In Vitro and *In Vivo* Anti-Inflammatory Activity of *Tetrastigma Sulcatum* Leaf Extract, Pure Compound and Its Derivatives

Ravindra Jagannath Waghole
Maharashtra Association for the Cultivation of Science: Agharkar Research Institute
Ashwini Vivek Misar
Maharashtra Association for the Cultivation of Science: Agharkar Research Institute
Neha Shashikant Kulkarni
Maharashtra Association for the Cultivation of Science: Agharkar Research Institute
Feroz Khan
CSIR-CIMAP: Central Institute of Medicinal and Aromatic Plants CSIR
Dattatraya Gopal Naik
MES Abasaheb Garware College
Sachin H Jadhav (shjadhav@aripune.org)
Maharashtra Association for the Cultivation of Science: Agharkar Research Institute
https://orcid.org/0000-0002-4752-4202

Research Article

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Abstract

The severity and perseverance of the inflammation have been demonstrated in many health conditions. The limitations of existing medications, propose the need for newer alternative anti-inflammatory medications. In our earlier studies, we demonstrated the topical anti-inflammatory potential of crude ethanolic extract of Tetrastigma sulcatum leaves and its fractions. In the present study, we further explored anti-inflammatory activity of T. sulcatum extract, fractions, pure compound and its derivatives using in vitro and in vivo bioassay techniques. We attempted to isolate a pure compound from leaf extract and was identified as Friedelan-3β-ol (CI) and its derivatives Friedelinol acetate (C II) and Friedelinol methyl ether (C III) were synthesized. Treatment with crude extract and its fractions demonstrated a significant reduction in the mRNA expression levels of pro-inflammatory cytokines (IL-1β, IL-6, TNF-α) and nitric oxide (NO) production in LPS-stimulated inflammation in RAW 264.7 cells. Likewise, compounds CI, CII and CIII showed a similar pattern of significant inhibition of proinflammatory cytokines and NO production. In vivo study in Carrageenan induced paw-inflammatory mice model demonstrated reduced paw oedema and proinflammatory cytokines levels in a dose-dependent manner upon treatment of extract, its fractions, pure compound (CI), and their derivatives (CII and CIII.). The docking study showed all the compounds (CI, CII and CIII) share common residues with Dexamethasone. TNF- α exhibited the most interacting residues with the compounds. The present study confirmed the T. sulcatum ’s anti-inflammatory activity, suggesting Friedelan-3β-ol as an active component in a crude extract.

Introduction

Inflammation is a fundamental part of the innate immune system, classified into acute or chronic inflammation (Šoltés and Kogan, 2014). Acute inflammation involves resident macrophages and dendritic cells, allowing immediate local response and signal other immune cells to locate injured tissue (Ansa and Ghosh, 2016). However, chronic inflammation is usually associated with many chronic human conditions and diseases, including arthritis, asthma, atherosclerosis, cancer, and autoimmune diseases. Inflammatory diseases are treated with conventional anti-inflammatory drugs such as steroidal (corticosteroids, glucocorticoids) and non-steroidal anti-inflammatory drugs (NSAIDs) (Ma et al., 2020; Pahawa et al., 2020). However, these synthetic drugs used against inflammation are reported to produce drug-related toxicities and adverse reactions complicating the treatment process (Ma et al., 2020; Pahawa et al., 2020). Considering severe concerns over the inflammation in human health and perseverance, the development of newer drugs with less or no side effects to treat chronic inflammation is of paramount importance.

Ancient knowledge of medicine is found to be hindered in applications of herbal medicines. Considering Indian medicinal systems, naturopathy and Ayurveda practices have gained increased attention and have become 'tradition to trend.' The practices, including herbal medicines, are becoming popular in preventive, promotional, and curative applications (Ekor 2013). Traditional plants as crude material or pure compounds play a significant role in discovering new drugs for treating various disease conditions (Ji et

al., 2009; Sofowora et al., 2013). Most herbal products contain novel and structurally diverse chemical compounds as secondary metabolites with biologically active properties (Ahmed et al., 2017). The common secondary metabolites isolated and reported to have medicinal properties are alkaloids (Wink, 2015), triterpenoids (Han and Bakovic, 2015), flavonoids (Panche et al., 2016), and coumarins (Jain and Joshi, 2012). Such herbal drugs with fewer side effects can have valuable applications in many diseases associated with inflammation.

The genus *Tetrastigma* belongs to the Vitaceae family, well-known for economically significant fruit crop variety, the Grape (*Vitis vinifera* L.) (Ma et al., 2021). *Tetrastigma* includes nearly 100 species distributed majorly in the Asian continent. Considering biodiversity evolution, conservation, and utilization, *Tetrastigma* is one of the genera that remains unexplored and poorly understood (Wen et al., 2018). Compared to other species, *T. hemsleyanum* is a widely studied species for its medicinal use (Ji et al., 2020; Zhu et al., 2020). Other species such as *T. angustifolia* (Junejo et al., 2020), *T. leucostaphylum* (Rudra et al., 2020) are scarcely reported as having medicinal activity. Amongst various *Tetrastigma* species, databases' Plants of the World' and 'eFlora of India' show *T. sulcatum* to be a naive Indian variety. Very few reports are available on the therapeutic activity of *T. sulcatum* other than one of our previous works (Waghole et al., 2015).

The presence of polyphenols and flavonoids in plant leaf extract has special significance in plant protection to control various pests and diseases (Mulat et al., 2019). Preliminary phytochemical studies revealed polyphenols, flavonoids, leucoanthocyanins, catechol tannins, syringing glycosides, tannins, raphides and, mucilage substances in the plant of *T. sulcatum*. Our previous report demonstrated the antioxidant and topical anti-inflammatory potential of crude ethanolic extract of *T. sulcatum* leaves and the fractions (Waghole et al., 2015). Another report showed antifungal activity of *T. sulcatum* (Law.) Gamble, particularly the essential oil from leaf extract, against the various fungi species such as *Fusarium oxysporum*, *F. moniliforme*, *F. equiseti*, *Aspargillus niger, Botrytis cinerea* (Jagannath and Gopal, 2016). Considering the medicinal properties and our preliminary studies, we are attempting to explore the anti-inflammatory competence of *T. sulcatum* leaves in the present study.

Given the aim, the leaf extract of *T. sulcatum* was extracted and fractionated. Subsequently, the significant compounds involved in the anti-inflammatory activities of *T. sulcatum* through bioassay-guided fractionation were identified. The anti-inflammatory activities of extracts, fractions and isolated pure compound and it's derivatives were evaluated using *in vitro* and *in vivo* bioassay techniques, and it was further confirmed by in silico studies.

Materials And Methods

1.1.Collection and authentication:

The leaves of *Tetrastigma sulcatum* (25.5 Kg) were collected from the experimental farm of Agharkar Research Institute at village Hol, Dist. Pune (Latitude 18.5282, Longitude 73.9677). The material was authenticated by the Botanical Survey of India (BSI), Pune – 411001. The material has been deposited at

the herbarium of Botanical Survey of India (BSI), Pune (Voucher specimen number 189329). It was identified as *Tetrastigma sulcatum* (Law.) Gamble, which belongs to the family Vitaceae.

1.2.Extraction and fractionation of the total crude ethanolic extract:

The total crude ethanolic extraction of *T. sulcatum* leaves and its fractionation was performed as mentioned in previous work (Waghole et al., 2015). In brief, extraction was carried out using Soxhlet apparatus to yield crude ethanolic extract (TSETOH) of *T. sulcatum* leaves. Further, the fractionation was carried out in the solvents of increasing polarity in the order hexane, toluene, and ethyl acetate, referred to as TSHEX, TSTOL and TSEA, respectively.

