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Original Article

Isolation, Characterization, and *in silico, in vitro* Evaluation of β-Sitosterol from *Smithia conferta* Sm. (Leguminosae)

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ABSTRACT

Background: An ethnomedicinal plant called *Smithia conferta* has long been used in traditional medicine to treat a variety of conditions associated with infection and inflammation. Nevertheless, despite its historical significance, insufficient research has been carried out to isolate, characterise, and analyse the biological activity of β-sitosterol from this plant, and the evaluation of its biological activity remains insufficiently explored. **Objectives:** The current investigation was designed to isolate and characterize β-Sitosterol from *Smithia conferta* and to evaluate its anti-inflammatory, antioxidant, and anti-arthritic potential using both *in silico* and *in vitro* methods. **Materials and Methods:** The aerial portions of *Smithia conferta* were extracted with petroleum ether and submitted to phytochemical screening and chromatographic separation. The isolated compound was studied by HPTLC finger printing, FTIR, ^{1}H NMR and mass spectroscopy. Molecular docking investigations were performed using PyRx 0.8 against the targets for inflammation and arthritis such as COX-2, LOX-5, TNF-α, MMP-13 and MMP-9. *In vitro* anti-inflammatory activity was tested by HRBC membrane stabilization and protein denaturation assays while antioxidant activity was investigated by DPPH and H_2O_2 scavenging assays. **Results:** β-Sitosterol was successfully separated and identified by spectral characterization and comparison with the literature. To the best of our knowledge, this is the initial account of β-sitosterol from *Smithia conferta*. Docking studies showed good binding affinities with inflammatory and arthritic targets. The chemical exhibited potent anti-inflammatory and antioxidant effects which are equivalent with the conventional medication. **Conclusion:** The existence of β-Sitosterol in *Smithia conferta* is confirmed by this study. *In vitro* and *in silico* studies confirm the therapeutic potential of isolated β-sitosterol from *Smithia conferta*.

Keywords: Anti-arthritic activity, Anti-inflammatory activity, β-Sitosterol, Molecular docking, *Smithia conferta*.

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INTRODUCTION

Arthritis is a group of diseases that are characterized by joint problems such as rheumatoid arthritis and osteoarthritis that can affect one or more joints (Arnett *et al.*, 1988; Gupta *et al.*, 2022). The current standard treatment of rheumatoid arthritis includes disease modifying anti-rheumatic drugs, analgesics, non-steroidal anti-inflammatory drugs and corticosteroids. However, these conventional therapies come with some side effects such as gastrointestinal ulcers and bleeding (Benjamin *et al.*, 2023; Li *et al.*, 2017). However, phytotherapy, the oldest medical approach across the globe has been used for a long time in rural India (Pandey *et al.*, 2013). The increasing use of herbal medicines is mainly owing to the limitations and adverse effects of traditional synthetic therapeutic agents. Herbal medicines have many benefits in the course of treating a wide variety of diseases (Kaushik *et al.*, 2021; Ghosh *et al.*, 2023). *Smithia conferta Sm.* (Fabaceae) is an ethnomedicinally significant plant widely used in rural and Ayurvedic practices. Classical literature including the authoritative text Indian Medicinal Plants by Kirtikar and Basu, documents the use of *Smithia conferta* in the management of rheumatism, indicating its traditional relevance in conditions characterized by joint pain, swelling, and inflammation (Kirtikar and Basu, 2003). Agarwal *et al.* reported significant anti-ulcer activity in this species, a pharmacological effect often attributed to underlying antioxidant and anti-inflammatory mechanisms (Agrawal *et al.*, 2010). Despite these traditional and scientific indications, the phytochemical constituents responsible for its medicinal properties remain poorly explored. There are only a few studies on the isolation of phytosterols from *Smithia conferta*, at present. To bridge this gap, β-sitosterol was isolated from *Smithia conferta*, characterized, and evaluated for its potential

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anti-arthritic activity using molecular docking simulations and *in vitro* approaches. Among the secondary metabolites derived from plants, β-sitosterol is among the most prevalent phytosterols and is renowned for having anti-inflammatory qualities. Importantly, Current research revealed strong mechanistic evidence for the anti-arthritic properties of β-sitosterol (Zhang *et al.*, 2020; Qian *et al.*, 2022). β-sitosterol was shown to inhibit proliferation, migration, and inflammatory activation of fibroblast-like synoviocytes (Tan *et al.*, 2025). This advanced mechanistic insight validates the ethnopharmacological relevance of *Smithia conferta* and further strengthens β-sitosterol as a promising therapeutic agent for inflammatory and arthritic disorders. The ethanomedicinal importance of *Smithia conferta* in treatment of inflammatory disorders has not been followed up with systematic studies on isolation, characterisation and biological evaluation of its phytoconstituents. Moreover, there is no complete report on the anti-arthritic potential of β-sitosterol isolated from this plant. Thus, the present study was aimed at isolation and characterization of β-sitosterol from *Smithia conferta* and to explore its potential as an anti-arthritic agent by molecular docking approaches along with *in vitro* anti-inflammatory and antioxidant evaluations. This unique report highlights the potential medicinal uses of *Smithia conferta*, focusing on a phytosterol extracted from the plant.

MATERIALS AND METHODS

Collection of Plant Materials

Smithia conferta was collected during Oct-Nov 2023 from India's Western Ghats region of Maharashtra. The collected sample was authenticated by the Taxonomy Department at The New College, Kolhapur, Maharashtra, India (Voucher No. V/N-Desai-01).

Preparation of Extracts

Powdered leaves were subjected to sequential extraction in a Soxhlet apparatus deploying absolute ethanol and petroleum ether for 24 hr. each. An aqueous extract was produced by macerating the leaves in water for 48 hr (Agrawal *et al.*, 2010). The yields for the petroleum ether, absolute ethanol, and aqueous extracts were 4.80 g, 8.50 g, and 10.8 g, respectively.

