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In silico, in vitro, and in vivo acute and sub-acute toxicity profiling of whole plant methanol extract of *Equisetum diffusum* D. Don from the sub-Himalayan West Bengal, India, having ethnobotanical uses

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Abstract

Background *Equisetum diffusum* D. Don commonly known as 'Himalayan horsetail', has been traditionally used in the treatment of back pain, bone fracture and dislocation, and arthritis by various tribal communities of India. Our previous study confirmed the anti-inflammatory efficacy of the plant through in silico, in vitro, and in vivo model studies. Therefore, the current research is focused on safety dose evaluation for the first-time of the whole-plant methanol extract (EDME) of *E. diffusum* through appropriate in silico, in vitro, and in vivo approaches.

Method The whole plant, along with its rhizomes, was collected, and the methanol extract was prepared. The in silico ADMET study was performed to predict the pharmacokinetics profile and toxicity of all the identified phytocompounds of EDME previously screened by GC–MS study. In vitro cytotoxicity study of EDME was performed using two cell lines: kidney (HEK293) and liver (Huh7) cell lines. The in vivo toxicity study of EDME was validated by the acute toxicity (OECD 423, 2002) and sub-acute toxicity assays (OECD 407, 2008) in the Wistar Albino rat model.

Results The in silico ADMET study of all 47 bioactives predicted good pharmacokinetic and low toxicity profiles. In vitro cytotoxicity showed higher IC_{50} values of EDME viz., 672 ± 15.7 µg/mL and 1698 ± 6.54 µg/mL for both kidney (HEK293) and liver (Huh7) cell lines, respectively, which were considered as low-toxic. Based on acute oral toxicity, the LD_{50} value of the extract was considered "non-toxic" up to a feeding range of 2000 mg/kg of body weight. The regular consumption of the extract for an extended period (28 days) was also qualified as safe based on the body and organ weight, hematological, biochemical, and histoarchitecture results in the sub-acute toxicity assay.

Conclusion The detailed in silico, in vitro, in vivo (acute and sub-acute oral toxicity) studies gave us a new insight to the safety dose evaluation of *Equisetum diffusum*, which may serve as a reliable documentation for undertaking the experimental validation of the ethnobotanical uses of the plant which would help in the field of drug development for the treatment of inflammation related complications.

Keywords *Equisetum diffusum* D. Don, ADME-Toxicity, HEK293, Huh7, Acute toxicity, Sub-acute toxicity

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Background

Medicinal plants are the major sources of drugs used in the management of various diseases. The use of traditional medication, particularly herbal, is widely in use in the treatment of various diseases in the developing countries [1]. Ayurveda, a 3000-year-old medicinal system, has been traditionally practiced in India [2]. About 70% of all Indians still rely on 'Ayurvedic herbal medication' for their primary healthcare [2]. The practice of this 'alternative medication' has attained more focus due to its easy availability, economical sustainability, minimum side effects, and success rates. The clinical results of traditional medication are also backed by its lower adverse effects compared to Western medicine [3, 4]. Although the use of herbal drugs is often safe with manageable side effects, yet it has its own limitations. The major limitations of herbal drugs are their unknown chemical composition, minimum knowledge of their biological activities, and experimental validation of their toxicological information [5, 6]. It necessitates, therefore, a proper scientific assay to evaluate the safe-dose and efficacy of the herbal crude plant products for its safe pharmaceutical applications.

Equisetum diffusum D. Don (English name: Himalayan horsetail; Nepali name: Kurkure Jhar; Family: *Equisetaceae*) is a native medicinal pteridophyte of the Himalayan mountains [7]. The plant is widely distributed in the hilly areas (1500 to 8500 feet) of tropical and subtropical regions, especially in south-east Asian countries including India, Nepal, Bangladesh, Pakistan, Vietnam, China, Tibet, and Japan [8, 9]. The stem of the plant is split by nodes that are joined by 5 to 6-long, hollow internodes that are 1.7 to 5.0 cm in length [8]. Owing to the abundance of silicic acid, this *Equisetum* species has various ethnomedicinal properties and has been used as a common folkloric medication by various tribal communities of the Eastern-Himalayan region of India [7]. The Adi community and Galo, Tagin, and Nyshi tribes of Arunachal Pradesh use the whole plant extract in the treatment of bone fracture [10, 11]. The plant has also been used by the ethnic groups of Jammu and Kashmir, Madhya Pradesh, and Sikkim states of India for treating arthritis, bone dislocation, and fracture [9, 12–14]. Due to its anti-inflammatory properties, the Mulam people of Guangxi, China, use the grounded fresh plant parts over the affected area, and the plant is considered as 'Vietnamese folk medicine' for its anti-inflammatory, diuretic, and hemostatic properties [15, 16].

Phytochemical studies on methanol extract of *E. diffusum* showed the presence of bioactive compounds such as tannin, saponin, phenols, and flavonoids [17, 18]. The presence of such phytochemicals also confirmed the antimicrobial and anti-fungal activities of the plant extract [19]. The methanol extract of the plant also showed good antioxidant activity against ABTS, ferrous chelation, and DPPH radical [17, 18]. Our recent findings confirmed the presence of 47 bio-active phyto-compounds through the GC–MS analysis of whole plant methanol extract [17]. Out of the 47 found, seven (7) possess anti-inflammatory and anti-arthritic properties (Oleic Acid, (Z)-, TMS derivative; Hexadecanoic acid, methyl ester; Stigmasta-3,5-dien-7-one; and 1,2-Benzenedicarboxylic acid, bis(2-ethylhexyl) esters) [20–23]. The anti-inflammatory activity of the plant was also confirmed by the in vitro anti-inflammatory (protein denaturation, heat-induced, and hypotonicity-induced haemolysis) and in vivo anti-inflammatory (carrageenan-induced paw edema) studies [17]. Despite the validation of the anti-inflammatory properties of *E. diffusum,* data related to toxicity profiling and safety dose evaluation of the plant were not been documented to date. So, the prime objective of the present study was to evaluate the detailed toxicity profiles of the whole plant methanol extract of *Equisetum diffusum* D. Don (EDME) through appropriate in silico, in vitro, and in vivo approaches.

Materials and methods

Plant material and preparation of *E. diffusum* whole plant methanol extract (EDME)

The whole plant of *Equisetum diffusum* D. Don has been collected from the foothills of the northern part of West Bengal (Darjeeling hilly region). The plant specimen was identified by Dr. R. K. Gupta, Scientist 'E' of the Central National Herbarium, Botanical Survey of India (BSI), Howrah, India. A voucher specimen number NBU/SS-002 was deposited in Central National Herbarium, BSI, India. The whole plant methanol extract of the *E. diffusum* (EDME) was prepared following a previously established procedure [17]. The extract (EDME) was then stored in an airtight container at 4 °C for future experimental usage. For animal feeding, the extract was reconstituted in 0.5% carboxymethylcellulose (CMC).

In silico toxicity study

ADME-toxicity study From the previous GC–MS data [17], the canonical SMILES (Simplified Molecular Input Line Entry System) format and 2D SDF (Structure Data File) format of all the phyto-compounds were retrieved from the NCBI (National Center for Biotechnology Information) PubChem database [24]. Then, in the Maestro flatform, ligands were prepared in the LigPrep module of Maestro (LigPrep, Maestro Version 12.5.139, Schrödinger, LLC, New York) using the OPLS3 (Optimized Potentials for Liquid Simulations) force field. The assumption of the pharmacokinetics profile and toxicity

prediction of all the potential compounds was conducted using the QikProp module of Maestro (QikProp, Maestro Version 12.5.139, Schrödinger, LLC, New York), Toxicity descriptor algorithm TOPKAT (Toxicity Prediction by Komputer Assisted Technology) in the BIOVIA Discovery Studio, and ADMETlab 2.0 server [25].

In vitro toxicity study

Cytotoxicity assay To determine the cytotoxicity of the plant extract, the spectrophotometric MTT (3-(4, 5-dimethyl thiazolyl-2)-2, 5-diphenyltetrazolium bromide) (HiMedia, India) test was conducted as per standard protocol with slight modifications [26]. Briefly, 5×10^3 HEK293 cells and 2×10^4 Huh7 cell line were cultured in DMEM (Dulbecco's Modified Eagle Medium) culture medium (HiMedia, India) with 10% FBS (Foetal Bovine Serum) and 1% antibiotic solution at 37 °C with 5% CO_2 in a CO_2 incubator for 24 h. After 24 h of incubation, each well was treated with different concentrations of EDME and incubated at 37 °C with 5% CO_2 for 24 h. Next, MTT (5 mg/ml) dissolved in Phosphate buffer saline (PBS) was added in each well and incubated for 3 h to allow it to form purple-colored formazan crystals. Finally, Dimethyl sulfoxide (DMSO) (Sigma Aldrich, USA) was added to dissolve the formazan crystals and was incubated for 15 min at room temperature. After the incubation period, absorbance was recorded at 570 nm/590 nm in a multi-mode microplate reader (BioTek SynergyTM H1, Vermont, USA). All experiments were replicated thrice independently to calculate cell viability using the following equation: Cell viability (%) = (OD Treated/OD Control) × 100.

