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IN VITRO ANTI-INFLAMMATORY POTENTIAL OF *ORTHOSIPHON THYMIFLORUS* (ROTH) SLEESEN EXTRACTS VIA INHIBITION OF CYCLOOXYGENASE, LIPOXYGENASE, AND PROTEIN DENATURATION

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ABSTRACT

Inflammation is a key pathological factor in many chronic diseases. The genus *Orthosiphon* is traditionally used for inflammatory conditions, but *O. thymiflorus* remains underexplored. This study evaluated the *in vitro* anti-inflammatory efficacy of chloroform and ethanolic extracts of *O. thymiflorus* whole plant. Anti-inflammatory activity was assessed using three complementary *in vitro* assays: Cyclooxygenase (COX) inhibition assay using LPS-stimulated RAW 264.7 macrophage cell lysates; Lipoxygenase (LOX) inhibition assay using the same cell line; and heat-induced egg albumin denaturation assay. Diclofenac sodium was used as the standard reference drug. Both extracts exhibited concentration-dependent inhibition across all assays. In the COX assay, the ethanolic extract demonstrated superior activity (33.51% inhibition at 100µg/mL) compared to the chloroform extract (25.95%). A similar trend was observed in the LOX assay (Ethanolic: 31.58%; Chloroform: 26.04% at 100 µg/mL). The albumin denaturation assay confirmed the membrane-stabilizing potential of both extracts, with ethanolic extract showing greater protective effect at higher concentrations. The ethanolic extract of *O.*

thymiflorus possesses significant anti-inflammatory activity, likely mediated through dual inhibition of COX and LOX pathways and stabilization of protein structures. These findings validate the traditional use of the plant and suggest its potential as a source for novel anti-inflammatory agents.

KEYWORDS: *Orthosiphon thymiflorus*; RAW 264.7 Macrophages; Cyclooxygenase (COX) Assay; Lipoxygenase (LOX) Assay; Albumin Denaturation; Anti-inflammatory; Diclofenac.

1. INTRODUCTION

Inflammation is a complex biological response of vascular tissues to harmful stimuli, including pathogens, damaged cells, or irritants. While acute inflammation is a protective mechanism essential for healing, unresolved chronic inflammation contributes to the pathogenesis of numerous debilitating diseases such as rheumatoid arthritis, inflammatory bowel disease, atherosclerosis, and even cancer.[1] The inflammatory cascade is driven by the metabolism of arachidonic acid (AA) via two principal enzymatic pathways: cyclooxygenase (COX) and lipoxygenase (LOX). These pathways generate potent lipid mediators, including prostaglandins (PGs) and leukotrienes (LTs), which orchestrate vasodilation, pain sensitization, and leukocyte recruitment.[2]

Current pharmacotherapy for inflammation relies heavily on non-steroidal anti-inflammatory drugs (NSAIDs) and corticosteroids. NSAIDs exert their effects primarily by inhibiting COX enzymes (COX-1 and COX-2), thereby reducing prostaglandin synthesis.[3] However, long-term use of conventional NSAIDs is frequently associated with significant adverse effects, particularly gastrointestinal ulceration, renal impairment, and cardiovascular risks, largely due to the non-selective inhibition of the gastroprotective COX-1 isoform.[4] Consequently, there is a sustained global interest in discovering novel anti-inflammatory agents from natural sources that may offer improved safety profiles and alternative mechanisms of action, such as dual inhibition of COX and LOX pathways.[5]

The genus *Orthosiphon* (family Lamiaceae) is a rich source of bioactive flavonoids, phenolic acids, and terpenoids. *Orthosiphon stamineus* (Java tea) is well-documented for its anti-inflammatory, antioxidant, and diuretic properties, traditionally used across Southeast Asia for kidney and urinary tract ailments.[6,7] *Orthosiphon thymiflorus* (Roth) Sleesen, a closely related species distributed in South India and parts of tropical Africa, has been utilized in regional Ayurvedic and folk medicine for similar indications, including joint pain, edema, and

mild urinary tract inflammation.[8,9] Despite its traditional relevance, scientific validation of the anti-inflammatory activity of *O. thymiflorus* remains extremely limited, with a scarcity of *in vitro* mechanistic studies.

