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Phytochemical and High Performance Liquid Chromatography (HPLC) Analysis of *Lawsonia inermis* Linn

Baburao B. Dhanawade[1], Laxmikant B. Dama[2] and Ajay S. Zinjade[3]

[1,2] *Department of Zoology, D. B. F. Dayanand College of Arts and Science, Solapur*
[3]*Department of Botany, Dr. Babasaheb Ambedkar Marathwada University, Chhatrapati Sambhajinagar.*
[1]E-mail- baburaodhanawade@gmail.com

ABSTRACT

Lawsonia inermis commonly known as Henna, is a plant belonging to Lythraceae family. It is one of the most effective medicinal plants and it has been using for treatment of wounds and burns. The plant is widely cultivated tropical region of Asia, Africa and the Americas, particularly in India, Pakistan, Iran and North Africa. The using of Henna leaves is very popular for Dye, Wool, Leather, Silk, Fingernails, Hair, Cosmetic as well as medicine in many countries. Henna leaves contain lots of different compounds and Lawsone is the main one. In current study, extraction with methanol solvent of *Lawsonia inermis* (Henna) leaves was performed different qualitative phytochemical test. The qualitative phytochemical analysis shows the contain such as, Glycosides, Alkaloids, Flavonoids, Steroids, Phenols, Terpenoids, Saponins, Carbohydrates, Proteins Starch and Flavanol are dominantly present in Methanol extract of *Lawsonia inermis* leaves and the absent of Tannin and Anthocyanin. The Lawsone was isolated by using High Performance Liquid Chromatography (HPLC) System. The potential applications *Lawsonia inermis* (Henna) in the pharmaceutical, cosmetic and food industries are also discussed, along with future research directions.

Keywords: Phytochemical, HPLC, *Lawsonia inermis*, Glycosides, Alkaloids, Flavonoids

INTRODUCTION

Plants have long been employed as a source of medicine throughout human history. The knowledge of the different medicinal values of plants has been passed down through the generations via observation and experimentation. However, from time to time, people started to be interested in knowing where the plant properties originate from and the scientific explanation for how those properties are capable of producing therapeutic effects (Mendoza, 2018). This is the beginning of phytochemistry which can be portrayed as a study of phytochemicals which involved chemicals that can be derived from plants. It deals with the structure and biological properties of secondary metabolites that responsible to give a therapeutic effect. Herbal medicines have wide biological and medicinal activities as they are in huge demand in different countries. Herbal medicines which contain natural substances promote good health and support wellness (Prasathkumar, et. al., 2021). To combat disease, medicinal plants are used as well as they are part of human society from ancient times (Manoharacharya and Nagaraju, 2016).

Lawsonia inermis Linn. (Henna) is a tropical and subtropical shrub, growing in North Africa, Middle East and Indian subcontinent. The powder made of dried crushed leaves is called Henna (Oumeish, 2001).

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The *Lawsonia inermis* is known by different names such as Hindi, Heena; Marathi, Mendi; Sanskrit, Medika and Dvivratna; Urdu, Mehendi and English, Egyptian privet. When applied in a form of paste onto hair or skin, it imparts a reddish-brown coloration lasting for up to twelve weeks. *Lawsonia inermis* is a perennial plant. The taxonomy of *Lawsonia inermis* is as follows: Kingdom, Plantae; Division, Magnoliophyta; Class, Magnoliopsida; Order, Meyrtales; Family, Lythraceae; Genus, *Lawsonia;* Species, *Lawsonia inermis* L. It is used as a traditional product with religious importance mainly for medicinal and cosmetic purposes (Warrier, 2001). *Lawsonia inermis* is a small tree or branched glabrous shrub, grow up to a height of 6.5 to 23 feet. Popularly its leaves are useful but other parts such as stem, roots, bark, flowers and seeds have many medicinal uses. It is used to colour the hair, skin and nails which is worldwide known as cosmetic agent. Leaves are elliptical in shape or broadly lanceolate, acute or obtuse measuring 1.3-3.2 cm by 0.6-1.6 cm, respectively. Flower character is white or rose-coloured, pedicels short and slender. Capsules are of 6 mm diameter, supported by persistent calyx and tipped and slightly veined outside. Seed capsules are red, about the size of a pea, globose with numerous tiny pyramidal, pitted brown seeds.