In vivo toxicity study

Experimental animals For the in vivo studies, Wistar albino rats of both sexes (8–12 weeks old; 120 ± 10 g) were used. Rats were purchased from an authorized animal dealer (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 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) (now CCSEA or Committee for 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 [27]. 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 dose groups. In the experimental dose groups, 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 at 30 min, 1 h, 2 h, 4 h, 6 h, 12 h, and 24 h and, thereafter, for 14 days for any delayed toxicological effects. Animals were observed daily for any behavioral change, such as consumption rate of food and water, aggressiveness, sedation, diarrhea, rising of fur, lethargy, mortality, and morbidity till the end of the experimental tenure. The body weight of the rats from each experimental group was recorded at day 0 followed by each week till the end of the experimental schedule. Based on acute toxicity results, the doses for the sub-acute toxicity study were selected.

Sub-acute toxicity studies Sub-acute toxicity test was performed according to the OECD guidelines 407 [28]. In this experimental set, Wistar albino rats (120 ± 10 g) of both sexes were categorized into 4 groups; each group contained 6 rats ($n=6$); which were further subdivided into 3 males and 3 females separately. Group I represented the normal control group and were administered with normal water for 28 days orally. Group II was considered as a low-dose group and received 250 mg/kg body weight of EDME. Group III was considered as a high- dose group and the animals in this group received 500 mg/kg body weight of EDME. Group IV served as vehicle control and was administered with 0.5% CMC orally for 28 days. All the animals from both the dose groups (group II and group III) received the treatment orally once daily at a fixed time continually for 28 days. During the experimental schedule, all the rats were observed for any abnormal behavioural signs, food intake, fur irritation, lethargic behaviour, mortality, and morbidity till the completion of the experiment. At the end of the experimental period, all the rats were kept fasted overnight (on day 28) and were sacrificed by cervical dislocation after euthanizing with sodium pentobarbital (60 mg/kg; i.p.) on the 29th day following standard procedure [29].

Body weight and relative body organ weight

Body weight from each experimental group was recorded before commencing the experiment on day 0. After starting the experimental tenure, body weight from each experimental group was measured at the end of each week till the end of the experimental schedule. On the 29th day, before the sacrifice, the final body weights were recorded, and immediately after the sacrifice, body organs (liver, kidneys, and spleen) were dissected out, and measured separately to obtain their absolute organ weight. From the absolute organ weight value, the relative organ weight of each animal in all the experimental groups was calculated by this formula [30]: Relative organ weight (ROW) = Absolute organ weight (g) × 100/ Body weight of rat on day of sacrifice (g).

Blood sample collection

Blood samples were collected by cardiac puncture into EDTA-coated vials (for hematological parameters) as well as in non-EDTA-coated vials (for serum parameters) following standard protocol [30].

Hematological analyses

For hematological parameters, collected blood samples (in EDTA-coated vials) were analysed using an automated hematology analyser (Sysmex XN-1000, Mumbai, India) following standard protocol [29]. The following parameters were considered for hematological analyses: red blood cell count (RBC), white blood cell count (WBC), hemoglobin (Hb) content, platelets (Pt) count, neutrophils (NP), lymphocytes (LC), monocytes (MC), eosinophils (EP), basophils (BP), hematocrit (HCT), Mean Red Blood Cell Volume (MCV), Mean Corpuscular Hb (MCH), Mean Corpuscular Hb Concentration (MCHC), and Mean Platelet cell Volume (MPV).

Biochemical parameters

For serum analyses, blood samples collected into non-EDTA coated vials were centrifuged (5000 rpm for 10 min, 4°C) and serum was collected. The collected serum samples were analysed for different biochemical parameters. To assess the liver function alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total protein content, and albumin content were measured. The measurement of urea and creatinine concentrations was done to evaluate kidney function. The amount of total cholesterol, triglyceride, HDL-C, and LDL-C were determined to evaluate the effect of the extract on the lipid profile. In addition, the amount of glucose and electrolytes (Sodium and Potassium) were also measured. All the biochemical parameters were measured using commercial Coral Kits (Coral clinical systems, India) following the manufacturers' protocol using a spectrophotometer (UV-1900i VIS-spectrophotometer, Shimadzu, Japan).

Histopathological examinations

Organs such as liver, kidney, spleen, stomach, and intestine were collected immediately after sacrifice from the rats of each group, washed with chilled PBS (pH 7.4), and fixed in 4% formalin. After fixation, the tissues were dehydrated using serial dilutions of ethanol, followed by embedding in paraffin wax. Tissues were then cut at 5 µm thickness and stained with hematoxylin–eosin. The tissues were then examined under a light microscope (Nikon Eclipse E200, Nikon, Tokyo, Japan) with 10X and 40X objectives and a scale bar was attached (100 µm and 25 µm) respectively [29].

Statistical analyses

Quantitative data concerning the body weight, and organ weight 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).

Results

ADME-toxicity analysis of EDME

The gas-chromatogram and phyto-chemical components of EDME identified from the GC–MS study are presented in Fig. 1 and Additional File 1. The reported phyto-compounds from EDME having anti-inflammatory and anti-arthritic activity are summarized in Additional File 2. The ADMET properties of 47 bio-active phyto-compounds identified from EDME through GC–MS analysis are presented in Additional File 3. From the ADMET data, it was found that all the compounds meet Lipinski's rule of five, and almost all of them were also found to have attained a good score of bioavailability. Solubility is an important parameter governing the absorption of the compound and its distribution in the body, and it was determined by the aqueous solubility values (QPlogS). Our results indicated that most of the compounds were highly soluble in water, and other polar solvents like methanol.

Caco-2 (human colon adenocarcinoma cell lines) permeability, MDCK (Madin-Darby Canine Kidney cells) permeability, human intestinal absorption, skin permeability levels, and P-glycoprotein substrate or inhibitor were used to predict the absorption level of the phyto-compounds. From our ADME results, it was predicted that most of the compounds (34 out of 47)

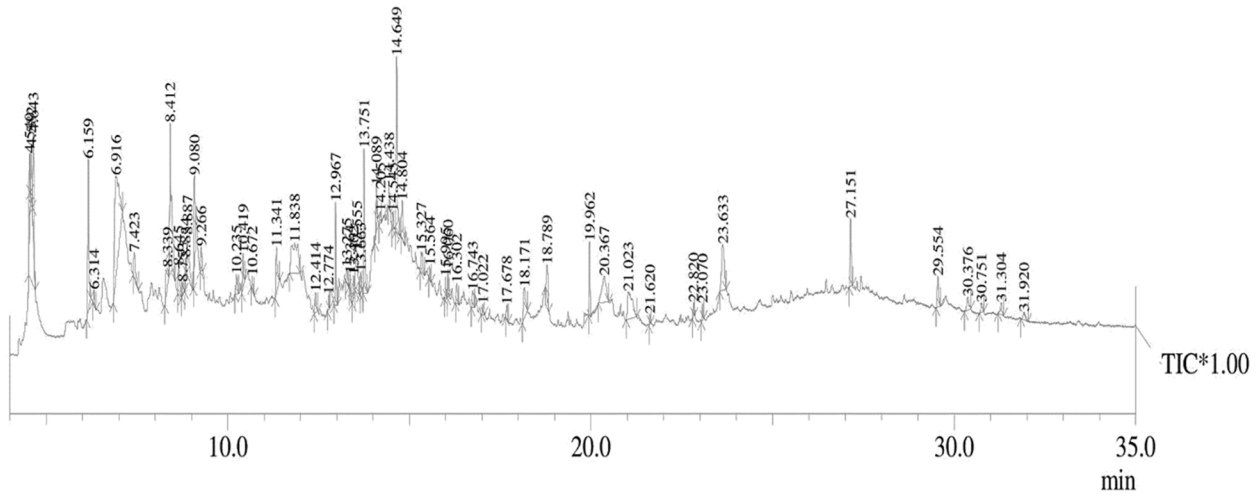

Fig. 1 GC–MS chromatogram of *Equisetum diffusum* D. Don whole plant methanol extract

of EDME seemed to have moderate-to-potent Caco-2 permeability values (> -5.15 log cm/s). Our results further indicated that all the compounds were highly passive for MDCK permeability (Papp > 20×10^{-6} cm/s). Similar trends were also noticed in the case of human intestinal absorption (HIA). Forty-four (44) phytocompounds of EDME showed a good HIA score (above 30%). Regarding skin permeability, it was found that most of the identified phytocompounds in the EDME were found to possess moderate-to-good skin-penetrability (log Kp value > -2.5 cm/h) (Additional File 3). The P-glycoprotein, also known as MDR1 or ABCB1, is a membrane-protein member of the ATP-binding cassette (ABC) transporter superfamily, involved in excreting drugs or other exogenous chemicals from cells. Our results suggested that the thirty-nine (39) phytocompounds of EDME could actively modulate the P-glycoprotein, which could participate in cellular transport. Moreover, all the short-listed phyto-compounds (7 out of 47) having anti-inflammatory and/or anti-arthritic properties were predicted to have good absorption properties. All the them showed moderate-to-potent Caco-2 permeability values (> -5.15 log cm/s), were found to be highly passive for MDCK permeability (Papp > 20×10^{-6} cm/s), and showed good HIA score (above 30%) (Additional File 4).

