. Both the protein denaturation and membrane stabilization test confirmed the anti-inflammatory activity of extracts. The *in vivo* carrageenan model also showed maximum inhibition of paw-edema in the protective group rats, thereby confirming the efficacy of the plant against inflammatory diseases. The quantitative phytochemical analysis of the methanolic extract also confirmed the presence of phenol, flavonoid, saponin, and tannins. The anti-lipid peroxidation and antioxidant properties of EDME also indicated its role in the medication of inflammatory disease. Moreover, GC-MS analysis of EDME indicated the presence of several bioactive phyto-components possessing potent anti-inflammatory activity against molecular targets. Our docking results indicate that the binding scores of bioactive components of EDME against inflammatory markers like, Cox-2, IL-6, and TNF-α were better than that of commercially available NSAIDs. Therefore, the efficacy of the *Equisetum diffusum* whole plant methanolic extract suggested a potential candidate for further exploration and drug development in treating inflammatory diseases.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

EDME: *Equisetum diffusum* whole plant methanolic extract; **NSAIDs:** Non-steroidal anti-inflammatory drugs; **CAMs:** Complementary and alternative medicines; **WHO:** World Health Organization; **GC-MS:** Gas chromatography-mass spectrometry; **CMC:** Carboxymethylcellulose; **DPPH:** 2, 2-diphenyl-1-picrylhydrazyl; **ABTS+:** 2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid); **FRAP:** Ferric reducing antioxidant power; **AIRF:** Advanced Instrumentation Research Facility; **TIC:** Total Ion Count; **COX:** Cyclooxygenase; **NCBI:** National Center for Biotechnology Information; **PDB:** Protein Data Bank; **PBS:** Phosphate buffer saline; **EDTA:** Ethylenediaminetetraacetic acid; **CPCSEA:** Committee for the Purpose of Control and Supervision of Experiments on Animals; **IAEC:** Institutional Animal Ethical Committee; **OECD:** Organization for Economic Cooperation and Development; **SEM:** Standard error mean; **SD:** Standard Deviation; **ANOVA:** One-way analysis of variance.

AUTHORS CONTRIBUTIONS

SB contributed to concept and designing of whole experiment; JJG designed all *in silico* studies; MSH performed and analyzed the data of *in vitro* plant activity; DM performed the *in silico* experiments and interpreted the data; SS and DM performed the *in vivo* experiments and analyzed the data; SS prepared and revised the manuscript; interpretation of the experimental outcome, critical revision of the manuscript and approval of the manuscript for publication were done by SB. All authors read and approved the final manuscript. SB: Soumen Bhattacharjee; JJG: John J. Georrge; MSH: Md Salman Haydar; DM: Debabrata Modak; SS: Sourav Sarkar.

SUMMARY

This study explores the anti-inflammatory activities of Equisetum diffusum D. Don whole plant methanolic extract (EDME) through *in silico*, *in vitro* and *in vivo* tests. The GC-MS analysis of EDME indicated the presence of several potential anti-inflammatory bioactives which was confirmed by docking results against prominent inflammatory markers like, Cox-2, IL-6, and TNF-α. The protein denaturation and membrane stabilization tests also confirmed the anti-inflammatory activities of the extract. The in vivo carrageenan model showed substantial inhibition of paw-edema in the protective group rats when compared to negative control group animals, thereby confirming the efficacy of the plant extract against inflammatory diseases.

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