AN EVALUATION OF PHYTOCHEMICAL, *INVITRO* ANTIOXIDANT AND ANTI-INFLAMMATORY ACTIVITIES OF LEAF AND BARK ETHANOLIC EXTRACT OF *MADHUCA LONGIFOLIA*

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Abstract**:** *Madhuca longifolia* is commonly found in Indian subcontinent and its parts have been used traditionally in indigenous medicine due to its medicinal value. The present study is focused on the phytochemical analysis of ethanolic extract leaf and bark of *M. longifolia* and found the presence of carbohydrates, tannins, phenols, flavonoids, cardiac glycosides, coumarins, quinones, steroids, and phytosterols. The phytochemical phenol, flavonoids and tannins were estimated as 166.75 ± 14.60 mg GAE/g dry weight, 307.93 ± 36.33 mg quercetin equivalents per gram of dry weight and 23.81 ± 10.00 mg/g respectively. The antioxidant activity was determined by DPPH radical scavenging activity and hydrogen peroxide scavenging activity. The DPPH free radical scavenging was estimated as 71.35 ± 0.003% at 250 µg/ml. The maximum hydrogen peroxide scavenging activity was recorded at 50 µg/ml as 49.34 ± 0.007%. The anti-inflammatory activity was determined by bovine serum albumin denaturation assay, and it was found as 44.55 ± 0.011% inhibition at 100 µg/ml. The current study revealed the diverse phytochemical constitutes, antioxidant and anti-inflammatory activity of ethanolic extract of leaf and bark of *M. longifolia.*

Keywords: *M. longifolia,* Antioxidant, Anti-inflammatory, Phytochemicals

1. INTRODUCTION

Medicinal plants have a vital role in traditional healthcare systems and continue to serve as important sources for the discovery of novel therapeutic agents. From the Ayurvedic era, herbal medicines have been considered the backbone of traditional medicine due to their significant pharmacological potential. It is estimated that more than 75% of the population in developing countries still relies on traditional medicinal practices for primary healthcare, emphasizing the need for scientific evaluation and validation of medicinal plants with therapeutic value (Chanchal DK & Sharma SK, 2024). *Madhuca longifolia* (Koenig) Macbr., belonging to the family Sapotaceae, is a medium-sized deciduous tree widely distributed across India. Commonly known as mahua or butter nut tree Iluppai in Tamil, the plant holds immense importance in traditional and folklore medicine and is often regarded as a "universal panacea" in Ayurveda.

Various parts of the plant, including flowers, leaves, bark, seeds, and fruits, are traditionally used for the treatment of a wide range of ailments such as epilepsy, diabetes mellitus, inflammation, bronchitis, ulcers, skin diseases, snakebite, and rheumatism (Kendre N & Wakte P, 2021). The flowers of *M. longifolia* are rich in vitamins A and C and are traditionally used for reducing sugar levels and improving general health. Flower preparations are also employed as tonics, analgesics, diuretics, cooling agents, aphrodisiacs, and astringents, and are used in the treatment of acute and chronic tonsillitis, pharyngitis, bronchitis, and gastrointestinal disorders (Fatma A et al, 2024). Fermented flower preparations are traditionally used to relieve labor pain, cough, cold, and urinary disorders. Additionally, flower juice has been applied for the treatment of various skin diseases and muscular pain (Santosh Shukla, 2024). Leaves of M. longifolia possess antioxidant properties and are traditionally used in the treatment of chronic bronchitis and other inflammatory conditions (Kurmoo et al., 2020).

The bark is known for its medicinal value in treating itching, swelling, fractures, bleeding gums, ulcers, rheumatism, and diabetes mellitus (Sandhu et al., 2018). It is also used as a remedy for snakebite and other inflammatory conditions. Seeds of *M. longifolia* are economically important as they are a rich source of edible lipids and are used for the extraction of mahua oil, which finds applications as edible fat, biofuel, and in pharmaceutical preparations. The oil has also demonstrated antioxidant and antimicrobial activities (Karthikeyan et al., 2024). Phytochemical investigations have revealed the presence of diverse bioactive compounds in different parts

of *M. longifolia*. The flowers contain sugars, vitamins, anthocyanins, betains, and organic acids. Bark constituents include oleanolic acid, α-spinasterol, α-amyrin acetate, erythrodiol, betulinic acid, and sesquiterpene alcohols (Sangeetha et al., 2016). Fruits are reported to contain quercetin, dihydroquercetin, β-sitosterol, and amyrin acetates (Shrivastava et al., 2018).