1.3.Purification of TSETOH

TSETOH (10 g) was dissolved in acetone (100 mL), and silica gel (60–120 mesh, 15 g) was added to it. TSETOH was adsorbed on silica gel. The adsorbed extract was loaded on an Medium Pressure Liquid Chromatography (MPLC) column of TLC silica gel G, column (2.6 x 46 cm). The column was eluted in to fractions with hexane (1.2 L), hexane/EtOAc (9.5:0.5,1 L), hexane/EtOAc (9:1 1.4 L), hexane/EtOAc (8.5:1.5,1 L), hexane/EtOAc (8:2, 0.6 L), hexane/EtOAc (7.5:2.5, 0.6 L), hexane/EtOAc (7:3 1 L), ethyl acetate (1 L) and methanol (0.4 L) at 4.8 mL/min, tR of 1: between 1-200 mL and 2: between 200–400 mL. TLC of all the fractions was recorded on pre-coated plates using 20 % ethyl acetate in a hexane solvent system. The plates were developed using an anisaldehyde–sulphuric acid. The fractions having the same Rf were combined. The major fractions 12–18 eluted with hexane/EtOAc ((9:1) were combined (0.56 g). The mixture (0.56 g) was dissolved in chloroform (10 mL), and silica gel (60–120 mesh, 0.56 g) was added to it. The solvent was carefully evaporated to remove the impurity of other compounds to prepare the dry pack column. The adsorbed dry powder was reloaded on a column of silica gel column. The column was eluted with hexane (0.5 L), hexane/EtOAc (9.5:0.5, 0.5 L), hexane/EtOAc (9:1, 0.6 L), hexane/EtOAc (8.5:1.5, 0.4 L), hexane/EtOAc (8:2, 0.5 L), hexane/EtOAc (7.5:2.5, 0.4 L), 7:3 (6 x 100 mL), ethyl acetate (0.3 L) and methanol (0.2 L). tR of 1: between 1-100 mL and 2: between 100–200 mL. Fractions 11–16 eluted with hexane/EtOAc (9:1) were combined and recrystallized with ether-methanol yielding white crystalline compound (0.271 g, I). This compound was further examined by TLC, recording the MP and optical rotation. The compound's structures obtained after column chromatography of the crude extract were established by spectroscopic and chemical conversions (supplementary 1). Compound I was identified as Friedelan-3β-ol. Further, Friedelinol acetate (C II) and friedelinol methyl ether (C III) were derived from compound I.

1.4.Cytotoxicity assay

RAW 264.7 (the mouse macrophage) cells were obtained from the National Centre for Cell Science (NCCS) Pune-411007. The MTT assay was performed to assess the cytotoxicity profile of *T. sulcatum* extracts and their fractions. Briefly, RAW 264.7 cells were seeded in 96-well plates overnight at a density 1 x 10^4 cells/well. The cells were treated with different concentrations of a) TSETOH and its fractions (50 and 100 µg/mL), b) pure compound (C I), and its derivatives (C II and C III) (0–2.5 µg/mL) for one hour.

Cells without any treatment under standard cell culture conditions served as a control. Cell proliferation was determined by the 3-(4,5-dimethylthiazol- 2-yl)-2,5-diphenyl tetrazolium bromide (MTT) colourimetric assay, as mentioned previously (Genc et al., 2019). The average optical density formed in control cells was taken as 100 % viability, and the results of treatments were expressed as a percentage of the control.

1.5.*In vitro* anti-inflammation studies

1.5.1.Nitrite (NO) Assay:

The RAW 264.7 cells were treated with lipopolysaccharide (LPS, 1 μg/mL) with or without different concentrations of a) TSETOH and its fractions (50 and 100 μg/mL), b) pure compound (C I) and its derivatives (2.5 and 5 μg/mL) and c) dexamethasone (10 μg/mL) for 24 h. Cells treated without LPS, and extract samples were utilized as the negative control, and cells treated with LPS alone were treated as the positive control. After 24 h of treatment, the cell culture supernatants were collected for nitrite assay. The concentration of nitrite in the supernatants was measured by the Griess reaction, as per the method described previously (Ahn et al. 2020).

1.5.2.Gene expression of pro-inflammatory cytokines

RNA isolation and cDNA synthesis

As mentioned in Sect. 2.4.1, RAW 264.7 cells were treated with LPS with or without test compounds and Dexamethasone for 24 h. After incubation, cell lysates were collected for total RNA isolation. Total RNA was isolated by PureLink®RNA mini kit (Invitrogen, USA), and the concentration of total RNA was detected by spectrophotometer (Nanodrop ND-100). Then cDNA Synthesis Kit (Invitrogen, USA) was used to reverse transcribe complementary DNA.

RT-PCR

Amplification of transcribed cDNA product was performed to determine the expression of IL-1, IL 1β, and TNFα using a thermal cycler (Roche). Quantitative real-time PCR was performed using SYBR Green Master Rox (Invitrogen, USA) at the standard conditions. The RT-PCR result was expressed as the ratio of optical density to GAPDH. Relative expression levels were calculated using the $2-\triangle\triangle Ct$ method (Livak and Schmittgen, 2001). The list of primers is given in Table 1.

Table 1
Primers list used in Real-time PCR experiments

Sl. No.	Gene	Primer Sequence (5'-3')
1.	GAPDH	5’agcctcgtcccgtagacaaaat and 5’tggcaacaatctccactttgc
2.	IL-1β	5 ‘aaatacctgtggccttgggc and 5’-cttgggatccacactctccag
3	TNF-α	5'gaccctcacactcagatcatcttct and 5'ccacttggtggtttgctacga
4	IL6	5’ acaaccacggccttccctactt and 5’ gtgtaattaagcctccgact

1.6.*In vivo* anti-inflammatory studies
Experimental animals

Swiss albino mice weighing 25 to 30 grams were selected for all the present study experiments and housed under standard environmental conditions. The mice were fed with standard mice feed and provided drinking water ad-libitum. Approval of the Institutional Animal Ethical Committee was obtained to perform the proposed experiments (Ref: ARI/IAEC/2015/04).

1.6.1.Acute oral toxicity study of *T. sulcatum* leaf extract (TSETOH) in mice

The acute oral toxicity study was performed in Swiss albino mice to assess TSETOH extract's safety using revised OECD guidelines (423, Dec 2001). The study was initiated with the limit test at the single highest dose of 2000 mg/kg body weight. The animals were grouped into solvent control and treated groups (n = 3). The test sample (crude extract) was suspended in Carboxy Methyl Cellulose (CMC, 1 %) and administered orally (2000 mg/kg). The control group animals received an equivalent quantity of CMC (1 %). All the animals were observed for clinical signs for 14 days, and the animals' body weights were taken at weekly intervals. On the 14th day, animals were sacrificed, and all organs were collected for gross pathological observations.

1.6.2.Anti-inflammatory effect in Carrageenan-induced inflammation

Swiss albino mice were divided into control, positive control, and treatment groups (n = 6). Positive control group animals received dexamethasone 10 mg/kg, and treatment group animals received 200, 400, 600 mg/kg doses of TSETOH and its fractions (TSHEX, TSTOL, and TSEA). The different set of animals used to test pure compound (C I) and its derivatives (C II and C III) in two doses (15 & 30 mg/kg). The negative control group animals received an only equal volume of saline. The treatment was given 30 minutes before the carrageenan injection. After 30 min of respective treatments, each mouse was treated with Carrageenan (2% in saline) subcutaneously at the planter region to the left hind paw to induce inflammation.

The inflamed paw volume was measured using Plethysmometer (Ugo Basil Plethysmometer, 7140), initially (0-time point, just before carrageenan injection) 1, 3, and 5 h after carrageenan injection by the plethysmographic method to study anti-inflammatory activity.

Further, paw tissue treated and controlled was homogenized, and mRNA was isolated. Gene expression (IL-1, IL 1β, and TNFα) at the mRNA level was evaluated, as mentioned in Sect. 2.5.2.

1.7.Molecular Docking studies:

Chemical structures were drawn with the Chem Draw Ultra 15.0. The energy minimization of structures was performed with MM2/MM3 molecular mechanics parameters until achieving the lowest stable energy state, using Chem3D 15.0. The 3D structure of standard drug Dexamethasone was downloaded from PubChem compound database of NCBI, USA (http://www.pubchem.ncbi.nlm.nih.gov). Protein crystallographic 3D structures (TNF-α (PDB: 2AZ5), IL-6 (PDB: 1ALU), and IL-1β) used as a target protein for Docking study were retrieved from the Brookhaven Protein Databank (PDB) (http://www.pdb.org). For molecular docking, protein structures were prepared for docking by adding Hydrogen atoms to achieve the structure's correct ionization. Flexible docking was performed by 'Auto Dock Vina' software, where we used the Lamarckian genetic algorithm for automated docking of the ligand into the active site of protein (Joshi et al., 2020).