Phytochemical studies on species from other regions have shown the richness of *Lawsonia inermis* in secondary metabolites such as flavonoids, tannins, coumarins, alkaloids, quinones, saponins, xanthones, sterols and terpenes (Dahake and Kamble, 2015). The phenolic profile of *Lawsonia inermis* consists mainly of caffeic, ellagic, ferulic, gallic, coumaric acids, lawsoniaside (1,3,4-trihydroxynaphthalene 1,4-di-β-D-gluco-pyronoside) and flavonoid glycosides (quercetin, kaempferol, rutin, myricetin, luteolin) (Dhaouadi, et. al., 2015 and Al-Snafi, 2019). The main colouring matter of the plant, Lawsone (2-hydroxy-1,4-naphtoquinone) is present in dried leaves at the concentration of 0.4% - 1.5% (Rahmoun, et. al., 2012 and Ziaei, et. al., 2016). Benzenoid derivatives (lawsoinermone, inermidioic acid, inermic acid) from the aerial part, two alkaloids (harmine and harmaline), five triterpenes including rosamutin, euscaphic acid, ursolic and arjunic acids from the leaves, and coumarins, lacoumarin (5-allyoxy-7-hydroxycoumarin) were characterized and isolated from *Lawsonia inermis* (Al-Snafi, 2019). Studies on henna and its components have revealed various pharmacological properties in the treatment and management of diseases such as leprosy, fever, leukorrhea, rheumatoid, arthritis, ulcers, heart diseases, wounds, blood infections, inflammations, diabetes, and headaches (Leonard, 2022 and Debapriya, et. al., 2020; Ameen et al., 2021).

The analgesic, hypoglycemic, hepatoprotective, immunostimulating, anti-inflammatory, antibacterial, antifungal, antivirus, anticancer, and anti-parasitic, etc. effects of the plant have been attributed to the presence of the phytochemicals mentioned above (Fathima, 2018). *Lawsonia inermis* is also well known, particularly for its purposes of accelerating growth and dyeing hair and nails due to Lawsone (Ziaei, et. al., 2016; Naini et al., 2013). This study complements the first, and aims to determine the qualitative and quantitative phytochemical profile potency of *Lawsonia inermis* fractions from Chhatrapati Sambhajinagar.

MATERIALS AND METHODS

Study Area

This study was conducted at the Department of Zoology, D. B. F. Dayanand College of Arts and Science, Solapur. All experiments were accomplished aseptically in the Department laboratory.

Collection of Plant

Fresh and healthy leaves of *Lawsonia inermis* were collected from Chhatrapati Sambhajinagar. The leaves were collected in the early morning in a sterilized paper bag with the help of sharp sterilized cutter. The paper bag was labelled properly by indicating the site of collection, date, time and then leaves sample were taken to the laboratory for further analysis (Fig. 1).

Authentication of Plant

The identification of Plant *Lawsonia inermis* Linn. (Family: Lythraceae) was authenticated by Plant Taxonomist Senior Professor Dr. Arvind S. Dhabe (Accession No. 00877), Department of Botany, Dr. Babasaheb Ambedkar Marathwada University, Chhatrapati Sambhajinagar (Maharashtra) and also through various literature survey comparisons.

Extract Preparation

The Fresh leaves were washed under running tap water to remove all the dust and air-dried in shade at room temperature for 4 weeks and ground with an electric grinder to obtain fine powder. The

powder was stored in a sealed airtight bottle at room temperature. The fine powder was extracted by Soxhlet extraction method by using the Methanol as a solvent. The 25 gm of fine powder was collected and enclosed in strong fitter paper pouches containing powder are placed within the Soxhlet thimble chamber. As an extraction 300ml solvent Methanol is taken in Round-bottom flask and heated using heating source such as heating mantle. The heating temperature is 40°C-50°C. The bottom flask's solvent vaporized in the condenser due to heat, and it subsequently drips back to the sample thimble. When the amount of liquid the siphon arm, the liquid contents once more drained into the bottom flask, and the siphon tube clear solution indicates that the procedure is finished. After Soxhlet extraction the solvent along with extract was collected and kept in a bottle. This bottle was tightly sealed and coated with adhesive tape and kept into the refrigerator at 4°C for further Studies.

Fig 1. Collection of *Lawsonia inermis* Linn

Qualitative Phytochemical Analysis

The Extracted solution was prepared from Methanol solvent. The obtained Extract solutions were subjected to phytochemical analysis based on standard methods (Fig. 2).

Test for Glycosides

Keller Killani Test (Cardiac glycosides): The test solution with few drops of glacial acetic acid in 2 ml of ferric chloride solution and concentrated sulphuric acid is added from the sides of the test tube which shows the separation between two layers, lower layer shows reddish brown and upper layer turns bluish green in colour.

Raymond's Test: Test solution treated with dinitrobenzene in hot methanolic alkali gives violet colour.

