IN VITRO ASSESSMENT OF ARGINASE INHIBITORY ACTIVITY OF *SCELETIUM TORTUOSUM* IN RAW 264.7 MACROPHAGE CELLS WITH RELEVANCE TO VARICOSE VEIN PATHOLOGY

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Abstract

Sceletium tortuosum is a medicinal plant traditionally recognized for its diverse pharmacological properties. The present study evaluated the arginase inhibitory and anti-inflammatory activities of its hydroalcoholic root extract in LPS-stimulated RAW 264.7 murine macrophages. Authenticated roots were shade-dried, powdered, and extracted using 70% hydroalcoholic solvent by maceration, yielding 21.6% of a dark brown semisolid extract. Preliminary phytochemical evaluation indicated the presence of alkaloids, flavonoids, phenolics, and other secondary metabolites. Cytotoxicity was assessed using the MTT assay, and the extract showed high cellular compatibility, with cell viabilities of 96.34 ± 0.64%, 90.54 ± 0.35%, and 88.36 ± 0.84% at 250, 500, and 1000 µg/mL, respectively. Arginase activity was successfully induced by LPS and 8-bromo-cAMP, increasing from 18.78 ± 0.37 to 87.45 ± 1.22 urea units. Treatment with the extract significantly reduced arginase activity to 30.76 ± 0.54 urea units, corresponding to 64.83% inhibition, compared with 72.76% for L-Norvaline. The extract also reduced nitrite production from 21.480 ± 0.341 to 16.397 ± 0.190 µM/mL, representing a 23.67% reduction. Restoration of total protein content further suggested improved cellular integrity. These findings demonstrate that *S. tortuosum* possesses significant arginase inhibitory and anti-inflammatory activity with low cytotoxicity. Its phytoconstituents, particularly mesembrine alkaloids, flavonoids, and phenolic compounds, may contribute to these effects. The extract therefore shows potential as a natural therapeutic candidate for inflammation-associated vascular disorders, including varicose vein pathology.

Key Words: *Sceletium tortuosum,* phytoconstituents, MTT assay, Cytotoxicity

INTRODUCTION

Varicose veins are a common manifestation of chronic venous disease, characterized by dilated, elongated, and tortuous superficial veins that predominantly affect the lower extremities. These

abnormal veins arise due to structural and functional failure of venous valves, which are responsible for maintaining unidirectional blood flow toward the heart. Under normal physiological conditions, venous return from the lower limbs is facilitated by a combination of competent valves, muscular contraction, and pressure gradients. However, when these valves become incompetent, retrograde blood flow occurs, leading to venous reflux and progressive venous dilation. This ultimately results in the visible bulging veins that define varicosity.

The condition is highly prevalent worldwide and represents a significant public health concern. Epidemiological data indicate that a substantial proportion of the adult population is affected, with prevalence increasing with age. Women are more commonly affected than men, likely due to hormonal influences, pregnancy-related changes, and differences in connective tissue composition. In addition, occupational factors such as prolonged standing or sitting, obesity, genetic predisposition, and sedentary lifestyle contribute significantly to disease development. Despite its high prevalence, varicose vein pathology is often underestimated, as it is frequently regarded as a cosmetic issue rather than a progressive vascular disorder

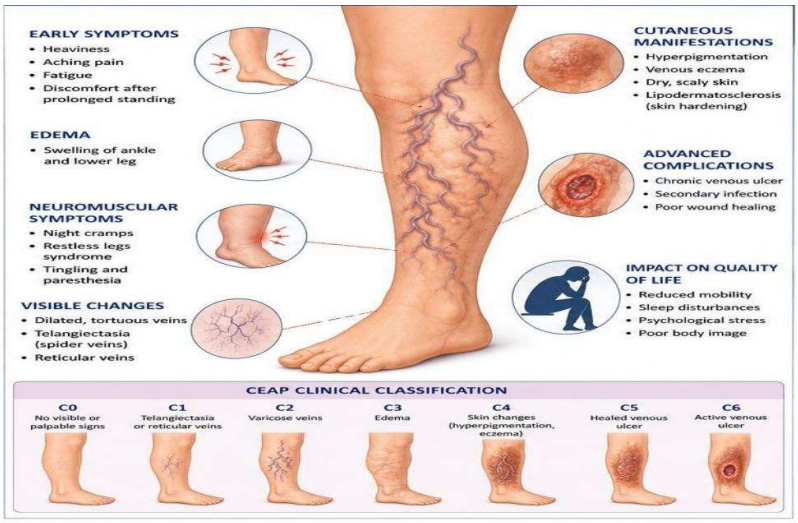

Figure 1: Clinical manifestation of varicose veins

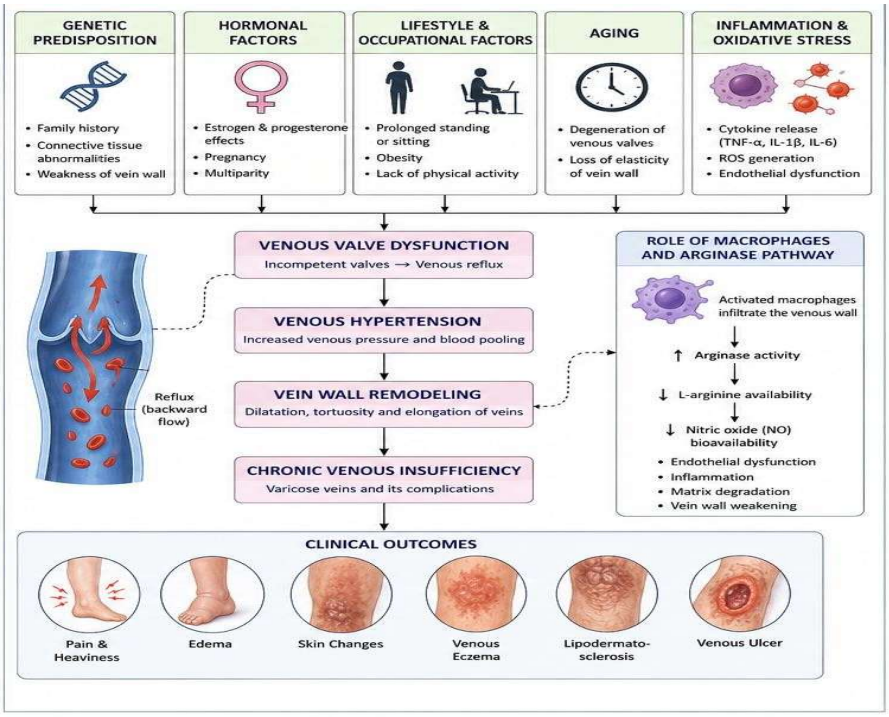

Figure 2: Etiology and pathogenesis of varicose veins

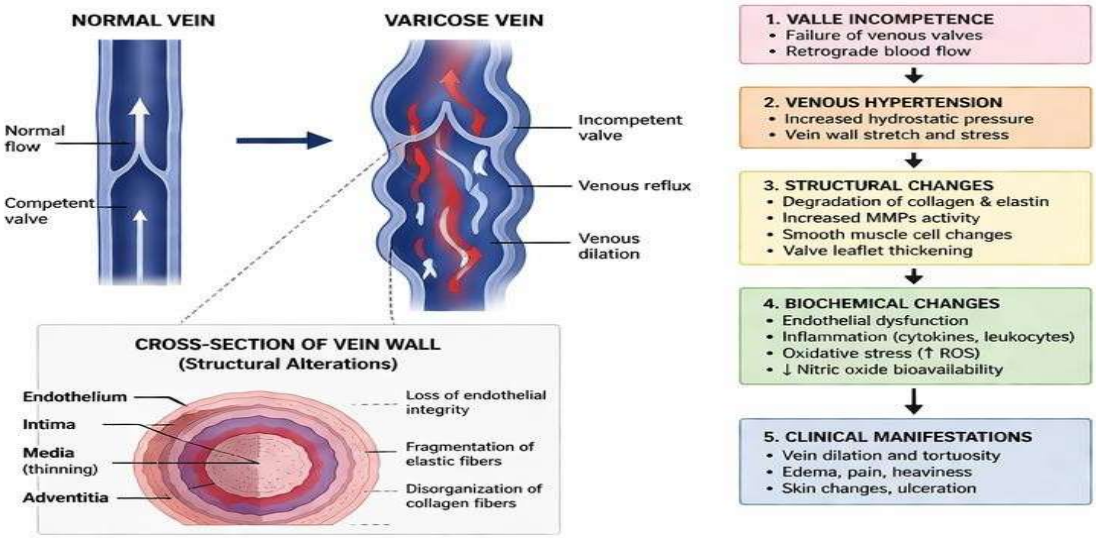

Figure 3: Pathogenesis of varicose veins

1.1. Role of Nitric Oxide in Vascular Function

Nitric oxide (NO) is a crucial endogenous signaling molecule that plays a central role in maintaining vascular homeostasis. It is a gaseous free radical synthesized from the amino acid

L-arginine by a family of enzymes known as nitric oxide synthases (NOS). Among the three isoforms of NOS—endothelial (eNOS), inducible (iNOS), and neuronal (nNOS)—endothelial nitric oxide synthase is particularly important in regulating vascular function. In healthy blood vessels, NO produced by endothelial cells diffuses into adjacent smooth muscle cells, where it activates soluble guanylate cyclase, leading to the formation of cyclic guanosine monophosphate (cGMP). This signaling cascade ultimately results in relaxation of vascular smooth muscle and vasodilation.

