In vitro and in vivo anti-inflammatory activity of *Tetrastigma sulcatum* leaf extract, pure compound and its derivatives

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Received: 11 May 2021 / Accepted: 14 November 2021 / Published online: 22 January 2022 © The Author(s), under exclusive licence to Springer Nature Switzerland AG 2021

Abstract

The severity and perseverance of inflammation have been demonstrated in many health conditions. The limitations of existing medications suggest the need for new alternative anti-inflammatory medications. In our earlier studies, we demonstrated the topical anti-inflammatory potential of the crude ethanolic extract of *Tetrastigma sulcatum* leaves and its fractions. In the present study, we further explored the 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 the leaf extract and identified it as a Friedelan-3β-ol (CI). Furthermore, Friedelinol acetate (C II) and friedelinol methyl ether (C III), derivatives of Friedelan-3β-ol (CI) were synthesised. LPS-induced inflammatory RAW 264.7 macrophages were used as in vitro model to study anti-inflammatory and anti-oxidative effects. Inflammation-induced oxidative damage was found to be restricted significantly \((P < 0.001)\), with scavenging activity and increased SOD activity of crude extract and fractions. Treatment with crude extract (TSETOH) and fractions (TSHEX, TSTOL) significantly reduced \((P < 0.001)\) the mRNA expression of pro-inflammatory cytokines (IL-1β, IL-6, TNF-α) and nitric oxide (NO) production in LPS-stimulated inflammation in RAW 264.7 cells in a dose-dependent manner. Likewise, compounds CI and CIII showed a similar pattern of significant inhibition \((P < 0.001)\) of pro-inflammatory cytokines and NO production in a dose-dependent manner. An in vivo study in a carrageenan-induced mouse paw oedema model demonstrated reduced paw oedema and pro-inflammatory cytokines in a dose-dependent manner upon treatment with the extract, its fractions, pure compound (CI), and their derivatives (CII, and CIII). The present study confirmed the anti-inflammatory activity of *T. sulcatum*, suggesting that Friedelan-3β-ol is an active component of the crude extract.

Keywords *Tetrastigma sulcatum* · Friedelan-3β-ol · DPPH assay · FRAP assay · SOD · Antioxidant activity · ROS · NO production · Inflammatory cytokines · Anti-inflammatory activity
Introduction

Inflammation is a fundamental part of the innate immune system and is classified as acute or chronic inflammation (Šoltés and Kogán 2014). Acute inflammation involves resident macrophages and dendritic cells, allowing immediate local response and signalling of other immune cells to locate injured tissue (Ansar 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; Pahwa et al. 2020). However, these synthetic drugs used against inflammation have been reported to produce drug-related toxicities and adverse reactions that complicate the treatment process (Ma et al. 2020; Pahwa et al. 2020). Considering the severe concerns over inflammation in human health and perseverance, developing newer drugs with fewer or no side effects to treat chronic inflammation is of paramount importance.

Ancient knowledge of medicine is hindered by the application of herbal medicines. Considering Indian medicinal systems, naturopathy and Ayurveda practices have gained increased attention and have become ‘tradition to trend’. Practices, including herbal medicines, are becoming popular in preventive, promotional, and curative applications (Ekor 2014). Traditional plants, such as crude material or pure compounds, play a significant role in the discovery of new drugs for the treatment of various diseases (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). Herbal drugs with fewer side effects can have valuable applications in many diseases associated with inflammation.

The genus Tetrasigma belongs to the Vitaceae family and is well known for its economically significant fruit crop variety, Grape (Vitis vinifera) (Ma et al. 2021). Tetrasigma includes nearly 100 species distributed mainly on the Asian continent. Tetrasigma is a genus that remains unexplored and poorly understood, considering its evolution, conservation, and utilisation (Wen et al. 2018). Compared to other species, T. hemsleyanum is a widely studied species for 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) have rarely been reported to have medicinal properties. Among various Tetrasigma species, databases ‘Plants of the World’ and ‘eFlora of India’ show T. sulcatum as a native Indian variety. However, very few reports are available on the therapeutic activity of T. sulcatum, other than our previous studies (Waghole et al. 2015).

