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Phytochemical Profiling Using GCMS and HPLC and Evaluation of Anti-oxidant, Anti-inflammatory and Anti-bacterial Activities of *Lawsonia Inermis*

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Abstract

Lawsonia inermis leaves are often used in traditional medicine for the treatment of various disorders. The main objectives of this study were to assess the phytochemical composition of the plant using various analytical techniques and also to investigate its pharmacological activities, including anti-oxidant, anti-inflammatory, and antimicrobial because little work had been reported on these aspects previously. Sequential extraction of dried leaves was performed using solvents, namely n-hexane, chloroform, methanol, and water. Physicochemical parameters were evaluated using United States Pharmacopeia (USP) 2015 guidelines. According to established procedures, primary and secondary metabolites were estimated. Mineral content estimation was done using atomic absorption spectroscopy. Analytical techniques, including FTIR, GCMS, and HPLC were employed for phytochemical profiling. For anti-oxidant activity, DPPH, FRAP, phosphomolybdenum assay, and H_2O_2 scavenging activity were performed. The protein denaturation method was used to investigate *in vitro* anti-inflammatory effect. Antimicrobial susceptibility testing was performed against several bacterial strains using the well diffusion method. Physicochemical analysis values fall within the USP 2015 range. In methanolic extract, flavonoids, glycosaponins, and polyphenols were found in higher concentrations. The results of analytical studies indicate the presence of various valuable compounds and minerals. The anti-inflammatory activity of the methanol extract was found to be 85.045% compared to 86.885% of diclofenac sodium as a standard. The methanolic extract showed significant antimicrobial activity at a concentration (400 mg/mL) comparable to the activity of the reference drug. The leaves of *Lawsonia inermis* extracts revealed the presence of bioactive constituents, which are known to exhibit medicinal as well as physiological activities. The plant has significant anti-oxidant, anti-inflammatory, and broad-spectrum antibacterial activity.

Keywords: *Lawsonia inermis*, Antibacterial activity, Anti-inflammatory activity, Anti-oxidant activity, Medicinal plants

Introduction

The use of medicinal plants to cure a variety of ailments is widespread throughout the world. Due to their pharmacological effects, it is believed that more than 50,000 plants are utilized to treat a variety of illnesses. Around 80% of individuals use medications made from natural sources to address different diseases worldwide [1]. Compared to

synthetic pharmaceuticals, medications made from plants are more readily available and have fewer negative effects [2]. According to a report by the WHO, most people around the world rely on herbal medicines and other complementary therapies because they are economical for patients. All ancient civilizations are reported to have had some herb to treat different diseases [3]. Given the significance of medicinal plants, *Lawsonia inermis* is the focus of the present research because of its extensive applications in traditional medicine.

Lawsonia inermis, usually referred to as henna or mehndi, is a member of the Lythraceae family. *Lawsonia inermis* is an evergreen, medium-sized branched shrub that grows up to 12 feet tall. The tiny flowers have four sepals, red or white stamens, and spear-shaped lobed. The fruit is a little brown capsule that contains 30–40 seeds. It is cultivated in Afghanistan, Bangladesh, Iran, Sudan, Libya, Egypt, Pakistan, UAE, India, Saudi Arabia, Somalia, and Turkey. In India, *Lawsonia inermis* is cultivated in the states of Gujarat and Uttar Pradesh [4]. Traditionally, henna is used on hands, feet, and hair due to its cooling effect and coloring properties. Especially in weddings, people apply it to the skin for beautiful designs. The main plant parts of henna are bark, leaves, stems, flowers, and roots, which are used in traditional medicines to treat various diseases such as arthritis, headaches, ulcers, jaundice, diarrhea, fever, leukemia, diabetes, and heart diseases. Like leaves, other plant parts such as seed pods and roots play an important role in the treatment of many diseases [5]. The seeds are effective as deodorants. The seeds are the best remedy for liver disorders and similar problems. The plant's flower extract is used in perfumes. The bark is used in the treatment of burns and is given internally for jaundice, enlarged spleen, and liver calculus. The root is best as a stimulant and is used to treat eye pain. The root is considered a powerful remedy for gonorrhea and herpes infections [6, 7]. Lawsone and lawsonoside are two of the most important phytochemicals discovered in different parts of plants. The phenolic compounds coumarins, flavonoids, and naphthoquinones are particularly present in *Lawsonia inermis* extracts. The prevalence of these components suggests the significance of the biological defenses of the plant against numerous illnesses [8, 9]. From the whole plant, the lawcoumarin was isolated. Lawsochrysin, luteolin, linarigenin, and linarisenin were also extracted from the plant leaves [10]. Lawsone possesses pharmacological qualities such as anticancer, antifungal, antibacterial, diuretic, anti-inflammatory, and analgesic [11]. As the plant contains potent phytochemicals, further scientific investigation is necessary to support the assertions made for why *Lawsonia inermis* is used in traditional medicine [12]. Therefore, the objectives of this research were to evaluate the plant's phytochemical composition and assess its pharmacological effects, such as its anti-oxidant, anti-inflammatory, and anti-microbial activities.