Previous phytochemical investigations on *O. thymiflorus* have revealed the presence of key secondary metabolites, including rosmarinic acid, eupatorin, sinensetin, and various triterpenoids, which are known to modulate inflammatory signaling pathways.[10,11] Given this phytochemical background and traditional usage, the present study was designed to evaluate the *in vitro* anti-inflammatory potential of chloroform and ethanolic extracts of *O. thymiflorus* whole plant using a multi-assay approach. This includes the assessment of COX and LOX enzyme inhibition using lipopolysaccharide (LPS)-stimulated RAW 264.7 murine macrophages and the evaluation of membrane stabilization activity via the albumin denaturation assay.

2. MATERIALS AND METHODS

2.1 Collection, Authentication, and Extraction

The whole plant of *Orthosiphon thymiflorus* (Roth) Sleesen was collected from Thiruvananthapuram, Kerala, India, and authenticated by a qualified botanist. A voucher specimen was retained. The shade-dried, powdered plant material (60 g) was subjected to successive Soxhlet extraction using chloroform followed by 95% ethanol. The extracts were concentrated under reduced pressure and dried to obtain the crude chloroform extract (OTCE) and crude ethanolic extract (OTEE). The extracts were stored at 4°C until analysis.

2.2 Chemicals and Reagents

Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), lipopolysaccharide (LPS, *E. coli* serotype 0111:B4), arachidonic acid, linoleic acid, and diclofenac sodium were procured from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals and reagents used were of analytical grade.

2.3 Cell Culture and Pre-treatment

The RAW 264.7 murine macrophage cell line was procured from the National Centre for Cell Science (NCCS), Pune, India. Cells were maintained in DMEM supplemented with 10% heat-inactivated FBS and 1% antibiotic-antimycotic solution at 37°C in a humidified 5% CO_2 incubator. For the assays, cells were grown to 60-70% confluence and then stimulated with LPS (1 µg/mL) to induce an inflammatory response. The LPS-stimulated cells were

simultaneously treated with varying concentrations (6.25, 12.5, 25, 50, 100 µg/mL) of the test extracts (OTCE and OTEE) or the standard drug diclofenac sodium. The plates were incubated for 24 hours. Following incubation, cell lysates were prepared for enzymatic assays.

2.4 Cyclooxygenase (COX) Inhibition Assay

COX activity was determined using the method described by Walker and Gierse with slight modifications.[12] Briefly, the cell lysate was incubated in Tris-HCl buffer (pH 8.0) containing 5 mM glutathione and 20 µg/mL hemoglobin for 1 minute at 25°C. The reaction was initiated by the addition of arachidonic acid (200 mM) and allowed to proceed for 20 minutes at 37°C. The reaction was terminated by adding 10% trichloroacetic acid in 1 N hydrochloric acid. Following centrifugation, 1% thiobarbiturate was added to the supernatant, and absorbance was measured at 632 nm using a UV-Visible spectrophotometer.

2.5 Lipoxygenase (LOX) Inhibition Assay

LOX activity was assayed based on the method established by Axelrod et al. with modifications.[13] The reaction mixture (2 mL total volume) consisted of Tris-HCl buffer (pH 7.4), 50 µL of cell lysate, and sodium linoleate (200 µL, 10 mg/mL). LOX activity was monitored spectrophotometrically at 234 nm, reflecting the formation of 5-hydroxyeicosatetraenoic acid (5-HETE) from linoleate.

2.6 Calculation of Percentage Inhibition

The percentage inhibition of COX and LOX enzyme activities by the test samples was calculated using the following formula.

% Inhibition = [(Absorbance of Control - Absorbance of Test) / Absorbance of Control] × 100

Where "Control" refers to the absorbance of the LPS-stimulated, untreated cell lysate.

2.7 Albumin Denaturation Assay

The membrane stabilization activity was evaluated using the heat-induced egg albumin denaturation method as described by Mizushima and Kobayashi with modifications.[14] The reaction mixture (5 mL) consisted of 0.2 mL of fresh egg albumin, 2.8 mL of phosphate-buffered saline (PBS, pH 6.4), and 2 mL of varying concentrations of the test extracts or standard diclofenac. The mixture was incubated at 37°C for 30 minutes and then heated in a water bath at 70°C for 15 minutes. After cooling, the absorbance was measured at 660 nm. A

lower absorbance value indicates a greater inhibition of protein denaturation and thus higher membrane stabilization potential.