1.8. Statistical Analysis:

All data obtained were expressed as the standard error of the mean (SEM), and one-way analysis of (ANOVA) was performed, followed by Bonferroni's Multiple Comparison Test. The Graph Pad Prism (version 5) software was used for the statistical analysis. $P < 0.05$ was considered statistically significant.

Results And Discussion

The adverse effects and cost of classically used steroids- or NSAID- based drugs as anti-inflammatory drugs have demanded an alternative to existing drugs. The application of herbal medicines as an anti-inflammatory agent would promise safety, better efficacy, and cost-effectiveness. Moreover, the generalised population is more biased towards herbal medicines because of various concerns such as 'natural is better, 'reluctance to allopathy,' religion, or cultural influences. In the current study, efforts have been made to evaluate the anti-inflammatory activity of *T. sulcatum*.

2.1.Extraction & Fractionation of the Total Crude Extract:

The pharmacologically active components are assessed with extract isolated from the medicinal plant parts, and further fractionation is carried out in different solvents. We first extracted the *T. sulcatum* leaves in ethanol to yield TSETOH extract. TSETOH extract was further fractionated to yield TSHEX, TSTOL, and TSEA. Soxhlet extraction of *T. sulcatum* leaves in the three experiments yielded total crude TSETOH extract (56.9 g, 7.59%) as a viscous and dark-coloured semisolid extract. A part of it (46 g) on fractionation yielded hexane soluble fraction (TSHEX, 18.3g, 39.8%), toluene soluble fraction (TSTOL, 14.7 g, 32%), ethyl acetate soluble fraction (TSEA, 4.3 g, 9.3%) leaving the insoluble fraction (TSRES, 8.7 g, 18.9 %).

2.2.Isolation and characterization of the pure compound and its derivatives

Developing novel anti-inflammatory agents with less or no side effects has remained a significant thrust area considering finding alternatives to NSAIDs. Natural products, especially medicinal plants, have remained a very successful avenue for discovering new therapeutic agents. Plants represent an enormous reservoir of new biological active molecules. The chemical structures of secondary metabolites of plants can be used as a model for new synthetic compounds. We attempted to isolate and characterize the active molecules from the leaf extract. Column chromatography of the total crude TSETOH extract yielded a pure crystalline compound. It was characterized by studying physical and spectral data. The details of the structure elucidation are as follows:

Compound I was observed to be white, crystalline, and has a sharp melting point. The elemental analysis suggested the molecular formula $C_{30}H_{52}O$. Further, the FTIR spectrum exhibited a strong band at 3620 cm^{-1}, indicating presence of hydroxyl bond in the molecule. ^{1}H NMR spectrum showed a broad one proton multiplet at δ 3.81, indicating the hydroxyl group's presence at the secondary carbon atom. The ^{13}C NMR (Supplementary Table 1) showed a signal of CH at δ 72.8 due to the carbon holding the hydroxyl group. These observations could easily establish the identity of compound I as 'Friedelan-3β-ol'. The structural assignment of compound I as Friedelan-3β-ol was further confirmed by comparing the physical and spectral data with literature (Chama et al., 2020; Gonçalves Pereira et al., 2020). We have identified a triterpene compound Friedelan-3β-ol as the important secondary metabolites in the crude ethanolic extract of *T. sulcatum* leaves. Thus, first-time we report the occurrence of bioactive Friedelan-3β-ol in *T. sulcatum* leaf extract. These findings will enrich the natural chemical library of the genus *Tetrastigma*.

Compound CII and CIII

Further, the Friedelinol acetate (C II) and friedelinol methyl ether (C III) derivatives of Friedelan-3β-ol (CI) were synthesized by known methods, and their structures were elucidated, similarly based on their physical and spectral data. Friedelan-3β-ol, on treatment with acetic anhydride in pyridine, yielded the acetate. Similarly, the reaction with trimethylorthoformate generated methyl ether. Structures of the derivatives were confirmed by recording their spectral data. The FTIR spectrum of acetate compound (C II) showed the diagnostic bands at 1740 and 1380 cm^{-1}. Further, the 1H NMR spectrum showed three proton singlet at δ 2.0 due to the acetate methyl in addition to the signals of tertiary methyls. The 13C NMR (Supplementary Table 1) and DEPT were in complete agreement with the structure. The spectral data of methyl ether compound (C III) was also in agreement with its structure. FTIR spectrum showed the absence of band due to hydroxyl, while its 1H NMR spectrum showed three proton singlet at δ 3.68 due to the methoxyl group. The 13C NMR (Supplementary Table 1) the spectral data was in complete agreement with those reported in the literature.

2.3.Cytotoxicity assay

For the biomedical application of any compound, the safety of the compound needs to be assessed. The cytotoxic effect of a) TSETOH extract and its fractions and b) Friedelan-3β-ol and its derivatives in RAW

264.7 cells using MTT assay was evaluated. It was observed that TSETOH and its fractions (0-100 µg/mL) were not associated with any toxic effects (Fig. 1A). Similarly, no changes in cell viability were observed by exposing RAW 264.7 cells to different concentrations (0–2.5 µg/mL) of the pure compound and its derivatives (Fig. 1B). This indicates that both extracts and pure compounds have no cytotoxic effect at the concentrations tested in our experimental conditions.

2.4.Evaluation of the *in vitro* anti- inflammatory effect

We further evaluated the anti-inflammatory effects of plant extracts and pure compounds *in vitro* using LPS induced inflammatory model. LPS model has been commonly used to study inflammation, as it mimics many inflammatory effects (Grylls et al., 2021).

The activity of crude leaf extract and its fractions

To investigate the anti-inflammatory effects of TSETOH and its fractions, we first examined the inhibitory effects of TSETOH extract and its fractions on LPS induced nitrite (NO) production in RAW 264.7 cells. Treatment of cells with TSETOH and its fractions (TSHEX and TSTOL) demonstrated a dose-dependent decrease in NO production. TSETOH and TSTOL at 100 µg/mL demonstrated the highest reduction in NO production. However, TSEA showed a low or negligible effect on NO production (Fig. 2A). NO is known as a pro-inflammatory mediator in different acute and chronic inflammatory diseases. The stress-induced production of NO in RAW 264.7 cells is related to inflammation. Stressed macrophages generate excessive inducible NO synthase (iNOS), which forms NO as a part of the inflammatory response and causes death by inducing apoptosis (Du et al., 2020).

Inflammation may lead to various cytokines, which act as essential mediators in inducing inflammatory effects. Among these inflammatory cytokines, IL-1β, IL-6, and TNF-α are considered the most crucial in mediating immunity and activating macrophages (Shen et al., 2018; Du et al., 2020). As the highest reduction in NO production was observed at 100 ug /mL, we further evaluated the effect of TSETOH and its fractions (100 µg/mL) on the LPS-stimulated expression of pro-inflammatory cytokines. The mRNA expressions of inflammatory markers IL-1β, IL-6, and TNF-α were measured by qPCR. It was observed that treatment of cells with TSETOH extract and its fractions (TSHEX and TSTOL) resulted in a significant decrease in expression levels of IL-1β, IL-6, and TNF-α compared to LPS stimulated cells which were not treated by extracts (Fig. 2B).

The activity of pure compound Friedelan-3β-ol and its derivatives

Similar to assessing extracts and fractions, the pure compound Friedelan-3β-ol and its derivatives were evaluated for anti-inflammatory effect on LPS induced NO production and expression of pro-inflammatory cytokines levels (IL 1-β, IL-6, and TNF-α) in RAW cells. The result demonstrated that Friedelan-3β-ol and its derivatives significantly inhibited NO production in a dose-dependent manner after treatment. (Fig. 3A). Similarly, expression levels of pro-inflammatory cytokines in LPS stimulated cells

significantly reduced compared to non-treated cells. The Friedelan-3β-ol and its derivative exhibited higher inhibitory effects on the expression of TNF-α as compared to the expression of IL 1-β and IL-6 (Fig. 3B).