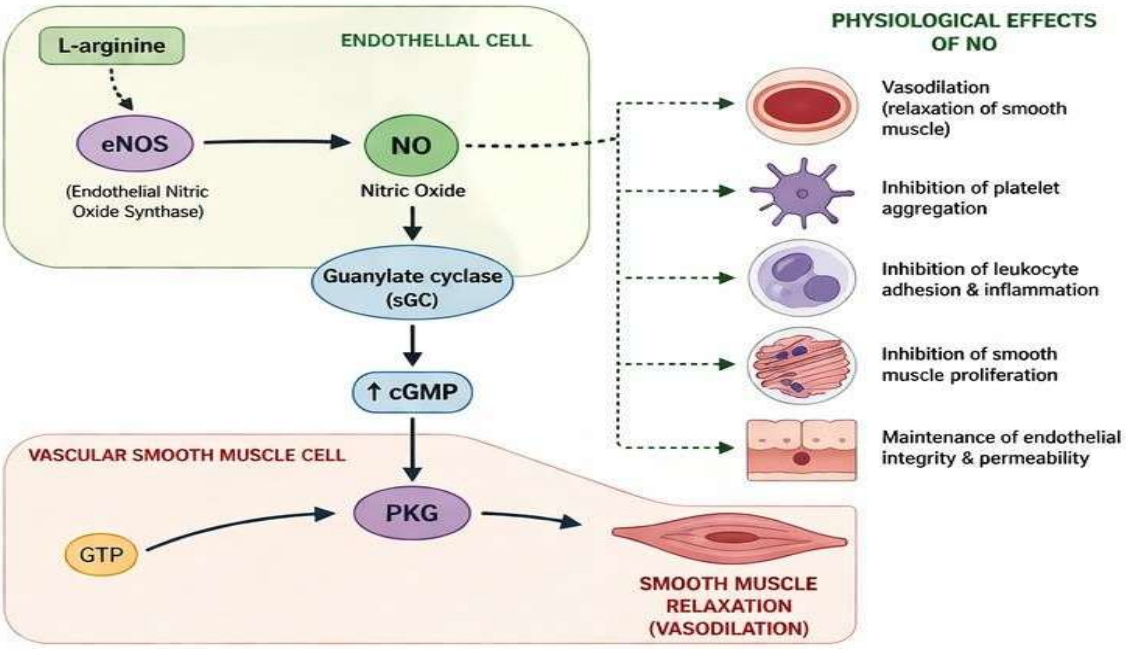

Figure 4: Nitric oxide mechanism in vascular function

PLANT PROFILE

1.1. Taxonomical classification

- **Kingdom** : Plantae
- **Subkingdom** : Tracheobionta
- **Division** : Magnoliophyta
- **Class** : Magnoliopsida
- **Order** : Caryophyllales
- **Family** : Aizoaceae
- **Genus** : *Sceletium*
- **Species** : *Sceletium tortuosum*

Figure 6: *Sceletium tortuosum*

Aim

To evaluate the arginase inhibitory activity of *Sceletium tortuosum* root extract in LPS and 8-bromo-cAMP induced RAW 264.7 macrophage cells, with relevance to varicose vein pathology.

Objectives

- To prepare and characterize the hydroalcoholic extract of *Sceletium tortuosum* roots.
- To assess the cytotoxicity of the extract on RAW 264.7 macrophage cells using the MTT assay.
- To induce arginase activity in RAW 264.7 cells using LPS and 8-bromo-cAMP.
- To evaluate the effect of *Sceletium tortuosum* extract on nitric oxide production using the Griess assay.
- To determine arginase activity by measuring urea formation in treated cells.
- To compare the activity of the extract with a standard arginase inhibitor (L-norvaline).
- To correlate arginase inhibition and nitric oxide modulation with mechanisms involved in varicose vein pathology, particularly endothelial dysfunction.
- To perform statistical analysis to determine the significance of the results.

SCOPE OF THE STUDY

The present study is designed to explore the pharmacological potential of *Sceletium tortuosum* root extract with specific emphasis on its ability to modulate arginase activity and nitric oxide metabolism in macrophage cells. The investigation is carried out using RAW 264.7 murine

macrophages as an established in vitro model to study cellular responses related to arginine metabolism. By employing LPS and 8-bromo-cAMP as stimulants, the study creates a controlled environment to induce arginase expression and mimic conditions associated with altered nitric oxide bioavailability.

The scope of this work primarily includes the preparation of a hydroalcoholic extract of *Sceletium tortuosum* roots and its evaluation for cytotoxicity to determine safe and effective concentration ranges. Within these limits, the extract is further assessed for its influence on key biochemical parameters, including nitric oxide production (measured as nitrite levels using the Griess assay) and arginase activity (determined through urea formation). These parameters are critical in understanding the balance between nitric oxide synthesis and arginase-mediated arginine metabolism, which plays an important role in vascular function.

A significant aspect of this study lies in correlating the observed biochemical effects with mechanisms relevant to varicose vein pathology. Varicose veins are associated with endothelial dysfunction, impaired nitric oxide signaling, and altered vascular tone. Since arginase competes with nitric oxide synthase for the common substrate L-arginine, increased arginase activity can reduce nitric oxide availability, contributing to vascular abnormalities. Therefore, the evaluation of arginase inhibition by *Sceletium tortuosum* extract provides a mechanistic basis to understand its potential role in improving vascular health. The study also includes comparison with a standard arginase inhibitor (L-norvaline), which helps in validating the observed effects and establishing the relative efficacy of the plant extract. Statistical analysis of the experimental data further ensures the reliability and significance of the findings. However, the scope of this study is limited to in vitro evaluation and does not extend to in vivo or clinical investigations. While the findings may provide valuable preliminary insights into the role of *Sceletium tortuosum* in modulating arginase activity and nitric oxide levels, further studies involving animal models and clinical trials would be necessary to confirm its therapeutic applicability in varicose vein management.

METHODS AND MATERIALS

1.1. Collection, authentication and preparation of extract

Roots of *Sceletium tortuosum* were collected from a suitable source during the appropriate season, cleaned thoroughly to remove adhering soil and debris, washed with distilled water, and shade-dried at room temperature for 10 days until a constant weight was obtained. The plant material was authenticated by a qualified taxonomist, and a voucher specimen was prepared and deposited in a recognized herbarium for future reference. The dried roots were then coarsely powdered using a mechanical grinder, sieved to obtain uniform particle size, and stored in an airtight container. About 100 g of the powdered material was subjected to hydroalcoholic extraction using 70% ethanol (ethanol:water, 70:30 v/v) by maceration for 72 hours with occasional stirring. The mixture was filtered through muslin cloth followed by Whatman filter paper, and the residue was re-extracted with fresh solvent to ensure maximum yield. The combined filtrates were concentrated under reduced pressure using a rotary

evaporator and further dried to obtain a semisolid extract, which was stored in a desiccator and preserved in an airtight container at 4°C until further use [67].

1.2. MTT Assay for Cytotoxicity Assessment

The MTT assay was performed following a previously reported method with slight modifications. The RAW 264.7 murine macrophage cell line was maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 100 µg/mL each of penicillin and streptomycin. Cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2. The cells were trypsinized, collected into a centrifuge tube, and resuspended in fresh culture medium. The cell suspension was seeded into 96-well tissue culture plates at a density of 1×10^5 cells/mL, with 200 µL of cell suspension added per well. The plates were incubated for 24–48 hours at 37°C in a 5% CO_2 incubator to allow proper cell attachment.

After incubation, the culture medium was carefully removed, and the cells were gently washed with sterile phosphate-buffered saline (PBS). The cells were then treated with different concentrations of *Sceletium tortuosum* extract (100, 250, and 500 µg/mL) prepared in serum-free DMEM. L-Norvaline was used as a standard reference compound. All treatments were carried out in triplicate, and the plates were incubated for 24 hours under standard culture conditions. Following the treatment period, 10 µL of MTT solution (5 mg/mL) was added to each well and incubated for an additional 2–4 hours to allow the formation of purple formazan crystals. After incubation, the supernatant was carefully aspirated, and the wells were gently rinsed with 200 µL of 1X PBS. To dissolve the formed formazan crystals, 100 µL of dimethyl sulfoxide (DMSO) was added to each well, and the plate was gently shaken for approximately 5 minutes. The absorbance was then measured at 570 nm using a microplate reader. The percentage cell viability and IC_{50} values were calculated [68].