2.8 Statistical Analysis

All experiments were performed in triplicate, and results were expressed as mean ± standard deviation (SD). The concentration required to produce 50% inhibition (IC_{50}) was calculated using linear regression analysis in Microsoft Excel.

3. RESULTS

3.1 Cyclooxygenase (COX) Inhibition Assay

Both chloroform and ethanolic extracts of *O. thymiflorus* exhibited a concentration-dependent inhibition of COX enzyme activity in LPS-stimulated RAW 264.7 cell lysates. The ethanolic extract (OTEE) demonstrated superior inhibitory potential compared to the chloroform extract (OTCE) across all tested concentrations. At the highest tested concentration of 100 µg/mL, the ethanolic extract achieved **33.51%** inhibition, whereas the chloroform extract showed **25.95%** inhibition. The standard drug, diclofenac sodium, exhibited a potent inhibitory effect with an IC_{50} value of **52.19µg/mL** (Figure 1, Table 1). The IC_{50} values for both plant extracts were determined to be >100 µg/mL, indicating mild to moderate COX inhibitory activity.

Table 3.1: Percentage Inhibition of COX Enzyme Activity by Extracts and Standard.

Concentration (µg/mL)	Diclofenac Sodium (%)	Chloroform Extract (%)	Ethanolic Extract (%)
6.25	11.84	2.47	4.77
12.5	21.50	5.12	8.51
25	35.86	10.29	14.50
50	52.22	16.54	23.31
100	70.06	25.95	33.51

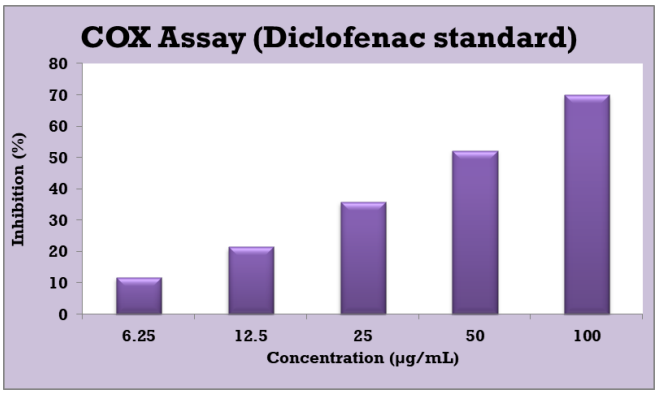

Figure 1: Percentage inhibition of COX enzyme activity by Diclofenac standard (Graphical Representation).

1) Sample Code: OTEE VHS 101 (chloroform extract).

Table 3.2: Absorbance values of chloroform extract.

Concentration (µg/mL)	Triplicate Values			Average OD
	OD 1	OD 2	OD 3	
Control (Without LPS)	0.233	0.223	0.218	0.225
Control (With LPS)	0.771	0.764	0.769	0.768
6.25	0.752	0.746	0.749	0.749
12.5	0.726	0.729	0.731	0.729
25	0.699	0.682	0.686	0.689
50	0.631	0.648	0.644	0.641
100	0.576	0.562	0.568	0.569

Table 3.3: Percentage inhibition of COX enzyme activity by chloroform extract.

Concentration (µg/mL)	Percentage of Inhibition
6.25	2.47
12.5	5.12
25	10.29
50	16.54
100	25.95
IC 50	**>100 µg/mL**

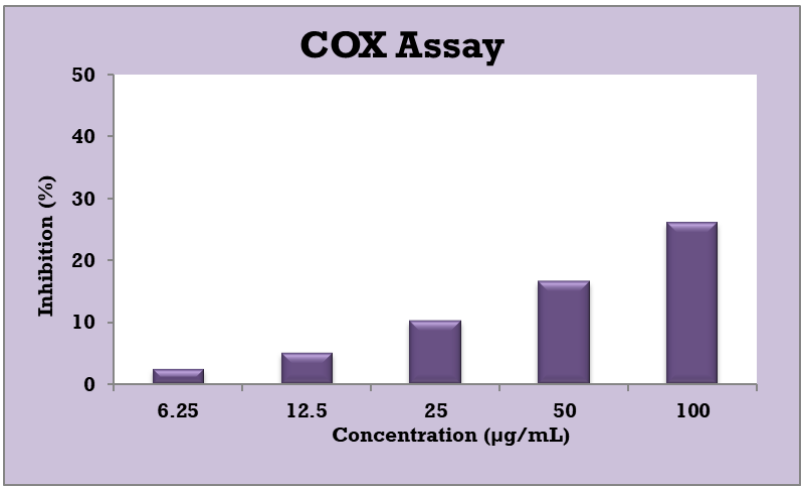

Figure 2: Percentage inhibition of COX enzyme activity by chloroform extract (Graphical Representation).

