

Phytopharmacological Investigation of *Leptadenia Reticulata* Root for Selective COX-1 and COX-2 Inhibition

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ABSTRACT

Leptadenia reticulata, a plant with a history of use in traditional medicine, was evaluated for its phytochemical composition and anti-inflammatory potential. Roots were collected, authenticated, and subjected to extraction using different solvents—ethanol, aqueous ethanol (80:20), hexane, and ethyl acetate. The extraction yields varied, with aqueous ethanol showing the highest yield (14.63%), followed by ethyl acetate (13.74%), ethanol (13.24%), and hexane (9.37%), reflecting solvent polarity's role in extraction efficiency. Phytochemical analysis revealed that ethanol and aqueous ethanol extracts were rich in flavonoids, tannins, phenolics, alkaloids, and glycosides, while hexane and ethyl acetate extracts contained terpenoids and steroids. The aqueous ethanol extract exhibited the highest total phenolic content (92.82 ± 1.89 mg GAE/g) and flavonoid content (86.91 ± 2.23 mg RE/g), correlating with strong antioxidant and anti-inflammatory potential. In COX inhibition assays, aqueous ethanol extract showed the strongest inhibition of COX-1 (IC_{50} of $34.57 \mu\text{g/mL}$) and COX-2 (IC_{50} of $31.38 \mu\text{g/mL}$), with flavonoids and phenolics primarily responsible for enzyme inhibition. These findings suggest that *Leptadenia reticulata*, particularly its aqueous ethanol extract, has substantial therapeutic potential as a natural anti-inflammatory agent, with selective COX-2 inhibition offering advantages in minimizing gastrointestinal toxicity. The study supports the plant's traditional use in managing inflammation and highlights its potential for further pharmacological and clinical exploration.

Keywords: *Leptadenia reticulata*, phytochemical composition, COX inhibition, anti-inflammatory, flavonoids, aqueous ethanol extract

INTRODUCTION

Inflammation is a complex physiological response to harmful stimuli, such as pathogens, tissue injury, or harmful irritants [1]. While acute inflammation is a protective mechanism that promotes healing, chronic inflammation is associated with numerous diseases, including arthritis, cardiovascular disorders, neurodegenerative diseases, and even cancer. Central to the process of inflammation is the cyclooxygenase (COX) enzymes, particularly COX-1 and COX-2, which catalyze the production of prostaglandins—lipid compounds that mediate inflammation [2, 3]. COX-1 is constitutively expressed and involved in the maintenance of normal physiological functions such as gastric mucosal protection and renal function [4], while COX-2 is inducible and primarily involved in the inflammatory response [5]. Due to their pivotal

roles in inflammation, these enzymes have become key targets for therapeutic intervention in inflammatory diseases. Non-steroidal anti-inflammatory drugs (NSAIDs), which inhibit COX activity, are commonly used to manage inflammation and pain [6]. However, long-term use of these drugs can lead to adverse effects, such as gastrointestinal irritation and renal damage, which has spurred interest in exploring safer, more natural alternatives.

In recent years, plant-based compounds have gained significant attention as potential sources of bioactive molecules that may serve as effective anti-inflammatory agents with fewer side effects [7]. Phytochemicals, which are naturally occurring chemical compounds found in plants, have been shown to possess a range of biological activities, including antioxidant, anti-inflammatory, and

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analgesic properties [8]. The identification and characterization of these compounds in medicinal plants are crucial steps in understanding their therapeutic potential and mechanism of action. *Leptadenia reticulata*, a member of the Asclepiadaceae family, is a perennial herb widely distributed across various regions of Africa and Asia [9]. Traditionally, the plant has been utilized in indigenous medicine systems for its broad-spectrum therapeutic properties, particularly for treating ailments associated with inflammation. In many cultures, the root of *Leptadenia reticulata* has been considered a valuable remedy for a variety of conditions, including rheumatism, joint pain, and swelling, which are often symptoms of underlying inflammatory disorders [10]. Despite its extensive use in ethnomedicine, the scientific validation of its pharmacological properties, especially its anti-inflammatory potential, remains relatively underexplored. Given the increasing interest in natural products for the development of novel therapeutic agents, *Leptadenia reticulata* presents a promising candidate for further investigation.

This study aims to fill this gap by evaluating the phytochemical composition of *Leptadenia reticulata* root extracts and assessing their anti-inflammatory potential through the inhibition of COX-1 and COX-2 enzymes. By extracting the bioactive compounds from the root and analyzing their chemical profile, we seek to identify key components that may be responsible for the plant's anti-inflammatory properties. The inhibition of COX enzymes will be assessed through established assays, which measure the reduction in prostaglandin production, a direct result of COX activity. These findings may provide a better understanding of how *Leptadenia reticulata* exerts its therapeutic effects and pave the way for its potential use in managing inflammatory conditions. The significance of this research extends beyond the identification of potential anti-inflammatory agents. The findings could contribute to the broader field of natural product-based drug discovery, where plant-derived compounds continue to play a vital role in the development of new treatments. Furthermore, the validation of traditional uses of *Leptadenia reticulata* through scientific investigation may promote the integration of indigenous knowledge with modern pharmacology, fostering the sustainable use of

medicinal plants in the development of new therapeutic strategies. As the global burden of chronic inflammatory diseases continues to rise, the need for novel, safe, and effective treatments has never been more pressing. This research, therefore, not only explores the pharmacological potential of *Leptadenia reticulata* but also contributes to the growing body of evidence supporting the role of plants in modern medicine.