Materials and Methods

Chemicals

Chloroform (Merck, Germany), n-hexane (BDH, UK), Methanol (Merck, Germany), Diclofenac Sodium (Merck Germany), Tetracycline (Pfizer), Egg albumin (fresh), DPPH (Sigma Aldrich, USA), Sodium Chloride (BDH, UK), Hydrochloric acid (BDH, UK), and Potassium dihydrogen phosphate(Merck, Germany) were utilized during experimental work.

Instruments

Gas Chromatography Mass Spectroscopy (GCMS, Agilent 7890B, USA), High Performance Liquid Chromatography

(HPLC, Agilent 1100, USA), UV/Visible Spectrophotometer (PG Instruments, India), Fourier Transform Infrared Spectroscopy (FTIR, Bruker, USA), Muffle Furnace (Gilson Company, USA), and Atomic Absorption Spectroscopy (AAS, Perkin Elmer 100, USA) were used in this study.

Microorganisms

In this study, anti-microbial effects were tested for gram-positive bacteria, including *Staphylococcus aureus* (ATCC 23235) *Streptococcus pneumoniae* (ATCC 49136), and gram-negative bacteria, including *Klebsiella pneumoniae* (ATCC 13883) and *Pseudomonas aeruginosa* (ATCC 10145). Further, anti-microbial effects against *Mycobacterium tuberculosis* (ATCC 25177) and *Peptostreptococcusanaerobius* (ATTC 27337) were also evaluated. All bacterial strains were obtained from an accredited laboratory at Sialkot Faiz Hospital, Sialkot, Pakistan. All bacteria were grown in agar broth at a temperature of 37 °C and after autoclaving, they were kept on an agar plate at a temperature of 40 °C.

Plant Collection and Authentication

Lawsonia inermis was authenticated by botanist Dr. Zaheer-ud-Din Khan at GC University, Lahore, Pakistan and issued a voucher number 3922. The powder is then stored in an airtight jar for future use.

Extraction of the Plant Material

Material (150 g) was collected and dried in shade for 21 days. A Soxhlet apparatus was used for extraction. To separate compounds with various polarities, solvents of increasing polarity, such as n-hexane, chloroform, methanol, and water were used. 1 L of each solvent was used for extraction purposes. The material was extracted sequentially with each solvent at 35-40 °C until the siphon tube becomes colorless. The extracted material for each solvent was collected separately. The remaining material was then extracted with water by hot extraction at 40 °C for 24 hours. Using a rotary evaporator, all extracts other than water were dried while the temperature was kept below the boiling point of each solvent. The freeze-drying process was used to dry the water extract. All extracts were gathered in pre-weighed, clean, labeled bottles, and they were all given time to dry in a 40 °C oven.

Proximate analysis

Quantitative analysis of *Lawsonia inermis* dry leaf powder was performed according to the method given in USP (2015). According to established protocols, the total ash, water-insoluble ash, acid-soluble ash, sulfated ash, moisture content, alcohol content, and water-soluble extractive values were computed [13].

Phytochemical Analysis

Lawsonia inermis dried leaf powder was used for phytochemical analysis, which evaluated the presence of metabolites. These were calculated using accepted techniques and are referred to as total proteins [14], total lipids [15], total polyphenols [16], carbohydrates [17], total flavonoids [18], total glycosaponins and total polysaccharides [19].

Mineral Content Analysis

Different metals and minerals were detected using atomic absorption spectroscopy and a flame photometer. Nitric acid (20% w/v) was used to clean the volumetric flask, followed by a deionized water rinse. A beaker was filled with 1 g of leaf powder. Then the sample was digested in 15 mL of a digestion solution and made up of a 1:3 (v/v)

concentration of concentrated nitric acid and hydrochloric acid. After that, the mixture was heated in a fume hood. At room temperature, the material was chilled and filtered. The final volume was made using deionized water and put in a stoppered bottle for analysis. The standard was made from stock solution at concentrations of 0, 1, 5, 10, 15, 20, 25, and 100 ppm [20].

Fourier Transform Infrared (FTIR) Spectroscopy

The plant leaf powder was analyzed using FTIR and spectra between 4000 cm^{-1} and 400 cm^{-1} were obtained.

Gas Chromatography–Mass Spectrometry (GC-MS) Analysis

A DB-5MS GC column and an inert mass selective detector (5977B) from Agilent (model number 7890B) were used for the analysis. A 2 µL sample injection was performed in split-less mode using an injector with a temperature of 250 °C and an interface temperature of 280 °C. It was planned to ramp up the oven temperature from 100 °C to 340 °C at a rate of 20 °C/min for one minute. Helium was used as the carrier gas and electron impact ionization was used in full-scan mode at -70 eV.