Inference: The sample studied exhibited cyclooxygenase (COX) inhibitory activity i.e. the sample has anti-inflammatory property. The maximum efficacy was elicited by the concentration **100 µg/mL**.

2) Sample Code: OTEE VHS 102 (Ethanolic extract)

Table 3.4: Absorbance values of ethanolic extract.

Concentration (µg/mL)	Triplicate Values			Average OD
	OD 1	OD 2	OD 3	
Control (Without LPS)	0.233	0.223	0.218	0.225
Control (With LPS)	0.771	0.764	0.769	0.768
6.25	0.729	0.731	0.734	0.731
12.5	0.700	0.702	0.706	0.703
25	0.652	0.655	0.663	0.657
50	0.583	0.594	0.590	0.589
100	0.516	0.502	0.514	0.511

Table 3.5: Percentage inhibition of COX enzyme activity by ethanolic extract.

Concentration (µg/mL)	Percentage of Inhibition
6.25	4.77
12.5	8.51
25	14.50
50	23.31
100	33.51
IC 50	**>100 µg/mL**

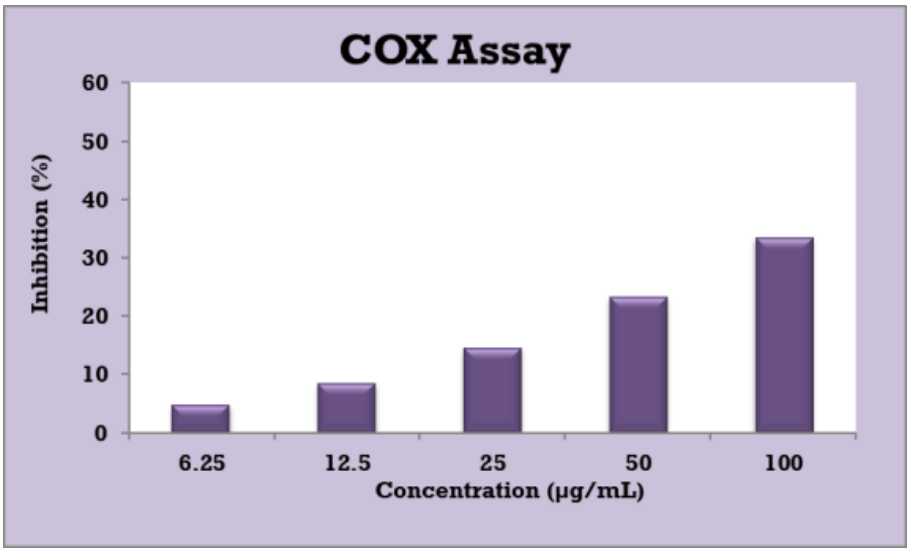

Figure 3: Percentage inhibition of COX enzyme activity by ethanolic extract (Graphical Representation).

Inference: The sample studied exhibited cyclooxygenase (COX) inhibitory activity i.e. the sample has anti-inflammatory property. The maximum efficacy was elicited by the concentration **100 µg/ml**.

3.2 Lipoxygenase (LOX) Inhibition Assay

Similar to the COX assay results, the extracts inhibited the LOX pathway in a concentration-dependent manner. The ethanolic extract again showed greater efficacy, with **31.58%** inhibition at 100 µg/mL, compared to **26.04%** for the chloroform extract at the same concentration. The standard drug diclofenac sodium exhibited **69.14%** inhibition at 100 µg/mL, with an IC_{50} of **60.22 µg/mL** (Table 2). The IC_{50} values for the plant extracts were >100 µg/mL.

Table 3.6: Percentage Inhibition of LOX Enzyme Activity by Extracts and Standard.

