Anti-Inflammatory and Enzyme Inhibitory Activity of Isolated Terpenoid Fractions from the Root Bark of *Withania somnifera* Against Lipoxygenase and Hyaluronidase

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Abstract

Inflammation is a complex physiological response mediated by various enzymes and inflammatory mediators that contribute to the development and progression of numerous acute and chronic diseases. The present study aimed to isolate terpenoid-rich fractions from the root bark of *Withania somnifera* and evaluate their anti-inflammatory potential through in vitro lipoxygenase and hyaluronidase inhibition assays. Root bark powder was extracted using a hydroalcoholic solvent system, followed by solvent fractionation and silica gel column chromatography to obtain terpenoid-enriched fractions. The isolated fractions were characterized using thin-layer chromatography (TLC), Fourier transform infrared spectroscopy (FTIR), and gas chromatography–mass spectrometry (GC–MS). Among the isolated fractions, TF-3 exhibited the most promising phytochemical profile and was selected for biological evaluation. GC–MS analysis indicated the presence of several putatively identified terpenoid constituents, including phytol, squalene, β-amyrin, lupeol, β-sitosterol, stigmasterol, and withanolide-related compounds. TF-3 demonstrated concentration-dependent inhibition of both lipoxygenase and hyaluronidase enzymes, achieving 84.67 ± 2.41% and 82.13 ± 2.26% inhibition, respectively, at 400 µg/mL. The IC_{50} values were determined to be 112.84 ± 4.16 µg/mL for lipoxygenase and 128.36 ± 5.04 µg/mL for hyaluronidase. These findings suggest that terpenoid-rich fractions from *Withania somnifera* possess significant enzyme-targeted anti-inflammatory activity and may serve as promising candidates for the development of plant-derived anti-inflammatory agents.

Keywords: *Withania somnifera*; terpenoid-rich fraction; anti-inflammatory activity; lipoxygenase inhibition; hyaluronidase inhibition; enzyme inhibition.

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1. Introduction

Inflammation is a fundamental biological response that protects the body against infection, injury, and various harmful stimuli. It involves a complex cascade of cellular and molecular events designed to eliminate damaging agents and initiate tissue repair. Although acute inflammation is essential for maintaining normal physiological functions, persistent or uncontrolled inflammation can contribute to the development of numerous chronic disorders, including rheumatoid arthritis, osteoarthritis, inflammatory bowel disease, asthma, cardiovascular diseases, neurodegenerative disorders, and certain types of cancer. The inflammatory process is regulated by a variety of mediators, enzymes, cytokines, and signalling

pathways that collectively determine the intensity and duration of the response.

Among the enzymes involved in inflammatory reactions, lipoxygenase and hyaluronidase play critical roles in the initiation and progression of inflammation. Lipoxygenase catalyzes the oxidation of arachidonic acid to produce leukotrienes and other bioactive lipid mediators that promote leukocyte recruitment, vascular permeability, and inflammatory signalling. Excessive activation of the lipoxygenase pathway has been implicated in several inflammatory and allergic disorders. Similarly, hyaluronidase catalyzes the degradation of hyaluronic acid, a major component of the extracellular matrix. Increased hyaluronidase activity results in the breakdown of connective tissue integrity, enhanced tissue permeability, edema formation, and facilitation of inflammatory cell migration. Therefore, inhibition of these enzymes has emerged as an attractive therapeutic strategy for controlling inflammation and preventing tissue damage.

Conventional anti-inflammatory drugs, including nonsteroidal anti-inflammatory drugs (NSAIDs) and corticosteroids, are widely used for the treatment of inflammatory conditions. However, prolonged use of these medications is frequently associated with adverse effects such as gastrointestinal irritation, ulceration, cardiovascular complications, renal toxicity, immunosuppression, and metabolic disturbances. These limitations have stimulated increasing interest in the discovery of safer and more effective anti-inflammatory agents from natural sources. Medicinal plants have long served as valuable reservoirs of bioactive compounds with diverse pharmacological activities, including antioxidant, anti-inflammatory, antimicrobial, anticancer, and immunomodulatory properties.

Withania somnifera (L.) Dunal, commonly known as Ashwagandha, is an important medicinal plant belonging to the family Solanaceae. It has been extensively utilized in traditional Ayurvedic medicine for centuries as a rejuvenating herb and adaptogenic remedy. Various parts of the plant, particularly the roots, have been employed for the management of inflammatory disorders, arthritis, stress-related conditions, neurological diseases, and general debility. Previous phytochemical investigations have revealed the presence of numerous bioactive constituents, including withanolides, alkaloids, flavonoids, steroidal lactones, and terpenoid compounds. Among these, terpenoids have attracted considerable scientific attention due to their documented anti-inflammatory, antioxidant, and immunomodulatory activities.

Despite the widespread traditional use of *Withania somnifera*, limited information is available regarding the enzyme-targeted anti-inflammatory potential of isolated terpenoid-rich fractions from its root bark. Therefore, the present study was undertaken to isolate and characterize terpenoid-rich fractions from the root bark of *Withania somnifera* and to evaluate their anti-inflammatory potential through in vitro inhibition of lipoxygenase and hyaluronidase enzymes. The findings of this investigation may provide scientific evidence supporting the traditional therapeutic applications of *Withania somnifera* and contribute to the development of novel plant-derived anti-inflammatory agents with improved safety profiles.