Legal's Test: The test solution treated with 1ml pyridine and 1ml sodium nitroprusside gives pink to red colour appears.

Test for Alkaloids

Mayer's Test: Test solution treated with Mayer's reagent (Potassium mercuric iodide) gives cream coloured precipitate.

Wagner's Test: The acidic test solution treated with Wagner's reagent (Iodine in potassium iodide) gives brown precipitate.
Hager's reagent: The acetic test solution treated with Hager's reagent (Saturated picric acid solution) gives yellow precipitate.

Test for Flavonoids

Ferric chloride Test: The test solution with few drops of ferric chloride solution shows intense green colour.

Shinoda Test: Test solution with few fragments of magnesium ribbon and concentrated hydrochloric acid shows pink to magenta red colour.

Zinc - Hydrochloric acid-reduction Test: Test solution with zinc dust and few drops of hydrochloric acid shows magenta red colour.

Alkaline reagent Test: Test solution when treated with sodium hydroxide solution shows increase in the intensity of yellow colour which becomes colourless on addition of few drops of dilute acid.

Lead acetate solution Test: Test solution with few drops of lead acetate solution (10% w/v) gives yellow precipitate.

Test for Steroids

Chloroform Test: The crude plant extracts (1 mg) was taken in a test tube and dissolved with chloroform (1 mL), then added equal volume of concentrated sulphuric acid to the test tube by sides. The upper layer in the test tube was turns into red and sulphuric acid layer showed yellow with green fluorescence. It showed the presence of steroids.

Salkowski's Test: The second portion of solution above was mixed with concentrated sulphuric acid carefully so that the acid formed a lower layer and the interface was observed for a reddish-brown colour.

Test for Phenols

Ferric Chloride Test: A small amount of the ethanolic extract was taken with 1 mL of water in a test tube and 1 to 2 drops of Iron III chloride (FeCl3) was added. A blue, green, red or purple colour is a positive test.

Test for Terpenoids

Salkowski's Test: When few drops of concentrated sulphuric acid is added to the test solution, shaken and allowed to stand, lower layer turns red indicating the presence of sterols.

Liebermann Burchard Test: The test solution treated with few drops of acetic anhydride and mixed well. When concentrated sulphuric acid is added from the sides of the test tube, it shows a brown ring at the junction of the two layers and the upper layer turns green.

Test for Saponins

Foam Test: Saponins when mixed with water and shaken shows the formation of foam which is stable at least for 15 min.

Haemolysis Test: 2 ml of 18%w/v sodium chloride in two test tubes were taken. To one test tube distilled water and to the other test tube 2 ml of filtrate were added and then few drops of blood was added to both the tubes. Mixed and observed the haemolysis under microscope.

Raymond's Test: Test solution treated with dinitrobenzene in hot methanolic alkali gives violet colour.

Test for Carbohydrates

Molisch's Test: Test solution with few drops of Molisch's reagent and two ml of concentrated sulphuric acid added slowly from the sides of the test tube shows a purple ring at the junction of two liquids.

Barfoed's Test: Test solution treated with Barfoed's reagent and boiling on a water bath shows brick red precipitate.

Benedict's Test: Test solution treated with Benedict's reagent and boiling on a water bath shows reddish brown precipitate.

Test for Proteins

Millon's Test: Test solution treated with Millon's reagent and heated on a water bath; protein is stained yellow on warming.

Xanthoproteic Test: Test solution treated with concentrated nitric acid and on boiling gives yellow precipitate.
Biuret Test: Test solutions treated with 40% sodium hydroxide and dilute copper sulphate solution gives blue colour.

Ninhydrin Test: Test solution treated with ninhydrin reagent gives blue colour.

Test for Starch

Starch Reagent: Test: 1ml of extract was added into 10ml of Nacl solution. After heating, starch reagent was added a blue purplish colour is a positive test for the presence of starch.

Test for Tannins

Gelatine Test: Plant Extract is dissolved in 5ml of distilled water and 1% gelatine solution and 10% Nacl. Reaction gives a white precipitate.

NaoH Test: 4 ml of 10% NaoH added into the 0.4ml of extract and shaken well formation of emulsion.

Lead acetate solution Test: Test solution with few drops of lead acetate solution (10% w/v) gives yellow precipitate.

Test for Anthocyanin

Hcl Test: 2ml of plant extract and 2 ml of 2N Hcl were mixed with few ml of ammonia gives the pink red solution turns into blue violet after addition of ammonia.