METHODS AND MATERIALS

Collection, authentication and preparation of *L. reticulata* root extracts

Leptadenia reticulata roots were collected ensuring proper identification based on morphological characteristics and consultation with botanical experts. Voucher specimens were prepared, and the plant material was authenticated. The collected roots were thoroughly washed to remove adhering soil and debris. Subsequently, they were air-dried in shade to retain the phytochemical constituents. The air-dried roots were coarsely powdered using a mechanical grinder to increase the surface area for efficient extraction. Coarsely powdered *Leptadenia reticulata* roots (100 g) were subjected to maceration in different solvents for 72 hours. The solvents used included ethanol, aqueous ethanol (80:20), hexane, and ethyl acetate. The extraction process was conducted at room temperature to ensure optimal extraction of bioactive compounds. After the maceration period, the extracts were filtered using Whatman filter paper to remove solid residues. The filtered extracts were concentrated using a rotary evaporator to remove the solvents and obtain crude extracts. The remaining extract was then solidified using a water bath at 50°C to obtain a semi-solid mass. The obtained extracts were transferred to airtight, light-resistant containers and stored in a cool and dry place for further use [11].

Preliminary phytochemical analysis

a) Test for proteins

Crude extract when boiled with 2ml of 0.2% solution of Ninhydrin, violet colour appeared suggesting the presence of amino acids and proteins.

b) Test for carbohydrates



Equal volume of Fehling A and Fehling B reagents were mixed together and 2ml of it was added to crude extract and gently boiled. A brick red precipitate appeared at the bottom of the test tube indicated the presence of reducing sugars.

c) Test for flavonoids

Crude extract was mixed with few fragments of magnesium ribbon and concentrated HCl was added drop wise. Pink scarlet colour appeared after few minutes which indicated the presence of flavonoids.

d) Test for saponins

Crude extract was mixed with 5ml of distilled water in a test tube and it was shaken vigorously. The formation of stable foam was taken as an indication for the presence of saponins.

e) Test for glycosides

Crude extract was mixed with each of 2ml of chloroform and 2ml of acetic acid. The mixture was cooled in ice. Carefully concentrated H₂SO₄ was added. A colour change from violet to blue to green indicated the presence of steroidal nucleus, i.e., glycone portion of glycoside.

f) Test for steroid

Crude extract was mixed with 2ml of chloroform and concentrated H₂SO₄ was added sidewise. A red colour produced in the lower chloroform layer indicated the presence of steroids. Another test was performed by mixing crude extract with 2ml of chloroform. Then 2ml of each of concentrated H₂SO₄ and acetic acid were poured into the mixture. The development of a greenish coloration indicated the presence of steroids.

g) Test for terpenoids

Crude extract was dissolved in 2ml of chloroform and evaporated to dryness. To this, 2ml of concentrated H₂SO₄ was added and heated for about 2 minutes. A grayish colour indicated the presence of terpenoids.

h) Test for alkaloids

Crude extract was mixed with 2ml of 1% HCl and heated gently. Mayer's And Wagner's reagents were then added to the mixture. Turbidity of the resulting

precipitate was taken as evidence for the presence of alkaloids.

i) Test for tannins

10 ml of bromine water was added to the 0.5 g aqueous extract. Decoloration of bromine water showed the presence of tannins [12, 13].

Estimation of total phenolic content

The total phenolic content of *Leptadenia reticulata* root extracts was determined using the Folin-Ciocalteu method, as described by Herald et al. (2012) and Vyas and Braganza (2018). Briefly, 20 µl of the plant extract (5 mg/ml) was added to the wells of a 96-well plate, followed by the addition of 80 µl of Folin-Ciocalteu reagent (diluted 1:10 with Milli-Q water). After a 6-minute incubation, 100 µl of 7.5% sodium carbonate (Na₂CO₃) solution was added to each well. The plate was covered and incubated in the dark for 90 minutes. Absorbance was measured at 765 nm using a Multiskan Go plate reader, with methanol as the blank. Gallic acid was used as the standard, and a calibration curve was prepared in the range of 12.5–200 µg/ml. The results were expressed as mg of Gallic acid equivalent (GAE) per gram of dry extract. Each standard and sample solution was analyzed in triplicate, with a sample control (sample without Folin-Ciocalteu reagent and sodium carbonate) included for comparison [14].

Estimation of total flavonoid content

The total flavonoid content of *Leptadenia reticulata* root extracts was determined using the Aluminium chloride method, as described by Herald et al. (2012) and Vyas and Braganza (2018). Briefly, 25 µl of the plant extract (1 mg/ml to 5 mg/ml) was added to the wells of a 96-well plate, followed by the addition of 10 µl of 5% sodium nitrite (NaNO₂). After 5 minutes, 15 µl of 10% aluminium chloride (AlCl₃) was added to the reaction mixture. After 6 minutes, 50 µl of 1 M sodium hydroxide (NaOH) was added, followed by 150 µl of Milli-Q water. The absorbance was measured at 510 nm against a methanol blank using a Multiskan Go plate reader. Rutin was used as the standard to generate a calibration curve in the range of 100–700 µg/ml. The results were expressed as mg of Rutin equivalent (RE) per gram of dry extract. Each sample was measured against a sample control



(sample solution without aluminium chloride and sodium nitrite) [14].

COX-1 Inhibition Assay for *Leptadenia reticulata* Root Extracts

The *Leptadenia reticulata* root extract was prepared by dissolving it in 100% DMSO to make a stock solution of 10 mg/ml. The extract was tested at concentrations of 10, 25, 50, 75, and 100 µg/ml, and each concentration was tested in triplicates. The COX-1 inhibition assay was performed using the commercial COX inhibitory screening assay kit (Cayman test kit-560131, Cayman Chemical Company), following the manufacturer's guidelines. The assay directly measures the production of PGF2α in the cyclooxygenase reaction. To initiate the reaction, 10 µl of the test extract (at each concentration) or vehicle control was mixed with 10 µl of ovine COX-1 enzyme solution. This mixture was diluted to 20 µl with 0.1 M Tris-HCl buffer (pH 8.0) and pre-incubated with the enzyme at 37°C for 15 minutes. Subsequently, 10 µl of 10 mM arachidonic acid (AA) was added to start the reaction, which was incubated at 37°C for an additional 2 minutes. The reaction was terminated by adding 50 µl of 1 N HCl and saturated stannous chloride. Diclofenac was used as the positive control for COX-1 inhibition at a concentration of 1µM. The amount of PGF2α produced was quantified spectrophotometrically via enzyme immunoassay (EIA). The inhibition of COX-1 by the extract was determined by comparing the PGF2α levels in the test wells with those in the positive control and vehicle controls [15].