High Performance Liquid Chromatography (HPLC) Analysis

For the test, reversed-phase HPLC was employed. A model 1100 pump from Agilent with a multi-solvent delivery system and an L-7400 ultraviolet (UV) detector were utilized for analysis. The column was an Agilent C18 (5 µm, 4.0 mm internal diameter, 250 mm). Gradient elution was carried out as follows: 0 min, 95:5, 10 min, 90:10, 40 min, 60:40, 55 min, 45:55, 60 min, 20:80, and 0:100; 65 min for the mobile phase made up of (A) 2% acetic acid (CH_3COOH) and (B) 0.5% acetic acid acetonitrile (CH_3CN), (50:50 V/V). Before use, the mobile phase was filtered through a 0.45 µm membrane filter under vacuum. There was a 1 mL/min flow. UV absorption was detected between 280 and 365 nm.

In VitroAnti-inflammatory Activity
Protein Denaturation Method

The reaction mixture (5 mL) contained 2 mL of the extract (1%), 2.8 mL of phosphate buffered saline, and 0.2 mL of fresh egg albumin. As a control, the same volume of double distilled water was used. The mixture was heated at 70 °C for 5 minutes after being incubated for 15 minutes at 37 ± 2 °C. After cooling, their absorbance was calculated using a blank (control) at a wavelength of 660 nm. Diclofenac sodium was utilized and handled as a reference agent. The following formula was used to determine the percentage inhibition of protein denaturation [21]:

$$\% \text{ inhibition} = \frac{\text{Absorbance (control)} - \text{Absorbance (sample)}}{\text{Absorbance (control)}} \times 100$$

In vitroAnti-oxidant ActivityDPPH (1, 1 biphenyl 2, piprylhydrazyl) Assay

1 mL of the sample solution was mixed with 2 mL of methanol and 1 mL of a 0.1 mM DPPH solution. The UV/Vis spectrophotometer at 517 nm measured the decrease in free radical concentration compared to a blank after 30 minutes. Additionally, a standard absorbance (vitamin C) and a control absorbance were calculated. All measurements were made three times, and the results were calculated using the formula below using the mean of the three measurements [22]:

$$\% \text{ inhibition} = \frac{\text{Absorbance (control)} - \text{Absorbance (sample)}}{\text{Absorbance (control)}} \times 100$$

Ferric Reducing Power (FRAP) assay

To determine the ferric acid reducing power, 1 mL of each sample solution (PH 6.7 and 0.2 M), 3 mL of phosphate buffer, and 1 mL of 1% potassium ferric cyanide were combined. The solution was cooled after 20 minutes at 50 °C of incubation, and 3.5 mL of 10% trichloroacetic acid was then added. To get the supernatant layer, the mixture was centrifuged at 3000 rpm for 10 minutes. After that, 2.5 mL of distilled water and 0.5 mL of ferric chloride (0.1%) were added to the supernatant layer, and the mixture was left to stand for 10 minutes. In order to calculate the absorbance at 700 nm, UV/Vis spectroscopy was used. The following formula was used to calculate the results after readings were taken in triplicate [21]:

$$\% \text{ Inhibition} = \frac{\text{Absorbance (control)} - \text{Absorbance (sample)}}{\text{Absorbance (control)}} \times 100$$

PhosphomolybdenumAnti-oxidant Assay

The Prieto et al. approach was used for this experiment, with a few changes. The reagent solution contains sulfuric acid (0.6 M, 1.5 mL), and ammonium molybdate (4 mM, 1.5 mL). The reagent solution was diluted to 4.5 mL, and 0.5 mL of the extract solution was added. The mixture was then incubated at 95 °C for 90 minutes. The absorbance was measured using a UV/Vis spectrophotometer at 695 nm in comparison to a blank after the mixture was chilled at room temperature. After three readings, the average was calculated. The following calculation was used to determine the percentage of inhibition [23].

$$\% \text{ Inhibition} = \frac{\text{Absorbance (control)} - \text{Absorbance (sample)}}{\text{Absorbance (control)}} \times 100$$

Hydrogen peroxide (H_2O_2) Radical Scavenging Capacity

With a few modifications, this test was carried out by Prieto et al. to compare the fraction of H_2O_2 scavenging in the standard and extract. 1 mL of the sample solution (50 µg/mL) was combined with 0.6 mL of H_2O_2 solution (40 mM in PB, pH 7.4) and 2.4 mL of phosphate buffer. After 10 minutes, absorbance at 230 nm was measured and compared to a blank. The following equation was used to assess and compute how much H_2O_2 the standard and extracts scavenged [23].

$$\% \text{ Inhibition} = \frac{\text{Absorbance (control)} - \text{Absorbance (sample)}}{\text{Absorbance (control)}} \times 100$$

Antibacterial Activity

Antibacterial activity was determined by the agar well diffusion method using two different extract concentrations, 200 mg/mL and 400 mg/mL. Five wells were prepared on agar plates, four of which were supplemented with plant extracts and one with the standard drug tetracycline [24].

Results and Discussion

Percentage Yield of Different Extracts of Lawsonia inermis

Lawsonia inermis dry leaf powder was extracted with four solvents: n-hexane, chloroform, methanol and water. The percentage of extract produced from different solvents was as follows: methanolic extract (14.26%) >water (6.8%) >n-hexane (2.7%) >chloroform extract (1.2%). Different chemicals have different polarities. Therefore, continuous sequential extraction using a gradient of polarity, from less polar to more polar, aids in the separation of compounds with various polarities. The current study's findings suggest that the methanolic fraction

may contain a greater number of compounds than the water because it can retain both polar and non-polar compounds, whereas the non-polar compounds will be separated in the n-hexane and chloroform extracts [25].