The *in vitro* study demonstrated that the ethanolic extract, fractions, pure compound Friedelan-3β-ol, and their derivatives significantly inhibited LPS-induced NO production and mRNA expression of pro-inflammatory cytokines IL-1β, IL-6, and TNF-α in RAW 264.7 macrophages. Reports suggest that flavonoid presence suppresses the TLR-4/NF-kB p65 signalling pathway, resulting in decreased gene expression of IL-1β, IL-6, and TNF-α and iNOS proteins during anti-inflammatory effect (Andrade et al., 2020). Macrophage activation induced by LPS causes mitogen-activated protein (MAP) kinases and NF-κB intracellular pathway (Hwang et al., 2017; Sanjeewa et al., 2019). This results in pro-inflammatory mediators such as cytokines or regulates iNOS expression by LPS (Hwang et al., 2017; Sanjeewa et al., 2019). The study exhibited a similar pattern of reduced NO production and pro-inflammatory cytokines reported in previous studies, such as *P. Subulate* aqueous extract (Genc et al., 2019), *O. Gratissimum* extract (Dzoyem et al., 2021), *L. Spinosa* leaf extract (Nguyen et al., 2020), showing anti-inflammation activity.

2.5.Acute oral toxicity study

An acute toxicity study of leaf extract and its fractions was carried out. It was found that the animals treated with the *T. sulcatum* leaf extract showed no mortality or any untoward signs or symptoms or abnormal behavioural changes during the 14 days observation period following dosing. The animals were found normal during the experimental period. The treated mice exhibited a regular pattern of body weight gain during 14 days period. There were no gross pathological changes observed in all animals' treatment groups than control (Table 3, Fig. 4).

Table 3
Acute toxicity of TSETOH extract at 2000 mg/kg as a single oral dose in mice

Parameters	7th day		14th day	
	Control	Experimental	Control	Experimental
Body Weight	30.17 ± 0.19	30.57 ± 0.73	32.30 ± 0.15	32.40 ± 0.37
SGPT (IU/L)	14.63 ± 1.2	13.56 ± 0.80	14.40 ± 1.06	13.60 ± 0.47
Creatinine (mg/dL)	0.35 ± 0.04	0.31 ± 0.03	0.39 ± 0.05	0.44 ± 0.04
Haemoglobin (g/dL)	14.76 ± 0.57	14.46 ± 0.31	15.76 ± 0.21	14.73 ± 0.31
RBC (million/µl)	7.32 ± 0.19	7.31 ± 0.15	7.65 ± 0.20	7.46 ± 0.15

2.6.Assessment of anti-inflammatory activity, *in vivo* mice model

Encouraged with the *in vitro* results, we further evaluated the anti-inflammatory effect of selective extracts and pure compounds *in vivo*. We used the carrageenan-induced mouse paw oedema model for *in vivo* studies. Carrageenan-induced mice paw oedema is a commonly used model to test systemic anti-

inflammatory activity (Banik et al., 2021; Kumar et al., 2020). *In vivo* studies demonstrated relative inflammatory activity represented as 'the ratio of treated to control measured paw volume.

The activity of crude leaf extract and its fractions

The TSETOH extract and its fraction (TSTOL, TSHEX), which showed significant anti-inflammatory activity in *in vitro* study, were further evaluated for *in vivo* anti-inflammatory activity. TSETOH extract and its fraction at the different doses (200, 400 and 600 mg/kg) showed a significant decrease in the Carrageenan induced mice to paw oedema volume in a dose-dependent manner at 1, 3 and 5 h compared with the control group. It was observed that at a higher dose, 600 mg/kg plant extracts showed a significant decrease in oedema volume comparable with standard anti-inflammatory drug such as Dexamethasone at five hours (Fig. 5A-C).

The activity of pure compound Friedelan-3β-ol and its derivatives

Like extract and fractions, Friedelan-3β-ol and its derivatives exhibited a significant decrease in carrageenan-induced paw oedema volume compared to carrageenan control in a dose-dependent manner. Friedelan-3β-ol and its methyl ether derivative at a dose rate of 30 mg/kg showed decreased carrageenan-induced paw oedema volume comparable with standard anti-inflammatory drug dexamethasone at five hours (Fig. 5D-E).

Gene expression study

Analogous to *in vitro* gene expression studies, reduced mRNA expression of IL-1β, IL-6, and TNF-α was observed in tissue homogenate of inflamed paw compared to the control group (Fig. 6).

Our previous study reported that the topical application of TSETOH and its fractions (TSHEX, TSTOL, TSEA) significantly reduced inflammation in the TPA-induced ear oedema model (Waghole et al., 2015). In the view of inflammation, the macrophages are antigen-presenting cells (APCs), which signals the immune system to produce various inflammatory mediators such as cytokines, nitric oxide (Kany et al., 2019). The current study reports increased pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α) and NO production in LPS stimulated inflammation. Carrageenan-induced inflammation in the paw mouse model revealed a similar pattern of NO production and expression of pro-inflammatory cytokines, including interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumour necrosis factor-α (TNF-α) (Ahn *et al.*, 2020). Various traditionally used herbal extracts were found to be a source of triterpenoids, carrying anti-inflammatory activity (Ou et al., 2019; Wu et al., 2021). Similar to the previous reports, the current study demonstrated significantly reduced IL-6, IL-1β, and TNF-α mRNA levels in tissue homogenates of inflamed paw tissues with anti-inflammatory leaf extract activity.

The pure compound Friedelan-3β-ol (CI) and its derivatives Friedelinol acetate (C II), friedelinol methyl ether (C III) also demonstrated the anti-inflammatory activity. The order of activity observed is Friedelinol > Friedelinol acetate > friedelin methyl ether. These findings suggest the presence of free hydroxyl groups

is needed for optimum activity. It was revealed that the triterpenoids exhibited better anti-inflammatory activity, which was found to agree with previous reports (Ou et al., 2019; Wu et al., 2021).

Further, the study report demonstrated the anti-inflammatory effect of leaf extract and its fractions comparative with Dexamethanasone (positive control). Dexamethasone (synthetic pregnane corticosteroid; a cortisol derivative) is a known anti-inflammatory and autoimmune condition drug (Black and Grodzinsky, 2019; Giles et al., 2018; Li et al., 2020). Therefore, the leaf extract of *T. sulcatum* can be taken as an anti-inflammatory drug candidate for future studies.

2.7.Molecular docking study

A strong correlation is observed in cytokine mediators and inflammation in inflammation-associated diseases such as traumas, autoimmune disease, bone pathologies (Kany et al., 2019). To get a better understanding of the structural-activity relationship, molecular docking was performed. The interaction study of compounds and Dexamethasone (a standard steroidal anti-inflammatory drug) was performed with TNF-α (PDB: 2AZ5), IL-6 (PDB: 1ALU), and IL-1β (PDB: 9ILB). The interaction study of IL-6 with compounds demonstrated that Friedelan-3β-methyl ether shared the most common residues (Thr-89, Glu-93, Thr-137, Asp-140, Asn-144, Leu-147, Asn-63, Tyr-97, Thr-143, Val-96, Pro-139) to the Dexamethasone, with higher docking energy of -7.1 kcal/mol^{-1}. Moreover, TNF-α docked interaction with ligands showed all the compounds (Friedelinol-3ß-ol, Friedelan-3ß-acetate, and Friedelan-3ß-methyl ether) share the common residues with Dexamethasone. Otherwise, IL-1β interaction revealed the common residues (Asn-7, Ser-5, Lys-63, Leu-62, Glu-64, Lys-65, Tyr-68, Tyr-90, Pro-91, Ser-43) with Friedelan-3ß-methyl ether alone, with docking energy of -7.3 kcal/mol-1 (Table 2, Fig. 7).