Phytochemical Analysis

To determine the existence of different phytochemical ingredients, all three produced extracts underwent preliminary phytochemical screening (Khandelwal, 2000; Agrawal *et al.*, 2010).

Isolation and purification

The petroleum ether extract of *Smithia conferta* Sm. was treated to column chromatography for the separation of the bio-active components. Stationary phase was silica gel (60-120 mesh). The column was packed by the wet slurry method of the column packing using n-hexane. A measured quantity of the extract was adsorbed on a little amount of silica gel, dried and carefully placed atop the column. The solvent system consisted of n-hexane:ether (97:3, v/v) and elution was carried out as described by Harborne (1998). Fractions were collected in succession and checked by TLC; Similar TLC profile fractions were combined and concentrated under reduced pressure. The obtained residue (47 mg) was a white crystalline substance and was submitted for further identification (Stahl, 1969). The isolated product was purified and characterized using High-Performance Thin-Layer Chromatography (HPTLC) with a β-sitosterol standard.

HPTLC (High-Performance Thin-Layer Chromatography) Fingerprint Analysis

Preparation of standard and sample solutions

A concentration of 1 mg/mL of β-sitosterol was obtained by dissolving 1 mg of the β-sitosterol in 1 mL of chloroform to create a reference standard solution. Sample solutions were prepared by dissolving the isolated fractions in chloroform and filtering before application.

Chromatographic Conditions

Aluminum-backed silica gel $60F_{254}$ precoated plates (200 × 100 mm; Merck) were used for HPTLC. A Linomat 5 sample applicator equipped with a Hamilton syringe was used to apply standard and sample solutions in 8 mm bands. The distance between the tracks was kept constant and the solvent front was allowed to travel up to 70 mm. Development was done in a 20 x 10 cm twin-trough chamber, soaked 20 min before development. Ethyl acetate:water:formic acid:acetic acid (5.2:2.6:1.1:1.1, v/v/v/v) was the mobile phase. The plates were allowed to air-dry at room temperature following development (Harborne, 1998; Uttu *et al.*, 2023).

Derivatization and Detection

The plates were derivatized with 10% methanoic sulfuric acid and air-dried. Visualization was accomplished in a CAMAG visualizing chamber and densitometric scanning was performed with a CAMAG TLC scanner. Data gathering and analysis were done by DESKTOP-TAG8IAQ software.

Isolation, identification and spectral studies of a compound

Identification of β-sitosterol was done by comparing the R_f value and densitometric peak with that of reference standard of β-sitosterol. The isolated substance from column chromatography was characterized for its structure by Fourier Transform Infrared (FTIR) and ^{1}H Nuclear Magnetic Resonance (NMR) spectroscopy. Using an FTIR spectrophotometer and the KBr pellet technique, FTIR analysis was performed in the 4000-400 cm^{-1} range. Absorption bands matching to the functional groupings have been registered. To further clarify the structure of the isolated molecule ^{1}H NMR spectroscopy was performed. Tetramethylsilane (TMS) was used as the internal standard

and the spectrum was obtained using a 400 MHz ^{1}H NMR spectrometer in Deuterated Chloroform ($CDCl_3$). The units used to express chemical changes were parts per million (δ, ppm).

Molecular Docking Study

In silico molecular docking simulations were carried out to assess β-sitosterol's binding interactions with target receptors: COX-2, LOX-5, TNF-α, MMP-13, and MMP-9. The Protein Data Bank (www.rcsb.org) provided the three-dimensional structures of these target receptors. The two-dimensional structure of β-sitosterol was developed using ChemDraw Professional 16.0 and optimized into its appropriate three-dimensional conformations. Discovery Studio Visualiser was used to create and visualise the receptor structures, which were then stored in PDB format. The docking computations were performed using the PyRx tool (Python Prescription 0.8) (Uttu *et al.*, 2023). Post-docking studies such as visualization of protein-ligand interactions and binding energies was performed using Discovery Studio.

In vitro Anti-inflammatory Activity

HRBC Membrane Stabilisation Assay

A sample of human blood was taken for the use and combined with Alsever's solution. The cells were pelleted by centrifugation and rinsed with isotonic saline. Prepared a 10% v/v suspension of HRBC. β-sitosterol and Diclofenac sodium at different doses (20-100 µg/mL) were combined with phosphate buffer, hyposaline and HRBC suspension. After being incubated at 37°C for 30 min, the mixtures were centrifuged. The supernatant was read at 560 nM for absorbance. Membrane stabilization (percentage) was estimated (Rastogi *et al.*, 2018).

Ethics Statement

Human blood used for the HRBC membrane stabilization assay was collected from healthy adult volunteers. The study was conducted *in vitro* in a laboratory setting using donated blood samples, and no interventions or clinical procedures were performed on the volunteers.

Inhibition of Protein Denaturation Assay

Reaction mixture including bovine serum albumin and varied concentrations (20-100 µg/mL) of test compounds and standard were made. After the pH was suitably adjusted, the samples were heated to 57°C for 3 min and then incubated at 37°C for 20 min. After cooling, absorbance at 660 nm was measured after phosphate buffer was introduced. Protein denaturation inhibition as a percentage was calculated (Ezung *et al.*, 2024).

In vitro Antioxidant Activity

DPPH assay method

The effective DPPH technique was utilized to evaluate the antioxidant activity of the isolated compound, β-sitosterol. 100 mL of ethanol was used to prepare a DPPH reagent at a concentration of 0.05 mM. The sample and standard ascorbic acid were produced at various concentrations of 20, 40, 60, 80, and 100 µg/mL. The standard test tubes were separated for 30 min after the sample was mixed with roughly 3 mL of freshly prepared DPPH reagent. Spectrophotometric measurements of each sample's and the standard's absorbance were made at 517 nm. The isolated compound's and the standard's radical scavenging activity was measured, and the method was applied to ascertain the percentage of DPPH inhibition (Gomathi *et al.*, 2024).

$$\text{DPPH inhibition percentage (\%)} = \frac{A - B}{A} \times 100$$

A: Blank absorbance values; B: Sample absorbance values.