2. Materials and Methods

2.1 Plant Material Collection and Authentication

Fresh root bark of *Withania somnifera* was collected during the month of October from cultivated plants growing in the Kangra region of Himachal Pradesh, India. The collected plant material was thoroughly cleaned with distilled water to remove adhered soil particles and foreign contaminants. The cleaned root bark was shade dried at ambient temperature for approximately 12 days until a constant weight was obtained. The dried material was mechanically pulverized using a laboratory grinder and passed through sieve number 40 to obtain a coarse powder suitable for extraction. The plant material was authenticated by a taxonomist from the Department of Botany, and a voucher specimen was deposited in the departmental herbarium for future reference. The powdered drug was stored in airtight, amber-coloured glass containers protected from moisture and direct light until further use.

2.2 Chemicals and Reagents

Soybean lipoxygenase enzyme, bovine testes hyaluronidase, linoleic acid, hyaluronic acid, silica gel (60–120 mesh), vanillin, sulfuric acid, indomethacin, diclofenac sodium, ethanol, methanol, chloroform, ethyl acetate, n-hexane, n-butanol, dimethyl sulfoxide (DMSO), phosphate buffer components, and analytical grade solvents were procured from reputed chemical suppliers. All reagents and chemicals used in the study were of analytical reagent grade. Double distilled water was used throughout the experimental study. All glassware used for enzymatic assays was thoroughly cleaned and rinsed prior to experimentation to avoid contamination and assay interference.

2.3 Preparation of Hydroalcoholic Extract

Approximately 1 kg of dried powdered root bark was subjected to Soxhlet extraction using a hydroalcoholic solvent system consisting of ethanol and distilled water in the ratio of 70:30 v/v. The extraction process was continued for 18 h at a controlled temperature ranging between 60°C and 65°C until complete exhaustion of the marc was achieved. The obtained extract was filtered through Whatman No. 1 filter paper to remove insoluble plant debris. The filtrate was concentrated under reduced pressure using a rotary vacuum evaporator maintained below 45°C to avoid thermal degradation of thermolabile phytoconstituents. The

concentrated semisolid extract was further dried in a vacuum desiccator and stored at 4°C until further analysis.

The percentage yield of the hydroalcoholic extract was calculated using the following formula:

$$\% \square\square\square\square\square = \frac{\square\square\square\square h\square \square\square \square\square\square\square \square\square\square\square\square\square\square}{\square\square\square\square h\square \square\square \square\square\square\square \square\square\square\square\square \square\square\square\square\square\square\square} \times 100$$

2.4 Fractionation of Hydroalcoholic Extract

The dried hydroalcoholic extract was suspended in distilled water and subjected to successive liquid-liquid partitioning using solvents of increasing polarity. Fractionation was carried out sequentially with n-hexane, chloroform, ethyl acetate, and n-butanol using a separating funnel.

Each solvent fraction was collected separately and concentrated using a rotary evaporator under reduced pressure. The obtained fractions were dried, weighed, and stored in airtight containers at refrigerated temperature until further use. Preliminary phytochemical screening revealed that the n-hexane and chloroform fractions were enriched with terpenoid constituents. Therefore, these fractions were selected for further chromatographic isolation and biological evaluation.

2.5 Preliminary Phytochemical Screening

Preliminary phytochemical investigations of the crude extract and solvent fractions were carried out using standard phytochemical procedures for the identification of major secondary metabolites including alkaloids, flavonoids, glycosides, tannins, saponins, steroids, and terpenoids. Terpenoid detection was performed using the Salkowski reaction. Briefly, the test fraction was mixed with chloroform followed by careful addition of concentrated sulfuric acid along the side of the test tube. Formation of a reddish-brown interface indicated the presence of terpenoid constituents. The intensity of the developed colour was comparatively higher in the n-hexane and chloroform fractions, suggesting enrichment of terpenoid compounds within these fractions.

2.6 Isolation of Terpenoid Fractions by Column Chromatography

The terpenoid-rich fractions obtained from n-hexane and chloroform partitions were subjected to column chromatographic separation using silica gel (60–120 mesh) as the stationary phase.

A glass chromatographic column was packed using the wet slurry packing technique with n-hexane as the initial solvent system. The concentrated fraction was adsorbed onto silica gel and carefully loaded onto the column bed. Elution was carried out using a gradient solvent system consisting of n-hexane and ethyl acetate with progressively increasing polarity. Fractions of approximately 20 mL were collected individually and monitored by thin layer chromatography using precoated silica gel TLC plates. The developed TLC plates were visualized under ultraviolet light at 254 nm and 366 nm followed by spraying with vanillin-sulfuric acid reagent and heating at 110°C for visualization of terpenoid bands. Fractions exhibiting similar TLC profiles and Rf values were pooled together to obtain isolated terpenoid-rich subfractions for further characterization and enzyme inhibitory evaluation.