Lawsone Quantification in *Lawsonia inermis* by High-Performance Liquid Chromatography (HPLC)

Chromatographic conditions

All other reagents, chemicals and solvents were used of analytical grade and HPLC grade. The Phenomenex C-18 column (250 mm × 4.6 mm having 5.0 µm particle size adjusted with a mobile phase consisting of water to acetonitrile (30:70, v/v)) were used. The mobile phase of pH 6.5 were used to run the HPLC. The flow rate of the run was kept at 1 ml/min with ambient temperature.

Preparation of Sample solution

The 1000 µg/ml stock solution was prepared by dissolving an accurately 25 mg of test sample in 25 ml methanol. The stock solution was further diluted to 10 µg/ml with methanol.

HPLC Analysis

The crude extract was obtained by *Lawsonia inermis* leaves. This extract was found to be soluble completely in Methanol solvent. The extract was screened for quantitative analysis which was carried by instrument DESIGN EXPERT® Version 7.0.2.8 (Stat-Ease Inc., Minneapolis, USA) to obtained phytochemical constituents by using HPLC technique at sophisticated analytical instrument.

RESULTS AND DISCUSSION

Qualitative Phytochemical Test

Lawsonia inermis has many traditional and commercial uses, the most common being as a dye for hair, skin and fingernails. As a dye and preservative for leather and cloth and as an antifungal. The results of the phytochemical analysis test carried out on *Lawsonia inermis* leaves confirmed the presence and absence of secondary plant metabolites. The carried out different qualitative phytochemical test such as, Glycosides, Alkaloids, Flavonoids, Steroids, Phenols, Terpenoids, Saponins, Carbohydrates, Proteins, Starch, Flavanol, Tannin and Anthocyanin. From the result was detected that 80% of the phytochemical test were present in *Lawsonia inermis* leaves. The qualitative phytochemical analysis showed the contain such as, Glycosides, Alkaloids, Flavonoids, Steroids, Phenols, Terpenoids, Saponins, Carbohydrates, Proteins Starch and Flavanol are dominantly present in methanol extract of *Lawsonia inermis* leaves. The qualitative phytochemical test analysis shows the absent of Tannin and Anthocyanin in the methanol extract of *Lawsonia inermis* leaves. This is because the level of maturity is an important factor that affects composition and number of phytochemical compounds in plant leaves. Methanol solvents confirmed to be more effective in extracting phytochemical compounds. The phytochemical compounds in *Lawsonia inermis* leaves are dominated by polar compounds, so the effective solvents for extracting phytochemical compounds are polar solvents such as Methanol (Table 1 & Fig. 2).

Lawsone Quantification in *Lawsonia inermis* by High-Performance Liquid Chromatography (HPLC)

The further phytochemical quantification of Lawsone in *Lawsonia inermis* was done from sophisticated analytical and instrumentation facility by using standard method of High-Performance Liquid Chromatography (HPLC). The chromatogram and its corresponding peak of crude extract of *Lawsonia inermis* was recorded at 202 nm wavelength. The chromatogram X-Axis shows how long each compound took to travel through the HPLC Column and Y-Axis represent the UV absorbance, which correlates to compound concentration. The chromatogram shows three distinct peaks of Lawsone compound presence. The first peak is small and Lawsone compound is minor and its area covered 8.151% in 2.497minute retention time. The second peak is the major peak, it indicating the most abundant Lawsone compound are present and its area covered 47.005% in 3.117-minute retention time similarly, the last third peak also a major component, nearly equal to the second peak, its area covered 44.8.44% in 17.735-minute retention time (Fig. 3).

The qualitative phytochemical analysis of *Lawsonia inermis* leaves revealed a rich profile of secondary metabolites, with 80% of tested compounds (Glycosides, Alkaloids, Flavonoids, Steroids, Phenols, Terpenoids, Saponins, Carbohydrates, Proteins, Starch, and Flavanol) detected in methanol extracts, while Tannins and Anthocyanins were

Fig 2. Qualitative Phytochemical Test of *Lawsonia inermis* Linn.