1.3. Induction and Treatment in RAW 264.7 Cells

RAW 264.7 murine macrophage cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and 1% antibiotic solution (penicillin and streptomycin). The cells were maintained at 37°C in a humidified incubator with 5% CO_2. For the experiment, cells were seeded into 96-well plates at an appropriate density and allowed to adhere for 24 hours. After attachment, the culture medium was removed and replaced with fresh serum-free medium prior to treatment. The cells were divided into four experimental groups as described above. The vehicle control group received a mixture of 80% ethanol and 20% propylene glycol. The induction control group was treated with lipopolysaccharide (LPS, 2 µg/mL) and 8-bromo-cAMP (0.5 mM) to stimulate arginase expression and inflammatory response. The test group was treated with *Sceletium tortuosum* extract at selected concentrations along with LPS (2 µg/mL) and 8-bromo-cAMP (0.5 mM). The standard group received L-Norvaline along with LPS and 8-bromo-cAMP, where L-Norvaline served as a reference arginase inhibitor. All treatments were performed in triplicate and incubated for 24 hours under standard culture conditions. Following incubation, the cells were processed for downstream assays such as nitric oxide estimation (Griess assay) and arginase activity measurement (urea estimation).

Table 2: Experimental design

Group	Treatment Description
Group I (Vehicle Control)	80% ethanol + 20% propylene glycol
Group II (Induction Control)	LPS (2 µg/mL) + 8-bromo-cAMP (0.5 mM)
Group III (Test Group)	*Sceletium tortuosum* extract + LPS (2 µg/mL) + 8-bromo-cAMP (0.5 mM)
Group IV (Standard Group)	L-Norvaline + LPS (2 µg/mL) + 8-bromo-cAMP (0.5 mM)

1.1. Estimation of Arginase Activity (Urea Assay)

Arginase activity was determined using a modified microplate method based on a previously reported procedure. RAW 264.7 cells were treated according to the experimental design, including vehicle control, induction control, test (*Sceletium tortuosum* extract with LPS and 8-bromo-cAMP), and standard (L-norvaline with LPS and 8-bromo-cAMP) groups. After the specified incubation period (24 hours), the cells were processed for arginase activity estimation. Following incubation, the culture medium was removed, and the cells were gently washed with phosphate-buffered saline (PBS) to remove residual media. The cells were then lysed using 300 µL of 0.5% Triton X-100 solution containing protease inhibitors. The lysates were kept under gentle agitation for 30 minutes at room temperature to ensure complete cell lysis. To activate arginase, 400 µL of 25 mmol/L Tris-HCl buffer (pH 7.4) and 100 µL of 10 mmol/L manganese chloride ($MnCl_2$) were added to the lysate. The mixture was then heated at 56°C for 10 minutes to facilitate enzyme activation.

Following activation, 50 µL of the lysate was mixed with 50 µL of L-arginine solution (0.5 mol/L, pH 9.7), and the reaction mixture was incubated at 37°C for 60 minutes to allow enzymatic hydrolysis of L-arginine to urea. The reaction was terminated by the addition of 800 µL of an acid stop solution composed of sulfuric acid (96%), phosphoric acid (85%), and distilled water in a ratio of 1:3:7 (v/v/v). Subsequently, 50 µL of α-isonitrosopropiophenone solution (9% w/v in ethanol) was added to each sample. The reaction mixtures were then heated at 100°C for 45 minutes to develop a colored complex for urea detection. After cooling to room temperature, the absorbance was measured at 550 nm using a microplate reader. The concentration of urea produced, which reflects arginase activity, was determined by comparison with a standard calibration curve prepared using serial dilutions of urea (0.039–5 µg/mL), processed under identical conditions. Arginase activity was expressed as µg of urea produced per mL or normalized to protein content where applicable. The inhibitory effect of *Sceletium tortuosum* extract on arginase activity was calculated in comparison to the induction

control group [69].**Estimation of Nitric Oxide Production (Griess Assay)**

Nitric oxide (NO) production was indirectly assessed by measuring the accumulation of nitrite (NO_2^-) in the culture supernatants using the colorimetric Griess reaction. RAW 264.7 cells were treated according to the experimental design, including vehicle control, induction control, test (*Sceletium tortuosum* extract with LPS and 8-bromo-cAMP), and standard (L-norvaline with LPS and 8-bromo-cAMP) groups. After the incubation period (24 hours), the culture supernatants were carefully collected from each well. In a 96-well plate, 60 µL of the collected supernatant was mixed with an equal volume (60 µL) of freshly prepared Griess reagent. The reaction mixture was incubated at room temperature for 10 minutes to allow the development of a pink-colored azo compound. Following incubation, the absorbance was measured at 550 nm using a microplate reader. The concentration of nitrite in the samples was determined by comparison with a standard calibration curve prepared using sodium nitrite solutions in the range of 1–10 µM. The results were expressed as µM of nitrite (NO_2^-) per well. A decrease in nitrite concentration in the test group indicates inhibition of nitric oxide production, suggesting anti-inflammatory activity of the *Sceletium tortuosum* extract [69].

1.2. Statistical analysis

All experiments were performed in triplicate, and the results were expressed as mean ± standard deviation (SD). Statistical analysis was carried out using Graph Pad Prism, and comparisons between groups were performed using one-way analysis of variance (ANOVA) followed by a suitable post hoc test. A value of $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

1.3. Extraction of *Sceletium tortuosum*

The roots of *Sceletium tortuosum* were successfully collected, authenticated, processed, and subjected to hydroalcoholic extraction using 70% ethanol by the maceration method. The extraction process yielded a dark brown semisolid mass with a characteristic odor and slightly sticky consistency. The concentrated extract was easily separated from the solvent and remained stable during storage at 4°C without any visible signs of degradation or microbial contamination.

The percentage yield of the hydroalcoholic root extract was calculated based on the initial weight of the dried powdered root material and the final weight of the dried extract obtained after solvent removal. The extraction of 100 g of powdered root material yielded 21.6 g of semisolid extract, corresponding to a percentage yield of 21.6%.

Table 3: Yield of Hydroalcoholic Extract of *Sceletium tortuosum* Roots

Parameter	**Observation**
Plant part used	Roots
Initial weight of powdered material	100 g
Solvent system	70% Ethanol
Extraction method	Maceration
Duration of extraction	72 h
Weight of dried extract obtained	21.6 g
Percentage yield	21.6%

The obtained extraction yield indicates efficient recovery of phytoconstituents from the root material. Hydroalcoholic solvents are widely employed in phytochemical extraction because they possess the ability to dissolve a broad range of polar and moderately non-polar compounds. The combination of ethanol and water enhances solvent penetration into plant tissues and facilitates the extraction of diverse bioactive constituents such as alkaloids, flavonoids, phenolic compounds, tannins, glycosides, and other secondary metabolites. Therelatively high yield observed in the present study suggests that the roots of *Sceletium tortuosum* contain substantial quantities of extractable phytochemicals.

The extraction efficiency obtained may be attributed to several factors, including the use of a hydroalcoholic solvent system, prolonged extraction time, repeated extraction cycles, and the fine powdered nature of the plant material. Maceration for 72 hours allowed adequate solvent contact with the plant matrix, thereby enhancing diffusion of soluble constituents into the extraction medium. Re-extraction of the marc further contributed to maximizing the recovery of bioactive compounds and minimizing loss of extractable material.

The yield obtained in the present investigation falls within the range generally reported for hydroalcoholic extraction of medicinal plant materials rich in secondary metabolites. Variations in extraction yield are influenced by factors such as plant age, geographical location, harvesting season, drying conditions, particle size, solvent polarity, extraction temperature, and extraction duration. Since *Sceletium tortuosum* contains a variety of phytoconstituents, including mesembrine alkaloids, flavonoids, and phenolic compounds, the hydroalcoholic solvent system likely facilitated their efficient extraction.

From a pharmacological perspective, a higher extraction yield is advantageous because it provides sufficient material for subsequent biological investigations and may indicate the presence of abundant bioactive constituents. The successful recovery of phytochemicals is particularly important for studies evaluating anti-inflammatory and arginase inhibitory

activities, as many plant-derived polyphenols and alkaloids are known to influence inflammatory pathways, oxidative stress responses, and enzyme regulation. Previous studies have demonstrated that phytochemicals possessing antioxidant and anti-inflammatory properties can modulate macrophage activation and reduce the production of inflammatory mediators associated with vascular dysfunction.

The hydroalcoholic extract obtained in the present study was therefore considered suitable for subsequent in vitro evaluation of arginase inhibitory activity in RAW 264.7 macrophage cells. The appreciable extraction yield of 21.6% suggests effective recovery of bioactive compounds that may contribute to the biological activities of *Sceletium tortuosum*. Furthermore, the use of a hydroalcoholic extraction approach is expected to provide a phytochemically diverse extract, thereby increasing the likelihood of identifying compounds capable of modulating arginase activity and inflammatory responses relevant to varicose vein pathology.