Proximate Analysis

To standardize the dried leaves powder, a proximate analysis was carried out. Table 1 provides an overview of the results. The analysis of moisture content was performed in accordance with the recommended method for botanical origin [13]. Analyzing the moisture content of a sample is a crucial physicochemical parameter. A low moisture content is required for powder to be stable for an extended period. *Lawsonia inermis'* moisture content was below 13% w/w and within the acceptable range [26]. Polar compounds, including phenols, tannins, glycosides, etc., were present as evidenced by their high alcohol and water solubility values [27]. Ash value analysis is crucial for determining the presence of foreign substances such as oxalates and silica. The estimated total ash value from the tests was 14%, which is less than the official range of 20% [28, 29].

Table 1. **Results of proximate analysis of *Lawsonia inermis* dried leaves powder.**

Proximate analysis	Content (% w/w)
Moisture contents	5.02 ± 0.43
Water soluble extractives	13.85 ± 0.27
Water insoluble ash	8.5 ± 0.24
Acid soluble ash	5.0 ± 0.56
Total ash content	14.0 ± 0.18
Alcohol soluble contents	11.5 ± 0.34
Primary metabolites	
Total lipids	1.34 ± 0.001
Total proteins	20.04 ± 0.5
Total carbohydrates	48.54 ± 0.4

Phytochemical Analysis

The estimation of primary and secondary metabolites was part of the phytochemical analysis. Lipids, proteins, and carbohydrates made up the majority of the estimated primary metabolites; Table 1 lists their distribution. Significant levels of lipids, proteins, and carbohydrates were found in the dry powder after analysis of key metabolites. Just like in earlier studies, the results of the current research imply that there were considerable levels of carbohydrates present. Plant nutrition appears to be influenced by the proximity of primary metabolites [28, 30]. The findings of secondary metabolites (total polyphenols, total flavonoids, total polysaccharides, and total glycosaponins) are summarized in Table 2. Analysis of secondary metabolites revealed that *Lawsonia inermis* dry leaf extract showed the highest amount of polyphenols (112.98 ± 0.033 g/mg) in the aqueous extract, while n-hexane showed the lowest amount of polyphenols (1.95 ± 0.012 g/mg). The methanol extract contains a significant amount of polyphenols (101.94 ± 0.011 mg/g) as well. Methanol extract showed the highest amount of flavonoids (93.81 ± 0.024 mg/g), while n-hexane had the least amount (1.34 ± 0.014 mg/g). The methanol extract contains the highest amount of glycosaponins (25.01 ± 0.033 mg/g), while n-hexane has the lowest amount (4.8 ± 0.017 mg/g) with polysaccharides (140.0 ± 024 mg/g) being the greatest in the water extract. Secondary metabolites were assumed to be the extraneous byproducts of plants [31]. One of the varied classes of secondary metabolites is polyphenols. In prior studies, it has been proven that polyphenols have anti-inflammatory [32], cardiovascular protective [33], anti-tumor, and gout reduction effects [34]. To assess the impact of phenolic compounds and their anti-oxidant actions, a study was conducted. According to the study's findings, phenolic chemicals also have an anti-

oxidant effect [35]. The importance of flavonoids in a range of medicinal, pharmacological, nutraceutical, and cosmetic applications is becoming more evident. The mechanisms by which the flavonoids work are currently unknown. But it's well known that items with a plant origin have a variety of biological properties [36].

Table 2. **Estimation of secondary metabolites in extracts.**

Extracts	Total Polyphenols (mg/g)	Total Glycosaponins (mg/g)	Total polysaccharides (g/g)	Total flavonoids (mg/g)
n-Hexane	1.95 ± 0.012	4.8 ± 0.017	15.0 ± 0.022	1.34 ± 0.014
Chloroform	11.01± 0.015	17.0 ± 0.190	34.0 ± 0.038	7.72 ± 0.02
Methanol	101.94 ± 0.011	38.8 ± 0.102	100.5 ± 0.006	93.81 ± 0.024
Water	112.98 ± 0.033	25.01 ± 0.033	140.0 ± 024	74.11 ± 0.01

Mineral Content Analysis of *Lawsonia inermis*Leaves

Numerous significant minerals were found in plants, according to the examination of their mineral composition (Table 3).

Table 3. **Results of mineral content analysis of *Lawsonia inermis* leaves.**

Element	Quantity (mg/g)
Fe	3.10
Cu	93.10
Cr	27.0
Zn	79.53
Ni	Not detected
Pb	0.05
Mg	0.91
Mn	275.0
P	229.54
Na	1553.0
K	3927.0
Ca	8.57

The results showed that plants are a rich source of precious minerals. As indicated in prior studies, numerous medical disorders are caused by a lack of minerals in the human body [37]. Maximum amounts of sodium, phosphorus, zinc, potassium, iron, calcium, and copper are present in the dried leaf powder of *Lawsonia inermis*. These minerals play a vital role in the maintenance of living organisms' physiology, such as calcium, which plays vital role in wound healing and blood coagulation. Magnesium is an essential component of plant chlorophyll and iron is required for the synthesis of hemoglobin [30, 38].