Seeds are rich in amino acids such as glycine, alanine, cysteine, leucine, and isoleucine, along with fatty acids including arachidic, oleic, linoleic, palmitic, stearic, and myristic acids (Devi et al., 2016). Leaves have been reported to contain sterols, flavonoids, carotenoids, glycosides, and xanthophylls, which contribute to their therapeutic potential (Shrivastava et al., 2018). Traditional medicine represents a comprehensive system of knowledge, skills, and practices based on indigenous beliefs and experiences used in the prevention, diagnosis, and treatment of physical and mental disorders. Despite the extensive traditional use of *M. longifolia*, systematic scientific studies validating its phytochemical composition and medicinal properties remain limited. The present study aims to analysis phytochemical constituents of leaf and bark extract of *M. longifolia*. This study also highlighted the antioxidant and anti-inflammatory activity of leaf and bark extract in invitro conditions.

2. Materials and Methods

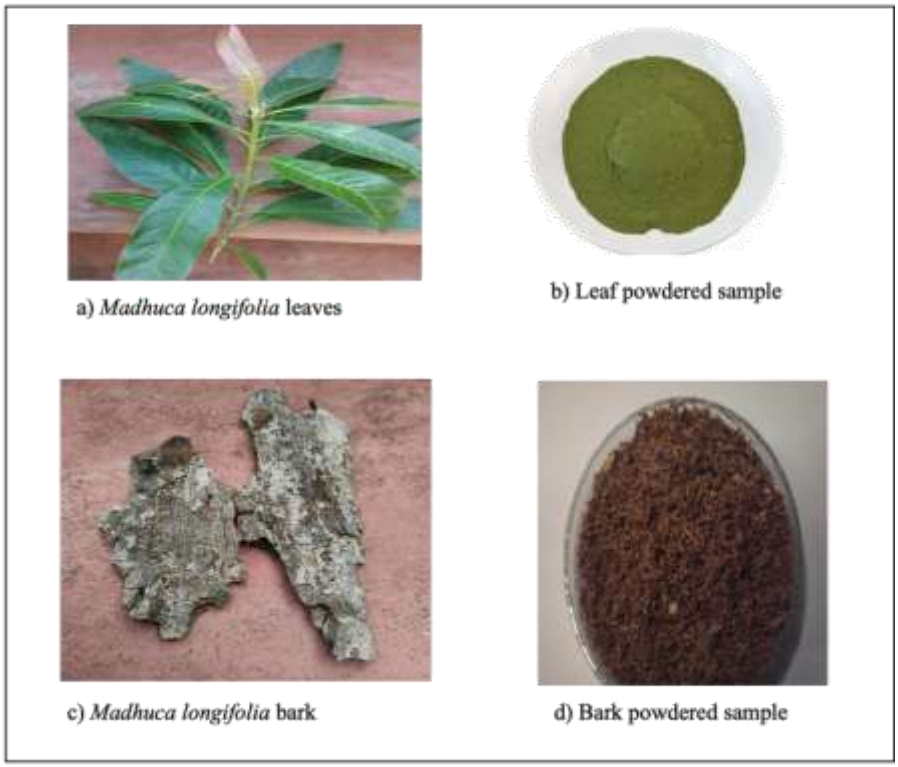
2.1 Collection and authentication of plant sample

The bark and leaf samples of M longifolia were collected from Arakkonam taluk, Ranipet district, Tamil Nadu, India. The plant material was authenticated by Dr. L. N. Sunil Kumar, Department of Pharmacognosy, Siddha Central Research Institute, Arumbakkam, Chennai, Tamil Nadu.