Scavenging of Hydrogen peroxide (H_2O_2)

The potential of β-sitosterol to scavenge hydrogen peroxide was studied by the following method: The concentration of H_2O_2 solution (40 mM) was estimated by spectrophotometric analysis. 0.6 mL of 40 mM H_2O_2 solution was combined with certain concentrations of β-sitosterol and standard ascorbic acid. The absorbance of H_2O_2 was measured after 10 min of incubation and compared to a blank solution that had phosphate buffer but no hydrogen peroxide (Arivarasu, 2023).

Statistical Analysis

All experiments were performed in triplicate (n=3), and the results are expressed as mean±Standard Deviation (SD). The IC_{50} values were calculated using GraphPad Prism version 5.0.

RESULTS

Preliminary Phytochemical Studies

Preliminary phytochemical screening of *Smithia conferta* leaf extracts showed different chemical profiles: Carbohydrates, amino acids, and flavonoids were found in the water extract; alkaloids, flavonoids, and carbohydrates were found in the alcoholic extract; and only steroids were found in the petroleum ether extract (Table 1) (Khandelwal, 2000; Agrawal *et al.*, 2010).

Spectral Analysis

Petroleum ether extract was used to isolate steroids from *Smithia conferta*. Column chromatographic separation of the petroleum ether extract of *Smithia conferta* Sm. resulted in the enrichment of phytoconstituents, which were subsequently analyzed by HPTLC (Figure 1).

The HPTLC fingerprint profile of separated fractions showed a strong band for β-sitosterol with R_f value of 0.33 which matched that of reference standard. Densitometric research revealed that β-sitosterol was concentrated mostly in A fractions, smaller quantities in B fractions and insignificant presence in C fractions. Similar Rf values and densitometric peak features of the

Table 1: **Preliminary phytochemical analysis of *Smithia conferta*.**

Sl. No.	Phytochemicals	Test	Aqueous extract	Alcoholic extract	Petroleum ether extract
1.	Carbohydrates	Molisch's test	Present	Present	Absent
2.	Reducing Sugar	Fehling's test	Present	Present	Absent
3.	Amino Acids	Ninhydrin test	Present	Absent	Absent
4.	Flavonoids	Sulphuric acid test	Present	Present	Absent
5.	Alkaloids	Dragendorff's test	Present	Present	Absent
6.	Steroids	Salkowski test	Absent	Absent	Present
7.	Tannins	Ferric chloride test	Absent	Absent	Absent
8.	Triterpenoids	Horizon test	Absent	Absent	Absent
9.	Saponine	Foam test	Absent	Absent	Absent
10.	Starch	Iodine test	Absent	Absent	Absent

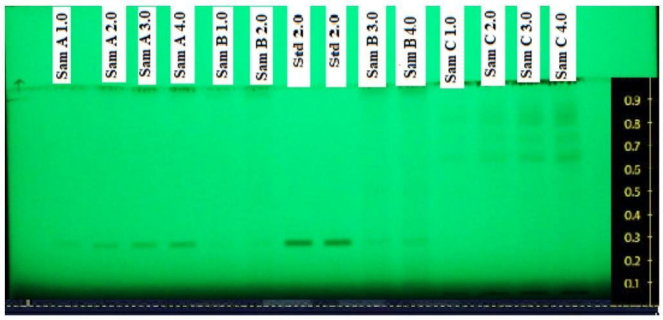


Figure 1: HPTLC fingerprint profile of β-sitosterol.

standard and isolated fractions confirmed this and assured the successful extraction and purification of β-sitosterol from *Smithia conferta* (Harborne, 1998; Stahl E, 1969). The results show the efficiency of column chromatography along with HPTLC as a dependable method for phytochemical isolation and confirmation. The FT-IR spectra (KBr) showed strong absorption peaks at 3432.67 cm^{-1} which corresponded to O-H stretching vibrations and 2937.06 cm^{-1}, which was assigned to aliphatic C-H stretching. The absorption band found at 1642.92 cm^{-1} indicated C=C stretching. More distinctive peaks at 1464.67 cm^{-1} (CH_2 bending), 1381.75 cm^{-1} (O-H deformation), 1062.59 cm^{-1} (cycloalkane vibrations) and 958.44 cm^{-1} (Figure 2) were seen with respect to literature data (Islam *et al.*, 2025).

The 1H NMR spectrum of isolated compound showed six methyl proton signals at δH 0.78-0.96 which are characteristic for the methyl groups of β-sitosterol. The proton resonance of the oxygenated methine at δH 3.58 (H-3) was consistent with a hydroxyl group at C-3 and the olefinic proton resonance at δH 5.46 (H-6) indicated a double bond between C-5 and C-6. A number of methylene and methine proton signals at δH 1.10-2.34 ppm were consistent with a steroidal ring system and an aliphatic side chain. The presence of a single hydroxyl functionality was further confirmed by a hydroxyl proton resonance at δ H 4.84. The spectral features are in good agreement with the reported 1H NMR data of β-sitosterol, confirming the identity of the isolated compound compared with the data reported in the literature (Uttu *et al.*, 2023).