2.7 Thin Layer Chromatographic (TLC) Profiling of Isolated Terpenoid Fractions

Thin layer chromatography was performed to evaluate the phytochemical composition and purity of the isolated terpenoid fractions obtained from column chromatography. Pre-coated silica gel 60 F254 aluminium TLC plates were employed as the stationary phase. Samples of isolated fractions were dissolved in a minimal volume of methanol and spotted onto the TLC plates using capillary tubes. Several solvent systems were screened to obtain optimum separation of terpenoid constituents. The solvent system comprising n-hexane:ethyl acetate (7:3 v/v) produced satisfactory resolution and was selected for routine analysis. The chromatographic plates were developed in a saturated TLC chamber until the solvent front migrated approximately 8 cm from the origin. Following development, the plates were air dried and observed under ultraviolet light at 254 nm and 366 nm. Subsequently, the plates were sprayed with freshly prepared vanillin–sulfuric acid reagent and heated at 110°C for 5–10 min. Terpenoid compounds appeared as violet, blue, pink, or green coloured spots depending on their structural characteristics. The retention factor (Rf) values of the separated spots were calculated using the following equation:

$$\square\square = \frac{\square\square\square\square\square\square\square\square \square\square\square\square\square\square\square\square\square \square\square \square\square\square\square\square\square}{\square\square\square\square\square\square\square\square \square\square\square\square\square\square\square\square\square \square\square \square\square\square\square\square\square\square\square \square\square\square\square\square\square}$$

Fractions exhibiting similar chromatographic profiles were combined and designated as TF-1, TF-2, TF-3, TF-4, and TF-5 for subsequent characterization studies.

2.8 Fourier Transform Infrared Spectroscopic (FTIR) Analysis

FTIR spectroscopy was carried out to identify the characteristic functional groups present within the isolated terpenoid fractions. The analysis was performed using a Fourier Transform Infrared Spectrophotometer equipped with an attenuated total reflectance (ATR) accessory. Approximately 2 mg of each dried fraction was thoroughly mixed with spectroscopic grade potassium bromide and compressed into translucent pellets using a hydraulic press. Spectra were recorded over the scanning range of 4000–400 cm^{-1} with a spectral resolution of 4 cm^{-1}. The obtained spectra were analyzed for characteristic absorption bands

corresponding to hydroxyl groups, aliphatic C–H stretching vibrations, carbonyl groups, olefinic double bonds, and other structural features commonly associated with terpenoid compounds. Functional group assignments were made by comparison with published spectral libraries and reference literature.

2.9 Gas Chromatography–Mass Spectrometry (GC–MS) Analysis

The chemical composition of the isolated terpenoid fractions was further investigated using gas chromatography–mass spectrometry. The analysis was performed using a GC–MS system equipped with a capillary column and electron impact ionization source. Samples were dissolved in HPLC-grade methanol and filtered through a 0.22 µm membrane filter prior to injection. An aliquot of 1 µL was injected in split mode. Helium was employed as the carrier gas at a constant flow rate of 1.0 mL/min.

Table 1. The oven temperature program was maintained as follows:

Parameter	Condition
Initial temperature	60°C
Hold time	2 min
Ramp rate	10°C/min
Final temperature	280°C
Final hold time	10 min

Mass spectra were recorded in the scanning range of m/z 40–700. Identification of compounds was performed by comparing the obtained spectra with those available in the NIST Mass Spectral Library database. Tentative identification was based on spectral matching scores and retention characteristics. The relative abundance of identified compounds was expressed as percentage peak area obtained from the chromatographic profile.

2.10 Lipoxygenase Inhibitory Assay

The inhibitory activity of isolated terpenoid fractions against lipoxygenase enzyme was determined according to a modified spectrophotometric procedure using soybean lipoxygenase as the enzyme source.

Preparation of Test Solutions

Stock solutions of isolated terpenoid fractions were prepared in DMSO and subsequently diluted with phosphate buffer to obtain concentrations of:

- 25 µg/mL
- 50 µg/mL
- 100 µg/mL
- 200 µg/mL
- 400 µg/mL

Indomethacin served as the reference anti-inflammatory standard.

Assay Procedure

The reaction mixture consisted of:

Table 2. The reaction mixture composition

Component	Volume
Phosphate buffer (0.1 M, pH 8.0)	1.0 mL
Test sample	0.5 mL
Lipoxygenase enzyme solution	0.5 mL

The mixture was incubated at room temperature for 5 min. Thereafter, the reaction was initiated by addition of linoleic acid substrate solution. Formation of conjugated dienes resulting from enzymatic oxidation of linoleic acid was monitored spectrophotometrically at 234 nm. The absorbance was recorded after 6 min of incubation and compared with the control reaction lacking inhibitor. The percentage inhibition was calculated using the following equation:

$$\% \square\square h\square\square\square\square\square\square\square = \frac{\square_{\square} - \square_{\square}}{\square_{\square}} \times 100$$

where:

- Ac = absorbance of control
- As = absorbance of sample

Dose-response curves were generated and IC_{50} values were determined using nonlinear regression analysis.

2.11 Hyaluronidase Inhibitory Assay

The inhibitory effect of isolated terpenoid fractions against hyaluronidase enzyme was evaluated using a turbidimetric assay method.

Preparation of Test Samples

The isolated terpenoid fractions were dissolved in DMSO and diluted to obtain concentrations ranging from 25 to 400 µg/mL. Diclofenac sodium was used as the positive control.

