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PHYTOCHEMICAL COMPONENTS, ANTIMICROBIAL PROPERTIES, AND ANTIOXIDANT ACTIVITIES OF *LAWSONIA INERMIS* LINN (HENNA) LEAF EXTRACT

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Abstract: The research aimed to evaluate the bioactive potential of henna ethanolic (HenEtOH) and henna hexane (HenH) crude extracts derived through maceration, and to compare their efficacy based on solvent polarity. Phytochemical screening indicated the presence of alkaloids, saponins, steroids, and phenolic compounds and the absence of terpenoids, flavonoids, and tannins in the ethanoic extract, while flavonoids and tannins were only detected in the hexane extract. Extraction yields were higher with ethanol, producing 41.43 g (20.70 ± 0.63 %) compared to 6.00 g (3.00 ± 0.12 %) with hexane. Antimicrobial activity analysis showed that HenEtOH exhibited broad-spectrum antibacterial and antifungal properties, with inhibition zones ranging from 14.00 ± 00 to 19.00 ± 00 mm. Conversely, HenH extract showed no inhibitory activity against any tested microorganisms. Antioxidant analysis revealed that HenEtOH exhibited superior antioxidant activity, with 94 ± 0.01 % inhibition. In contrast, HenH displayed lower antioxidant. The findings demonstrated that solvent polarity greatly influences extraction efficiency and bioactivity, with the ethanol extract (HenEtOH) showing higher yield and stronger biological effects than the hexane extract (HenH).

Keywords: *bioactive compounds, ethanolic extract, extraction efficiency, polarity, screening*

INTRODUCTION

Lawsonia inermis Linn, commonly referred to as henna, is a flowering plant renowned for its dye-producing leaves. This perennial shrub, native to tropical and subtropical regions, has been used for centuries for various purposes, including body art, hair coloring, and even as a natural remedy. The leaves of *Lawsonia inermis* are rich in lawsone, a compound responsible for the plant's characteristic reddish-brown pigment. Historically, henna has held significant cultural importance in numerous societies, symbolizing joy and celebration during weddings and festivals. Its sustainable cultivation and versatile applications continue to make it a popular choice in modern times for both cosmetic and therapeutic use. The henna plant, belonging to the *Lythraceae* family, is a flowering species that can be characterized as a shrub or small tree. It typically grows to a height of two to six meters. The leaves are characterized by their small size and elliptical shape, arranged oppositely along the stem, and feature a sleek, glossy texture. The flowers emit a delightful fragrance and display colors ranging from white to pinkish hues, forming beautiful terminal panicles. The fruit takes the form of a compact, brownish capsule packed with a multitude of seeds. This plant is grown in various areas for its ornamental value and as a source of commercial dye, as noted by Gupta [1]. This species predominantly inhabits the tropical, sub-tropical, and semi-arid regions of Africa, specifically within the tropical Savannah and arid zones. Additionally, it can be found in southern Asia and northern Australia, as noted by Amit *et al.* [2]. *L. inermi*s, commonly known as henna, has found extensive use across various countries, including Sudan, India, and regions of the Middle East, for both traditional medicinal applications and cosmetic uses. Henna, a natural dye derived from the *Lawsonia inermis* plant, is commonly referred to by several names, including henna, the henna tree, the mignonette tree, and the Egyptian privet. This plant represents the only species within the Lawsonia genus [3]. Henna has a long-standing history that dates back to ancient times, serving as a natural dye used for coloring the skin, hair, and nails, in addition to being used on various fabrics such as silk, wool, and leather. The term is also applied to various skin and hair coloring products, including black henna and neutral henna. It is important to note that neither of these products is sourced from the actual henna plant [4, 5].

Previous analytical studies revealed that, Henna plant contained mainly lawsone which is responsible for brown dye. Henna does not stain the skin until lawsone molecules are released from the leaves. When dried henna leaves are crushed into a paste, they can stain the skin. The lawsone gradually migrates from the paste into the outer skin layer, where it binds to skin proteins, producing a lasting stain. Because coarse crushed leaves are difficult to use for detailed designs, henna is typically processed into a fine powder by drying [6], milling, and sifting the leaves. This powder is then mixed with liquids such as water, lemon juice, or strong tea, along with other ingredients depending on cultural or traditional practices. Many previous studies have shown that henna contains a variety of phytochemical compounds besides lawsone, found in different parts of the plant, including flavonoids, tannins, and coumarins [7]. These compounds have demonstrated biological activities such as anti-inflammatory and antimicrobial effects [8], antioxidant properties [9], and anticancer potential [10]. Currently, the global landscape is experiencing an increasing prevalence of chronic non-communicable diseases (NCDs), including diabetes, cardiovascular diseases, strokes, and various forms of cancer. According to the World Health Organization [11], these conditions are responsible for