The presence of polyphenols and flavonoids in plant leaf extracts has special significance in plant protection to control various pests and diseases (Mulat et al. 2020). Polyphenols, flavonoids, catechol tannins, leucoanthocyanins, syringing glycosides, raphides, tannins, and mucilage substances have been identified in preliminary phytochemical studies of T. sulcatum (Waghole et al. 2015). Our previous report demonstrated the antioxidant and topical anti-inflammatory potential of crude ethanolic extract of T. sulcatum leaves and its fractions (Waghole et al. 2015). Another report showed the antifungal activity of T. sulcatum (Law.) Gamble, particularly the essential oil from leaf extract, against various fungal species such as Fusarium oxysporum, M. moniliforme, F. equiseti, Aspergillus niger, and Botrytis cinerea (Jagannath and Gopal, 2016). Considering the medicinal properties and our preliminary studies, we attempted to explore the anti-inflammatory effects of T. sulcatum leaves in the present study.

Therefore, the leaf extract of T. sulcatum was extracted and fractionated. Subsequently, the significant compounds involved in the anti-inflammatory activities of T. sulcatum were identified through bioassay-guided fractionation. The anti-inflammatory and antioxidant activity of the extract, fractions, and isolated pure compounds were evaluated using in vitro and in vivo bioassay techniques.

Materials and methods

Collection and authentication

The leaves of Tetrasigma sulcatum (25.5 kg) were collected from the experimental farm of the 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-411,001. The material has been deposited at the herbarium of the Botanical Survey of India (BSI), Pune (Voucher specimen number 189329). It was identified as Tetrasigma sulcatum (Law.) Gamble belongs to the family Vitaceae.

Extraction and fractionation of the total crude ethanolic extract

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

**Purification of TSETOH**

TSETOH (10 g) was dissolved in acetone (100 mL), and silica gel (60–120 mesh, 15 g) was added. TSETOH was adsorbed onto the silica gel. The adsorbed extract was loaded on a Medium Pressure Liquid Chromatography (MPLC) column of TLC silica gel G (2.6 × 46 cm). The column was eluted into 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 1 L), ethyl acetate (1 L), and methanol (0.4 L) at 4.8 mL/min, tR of 1:–1–200 mL and 2:–200–400 mL. TLC of all fractions was recorded on pre-coated plates using 20% ethyl acetate in a hexane solvent system. The plates were developed using anisaldehyde-sulfuric acid. Fractions with 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. The solvent was carefully evaporated to remove impurities from the other compounds to prepare a dry pack column. The adsorbed dry powder was loaded onto a column of silica gel columns. 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 × 100 mL), ethyl acetate (0.3 L), and methanol (0.2 L). tR of 1: between 1 and 100 mL and 2: between 100 and 200 mL. Fractions 11–16 eluted with hexane/EtOAc (9:1) were combined and recrystallized with ether methanol to yield a white crystalline compound (0.271 g, I). This compound was further examined by TLC, MP recording, and optical rotation. The structures of the compounds obtained after column chromatography of the crude extract were determined by spectroscopic and chemical conversions (Supplementary 1). Compound I was identified as Friedelan-3β-ol. Furthermore, friedelinol acetate (C II) and friedelinol methyl ether (C III) were derived from compound I.

**Cytotoxicity assay**

Mouse macrophages (RAW 264.7) 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 of 1 × 10⁴ cells/well. The cells were treated with different concentrations of a) TSETOH and its fractions (10, 25, 50, 100, and 200 µg/mL), b) pure compound (C I), and its derivatives (C-II and C-III) (0.5, 1.5, 2.5 and 5.0 µg/mL) for 24 h. Cells without any treatment under standard cell culture conditions served as controls. Cell proliferation was determined using the 3-(4,5-dimethylthiazol-2-yl)–2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay, as described previously (Genc et al. 2019). The average optical density formed in control cells was considered to be 100% viable, and the treatments were expressed as a percentage of the control.

**Determination of antioxidant activity**

**DPPH and FRAP assay**

The antioxidant potential of the extract and fractions were assessed using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ferric reducing antioxidant power (FRAP) assays, as described previously (Waghole et al. 2015). Butylated hydroxy toluene (BHT) (1, 5, 10, 20, 40, and 80 µg/mL) was used as the standard for both assays.

**ROS staining**

In vitro oxidative damage was assessed by ROS staining performed as per the Image-IT™ LITE Green Reactive Oxygen Species Detection Kit (Invitrogen). The RAW 264.7 cells were treated with lipopolysaccharide (LPS, 1 µg/mL) with or without different concentrations of (a) TSETOH and its fractions (10, 50, 100, and 200 µg/mL), (b) pure compound (C I) and its derivatives (0.5, 1.5, 2.5 and 5.0 µg/mL), and (c) dexamethasone (10 µg/mL) for 24 h. Cells treated with LPS alone were considered as a positive control for ROS generation, inducing oxidative stress.