Table 2

Revealing the binding affinity (kcal mol− 1) and residues interaction of control compound (Dexamethasone) and other compounds (Friedelan-3-ol, Friedelan-3ß-acetate, and Friedelan-3ß-methyl ether) against anti-inflammatory targets TNF- (PDB: 2AZ5), IL-6 (PDB: 1ALU), and IL-1 (PDB: 9ILB). The residues in bold share common with Dexamethasone at respective targets.

βαβ			
Protein	**Ligands**	**Binding affinity (kcal mol^{-1})**	**Pocket residues**
IL-6 (PDB: 1ALU)	**Dexamethasone**	-6.6	Leu-147, Tyr-97, Asn-144, Val-96, Pro-139, Asp-140, Thr-138, Thr-89, Thr-137, Glu-93, Thr-143, Asn-63
	Friedelan-3β-ol	**-7.3**	Asp-134, Leu-133, Lys-70, **Thr-89**, **Glu-93**, Lys-86, Asp-71, Val-85, Thr-82, Glu-81
	Friedelan-3β-acetate	-7.1	**Thr-89**, Ile-136, **Thr-137**, Lys-70, Thr-82, Asp-71, Leu-133, Asp-134, Val-85, Lys-86
	Friedelan-3β-methyl ether	-7.1	**Glu-93**, **Asp-140**, **Asn-144**, **Leu-147**, Lys-150, Leu-62, Asn-61, **Asn-63**, **Tyr-97**, **Thr-143**, **Val-96**, **Pro-139**
TNF-α (PDB: 2AZ5)	**Dexamethasone**	-8.1	Leu-(C)-57, Ile-(C)-58, Tyr-(C)-59, Gly-(C)-122, Leu-(D)-57, Tyr-(D)-59, Gly-(C)-121, Leu-(C)-120, Tyr-(C)-119, Tyr-(D)-151, Gln-(D)-61, Tyr-(D)-119, Ser-(D)-60, Leu-(D)-120, Gly-(D)-121
	Friedelan-3β-ol	-9.7	**Leu-(D)-120**, **Leu-(C)-57**, **Gly-(C)-121**, **Leu-(D)-57**, **Tyr-(C)-59**, **Leu-(C)-120**, **Tyr-(D)-59**, Leu-(B)-55, **Gly-(D)-121**, **Tyr-(D)-119**, **Tyr-(C)-119**
	Friedelan-3β-acetate	-9.5	**Tyr-(D)-151**, **Tyr-(C)-59**, **Gly-(C)-121**, Val-(B)-123, Val-(D)-123, **Leu-(B)-157**, **Leu-(D)-57**, Leu-(B)-55, **Leu-(C)-120**, **Tyr-(C)-119**, **Tyr-(D)-59**, **Ser-(D)-60**, **Leu-(D)-120**, **Gly-(D)-121**
	Friedelan-3β-methyl ether	-10.4	Leu-(B)-55, **Tyr-(D)-119**, **Leu-(C)-120**, **Ile-(C)-58**, **Gly-(C)-121**, Leu-(C)-57, **Gly-(C)-122**, **Tyr-(C)-59**, **Leu-(D)-57**, **Tyr-(D)-59**, **Tyr-(C)-119**, **Gly-(D)-121**, **Leu-(D)-120**, Leu-(B)-55,
IL-1β (PDB: 9ILB).	**Dexamethasone**	-7.3	Leu-67, Leu-62, Tyr-90, Tyr-68, Gly-61, Pro-91, Ser-5, Asn-7, Glu-64, Lys-63, Ser-43, Lys-65
	Friedelan-3β-ol	-7.5	Thr-137, Gly-136, Gly-135, Trp-120, Leu-134, Leu-80, Pro-78, Phe-133, Val-132, Pro-131, Thr-79
	Friedelan-3β-acetate	-7.9	Lys-77, Leu-134, Pro-78, Leu-80, Ser-125, Asp-142, Met-130, Pro-131, Phe-133, Thr-79, Trp-120
	Friedelan-3β-methyl ether	-7.3	Arg-4, Ala-1, **Asn-7**, **Ser-5**, **Lys-63**, **Leu-62**, **Glu-64**, **Lys-65**, **Tyr-68**, **Tyr-90**, **Pro-91**, **Ser-43**

The docking study and experimental results demonstrated that significant inhibition of cytokines by CI and CIII. The *in-silico* data suggests TNF-α shares most interaction residues of either CI, CII or CIII. The significant reduction of TNF-α mRNA expression levels *in vitro* is in concurrence with *in-silico* data. Moreover, the highest binding energy (-10 kcal/mol^{-1}) was observed to be associated with TNF-α

interactions. A similar study reported (Dayakar et al., 2017) the higher selectivity towards TNF-α, suggesting NF-kB pathway activation by anti-inflammatory agents. In the present study, the reduction of pro-inflammatory cytokines has been observed, suggesting the NF-kB pathway's involvement (Andrade et al., 2020).

Conclusion

T. sulcatum extract and fractions exhibited potent anti-inflammatory activity *in vitro* and *in vivo*. We first time demonstrated that *T. sulcatum* leaf extract is a good source of Friedelan-3β-ol. Further, we showed that Friedelan-3β-ol is the active component responsible for the anti-inflammatory potential of *T. sulcatum*. Moreover,the *in-silico* studies revealed that all the compounds (CI, CII and CIII) share common residues with Dexamethasone. TNF- α exhibited the most interacting residues with the compounds.. This information will aid in designing drugs in inflammatory disease to target specific residues in the 3D conformation. Thus, it can be concluded that *T. sulcatum* leaf extract exhibits anti-inflammatory potential to treat inflammation. However, T. sulcatum plant extract's anti-inflammatory mechanism, including various signalling, immune regulatory pathways, would help explore potential in future medicine in inflammatory diseases.

Declarations

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Author contributions:

RJW and AVM carried out experimentation. FK carried out molecular docking studies. SHJ, NK, RJW and DGN executed data analysis and write-up of the manuscript.

Declaration of competing interest:

The authors declare that there are no conflicts of interest.

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Figures

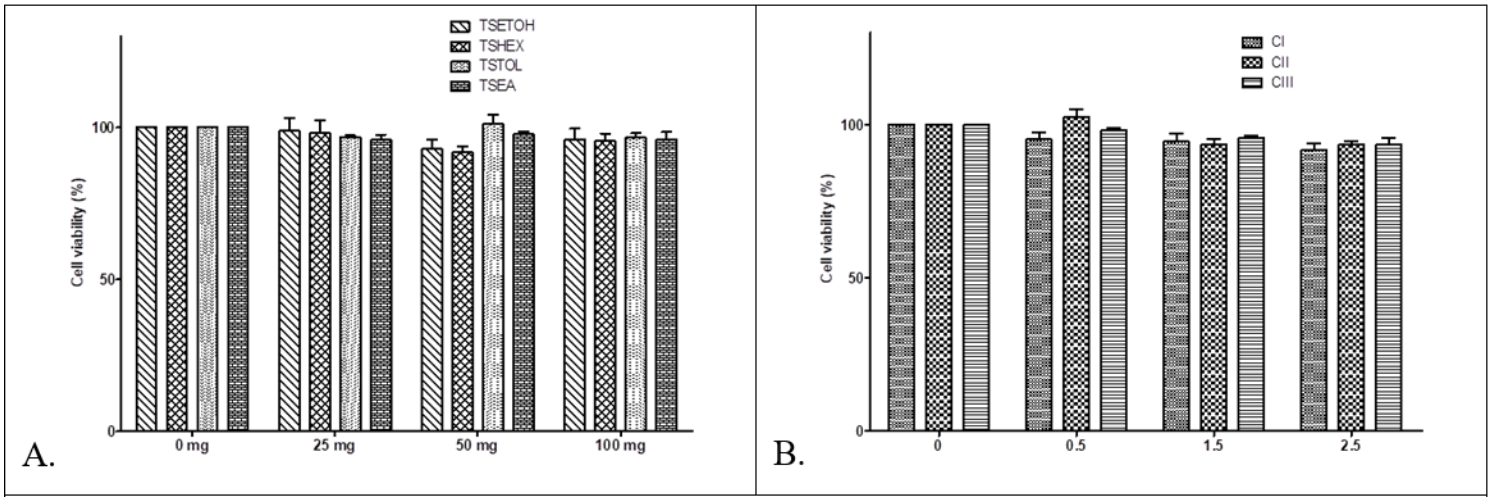


Figure 1

Cytotoxicity assay on the viability of RAW 264.7 (macrophage) cells using MTT. Effect of A. T. sulcatum extract (TSETOH) and their fractions (TSHEX, TSTOL, TSEA), 0 -100 µg/mL, B. Pure compound CI: Friedelan-3β-ol and its fractions, CII: Friedelinol acetate, CIII: friedelinol methyl ether; 0 - 2.5 µg/mL. Representatives of three independent experiments. Values are represented as mean ± S.E.M.