Molecular Docking Studies

In this study, we assessed the potential anti-inflammatory and anti-arthritic binding affinity and interactions of the compound β-sitosterol with several inflammatory and arthritic target proteins namely COX-2, LOX-5, TNF-α, MMP-13 and MMP-9 (PDB: 5KIR, 6NCF, 2AZ5, 1XUC and 1GKC respectively. These proteins are implicated in mediating inflammation and arthritic diseases and are implicated in many pathological and physiological processes such as cell signaling, immunological responses, pain and cartilage degradation. Molecular docking was used to determine the binding energy of β-sitosterol with each receptor and the hydrogen bonds and hydrophobic interactions between the drug and target residues were analyzed. Figure 3A shows the docking pose of β-sitosterol with COX-2. β-Sitosterol showed binding affinity to COX-2 with an energy of −10.2 kcal.mol−1 with 1 hydrogen bond (GLY A:225), 3 hydrophobic/π-alkyl interactions (LEU B: 145, PHE B: 142 (with 2 π-alkyl interactions)) and 19 van der Waals interactions. The docking conformation of β-sitosterol with LOX-5 is shown in Figure 3B. The binding energy of β-sitosterol docked into the active site of 5-lipoxygenase (5-LOX) PDB ID: 6NCF was −8.3 $kcal{\cdot}mol^{-1}$. It has hydrophobic interaction with LEU B:475, ILE B:454, PRO B:451, LYS B:455, LEU B:476 residues and has 8 van der waal contacts. No hydrogen bonding was observed (Hsu *et al.*, 2020). Docking studies of TNF-α (PDB ID: 2AZ5) with β-sitosterol showed a binding energy of −6.3 kcal/mol, Interaction analysis showed that β-sitosterol mainly formed hydrophobic interactions, including π-alkyl interactions with TYR A:59 and ILE A:155, and an alkyl interaction with LEU A:120. The absence of hydrogen bond formation in the binding of β-sitosterol to TNF-α in Figure 3C indicates that the main mechanism is hydrophobic stabilization. The docking configuration of MMP-13

with β-sitosterol in Figure 3D. β-Sitosterol exhibited a binding energy of −8.6 kcal·mol^{-1} when docked to MMP-13.

The interaction study showed development of one conventional hydrogen bond with LYS A:112 and several alkyl/π-alkyl and π-type interactions with residues such as PHE B:107, PHE B:189, TYR B:176 and PRO A/B (PRO A:108, PRO A:191, PRO B:193). Furthermore, many van der Waals contacts were identified with the surrounding residues, including SER A:114, GLY A:192, LEU A:111 and ASN A:194. Figure 3E shows the docking configuration of MMP-9 with β-sitosterol. The docking prediction of β-sitosterol with MMP-9 (PDB: 1GKC) showed a binding affinity of -7.2 kcal/mol. The ligand formed multiple hydrophobic and π-alkyl interactions with key amino acid residues such as Tyr393, Tyr423, Phe425 and Lys214, which helped to stabilize its orientation in the active pocket (Zahid *et al.*, 2023). The results suggest the involvement of β-sitosterol in modifying the activity of matrix metalloproteinases contributing to its anti-arthritic and anti-inflammatory capabilities.

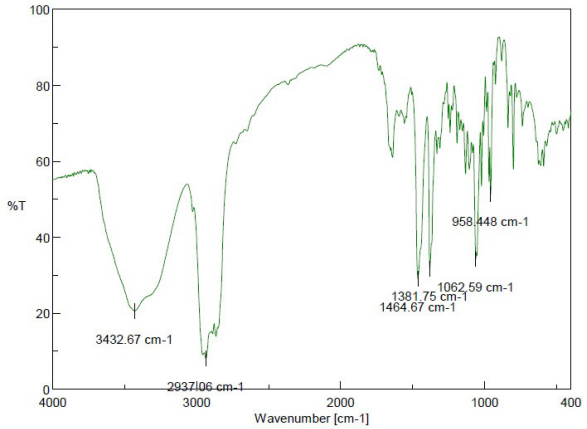


Figure 2: FTIR of β-sitosterol.

Anti-inflammatory Study

Stabilization of membrane activity by HRBC

The IC_{50} values obtained in this experiment in Table 2 show that diclofenac sodium has stronger membrane stabilizing activity and effectiveness than β-sitosterol. These findings highlight the membrane stablising potential of plant isolated β-sitosterol in a dose dependent manner with moderate efficacy IC_{50} values of 62.57 µg/mL as compared to the more potent standard diclofenac sodium IC_{50} 47.08 µg/mL, in Figure 4, which is in accordance with the reports on phytosterols from medicinal plants such as *Proboscidea louisianica* and *Jatropha tanjorensis* (Nakka *et al.*, 2025; Omoboyowa, 2021; Paniagua-Pérez *et al.*, 2016).