70 % of deaths worldwide. The viability of synthetic drugs is diminishing, largely due to a range of concerning side effects. These include issues related to toxicity, the development of drug resistance, escalating costs, dependence on non-renewable resources, and negative effects on the environment [12]. This scenario highlights the necessity for alternative treatment options, particularly those derived from medicinal plants. The medicinal plants found in Sudan exhibit substantial potential, primarily due to the rich diversity of phytochemical compounds present. This diversity is largely shaped by the varying geographic factors within the region. As a result, this study focuses on cultivating *L. inermis* in Sudan and creating leaf extracts using the maceration method, which employs a variety of organic solvents, including n-hexane and ethanol. Additionally, this study seeks to analyze the phytochemical components present and assess their diverse pharmacological effects, particularly focusing on antibacterial and antioxidant properties. Research on the health benefits of Sudanese *L. inermis* may provide valuable insights into this topic, making it a promising avenue for the development of natural treatment agents while also reducing the usage of manufactured medications.

MATERIALS AND METHODS

Materials

Analytical-grade chemicals and reagents were used throughout the experimental procedures. Wagner's reagent was purchased from local chemicals company in Khartoum town (Sudan). Sodium hydroxide (≥ 98 % purity, pellets, reagent grade) was obtained from Honeywell Chemicals (UK). Potassium hydroxide (97 % purity, reagent grade, powder) was procured from Sigma-Aldrich. Chloroform (99.9 % purity, moisture content ≤0.01 %) was supplied by Henan GP Chemicals (China). Ethanol (99.9 % purity, industrial/electronic grade) was sourced from manufacturers in Malaysia, as listed in the Global Suppliers Directory. Normal hexane (99 % purity, HPLC grade) and Dragendorff's reagent (≥ 98 % purity) were supplied by Auraiya Laboratory Chemicals Pvt. Ltd. (India). Dimethyl sulfoxide (DMSO, ≥ 99 % purity, reagent grade) was purchased from Sigma and supplied via local distributors. Sulfuric acid (98 % w/w purity), ferric chloride (analytical grade), were also sourced from Sigma through local suppliers and distilled water (lab-distilled).

Methods

Collecting and authenticating of botanical samples

Samples of candidate *L. inermis* plants were collected in October 2022, from the city of Omdurman, located in Khartoum, Sudan. The plant specimens underwent identification and confirmation at the Herbarium in the Department of Botany, College of Science and Technology at Omdurman Islamic University. This process was conducted by staff members from the Botany Laboratory associated with the department.

Preparation of L. inermis leaf extracts

The plant samples for the research were cleaned by rinsing in tap water and followed by three washes with distilled water. They were air-dried at 26 °C until completely dry and then ground into a fine powder. For extraction, two hundred grams of the dried plant material were macerated in 500 mL of ethanol, and an additional 200 g of the same powdered sample was extracted with 500 mL of *n*-hexane at room temperature for 48 hours. The extracts were filtered using Whatman filter paper and cotton wool. After this, the samples were freeze-dried. The crude extracts were dissolved in dimethyl sulfoxide (DMSO) and stored in a refrigerator at 4 °C for later use.

Phytochemical analysis

The study analyzed extracts from the *L. inermis* plant using ethanol and n-hexane. It followed a standard procedure reported by Ukey and Gogle [13] to detect active compounds including alkaloids and flavonoids, saponins, tannins, terpenoids, steroids, and phenolic compounds.

Alkaloids (Wagner's test)

In the experiment, 1 mL of the extract was mixed with 2 mL of Wagner's reagent, and the mixture was stirred well. Following this reaction, the obtaining of a distinct reddish-brown or orange precipitate indicates the presence of alkaloids.

Flavonoids

Two milliliters of sodium hydroxide solution (0.5 M) were added to the 5 mL extract. The first thing noticed was a brilliant yellow hue, but it later disappeared. The presence of flavonoids is evidenced by discoloration/becoming colorless upon addition of dilute acid.

Saponins (Frothing test)

A test tube was prepared by combining 5 milliliters of each extract with an equal volume of 5 milliliters of distilled water. The mixture was agitated energetically while keeping a close eye on the formation of a consistent and enduring foam.