**SOD activity**

Superoxide dismutase (SOD) activity was assessed using the SOD kit (Sigma), which is represented as the percentage inhibition rate of ROS. Oxidative stress-induced cells treated with LPS alone were considered as a positive control for ROS generation, inducing oxidative stress.
LPS-treated cells were used as the positive control. After 24 h of treatment, cell culture supernatants were collected for the nitrite assay. The nitrite concentration in the supernatants was measured using the Griess reaction, as per the method described previously (Ahn et al. 2021).

**Gene expression of pro-inflammatory cytokines**

RNA isolation and cDNA synthesis As mentioned in Sect. Determination of antioxidant activity, 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 using the PureLink®RNA Mini Kit (Invitrogen, USA), and the concentration of total RNA was determined using a spectrophotometer (Nanodrop ND-100). The cDNA Synthesis Kit (Invitrogen, USA) was used to reverse transcribe the complementary DNA.

RT-PCR The transcribed cDNA product was amplified to determine the expression of IL-6, IL-1β, and TNFα using a thermal cycler (Roche). Quantitative real-time PCR was performed using SYBR Green Master Rox (Invitrogen, USA) under standard conditions. The RT-PCR results were expressed as the ratio of optical density to GAPDH. Relative expression levels were calculated using the $2^{-\Delta\Delta C_{t}}$ method (Livak and Schmittgen 2001). The primers used are listed in Table 1.

**In vivo anti-inflammatory studies**

**Experimental animals**

Swiss albino mice weighing 25-30 g were selected for all the present study experiments and housed under standard environmental conditions. The mice were fed standard mouse feed and provided with drinking water ad libitum.

**Toxicological evaluation**

The toxicological evaluation was performed in Swiss albino mice to assess the safety of TSETOH extract using the revised OECD guidelines (423, Dec 2001). The animals were grouped into control and treated groups ($n = 3$). The test sample (crude extract) was suspended in carboxymethyl cellulose (CMC, 1%) and administered orally (2000 mg/kg). The control group received an equivalent quantity of CMC (1%). All animals were observed for clinical signs for 14 days, and their body weights were recorded weekly. On the 14th day, the animals were killed, and all organs were collected for gross pathological examination.

**Anti-inflammatory effect in Carrageenan-induced inflammation**

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

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

Furthermore, paw tissues treated and controlled were homogenised, and the mRNA was isolated. Gene expression (IL-1β, IL-6, and TNFα) at the mRNA level was evaluated as described in Sect. Gene expression of pro-inflammatory cytokines.

**Statistical analysis**

All data obtained were expressed as the standard error of the mean (SEM), and two-way or one-way analysis of variance (ANOVA) was performed, followed by Bonferroni’s multiple comparison test. GraphPad Prism (version 5) software was used for the statistical analyses. Statistical significance was set at $p < 0.05$.

**Table 1 Primer list used in real-time PCR experiments**

| Sl. no. | Gene     | Primer sequence (5’-3’)                                      |
|---------|----------|-------------------------------------------------------------|
| 1       | GAPDH    | 5’agcgctggccctagacaaat and 5’gcaagaactccaccattgc            |
| 2       | IL-1β    | 5’aatacatggtgctgggc and 5’cttggcatccacactcccg                |
| 3       | TNF-α    | 5’gaccccaacactgacatctctt and 5’ccacctgtttgcgtctcga          |
| 4       | IL6      | 5’acaaccagggctctccctactt and 5’gtgtaattaaggctttccgact       |
Results

Extraction and fractionation of the total crude extract

The pharmacologically active components were assessed with extracts isolated from medicinal plant parts, and further fractionation was carried out in different solvents. We first extracted T. sulcatum leaves in ethanol to yield the TSETOH extract. The 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, 18.3 g, 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%).

Isolation and characterisation of the pure compound and its derivatives

We isolated and characterised the active molecules present in leaf extracts. Column chromatography of the total crude TSETOH extract yielded a pure crystalline compound. This was characterised by studying the physical and spectral data. The details of the structure elucidation are as follows:

Compound I was white, crystalline, and had a sharp melting point. Elemental analysis suggested the molecular formula of C30H52O. Furthermore, the FTIR spectrum exhibited a strong band at 3620 cm⁻¹, indicating the presence of a hydroxyl bond in the molecule. The 1H NMR spectrum showed three proton singlets at δ 3.68 due to the methoxyl group. The 13C NMR (Supplementary Table 1) spectral data agreed with those reported in the literature.