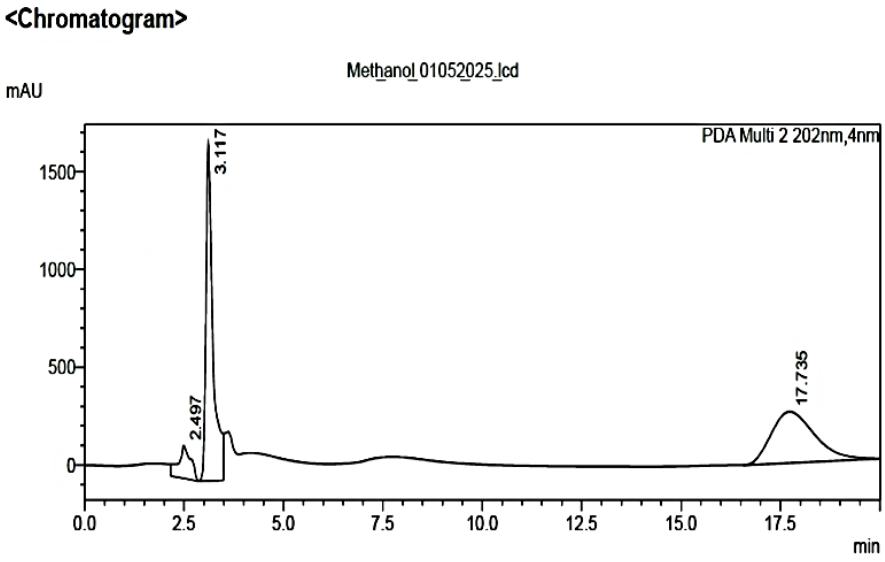

Fig 3. HPLC Chromatogram of Lawsone

notably absent, potentially due to leaf maturity influencing phytochemical composition. The efficacy of methanol as an extraction solvent underscores the polar nature of these bioactive compounds. HPLC quantification of Lawsone—the primary naphthoquinone responsible for the plant's dyeing and antifungal properties—demonstrated its substantial presence, with chromatograms at 202 nm revealing three distinct peaks: a minor component (8.15% area at 2.50 min) and two major peaks at 3.12 min (47.01% area) and 17.74 min (44.84% area), collectively indicating high Lawsone concentration. This robust phytochemical profile, dominated by polar metabolites and significant Lawsone yield, scientifically validates *Lawsonia inermis*'s traditional applications in hair dyeing, leather preservation, and antifungal treatments, while highlighting methanol's superiority for extracting its bioactive constituents.

Table 1. Qualitative Phytochemical Characteristics Test of *Lawsonia inermis* with Methanol Solvent

Sr. No.	Chemical Constituents	Tests Name	Observation
1	**Glycosides**	Keller Killani Test	+
		Raymond's Test	+
		Legal's Test	-
2	**Alkaloids**	Mayer's Test	+
		Wagner's Test	-
		Hager's Reagent Test	-
3	**Flavonoids**	Ferric Chloride Test	+
		Shinoda Test	-
		Zinc – Hydrochloric Acid-reduction Test	-
		Alkaline Reagent Test	+
		Lead Acetate Solution Test	+
4	**Steroids**	Chloroform Test	+
		Salkowski's Test	+
5	**Phenols**	Ferric Chloride Test	+
6	**Terpenoids**	Salkowski's Test	+
		Liebermann Burchard Test	-
7	**Saponins**	Foam Test	+
		Hemolysis Test	-
		Raymond's Test	-
8	**Carbohydrates**	Molisch's Test	+
		Barfoed's Test	-
		Benedict's Test	+
9	**Proteins**	Millon's Test	-
		Xanthoproteic Test	-
		Biuret Test	+
		Ninhydrin Test	-
10	**Starch**	Starch Reagent Test	+
11	**Flavanol**	Ferric Chloride Test	+
		Shinoda Test	-
		Zinc – Hydrochloric Acid-reduction Test	-
		Alkaline Reagent Test	+
12	**Tannins**	Gelatin Test	-
		NaOH Test	-
13	**Anthocyanin**	Hcl Test	-

CONCLUSION

Lawsonia inermis is a universal herbal medicine that acts as colouring agent and also as a product with diverse pharmacological activity. It is a unique source of various phytochemicals. The qualitative phytochemical study confirmed the presence of Glycosides, Alkaloids, Flavonoids, Steroids, Phenols, Terpenoids, Saponins, Carbohydrates, Proteins Starch and Flavanol are dominantly present in methanol extract of *Lawsonia inermis* leaves and the absent of Tannin and Anthocyanin. The Lawsone was isolated by using High Performance Liquid Chromatography (HPLC) System. The chromatogram and its corresponding peak of crude extract of *Lawsonia inermis* was recorded at 202 nm wavelength. Lawsone quantification study was very useful to draw the usage pattern of henna powder for the consumer to follow the correct methods to achieve the optimum active potential during the colouring practice and get the vibrant colour delivery. Further evaluation needs to be carried out on *Lawsonia inermis* in order to explore the concealed areas and their practical clinical applications, which can be used for the welfare of the mankind.

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Conflicts of Interest

Authors declare that there is no conflict of interests regarding the publication of this paper.

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