2.2 Preparation of extracts

The collected bark and leaves were shade dried for 4–5 days and then ground separately into a fine powder. The powdered samples were sieved to remove coarse particles. About 7 g of bark and leaf powder were weighed separately and mixed well. The mixture was soaked in 60 ml of ethanol and incubated. The extract was filtered, concentrated, and stored under refrigeration for further phytochemical analysis.

Figure 1: Preparation of Leaf and bark sample



2.3 Qualitative phytochemical analysis

2.3.1 Test for Carbohydrates

Carbohydrates are treated with concentrated sulphuric acid; it undergoes dehydration to form furfural derivatives. These derivatives react with α-naphthol (Molisch's reagent) to produce a purple or reddish-violet colored ring at the interface of the two liquids. The formation of this ring indicates the presence of carbohydrates (Harborne, 1998).

2.3.2 Test for Tannins

A few drops of freshly prepared 5% ferric chloride solution was added to the extract and mixed gently. In the presence of ferric chloride, hydrolysable tannins produce a blue-black coloration, while condensed tannins yield a greenish-black color. The formation of these characteristic colors indicates the presence of tannins in the plant extract (Evans, 2009).

2.3.3 Test for Saponins

A small quantity of the plant extract was diluted with distilled water and shaken vigorously in a test tube for about 15 minutes. The formation of a stable and persistent froth indicated the presence of saponins. The froth formation is due to the soap-like properties of saponins, which reduce surface tension and produce foam, thereby confirming the presence of saponins in the plant extract (Sofowora, 1993).

2.3.4 Test for Flavonoids

A few drops of 10% sodium hydroxide solution were added to the plant extract and mixed gently. This resulted in the formation of an intense yellow coloration. Subsequently, a few drops of dilute hydrochloric acid were added, which caused the yellow color to disappear. This reaction confirms the presence of flavonoids in the plant extract due to the formation of flavonoid anions in alkaline conditions (Mbaebie, 2005).

2.3.5 Test for Alkaloids

Alkaloids are basic nitrogen-containing compounds that react with specific acidic reagents to form insoluble colored or precipitated complexes. A few drops of Mayer's reagent were added to the plant extract. The formation of a cream or white precipitate due to alkaloid–reagent complex formation confirms the presence of alkaloids in the plant extract (Harborne, 1998).

2.3.6 Test for Quinones

Quinones are oxidized aromatic compounds that react with strong alkaline solutions to form colored complexes. In this test, sodium hydroxide solution was added to the plant extract. The formation of a red or green coloration occurs due to the formation of quinone salts or ionized quinone derivatives. The development of this characteristic color indicates the presence of quinones in the plant extract (Evans, 2009)

2.3.7 Test for Glycosides

Glycosides consist of a sugar moiety linked to a non-sugar aglycone. Under acidic conditions, glycosides undergo hydrolysis to release the aglycone, which subsequently reacts with specific reagents to produce characteristic color red or pink. The formation of these colored complexes confirms the presence of glycosides in the plant extract. (Sofowora,1993).

2.3.8 Test for Cardiac Glycoside

The plant extract was treated with glacial acetic acid followed by the addition of a few drops of freshly prepared 5% ferric chloride solution. This mixture was carefully layered with concentrated sulphuric acid. After standing for about 10 minutes, the formation of a brown ring at the interface indicated the presence of cardiac glycosides in the plant extract (Gokhale, 2010).

2.3.9 Test for Terpenoids

Terpenoids react with concentrated sulphuric acid in the presence of chloroform to undergo sulphonation and form conjugated carbocation complexes. These complexes produce a reddish-brown coloration at the interface of the two layers. The formation of this characteristic color indicates the presence of terpenoids in the plant extract (Harborne, 1998).

2.3.10 Test for Triterpenoids

The plant extract was treated with chloroform, followed by the careful addition of concentrated sulphuric acid along the side of the test tube to form a separate layer. The development of a yellow coloration in the lower layer indicated the presence of triterpenoids in the plant extract (Evans, 2009).