Concentration (µg/mL)	Diclofenac Sodium (%)	Chloroform Extract (%)	Ethanolic Extract (%)
6.25	6.84	1.40	4.85
12.5	14.34	4.44	7.41
25	30.03	9.22	13.66
50	47.96	14.42	21.07
100	69.14	26.04	31.58

Table 3.7: Percentage inhibition of LOX enzyme activity by Diclofenac standard.

Concentration (µg/mL)	Percentage of Inhibition
6.25	6.84
12.5	14.34
25	30.03
50	47.96
100	69.14
IC 50	**60.22 µg/mL**

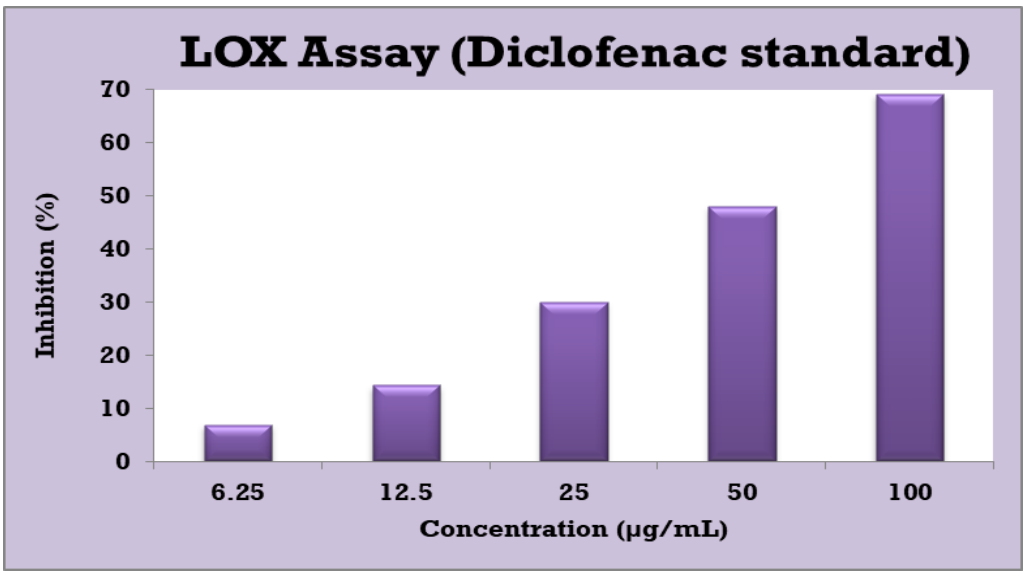

Figure 4: Percentage inhibition of LOX enzyme activity by Diclofenac standard (Graphical Representation)

1) Sample 1: Chloroform extract

Table 3.8: Absorbance values of chloroform extract.

Concentration (µg/mL)	Triplicate Values			Average OD
	OD 1	OD 2	OD 3	
Control (Without LPS)	0.060	0.065	0.068	0.064
Control (With LPS)	0.572	0.564	0.577	0.571
6.25	0.568	0.565	0.556	0.563
12.5	0.543	0.548	0.546	0.546
25	0.511	0.526	0.518	0.518
50	0.489	0.485	0.492	0.489
100	0.425	0.422	0.420	0.422

Table 3.9: Percentage inhibition of LOX enzyme activity by chloroform activity.

Concentration (µg/mL)	Percentage of Inhibition
6.25	1.40
12.5	4.44
25	9.22
50	14.42
100	26.04
IC 50	**>100 µg/mL**