MTT assay

The cytotoxicity of the hydroalcoholic root extract of *Sceletium tortuosum* was evaluated in RAW 264.7 murine macrophage cells using the MTT assay. Cell viability was assessed after 24 h of exposure to different concentrations of the extract and compared with the standard compound L-Norvaline. The MTT assay is a widely accepted colorimetric method used to determine cellular metabolic activity and viability based on the reduction of MTT into insoluble purple formazan crystals by mitochondrial dehydrogenase enzymes present in viable cells. The quantity of formazan produced is directly proportional to the number of metabolically active cells and serves as an indicator of cell survival following treatment.

The results of the MTT assay of L-Norvaline and *Sceletium tortuosum* extract exhibited high cell viability across all tested concentrations, indicating low cytotoxicity toward RAW 264.7 macrophages. The percentage viability remained above 88% even at the highest concentration tested, demonstrating that the treatments did not produce substantial toxic effects on macrophage cells.

Table 4: Effect of *Sceletium tortuosum* Extract on RAW 264.7 Cell Viability

Concentration (µg/mL)	L-Norvaline (% Cell Viability ± SD)	*S. tortuosum* (% Cell Viability ± SD)
250	98.34 ± 0.32	96.34 ± 0.64
500	96.25 ± 0.22	90.54 ± 0.35
1000	94.39 ± 0.71	88.36 ± 0.84

The results indicate a concentration-dependent reduction in cell viability for both treatments. However, the decline was relatively small and remained within acceptable limits for in vitro pharmacological investigations. L-Norvaline exhibited excellent cellular compatibility, maintaining viability above 94% even at 1000 µg/mL. Similarly, the *Sceletium tortuosum*

extract maintained cell viability above 88% at the highest tested concentration, indicating that the extract exerted only mild effects on cellular metabolic activity.

At 250 µg/mL, *Sceletium tortuosum* extract demonstrated a cell viability of 96.34%, which was comparable to that observed with L-Norvaline (98.34%). The minimal reduction in viability suggests that the extract does not adversely affect macrophage survival at lower concentrations. Such findings are particularly important because subsequent evaluation of arginase inhibitoryactivity requires concentrations that do not interfere with normal cellular function. The observed viability above 95% indicates that the extract can be safely utilized for biological investigations without causing significant cellular damage.

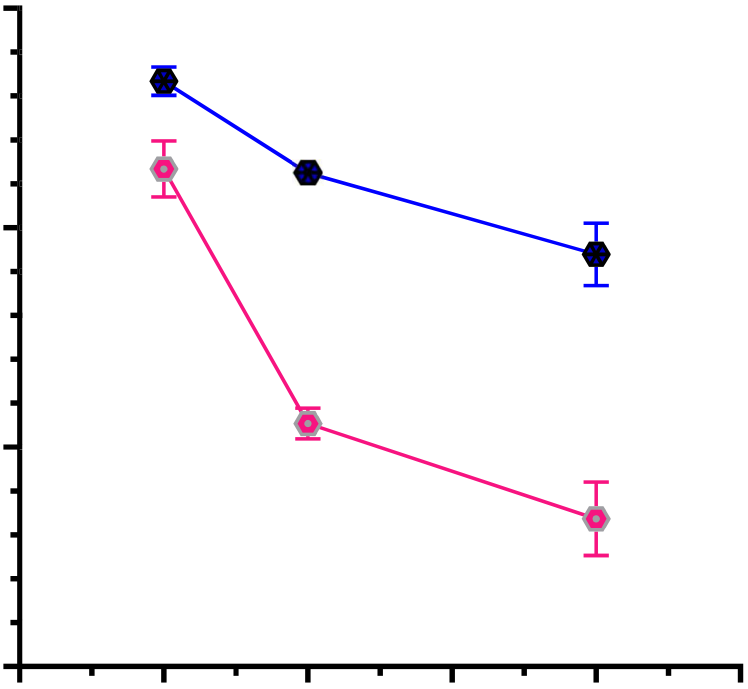

Figure 7: Effect of *Sceletium tortuosum* Extract on RAW 264.7 Cell Viability

At 500 µg/mL, a slight decrease in viability was observed in both treatment groups. Cells treated with *Sceletium tortuosum* extract exhibited 90.54% viability, whereas those treated with L-Norvaline maintained 96.25% viability. Although a reduction in viability was evident, more than 90% of cells remained metabolically active. This observation suggests that the extract possesses good biocompatibility and does not induce substantial mitochondrial dysfunction or membrane damage within the tested concentration range.

The highest concentration tested (1000 µg/mL) resulted in cell viability values of 94.39% and 88.36% for L-Norvaline and *Sceletium tortuosum*, respectively. Despite the concentration-dependent decline, viability remained considerably above the generally accepted cytotoxicity threshold of 70–80% commonly used in cell culture studies. These findings indicate that the

extract does not exert significant toxic effects even at elevated concentrations and can therefore be considered safe for further mechanistic investigations.

The concentration-dependent decrease in viability observed with the extract may be attributed to the presence of bioactive phytochemicals capable of interacting with cellular metabolic pathways. *Sceletium tortuosum* is known to contain several alkaloids, including mesembrine, mesembrenone, mesembrenol, and mesembranol, as well as phenolic and flavonoid compounds. While these phytoconstituents possess numerous beneficial pharmacological activities, higher concentrations may produce mild alterations in cellular metabolism, resulting in a modest reduction in MTT reduction capacity. However, the reduction observed in the present study was not sufficient to indicate significant cytotoxicity.

The maintenance of high cell viability across all tested concentrations suggests that the extract is well tolerated by RAW 264.7 macrophages. This observation is particularly relevant because macrophages play a critical role in inflammatory processes associated with vascular disorders, including varicose veins. Preservation of macrophage viability ensures that any subsequent effects observed in arginase inhibition studies are likely to be related to pharmacological modulation rather than nonspecific cellular toxicity.

The findings also support the hypothesis that the bioactive constituents present in *Sceletium tortuosum* may exert immunomodulatory effects without compromising cell survival. Previous studies have reported antioxidant and anti-inflammatory activities of *Sceletium tortuosum*, which may contribute to its protective effects against oxidative and inflammatory stress. The presence of antioxidant phytochemicals could help maintain cellular integrity and reduce oxidative damage, thereby contributing to the high viability observed in treated cells.

An important outcome of the present study is that the IC_{50} value was not reached within the tested concentration range. Since cell viability remained above 88% even at 1000 µg/mL, the concentration required to reduce viability by 50% would be expected to be substantially higher than the maximum concentration tested. This indicates a wide safety margin and confirms the low cytotoxic potential of the extract. Consequently, the tested concentrations were considered appropriate for subsequent arginase inhibitory and anti-inflammatory investigations.

1.1. Arginase inhibition assay

The effect of *Sceletium tortuosum* extract on arginase activity was evaluated in LPS and 8-bromo-cAMP stimulated RAW 264.7 macrophages using a urea estimation assay. Arginase activity was determined by measuring the amount of urea produced following enzymatic hydrolysis of L-arginine.

Table 5: Effect of *Sceletium tortuosum* Extract on Total Protein Content and Arginase Activity in RAW 264.7 Macrophages

Group	**Treatment**	**Total Protein Content (µg/mL) (Mean ± SD)**	**Arginase Activity (Urea Units) (Mean ± SD)**
G1	Vehicle Control	36.87 ± 0.35	18.78 ± 0.37
G2	LPS + 8-bromo-cAMP	19.13 ± 0.35	87.45 ± 1.22
G3	*Sceletium tortuosum* Extract (1000 µg/mL) + LPS + 8-bromo-cAMP	80.10 ± 0.70	30.76 ± 0.54
G4	L-Norvaline + LPS + 8-bromo-cAMP	86.87 ± 0.60	23.82 ± 0.34

The vehicle control group exhibited basal arginase activity of 18.78 ± 0.37 urea units. Treatment with LPS and 8-bromo-cAMP significantly increased arginase activity to 87.45 ± 1.22 urea units, confirming successful induction of arginase expression in RAW 264.7 macrophages.

Treatment with *Sceletium tortuosum* extract markedly reduced arginase activity to 30.76 ± 0.54 urea units. The standard inhibitor L-Norvaline further reduced arginase activity to 23.82 ± 0.34 urea units. Both treatments demonstrated substantial suppression of enzyme activity when compared with the induction control group.

Total protein content was also markedly altered following induction. The induction control group showed reduced protein content (19.13 ± 0.35 µg/mL), whereas treatment with *Sceletium tortuosum* and L-Norvaline restored protein levels to 80.10 ± 0.70 µg/mL and 86.87 ± 0.60 µg/mL, respectively.The percentage inhibition of arginase activity relative to the induction control was calculated as 64.83% for *Sceletium tortuosum* extract and 72.76% for L-Norvaline.

The present investigation demonstrated that stimulation of RAW 264.7 macrophages with LPS and 8-bromo-cAMP resulted in a substantial increase in arginase activity. This observation confirms successful activation of inflammatory pathways and induction of arginase expression in macrophages. Increased arginase activity is a characteristic feature of activated macrophages and contributes significantly to inflammatory tissue remodeling and vascular dysfunction.