FourierTransform Infrared Spectroscopy

The FTIR spectra of dried leaves powder of *Lawsonia inermis* is presented in Fig. 1. The presence of phenol and alcohol was indicated by a peak in the plant powder's FTIR spectrum at 2700 cm^{-1} (OH stretching and H-bonding). Alkanes are present, as shown by the peak between 3000 $cm^{-1}$and 2850 cm^{-1}(medium, C-H stretching). Esters and saturated aliphatic functional groups are present, as shown by the C=O stretch at 1735 cm^{-1}. This shows that an aromatic band (C-C extension) exists at 1602 cm^{-1}. The presence of alcohol, carboxylic acid and ether moiety was indicated by the peak between 1320 $cm^{-1}$and 1000 cm^{-1} (strong, C-O stretching) [30].

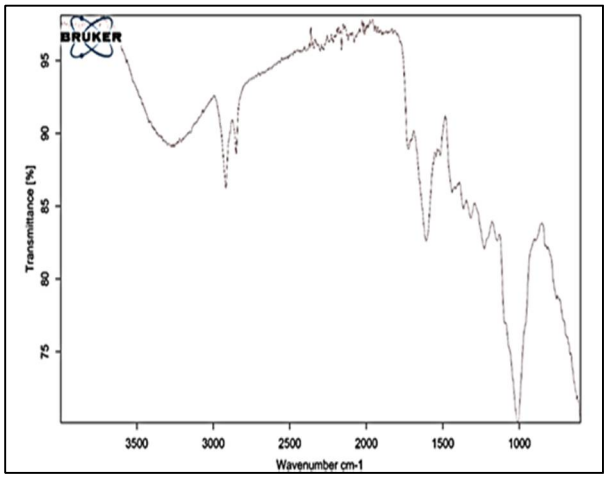

Figure 1. **FTIR Spectrum of *Lawsonia inermis* leaves powder**

Gas Chromatography Mass Spectrometry Analysis

GC-MS analysis shows approximately 100 compounds (Fig. 2). Compounds that showed an appreciable % area are summarized in Table 4. A study indicates 1,2,3-benzenetriol (10.86% area) showed a significant anti-oxidant effect [39]. 2(H)-furanone, dihydro-3-hydroxy-4,4-dimethyl- with molecular weight (130.1418) identified at peak number 5 having an area under the curve (5.29%). An earlier study showed that this chemical possesses anti-bacterial activity, which is caused by the suppression of biofilm production, particularly in gram-negative bacteria [40-42]. D- allose (4.05% area), 1(3H) - Isobenzofuranone (2.16% area), furneol (1.65% area) and benzofuran (1.75% area) showed substantial anti-bacterial and antioxidant effects in earlier studies [36-39, 43]. Glycerin is found at peak number 9 with an area under curve (3.74%). It is widely used in lubricants and as an essential ingredient in many cough syrups, expectorants, ointments, anesthetics, expectorants, and lozenges [44, 45]. 1(3H)-Isobenzofuranone at peak number 25 with molecular weight 134.1320 (g/mol) is identified. It is mainly used in lacquers, paints, and textiles [46].