Cytotoxicity assay

The cytotoxic effect of TSETOH extract, its fractions, Friedelan-3β-ol, and its derivatives was evaluated in RAW 264.7 cells using the MTT assay. TSETOH and its fractions (10, 25, 50, 100, and 200 µg/mL) were not associated with any toxic effects (Fig. 1A). Similarly, no toxicity was observed when RAW 264.7 cells were treated with different concentrations (0.5, 1.5, 2.5 and 5.0 µg/mL) of the pure compound and its derivatives (Fig. 1B).

Evaluation antioxidant activity

The FRAP assay (Fig. 2 A–C) demonstrated the reducing power of the extract, its fractions, and pure compounds in a dose-dependent manner. The TSTOL fraction demonstrated the highest reducing power, whereas TSHEX showed the lowest activity. Similarly, CI and CIII exhibited a higher reducing power than CII.

Radical scavenging activity was represented as the percentage inhibition of free ROS and reported in terms of IC50 value (Table 2). The IC50 concentration of BHT was found to be about 25.44 µg/mL (Fig. 2D, Table 2). Crude extract exhibited a lower IC50 value of approximately 47.15 µg/mL showing higher radical scavenging activity. In contrast, the TSEA fraction showed the highest IC50 dose of approximately 231.45 µg/mL. CIII demonstrated the lowest scavenging activity with an IC50 dose of approximately 30.61 µg/mL, followed by an IC50 value of 54.48 µg/mL and 97.67 µg/mL, for CII and CI, respectively.

Increased green fluorescence of ROS (Fig. 3A) was noted in macrophages upon LPS treatment compared to the control, indicating the induction of oxidative stress. Dexamethasone reduced free ROS compared to LPS-treated cells. Similarly, treatment with crude extract TSETOH, fraction TSHEX, and pure compounds CI, CII, and CIII showed reduced free ROS. However, the TSEA fraction demonstrated the presence of free ROS in the cells. LPS-treated macrophages exhibited reduced SOD activity compared to the control cells. SOD enzyme activity was observed to be significantly (P < 0.001) increased upon treatment with dexamethasone, crude extract, its fractions, and pure compounds, compared to LPS-treated cells (Fig. 3B, C).

Evaluation of the in vitro anti-inflammatory effect

We further evaluated the anti-inflammatory effect of the leaf extract and pure compounds in vitro using an LPS-induced inflammatory model.
The activity of crude leaf extract and its fractions

To investigate the anti-inflammatory effect of TSETOH and its fractions, we first examined the inhibitory effect of TSETOH extract and its fractions on LPS-induced nitrite (NO) production in RAW 264.7 cells. Treatment of cells with TSETOH, TSHEX, and TSTOL at 200 µg/mL demonstrated the highest reduction in NO production. TSEA showed a low or negligible inhibitory effect on NO production at 10, 50, and 100 µg/mL (Fig. 4A), whereas at 200 µg/mL, significant inhibition of NO production was noted.

Furthermore, we evaluated the effect of TSETOH and its fractions (10, 50, 100, and 200 µg/mL) on the LPS-stimulated expression of pro-inflammatory cytokines. The mRNA expression levels of the 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, TSTOL, and TSEA) resulted in a significant decrease in the expression levels of IL-1β (Fig. 4B), IL-6 (Fig. 2C), and TNF-α (Fig. 4D) compared to that in LPS-stimulated cells in a dose-dependent manner. Non-significant inhibition of pro-inflammatory cytokines was observed at a lower concentration (10 µg/mL). At higher treatment concentrations (TSETOH, TSHEX, TSTOL, at 100 and 200 µg/mL), a statistically significant reduction was noted in pro-inflammatory cytokines.
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 their anti-inflammatory effects on LPS-induced NO production and expression of pro-inflammatory cytokines levels (IL-1β, IL-6, and TNF-α) in RAW cells. The results demonstrated that Friedelan-3β-ol (CI) and its derivative...
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(CIII) significantly inhibited NO production in a dose-dependent manner after treatment. (Fig. 5A). Similarly, statistically significant inhibition of pro-inflammatory cytokine expression was observed in a dose-dependent manner, with the highest expression observed at 5 µg/mL (Fig. 5B, D). However, derivative CII showed a non-significant inhibitory effect on NO production and relative mRNA expression of IL-6 and TNF-α.