LPS acts through Toll-like receptor-4 (TLR4) signaling, leading to activation of several intracellular pathways including NF-κB and MAPK cascades. These pathways promote transcription of inflammatory mediators and enzymes involved in macrophage activation. The addition of 8-bromo-cAMP further enhances arginase expression through cyclic AMP-

mediated signaling pathways. Consequently, the induction control group exhibited the highest arginase activity among all experimental groups.

Treatment with *Sceletium tortuosum* extract produced a pronounced reduction in arginase activity. The decrease from 87.45 ± 1.22 to 30.76 ± 0.54 urea units demonstrates significant suppression of enzymatic activity. The extent of inhibition approached that observed with the standard inhibitor L-Norvaline, indicating substantial arginase inhibitory potential of the extract.

The observed activity may be attributed to the presence of bioactive alkaloids such as mesembrine, mesembrenone, mesembrenol, and mesembranol, together with phenolic and flavonoid constituents. These compounds possess well-documented antioxidant and anti-inflammatory properties and may regulate inflammatory signaling pathways involved in arginase induction.

An important observation was the restoration of total protein content in extract-treated cells. The induction control group displayed markedly reduced protein levels, whereas treatment with *Sceletium tortuosum* restored protein content to values exceeding those observed in the vehicle control group. This finding suggests preservation of cellular integrity and maintenance of normal metabolic activity. The protein restoration data are consistent with the MTT assay findings, which demonstrated high cellular viability following extract treatment.Arginase plays a critical role in regulating L-arginine metabolism. Increased arginase activity reduces the availability of L-arginine for nitric oxide synthase (NOS), thereby decreasing nitric oxide production. Reduced nitric oxide levels contribute to endothelial dysfunction, impaired vascular relaxation, oxidative stress, and chronic inflammation. Therefore, inhibition of arginase may help restore nitric oxide bioavailability and improve vascular homeostasis.

The present findings are particularly relevant to varicose vein pathology. Chronic venous disease is characterized by persistent inflammation, oxidative stress, endothelial dysfunction, leukocyte infiltration, and abnormal vascular remodeling. Elevated arginase activity has been implicated in many of these pathological processes. Consequently, suppression of arginase activity may represent a promising therapeutic approach for limiting vascular inflammation and preserving venous function.

The ability of *Sceletium tortuosum* extract to significantly inhibit arginase activity while maintaining cell viability suggests that the extract may possess therapeutic potential as a natural arginase-modulating agent. These findings support its further investigation as a candidate for the management of inflammatory vascular disorders including varicose veins.

1.2. Proposed mechanism of action of arginase inhibition

The results of the present study demonstrated that the hydroalcoholic root extract of *Sceletium tortuosum* significantly reduced arginase activity in LPS and 8-bromo-cAMP stimulated RAW 264.7 macrophages. Although the precise molecular mechanism requires further investigation,

the observed activity may be explained through a combination of direct arginase inhibition, modulation of inflammatory signaling pathways, antioxidant effects, regulation of macrophage function, and restoration of nitric oxide bioavailability. These mechanisms collectively contribute to the attenuation of inflammatory responses and may have particular relevance in the management of varicose vein pathology.

1.2.1. Role of Arginase in Inflammation and Vascular Dysfunction

Arginase is a manganese-dependent enzyme that catalyzes the hydrolysis of L-arginine into L-ornithine and urea. Two isoforms of arginase have been identified in mammals, namely arginase I and arginase II. Arginase I is primarily localized in the cytosol and is abundantlyexpressed in the liver, whereas arginase II is predominantly mitochondrial and is distributed in various tissues including vascular endothelial cells, smooth muscle cells, and immune cells.

In addition to its physiological role in the urea cycle, arginase plays a critical role in regulating immune responses and vascular homeostasis. One of the most important aspects of arginase function is its competition with nitric oxide synthase (NOS) for the common substrate L-arginine. Under normal physiological conditions, nitric oxide synthase utilizes L-arginine to generate nitric oxide (NO), a key signaling molecule responsible for vasodilation, inhibition of platelet aggregation, suppression of leukocyte adhesion, and maintenance of endothelial integrity.

During inflammation, increased arginase expression accelerates the metabolism of L-arginine into ornithine and urea, thereby reducing substrate availability for nitric oxide synthesis. This imbalance contributes to decreased nitric oxide production, endothelial dysfunction, oxidative stress, and vascular injury. Consequently, excessive arginase activity has been implicated in several inflammatory and vascular disorders, including atherosclerosis, hypertension, diabetes-associated vascular complications, and chronic venous diseases such as varicose veins.

1.2.2. Modulation of LPS-Induced Inflammatory Signaling

The inflammatory model employed in the present study involved stimulation of RAW 264.7 macrophages with lipopolysaccharide (LPS) and 8-bromo-cAMP. LPS is a potent endotoxin derived from the outer membrane of Gram-negative bacteria and serves as a powerful activator of macrophages.

Upon exposure to LPS, Toll-like receptor 4 (TLR4) present on macrophage surfaces becomes activated. TLR4 activation initiates a cascade of intracellular signaling events involving adaptor proteins such as MyD88 and TRIF. These pathways subsequently activate nuclear factor-kappa B (NF-κB), activator protein-1 (AP-1), and mitogen-activated protein kinase (MAPK) signaling pathways.

Activation of these signaling pathways results in increased transcription of numerous inflammatory genes, including cytokines such as TNF-α, IL-1β, IL-6, inducible nitric oxide synthase, cyclooxygenase-2, and arginase. Consequently, macrophages adopt an activated inflammatory phenotype characterized by enhanced production of inflammatory mediators and increased enzyme expression.

The phytoconstituents present in *Sceletium tortuosum* may interfere with these signaling pathways at multiple levels. Several plant-derived alkaloids and polyphenolic compounds are known to suppress NF-κB activation by preventing phosphorylation and degradation of IκB proteins. Inhibition of NF-κB translocation to the nucleus reduces the transcription of inflammatory genes and consequently limits arginase induction.

Similarly, inhibition of MAPK pathways including ERK, JNK, and p38 MAPK may contribute to reduced expression of inflammatory mediators. Through suppression of these pathways, *Sceletium tortuosum* may effectively attenuate macrophage activation and decrease arginase production.

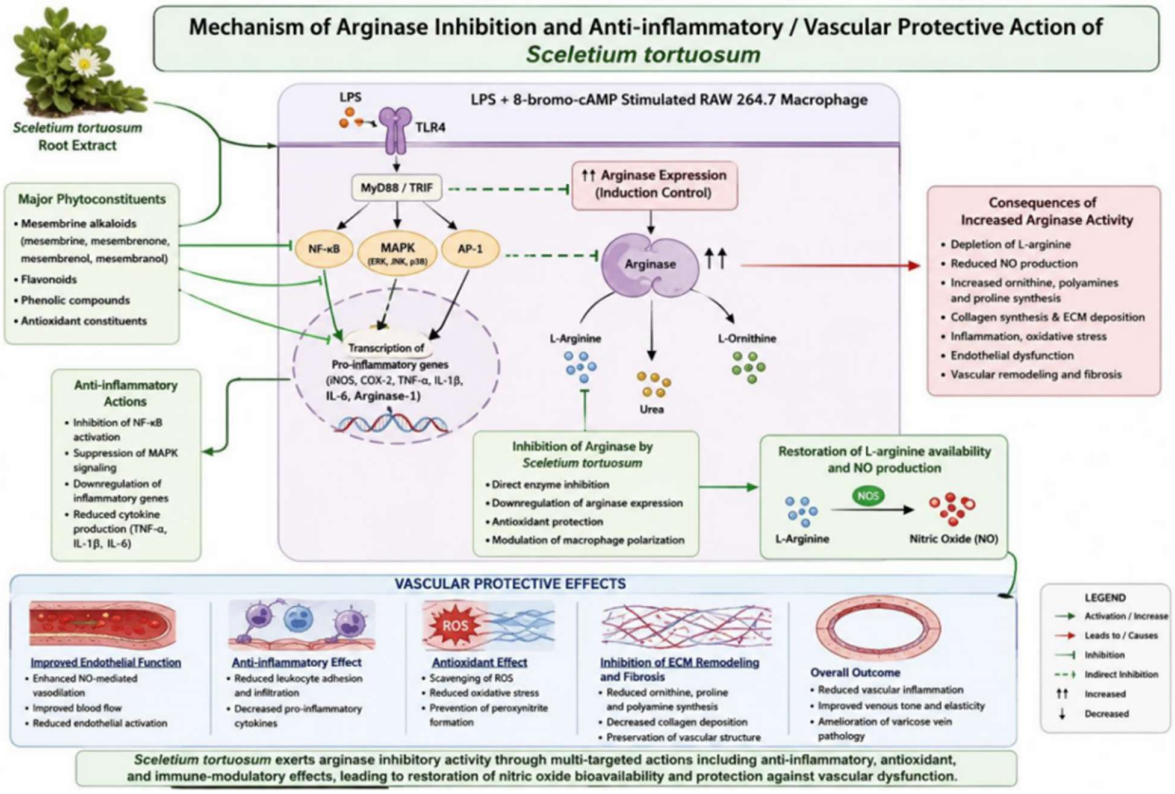

Figure 8: Proposed mechanism of action of *S. tortuosum* extract on arginase inhibition

1.1.1. Antioxidant-Mediated Suppression of Arginase Activity

Oxidative stress is closely associated with inflammatory responses and vascular dysfunction. Activated macrophages generate excessive amounts of reactive oxygen species (ROS),

including superoxide anions, hydrogen peroxide, and hydroxyl radicals. These reactive molecules contribute to cellular injury, activation of inflammatory signaling pathways, and induction of enzyme systems involved in tissue remodeling.