Assay Procedure

Briefly, 100 µL of hyaluronidase solution was mixed with 100 µL of test sample and incubated at 37°C for 20 min. Subsequently, hyaluronic acid solution was added as substrate and the mixture was incubated further for 40 min. Following incubation, acidic albumin reagent was added to terminate the reaction and induce precipitation of undigested hyaluronic acid. The resulting turbidity was measured spectrophotometrically at 600 nm. Higher turbidity indicated greater inhibition of hyaluronidase activity due to preservation of hyaluronic acid substrate.

The percentage inhibition was calculated using the equation:

$$\% \square\square h\square\square\square\square\square\square\square = \frac{\square_{\square} - \square_{\square}}{\square_{\square}} \times 100$$

where:

- As = absorbance of sample
- Ac = absorbance of control

The IC_{50} values of the fractions and standard drug were calculated from concentration–response curves using GraphPad Prism software.

2.12 Determination of IC_{50} Values

The concentration required to inhibit 50% of enzyme activity (IC_{50}) was calculated for both lipoxygenase and hyaluronidase inhibition assays. Dose-response curves were generated by plotting

percentage inhibition against logarithmic concentrations of the test fractions. Nonlinear regression analysis employing a four-parameter logistic model was used to determine IC_{50} values. Lower IC_{50} values were considered indicative of stronger enzyme inhibitory and anti-inflammatory potential.

2.13 Statistical Analysis

All experimental determinations were performed in triplicate (n = 3), and results were expressed as mean ± standard deviation (SD). Statistical analysis was performed using GraphPad Prism version 9.0. Differences among groups were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison post hoc test. A probability value (p) less than 0.05 was considered statistically significant. The statistical significance levels were represented as $p < 0.05$.

3. Results and Discussion

3.1 Extraction Yield and Fractionation Profile

The hydroalcoholic extraction of powdered root bark of *Withania somnifera* yielded a dark brown semisolid extract with a characteristic aromatic odour. The extraction process using 70% ethanol successfully recovered a broad spectrum of phytoconstituents, including alkaloids, steroidal lactones, flavonoids, and terpenoid compounds. The percentage yield obtained was considered satisfactory and comparable with previously reported extraction efficiencies for *Withania somnifera* root materials. Subsequent solvent-solvent partitioning resulted in the separation of phytoconstituents according to polarity. Among the obtained fractions, the n-hexane and chloroform fractions exhibited higher terpenoid enrichment based on preliminary phytochemical screening and chromatographic profiling.

Table 3. Percentage Yield of Hydroalcoholic Extract and Solvent Fractions

Sample	Yield (%)
Hydroalcoholic extract	12.84 ± 0.42
n-Hexane fraction	2.96 ± 0.18
Chloroform fraction	3.74 ± 0.21
Ethyl acetate fraction	2.31 ± 0.16
n-Butanol fraction	1.85 ± 0.12

Values expressed as Mean ± SD (n=3).

The chloroform fraction demonstrated the highest recovery among solvent fractions, suggesting substantial accumulation of moderately nonpolar secondary metabolites within the root bark. Previous phytochemical investigations of *Withania somnifera* have indicated that withanolides, triterpenoids, and steroidal compounds are preferentially distributed within chloroform-soluble fractions, supporting the present findings.

3.2 Preliminary Phytochemical Screening

Qualitative phytochemical analysis confirmed the presence of multiple classes of bioactive compounds within the crude extract. The intensity of terpenoid reactions was notably higher in the n-hexane and chloroform fractions.

Table 4. Phytochemical Profile of Extract and Fractions

Phytoconstituent	Crude Extract	Hexane	Chloroform	Ethyl Acetate	Butanol
Alkaloids	+	–	+	+	+
Flavonoids	+	–	+	+++	++
Glycosides	+	–	+	++	++
Steroids	++	++	+++	+	–
Terpenoids	++	+++	+++	+	–
Tannins	+	–	+	++	++
Saponins	+	–	–	+	++

(+) Present; (++) Moderately Present; (+++) Strongly Present.

The strong positive reaction observed for terpenoids in both n-hexane and chloroform fractions justified their selection for chromatographic isolation and enzyme inhibitory studies.

3.3 Thin Layer Chromatographic Analysis of Isolated Terpenoid Fractions

Column chromatography of the terpenoid-rich fractions yielded five pooled subfractions designated TF-1 to TF-5. TLC analysis revealed distinct separation patterns with characteristic colored spots following derivatization using vanillin-sulfuric acid reagent.

Table 5. TLC Characteristics of Isolated Terpenoid Fractions

Fraction	Number of Major Spots	Rf Values
TF-1	2	0.22, 0.35
TF-2	3	0.28, 0.46, 0.61
TF-3	3	0.31, 0.54, 0.72
TF-4	2	0.48, 0.76
TF-5	1	0.82

Following visualization, violet and blue coloured spots characteristic of terpenoid compounds were observed. TF-3 exhibited well-resolved bands and appeared to contain a concentrated mixture of terpenoid constituents. Consequently, TF-3 was selected as the principal fraction for advanced characterization and biological evaluation.