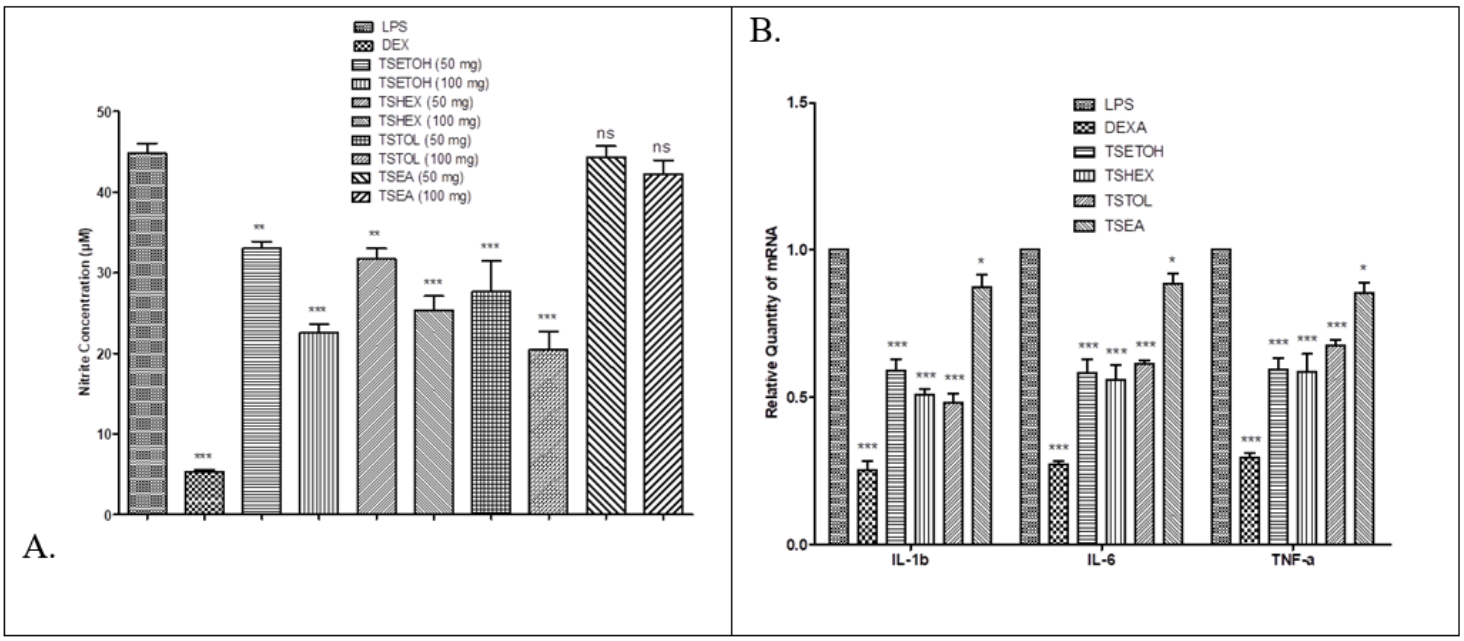


Figure 2

In vitro Anti-inflammatory effect of A) crude extract (TSETOH) and its fractions (TSHEX, TSTOL, TSEA) on LPS induced inflammation RAW 264.7 macrophages, upon 24 h treatment. examethasone (DEX, 10 µg/mL) was used as a positive control, a known anti-inflammatory agent. Nitrite production and pro-inflammatory cytokines (IL) were assessed as markers for inflammation in cells. A. Nitrite assay; representing Nitrite concentration (µM) against treatment with TSETOH, TSHEX, TSTOL, and TSEA (50 and 100 µg/mL); B. Relative mRNA levels of IL-1β, IL-6, and TNF-α upon treatment of TSETOH, TSHEX, TSTOL, and TSEA (100 µg/mL). Results are representative of three independent experiments. Represented as mean ± S.E.M. Statistical analysis: Two way ANOVA (*P < 0.05; ** P < 0.01; *** P < 0.001 and ns: not significant).

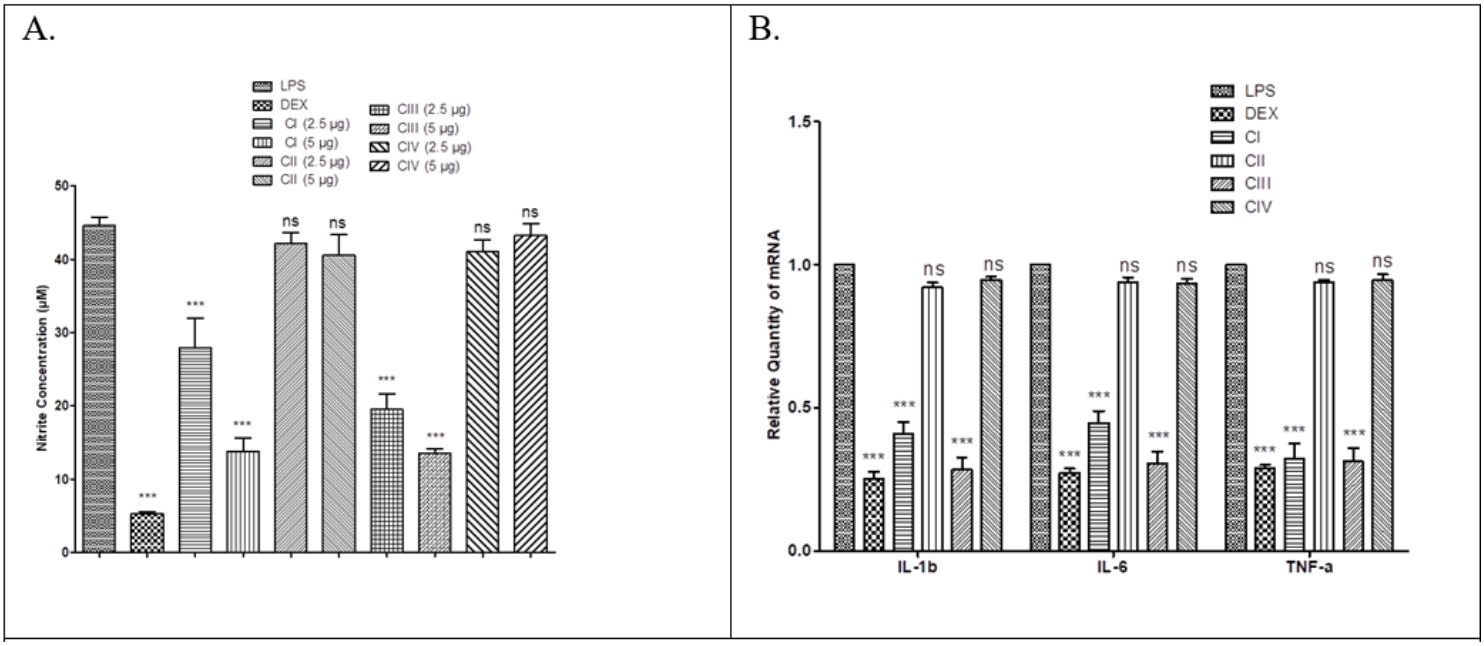


Figure 3

In vitro Anti-inflammatory effect of Pure compound (CI) and its fractions (CII and CIII) on LPS induced inflammation RAW 264.7 macrophages, upon 24 h treatment. Dexamethasone (DEX, 10 µg/mL) was used as a positive control, a known anti-inflammatory agent. Nitrite production and pro-inflammatory cytokines (IL) were assessed as markers for inflammation in cells. A. Nitrite assay; representing Nitrite concentration (µM) against treatment with CI, CII, CIII, and CIV (2.5 and 5 µg/mL); B. Relative mRNA levels of IL-1β, IL-6, and TNF-α upon treatment of CI, CII, CIII and CIV (5 µg/mL). Results are representative of three independent experiments. Represented as mean ± S.E.M. Statistical analysis: One way ANOVA with Bonferroni's Multiple Comparison Test statistical tests (*P < 0.05; ** P < 0.01; *** P < 0.001 and ns: not significant).