COX-2 Inhibition Assay for *Leptadenia reticulata* Root Extracts

The COX-2 inhibition assay for *Leptadenia reticulata* root extract followed a similar procedure as COX-1. The extract was dissolved in 100% DMSO to a stock concentration of 10 mg/ml and tested at concentrations of 10, 25, 50, 75, and 100 µg/ml in triplicates. The commercial COX inhibitor screening assay kit (Cayman test kit-560131, Cayman Chemical Company) was used to measure the inhibition of human recombinant COX-2 activity. 10 µl of the test extract (at each concentration) or vehicle control was mixed with 10 µl of human recombinant COX-2 enzyme solution and diluted to 20 µl with 0.1 M Tris-

HCl buffer (pH 8.0). The enzyme solution was pre-incubated at 37°C for 15 minutes. The reaction was then initiated by adding 10 µl of 10 mM arachidonic acid (AA), followed by a 2-minute incubation at 37°C. The reaction was stopped by the addition of 50 µl of 1 N HCl and saturated stannous chloride. Diclofenac (1µM) was used as the positive control for COX-2 inhibition. The PGF2α levels produced in the reaction were quantified spectrophotometrically using enzyme immunoassay (EIA). The inhibition of COX-2 was assessed by comparing the PGF2α production in the test samples with those in the positive control and vehicle controls. The percentage inhibition of COX-2 by the extract was determined by analyzing the reduction in PGF2α levels relative to the control [15].

RESULTS AND DISCUSSION

Extraction of *L. reticulata* roots

The roots of *Leptadenia reticulata* were successfully collected, authenticated, and processed for extraction using various solvents. The methodical approach ensured the preservation of phytoconstituents by employing shade drying and cold maceration techniques. Four different solvents—ethanol, aqueous ethanol (80:20), hexane, and ethyl acetate—were used to extract a broad spectrum of phytochemicals based on their polarity range.

The percentage yield of the crude extracts obtained from 100 g of dried root powder is presented in Table 1.

Table 1: Percentage Yield of *Leptadenia reticulata* Root Extracts

Solvent Used	% Yield (w/w)
Ethanol	13.24%
Aqueous Ethanol	14.63%
Hexane	9.37%
Ethyl Acetate	13.74%

The highest yield was obtained from the aqueous ethanol extract (14.63%), followed by ethyl acetate (13.74%) and ethanol (13.24%). The hexane extract yielded the least (9.37%), consistent with the lower solubility of phytochemicals in non-polar solvents. Semi-polar to polar solvents, like aqueous ethanol, efficiently extract a range of phytochemicals such as glycosides, flavonoids, and alkaloids. Hexane's lower



yield suggests non-polar compounds, like fixed oils and terpenoids, are less abundant or extractable. These findings align with previous studies showing aqueous ethanol's superior extraction efficiency for both hydrophilic and lipophilic compounds, especially when using cold maceration to preserve sensitive bioactives.

Preliminary Phytochemical analysis

Ethanol Extract

The ethanol extract of *Leptadenia reticulata* roots yielded 13.24%, highlighting ethanol's effectiveness for extracting various phytochemicals. Phytochemical screening identified carbohydrates, flavonoids, saponins, glycosides, terpenoids, alkaloids, and tannins, but no proteins or steroids. The presence of flavonoids and tannins suggests potential antioxidant and anti-inflammatory benefits. Saponins and alkaloids may contribute to immunomodulatory, antimicrobial, and cytotoxic activities, supporting the plant's traditional medicinal uses. Cardiac and steroidal glycosides point to possible cardiotonic effects. The absence of proteins and steroids may be

due to ethanol's limited solubility for larger molecules. This extract shows promise for further biological evaluation, especially for adaptogenic and antioxidant activities.

Aqueous Ethanol Extract

The aqueous ethanol extract (80:20 ethanol:water) yielded the highest at 14.63%, due to the solvent's ability to solubilize both polar and semi-polar compounds. Phytochemical analysis revealed proteins, carbohydrates, flavonoids, saponins, glycosides, terpenoids, alkaloids, and tannins, with no steroids. Notably, proteins were only detected in this extract, highlighting water's role in extracting hydrophilic macromolecules. The abundance of flavonoids, saponins, and tannins suggests antioxidant and anti-inflammatory potential, while alkaloids and glycosides support analgesic, antimicrobial, and cardiovascular benefits. This diverse phytochemical profile indicates the extract's broad therapeutic potential, making it a strong candidate for further pharmacological studies.

Table 2: Preliminary Phytochemical analysis

Phytochemical Test	Ethanol Extract	Aqueous Ethanol Extract	Hexane Extract	Ethyl Acetate Extract
Proteins	—	+	—	—
Carbohydrates	+	+	—	+
Flavonoids	+	+	—	+
Saponins	+	+	—	—
Glycosides	+	+	—	+
Steroids	—	—	+	+
Terpenoids	+	+	+	+
Alkaloids	+	+	—	+
Tannins	+	+	—	+

Hexane Extract

The hexane extract of *Leptadenia reticulata* yielded the lowest at 9.37%, reflecting hexane's limited ability to solubilize polar compounds. Phytochemical screening revealed only terpenoids and steroids, with no carbohydrates, proteins, flavonoids, saponins, glycosides, alkaloids, or tannins. This suggests hexane primarily extracted non-polar substances like triterpenoids and phytosterols, which are linked to anti-inflammatory, antitumor, and hormonal

activities. While the extract's yield and diversity were limited, its bioactives may still offer therapeutic potential in lipid metabolism and hormone regulation. The absence of hydrophilic compounds aligns with hexane's non-polar nature. Despite the low yield, this extract is valuable for studying lipophilic phytochemicals related to inflammation and steroidogenesis.