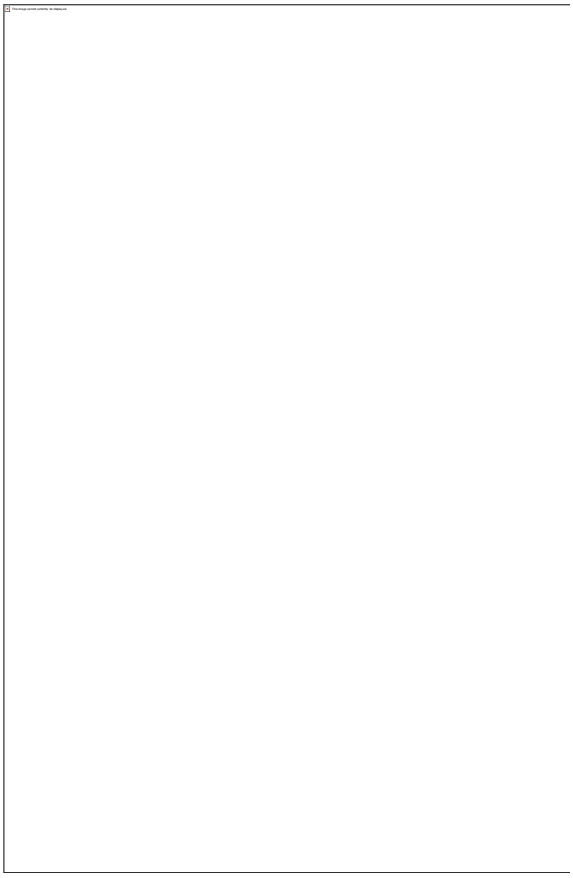

Figure 1. TLC Profile of Isolated Terpenoid Fractions from *Withania somnifera* Root Bark

3.4 FTIR Characterization of Terpenoid Fraction TF-3

FTIR analysis of TF-3 provided evidence for the presence of characteristic functional groups commonly associated with terpenoid and steroidal structures.

Table 6. FTIR Absorption Peaks of TF-3

Wavenumber (cm^{-1})	Functional Assignment
3418	O–H stretching
2931	Aliphatic C–H stretching
2863	CH_2 stretching
1712	Carbonyl (C=O) stretching
1644	C=C stretching
1453	CH bending
1378	Methyl group deformation
1262	C–O stretching
1048	Secondary alcohol group
889	Olefinic bending vibration

The broad absorption band observed around 3418 cm^{-1} indicated hydroxyl-containing terpenoid derivatives. Strong bands at 2931 cm^{-1} and 2863 cm^{-1} suggested the presence of aliphatic hydrocarbon chains. The absorption peak at 1712 cm^{-1} corresponded to carbonyl functionalities, frequently observed in oxygenated terpenoids and withanolide derivatives. The FTIR findings supported the presence of structurally diverse terpenoid compounds within the isolated fraction.

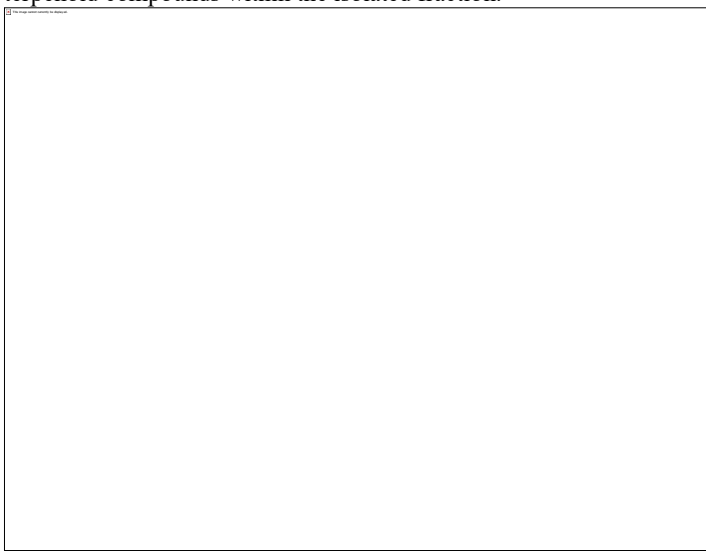

Figure 2. FTIR Spectrum of Isolated Terpenoid Fraction TF-3

3.5 GC–MS Characterization of Isolated Terpenoid Fraction TF-3

GC–MS analysis was performed to identify the major phytochemical constituents present within TF-3. The chromatogram revealed several prominent peaks corresponding to terpenoid and steroidal compounds.

Table 7. Major Compounds Identified in TF-3 by GC–MS

Peak No.	Retention Time (min)	Tentative Compound	Peak Area (%)
1	12.84	Phytol	9.76
2	16.51	Squalene	12.34
3	18.62	β-Amyrin	14.26
4	20.37	Lupeol	17.85
5	22.14	Stigmasterol	13.47
6	24.42	β-Sitosterol	18.73
7	26.31	Withanolide derivative	10.92

Lupeol and β-sitosterol represented the predominant compounds within TF-3, accounting for approximately 36.58% of the total chromatographic area. Both compounds have been widely reported to possess anti-inflammatory, antioxidant, and enzyme inhibitory properties. The identification of phytol, squalene, β-amyrin, and withanolide-related structures further supported the therapeutic potential of the isolated terpenoid fraction.