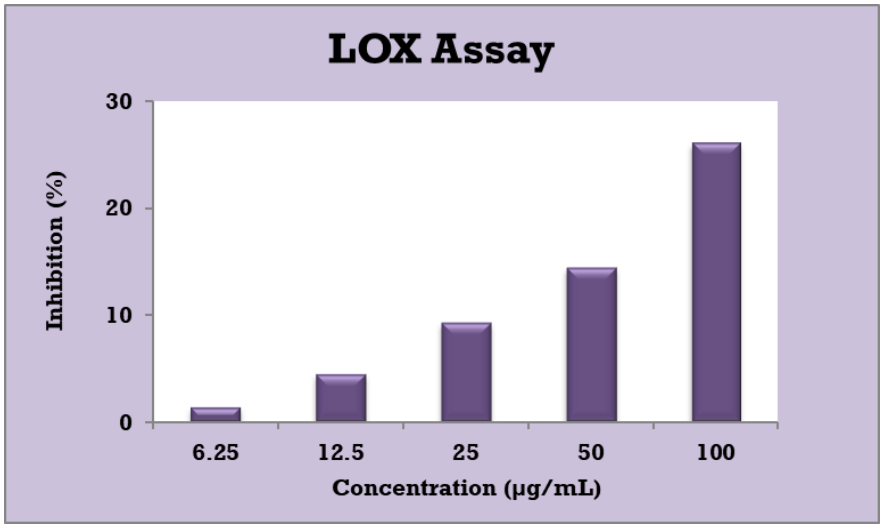

Figure 5: Percentage inhibition of LOX enzyme activity by chloroform extract (Graphical Representation).

Inference: The sample studied exhibited lipoxygenase (LOX) inhibitory activity i.e. the sample has anti-inflammatory property. The maximum efficacy was elicited by the concentration **100 µg/mL**.

2) Sample 2 :Ethanolic extract

Table 3.11: Absorbance values of ethanolic extract.

Concentration (µg/mL)	Triplicate Values			Average OD
	OD 1	OD 2	OD 3	
Control (Without LPS)	0.060	0.065	0.068	0.064
Control (With LPS)	0.572	0.564	0.577	0.571
6.25	0.542	0.540	0.548	0.543
12.5	0.522	0.531	0.533	0.529
25	0.491	0.495	0.493	0.493
50	0.455	0.451	0.446	0.451
100	0.386	0.391	0.395	0.391

Table 3.12: Percentage inhibition of LOX enzyme activity by ethanolic extract.

Concentration (µg/mL)	Percentage of Inhibition
6.25	4.85
12.5	7.41
25	13.66
50	21.07
100	31.58
IC 50	**>100 µg/mL**

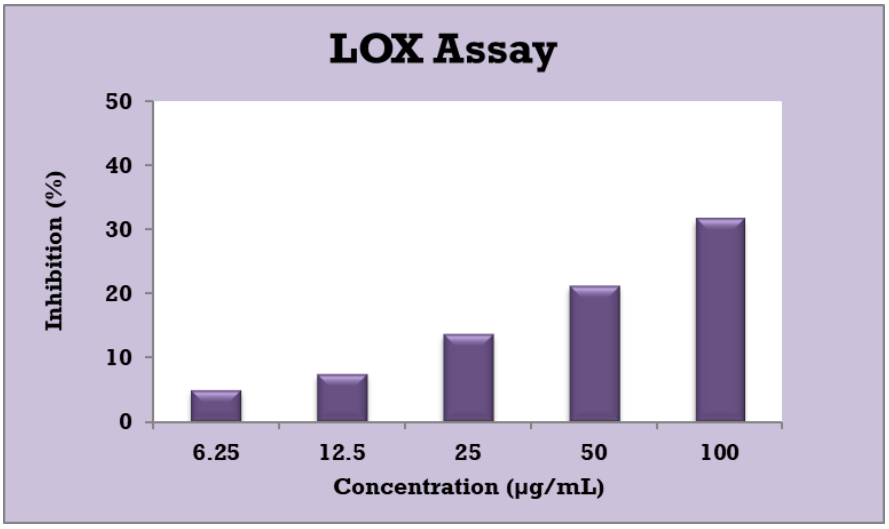

Figure 6: Percentage inhibition of LOX enzyme activity by ethanolic extract (Graphical Representation).

Inference: The sample studied exhibited lipoxygenase (LOX) inhibitory activity i.e. the sample has anti-inflammatory property. The maximum efficacy was elicited by the concentration **100 µg/mL**.

Figure 7: Image of RAW 264.7 cell lines used in LOX & COX Assay.

3.3 Albumin Denaturation Assay

The ability of the extracts to prevent heat-induced denaturation of egg albumin was assessed as a measure of membrane stabilization. The optical density (OD) decreased with increasing concentrations of both extracts, indicating a protective effect against protein denaturation. At 100 µg/mL, the ethanolic extract showed an OD of **0.983**, while the chloroform extract showed **0.914**, both significantly lower than the control, confirming their anti-denaturation potential.

Figure 8: Different concentrations of the mixture.

Table 3.13: Albumin denaturation Assay.