The roots of *Sceletium tortuosum* contain various antioxidant constituents capable of scavenging free radicals and reducing oxidative stress. Phenolic compounds and flavonoids present in the extract can neutralize reactive oxygen species before they damage cellular components.

Reduction of oxidative stress may indirectly suppress arginase expression by limiting redox-sensitive signaling pathways. Oxidative stress is known to activate NF-κB and MAPK pathways, which promote inflammatory gene expression. Therefore, antioxidant activity may contribute significantly to the reduction in arginase activity observed in the present study.

Furthermore, excessive ROS can react with nitric oxide to form peroxynitrite, thereby reducing nitric oxide bioavailability. By reducing oxidative stress, *Sceletium tortuosum* may preserve nitric oxide levels and support endothelial function.

1.1.2. Restoration of Nitric Oxide Bioavailability

One of the most important consequences of arginase inhibition is the restoration of nitric oxide production. Nitric oxide is a critical regulator of vascular physiology and plays an essential role in maintaining normal blood vessel function.

Under inflammatory conditions, elevated arginase activity reduces intracellular L-arginine concentrations, limiting substrate availability for nitric oxide synthase. This phenomenon is commonly referred to as the "arginine steal" effect. As arginase consumes increasing amounts of L-arginine, nitric oxide synthesis becomes progressively impaired.

By inhibiting arginase activity, *Sceletium tortuosum* may increase intracellular L-arginine availability, thereby enhancing nitric oxide production. Increased nitric oxide levels can promote vasodilation, improve blood flow, inhibit platelet activation, suppress leukocyte adhesion, and reduce endothelial inflammation.

Restoration of nitric oxide signaling is particularly important in chronic venous disease, where endothelial dysfunction contributes significantly to disease progression. Improved nitric oxide

availability may help counteract vascular abnormalities associated with varicose veins and support maintenance of vascular homeostasis.

1.1.3. Regulation of Macrophage Polarization

Macrophages exhibit remarkable functional plasticity and can adopt different activation states depending on environmental stimuli. Two major phenotypes are commonly recognized: classically activated M1 macrophages and alternatively activated M2 macrophages.

M1 macrophages produce large amounts of pro-inflammatory cytokines and reactive oxygen species and are involved in host defense and inflammatory responses. M2 macrophages are associated with tissue repair, wound healing, extracellular matrix remodeling, and expression of arginase.

Although arginase expression is often associated with M2 polarization, excessive activation of arginase-mediated pathways can promote pathological tissue remodeling and fibrosis. Increased ornithine production stimulates synthesis of polyamines and proline, which are essential precursors for collagen formation and extracellular matrix deposition.

The bioactive compounds present in *Sceletium tortuosum* may regulate macrophage polarization and maintain a balanced immune response. By modulating cytokine production and intracellular signaling pathways, the extract may prevent excessive activation of pathways that contribute to chronic inflammation and abnormal tissue remodeling.

1.1.4. Prevention of Extracellular Matrix Remodeling and Fibrosis

One of the downstream consequences of excessive arginase activity is increased production of ornithine. Ornithine serves as a precursor for polyamines and proline, molecules that play essential roles in cell proliferation, collagen synthesis, and extracellular matrix formation.

Persistent activation of arginase can therefore promote excessive collagen deposition and fibrosis. In vascular tissues, abnormal extracellular matrix remodeling contributes to structural alterations of the vessel wall, reduced elasticity, and progressive deterioration of vascular function.Varicose veins are characterized by significant remodeling of the venous wall, including changes in collagen and elastin content, smooth muscle dysfunction, and chronic inflammatory infiltration. Excessive arginase activity may contribute to these pathological alterations by promoting fibrotic processes and abnormal tissue repair.

By reducing arginase activity, *Sceletium tortuosum* may limit excessive ornithine production and subsequently reduce collagen accumulation and vascular remodeling. Such effects may help preserve normal venous architecture and slow disease progression.

1.1.5. Relevance to Varicose Vein Pathology

The findings of the present study have particular significance in the context of varicose vein pathology. Chronic venous disease is increasingly recognized as an inflammatory vascular disorder involving endothelial dysfunction, leukocyte recruitment, oxidative stress, cytokine release, and extracellular matrix remodeling.

Increased arginase activity contributes to several of these pathological mechanisms by reducing nitric oxide bioavailability and promoting fibrotic changes within the venous wall. Therefore, arginase inhibition has emerged as a promising therapeutic target for the management of chronic venous disorders.

The substantial reduction in arginase activity observed following treatment with *Sceletium tortuosum* suggests that the extract may interrupt multiple pathological pathways simultaneously. Through suppression of inflammatory signaling, reduction of oxidative stress, restoration of nitric oxide production, regulation of macrophage activation, and prevention of abnormal extracellular matrix remodeling, the extract may provide comprehensive vascular protection.

1.2. Nitric Oxide Estimation by Griess Assay

Nitric oxide production in RAW 264.7 macrophage cells was assessed indirectly by measuring total nitrite accumulation in the culture supernatant using the Griess reagent method. Since nitric oxide is an unstable free radical with a very short half-life, its stable oxidation product, nitrite, is commonly quantified as an indirect marker of nitric oxide generation. In the present study, nitrite levels were measured after 24 hours of treatment in control, LPS-induced, *Sceletium tortuosum*-treated, and L-Norvaline-treated groups.The results demonstrated that stimulation with LPS markedly increased nitrite production in RAW 264.7 macrophages. The control group showed a basal total nitrite concentration of 9.472
± 0.143 µM/mL. In contrast, the LPS-treated induction control group showed a substantial increase in total nitrite concentration to 21.480 ± 0.341 µM/mL. This increase confirmed successful activation of macrophages and induction of inflammatory nitric oxide production.

Treatment with *Sceletium tortuosum* extract at 1000 µg/mL reduced total nitrite concentration to 16.397 ± 0.190 µM/mL when compared with the LPS-induced group. This indicates that the extract was able to suppress LPS-induced nitric oxide production in activated macrophages. The standard group treated with L-Norvaline showed a total nitrite concentration of 9.256 ±
0.125 µM/mL, which was close to the basal control value, indicating strong suppression of nitrite production.

Table 6: Effect of *Sceletium tortuosum* Extract on Total Nitrite Production in LPS-Stimulated RAW 264.7 Macrophages

Group	**Treatment**	**Total Nitrite (µM/mL) Mean ± SD**
G1	Control	9.472 ± 0.143
G2	LPS (1 µg/mL)	21.480 ± 0.341
G3	*Sceletium tortuosum* extract (1000 µg/mL) + LPS	16.397 ± 0.190
G4	L-Norvaline (1000 µg/mL) + LPS	9.256 ± 0.125

Values are expressed as Mean ± SD, n = 3.
The percentage reduction in nitrite production was calculated in comparison with the LPS-induced control group. *Sceletium tortuosum* extract reduced nitrite production by approximately 23.67%, whereas L-Norvaline reduced nitrite production by approximately 56.91% when compared with the LPS group.

Table 7: Percentage Reduction of Total Nitrite Production

Group	**Total Nitrite (µM/mL)**	**Percentage Reduction Compared with LPS Control**
LPS control	21.480	—
Sceletium tortuosum extract	16.397	23.67%
L-Norvaline	9.256	56.91%

The reduction in nitrite concentration in the *Sceletium tortuosum*-treated group indicates that the extract possesses anti-inflammatory potential by suppressing nitric oxide generation in activated macrophages. Although the inhibitory effect was lower than that observed with L-Norvaline, the extract produced a clear reduction in LPS-induced nitrite production, supporting its possible role in modulating macrophage-mediated inflammatory responses.

In the present investigation, LPS treatment significantly increased total nitrite production from 9.472 ± 0.143 µM/mL in the control group to 21.480 ± 0.341 µM/mL in the induction control group. This marked increase indicates that LPS successfully activated

macrophages and stimulated nitric oxide production. LPS is a potent inflammatory stimulus that activates macrophages through Toll-like receptor 4 signaling. This activation leads to downstream stimulation of inflammatory transcription factors, particularly nuclear factor-kappa B and mitogen-activated protein kinase pathways. These pathways promote the expression of inducible nitric oxide synthase, which catalyzes the conversion of L-arginine into nitric oxide.