The volume of distribution (VD), fraction unbound (Fu) in the plasma, and blood–brain barrier (BBB) penetration values were used to predict the distribution of compounds. The VD is an important parameter to describe the in vivo distribution of drugs. Our results showed that the distribution volume (VD) of all the compounds was high and within the predicted range (between 0.04–20 L/kg). The efficacy of a given compound may be affected by the degree to which it binds to proteins within the blood; the more the bond, the less efficiently it can traverse cellular membranes or diffuse through. Most of the compounds of EDME (26 out of 47) showed good Fu value, indicating probable unbound plasma-protein interactions. The blood–brain barrier (BBB) membrane permeability also showed promising results; 38 out of 47 compounds were permeable to cross the BBB (logBB > 0.3). The compounds like phytol, TMS derivative [PCID 5372684], and 13-Docosenoic acid, (Z)-, TMS derivative [PCID 91696405] had the highest distribution volume (logVD) of 5.894 L/kg and 6.098 L/kg, respectively, and are supposed to show the more successful distribution in the tissues (Additional File 3). Moreover, all the 7 short-listed compounds were also predicted to have good Distribution properties. The BBB membrane permeability (logBB > 0.3) and distribution volume (VD between 0.04–20 L/kg) of all 7 compounds except 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl- [PCID 119838] was high and within the predicted range (Additional File 4).

Cytochrome P450 is one of the most important drug-metabolising enzymes in the liver. The two main subtypes of cytochrome P450 are CYP2D6 and CYP3A4. The inhibition of these subtypes could lead to various adverse effects due to drug toxicity. Our results found that most of the compounds were non-inhibitors/non-substrates for the two subtypes; only compound 5,6,7,7-Tetramethyl-3,5-octadien-2-one [PCID 5371333] was a substrate for both CYP2D6 and CYP3A4. All the compounds except 9-Octadecenoic acid (z)-, methyl ester [PCID 5364509] were predicted to be non-inhibitors of both CYP2D6 and CYP3A4 subtypes (Additional File 4). The predicted Metabolism results of the short-listed compounds were found to be promising. All the 7 compounds were found

to be non-inhibitors/non-substrates for the two subtypes: CYP2D6 and CYP3A4, except Stigmasta-3,5-dien-7-one [PCID 12444466] and 1,2-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester [PCID 8343] (Additional File 4).

The clearance rate (CL) and half-life of the compound ($T_{1/2}$) were predicted to know the probable toxicity in the excretion route. The results of CL predicted 19 compounds to have a good-to-moderate clearance rate. The compounds like 24-Norursa-3,12-diene [PCID 91735342] and Stigmast-4-en-3-one [PCID 5484202] had the highest clearance rate (ml/min/kg) of 18.721 ml/min/kg and 15.901 ml/min/kg, respectively, and are supposed to be actively excreted from the kidney cells (Additional File 3). The half-life ($T_{1/2}$) data predicted that out of the 47 compounds, 20 compounds showed good $T_{1/2}$ values (with $T_{1/2} \leq 3$) and were classified as $T_{1/2+}$, while 11 compounds showed moderate $T_{1/2}$ values (with $T_{1/2}$ between 0.3–0.7). Moreover, out of the 7 short-listed compounds, 3 showed good clearance rate (CL) and good half-life ($T_{1/2}$) value (Additional File 4). However, only one compound 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl- [PCID 119838] showed poor clearance rate ($CL < 5$) and poor half-life ($T_{1/2} > 3$) value (Additional File 4).

The AMES toxicity, hERG inhibition, human hepatotoxicity (H-HT), rat oral acute toxicity (ROA), LD_{50} in the rat (g/kg body weight), and skin sensitisation parameters were predicted to find out the probable toxicity profile of the identified phyto-compounds from EDME. The AMES toxicity test predicts for mutagenicity of any compound. AMES toxicity results predicted that all the compounds of EDME to be AMES-negative, i.e., non-mutagenic, except 4H-Pyran-4-one, 2,3-Dihydro-3,5-dihydroxy-6-methyl- [PCID 119838]. The hERG potassium channel inhibition predicts the rate of cardiotoxicity of any drug. Our results suggest that the majority of the compounds (93%) do not inhibit the hERG potassium channel (Additional File 3). Human hepatotoxicity (H-HT) predicts the overall rate of liver damage caused by any drug molecules. Our results suggested that all the phytocompounds from EDME were H-HT negative (-), i.e., non-toxic to the liver. Rat oral acute toxicity (ROA) is another parameter for predicting the safety evaluation of any drug in the rodent model. From our predicted ROA results, it was depicted that all the phytocompounds of EDME (100%) had high ROA value (> 500 mg/kg), which was classified as 'non-toxic' in nature. Skin sensitisation results predicted that most of the compounds (68%) were 'non-sensitiser' (Additional File 3). The predicted toxicity results of the short-listed compounds were found to be promising. Except for one compound Stigmasta-3,5-dien-7-one [PCID 12444466], all other 6 were found to be non-inhibitor of hERG potassium channel (non-toxic to the heart) and non-sensitiser to the skin (Additional File 4). All the 7 compounds were found to be non-toxic to the liver (H-HT negative) and showed a high ROA value (> 500 mg/kg) (Additional File 4).

In vitro cytotoxicity assay of EDME

The potential cytotoxicity of EDME was performed against a kidney (HEK293) cell line and a liver (Huh7) cell line by MTT cell-viability assay. EDME at 25–1800 µg/mL concentrations were used and cell viability results were presented in Fig. 2 and summarized in Additional

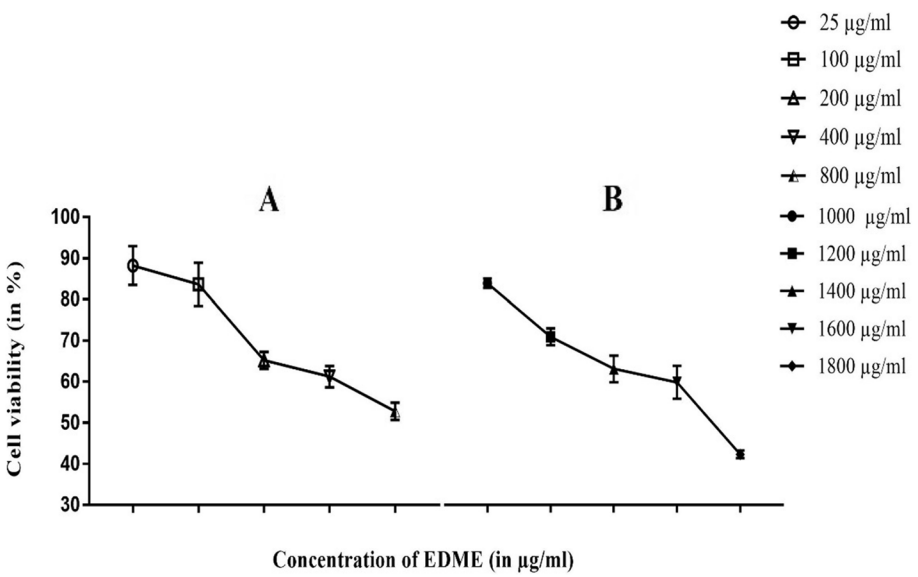

Fig. 2 Cytotoxicity of *E. diffusum* whole plant methanol extract (EDME) against two cell lines. **A** HEK293, kidney cell line; **B** Huh7, liver cell line

File 5. From our results, it was observed that with the increase in extract concentration, the cell viability decreased (Fig. 2). EDME were found to be toxic at higher than 400 µg/mL concentration for the HEK293 cell line and for the Huh7 cell line, it was found to be higher than 1600 µg/mL concentration (Fig. 2 and Additional File 5). However, the kidney cell line retained higher than 80% cell viability when exposed to an extract concentration of less than 100 µg/mL. Moreover, up to 1000 µg/mL EDME concentration, the liver cell line retained higher than 80% cell viability. The concentrations of EDME responsible for a 50% reduction in cell viability (IC_{50}) was found to be around 672 ± 15.7 µg/mL and 1698 ± 6.54 µg/mL for the HEK293 cell line and Huh7 cell line, respectively (Additional File 5).