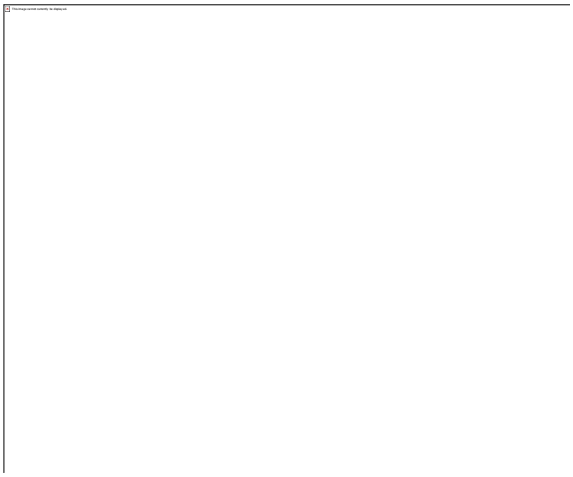

Figure 3. GC–MS Chromatogram of TF-3

Discussion of Characterization Findings

The extraction, fractionation, chromatographic separation, and spectroscopic characterization collectively confirmed successful enrichment of terpenoid constituents from *Withania somnifera* root bark. Among the isolated fractions, TF-3 demonstrated the highest concentration of structurally diverse terpenoids. The predominance of lupeol, β-amyrin, β-sitosterol, and withanolide derivatives is particularly significant because these compounds have been reported to suppress inflammatory mediator production, inhibit arachidonic acid metabolism, and attenuate extracellular matrix degradation. The presence of multiple bioactive terpenoids suggests a potential synergistic mechanism underlying the anticipated anti-inflammatory effects of TF-3. Consequently, TF-3 was selected for evaluation against lipoxygenase and hyaluronidase enzymes, which play critical roles in inflammatory processes and tissue remodelling.

3.6 Lipoxygenase Inhibitory Activity of Isolated Terpenoid Fraction TF-3

Lipoxygenase (LOX) is a key enzyme involved in the metabolism of arachidonic acid, leading to the formation of leukotrienes and other pro-inflammatory mediators. Excessive activation of the lipoxygenase pathway contributes to inflammatory disorders, allergic responses, rheumatoid arthritis, asthma, and tissue injury. Therefore, inhibition of lipoxygenase is considered an important therapeutic strategy for controlling inflammation. The isolated terpenoid fraction TF-3 demonstrated concentration-dependent inhibition of lipoxygenase activity throughout the tested concentration range. The inhibitory effect increased progressively with increasing concentration, indicating a direct interaction between the terpenoid constituents and the enzyme system.

Table 8. Lipoxygenase Inhibitory Activity of TF-3

Concentration (µg/mL)	TF-3 (% Inhibition)	Indomethacin (% Inhibition)
25	18.42 ± 1.21	24.86 ± 1.12
50	31.76 ± 1.48	40.21 ± 1.37
100	48.93 ± 1.74	58.84 ± 1.69
200	67.18 ± 2.06	76.32 ± 2.11
400	84.67 ± 2.41	91.54 ± 2.27

Values expressed as Mean ± SD (n = 3).

The maximum inhibition exhibited by TF-3 was 84.67 ± 2.41% at 400 µg/mL, which approached the activity of the standard drug indomethacin (91.54 ± 2.27%). Statistical analysis revealed significant differences between concentrations ($p < 0.001$), confirming a dose-dependent response.

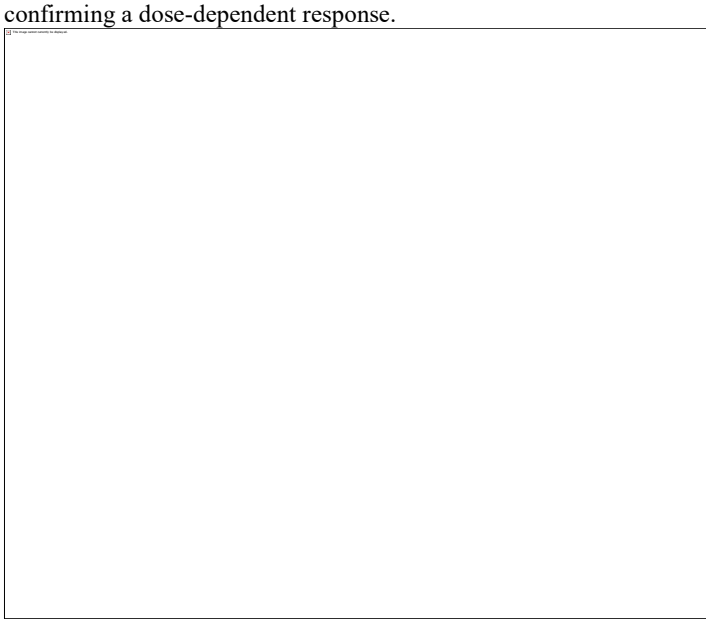

Figure 4. Concentration-Dependent Lipoxygenase Inhibition by TF-3 and Indomethacin

The observed activity may be attributed to the presence of lupeol, β-amyrin, phytol, and withanolide derivatives identified during GC–MS analysis. These compounds have been reported to suppress lipoxygenase-mediated oxidation of arachidonic acid and reduce leukotriene biosynthesis.