Tannins (Ferric chloride reagent)

A 0.5 milliliter portion of each extract was combined with 10 milliliters of distilled water in a test tube. Following this, several drops of 1 % solution of ferric chloride were introduced. The combination was subsequently observed for any alterations in color, particularly seeking indications of a blue or greenish-black tint.

Test for terpenoids and steroids (Salkowski's test)

A mixture was prepared by adding 0.5 mL of each extract to 2 mL of chloroform, followed by the gradual addition of 3 mL of concentrated sulfuric acid (H_2SO_4 98 %) to form a separate layer. The appearance of a reddish-brown coloration at the interface suggests the possible presence of terpenoids and steroids.

Phenolic compounds

A 1 mL aliquot of each extract was mixed with 2 mL of distilled water, followed by the addition of 120 µL of 10 % ferric chloride solution. The mixture was observed for the development of blue or green coloration, indicative of phenolic compounds.

Antimicrobial activity

Evaluated microorganisms: bacteria and fungi

Five prevalent bacterial strains and two fungal species were obtained from the Microbiology laboratory located within the Faculty of Medical Laboratory Sciences at Omdurman Islamic University. The study identified a range of microorganisms, classified as Gram-positive (G^+) and Gram-negative (G^-) bacteria. Notable gram-positive bacteria included *Bacillus subtilis* and *Staphylococcus aureus*. In contrast, the gram-negative bacteria featured in the study were *Pseudomonas aeruginosa*, *Salmonella typhi*, and *Escherichia coli*. Additionally, the research also highlighted the presence of fungi, specifically *Aspergillus niger* and *Candida albican*s. The bacterial and fungal species underwent a process of activation and cultivation on nutrient agar three separate times. Following these procedures, they were stored on nutrient agar slants at a temperature of 4 °C for preservation. Additional cultivation and experimentation were conducted using nutrient agar medium.

Bacterial suspension preparation

One milliliter sample from a 24-hour broth culture containing the test microorganisms. were placed on nutrient agar slopes. They were incubated at 37 °C for a period of 24 hours. After incubation, the bacteria were washed with 100 mL of sterile normal saline to create a suspension with a concentration of about 108 to 109 colony-forming units per milliliter. Samples of one milliliter from a 24-hour broth culture containing the test microorganisms were meticulously introduced onto nutrient agar slopes. These were then incubated at a controlled temperature of 37 °C for a period of 24 hours. Subsequently, the bacterial proliferation was gathered and thoroughly washed using 100 milliliters of sterile normal saline. This process aimed to produce a suspension with an estimated concentration ranging from 108 to 109 colony-forming units per milliliter. The plates were left to stand at room temperature for 2 hours, permitting the drops to dry thoroughly before being incubated at 37 °C for a period of 2 hours. Following the incubation phase, the quantity of colonies that formed within each drop was documented. To determine the viable count of the stock suspension in terms of colony-forming units per milliliter, the average colony count for a 0.02 milliliter drop was multiplied by a factor of 50 and by the dilution factor. Each time a new stock suspension was prepared, all experimental parameters were standardized to guarantee that the resulting suspensions produced closely comparable viable counts.

Fungal suspension preparation

The fungal samples were grown on Sabouraud dextrose agar (SDA) and incubated at a temperature of 25 °C for a duration of four days. Subsequently, the fungal culture was gathered, they were thoroughly rinsed with sterile normal saline and suspended in 100 mL of sterile normal saline solution. The suspension was then stored refrigerated until needed for further analysis.

Assessment of the antibacterial activity of extracts using in vitro testing methods

As an alternative to the cup-plate agar diffusion method, the antibacterial activity was assessed using the broth microdilution method [14]. Serial twofold dilutions of each extract were prepared in Mueller–Hinton broth in sterile 96-well microtiter plates to obtain concentrations ranging from 1,000 $\mu g \cdot mL^{-1}$ to 7.8 $\mu g \cdot mL^{-1}$. Each well was

inoculated with 100 µL of a standardized bacterial suspension (approximately 10^6 CFU·mL^{-1}). The plates were then incubated at 37 °C for 24 hours. Bacterial growth was assessed visually and by measuring optical density at 600 nm using a microplate reader. The minimum inhibitory concentration (MIC) was determined as the lowest concentration of the extract that completely inhibited visible bacterial growth.

Evaluation of extracts for antifungal efficacy through in vitro testing

The method employed for bacteria has also been adapted for this context. Nonetheless, nutrient agar was substituted by Sabouraud dextrose agar. The inoculated medium was maintained at a temperature of 25 ºC for a duration of two days to promote the proliferation of *Candida albicans*.