Ethyl Acetate Extract



The ethyl acetate extract yielded 13.74%, indicating effective extraction with a moderately polar solvent. Phytochemical analysis identified carbohydrates, flavonoids, glycosides, steroids, terpenoids, alkaloids, and tannins, but no proteins or saponins. The semi-polar solvent extracted both lipophilic and hydrophilic compounds, explaining the diverse bioactives. Flavonoids and tannins suggest antioxidant and anti-inflammatory properties, while glycosides and alkaloids offer cardioprotective and antimicrobial benefits. Steroids and terpenoids contribute to hormonal and anti-inflammatory effects. The absence of proteins and saponins is due to their poor solubility in ethyl acetate. This extract's balanced profile and yield make it promising for further pharmacological testing.

Estimation of total phenol contents

The total phenolic content (TPC) of *Leptadenia reticulata* root extracts was evaluated using the Folin–Ciocalteu colorimetric method, and the results were expressed in terms of mg of Gallic Acid Equivalent (GAE) per gram of dry extract. Among the four extracts tested, the aqueous ethanol extract showed the highest TPC value at 97.82 ± 1.89 mg GAE/g, followed by the ethanol extract (84.36 ± 2.14 mg GAE/g) and the ethyl acetate extract (76.55 ± 1.45 mg GAE/g). The hexane extract exhibited the lowest phenolic content, with a value of 18.74 ± 0.97 mg GAE/g.

Table 3: Estimation of total phenol content

Extract	TPC (mg GAE/g extract)
Ethanol	84.36 ± 2.14
Aqueous Ethanol (80:20)	97.82 ± 1.89
Hexane	18.74 ± 0.97
Ethyl Acetate	76.55 ± 1.45

These findings clearly demonstrate the role of solvent polarity in the extraction efficiency of phenolic compounds. Aqueous ethanol, being a mixture of polar solvents (water and ethanol), facilitates the dissolution of a broader range of phenolic constituents, particularly those that are moderately to highly polar. This corresponds well with the earlier phytochemical analysis, where the aqueous ethanol extract showed a wider range of bioactive

compounds. The ethanol extract also displayed substantial phenolic content, supporting its potential antioxidant capacity. The ethyl acetate extract, although less polar than ethanol or aqueous ethanol, still managed to extract a significant amount of phenolics, possibly due to the presence of mid-polar compounds. In contrast, the hexane extract, being non-polar, extracted very few phenolics, as these compounds are generally polar and more soluble in aqueous or alcoholic solvents. Phenolic compounds are widely recognized for their antioxidant, anti-inflammatory, and anti-carcinogenic properties. The high TPC values observed in the aqueous ethanol and ethanol extracts suggest that these could offer substantial therapeutic benefits, especially in combating oxidative stress-related disorders.

Estimation of total flavonoid contents

The total flavonoid content (TFC) of *Leptadenia reticulata* root extracts was quantitatively assessed using the Aluminium chloride colorimetric assay, with results expressed in mg of Rutin Equivalent (RE) per gram of dry extract. Among the tested extracts, the aqueous ethanol extract exhibited the highest flavonoid content, amounting to 86.91 ± 2.23 mg RE/g, followed by the ethanol extract (72.48 ± 1.96 mg RE/g) and the ethyl acetate extract (67.32 ± 1.78 mg RE/g). As expected, the hexane extract showed the lowest flavonoid concentration, with a value of 15.64 ± 0.85 mg RE/g, highlighting the polarity-dependent solubility of flavonoid compounds.

Table 4: Estimation of total flavonoid content

Extract	TFC (mg RE/g extract)
Ethanol	72.48 ± 1.96
Aqueous Ethanol (80:20)	86.91 ± 2.23
Hexane	15.64 ± 0.85
Ethyl Acetate	67.32 ± 1.78

These results reinforce the strong flavonoid-extraction capacity of hydroalcoholic solvents, especially aqueous ethanol, which provides an optimal polarity range for both glycosidic and aglyconic flavonoid compounds. Flavonoids, including flavonols and flavones, are known for their remarkable antioxidant, hepatoprotective, and anti-inflammatory properties. The high levels observed in the aqueous ethanol and ethanol extracts align well



with earlier phytochemical screening that confirmed the presence of flavonoids in these fractions. The moderate TFC in the ethyl acetate extract may be attributed to the extraction of mid-polar flavonoid aglycones, while the minimal flavonoid content in the hexane extract can be explained by the non-polar nature of the solvent, which is not conducive to the solubilization of flavonoid structures that typically contain multiple hydroxyl groups.

The elevated flavonoid content in both aqueous ethanol and ethanol extracts supports the traditional use of *Leptadenia reticulata* in Ayurvedic medicine for rejuvenation and immunomodulation. These

findings, combined with previously reported phenolic content and phytochemical profiles, suggest that these two extracts may exhibit strong antioxidant potential.

Invitro COX-1 inhibition assay

The cyclooxygenase-1 (COX-1) inhibition assay was performed to evaluate the anti-inflammatory potential of various *Leptadenia reticulata* root extracts. The % inhibition of COX-1 activity was determined at concentrations ranging from 10 to 100 $\mu\text{g/mL}$, and the IC_{50} values were calculated for each extract. Diclofenac (1 μM) was used as a standard and showed 100% inhibition, serving as a positive control.