2.3.11 Test for Phenols

The plant extract, an equal volume of distilled water was added, followed by a few drops of 10% ferric chloride solution. The mixture was allowed to stand for 10 minutes. The formation of a blue or green coloration indicated the presence of phenolic compounds in the plant extract (Sofowora,1993).

2.3.12 Test for Coumarins

The plant extract, a few drops of 10% sodium hydroxide solution were added and allowed to stand for 10 minutes. The appearance of yellow coloration or fluorescence confirmed the presence of coumarins in the plant extract (Khandelwal, 2008).

2.4 Quantitative estimation of phytochemicals

2.4.1 Phenol

The total phenolic content of the plant extract was determined using the Folin–Ciocalteu method. A few drops of Folin–Ciocalteu reagent were added to each tube and mixed well, followed by the addition of 500 µL of sodium carbonate (Na_2CO_3) solution. The contents were mixed thoroughly and incubated at room temperature for 10 minutes. The UV–Visible spectrophotometer was preheated, and the absorbance of the samples was measured at 760 nm using the prepared solution. The total phenolic content was calculated based on the absorbance values obtained and expressed using gallic acid as the standard (Ochuko & Edeoga, 2001).

2.4.2 Flavonoid

Total flavonoid content was measured by the aluminium chloride colorimetric assay. Briefly, the reaction mixture contains 1ml of extract and 4ml of distilled water in a 10ml volumetric flask. Add, 0.3ml of 5% sodium nitrite and after 5 minutes, 0.3ml of 10% aluminium chloride solution added. After 6 minutes, 2ml of 1M sodium hydroxide was added and diluted to 10ml with distilled water. The UV–Visible spectrophotometer was preheated for 15 minutes, and absorbance was recorded at 415 nm. The absorbance of the reaction mixture was measured using a UV–Visible spectrophotometer, and the total flavonoid content was calculated based on the standard curve obtained. (Chen, 2002).

2.4.3 Tannin

Total tannin content was measured by the folin-ciocalteu assay. 100 µL of Folin–Ciocalteu reagent (1:10 dilution) was added, followed by the addition of 1mL of sodium carbonate solution. The contents were mixed thoroughly and incubated at room temperature for 20 minutes. The UV–Visible spectrophotometer was preheated, and the absorbance of the reaction mixture was measured at 640 nm (Ochuko & Edeoga, 2001).

2.5 Antioxidant activity

2.5.1 DPPH Free Radical Scavenging Assay

The free radical scavenging activity of the plant extract was determined using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method. A stock solution of DPPH was prepared in ethanol, and different concentrations of the plant extract were prepared. An aliquot of the extract was mixed with DPPH solution and incubated in the dark at room temperature for 10 minutes. The absorbance was measured using a UV–Visible spectrophotometer at 580 nm. Ascorbic acid was used as the standard antioxidant (Lamela-Raventós & Waterhouse,1999).

2.5.2 Hydrogen Peroxide Free Radical Scavenging Assay

The hydrogen peroxide scavenging activity of the plant extract was determined using a spectrophotometric method. A solution of hydrogen peroxide was prepared in phosphate buffer (pH 7.4). Various concentrations of the plant extract were added to the hydrogen peroxide solution and incubated at room temperature for 10 minutes. The absorbance of the reaction mixture was measured at 230 nm using a UV–Visible spectrophotometer (Kunchandy & Rao, 1990).

2.6 Anti-inflammatory activity

2.6.1 Bovine Serum Albumin (BSA) Denaturation Assay

The anti-inflammatory activity of the extract was evaluated using the BSA denaturation assay. Phosphate-buffered saline was added to each tube to adjust the final volume to 500 µL. The reaction mixtures were incubated in a water bath at 60°C for 10 minutes, followed by cooling to room temperature. The UV–Visible spectrophotometer was preheated for 15 minutes, and the absorbance was measured to determine the extent of protein denaturation (Leelaprakash, 2011).

3. RESULT AND DISCUSSION

3.1 Preparation of Extraction

The ethanolic extract of the bark and leaves of *M. longifolia* was prepared and the extract was subsequently dried. The crude extract yield was found to be 400 mg.