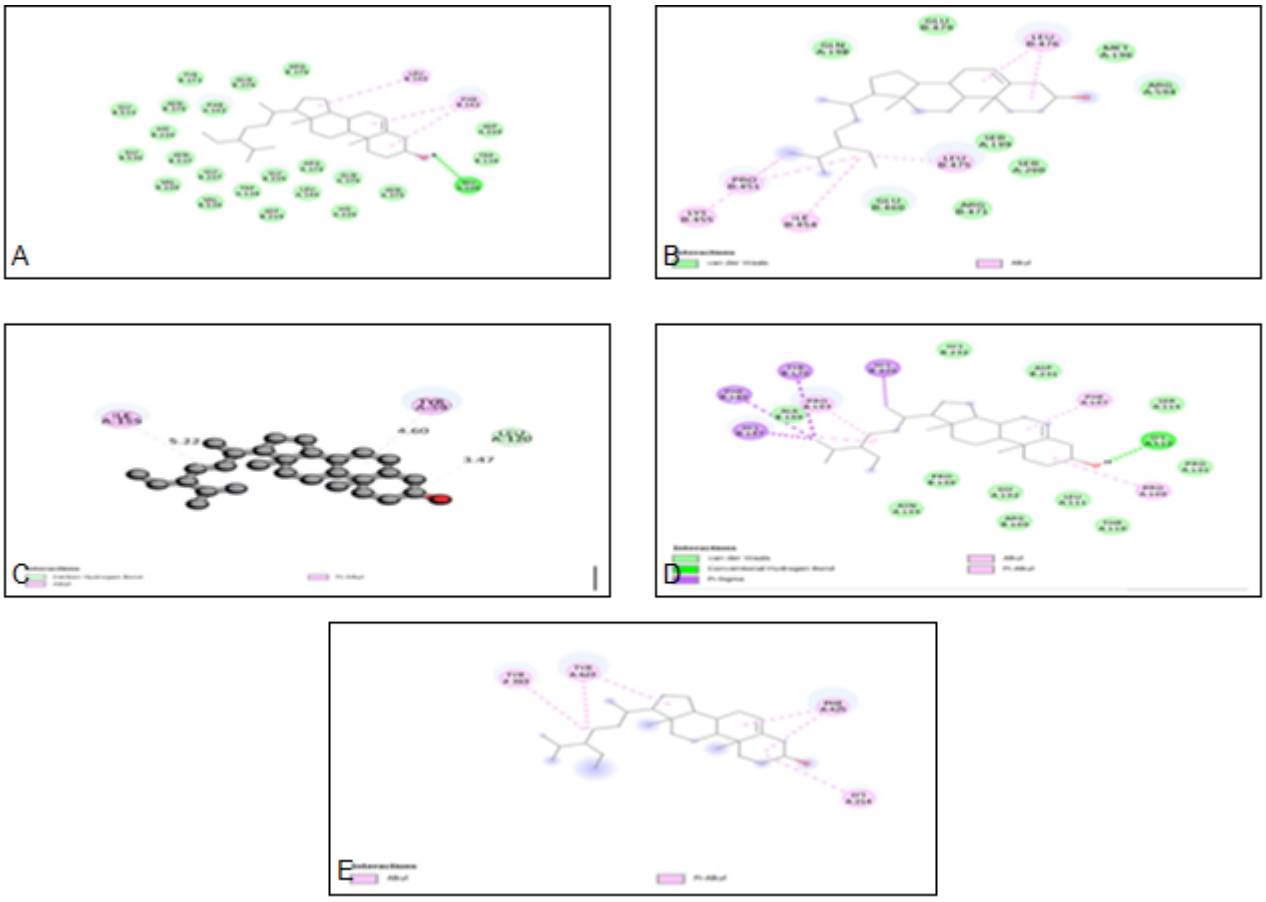


Figure 3: Molecular docking interactions of β-sitosterol with inflammatory and arthritic targets. (A) COX-2, (B) 5-LOX, (C) TNF-α, (D) MMP-13, and (E) MMP-9.

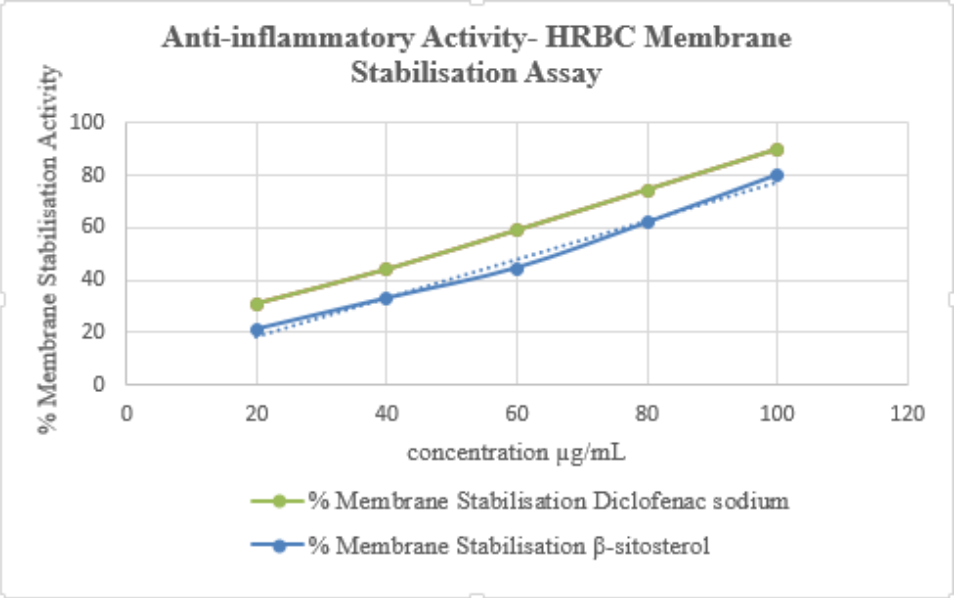


Figure 4: HRBC membrane stabilization assay of Diclofenac sodium and β-sitosterol. Data are presented as mean±SD (*n*=3).

Table 2: Anti-inflammatory activity of β-sitosterol in HRBC membrane stabilization activity.

Concentration (µg/mL)	Diclofenac sodium (% Stabilization)	β-Sitosterol (% Stabilization)
20	30.810±2.10	21.076±2.14
40	43.899±2.16	32.924±2.15
60	58.925±3.16	44.518±3.21
80	74.498±3.15	61.874±3.27
100	90.021±4.17	80.138±4.21
IC_{50} (µg/mL)	47.08	62.57

Values are expressed as mean±SD (*n*=3). IC_{50} (µg/mL): Diclofenac sodium: 47.08; β-sitosterol: 62.57.