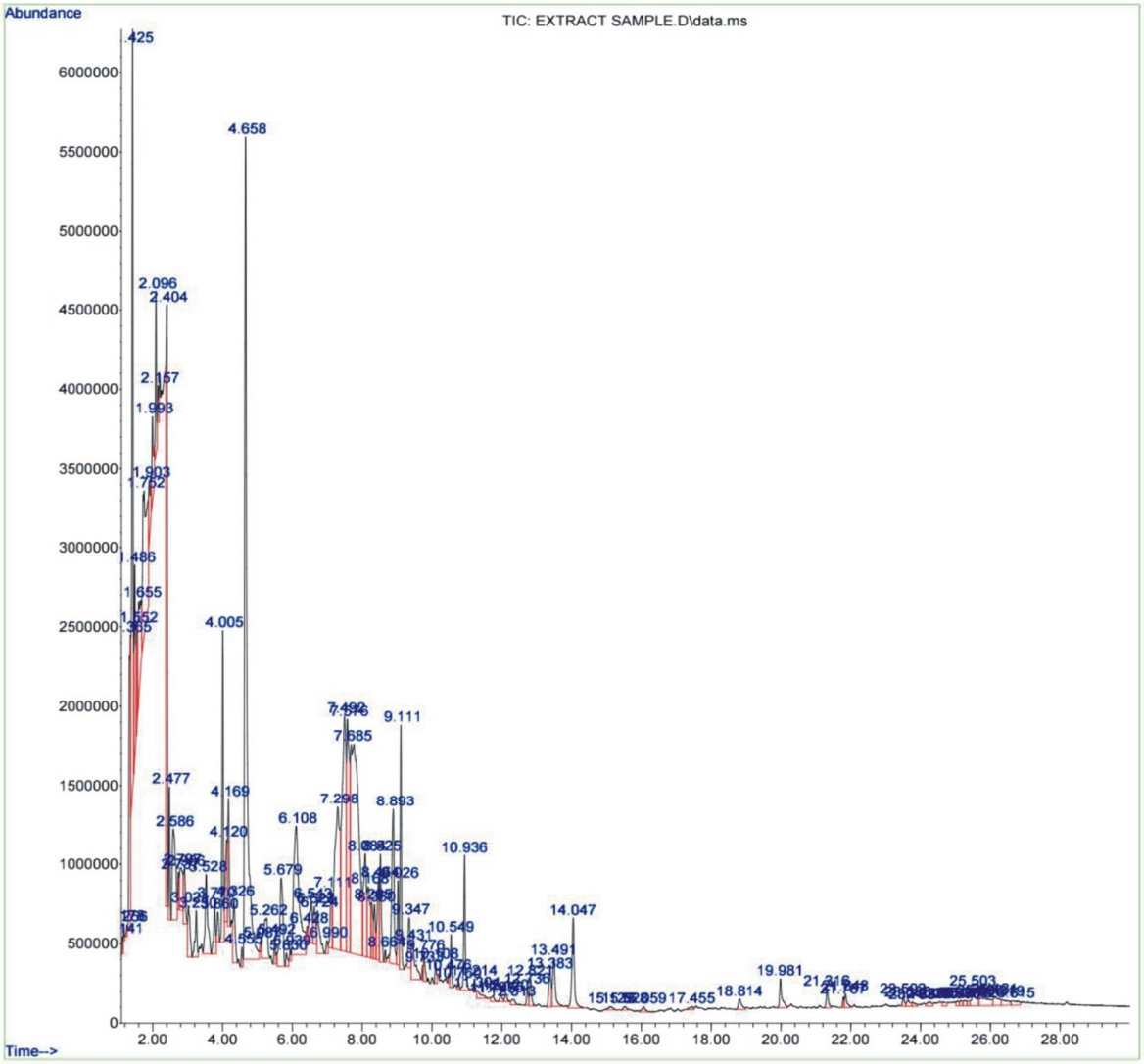

Figure 2. **GCMS chromatogram of dried leaves extract of *Lawsonia inermis***

Table 4. **Compounds detected in GCMS Analysis.**

Peak No.	RT (min)	Name of the compound	Molecular formula	Molecular weight (g/mol)	Area (%)
1	1.141	2-propamine, 1-methoxy	C_4H_8O	88.1051	0.08
5	1.425	2(H)-Furanone,dihydro-3-hydroxy-4,4-dimethyl-	$C_6H_{10}O_3$	130.1418	5.29
6	1.486	Furaneol	$C_6H_8O_3$	128.127	1.65
9	1.752	Glycerin	$C_3H_8O_3$	92.09	3.74
10	1.903	Hexane, 3-iodo-	C_6H_{13}	212.07	0.59
11	1.993	2-propanamine, N-methyl–N-nitroso-	$C_4H_{10}N_2O$	102.14	0.81
12	0.296	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	$C_6H_8O_4$	144.12	0.89
13	2.157	1-Penten-3-ol, 2-methyl	$C_6H_{12}O$	100.16	0.23
14	2.404	4H-Pyran-4-one, 3,5-dihydroxy-2-methyl-	$C_6H_6O_4$	142.1094	4.32
15	2.477	Catechol	$C_6H_6O_2$	110.1	0.77
16	2.586	Benzofuran	C_8H_6O	118.0	1.75
18	2.797	4-Vinylphenol	C_8H_8O	120.15	0.80
19	2.906	Octane,1-(ethenylthio)-	$C_{10}H_{20}S$	172.33	0.59
20	3.021	Diisopropyl sulfide	$C_6H_{14}S$	118.24	0.66
21	3.250	Butanoic acid, pentyl ester	$C_9H_{18}O_2$	158.24	0.48
22	3.528	2-methoxy-4-vinylphenol	$C_9H_{10}O_2$	150.17	1.14
23	3.770	Hydroquionone	$C_6H_6O_2$	110.11	0.52
25	4.005	1(3H) –Isobenzofuranone	$C_8H_6O_2$	134.1320	2.16
27	4.169	3-Butylisobenzofuran-1(3H)- one	$C_{12}H_{14}O$	190.2384	1.00
28	4.326	Butanoic acid, 2-methyl-, 1-methylethyl ester	$C_8H_{16}O$	144.2114	0.76
29	4.555	Benzaldehyde, 3-hydroxy-4-methoxy-	C_8H_8O	152.1473	0.14
30	4.658	1,2,3-Benzenetriol	$C_6H_6O_3$	126.11	10.86
31	5.087	4-vinylbenzene-1,2-diol	C_8H_{80}	136.14800	0.08
32	5.262	3-Buten-2-one, 4-(2-furanyl)	$C_8H_8O_2$	136.15	0.99
34	5.679	Tetralin-1,4-dione	$C_{10}H_8O_2$	160.169	1.56
35	5.830	2-Acetylbenzoic acid	$C_9H_8O_3$	164.16	0.15
36	5.939	Hexaethylene glycol	$C_{12}H_{26}O_7$	282.33	0.08
37	6.18	D- Allose	$C_6H_{12}O_6$	180.16	4.05
39	6.543	Fumaric acid, ethyl 2-proplphen	$C_{15}H_{18}O$	262.30	0.67
40	6.622	Phthalimidine	$C_8H_5NO_2$	147.13	2.00