**Toxicological evaluation of *T. sulcatum* leaf extract**

Toxicological evaluation of *T. sulcatum* leaf extract was carried out. It was found that the animals treated with the *T. sulcatum* leaf extract showed no mortality or any untoward signs, symptoms, or abnormal behavioural changes during the 14 days observation period following dosing. All the animals were normal throughout the experimental period. The treated mice exhibited a regular pattern of body weight gain during 14 days period. Histopathological examination showed a typical distinct red-white pulp of the spleen and an intact glomerulus with distal and proximal tubules of the kidney. Integral hepatocytes, a central portal vein of the liver, and brain neuronal cells showed no toxicity (Table 3, Fig. 6).

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

We used a carrageenan-induced mouse paw oedema model for in vivo studies. 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 (TSHEX, TSTOL), which showed significant anti-inflammatory activity in vitro, were further evaluated for in vivo anti-inflammatory activity. TSETOH extract and its fraction at different doses (200, 400, and 600 mg/kg) showed a significant decrease in 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 to standard anti-inflammatory drugs such as dexamethasone at 5 h (Fig. 7A–C).

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

Similar to the extracts and fractions, Friedelan-3β-ol and its derivatives exhibited a significant decrease in carrageenan-induced paw oedema volume compared to the control in a dose-dependent manner. Friedelan-3β-ol and its derivatives (CII, CIII) at a dose rate of 30 mg/kg showed a decrease in carrageenan-induced paw oedema volume comparable to that of the standard anti-inflammatory drug dexamethasone at five hours (Fig. 7D, 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 homogenates of inflamed paw compared to the control group (Fig. 8).

**Discussion**

The development of novel anti-inflammatory agents with less or no side effects has remained a thrust area, considering finding an alternative to NSAIDs. Plants represent an enormous reservoir of biologically active molecules. Many reports have demonstrated anti-inflammatory drugs targeting the inhibition of releasing factors, such as pro-inflammatory cytokines and nitric oxide, with plant extract (Pranweerapaiboon et al. 2020). Our preliminary study (Waghole et al. 2015) demonstrated the topical anti-inflammatory potential of the crude extract of *T. sulcatum* leaves in a TPA-induced ear oedema model. Given its potency as an anti-inflammatory agent, efforts have been made in the phytochemical investigation of *T. sulcatum* on the ethanolic extract of leaves in the present study.

The compound was isolated from a crude extract of leaves (TSETOH), named CI, and its structural assignment was confirmed by analytical spectroscopy (FTIR, 1H NMR, and 13C NMR). We identified the triterpene compound Friedelan-3β-ol as an important secondary metabolite in the crude ethanolic extract of *T. sulcatum* leaves (Chama et al. 2020; Gonçalves Pereira et al. 2020). Thus, for the first time,

| IC₅₀ (µg/mL)          |               |
|----------------------|---------------|
| BHT                  | 25.44 ± 0.36  |
| TSETOH               | 47.15 ± 1.46  |
| TSTOL                | 129.16 ± 1.63 |
| TSHEX                | 56.63 ± 1.09  |
| TSEA                 | 231.45 ± 1.01 |
| CI                   | 97.67 ± 2.42  |
| CII                  | 54.48 ± 2.41  |
| CIII                 | 30.61 ± 9.21  |
### Table

| Treatment | Hoechst 33342 | ROS | Merged |
|-----------|---------------|-----|--------|
| Control   | ![Control Image](image1.png) | ![Control Image](image2.png) | ![Control Image](image3.png) |
| LPS       | ![LPS Image](image4.png) | ![LPS Image](image5.png) | ![LPS Image](image6.png) |
| Dex       | ![Dex Image](image7.png) | ![Dex Image](image8.png) | ![Dex Image](image9.png) |
| TSETOH    | ![TSETOH Image](image10.png) | ![TSETOH Image](image11.png) | ![TSETOH Image](image12.png) |
| TSHEX     | ![TSHEX Image](image13.png) | ![TSHEX Image](image14.png) | ![TSHEX Image](image15.png) |