Acute toxicity studies

All the animals were alive till the experimental tenure (14 days) and no death or changes in the behavioural physiology were observed (Additional File 6). The body weight data also showed no significant alternation after single-day exposure to EDME among different experimental groups (Fig. 3). 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 the low dose group (250 mg/kg body weight or 1/8th of LD_{50} value) and the high dose group (500 mg/kg body weight or 1/4th of LD_{50} value) were selected for further sub-acute toxicity study.

Sub-acute toxicity studies

All the animals survived throughout the experimental schedule (28 days) and no observable abnormal behaviour was noticed in the experimental dose, vehicle, and normal control groups (data not shown).

Effect of EDME on body weight

The initial, final, and gain in body weights in both male and female rats from all the experimental groups were shown in Table 1. From our results, it was depicted that all experimental groups exhibited typical weight gains during the experimental schedule. There was no significant difference ($p < 0.05$) in body weight gain as observed

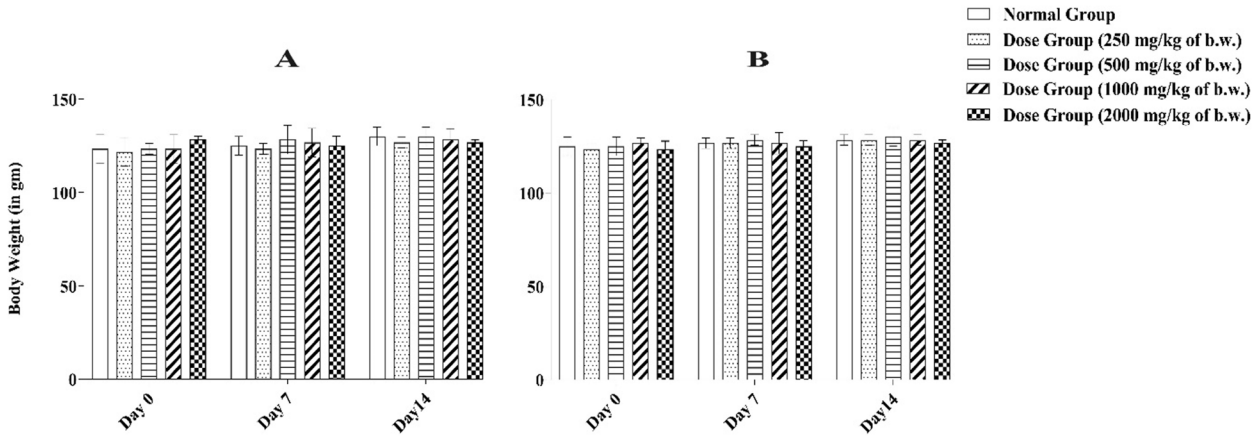

Fig. 3 Body weight of rat of both sexes during acute toxicity study with different doses of EDME (**A**-Male; **B**-Female)

Table 1 Body weight of rat during sub-acute toxicity assay with different doses of EDME

Gender	Group	Initial Weight (g)	Final Weight (g)	Weight gain (g)
Male	Normal Control	126.70 ± 4.41	145.30 ± 9.84	18.67 ± 5.78
	Vehicle Control	118.30 ± 3.33	133.30 ± 2.67	15.00 ± 3.06
	Low Dose (250 mg/kg)	118.30 ± 3.30	136.00 ± 5.77	17.67 ± 3.33
	High Dose (500 mg/kg)	123.30 ± 4.41	140.00 ± 6.42	16.67 ± 2.73
Female	Normal Control	125.00 ± 5.77	143.00 ± 6.81	18.00 ± 1.73
	Vehicle Control	125.00 ± 5.77	142.00 ± 6.81	17.00 ± 1.73
	Low Dose (250 mg/kg)	125.00 ± 2.89	140.70 ± 3.48	15.67 ± 0.67
	High Dose (500 mg/kg)	123.30 ± 4.41	137.30 ± 4.67	14.00 ± 1.53

Values were expressed as mean ± SEM of 6 animals per group (3 males and 3 females in each group), $p < 0.05$ (Two-way ANOVA was performed following Dunnett's multiple comparison test)

in the EDME-fed experimental group of both sexes when compared with the normal control group. Rats of the vehicle control group of both sexes also seemed to have no significant difference in body weight gain when compared to the normal control group rats.

Effect of EDME on organ weight

The absolute and relative body organ weights of both male and female rats are represented in Table 2. The relative organ weight of the liver, kidney, and spleen of the experimentally treated group of both sexes seemed to have no significant difference ($p < 0.05$) compared to the normal control group. In female high-dose group rats, when we measured the absolute organ weight of the liver, an apparent weight loss was observed from the normal control group; however, this weight loss seemed to be non-significant when compared with the normal control ($p < 0.05$). However, no significant differences were noticed when the absolute weight of other organs of both sexes was compared with the normal control group.

Effect of EDME on hematological parameters

The hematological parameters of both male and female rats are presented in Table 3. In both EDME-fed experimental male and female rats, the hematological parameters did not show any significant difference ($p < 0.05$) when compared with the normal control group. All the hematological data seemed to be within the normal range, so no significant difference was noticed within the experimental groups compared to the normal control group.

Effect of EDME on biochemical parameters

The biochemical parameters of both sexes were summarised in Table 4. During the 28-day treatment period, all the biochemical parameters of both male and female rats were within the reference range for rats. The serum AST and ALP levels in both sexes increased in the extract-fed groups. However, those did not show any significant difference ($p < 0.05$) compared to the normal control group. The ALT, total protein, and albumin data also showed no significant difference among the EDME-treated group of both sexes compared to the normal control group. Similar normalcy was observed in serum urea and creatinine levels in both sexes from all the experimental groups compared to the normal control group. EDME also exhibited non-significant differences in the lipid profile parameters in all the experimental groups when compared to the normal control group. A similar trend was also observed in the serum electrolytes levels, and these parameters also showed non-significant changes when compared with the normal control group. However, in the case of the male high-dose group, the fasting glucose level was slightly lower than the other EDME-treated experimental groups, but the value seemed to be non-significant ($p < 0.05$) when compared to the normal control group.

Effect of EDME on histopathological change

The images obtained from the histological sections (transverse) of all the experimental EDME-treated groups and the vehicle group showed no significant observable structural changes when compared with the normal control group. The liver sections (Fig. 4A-D) of all the experimental groups showed well-organized cellular structures with prominent hepatocytes, sinusoids with Kupffer's cells, having distinct central vein along with prominent hepatic portal vein, and hepatic artery were seen without any histoarchitectural abnormalities. The kidney sections from all the experimental groups (Fig. 4E-H) also showed well-organized Bowman's capsule cells with well-vascularised glomeruli along with distinct urinary space. The areas of

Table 2 Effect of oral administration of EDME on absolute and relative organ weight of rats

Gender	Group	Absolute Organ Weight (in g)			Relative Organ Weight (in g)		
		Liver (g)	Kidney (g)	Spleen (g)	Liver (g)	Kidney (g)	Spleen (g)
MALE	Normal Control	5.04 ± 0.52	1.29 ± 0.16	0.47 ± 0.03	3.45 ± 0.14	0.88 ± 0.05	3.26 ± 0.04
	Vehicle Control	4.33 ± 0.29	1.07 ± 0.03	0.44 ± 0.07	3.24 ± 0.16	0.81 ± 0.03	3.34 ± 0.47
	Low Dose	4.30 ± 0.58	1.08 ± 0.12	0.44 ± 0.13	3.14 ± 0.31	0.79 ± 0.06	3.13 ± 0.82
	High Dose	5.00 ± 0.41	1.08 ± 0.14	0.44 ± 0.08	3.56 ± 0.13	0.77 ± 0.09	3.08 ± 0.39
FEMALE	Normal Control	4.69 ± 0.38	1.26 ± 0.09	0.50 ± 0.03	3.27 ± 0.13	0.87 ± 0.02	3.55 ± 0.09
	Vehicle Control	4.79 ± 0.96	1.10 ± 0.08	0.45 ± 0.03	3.33 ± 0.52	0.77 ± 0.02	3.18 ± 0.06
	Low Dose	4.56 ± 0.23	1.09 ± 0.11	0.49 ± 0.03	3.24 ± 0.10	0.77 ± 0.05	3.47 ± 0.16
	High Dose	3.79 ± 0.12	1.07 ± 0.07	0.49 ± 0.01	2.76 ± 0.03	0.77 ± 0.02	3.64 ± 0.06