Antioxidant activity

Assessment of DPPH radical scavenging efficiency

The assessment of the antioxidant effect was conducted by analyzing its ability to donate hydrogen or neutralize free radicals, utilizing the stable radical DPPH as a measurement tool. The experiments were performed in accordance with the methodology established by Ghannam *et al.* [15], incorporating slight modifications. Active samples can convert the stable DPPH radical into yellow-colored diphenyl-picryl hydrazine.

Assay

The test samples were allowed to react with 2 mL of a 300 µM solution of 2,2-di(4-tert-octylphenyl)-1-picrylhydrazyl (DPPH), a stable free radical. The preparation took place in ethanol, but the test samples were dissolved in dimethyl sulfoxide (DMSO). The test was carried out and incubated in a 96-well plate. Following incubation, the absorbance at 517 nm was measured using a UV-Vis spectrophotometer (Model UV-1800, Shimadzu, Japan; wavelength range: 37 °C for 30 minutes). By comparing the sample's absorbance to the absorbance of a control that was treated with DMSO, the percentage of DPPH radical scavenging activity was calculated. To ensure repeatability, every trial was performed in triplicate. The percentage of DPPH radical scavenging activity was calculated using the formula below.

$$DPPH\ radical\ scavenging\ (\%) = 100 - \{(Ac - At) / Ac\} \times 100 \quad (1)$$

where: *Ac* = absorbance of the control; *At* = absorbance of the test compound.

Statistical evaluation

All data was presented as mean values accompanied by their standard deviation. The analysis of statistical data for all assay results was performed utilizing Microsoft Excel software (2016).

RESULTS AND DISCUSSION

This study evaluates the effectiveness of various solvents in extracting phytochemical components from *L. inermis* leaves and assesses the bioactivity of the resulting extracts, including their antioxidant properties.

Extract yield (% yield)

Yield extraction refers to the quantity of yield generated from an investment or asset. Table 1 presents the percentage yield (% yield) and physical characteristics of the two crude *Lawsonia inermis* leaf extracts obtained by maceration using two different solvents.

Table 1. *Percentage yield of crude extracts obtained from L. inermis leaves*

Extract	Visual characteristics	Yield [g]	% Yield
HenEtOH	Dark green powder	41.43	20.70 ± 0.63
HenH	Dark green viscous residue	6.00	3.00 ± 0.12

Notes: HenEtOH = Ethanolic extract; HenH = Hexane extract

The HenEtOH extract was observed to take on a dark green powdery texture, whereas the HenH extract presented itself as a thick green crude residue. The highest yields recorded for HenEtOH and HenH were determined to be 41.43 and 6.00 g, respectively. The percentage yields were measured at 20.70 ± 0.63 and 3.00 ± 0.12 for each extract. Research utilizing the maceration method with various polar solvents demonstrated that the extraction efficiency of phytochemical compounds from leaf samples was significantly higher in highly polar solvents such as ethanol, in comparison to non-polar solvents such as chloroform and hexane. The findings align with earlier research suggesting that the maceration process using ethanol produces a greater yield than that obtained with hexane. This aligns with the concept that solvents have the ability to dissolve bioactive compounds that possess similar polarities, as noted by Peiris *et al.* [16].

Analysis of phytochemicals in *Lawsonia inermis* leaf extracts

The identified phytochemical compounds include alkaloids, flavonoids, saponins, tannins, terpenoids, steroids, and phenolic compounds are illustrated in Table 2.

Table 2. *Phytochemical analysis results of Lawsonia inermis leaf extracts*

Extract	Phytochemical constituents					
	Alkaloids	Terpenoids/ Steroids	Flavonoids	Tannins	Saponins	Phenolic compounds
HenEtOH	+	-	-	-	+	+
HenH	-	-	+	+	-	-

Notes: + = Found; - = Not found

Qualitative analysis of phytochemical constituents of the crude ethanolic extract of *L. inermis* leaf has demonstrated the identification of several bioactive compounds, including alkaloids, saponins, and phenolic compounds. However, the analysis indicated the absence of terpenoids, flavonoids, and tannins in the extract. The hexane extract was found to contain flavonoids and tannins, while being devoid of other identified components.

Antimicrobial activity of *Lawsonia inermis* leaf extracts versus standard organisms

To evaluate the antibacterial properties of the two extracts, the inhibition zones were assessed using the broth microdilution method. The findings acquired are illustrated in Table 3.