3.2 Phytochemical analysis

3.2.1Qualitative analysis

The qualitative phytochemical screening of ethanolic bark and leaf extract of *M. longifolia* revealed the presence of several bioactive compounds. Carbohydrates, tannins, phenols, flavonoids, cardiac glycosides, coumarins, quinones, steroids, and phytosterols were detected in the extract. In contrast, saponins, glycosides, terpenoids, and triterpenoids were found to be absent. Table 1 shows the qualitative phytochemical screening of ethanolic bark and leaf extract of *M. longifolia.*

Table 1: Phytochemical screening of ethanolic extract of leaf and bark of *Madhuca longifolia*

S. No	Phytochemicals	Result
1.	Carbohydrates	+
2.	Tannins	+
3.	Saponins	_
4.	Flavonoids	+
5.	Alkaloids	+
6.	Quinones	+
7.	Glycosides	_
8.	Cardiac Glycosiides	+
9.	Terpenoids	_
10.	Triterpenoids	_
11.	Phenols	+
12.	Coumarins	+
13.	Steroids and Phytosteroids	+
14.	Phlobatannins	_
15.	Anthraquinones	_

Similar observations were reported by Thirumalaisamy et al, who demonstrated the presence of alkaloids, carbohydrates, proteins, tannins, and triterpenoids in the methanolic extract of *M. longifolia*. However, gums and mucilage as well as fixed oils and fats were reported to be absent, which is consistent with the findings of the present study.

In agreement with these findings, Joshi (2013) et al. reported that phytochemical screening of *M. longifolia* leaf and flower extracts prepared using n-hexane, ethyl acetate, methanol, and ethanol revealed the presence of tannins. These compounds are known to possess notable cytotoxic and antitumor properties. According to the present investigation, the experimental plant sample contained tannins along with other important phytoconstituents.

3.3. Quantitative analysis

3.3.1 Phenol

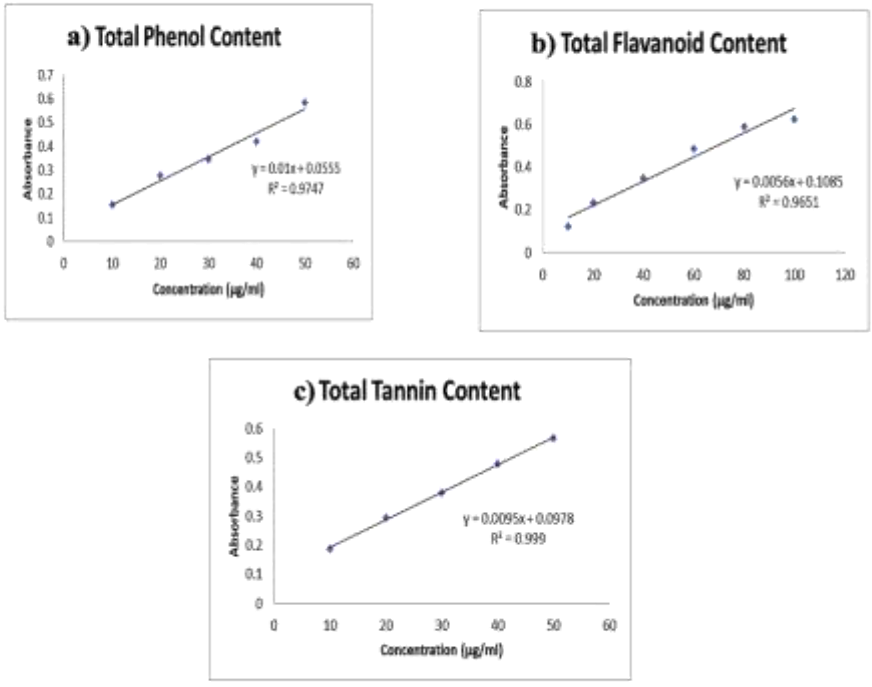
The total phenolic content of the ethanolic extract of *M longifolia* was determined using gallic acid as a standard. At a concentration of 10 mg/mL, the extract exhibited phenolic content of 166.75 ± 14.60 mg gallic acid equivalent (GAE)/g dry weight (Table 2). The estimation was based on absorbance values obtained from the standard calibration curve (Figure 2).