High Performance Liquid Chromatography Analysis

The results of the HPLC analysis of the methanolic extract of the plant leaves are demonstrated in Fig. 3. Peaks at 3, 4, and 12 exhibited the presence of gallic acid (RT= 2.349), tannic acid (RT= 2.704) and quercetin (RT= 62.407), respectively, in the methanolic extract. Numerous in vivo and in vitro studies have demonstrated how quercetin works. Quercetin, a potent antioxidant, is now found in many therapeutic products. It is possible to conduct additional studies on the effects of quercetin on a number of different diseases [47]. The growth of various Gram-positive and Gram-negative bacteria, fungi, and viruses has been shown to be inhibited by quercetin. Quercetin has been proposed for use as an

antiviral in various studies due to its ability to inhibit the early stages of viral infection, interact with proteases essential for viral replication, and reduce infection-related inflammation. Quercetin may also be useful when used with other medications to increase their benefits or interact with them in a synergistic way in order to minimize their side effects and related toxicity [48]. The biological effects of polyphenols, which are naturally occurring antioxidants, include ulcer healing, cholesterol lowering, antibacterial, anticancer, antiviral, and antifungal properties. In the plant world, trihydroxybenzoic acid, often known as gallic acid, is a frequently present metabolite. As a result of its potent antioxidant and free radical scavenging properties, it can guard biological cells, tissues, and organs from harm caused by oxidative stress [49]. Because pharmaceuticals containing gallic acid can have a wide range of therapeutic effects, including antioxidants, anticancer, antibacterial, chondroprotective effects, carbonic anhydrase inhibitors, anti-ulcerogenic effects, and cathepsin D inhibitors, gallic acid has developed into a key component in the creation of novel pharmacological drugs [50].

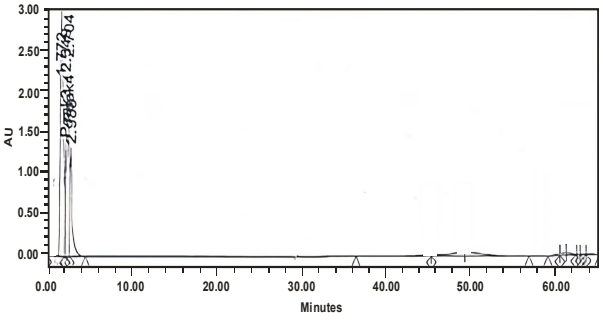

Figure 3. **HPLC analysis of methanol extract**

Tannic acid belongs to the same polyphenolic family as phenolic acid and can be acquired from plants, either as a pure chemical or as a component of a plant extract. Due to its unique properties, tannic acid has been the focus of various studies looking at how it might be added to biopolymer materials. It has remarkable biological characteristics, including speeding up cell proliferation, tissue regeneration, and wound healing, in addition to antibacterial and antiviral activities [51].

In vitro anti-inflammatory activity

The results showed (Fig. 4) that methanolic extract has the maximum value of percentage inhibition (86.885%) while n-hexane extract has the minimum value of percentage inhibition (35.8%). The standard drug diclofenac showed 85.045% inhibition. The actual measurement of protein stabilization compared to the control is the percentage of protein denaturation inhibition. A comparative evaluation of methanolic extract (86.88%) and diclofenac extract (85.045%) reveals that methanolic extract had better efficacy than the reference drug. Inflammation is a defensive mechanism of the body that may be induced by various disorders, i.e., rheumatoid arthritis, gout, inflammatory bowel disease, conjunctivitis, heart disease, and neurodegenerative disorders [52]. *Lawsonia inermis* leaves contain a variety of significant phytochemicals, including flavonoids, glycosaponins, and phenols that are beneficial in lowering the inflammatory response. Despite the fact that medications derived from natural sources are believed to be safe, *in vivo* and clinical studies will eventually be required to prove the plant's safety, effectiveness, and potential for therapeutic use [30, 53].

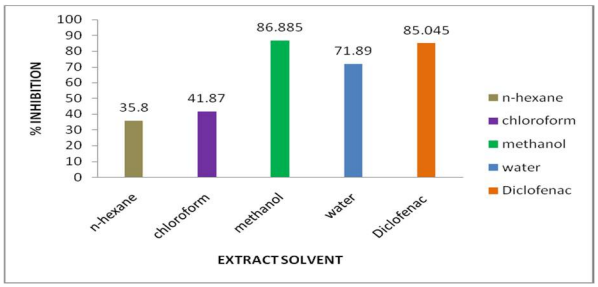

Figure 4. **Anti-inflammatory effect of *Lawsonia inermis* different extracts**

Anti-oxidant Activity

The anti-oxidant activity of the plant was tested by different assays, including DPPH, FRAP, phosphomolybdenum, and H_2O_2 radical scavenging activity. The study outcomes (Table 5) have shown that the methanolic extract showed maximum anti-oxidant activity as compared to the other extracts and results are comparable with the standard drug (Vitamin C).