Nitric oxide has dual biological roles. Under physiological conditions, it participates in host defense, vascular regulation, and immune signaling. However, excessive nitric oxide production during inflammation can contribute to tissue injury. In activated macrophages, large amounts of nitric oxide are produced through inducible nitric oxide synthase. This excessive nitric oxide may react with superoxide radicals to form peroxynitrite, a highly reactive nitrogen species capable of damaging proteins, lipids, DNA, and cellular membranes. Therefore, increased nitrite accumulation in the LPS group reflects an inflammatory response and oxidative/nitrosative stress in macrophages.

Treatment with *Sceletium tortuosum* extract reduced total nitrite production to 16.397 ± 0.190 µM/mL. This reduction suggests that the extract attenuated LPS-induced macrophage activation and suppressed nitric oxide generation. The decrease in nitrite level indicates that the extract may interfere with inflammatory signaling pathways responsible for inducible nitric oxide synthase expression or activity. The observed effect supports the anti-inflammatory potential of *Sceletium tortuosum* in macrophage-mediated inflammatory conditions.The reduction in nitrite production by *Sceletium tortuosum* is particularly important in the context of varicose vein pathology. Varicose veins and chronic venous disease are not merely mechanical disorders caused by venous valve incompetence; they are also associated with chronic inflammation, endothelial dysfunction, oxidative stress, leukocyte recruitment, macrophage infiltration, and extracellular matrix remodeling. Activated macrophages present within inflamed vascular tissues can release nitric oxide, cytokines, reactive oxygen species, and matrix metalloproteinases. These mediators contribute to venous wall injury, degradation of extracellular matrix, smooth muscle dysfunction, and progressive vascular remodeling.

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metalloproteinases. These mediators contribute to venous wall injury, degradation of extracellular matrix, smooth muscle dysfunction, and progressive vascular remodeling.

The ability of *Sceletium tortuosum* extract to reduce nitrite production suggests that it may help regulate excessive inflammatory mediator release from macrophages. By decreasing nitric oxide overproduction, the extract may reduce nitrosative stress and protect vascular cells frominflammatory damage. This effect may be beneficial in chronic venous disorders where persistent inflammation contributes to disease progression.

L-Norvaline, used as the standard compound in the present study, reduced total nitrite concentration to 9.256 ± 0.125 μM/mL. This value was nearly comparable to the control group, indicating strong suppression of LPS-induced nitrite production. L-Norvaline is known primarily as an arginase inhibitor. By modulating L-arginine metabolism, it can influence the balance between arginase and nitric oxide synthase pathways. The strong reduction observed with L-Norvaline indicates effective regulation of inflammatory nitrogen metabolism in activated macrophages.

The comparison between *Sceletium tortuosum* and L-Norvaline shows that the plant extract produced a moderate but meaningful reduction in nitrite production. While L-Norvaline exhibited stronger suppression, *Sceletium tortuosum* demonstrated a clear inhibitory effect, reducing nitrite production by 23.67% compared with the LPS group. This finding is significant because crude plant extracts contain complex mixtures of phytoconstituents and may exert their effects through multiple complementary mechanisms rather than one specific target.

The anti-inflammatory activity of *Sceletium tortuosum* may be attributed to its phytochemical constituents, especially mesembrine-type alkaloids, flavonoids, phenolic compounds, and antioxidant molecules. These compounds may suppress inflammatory mediator production by inhibiting activation of NF-κB and MAPK signaling pathways, reducing oxidative stress, and modulating macrophage responses. Phenolic and flavonoid compounds are particularly known for their ability to scavenge free radicals and suppress inflammatory enzyme expression. Alkaloids may also contribute to immunomodulatory effects by regulating intracellular signaling pathways.

Another important point is that the MTT assay performed previously showed that *Sceletium tortuosum* maintained high cell viability in RAW 264.7 macrophages. Therefore, the reduction in nitrite production observed in the present assay is unlikely to be due to nonspecific cytotoxicity. Instead, it suggests a true pharmacological modulation of inflammatory nitric oxide production. This is essential for interpreting anti-inflammatory results because a decrease in nitrite level caused by cell death would not indicate genuine anti-inflammatory activity. Since the extract demonstrated acceptable cell viability, its nitrite-lowering effect can be considered biologically relevant.Overall, the Griess assay results indicate that *Sceletium tortuosum* extract attenuated LPS-induced nitric oxide production in RAW 264.7 macrophages. The extract reduced total nitrite accumulation,

suggesting inhibition of inflammatory macrophage activation and possible suppression of inducible nitric oxide synthase-mediated nitric oxide generation. These findings support the potential role of *Sceletium tortuosum* as a natural anti-inflammatory agent with relevance to vascular inflammatory disorders such as varicose veins.

1.1. Proposed Mechanism of Action of nitric oxide

The possible mechanism of action of *Sceletium tortuosum* in reducing total nitrite production may involve multiple interconnected pathways related to macrophage activation, inflammatory signaling, oxidative stress, and L-arginine metabolism.

When RAW 264.7 macrophages are exposed to LPS, the LPS molecule binds to Toll-like receptor 4 on the macrophage surface. This receptor activation triggers intracellular signaling through adaptor proteins such as MyD88 and TRIF. These signaling molecules activate downstream pathways including NF-κB and MAPK. Once activated, NF-κB translocates into the nucleus and promotes transcription of inflammatory genes, including inducible nitric oxide synthase. Inducible nitric oxide synthase then catalyzes the conversion of L-arginine into nitric oxide and citrulline. The nitric oxide produced rapidly undergoes oxidation to form nitrite, which is detected by the Griess reaction.

In the LPS-induced group, this pathway was strongly activated, resulting in elevated nitrite production. The increase in total nitrite reflects enhanced inducible nitric oxide synthase activity and inflammatory macrophage activation. Excess nitric oxide produced under such inflammatory conditions may react with reactive oxygen species, particularly superoxide radicals, to form peroxynitrite. Peroxynitrite can damage cellular proteins, lipids, and nucleic acids and may further amplify inflammatory injury.

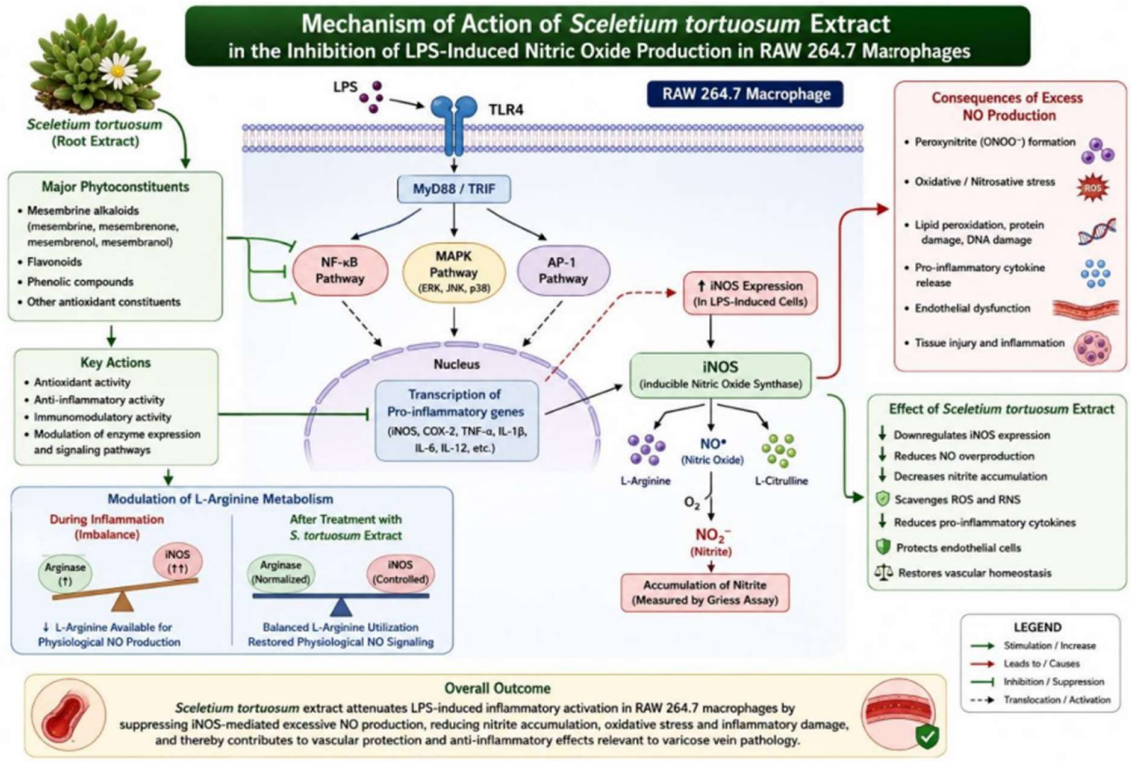

Figure 10: Proposed mechanism for inhibition of nitric oxide production

Sceletium tortuosum extract may interfere with this inflammatory cascade at several levels. Firstly, the phytoconstituents present in the extract may suppress activation of NF-κB. Inhibition of NF-κB prevents the transcriptional upregulation of inducible nitric oxide synthase, thereby reducing nitric oxide production. When less inducible nitric oxide synthase is expressed, less nitric oxide is produced from L-arginine, leading to lower nitrite accumulation in the culture supernatant.