Phenolic compounds are recognized as important chemo preventive phytochemicals due to their antioxidant and anticancer properties. The phenolic content showed a concentration-dependent increase, indicating a positive correlation between extract concentration and phenolic yield.

3.3.2 Flavonoids

The total flavonoid content of the ethanolic extract of *M. longifolia* was estimated using quercetin as the reference standard, and the absorbance values obtained were compared accordingly. At a concentration of 10 mg/ml, the extract exhibited a total flavonoid content of 307.93 ± 36.33 mg quercetin equivalents per gram of dry weight. The absorbance data clearly confirmed the presence of flavonoids in the plant extract. Figure: 2 shows the standard calibration curve and table: 2 shows the total flavonoid content present in *M.longifolia*.

Figure:2 Standard graphs of calibration line for different phytochemical standards a) Calibration line for Phenols b) Calibration line for Flavonoids c) Calibration line for Tannins.



An increasing trend in flavonoid content was observed with increasing extract concentration, indicating a concentration-dependent enhancement. Furthermore, the extract demonstrated a comparatively higher flavonoid content than total phenolic compounds, suggesting flavonoids as the predominant phytoconstituents in *M. longifolia* bark and leaves.

Supporting these findings, a study by Koomson et al. reported that phytochemical screening of *M. longifolia* leaf and flower extracts prepared using n-hexane, ethyl acetate, methanol, and ethanol revealed the presence of flavonoids. These compounds, recognized as natural biological response modifiers, have been documented to exhibit significant antimicrobial, anti-allergic, anti-inflammatory, and anticancer activities.

3.3.3 Tannins

The total tannin content of the ethanolic extract was also determined using tannic acid as the reference standard. At a concentration of 10 mg/ml, the tannin content, expressed as tannic acid equivalents per gram of dry sample, was found to be 23.81 ± 10.00 mg/g (Table: 2 and Figure: 3). The presence of tannins was confirmed through absorbance measurements, although their levels were comparatively lower than those of phenolics and flavonoids.

A gradual increase in tannin content was observed with increasing extract concentration; however, the overall levels remained relatively low. Qualitative analysis further supported the presence of tannins, as the formation of a white precipitate was observed when a few drops of the extract were shaken with acetate solution.

Table 2: Estimation of Total Phenols, Flavonoids and Alkaloids content in ethanolic extract of leaf and bark of *M longifolia*

Phytochemicals	Leaf and Bark extract
Phenols	166.75 ± 14.60
Flavonoids	307.93 ± 36.33
Tannins	23.81 ± 10.00

Total Phenols, Flavonoid and Tannins content values are expressed at mean standard deviation (n=3).

3.4 Antioxidant Properties

3.4.1 DPPH Assay

The antioxidant activity of the ethanolic extract was evaluated using the DPPH free radical scavenging assay. The results showed a concentration-dependent increase in radical scavenging activity for both the plant extract and ascorbic acid. At concentrations ranging from 50 to 250 µg/mL, the ethanolic extract exhibited percentage inhibition values from 28.56 ± 0.037% to 71.35 ± 0.003%.

The maximum DPPH radical scavenging activity was observed at 250 µg/mL, where the ethanolic extract demonstrated 71.35 ± 0.003% (Table 3). Supporting these observations, a study conducted by Jose et al. reported significant antioxidant activity of the ethanol bark extract of *M. longifolia* using the DPPH assay. The extract exhibited notable free radical scavenging activity against DPPH radicals, with maximum absorption observed at 517 nm. The DPPH assay is widely regarded as a reliable method for evaluating antioxidant potential, as compounds capable of scavenging DPPH radicals result in a measurable decrease in absorbance at 517 nm.