Protein Denaturation Assay

Protein denaturation was inhibited in a concentration-dependent manner by β-sitosterol, and the inhibition increased from 22.93±2.12% at 20 µg/mL to 60.41±4.15% at 100 µg/mL. Diclofenac sodium in the same concentration range showed stronger inhibitory activity in Table 3, which was between 34.44±2.11% and 79.12±4.18%. The IC_{50} of β-sitosterol and diclofenac sodium were 74.41 µg/mL and 46.80 µg/mL, respectively, which indicates that β-sitosterol has strong anti-denaturation activity but is less powerful than the standard drug in Figure 5 (Modak *et al.*, 2021).

Antioxidant Studies

DPPH Assay

The DPPH radical scavenging activity of β-Sitosterol was concentration dependent and inhibition increased from 15.24±2.14% at 20 µg/mL to 57.62±4.12% at 100 µg/mL. The antioxidant ascorbic acid showed better scavenging activity (range 32.28±2.12% to 90.01±4.14%) at the quantities evaluated in Table 4. The IC_{50} values of beta-sitosterol and ascorbic acid were 80.30 µg/mL and 39.98 µg/mL respectively, demonstrating a moderate antioxidant activity of β-sitosterol although less potent than the standard antioxidant (Figure 6) (Arivarasu, 2023).

Hydrogen Peroxide (H_2O_2) Scavenging Assay

The β-Sitosterol scavenging activity of hydrogen peroxide was increased in a concentration-dependent manner with inhibition of 13.38±2.12% at 20 µg/mL and 63.49±4.21% at 100 µg/mL. The conventional antioxidant, ascorbic acid demonstrated better scavenging activity (33.68±2.12% to 89.53±4.13%) at the concentrations studied (Table 5). Ascorbic acid and β-sitosterol had IC_{50} values of 47.16 µg/mL and 81.85 µg/mL, respectively. Indicating substantial H_2O_2-scavenging activity for β-sitosterol; however, it was less potent than the standard antioxidant, as illustrated in Figure 7 (Arivarasu, 2023; Gomathi & Maneemegalai, 2024).

DISCUSSION

In this work, β-sitosterol is extracted and characterized from *Smithia conferta* petroleum ether extract and its anti-inflammatory, antioxidant and anti-arthritic potential by *in vitro* studies and molecular docking. Only the petroleum ether extract contained steroids, according to preliminary phytochemical screening, which supports its selection for phytosterol extraction. Similar observations have been reported for various medicinal plants where non-polar solvent preferentially extracts sterol-rich fractions (Harborne, 1998; Stahl, 1969). The isolated chemical was identified by HPTLC, FTIR and ^1H NMR analysis. Confirmation of successful isolation and purification was done by the HPTLC chromatogram which showed similar R_f value with standard β-sitosterol. Furthermore,

FTIR spectra revealed the presence of characteristic absorption bands of hydroxyl, aliphatic C-H and olefinic functional groups while the 1H NMR spectrum showed signals diagnostic of the β-sitosterol skeleton. These results are in good accord with the spectrum features of β-sitosterol from medicinal plants previously identified (Uttu *et al.*, 2023; Islam *et al.*, 2025).

Molecular docking experiments revealed that β-sitosterol showed good interaction with key inflammatory and arthritic targets notably COX-2, LOX-5, TNF-α, MMP-13 and MMP-9. Among the examined targets, β-sitosterol displayed the highest binding affinity towards COX-2 (-10.2 kcal/mol) followed by MMP-13 (-8.6 kcal/mol), and LOX-5 (-8.3 kcal/mol). Formation of hydrogen bonds, hydrophobic interactions and substantial van der Waals contact indicate stable ligand-protein complexes. COX-2 and LOX-5 are important enzymes responsible for production of prostaglandins and leukotriene's which play essential roles in inflammatory reactions. Thus, inhibition of these enzymes might be involved in the anti-inflammatory effect of β-sitosterol.

Table 3: Anti-inflammatory activity of β-sitosterol in protein denaturation assay.

Concentration (µg/mL)	Diclofenac sodium (% Inhibition)	β-Sitosterol (% Inhibition)
20	34.439±2.11	22.928±2.12
40	46.604±2.23	35.485±2.20
60	58.134±2.27	44.454±2.23
80	68.403±3.15	53.312±3.12
100	79.124±4.18	60.406±4.15
IC_{50} (µg/mL)	46.80	74.41

Values are expressed as mean±SD (n=3). IC_{50} (µg/mL): Diclofenac sodium: 46.80; β-sitosterol: 74.41.

Similar computational investigations have documented potent interactions of β-sitosterol with inflammatory mediators and emphasized its therapeutic potential against rheumatoid arthritis and chronic inflammatory diseases (Wang and Mao, 2024; Jagtap *et al.*, 2025). Interaction of β-sitosterol with TNF-α is of special interest as TNF-α is one of the key pro-inflammatory cytokines involved in the pathophysiology of rheumatoid arthritis. The prevalence of hydrophobic contacts observed in the present investigation supports a sustained binding in the receptor pocket and a possible modification of TNF-α mediated signaling pathways. Similar outcomes have been reported in structure-based studies for TNF-α inhibitors where hydrophobic stabilization played a role in ligand binding (Chen *et al.*, 2017). Furthermore, the remarkable binding affinities toward MMP-13 and MMP-9 indicate the potential of β-sitosterol to interfere with the matrix metalloproteinase-mediated cartilage degradation. The high levels of MMP activity are involved in joint destruction and disease development in arthritis and inhibition of these enzymes may have preventive benefits against tissue damage (Liu *et al.*, 2021). The HRBC membrane stabilization experiment also confirmed the anti-inflammatory action of β-sitosterol. At 62.57 µg/mL, the isolated molecule exhibited concentration-dependent membrane stability. Even though diclofenac sodium was more potent, β-sitosterol provided considerable protection against haemolysis. Stabilization of erythrocyte membranes is believed to be similar to stabilization of lysosomal membranes which prevents release of inflammatory mediators and tissue damaging enzymes during inflammation. Similar membrane-protective properties have been observed for β-sitosterol-containing extracts from *Jatropha tanjorensis* and other medicinal plants (Omoboyowa 2021; Nakka *et al.* 2025). The present findings, thus, validate the role of β-sitosterol as a natural anti-inflammatory agent.