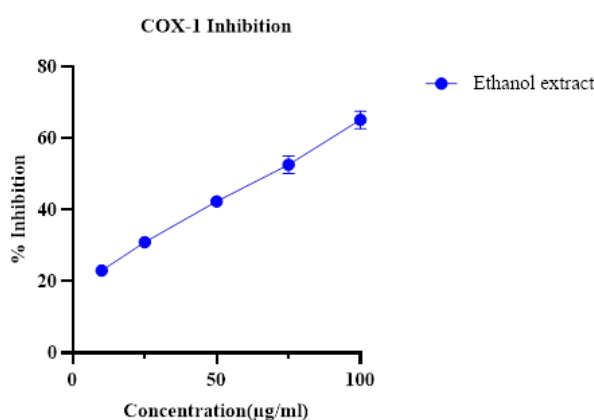


Figure 1: Effect of ethanol extract on COX-1 inhibition

Aqueous Ethanol Extract

Among all the tested extracts, the aqueous ethanol extract exhibited the most potent COX-1 inhibitory activity, with an IC_{50} value of 34.57 $\mu\text{g/mL}$. At the highest tested concentration (100 $\mu\text{g/mL}$), it achieved $72.34 \pm 2.48\%$ inhibition, demonstrating a strong, dose-dependent inhibition pattern. The enhanced efficacy of this extract can be attributed to its enriched phytochemical profile, particularly the high content of polyphenols and flavonoids, as previously confirmed in the total phenolic and flavonoid content assays. These classes of compounds are well documented for

their ability to modulate inflammatory enzymes, including COX isoforms, through direct enzyme inhibition or indirect antioxidant mechanisms. The hydroalcoholic nature of the solvent system (80:20 ethanol:water) likely enhanced the solubility and extraction of both polar and semi-polar bioactive compounds that act synergistically to inhibit COX-1. These findings support the traditional use of *Leptadenia reticulata* in managing inflammation-related conditions and highlight this extract as a promising candidate for further anti-inflammatory investigations.

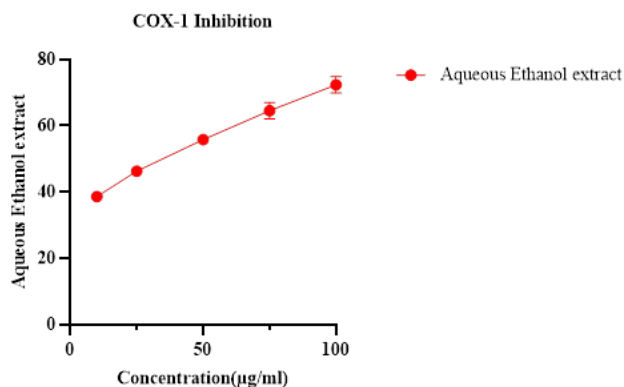


Figure 2: Effect of aqueous ethanol extract on COX-1 inhibition

Ethanol Extract

The ethanol extract of *Leptadenia reticulata* also showed significant COX-1 inhibitory activity, with an IC_{50} of 66.92 $\mu\text{g/mL}$. At 100 $\mu\text{g/mL}$, it reached $65.15 \pm 2.43\%$ inhibition, following a clear concentration-dependent trend. Though slightly less effective than the aqueous ethanol extract, its performance still indicates notable anti-inflammatory potential. Ethanol, being a polar organic solvent, efficiently extracts flavonoids, phenolic acids, and alkaloids, many of which are known to interfere with prostaglandin synthesis by inhibiting COX enzymes. The substantial COX-1 inhibition may also be linked to the presence of steroidal components, saponins, and terpenoids, previously identified in the preliminary phytochemical screening. The ethanol extract's moderate IC_{50} suggests that while it is effective, it may benefit from either fractionation or combination with synergistic agents to enhance its therapeutic index.

Ethyl Acetate Extract

The ethyl acetate extract demonstrated a moderate inhibitory effect, with an IC_{50} of 111.32 $\mu\text{g/mL}$ and maximum inhibition of $46.97 \pm 1.82\%$ at the highest concentration tested. Ethyl acetate, being a mid-polar solvent, likely extracted a different phytochemical spectrum rich in aglycone flavonoids, phenolic esters, and some terpenoids. While these compounds do possess anti-inflammatory capabilities, their concentration and potency within this extract appear to be less pronounced compared to the ethanol-based extracts. The moderate inhibition seen here may indicate that the ethyl acetate extract contains bioactive components in suboptimal concentrations or that they require further purification for enhanced activity. Nonetheless, its intermediate profile still suggests potential use in mild inflammatory conditions or as an adjunct to other bioactive extracts.