CONCENTRATION (µg/ml)	OPTICAL DENSITY OF ETHANOL EXTRACT	OPTICAL DENSITY OF CHLOROFORM EXTRACT
10µg/ml	1.839	1.731
20µg/ml	1.613	1.442
40µg/ml	1.366	1.283
80µg/ml	1.248	1.211
100µg/ml	0.983	0.914

OBSERVATION

It was found that the optical density wass decreased upon increased concentration of both ethanolic and choloform extract.

Inference

This indicates a decrease in protein denaturation on increasing concentration and thereby providing good anti-inflammatory activity.

4. DISCUSSION

Inflammation is mediated by a complex network of signaling molecules, among which the metabolites of arachidonic acid play a pivotal role. The dual inhibition of COX and LOX pathways is a therapeutically attractive strategy as it blocks the production of both prostaglandins (via COX) and leukotrienes (via LOX), while potentially avoiding the shunting of arachidonic acid metabolism toward the alternate pathway—a phenomenon linked to the gastrointestinal side effects of selective COX inhibitors.[5] The present study provides the first *in vitro* evidence that extracts of *Orthosiphon thymiflorus* possess the capacity to inhibit both of these critical pro-inflammatory enzymes.

The observed anti-inflammatory activity can be attributed to the rich phytochemical profile of the plant, particularly the presence of flavonoids (e.g., eupatorin, sinensetin) and phenolic acids (e.g., rosmarinic acid). Previous studies on related *Orthosiphon* species have demonstrated that flavonoids can suppress the expression of inducible COX-2 and the production of pro-inflammatory cytokines such as TNF-α and IL-6 in macrophages.[15,16] Rosmarinic acid, a major constituent in many Lamiaceae members, is a well-known inhibitor of the complement system and LOX activity, and it also reduces oxidative stress, a key amplifier of the inflammatory response.[17]

The ethanolic extract consistently outperformed the chloroform extract in all three assays. This is likely due to the higher polarity of ethanol, which facilitates the extraction of a broader spectrum of polar bioactive compounds, including flavonoid glycosides and phenolic acids, which are largely responsible for the observed bioactivity. The preliminary phytochemical screening of the extracts (data not shown) confirmed the presence of abundant phenols, flavonoids, and terpenoids in the ethanolic extract.

The albumin denaturation assay serves as a reliable *in vitro* model for evaluating the membrane-stabilizing potential of anti-inflammatory agents. Protein denaturation is a well-recognized cause of cellular damage and inflammation in conditions like rheumatoid arthritis.[14] The ability of *O. thymiflorus* extracts to significantly inhibit heat-induced albumin denaturation suggests that part of its anti-inflammatory mechanism involves protecting cellular and lysosomal membranes from injury, thereby limiting the release of inflammatory proteases and mediators. This activity aligns with the traditional use of the plant in managing joint pain and swelling.[8]

While the IC_{50} values of the crude extracts were higher than that of the standard drug diclofenac, this is expected for crude natural product mixtures compared to pure, isolated compounds. Bioactivity-guided fractionation of the ethanolic extract could lead to the isolation of more potent fractions or pure compounds with enhanced COX/LOX inhibitory activity. Furthermore, the dual inhibition exhibited by the extract, albeit moderate, holds promise for developing safer anti-inflammatory agents with reduced gastrointestinal liability.

5. CONCLUSION

This study provides the first *in vitro* evidence that *Orthosiphon thymiflorus* whole plant extracts possess significant anti-inflammatory activity. The ethanolic extract demonstrated superior efficacy compared to the chloroform extract, achieving 33.51% COX inhibition and 31.58% LOX inhibition at 100 µg/mL. Both extracts exhibited concentration-dependent inhibition across all three complementary assays, confirming their ability to modulate key inflammatory pathways. The dual inhibition of COX and LOX enzymes, coupled with membrane stabilization demonstrated in the albumin denaturation assay, supports the traditional use of this plant in managing inflammatory conditions. The observed activity is attributable to the rich phytochemical profile, particularly flavonoids and phenolic compounds present abundantly in the ethanolic extract. These findings scientifically validate the ethnopharmacological relevance of *O. thymiflorus* and highlight its potential as a promising natural source for developing novel, dual-acting anti-inflammatory agents with potentially improved safety profiles. Further *in vivo* investigations are warranted to establish clinical efficacy and safety.

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