Secondly, the extract may modulate MAPK signaling pathways, including ERK, JNK, and p38 MAPK. These pathways are important regulators of inflammatory mediator production in macrophages. Suppression of MAPK activation can reduce the expression of inflammatory enzymes and cytokines, including inducible nitric oxide synthase, TNF-α, IL-1β, and IL-6. Therefore, inhibition of MAPK signaling may contribute to the reduced nitrite production observed in the *Sceletium tortuosum*-treated group.

Thirdly, antioxidant constituents present in *Sceletium tortuosum* may reduce oxidative stress in activated macrophages. LPS stimulation increases the generation of reactive oxygen species, which can further activate inflammatory signaling pathways. By scavenging reactive oxygen

species, the extract may reduce redox-sensitive activation of NF-κB and MAPK. This antioxidant effect may indirectly suppress inducible nitric oxide synthase expression and reduce nitric oxide production.

Fourthly, *Sceletium tortuosum* may regulate the balance between arginase and nitric oxide synthase pathways. Both arginase and nitric oxide synthase utilize L-arginine as a common substrate. Arginase converts L-arginine into ornithine and urea, whereas nitric oxide synthase converts L-arginine into nitric oxide and citrulline. In inflammatory conditions, dysregulation of L-arginine metabolism contributes to abnormal nitric oxide production, oxidative stress, and vascular dysfunction. The previously observed arginase inhibitory activity of *Sceletium tortuosum* suggests that the extract may influence L-arginine metabolism and help normalize macrophage responses.

However, the interpretation of nitric oxide modulation requires careful understanding. Nitric oxide is protective at physiological levels but harmful when excessively produced during inflammation. In vascular tissues, endothelial nitric oxide supports vasodilation and vascular homeostasis. In contrast, excessive nitric oxide produced by activated macrophages through inducible nitric oxide synthase contributes to inflammatory and nitrosative injury. Therefore, the beneficial effect of *Sceletium tortuosum* is not simply the complete suppression of nitric oxide, but rather the regulation of excessive inflammatory nitric oxide production while potentially preserving physiological nitric oxide signaling.

This distinction is highly relevant to varicose vein pathology. In chronic venous disease, endothelial dysfunction, leukocyte adhesion, macrophage infiltration, and oxidative stress play major roles in disease progression. Excessive inflammatory nitric oxide and reactive nitrogen species may contribute to endothelial injury, venous wall weakening, extracellular matrix degradation, and tissue remodeling. By reducing LPS-induced nitrite production, *Sceletium tortuosum* may limit macrophage-derived inflammatory stress and reduce vascular injury.

At the same time, inhibition of excessive arginase activity may improve L-arginine availability for endothelial nitric oxide synthase under vascular conditions, thereby supporting physiological nitric oxide-mediated vasodilation. Thus, *Sceletium tortuosum* may exert a balancing effect on nitric oxide biology: reducing harmful inflammatory nitric oxide overproduction from macrophages while supporting vascular nitric oxide availability through arginase modulation.

SUMMARY AND CONCLUSION

- The roots of *Sceletium tortuosum* were successfully collected, authenticated, shade-dried, powdered, and extracted using 70% hydroalcoholic solvent by the maceration method. The extraction process yielded a dark brown semisolid extract with a percentage yield of 21.6%, indicating efficient recovery of bioactive phytoconstituents from the plant material.

- The hydroalcoholic extraction method proved suitable for isolating a broad spectrum of phytochemicals, including alkaloids, flavonoids, phenolic compounds, and other secondary metabolites that are potentially responsible for the biological activities of *Sceletium tortuosum*.
- Cytotoxicity evaluation was performed using the MTT assay in RAW 264.7 murine macrophage cells. The extract demonstrated high cellular compatibility, maintaining cell viability of 96.34 ± 0.64%, 90.54 ± 0.35%, and 88.36 ± 0.84% at concentrations of 250,
  500, and 1000 µg/mL, respectively.
- The standard compound L-Norvaline exhibited cell viability values of 98.34 ± 0.32%, 96.25 ± 0.22%, and 94.39 ± 0.71% at corresponding concentrations. The results confirmed that both the extract and standard possessed minimal cytotoxicity toward RAW 264.7 macrophages.
- Since cell viability remained above 88% even at the highest concentration tested, the extract was considered safe for subsequent anti-inflammatory and arginase inhibition studies. Furthermore, the IC_{50} value was not attained within the tested concentration range, indicating low cytotoxic potential.
- Arginase activity was successfully induced in RAW 264.7 macrophages using LPS and 8-bromo-cAMP. The induction control group exhibited a marked increase in arginase activity (87.45 ± 1.22 urea units) compared with the vehicle control (18.78 ± 0.37 urea units), confirming successful activation of inflammatory pathways.
- Treatment with *Sceletium tortuosum* extract (1000 µg/mL) significantly reduced arginase activity to 30.76 ± 0.54 urea units, indicating substantial inhibition of the enzyme in activated macrophages. The reduction demonstrated the ability of the extract to regulate L-arginine metabolism under inflammatory conditions.
- The standard inhibitor L-Norvaline reduced arginase activity to 23.82 ± 0.34 urea units, validating the assay system and providing a positive control for comparison. The activity of the extract approached that of the standard inhibitor, suggesting considerable arginase inhibitory potential.The percentage inhibition of arginase activity produced by *Sceletium tortuosum* was approximately 64.83%, while L-Norvaline produced an inhibition of approximately 72.76%. These findings indicate that the extract possesses strong enzyme-modulating activity in activated macrophages.
- Total protein estimation revealed a marked reduction in protein content in the induction control group (19.13 ± 0.35 µg/mL) compared with the vehicle control (36.87 ± 0.35 µg/mL). This reduction reflects cellular stress associated with inflammatory activation.
- Treatment with *Sceletium tortuosum* restored total protein content to 80.10 ± 0.70 µg/mL, whereas L-Norvaline increased protein levels to 86.87 ± 0.60 µg/mL. The restoration of protein content suggests preservation of cellular integrity and metabolic activity following treatment.

- Nitric oxide production was indirectly assessed through measurement of total nitrite concentration using the Griess assay. The induction control group exhibited a significant increase in nitrite production (21.480 ± 0.341 μM/mL) compared with the untreated control (9.472 ± 0.143 μM/mL), confirming successful induction of inflammatory nitric oxide synthesis.
- Treatment with *Sceletium tortuosum* extract reduced total nitrite concentration to 16.397 ± 0.190 μM/mL, demonstrating suppression of excessive nitric oxide production in activated macrophages. This reduction indicates significant anti-inflammatory activity of the extract.
- L-Norvaline further reduced nitrite concentration to 9.256 ± 0.125 μM/mL, producing values comparable to the untreated control group. This observation confirms the effectiveness of the standard treatment in regulating inflammatory nitric oxide generation.
- The percentage reduction in nitrite production achieved by *Sceletium tortuosum* was approximately 23.67%, whereas L-Norvaline produced a reduction of approximately 56.91%. These results indicate that the extract effectively attenuated macrophage-mediated inflammatory responses.
- The observed biological activities may be attributed to the presence of mesembrine alkaloids, flavonoids, phenolic compounds, and antioxidant constituents present in *Sceletium tortuosum*. These phytochemicals are known to modulate inflammatory signaling pathways and protect cells against oxidative damage.
- Mechanistically, the extract appears to suppress LPS-induced macrophage activation by regulating NF-κB and MAPK signaling pathways, reducing inducible nitric oxide synthase expression, inhibiting arginase activity, and decreasing inflammatory mediator production.Arginase inhibition by *Sceletium tortuosum* may enhance intracellular L-arginine availability and help restore nitric oxide homeostasis. This mechanism is particularly relevant in vascular inflammatory conditions where dysregulated arginase activity contributes to endothelial dysfunction and tissue remodeling.
- The reduction in excessive nitric oxide production, together with suppression of arginase activity, suggests that the extract possesses dual anti-inflammatory and immunomodulatory effects. These activities may help attenuate oxidative stress, nitrosative stress, and inflammatory vascular injury.
- Overall, the findings of the present study demonstrate that the hydroalcoholic root extract of *Sceletium tortuosum* possesses significant arginase inhibitory and anti-inflammatory activity in LPS-stimulated RAW 264.7 macrophages. The extract exhibited low cytotoxicity, effectively reduced arginase activity and nitrite production, restored protein content, and showed activity approaching that of L-Norvaline, supporting its potential as a natural therapeutic candidate for inflammation-associated vascular disorders with particular relevance to varicose vein pathology.

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