**Fig. 3** In vitro assessment of oxidative stress and antioxidant potential. **A** Representative live macrophage cell images for ROS detection, green fluorescence demonstrates free radicals ROS and Hoechst stains cellular nucleus blue. LPS-induced cells were used as positive control followed with treatment of cells with extract (TSETOH), fractions (TSTOL, TSHEX, and TSEA) and pure compounds (CI, CII, and CIII). Control cells are without any treatment either ROS or other test compounds. **B** Determination of SOD activity. 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). Compared to LPS control all other treatment showed reduced free ROS comparable to control cells. SOD activity was significantly increased upon treatment with extract (TSETOH), fractions (TSTOL, TSHEX, and TSEA) and pure compounds (CI, CII, and CIII) compared to LPS.
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Fig. 3 (continued)
Fig. 3 (continued)
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*. Derivatives of Friedelan-3β-ol, CII, and CIII were synthesised, and their structures were confirmed using physical and spectral data. The comparative anti-inflammatory efficacy of TSETOH,
Fig. 4 (continued)

C

Relative Quantity of mRNA

D

Relative Quantity of mRNA
Fig. 5  In vitro anti-inflammatory effect of pure compound (CI) and its derivatives (CII and CIII) on LPS-induced inflammation in RAW 264.7 macrophages, after 24 h of 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 of inflammation in the cells. A. Nitrite assay, representing nitrite concentration (µM) against treatment with CI, CII, and CIII (0.5, 1.5, 2.5, and 5 µg/mL). Relative mRNA levels of B. IL-1β, C. IL-6, and D. TNF-α upon treatment with CI, CII, and CIII (0, 0.5, 1.5, 2.5, and 5 µg/mL). Results are representative of three independent experiments. Represented as mean ± S.E.M. Statistical analysis: two way ANOVA with Bonferroni's Multiple Comparison Test statistical tests (*P<0.05; **P<0.01; ***P<0.001 and ns: not significant). Except for CII, the CI and CIII compounds showed significant inhibition of nitrite production at 1.5, 2.5, and 5 µg/mL. Statistically significant inhibition of pro-inflammatory cytokines at 5 µg/mL and others demonstrated inhibition in a dose-dependent manner.
Fig. 5 (continued)
TSHEX, TSTOL, and TSEA, along with compounds CI, CII, and CIII, was evaluated in the current study.

Macrophage activation causes mitogen-activated protein (MAP) kinases and NF-κB intracellular pathways under inflammatory conditions induced by LPS (Grylls et al. 2021; Hwang et al. 2017; Kany et al. 2019; Sanjeewa et al. 2019). This results in pro-inflammatory mediators, such as cytokines, or regulates iNOS expression (Hwang et al. 2017; Sanjeewa et al. 2019). Oxidative stress is a major injury to cells caused by an inflammatory response (Baek et al. 2020). Free reactive oxygen species (ROS) are involved in oxidative damage of cells. We have previously reported the scavenging activity of crude extract and its fractions using DPPH and FRAP assays (Waghole et al. 2020). Our in vitro study demonstrated the antioxidant potential of the extract and its fractions with lower reducing power or SOD activity (Banik et al. 2021). Analogous scavenging activity has been found in various extracts, such as M. oleifera leaves (Xu et al. 2019), green coffee beans (Pergolizzi et al. 2020), and Phlomis rigida Labill. (Okur et al. 2021), used as an anti-inflammatory agent. Various antioxidant enzymes, such as superoxide dismutase (SOD), detoxify ROS and restrict the accumulation of ROS within mitochondria, avoiding cellular oxidative damage (Gasparini et al. 2017). Our in vitro study demonstrated the antioxidant potential of the extract and its fractions with reduced ROS in live cells and increased SOD activity. The results showed that TSETOH, TSTOL, TSHEX, CI, and CIII demonstrated antioxidant potential, which is beneficial for avoiding cellular damage to cells. However, the TSEA fraction and CII compound showed lower antioxidant potential activity, with lower reducing power or SOD activity.