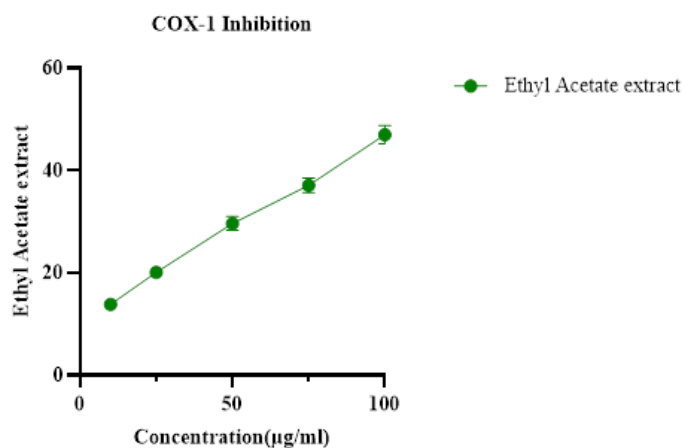


Figure 3: Effect of ethyl acetate extract on COX-1 inhibition

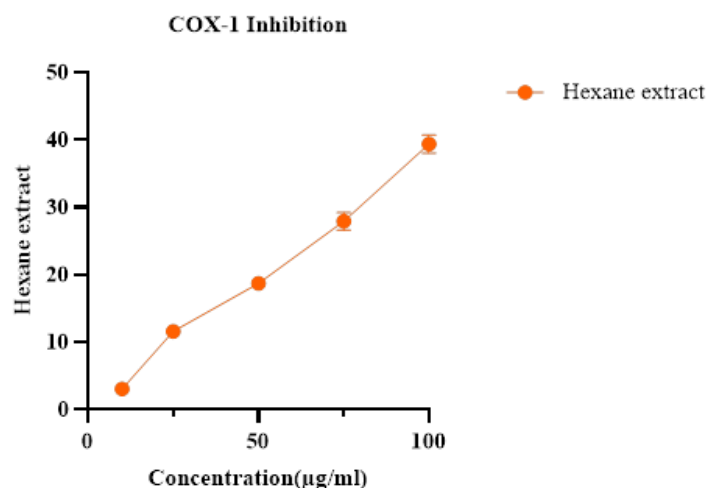


Figure 4: Effect of hexane extract on COX-1 inhibition

Hexane Extract

The hexane extract showed the least COX-1 inhibition, with an IC_{50} value of 131.29 $\mu\text{g/mL}$ and only $39.41 \pm 1.36\%$ inhibition at 100 $\mu\text{g/mL}$. Hexane, being a non-polar solvent, predominantly extracts lipophilic constituents such as fats, waxes, and non-polar terpenoids. These compounds, while beneficial in other pharmacological contexts, are generally less

effective in modulating cyclooxygenase activity unless present in very high concentrations. The weak performance of the hexane extract in the COX-1 assay correlates with its low total phenolic and flavonoid contents, suggesting a limited presence of conventional anti-inflammatory phytochemicals. However, this extract may still possess other bioactivities that are independent of COX inhibition, warranting further exploration in other models.

Table 5: Effect of COX-1 Inhibition of *L. reticulata* root extracts

Concentration ($\mu\text{g/mL}$)	Ethanol extract	Aqueous Ethanol extract	Hexane extract	Ethyl acetate extract
10	23.05 ± 0.26	38.65 ± 0.31	3.06 ± 0.14	13.83 ± 0.35
25	30.97 ± 0.29	46.27 ± 1.42	11.61 ± 0.23	20.05 ± 0.26
50	42.39 ± 1.37	55.84 ± 1.44	18.73 ± 0.34	29.61 ± 1.38
75	52.61 ± 1.41	64.55 ± 2.51	27.95 ± 1.29	37.08 ± 1.40
100	65.15 ± 2.43	72.34 ± 2.48	39.41 ± 1.36	46.97 ± 1.82

Mechanism of Action of COX-1 Inhibition by *Leptadenia reticulata* Root Extracts

Cyclooxygenase-1 (COX-1) is a constitutively expressed isoform of the COX enzyme family that plays a crucial role in maintaining physiological functions such as gastric mucosal protection, platelet aggregation, and renal perfusion. Inhibition of COX-1 is typically associated with non-selective NSAIDs and is often linked to gastrointestinal and renal side effects. The extracts of *Leptadenia reticulata* demonstrated varying degrees of COX-1 inhibition, primarily driven by their phytochemical content,

solubility profiles, and interaction potential with the enzyme's active site.

The COX-1 inhibitory activity observed, particularly in the aqueous ethanol and ethanol extracts, can be attributed to the presence of polyphenols, flavonoids, saponins, and tannins. These compounds are known to exert competitive or mixed-mode inhibition by binding to the cyclooxygenase (peroxidase) domain of the enzyme, where arachidonic acid is normally converted to prostaglandin G₂ (PGG₂). Flavonoids such as quercetin, kaempferol, and luteolin – which have been reported in related plant species – possess the structural mimicry necessary to occupy the

substrate-binding pocket and form hydrogen bonds with amino acid residues like Tyr385, Ser530, and Arg120, thereby impeding arachidonic acid access.

Moreover, phenolic compounds may stabilize the inactive form of COX-1 or interfere with the heme group that is essential for the enzyme's peroxidase activity. Tannins, due to their astringent and protein-binding nature, may exert indirect inhibitory effects by precipitating protein subunits or altering local enzyme conformation. Saponins, although not direct inhibitors of the enzyme, may modulate membrane permeability and enzyme localization, contributing to partial inhibition.

The weaker COX-1 inhibition noted in ethyl acetate and hexane extracts is consistent with their limited polar phytoconstituent content. These extracts are rich in terpenoids, steroids, and non-polar lipids, which typically do not interact effectively with the polar regions of the COX-1 active site. However, some terpenoids may cause mild interference via membrane fluidity modulation or by disrupting intracellular signaling pathways that indirectly affect enzyme activity.

In summary, the mechanism of COX-1 inhibition by *Leptadenia reticulata* extracts involves a combination of direct active site binding, allosteric modulation, and structural disruption, with the polar extracts (especially aqueous ethanol) showing the most potent inhibitory action due to the presence of structurally favorable and bioactive phytochemicals. Nonetheless, because COX-1 inhibition is associated with physiological prostaglandin suppression, these effects must be interpreted cautiously regarding therapeutic relevance.

Invitro COX-2 inhibition assay

Aqueous Ethanol Extract

The aqueous ethanol extract of *Leptadenia reticulata* demonstrated the most potent COX-2 inhibitory activity, with an IC_{50} value of 31.38 $\mu\text{g/mL}$, outperforming all other solvent extracts. At the highest tested concentration of 100 $\mu\text{g/mL}$, the extract achieved $72.34 \pm 2.46\%$ inhibition. The extract displayed a strong concentration-dependent inhibitory profile, indicative of effective interaction with the COX-2 enzyme.