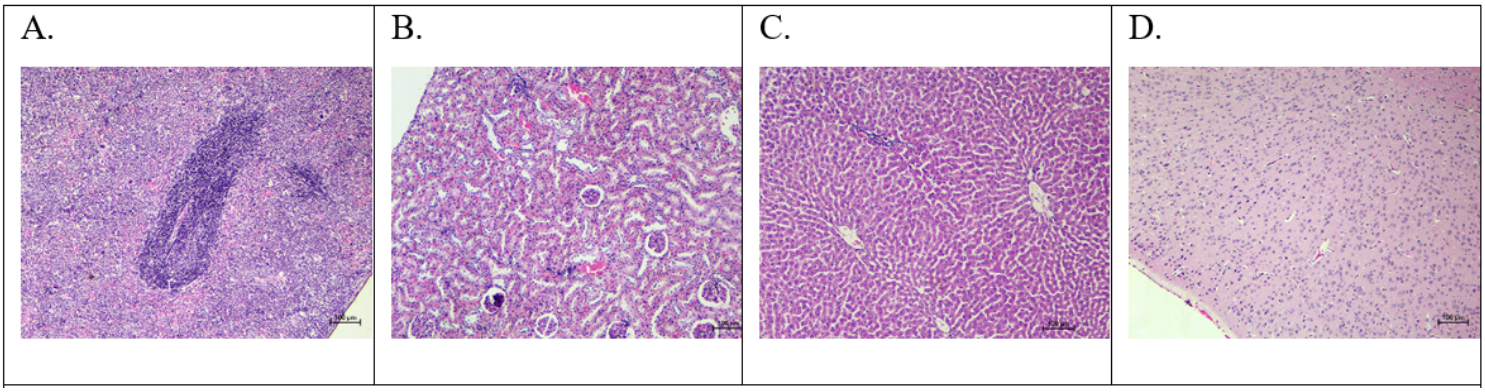


Figure 4

Histopathologic features representative of acute toxicity study of T. sulcatum's ethanolic crude extract (TSETOH) on A) Spleen; B) Kidney; C) Liver; D) Brain of Swiss albino mice, after 14 days of treatment.

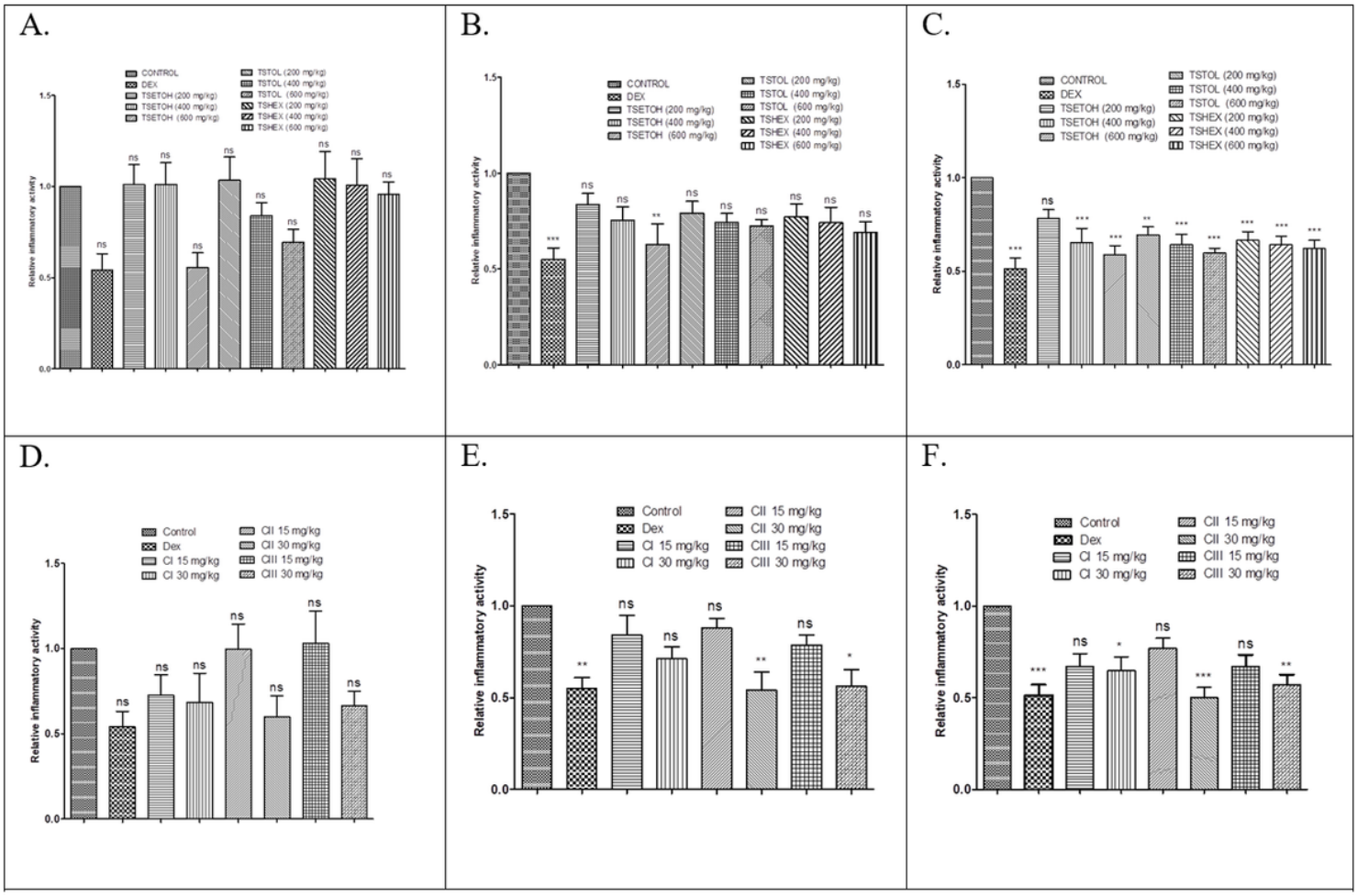


Figure 5

In vivo anti-inflammatory activity represented as a ratio of inflamed volume to control in Carrageenan induced paw-inflammation Swiss mice model. 5A-C. Effect of leaf crude extract (TSETOH) and its fractions (TSHEX, TSTOL, TSEA) corresponding to A) 1 h, B) 3 h,s and C) 5 h; 5D-F. Effect of pure compound (CI) and its fractions (CII and CIII) represented as a relative inflammatory activity of respective test sample, corresponding to D) 1 h, E) 3 h and F) 5 h. Data represented as mean ± S.E.M (n=6). Statistical analysis: One way ANOVA with Bonferroni's Multiple Comparison Test statistical tests (*P < 0.05; ** P < 0.01; *** P < 0.001 and ns: not significant).