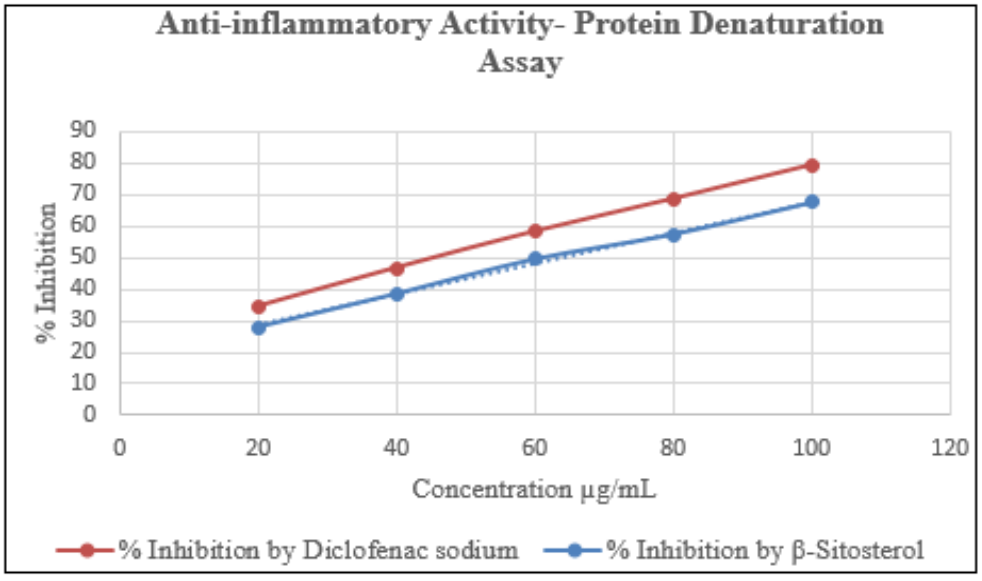


Figure 5: Protein denaturation assay of Diclofenac sodium and β-sitosterol. Data are presented as mean±SD (n=3).

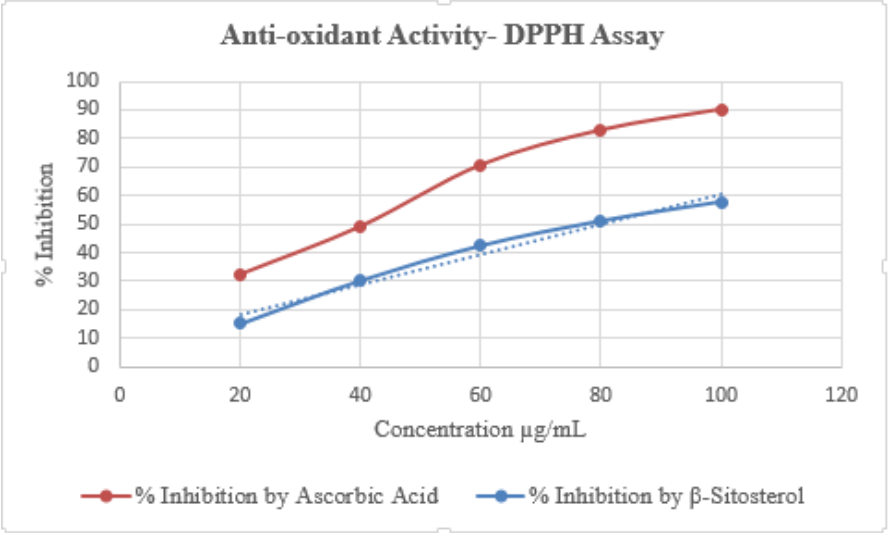


Figure 6: DPPH assay of Ascorbic acid and β-sitosterol. Data are presented as mean±SD (*n*=3).

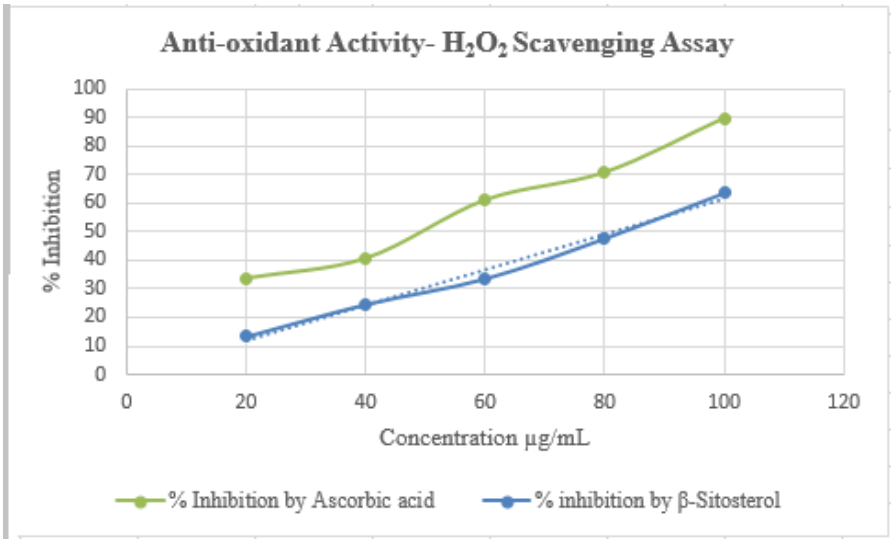


Figure 7: Hydrogen Peroxide (H_2O_2) Scavenging assay of Ascorbic acid and β-sitosterol. Data are presented as mean±SD (*n*=3).