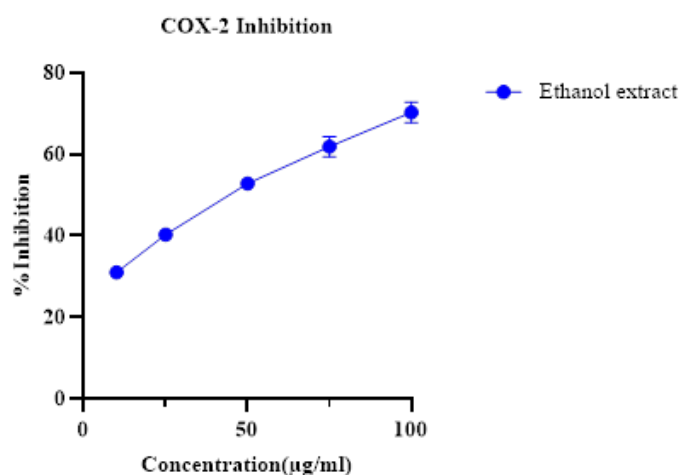


Figure 5: Effect of ethanol extract on COX-2 inhibition

The high potency is likely due to the extract's enriched phytoconstituents, particularly flavonoids and polyphenolic compounds, which are known to selectively inhibit COX-2 by blocking its active site or reducing its expression. The solvent system of 80:20 ethanol to water provides a polarity gradient that favors the extraction of both polar and semi-polar bioactives, facilitating the presence of flavones,

flavonols, tannins, and glycosidic phenolics. These compounds may be acting synergistically to exert a significant anti-inflammatory effect by modulating COX-2-mediated prostaglandin synthesis, a key pathway in inflammation. The extract's activity suggests selective COX-2 inhibition over COX-1, making it a promising natural anti-inflammatory agent with potentially fewer gastric side effects.



Ethanol Extract

The ethanol extract also exhibited considerable COX-2 inhibition, with an IC_{50} of 44.09 $\mu\text{g/mL}$ and a maximum inhibition of $70.28 \pm 2.52\%$ at 100 $\mu\text{g/mL}$. The extract showed a well-defined dose-response curve, suggesting consistent bioactivity across concentrations. Ethanol, being a polar solvent, is effective in extracting a wide range of phytochemicals, including alkaloids, steroids, flavonoids, and terpenoids—many of which have established COX-2 inhibitory mechanisms. The

slightly lower activity compared to the aqueous ethanol extract could be due to the absence of the water component, which might reduce the solubility of certain glycosidic flavonoids and tannins. Nevertheless, the ethanol extract's strong activity confirms that it is rich in bioactive constituents capable of modulating inflammatory pathways, supporting its traditional medicinal use. The COX-2 inhibitory effect of this extract also highlights its potential for developing plant-based therapeutics with fewer gastrointestinal complications than non-selective NSAIDs.

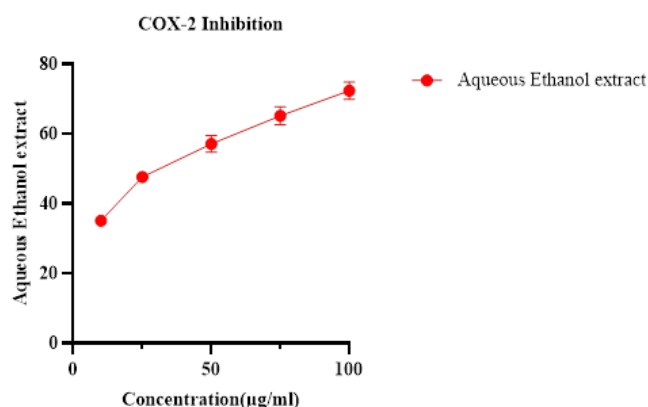


Figure 6: Effect of aqueous ethanol extract on COX-2 inhibition

Ethyl Acetate Extract

The ethyl acetate extract showed moderate COX-2 inhibition, achieving $29.41 \pm 2.45\%$ inhibition at 100 $\mu\text{g/mL}$, with an IC_{50} value of 199.89 $\mu\text{g/mL}$. The extract exhibited a less steep dose-response curve, indicating lower potency and efficacy in inhibiting COX-2 compared to the ethanol-based extracts. Ethyl acetate, a semi-polar solvent, tends to extract mid-polar compounds such as aglycone flavonoids, phenolic esters, and certain terpenoids. While these

compounds possess bioactivity, their concentration and affinity toward the COX-2 enzyme might be suboptimal in this fraction. The moderate inhibition observed may result from partial extraction of flavonoid aglycones and certain non-glycosylated phenolics. Despite its lower potency, the ethyl acetate extract still demonstrated relevant biological activity and could be explored further in combinational studies or subjected to fractionation to isolate active constituents.

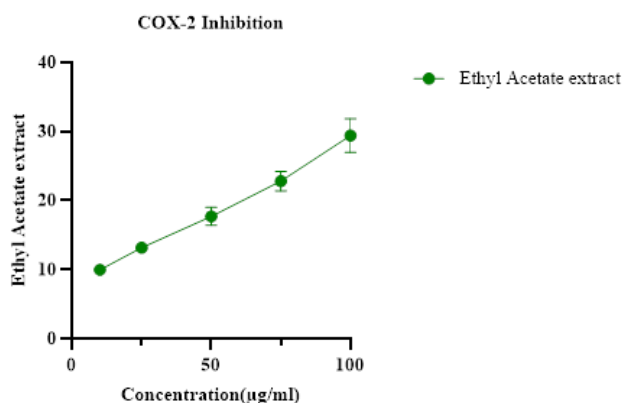


Figure 7: Effect of ethyl acetate extract on COX-2 inhibition



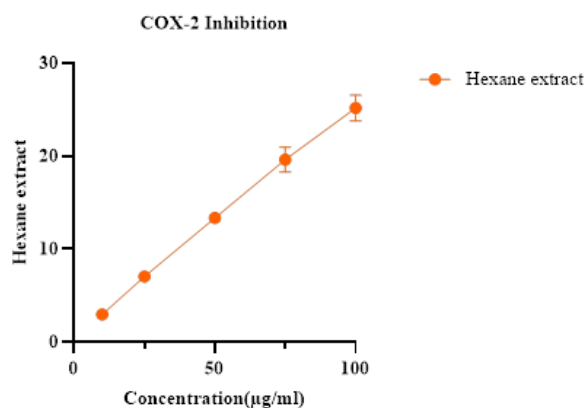


Figure 8: Effect of hexane extract on COX-2 inhibition

Hexane Extract

The hexane extract displayed the lowest COX-2 inhibition, with only $25.17 \pm 1.38\%$ inhibition at 100 $\mu\text{g/mL}$ and an IC_{50} of 290.21 $\mu\text{g/mL}$, indicating minimal activity in this assay. Hexane, being non-polar, primarily extracts lipophilic components such as fats, waxes, and non-polar terpenoids, which generally lack strong interaction with the polar binding site of COX-2. The low inhibition values reflect a limited presence of polyphenolic or flavonoid