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). Furthermore, inflammation may lead to various cytokines, such as IL-1β, IL-6, and TNF-α, which are crucial in mediating immunity and activating macrophages (Shen et al. 2018; Du et al. 2020). In the present study, to assess anti-inflammatory activity, nitrite (NO) production and relative mRNA expression of IL-1β, IL-6, and TNF-α were used as markers of anti-inflammatory activity in LPS-induced inflammatory macrophages. Reports suggest that flavonoid presence suppresses the TLR-4/NF-κB p65 signalling pathway, resulting in down-regulation of IL-1β, IL-6, and TNF-α gene expression and iNOS proteins during anti-inflammatory effects (Andrade et al. 2020). A similar pattern of reduced NO production and pro-inflammatory cytokines has been reported in previous studies, demonstrating the anti-inflammatory activity of plant extracts, such as P. subulate aqueous extract (Genc et al. 2019), O. gratissimum extract (Dzoyem et al. 2021), and L. spinosa leaf extract (Nguyen et al. 2020). In the present study, in vitro anti-inflammatory bioassays demonstrated that TSETOH, TSHEX, and TSTOL, together with CI and CIII, 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.

Encouraged by the in vitro results, we further evaluated the anti-inflammatory effects of the selective extracts and pure compounds in vivo. A limit test (2000 mg/kg), according to OECD/OCDE guidelines 423, demonstrated that the extract is safe for in vivo studies. The carrageenan-induced mouse paw oedema model is commonly used to test systemic anti-inflammatory activity (Banik et al. 2021; Kumar et al. 2020). Analogous to the LPS-induced in vitro inflammation model, the carrageenan-induced mouse paw oedema model was established, and test treatments in proportions of 1:10, 1:20, and 1:30 to a limit test dose were administered. 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. 2021). Various traditionally used herbal extracts are a source of triterpenoids with anti-inflammatory activity (Ou et al. 2019; Wu et al. 2021). Similar to 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. Furthermore, the pure compound Friedelan-3β-ol (CI) and

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

| Parameters          | 7th day Control | Experimental | 14th day 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  |

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its derivatives, Friedelin acetate (C II) and friedelinol methyl ether (C III), also demonstrated anti-inflammatory activity. Friedelan-3β-ol, a compound isolated from crude ethanolic extract, was found to exhibit anti-inflammatory activity. Furthermore, the derivative CIII, friedelin methyl ether, demonstrated maximum activity in vitro and in vivo. Triterpenoids have been shown to exhibit better anti-inflammatory activity, which is in agreement with previous reports (Ou et al. 2019; Wu et al. 2021).

Dexamethasone (synthetic pregnane corticosteroid; a cortisol derivative) is a known drug against inflammatory and autoimmune conditions (Black and Grodzinsky 2019; Giles et al. 2018; Li et al. 2020). The current study demonstrated the antioxidant and anti-inflammatory effects of leaf extract and its fractions compared to dexamethasone (positive control). Altogether, the leaf extract of *T. sulcatum* TSETOH, and particularly fractions TSHEX and TSTOL, along with isolated CI compound and its derivative CIII, can be considered as anti-inflammatory drug candidates for future studies.

**Conclusion**

*T. sulcatum* leaf extract (TSETOH) and fractions (TSHEX, TSTOL) exhibited potent anti-inflammatory activity in vitro and in vivo. It has been for the first time demonstrated that *T. sulcatum* leaf extract is a good source of Friedelan-3β-ol, the active component responsible for the anti-inflammatory potential of *T. sulcatum*. Thus, it can be concluded that *T. sulcatum* leaf extract exhibits anti-inflammatory potential to treat inflammation, significantly inhibiting nitrite production with downregulation of IL-6, IL-1β, and TNF-α mRNA levels. Furthermore, crude extract and fractions exhibited potent antioxidant activity, which is helpful in restricting oxidative damage induced by inflammation. However, the anti-inflammatory mechanism of *T. sulcatum* plant extract, including various signalling and immune regulatory pathways, would aid in the exploration of future therapies for inflammatory diseases.
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Funding
This research has not received financial assistance from any funding agencies in the public, commercial, or non-profit sectors.

Acknowledgements
The authors thank Dr. P.P. Kulkarni, Scientist, Bioprospecting Group, for providing the necessary materials and instruments to conduct cell culture work and Dr. Dnyanesh Ranade for assisting in carrying out RT-PCR work.

Author contributions
RJW, AVM, and NK performed the experiments. SHI, NK, RJW, FK, and DGN performed the data analysis and wrote the manuscript.

Funding
This research has not received financial assistance from any funding agencies in the public, commercial, or non-profit sectors.

Declarations
Conflict of interest
The authors declare that there are no conflicts of interest.

Ethics approval
The study was approved by the Institutional Animal Ethics Committee of Agharkar Research Institute, Pune (India). All the animal experiments were approved (Ref: ARI/IAEC/2015/04) and Performed as per the standard OECD/OCDE guidelines 423.

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