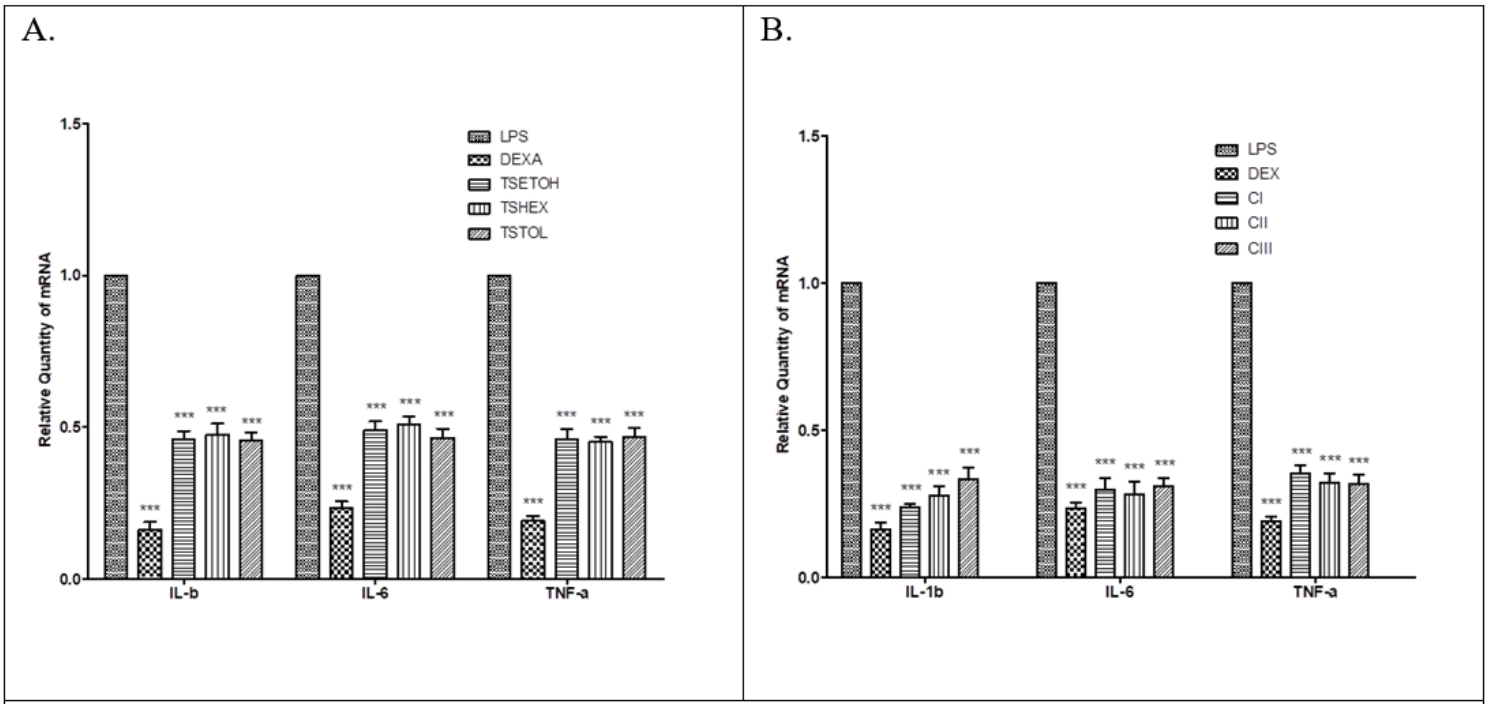


Figure 6

Relative mRNA levels of IL-1β, IL-6, and TNF-α upon treatment (5 h) of A. Leaf crude extract (TSETOH) and its fractions (TSHEX, TSTOL, and TSEA), 600 mg/kg B. Pure compound (CI) and its fractions (CII and CIII), 30 mg/kg Data represented as mean ± S.E.M (n=6). Statistical analysis: One way ANOVA with Bonferroni's Multiple Comparison Test statistical tests (*P < 0.05; ** P < 0.01; *** P < 0.001 and ns: not significant).

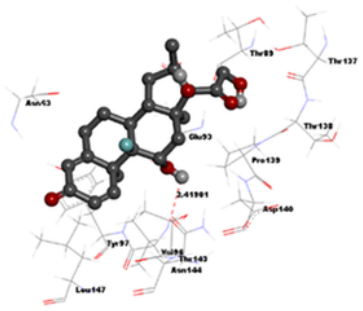
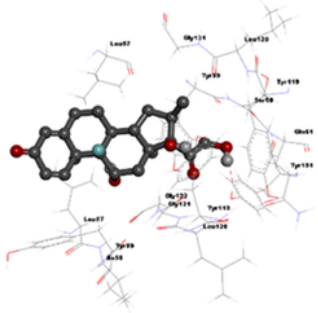
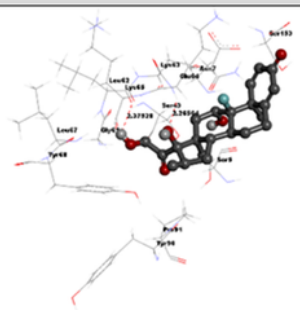
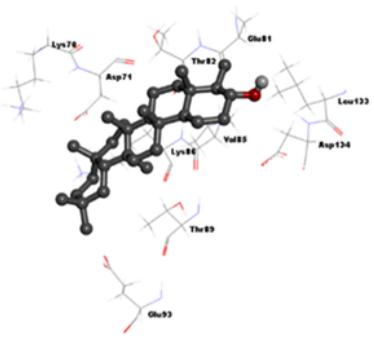
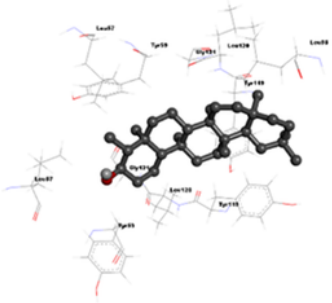
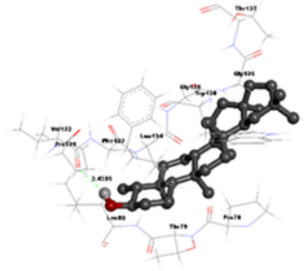
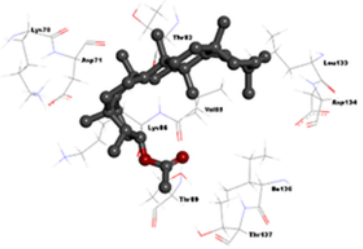
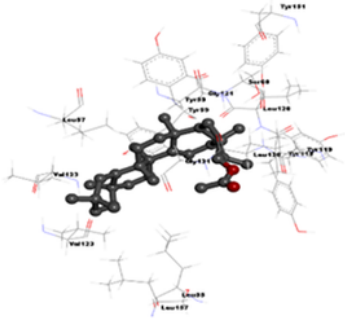
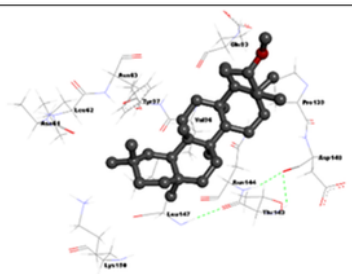
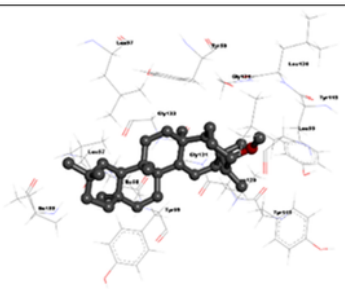
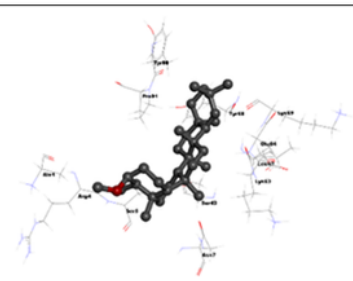
Protein	IL-6 (PDB: 1ALU)	TNF-α (PDB: 2AZ5),	IL-1β (PDB: 9ILB)
Dexamethasone (Control drug)			
Friedelan-3β-ol			
Friedelan-3β-acetate			
Friedelan-3β-methyl ether			

Figure 7

Docking visualization of Friedelan-3β-ol, Friedelan-3ß-acetate, Friedelan-3ß-methyl ether and standard drug dexamethasone against anti-inflammatory targets TNF-α (PDB: 2AZ5), IL-6 (PDB: 1ALU) and IL-1β (PDB: 9ILB)

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- GraphicalAbstract.png
- Supplementary.doc