Protein denaturation is a well-known mechanism associated with inflammatory and arthritic diseases as denatured proteins may be autoantigens and induce immune responses. In the present work, β-sitosterol showed the inhibition of protein denaturation in a concentration-dependent manner with IC_{50} value of 74.41 µg/mL. The inhibition found, although lower than that of diclofenac sodium, shows considerable potential to protect proteins from stress-induced structural changes. Anti-denaturation activity has also been identified in plant derived anti-inflammatory chemicals and phytosterols indicating their therapeutic potential in inflammatory illnesses (Modak *et al.*, 2021; Paniagua-Pérez *et al.*, 2016).

Oxidative stress is directly related to the beginning and progression of inflammation and arthritis by high levels of reactive oxygen species. Antioxidants capable of scavenging free radicals could therefore be of benefit in the management of inflammatory illnesses. In DPPH and hydrogen peroxide scavenging tests, β-sitosterol demonstrated concentration-dependent antioxidant activity. The substance showed strong free radical scavenging action, but it was less potent than ascorbic acid. The DPPH and H_2O_2 assays yielded IC_{50} values of 80.29 µg/mL and 81.84 µg/mL, respectively. The results are in agreement with the earlier research showing antioxidant activity of β-sitosterol and phytosterol rich fractions from medicinal plants (Arivarasu, 2023; Gomathi and Maneemegalai, 2024). The antioxidant activity reported in the present work may contribute synergistically to anti-inflammatory benefits by lowering oxidative stress-generated cellular damage.

CONCLUSION

In conclusion, the strong correlation between the results of molecular docking and the biological activities *in vitro* indicates that β-sitosterol acts through multiple mechanisms, such as

Table 4: Antioxidant activity of β-sitosterol in DPPH assay.

Concentration (µg/mL)	Ascorbic acid (% Inhibition)	β-Sitosterol (% Inhibition)
20	32.281±2.12	15.242±2.14
40	49.043±2.43	30.283±2.41
60	70.564±3.12	42.364±3.13
80	82.773±3.41	50.981±3.43
100	90.012±4.14	57.624±4.12
IC_{50} (µg/mL)	39.97	80.29

Values are expressed as mean±SD (n=3). IC_{50} (µg/mL): Ascorbic acid: 39.97; β-sitosterol: 80.29.

Table 5: Antioxidant activity of β-sitosterol in Hydrogen Peroxide (H_2O_2) Scavenging assay.

Concentration (µg/mL)	Ascorbic acid (% Inhibition)	β-Sitosterol (% Inhibition)
20	33.678±2.12	13.383±2.12
40	40.656±2.33	24.624±2.27
60	60.984±3.14	33.581±3.13
80	70.623±3.44	47.622±3.41
100	89.533±4.13	63.494±4.21
IC_{50} (µg/mL)	47.15	81.84

Values are expressed as mean±SD (n=3). IC_{50} (µg/mL): Ascorbic acid: 47.15; β-sitosterol: 81.84.

inhibition of inflammatory mediators, stabilization of cellular membranes, prevention of protein denaturation, and scavenging of reactive oxygen species. The present findings scientifically justify the use of *Smithia conferta* in traditional medicine and also identify β-sitosterol as one of the key bioactive compounds responsible for anti-inflammatory and anti-arthritic activity.

β-sitosterol was successfully isolated and characterized from the petroleum ether extract of *Smithia conferta* utilizing chromatographic and spectroscopic techniques, viz HPTLC, FTIR, and ^{1}H NMR analyses. *In silico* and *in vitro* studies revealed that the isolated compound displayed excellent anti-inflammatory, antioxidant, and anti-arthritic potential. Molecular docking experiments indicated high binding affinities of β-sitosterol towards important inflammatory and arthritic targets, namely COX-2, LOX-5, TNF-α, MMP-13 and MMP-9, indicating its potential to influence various pathways involved in inflammation and joint degeneration. *In vitro* studies also demonstrated its biological activity by considerable HRBC membrane stabilization, suppression of protein denaturation, and free radical scavenging capabilities in DPPH and hydrogen peroxide assays. The molecule showed a clearly dosage-dependent response in all the assays, but β-sitosterol showed less activity than the conventional medicines. The reported pharmacological actions may be due to the synergistic anti-inflammatory and antioxidant capabilities of β-sitosterol, which may play a role in its protective effects against inflammatory and arthritic diseases.

Overall, the results provide scientific proof for the traditional medicinal usage of *Smithia conferta* and reveal β-sitosterol as a key bioactive element with therapeutic potential. Further *in vivo* studies and clinical trials are needed to determine the safety, efficacy, and mechanism of action for the development of plant-based anti-inflammatory and anti-arthritic medicines.

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ABBREVIATIONS

HPTLC: High-Performance Thin-Layer Chromatography; **FTIR:** Fourier-Transform Infrared Spectroscopy; **1H NMR:** Proton Nuclear Magnetic Resonance; **R_f:** Retention factor; **COX-2:** Cyclooxygenase-2; **LOX-5:** Lipoxygenase-5; **TNF-α:** Tumor Necrosis Factor-α; **MMP:** Matrix Metalloproteinase; **HRBC:** Human Red Blood Cell; **DPPH:** 2,2-diphenyl-1-picrylhydrazyl; **IC_{50}:** Half maximal inhibitory concentration; **SD:** Standard Deviation (statistics).

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS CONTRIBUTION

AAJ: Concept, design, manuscript review; **DJD:** Literature search, experimental studies, data acquisition, manuscript preparation, manuscript editing; **RBR:** Data analysis.

SUMMARY

Future studies focusing on *in vivo* evaluation, formulation optimization, and clinical trials are warranted to establish the therapeutic potential of β-sitosterol and facilitate its development as a novel drug candidate for inflammatory and arthritic diseases.

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