Table 5. **Effect of *Lawsonia inermis* leaves extracts on different *invitro* activities.**

Assay	Absorbance of extracts/standard (As)	Activity (%)
DPPH		
Control (Ac)	-	-
n-Hexane	1.78	24.57
Chloroform	1.87	20.76
Methanol	0.307	86.99
Water	0.91	61.44
Standard (Vitamin C)	0.293	87.58
FRAP		
Control (Ac)	-	-
n-Hexane	1.78	26.14
Chloroform	1.76	26.97
Methanol	0.371	84.6
Water	0.98	59.33
Standard (Vitamin C)	0.29	87.96
Phosphomolybdenum antioxidant activity		
Control (Ac)	-	-
n-Hexane	1.53	40.23
Chloroform	1.21	52.7
Methanol	0.51	80.07
Water	0.592	76.87
Standard	0.458	82.01
H_2O_2 scavenging capacity		
Control (Ac)	-	-
n-Hexane	2.05	10.4
Chloroform	1.56	31.8
Methanol	0.415	81.87
Water	0.89	61.13
Standard	0.321	85.98

Numerous studies have established that the polyphenols and flavonoids present in the plant's leaves are what give it its antioxidant properties. Reactive oxygen species (ROS), which are harmful to health, are created during metabolism. When antioxidants are present, the body usually eliminates these free radicals [54]. However, if the body is unable to get rid of free radicals, it could lead to long-term degenerative diseases like cancer, arthritis, and cardiac issues. Natural antioxidants found in medicinal plants are a significant source of health benefits for humans as well as being crucial to plant survival [21].

Antibacterial Assay

Lawsonia inermis different extracts showed substantial antimicrobial activity (Table 6) at a concentration of 400 mg/mL. The results were interpreted according to the Clinical and Laboratory Standards Institute (CLSI) guidelines, 2021. The antimicrobial activity was found in the order of methanol > water > chloroform > hexane extract. Among the entire extracts, methanol showed the maximum activity and results are comparable to the reference drug (tetracycline). Plants are thought to have dynamic chemicals that provide defense against a variety of microbes, parasites, and protozoa. The leaf extract of *Lawsonia inermis* has been found to contain certain bioactive components that show medicinal and physiological activities. Its antimicrobial properties could be attributed to a phytochemical called quercetin. Previous research has demonstrated that quercetin prevents the development of a variety of fungi, gram-positive and gram-negative bacteria and viruses. The process by which it has antibacterial effects involves mitochondrial malfunction, cell membrane damage, altered membrane permeability, suppression of protein and nucleic acid synthesis, down regulation of virulence factor expression, and prevention of biofilm formation [55].

Table 6. **Antibacterial activity of *Lawsonia inermis* in various extracts.**

Extract/ Drug	Zone of inhibition (mm)					
	Gram +ve bacteria		Gram –ve bacteria		Aerobic bacteria	Anaerobic bacteria
	Styphylococ cusaureus	*Streotococcusp nuemoniae*	*Klebsiellapneum oniae*	*Pseudomonas aeroginosa*	*Mycobacterium tuberculosis*	*Peptostreptococ cusanaerobius*
N-hexane	6.0	6.2	5.2	5.0	5.5	4.5
Chloroform	7.0	7.3	6.4	6.0	6.3	6.0
Methanol	13.0	13.2	12.7	12.5	8.1	9.2
Water	8.1	7.4	5.3	2.5	6.9	6.3
Tetracycline	13.5	13.0	13.7	12.9	9.1	10.3

Conclusion

Worldwide, medicinal plants are used extensively to treat a wide range of illnesses because they are seen as secure, efficient, and available. In traditional medicine, *Lawsonia inermis* has a long history of demonstrating powerful pharmacological effects. The plant leaf extracts used in the current investigation revealed the presence of several bioactive substances like polyphenols, flavonoids, glycosaponins, and tannins which are known to have physiological and therapeutic effects. Numerous minerals were found i.e., Fe, Zn, K, Na, and Ca, which are necessary to support human physiology. Important volatile compounds such as 1,2,3-benzenetriol, furneol, furanone, D-allose, isobenzofuranone, etc. that had prominent biological activity in earlier studies were found by GCMS analysis. The methanolic extract of the plant's leaf had noticeably higher antioxidant, anti-inflammatory, and broad-spectrum antibacterial activity than the others. As the plant has demonstrated the presence of many bioactive chemicals and shown therapeutic effects as well, it is encouraged to conduct more research in the future to establish the plant's usefulness and safety when used in therapeutic contexts.

Conflict of Interest statement

The author declared no conflict of interest among them.

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