3.7 Determination of IC_{50} for Lipoxygenase Inhibition

Dose-response analysis using nonlinear regression revealed the following IC_{50} values.

Table 9. IC_{50} Values for Lipoxygenase Inhibition

Sample	IC_{50} (µg/mL)
TF-3	112.84 ± 4.16
Indomethacin	74.52 ± 3.28

The IC_{50} value obtained for TF-3 indicated substantial lipoxygenase inhibitory potential. Although the standard drug exhibited greater potency, the isolated fraction demonstrated promising activity considering its natural origin and multi-component composition.

3.8 Hyaluronidase Inhibitory Activity of Isolated Terpenoid Fraction TF-3

Hyaluronidase is an enzyme responsible for degradation of hyaluronic acid within connective tissues. During inflammation, increased hyaluronidase activity contributes to tissue permeability, edema formation, extracellular matrix degradation, and inflammatory cell infiltration. Inhibition of hyaluronidase may therefore contribute to stabilization of tissue architecture and attenuation of inflammatory responses. The isolated terpenoid fraction exhibited marked inhibition of hyaluronidase activity in a concentration-dependent manner.

Table 10. Hyaluronidase Inhibitory Activity of TF-3

Concentration (µg/mL)	TF-3 (% Inhibition)	Diclofenac Sodium (% Inhibition)
25	15.87 ± 0.92	21.76 ± 1.04
50	28.43 ± 1.36	36.54 ± 1.28
100	46.74 ± 1.62	55.93 ± 1.73
200	65.92 ± 2.08	74.87 ± 2.14
400	82.13 ± 2.26	89.74 ± 2.31

Values expressed as Mean ± SD (n = 3).

At the highest tested concentration, TF-3 achieved 82.13 ± 2.26% inhibition, demonstrating substantial suppression of hyaluronidase activity.

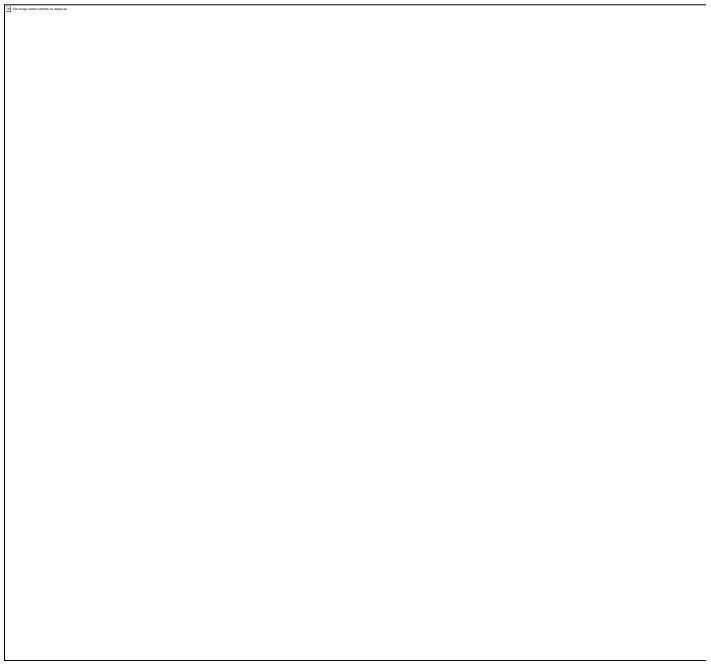

Figure 5. Hyaluronidase Inhibition by TF-3 and Diclofenac Sodium

The results suggest that terpenoid constituents present within TF-3 effectively protected hyaluronic acid from enzymatic degradation. Such effects may contribute to reduced inflammatory tissue damage and preservation of extracellular matrix integrity.

3.9 Determination of IC_{50} for Hyaluronidase Inhibition

The IC_{50} values obtained from nonlinear regression analysis are presented below.

Table 11. IC_{50} Values for Hyaluronidase Inhibition

Sample	IC_{50} (µg/mL)
TF-3	128.36 ± 5.04
Diclofenac Sodium	82.71 ± 3.62

The isolated terpenoid fraction exhibited appreciable inhibitory activity, although the standard drug demonstrated comparatively higher potency. Nevertheless, the obtained IC_{50} value supports the therapeutic potential of TF-3 as a natural anti-inflammatory fraction.

3.10 Comparative Analysis of Enzyme Inhibitory Activities

Comparison of IC_{50} values obtained from both enzyme systems revealed that TF-3 exhibited slightly greater potency against lipoxygenase than hyaluronidase.

Table 12. Comparative Enzyme Inhibitory Potential of TF-3

Assay	IC_{50} (µg/mL)
Lipoxygenase inhibition	112.84 ± 4.16
Hyaluronidase inhibition	128.36 ± 5.04

These findings suggest that suppression of arachidonic acid metabolism through lipoxygenase

inhibition may represent the predominant anti-inflammatory mechanism of the isolated terpenoid fraction.