Values were expressed as mean ± SEM, $n = 6$ animals/group (3 males and 3 females), $p < 0.05$ (ANOVA/ Dunnett's test)

Table 3 Effect of oral administration of EDME on hematological parameters of rats

Gender	Parameters	Reference range #	Normal Control	Vehicle Control	Low Dose (250 mg/kg)	High Dose (500 mg/kg)
MALE	WBC count (×10^3/uL)	1.98–11.06	9.80 ± 0.12	9.90 ± 0.30 ($p = 0.96$; ns)	9.79 ± 0.33 ($p > 0.99$; ns)	10.10 ± 0.48 ($p = 0.55$; ns)
	RBC count (×10^6/uL)	4.60–9.19	7.56 ± 0.36	7.39 ± 0.21 ($p = 0.80$; ns)	7.27 ± 0.19 ($p = 0.47$; ns)	6.95 ± 0.29 ($p = 0.06$; ns)
	Hemoglobin (g/dL)	10.00–16.70	13.60 ± 0.35	13.70 ± 0.15 ($p = 0.96$; ns)	13.40 ± 0.40 ($p = 0.84$; ns)	13.20 ± 0.25 ($p = 0.31$; ns)
	Platelet (×10^3/uL)	574–1253	617 ± 29.70	615 ± 49.00 ($p > 0.99$; ns)	711 ± 39.00 ($p = 0.10$; ns)	686 ± 68.00 ($p = 0.26$; ns)
	Neutrophil (%)	5.30–38.10	20.70 ± 1.65	29.10 ± 2.45 ($p = 0.07$; ns)	18.60 ± 5.80 ($p = 0.85$; ns)	20.00 ± 4.20 ($p > 0.99$; ns)
	Lymphocyte (%)	66.60–90.30	74.90 ± 3.75	66.60 ± 0.95 ($p = 0.10$; ns)	77.90 ± 6.15 ($p = 0.72$; ns)	77.50 ± 4.20 ($p = 0.78$; ns)
	Monocyte (%)	0.80–3.80	3.70 ± 2.20	3.10 ± 0.80 ($p = 0.87$; ns)	2.50 ± 0.00 ($p = 0.49$; ns)	1.60 ± 0.10 ($p = 0.14$; ns)
	Eosinophil (%)	0.20–3.50	0.40 ± 0.10	1.00 ± 0.60 ($p = 0.13$; ns)	0.85 ± 0.25 ($p = 0.29$; ns)	0.40 ± 0.00 ($p > 0.99$; ns)
	Basophil (%)	0.00–0.80	0.40 ± 0.00	0.30 ± 0.10 ($p = 0.40$; ns)	0.20 ± 0.10 ($p = 0.05$; ns)	0.50 ± 0.10 ($p = 0.40$; ns)
	HCT (%)	39.60–52.50	45.90 ± 2.05	47.10 ± 0.90 ($p = 0.48$; ns)	45.50 ± 0.70 ($p = 0.97$; ns)	44.50 ± 0.60 ($p = 0.43$; ns)
	MCV (fL)	50.00–77.70	61.00 ± 5.65	63.80 ± 0.60 ($p = 0.51$; ns)	62.70 ± 0.65 ($p = 0.81$; ns)	66.00 ± 0.25 ($p = 0.15$; ns)
	MCH (pg)	16.00–23.10	18.10 ± 1.35	18.40 ± 0.30 ($p = 0.87$; ns)	18.50 ± 0.05 ($p = 0.83$; ns)	19.50 ± 0.20 ($p = 0.08$; ns)
	MCHC (g/dL)	28.20–34.10	29.70 ± 0.55	28.90 ± 0.20 ($p = 0.08$; ns)	29.60 ± 0.35 ($p = 0.97$; ns)	29.60 ± 0.20 ($p > 0.99$; ns)
	MPV (fL)	6.20–9.40	8.55 ± 0.35	8.50 ± 0.30 ($p > 0.99$; ns)	8.05 ± 0.35 ($p = 0.18$; ns)	8.60 ± 0.20 ($p > 0.99$; ns)
FEMALE	WBC count (×10^3/uL)	1.13–7.49	7.09 ± 0.87	7.28 ± 1.08 ($p > 0.99$; ns)	7.37 ± 1.10 ($p = 0.72$; ns)	7.81 ± 1.70 ($p = 0.77$; ns)
	RBC count (×10^6/uL)	7.07–9.03	7.04 ± 0.14	7.3 ± 0.19 ($p = 0.74$; ns)	7.76 ± 0.69 ($p = 0.11$; ns)	7.21 ± 0.11 ($p = 0.90$; ns)
	Hemoglobin (g/dL)	8.60-15.38	12.30 ± 0.70	12.60 ± 0.10 ($p = 0.90$; ns)	13.60 ± 1.08 ($p = 0.10$; ns)	12.90 ± 0.15 ($p = 0.60$; ns)
	Platelet (×10^3/uL)	599–1144	659 ± 20.50	653 ± 40.00 ($p > 0.99$; ns)	604 ± 70.10 ($p = 0.61$; ns)	680 ± 97.20 ($p = 0.96$; ns)
	Neutrophil (%)	7.10–33.20	25.80 ± 2.70	24.60 ± 0.60 ($p = 0.98$; ns)	22.90 ± 9.10 ($p = 0.84$; ns)	21.70 ± 4.20 ($p = 0.67$; ns)
	Lymphocyte (%)	62.20–90.00	69.10 ± 1.15	69.80 ± 2.00 ($p > 0.99$; ns)	72.90 ± 8.50 ($p = 0.70$; ns)	75.00 ± 5.60 ($p = 0.41$; ns)
	Monocyte (%)	0.80–3.90	3.48 ± 0.97	3.50 ± 1.00 ($p > 0.99$; ns)	2.05 ± 0.75 ($p = 0.15$; ns)	1.80 ± 0.42 ($p = 0.09$; ns)
	Eosinophil (%)	0.50–4.50	0.55 ± 0.45	1.65 ± 0.45 ($p = 0.31$; ns)	1.80 ± 1.30 ($p = 0.23$; ns)	1.50 ± 0.80 ($p = 0.41$; ns)
	Basophil (%)	0.00–0.80	0.23 ± 0.15	0.36 ± 0.15 ($p = 0.52$; ns)	0.26 ± 0.15 ($p = 0.98$; ns)	0.33 ± 0.05 ($p = 0.70$; ns)
	HCT (%)	37.90–49.90	42.50 ± 1.35	42.30 ± 0.10 ($p > 0.99$; ns)	45.10 ± 1.67 ($p = 0.05$; ns)	42.80 ± 0.75 ($p = 0.98$; ns)
	MCV (fL)	50.00–60.00	60.30 ± 0.70	58.10 ± 1.45 ($p = 0.52$; ns)	62.60 ± 3.80 ($p = 0.51$; ns)	59.40 ± 2.00 ($p = 0.93$; ns)
	MCH (pg)	16.00–23.10	17.50 ± 0.65	17.30 ± 0.60 ($p > 0.99$; ns)	18.40 ± 1.30 ($p = 0.41$; ns)	17.90 ± 0.55 ($p = 0.88$; ns)
	MCHC (g/dL)	28.20–34.10	28.90 ± 0.80	29.80 ± 0.30 ($p = 0.20$; ns)	29.40 ± 0.25 ($p = 0.66$; ns)	30.10 ± 0.70 ($p = 0.08$; ns)
	MPV (fL)	6.20–9.80	8.20 ± 0.20	8.25 ± 0.25 ($p = 0.98$; ns)	8.15 ± 0.25 ($p = 0.98$; ns)	8.50 ± 0.10 ($p = 0.26$; ns)

Values were expressed as mean ± SD, $n = 6$ animals/group (3 males and 3 females). Analysis was done by one-way ANOVA following the Dunnett's post hoc test, where values of $p < 0.05$ were taken to indicate a statistical difference, as compared to the normal control group rats, where 'ns' indicates $p > 0.05$ # [31, 32]

HCT Hematocrit, *MCV* Mean Red Blood Cell Volume, *MCH* Mean Corpuscular Hb, *MCHC* Mean Corpuscular Hb Concentration, *MPV* Mean Platelet cell Volume

the spleen (Fig. 4I-L) also showed normal histoarchitecture with well-developed splenic follicles (white pulps) and splenic sinusoids (red pulps) among all the experimental groups. The stomach sections (Fig. 4M-P) showed prominent mucosal and sub-mucosal lining with well-developed gastric glands found between the experimental dose groups and the normal control group. The intestinal sections (Fig. 4Q-T) also showed prominent epithelial lining (sub-mucosal layer) with well-developed intestinal villi and duodenal gland (Brunner's gland) among the experimental groups.