Table 3. *Antimicrobial activity results of Lawsonia inermis leaf extracts against standard organisms*

Extract	**HenEtOH**					**HenH**				
Concentration [mg·mL^{-1}]	**100**	**50**	**25**	**12.5**	**6.2**	**100**	**50**	**25**	**12.5**	**6.2**
	Inhibition zones (mm)									
Bs.	14±1	11±1	Niz	Niz	Niz	Niz	Niz	Niz	Niz	Niz
Sa.	11±1	11±1	Niz	Niz	Niz	Niz	Niz	Niz	Niz	Niz
Ec.	17±1	8±1	Niz	Niz	Niz	Niz	Niz	Niz	Niz	Niz
St.	18±1	12±1	Niz	Niz	Niz	Niz	Niz	Niz	Niz	Niz
An	14±1	Niz	Niz	Niz	Niz	Niz	Niz	Niz	Niz	Niz
Ca	19±1	11±1	9±1	Niz	Niz	Niz	Niz	Niz	Niz	Niz

Notes: Niz = No inhibition zone; ***Bs.*** = *Bacillus subtili*; ***Sa.*** = *Staphylococcus aureus*; ***Ec.*** = *Escherichia coli*; ***St.*** = *Salmonella typhi*; ***An.*** = *Aspergillus niger*; ***Ca.*** = *Candida albicans*

The effectiveness of the extracts was first evaluated through the cup-plate agar diffusion method. This evaluation focused on the efficacy of the extracts against two Gram-positive bacterial strains: *Bacillus subtilis* and *Staphylococcus aureus*. Additionally, it also evaluated the activity against two Gram-negative bacterial strains: *Escherichia coli* and *Salmonella typhi.* Besides the methodology employed for bacterial evaluations has been adapted to assess the antifungal effects of extracts on two types of fungi: *Aspergillus niger* and *Candida albicans*. In a significant change, the experimental framework transitioned from nutrient agar to Sabouraud dextrose agar. Table 3 presents the recorded measurements conducted at different concentrations, specifically 100, 50, 25, 12.5, and 6.2 mg/L, for both HenEtOH and HenH. The HenEtOH extracts exhibited antibacterial activity against all bacterial strains evaluated in the study. The study revealed that the extract displayed notable effectiveness in combating *Escherichia coli*, *Salmonella typhi*, and *Bacillus subtilis*, producing inhibition zones measuring 17.00 ± 00, 18 ± 00, and 14 ± 00 mm, respectively. Furthermore, HenEtOH displayed improved effectiveness against *Candida albicans* and *Aspergillus niger*, showcasing inhibition zones measuring 19.00 ± 00 and 14.00 ± 00 mm, respectively. In a notable contrast, HenH showed no activity against the tested organisms.

Assessment of DPPH free radical scavenging activity

DPPH radical scavenging assay

The antioxidant properties of the two crude extracts were evaluated using the DPPH method, following the procedure described by Ghannam *et al.* [15]. The findings are summarized in Table 4.

Table 4. *DPPH radical scavenging activities values of L. inermis leaf extracts: HenEtOH and HenH*

Sample	RSD% ± SD (DPPH)
HenEtOH	94±0.01
HenH	77±0.03
Propyl gallate (STD)	94±0.01

The HenEtOH extract exhibited remarkable antioxidant properties, recording an outstanding inhibition rate of 94 ± 0.01 %. This result aligns with the recognized standard inhibition value associated with propyl gallate. In contrast, the HenH extract demonstrated the lowest level of antioxidant activity, with an inhibition value of 77 ± 0.03 %.

Based on the results summarized in Table 4, the HenEtOH extract has the most significant antioxidant activity, while the HenH extract has relatively diminished antioxidant capabilities. This characteristic, attributed to the capacity to give hydrogen atoms, facilitated the existence of various phenolic and flavonoid compounds that interact with DPPH radicals. These compounds feature aromatic rings that incorporate hydroxyl groups. These hydroxyl groups can stabilize free radicals by donating electrons to their structure, thereby inhibiting the generation of new radicals [17].

CONCLUSIONS

The study concluded that *Lawsonia inermis* Linn leaf extracts exhibit significant bioactive potential, with the ethanolic extract (HenEtOH) demonstrating superior phytochemical richness, antimicrobial efficacy, and antioxidant activity compared to the hexane extract (HenH). These findings underscore the influence of solvent polarity on extraction efficiency and support the use of ethanol as a preferred solvent for obtaining biologically active compounds from studied medicinal plants.

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CONFLICTS OF INTEREST

There are no potential conflicts of interest in terms of finance, personal relationships, academics, or anything else that may have affected the way this study was carried out or its conclusions. All the data presented in the study are accurate and devoid of manipulation or prejudice, and the study was carried out in an ethical and open manner.

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