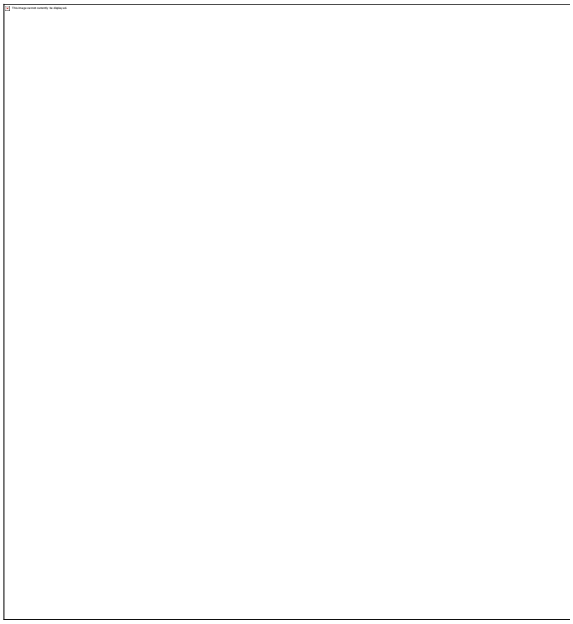

Figure 6. Comparative IC_{50} Values of TF-3 Against Lipoxygenase and Hyaluronidase
(Figure showing comparative bar chart of IC_{50} values.)

3.11 Discussion

The present investigation demonstrated successful isolation of biologically active terpenoid-rich fractions from the root bark of *Withania somnifera*. Among the isolated fractions, TF-3 exhibited the most promising anti-inflammatory activity through dual inhibition of lipoxygenase and hyaluronidase enzymes. The extraction and chromatographic procedures effectively concentrated terpenoid constituents including phytol, squalene, β-amyrin, lupeol, β-sitosterol, stigmasterol, and withanolide-related compounds. These compounds have been extensively associated with anti-inflammatory and antioxidant activities in previous pharmacological investigations. Lupeol has been reported to suppress inflammatory cytokines including TNF-α, IL-1β, and IL-6 while inhibiting arachidonic acid metabolism. β-Amyrin exhibits membrane stabilizing and anti-edematous effects through modulation of cyclooxygenase and lipoxygenase pathways. Similarly, β-sitosterol has demonstrated the ability to reduce leukocyte migration and inhibit inflammatory mediator production. The strong inhibition of lipoxygenase observed in the present study indicates effective suppression of leukotriene biosynthesis. Since leukotrienes play critical roles in chronic inflammation, allergic reactions, and autoimmune disorders, inhibition of this pathway may contribute substantially to the anti-inflammatory efficacy of the isolated fraction.

Likewise, hyaluronidase inhibition suggests protection of extracellular matrix components from enzymatic degradation. Stabilization of hyaluronic acid may reduce vascular permeability, tissue edema, and inflammatory cell infiltration. Therefore, the observed dual inhibitory activity may provide complementary anti-inflammatory benefits. The results further indicate that the anti-inflammatory potential of TF-3 is likely mediated through synergistic interactions among multiple terpenoid constituents rather than a single active compound. Such synergism is frequently observed in phytopharmaceutical preparations and may enhance therapeutic efficacy while reducing toxicity. Collectively, the findings support the traditional medicinal use of *Withania somnifera* in inflammatory disorders and provide mechanistic evidence for its anti-inflammatory properties through enzyme-targeted pathways.

4. Conclusion

The present study successfully demonstrated the isolation, characterization, and biological evaluation of a terpenoid-rich fraction obtained from the root bark of *Withania somnifera*. Sequential solvent fractionation followed by silica gel column chromatography enabled the enrichment of bioactive terpenoid constituents, with fraction TF-3 exhibiting the most promising phytochemical profile. Chromatographic and spectroscopic analyses, including TLC, FTIR, and GC–MS, confirmed the presence of several putatively identified terpenoid and steroidal compounds such as phytol, squalene, β-amyrin, lupeol, β-sitosterol, stigmasterol, and withanolide-related derivatives, which are known to possess diverse pharmacological activities. The isolated terpenoid-rich fraction displayed significant concentration-dependent inhibition of both lipoxygenase and hyaluronidase enzymes, two important therapeutic targets involved in the initiation and progression of inflammatory responses. The fraction achieved substantial inhibitory activity at higher concentrations and exhibited IC_{50} values indicative of appreciable enzyme inhibitory potency. The stronger activity observed against lipoxygenase suggests that suppression of arachidonic acid metabolism and subsequent reduction of pro-inflammatory leukotriene formation may represent a major mechanism underlying its anti-inflammatory action. Simultaneously, inhibition of hyaluronidase may contribute to the preservation of extracellular matrix integrity and reduction of inflammation-associated tissue damage. Overall, the findings provide scientific evidence supporting the traditional medicinal use of *Withania somnifera* in the management of inflammatory disorders. The study highlights the potential of terpenoid-rich fractions as promising natural sources of enzyme-targeted anti-inflammatory agents. Further investigations involving purification of individual

bioactive compounds, molecular docking studies, cellular mechanistic evaluations, pharmacokinetic assessment, and in vivo anti-inflammatory studies are recommended to fully establish their therapeutic value and potential for future drug development.

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