constituents, which are typically responsible for COX-2 enzyme modulation. While non-polar terpenoids and phytosterols may exhibit anti-inflammatory effects through other pathways (e.g., NF- κ B or TNF- α inhibition), their role in direct COX-2 inhibition appears negligible in this context. Therefore, the hexane extract may be more suitable for other pharmacological evaluations, such as lipid-modulating or antimicrobial studies, rather than anti-inflammatory purposes.

Table 6: Effect of COX-2 Inhibition of *L. reticulata* root extracts

Concentration ($\mu\text{g/mL}$)	Ethanol extract	Aqueous Ethanol extract	Hexane extract	Ethyl acetate extract
10	31.01 ± 1.28	35.09 ± 1.35	2.97 ± 0.12	9.97 ± 0.26
25	40.29 ± 1.33	47.64 ± 1.39	7.04 ± 0.21	13.18 ± 0.29
50	52.86 ± 1.41	57.09 ± 2.44	13.34 ± 0.34	17.73 ± 1.32
75	61.91 ± 2.48	65.15 ± 2.51	19.62 ± 1.36	22.83 ± 1.40
100	70.28 ± 2.52	72.34 ± 2.46	25.17 ± 1.38	29.41 ± 2.45

Mechanism of Action of COX-2 Inhibition by *Leptadenia reticulata* Root Extracts

Cyclooxygenase-2 (COX-2) is an inducible isoform expressed primarily during inflammatory processes, tissue injury, and tumorigenesis. Unlike COX-1, it is not typically involved in physiological maintenance but rather in pathological prostaglandin production, particularly prostaglandin E₂ (PGE₂), which promotes pain, fever, and inflammation. Inhibition of COX-2 is a prime therapeutic target for anti-inflammatory and analgesic agents, and selective COX-2 inhibitors are known to reduce inflammation with minimal gastrointestinal side effects.

The root extracts of *Leptadenia reticulata*, especially the aqueous ethanol and ethanol extracts, demonstrated strong inhibition of COX-2 activity, and this can be mechanistically explained by the rich presence of flavonoids, polyphenols, glycosides, and tannins, all of which are well-documented for their COX-2 modulating potential. The mechanism of action primarily involves dual-level interference: (1) Direct enzymatic inhibition and (2) downregulation of COX-2 gene expression.

1. Direct Inhibition of COX-2 Enzyme:

Several flavonoids and phenolic compounds act as substrate analogs, competing with arachidonic acid



for access to the COX-2 active site. Unlike COX-1, COX-2 has a larger and more flexible side pocket due to valine substitutions at positions where COX-1 contains bulkier isoleucine residues (e.g., Val523 in COX-2 vs. Ile523 in COX-1). This structural difference makes COX-2 more accessible to bulky flavonoids and glycosides, which can bind with higher affinity and specificity. These compounds form π - π stacking interactions and hydrogen bonds with residues like Arg513, His90, and Ser530, thereby impeding the catalytic cycle of prostaglandin synthesis.

2. Transcriptional Suppression:

The extracts also likely exert anti-inflammatory effects via modulation of transcription factors that control COX-2 expression. Flavonoids and polyphenols can inhibit nuclear factor-kappa B (NF- κ B) and activator protein-1 (AP-1), both of which are key regulators of COX-2 gene induction in response to pro-inflammatory cytokines like IL-1 β and TNF- α . Additionally, inhibition of the MAPK/ERK signaling pathway could further suppress COX-2 mRNA transcription and translation, reducing overall protein expression and downstream prostaglandin production.

3. Synergistic Mechanisms:

In some cases, saponins and tannins may enhance the permeability of cell membranes or interfere with phospholipase A2 activity, indirectly reducing arachidonic acid availability—the precursor for COX enzymes. This upstream blockade contributes synergistically to reduced COX-2 substrate turnover.

In contrast, the hexane and ethyl acetate extracts exhibited only modest to low COX-2 inhibition. This is likely due to the absence of strong COX-2 inhibitors like polyphenols or glycosylated flavonoids in these non-polar extracts. Their minimal effect may arise from terpenoid-mediated disruption of inflammatory signaling cascades, though not from direct COX-2 inhibition.

The COX-2 inhibitory mechanism of *Leptadenia reticulata* extracts is multi-faceted, involving direct active site occupancy, transcriptional repression, and modulation of upstream signaling, with the aqueous ethanol extract being the most effective. These properties suggest strong potential for anti-

inflammatory therapeutic development with preferential COX-2 selectivity, minimizing gastrointestinal side effects typical of traditional NSAIDs.

CONCLUSION

Leptadenia reticulata root extracts, particularly from aqueous ethanol, demonstrate significant pharmacological potential, supporting the plant's traditional use in treating inflammatory conditions. The study highlights the critical role of solvent polarity in the extraction of bioactive compounds, with polar solvents like aqueous ethanol yielding the highest concentrations of phenolics and flavonoids, which are directly linked to potent anti-inflammatory activity. The strong COX-1 and COX-2 inhibitory effects observed in these extracts, particularly in aqueous ethanol, suggest a promising natural alternative for inflammation management with reduced side effects. These findings validate the therapeutic value of *Leptadenia reticulata* and provide a solid foundation for future research into its clinical applications, especially for selective COX-2 inhibition in inflammatory diseases

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AUTHOR CONTRIBUTIONS

All authors contributed significantly to the conception and design of the study, formulation development, data acquisition, analysis, and interpretation.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest associated with this work.

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