3.4.2 Hydrogen Peroxide Free Radical Scavenging Assay

The hydrogen peroxide scavenging activity of the ethanolic extract of *M. longifolia* was evaluated by recording absorbance values at different sample concentrations and the results were depicted in table 4. The results demonstrated a dose-dependent increase in hydrogen peroxide scavenging activity for the plant extract. The ethanolic extract showed percentage scavenging activity ranging from 17.76 ± 0.134% to 49.34 ± 0.007% at concentrations of 10–50 µg/ml. The highest hydrogen peroxide scavenging activity of the ethanolic extract was observed at 50 µg/ml, with an inhibition of 49.34 ± 0.007%.

Table 3: **DPPH radical scavenging activity of ethanolic extract of *Medhuca longifolia* and values are expressed at mean standard deviation (n=3)**

Concentration µg/ml	% inhibition
50	28.56 ± 0.037
100	35.38 ± 0.028
150	45.96 ± 0.020
200	60.87 ± 0.015
250	71.35 ± 0.003

Table 4: Hydrogen Peroxide radical scavenging activity of ethanolic extract of *Medhuca longifolia* and values are expressed at mean standard deviation (n=3)

Concentration µg/ml	% Inhibition
10	17.76 ± 0.134
20	26.97 ± 0.007
30	39.47 ± 0.028
40	46.05 ± 0.014
50	49.34 ± 0.007

3.5 Anti-Inflammatory

3.5.1 BSA Denaturation Assay

The anti-inflammatory activity was evaluated using the BSA assay by measuring absorbance at different sample concentrations. The results indicated a concentration-dependent increase in inhibition of protein denaturation for the plant extract. The ethanolic extract showed percentage inhibition ranging from 23.71 ± 0.089% to 44.55 ± 0.011% at concentrations of 20–100 µg/ml. The maximum anti-inflammatory activity of the ethanolic extract was observed at 100 µg/mL, showing 44.55 ± 0.011% inhibition.

In support of these findings, Lopes et al. reported that hydrogen peroxide plays a dual role as both a pro-inflammatory and anti-inflammatory mediator, depending on the biological context. Their study highlighted the involvement of H_2O_2 in inflammatory pain modulation and suggested its potential as a therapeutic target, particularly through the regulation of neutrophil activation and the NF-κB signaling pathway. They also have reported that bovine serum albumin exhibits anti-inflammatory activity through its antioxidant properties. This activity is attributed to its ability to directly scavenge free radicals and inhibit free radical–induced oxidative stress via multiple ligand-binding sites and radical-trapping mechanisms. The present findings further support the antioxidant potential of *M. longifolia* through its ability to scavenge hydrogen peroxide radicals.

Table 5: Anti-inflammatory activity of ethanolic extract of *Medhuca longifolia* and values are expressed at mean standard deviation (n=3)

Concentration µg/ml	% Inhibition
20	23.71 ± 0.089
40	26.29 ± 0.079
60	30.11 ± 0.045
80	34.04 ± 0.048
100	44.55 ± 0.011

4. CONCLUSIONS

The present study evaluated the phytochemical, antioxidant, and anti-inflammatory properties of ethanolic extract of leaves and bark of *M.longifolia*. The qualitative analysis confirmed the presence of important bioactive compounds such as alkaloids, flavonoids, tannins, saponins, terpenoids, phenols, and glycosides, while the quantitative estimation showed a high content of phenolic and flavonoid compounds that contribute to its strong biological activity. The antioxidant assays, including hydrogen peroxide scavenging and DPPH free radicals reducing methods revealed that a remarkable ability to neutralize free radicals and reduce oxidative stress in a concentration-dependent manner. The anti-inflammatory activity assay showed that the ethanolic extract significantly inhibit protein denaturation, demonstrating its potential to reduce inflammation naturally. These results suggest that *M.longifolia* has excellent antioxidant and anti-inflammatory properties due to the synergistic action of its phytoconstituents. The present findings support its traditional medicinal uses and highlight its potential as a natural source for developing safe, plant-based therapeutic agents and it can be considered a promising medicinal plant for preventing oxidative stress and inflammation-related diseases.

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