Discussion

Many medicinal herbs have been used as a therapeutic source in treating various human ailments throughout the world [34]. For many years, herbal and traditional medicines have been considered safe, leading often to their indiscriminate use in humans. The lack of proper knowledge regarding their chemical composition and lack of awareness regarding their safety dose and toxicity also prompted such indiscriminate use. With a view to identify potential drug targets, there is an urgent need to study the chemical composition, safety

Table 4 Effect of oral administration of EDME on Biochemical parameters of rats

Gender	Parameters	Reference range[#]	Normal Control	Vehicle Control	Low Dose (250 mg/kg)	High Dose (500 mg/kg)
MALE	AST (U/L)	74–143	104.00 ± 8.49	106.00 ± 9.87 ($p > 0.99$; ns)	110.00 ± 13.20 ($p = 0.82$; ns)	111.00 ± 9.36 ($p = 0.75$; ns)
	ALT (U/L)	18–45	39.00 ± 6.66	39.70 ± 4.15 ($p > 0.99$; ns)	39.10 ± 6.77 ($p > 0.99$; ns)	42.00 ± 8.00 ($p = 0.90$; ns)
	ALP (U/L)	62–230	164.00 ± 9.25	165.00 ± 19.40 ($p > 0.99$; ns)	182.00 ± 11.30 ($p = 0.30$; ns)	181.00 ± 10.60 ($p = 0.33$; ns)
	Total protein (g/dL)	5.20–7.10	6.66 ± 0.79	6.13 ± 0.44 ($p = 0.62$; ns)	6.72 ± 0.23 ($p > 0.99$; ns)	6.62 ± 0.82 ($p > 0.99$; ns)
	Albumin (g/dL)	3.40–4.80	4.56 ± 0.14	4.64 ± 0.21 ($p = 0.99$; ns)	4.43 ± 0.38 ($p = 0.96$; ns)	4.25 ± 0.72 ($p = 0.72$; ns)
	Urea (mg/dL)	12.30–24.60	19.30 ± 2.04	21.90 ± 2.20 ($p = 0.31$; ns)	22.00 ± 1.84 ($p = 0.29$; ns)	22.30 ± 1.72 ($p = 0.22$; ns)
	Creatinine (mg/dL)	0.20–0.50	0.36 ± 0.02	0.35 ± 0.01 ($p = 0.64$; ns)	0.35 ± 0.01 ($p = 0.64$; ns)	0.37 ± 0.01 ($p = 0.94$; ns)
	Cholesterol (mg/dL)	37–85	51.50 ± 2.95	49.10 ± 4.22 ($p = 0.85$; ns)	48.10 ± 4.86 ($p = 0.70$; ns)	47.30 ± 5.42 ($p = 0.55$; ns)
	Triglyceride (mg/dL)	20–114	36.10 ± 2.95	33.70 ± 5.27 ($p = 0.84$; ns)	33.90 ± 5.09 ($p = 0.86$; ns)	28.90 ± 3.58 ($p = 0.17$; ns)
	HDL-C (mg/dL)	36.78–54.65	38.20 ± 0.25	37.60 ± 1.44 ($p = 0.77$; ns)	37.30 ± 1.17 ($p = 0.54$; ns)	36.40 ± 0.59 ($p = 0.12$; ns)
	LDL-C (mg/dL)	15.58–35.09	31.80 ± 1.02	31.60 ± 1.97 ($p > 0.99$; ns)	32.50 ± 1.75 ($p = 0.93$; ns)	31.40 ± 2.07 ($p = 0.99$; ns)
	Glucose (mg/dL)	70–208	87.80 ± 4.43	82.00 ± 8.45 ($p = 0.67$; ns)	82.00 ± 8.70 ($p = 0.66$; ns)	80.60 ± 7.04 ($p = 0.52$; ns)
	Sodium (mmol/L)	142–151	147.00 ± 3.04	146.00 ± 3.94 ($p = 0.86$; ns)	145.00 ± 1.61 ($p = 0.63$; ns)	143.00 ± 2.75 ($p = 0.30$; ns)
	Potassium (mmol/L)	3.82–5.55	4.84 ± 0.11	4.81 ± 0.17 ($p > 0.99$; ns)	4.81 ± 0.23 ($p > 0.99$; ns)	4.75 ± 0.21 ($p = 0.89$; ns)
FEMALE	AST (U/L)	65–203	103.00 ± 4.07	113.00 ± 12.60 ($p = 0.61$; ns)	118.00 ± 13.40 ($p = 0.33$; ns)	121.00 ± 13.90 ($p = 0.22$; ns)
	ALT (U/L)	13–56	51.50 ± 10.20	52.70 ± 9.69 ($p > 0.99$; ns)	53.10 ± 4.84 ($p = 0.99$; ns)	53.40 ± 4.89 ($p = 0.98$; ns)
	ALP (U/L)	26–147	129.00 ± 6.73	136.00 ± 7.48 ($p = 0.45$; ns)	138.00 ± 3.82 ($p = 0.26$; ns)	138.00 ± 4.85 ($p = 0.23$; ns)
	Total protein (g/dL)	5.50–7.70	6.55 ± 0.74	5.82 ± 0.29 ($p = 0.32$; ns)	5.95 ± 0.78 ($p = 0.46$; ns)	6.07 ± 0.14 ($p = 0.61$; ns)
	Albumin (g/dL)	3.60–5.50	4.39 ± 0.54	4.80 ± 0.17 ($p = 0.58$; ns)	4.00 ± 0.48 ($p = 0.62$; ns)	4.43 ± 0.51 ($p > 0.99$; ns)
	Urea (mg/dL)	13.20–27.10	22.90 ± 1.88	24.10 ± 0.53 ($p = 0.82$; ns)	24.80 ± 2.97 ($p = 0.53$; ns)	25.00 ± 1.84 ($p = 0.45$; ns)
	Creatinine (mg/dL)	0.20–0.60	0.38 ± 0.01	0.36 ± 0.01 ($p = 0.53$; ns)	0.40 ± 0.01 ($p = 0.26$; ns)	0.39 ± 0.02 ($p = 0.52$; ns)
	Cholesterol (mg/dL)	24–73	43.90 ± 1.83	42.60 ± 5.59 ($p = 0.97$; ns)	41.30 ± 5.85 ($p = 0.83$; ns)	41.10 ± 3.91 ($p = 0.81$; ns)
	Triglyceride (mg/dL)	14–46	30.60 ± 4.10	30.40 ± 5.22 ($p > 0.99$; ns)	28.90 ± 4.04 ($p = 0.94$; ns)	29.80 ± 4.47 ($p > 0.99$; ns)
	HDL-C (mg/dL)	37.00–68.73	41.70 ± 1.72	39.50 ± 1.85 ($p = 0.31$; ns)	38.60 ± 1.15 ($p = 0.12$; ns)	38.80 ± 1.78 ($p = 0.15$; ns)
	LDL-C (mg/dL)	12.21–27.36	23.60 ± 0.73	24.90 ± 2.01 ($p = 0.47$; ns)	22.40 ± 1.08 ($p = 0.52$; ns)	22.20 ± 0.61 ($p = 0.43$; ns)
	Glucose (mg/dL)	76–175	77.80 ± 3.54	76.10 ± 10.30 ($p = 0.97$; ns)	75.40 ± 4.42 ($p = 0.93$; ns)	75.40 ± 2.20 ($p = 0.92$; ns)
	Sodium (mmol/L)	140–150	144.00 ± 4.80	144.00 ± 3.37 ($p = 0.98$; ns)	143.00 ± 4.10 ($p = 0.96$; ns)	142.00 ± 1.41 ($p = 0.78$; ns)
	Potassium (mmol/L)	3.31–4.90	4.74 ± 0.12	4.68 ± 0.06 ($p = 0.82$; ns)	4.75 ± 0.14 ($p > 0.99$; ns)	4.65 ± 0.09 ($p = 0.62$; ns)

Values expressed as mean ± SD, $n = 6$ animals/group (3 males and 3 females). Analysis was done by one-way ANOVA following the Dunnett's post hoc test, where values of $p < 0.05$ were taken to indicate a statistical difference, as compared to the normal control group rats, where 'ns' indicates $p > 0.05$ # [32, 33]

AST aspartate aminotransferase, *ALT* alanine aminotransferase, *ALP* alkaline phosphatase, *HDL-C* high-density lipoprotein cholesterol, *LDL-C* low-density lipoprotein cholesterol

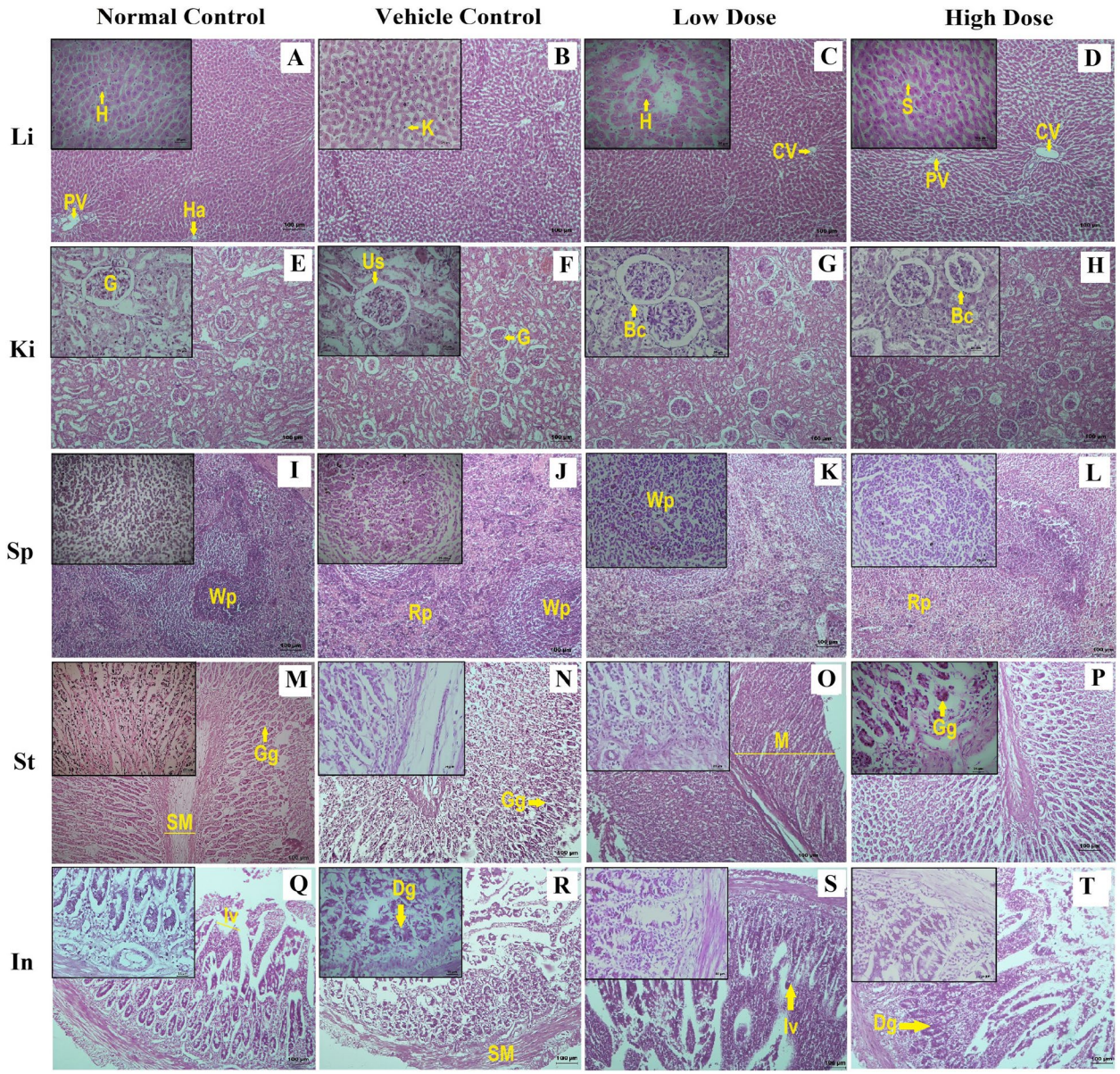

Fig. 4 Histological sections of major organs of different experimental groups. 'Li' denotes Liver (**A-D**); 'Ki' denotes Kidney (**E–H**); 'Sp' denotes Spleen (**I-L**); 'St' denotes Stomach (**M-P**) and 'In' denotes Intestine (**Q-T**). The first column represents normal control group, followed by vehicle control group, low dose group, and high dose group. The main images represent sections observed under 10X magnification; insets represent 40X magnified images. Scale bars are 100 µm in 10X and 25 µm in 40X. Following yellow markings are the labelling of histological structures: Hepatocytes (H); Hepatic artery (Ha); Portal vein (PV); Sinusoids (S); Kupffer cell (K); Central vein (CV); vascularised Glomeruli (G); Urinary space (Us); Bowman's capsule (Bc); White pulp (Wp); Red pulp (Rp); Gastric gland (Gg); Mucosal layer (M); Sub-mucosal layer (SM); Intestinal villi (Iv); Duodenal/ Brunner's gland (Dg)

dose determination, and detailed toxicity profiling of the plants [30]. The principal aim of the present study was to evaluate the safety dose of *Equisetum diffusum* whole plant methanol extract (EDME) through appropriate in silico, in vitro, and in vivo approaches.

The use of in silico studies in determining the toxicity of any drug/plant-based compounds has increased due to their cost and time-effectiveness. Post GC–MS, in silico approach, has been commonly employed for the initial screening of phytochemicals from a medicinal plant [35].

The previously reported GC–MS analysis of the whole plant methanol extract revealed the presence of 47 potent bioactive compounds in the EDME [17]. Out of 47, compounds like Hexadecanoic acid, methyl ester; Oleic Acid, (Z)-, TMS derivative; and 1,2-Benzenedicarboxylic acid, bis(2-ethylhexyl)ester) were found to have potent anti-inflammatory and anti-arthritic activity [20, 21, 36, 37]. The proper in silico pharmacokinetics and toxicity profiling of these bioactives are the prerequisites before evaluating their efficacy in in vitro and in vivo models [38]. The potentiality of a promising drug usually depends upon its absorption, distribution, metabolism, excretion, and toxicity (ADMET) characteristics. Therefore, the ADMET parameters were estimated to determine the probability of pharmacokinetics and toxicity properties of the *E. diffusum* methanol extract (EDME). From our study, it was predicted that all the 47 bio-active phytocompounds (100%) conform to the Lipinski rule and have a good bioavailability score, suggesting that all the phytocompounds have good potential to be an ideal drug [38, 39]. The Absorption results indicated that 93% of the compounds had reasonable absorption rates. The Distribution results showed that the distribution volume of all the compounds (100%) was high, and 81% of the compounds showed a good BBB permeability. CYP2D6 and CYP3A4 subtypes are responsible for the metabolism of about 60% of xenobiotics, including drugs, steroids, and carcinogens from our body [38]. Our results also showed that almost all the compounds (~98%) were non-inhibitors/non-substrates for both CYP2D6 and CYP3A4 subtypes, similar to the effects of the identified phytochemical constituents from the *Eruca sativa* crude extract [38]. However, the excretion results predict that only 40% of the compounds have a reasonable clearance rate (CL). This might be due to the high molecular weight of some of the phyto-compounds. The Toxicity results showed that all the compounds (100%) were non-mutagenic, i.e., AMES negative and H-HT negative (-), i.e., non-toxic to the liver, which was further validated from our in vitro cytotoxicity assays. The short-listed 7 compounds have an acceptable toxicity profile. Therefore, all our ADME-Toxicity data predicts the non-toxic nature of the identified bioactives from the *E. diffusum* whole plant methanol extract. Its usage as a drug can also be confirmed by this in silico approach.

To validate the results of in silico toxicity, the in vitro toxicity study was assessed. In this respect, the in vitro cytotoxicity test is helpful for preliminary assessment to determine the safety dose evaluation of plant species, which lays the foundation that supports its usage for in vivo animal tests regarding toxicity [40]. In our cytotoxicity study, we used two cell lines: the human embryonic kidney 293 cells (HEK293) and the human liver cell line (Huh7). Our results suggested low levels of cytotoxicity of crude methanol extract of *E. diffusum* in both the kidney and liver cell lines. This result also corroborated the previously conducted studies on cytotoxicity of 3 solvent extracts (n-hexane, ethyl acetate, and methanol) of *E. diffusum* aerial parts in the human intestinal epithelial cell line (FHs 74 Int), where the authors found higher IC_{50} values in the case of methanol (124.7 ± 23.0 µg/ml) compared with the other two solvents [16]. The cytotoxicity effect of the 70% methanol and ethanol extracts of *E. hyemale* also corroborated our results [41]. All these extracts (methanol) thus, required higher IC_{50} values to produce a cytotoxicity effect to the cell population indicating their safety dose evaluation.

To corroborate the results of in vitro cytotoxicity, the in vivo toxicity was designed. The acute oral toxicity test was conducted to evaluate the single-time exposure of the plant products [42]. The single-time exposure of EDME showed no adverse effect at a dose of 2000 mg/kg b.w. and was considered 'non-toxic.' Since the LD_{50} value of EDME was more than 2000 mg/kg b.w., the EDME could be assigned as a 'low toxic product' and thus falls in the Class 5 category drug. However, to evaluate the long-term exposure of plant products, the sub-acute toxicity test is essential [6]. This sub-acute toxicity testing is vital in assessing the gradual accumulation of extracts on target tissues and organs, and hematological or biochemical effects in experimental animals. In our study, the long-term exposure (28-day) to EDME showed no mortality, and the body and organ weight, hematological, biochemical, and histological structure of vital organs in the extract-fed rats also confirmed the low toxicity of the plant extract when compared with the normal control group rats.

Body-weight-gain and organ weight serve as good indicators of health status, including physiological and pathological conditions of experimental animals [43–45]. Any parameter alteration indicates vital signs for toxicity [6]. In this study, the body-weight gain and relative weight of major organs like the liver, kidney, and spleen of all animal of the extract-treated groups did not show any significant difference when compared to the normal control group, suggesting the non-toxic nature of EDME.

The evaluation of hematological parameters is essential for determining the toxic effects of plant extract in the blood of animals. These hematological parameters also indicate the physiological and pathological status of any animal [46]. Any toxic substance entering blood will alter the normal range of its cellular components by affecting the normal function of the bone marrow, leading to disability of normal body function [6]. In our study, all the hematological values were found to be within the normal range, and administration of EDME for a 28-day long experimental tenure does not show any significant

alterations in hematological parameters when compared with the normal control group of both sexes.

The biochemical parameters also provide essential information regarding the toxic effects of any drug in primary tissues like the liver and kidney [6]. Any toxic substance like a drug or plant extract entering the body is first metabolized in the liver, and the resulting waste products are excreted by the kidney. So, these two major organs are the primary target of toxic substances to cause injury leading to chronic disability [42]. To assess the health status of the liver, some of the significant hepatic damage markers like serum AST, ALT, and ALP levels were measured. Any increment of these markers is an indicator of hepatic injury. In our study, the AST and ALP levels in the dose group of both sexes have increased compared to the normal. However, these differences have been within the normal range. To evaluate kidney function, the urea and creatinine level in the blood was studied. The increment of these markers indicates the sign of kidney damage [47]. In the present study, administration of EDME did not alter the serum urea and creatinine levels in both the dose groups when compared with the control group, suggesting the non-toxic nature of EDME to kidneys in experimental animals. The measurement of sodium and potassium is a good indicator of determining the heart, muscle, and neural functions [42]. In our study, the level of these two ions of both dose groups also showed no significant difference compared to the normal control group. The cholesterol and triglyceride levels also backed the non-toxic nature of EDME in the case of the extract-treated group. Both the dose and normal control groups showed non-significant changes in cholesterol and triglyceride levels. However, the glucose level in the case of the dose group has decreased marginally, indicating the hypoglycemic activity of EDME; however, the decrease is statistically insignificant.

The liver and kidneys are the two major organs that play an essential role in the metabolism and excretion of drugs or plant products from our body. Any xenobiotics entering the body may cause toxicity or cell damage in these organs [48]. The histoarchitecture of the liver was also substantiated by the non-adverse effects of the EDME as shown in the assays on the biochemical markers (ALT, AST, and ALP), which showed non-significant changes, compared with the normal control group. The kidney architecture of the extract-fed rat groups also showed well-organised Bowman's capsule with glomeruli and did not show any abnormality. The histological assessment of other significant organs such as the spleen, stomach, and intestine from the extract-treated group also confirmed the low toxicity of EDME at the micro-anatomy level since no abnormalities were observed in their histoarchitecture when compared with the normal control group rats, suggesting the non-toxic nature of the plant.

Conclusion

In summary, the current study confirmed the safety dose evaluation and non-toxic nature of the whole plant methanol extract of *Equisetum diffusum* D. Don. The in silico ADME-Toxicity results of the 47 phyto-compounds suggested suitable pharmacokinetic properties and low to moderate toxicity. The in vitro cytotoxicity also confirms the non-toxicity of EDME in both kidney and liver cell lines. The in vivo acute oral toxicity of *E. diffusum* whole plant methanol extract (EDME) was considered non-toxic up to a dose of 2000 mg/kg body weight upon single day exposure. In brief, the safety dose evaluation of EDME following its regular consumption for an extended period (28 days) was also considered safe, since no significant alterations in body weight gain, absolute and relative organ weights, hematological parameters, and biochemical parameters were observed in both sexes. Furthermore, histoarchitecture of major organs like the liver, kidney, spleen, stomach, and intestine, showed no significant structural abnormalities after prolonged administration of the extract, suggesting the non-toxic nature upon its extended use. Hence, all our findings confirmed the safety dose evaluation of the plant, thereby justifying its short and long-term folklore uses of an oral dose and also laid the foundation for initiating experimental validation of the plant's ethnobotanical uses, potentially contributing to drug development for inflammation-related complications.

Abbreviations

EDME	*Equisetum diffusum* whole plant methanol extract
GC-MS	Gas Chromatography-Mass Spectrometry
SMILES	Simplified Molecular Input Line Entry System
SDF	Structure Data File
NCBI	National Center for Biotechnology Information
OPLS3	Optimized Potentials for Liquid Simulations
TOPKAT	Toxicity Prediction by Komputer Assisted Technology
MTT	(3-(4, 5-Dimethyl thiazolyl-2)-2, 5-diphenyltetrazolium bromide
DMEM	Dulbecco's Modified Eagle Medium
FBS	Foetal Bovine Serum
PBS	Phosphate buffer saline
DMSO	Dimethyl sulfoxide
IAEC	Institutional Animal Ethical Committee
CPCSEA	Committee for the Purpose of Control and Supervision of Experiments on Animals
OECD	Organization for Economic Cooperation and Development guidelines
RBC	Red blood cell count
WBC	White blood cell count
Hb	Haemoglobin
Pt	Platelets
NP	Neutrophils
LC	Lymphocytes
MC	Monocytes
EP	Eosinophils
BP	Basophils
HCT	Hematocrit

MCV	Mean Red Blood Cell Volume
MCH	Mean Corpuscular Hb
MCHC	Mean Corpuscular Hb Concentration
MPV	Mean Platelet cell Volume
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
ALP	Alkaline phosphatase
HDL-C	High-density lipoprotein cholesterol
LDL-C	Low-density lipoprotein cholesterol
SEM	Standard Error Mean
SD	Standard Deviation
ANOVA	Analysis of variance

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12906-024-04606-y.

Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Supplementary Material 4

Supplementary Material 5

Supplementary Material 6

Supplementary Material 7

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Authors' contributions
SBH contributed to the concept and designing of the whole experiment; JJG designed in silico studies; RB and SB designed the in vitro MTT assay part; SS and DM performed the in silico experiments and interpreted the data; AB and MI performed and analyzed the data of in vitro MTT assays. SS, DM, and SKR 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 SBH. All authors read and approved the final manuscript. SBH: Soumen Bhattacharjee; JJG: John J. Georrge; RB: Rinku Baishya; SB: Sujoy Bose; SS: Sourav Sarkar; DM: Debabrata Modak; AB: Anupam Biswas; MI: Mafidul Islam; SKR: Sudipta Kumar Roy.

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Availability of data and materials
All the materials will be available for research purposes if requested to the corresponding author.

Declarations

Ethics approval and consent to participate
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. The ARRIVE guidelines were followed in reporting the study (Additional File 7).

Consent for publications
Not applicable.

Competing interests
The authors declare no competing interests.

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