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Lawsonia inermis seeds cotyledon and coat extracts as a potential antimicrobial agent

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Abstract

Background: The plant *Lawsonia inermis*, which is a member of the Lythraceae family, has long been used to cure a number of diseases. Previous studies have demonstrated the antibacterial capabilities of the plant's components. However, neither the efficiency of these extracts on bacterial strains that are antibiotic-resistant nor a systematic analysis of the extracts from the various seed components have been conducted.

Methods: The coat part was separated from the cotyledon. Each part was pulverized and extracted with ethanol, acetone, and hexane. The inhibitory effects of the resulting extracts were tested on three pathogenic bacterial strains and a fungus. The effect of the extracts on antibiotic-resistant bacteria was also evaluated.

Results: When tested against pathogenic bacteria *(Bacillus subtilis, Staphylococcus aureus, Klebsiella pneumonia, and Candida albicans*), *L. inermis* seed parts (cotyledon and coat) showed varying levels of antibacterial and antifungal activity. In which the ethanolic extract outperformed the acetonic extract in effectiveness. The minimal inhibitory concentration (MIC) for each pathogenic microorganism was established. Utilizing the extract yield, total antibacterial activity (TAA) was calculated. *Lawsonia inermis* seed components inhibited antibiotic-resistant strains of *S. aureus and P. aeruginosa*, with strong antibacterial activity seen in aqueous extracts of their cotyledons and coats.

Conclusion: We summarize that *Lawsonia inermis* seed extracts, which have historically been used as secure antimicrobials for human healthcare and cosmetics have the potential to replace current antimicrobial agents that are no longer effective. Moreover, may be a promising source for the isolation of potent drugs for the treatment of bacterial diseases.

Introduction

Different parts of medicinal plants, such as leaves, roots, seeds, pods, barks, and fruits, are commonly used in medical practice by native peoples to prevent and treat a wide range of ailments, from common colds to cancer [1,2]. Despite advances in chemical synthesis, about 80% of the world's population still depends on plants for health care. Furthermore, about 25% of prescription medications are derived either explicitly or implicitly from plants [3,4]. Antimicrobial resistance (AMR) occurs when bacteria, viruses, fungi, and parasites evolve and no longer respond to drugs, rendering the treatment ineffective. It has emerged as a significant global public health concern, particularly for antibiotics [5]. As drug-resistant pathogens become more common, it is essential to discover and isolate novel bioactive compounds or drugs from safer sources. Apart from plant bioactive secondary metabolite drugs, many plant peptides and proteins have been investigated as potential future drugs, with a few currently in clinical trials [6,7]. *Lawsonia inermis* is a small tree that grows to a height of 2-6 meters. In the Muslim world, it is known as *henna*, and it has been used cosmetically and medicinally in folk medicines since ancient times. [8,9]. Antioxidant and anti-inflammatory properties are among the biological effects detected using *Lawsonia inermis* extracts [10], as antibacterial [11] and anticancer properties [12]. Although henna seeds' total extract has long been known to have antibacterial and antifungal properties, a thorough study using the plant's soluble protein extract still needs to be done.

Furthermore, whole plant seeds have been used in studies on the plant's antimicrobial properties [13]. We found in a pilot study using fractionated henna seeds that extracts from the seeds' coat and cotyledons have various antimicrobial properties. As a result, it was appealing to conduct this study to learn more about the extent of these variations in terms of the pathogenic microbes used.

Methods

Plant materials and microbial strains

The wildly growing *Lawsonia inermis* (henna) plants were collected from different areas of Khartoum State, Sudan. The seeds were collected, cleaned, ground, and stored at 4°C until further use. The following microbial strains were obtained from the National Research Center (NRC), Khartoum, Sudan: (gram-positive bacteria (*Bacillus subtilis* NCTC 8236 and *Staphylococcus aureus* ATCC 25923), gram-negative bacteria (*Klebsiella pneumonia* ATCC 53657), and the fungus (*Candida albicans* ATCC 7596). Also, four bacterial-resistant isolates of *S. aureus* (R4, R10, R11, and R14) and a single bacterial-resistant strain of *Pseudomonas aeruginosa* (R5) were tested. All chemical reagents and materials are of analytical or the highest molecular grade available.

Extraction of secondary metabolites

The whole seeds of *L. inermis* are lightly ground with a pestle and mortar. The cotyledons and seed coats are separated and ground into a fine powder. Three different extractions were performed using different organic solvents. 25 g cotyledons and seed coat powder were dissolved in 250 ml of n-hexane, 50% acetone, and 70% ethanol, respectively. Using Soxhlet, each extraction was performed for 72 h at solvent boiling points of 78.37°C, 56°C, and 69°C, respectively. The resulting extract was filtered through Whatman #1 paper and left at room temperature for 72 h for the solvent to evaporate completely.

Protein extraction and fractionation

The total protein crude extract was performed as described by Awadallah and colleagues [14]. Briefly, 500g each of *L. inermis* seed cotyledon and seed coat powder was defatted using n-hexane (1g powder/ 5ml n-hexane) under continuous stirring for 4h at 4°C. Then 5g of defatted powder was suspended in 25 mL of 20mM Tris-HCl buffer pH 7.5, prepared in 0.145 M NaCl. The clear protein extract obtained following the centrifugation at 6000 *rpm* was denoted as fraction A for crude protein extract obtained from seed (FrcA-S) and seed coat (FrcA-SC), respectively. The protein extracts were dialyzed exhaustively against distilled water using dialysis tubes cut off 3 and or 10 kDa. The dialysates were then lyophilized to dryness and dissolved in a minimal amount of 20mM Tris-HCl, pH 7.5 prepared in 0.150 mM NaCl, and preserved at -20°C till further use.

Estimation of protein concentration

The protein content was estimated using the Bradford colorimetric assay at 595 nm using bovine serum albumin (BSA) as the standard [15].

Antimicrobial bioactivity of the *Lawsonia inermis* extracts

Bacterial strains were sub-cultured at 37°C in nutrient agar plates and allowed to grow overnight. The colonies were then harvested and emulsified in sterile normal saline. The turbidity was adjusted to equivalent to McFarland 0.5, then serially diluted to attain a viable cell count of 106 CFU/ml. To determine the minimum inhibitory concentration (MIC) for all extracts, the secondary metabolites were prepared in DMSO 40% to obtain concentrations of 15 mg/mL, which were then serially diluted (15 – 0.08 mg/mL). The total protein extract (FrcA-S and FrcA-SC) was prepared in Tris-HCl 20 mM buffer (8.3 µg/100µL). The antimicrobial activity

of all extracts was evaluated using the well diffusion method, which involved placing 100 uL of each extract into 8-mm wells and incubating them for 24 hours at 37 °C [16]. The inhibition zones were measured, and the MIC was determined as the lowest concentration for each extract, resulting in no visible inhibitory zone.

Statistical analysis

The inhibition zones were measured in millimeters (mm) as (means ± SD). The significance of the results was evaluated using the analysis of variance (ONE-WAY ANOVA) at a P value of 0.05. The analysis was performed using SPSS Version-26 (IBMSPSS Statistics for Windows, Version 26.0. Armonk, NY: IBM Corp, USA).

Results

The effect of *L. inermis* secondary metabolites extracts

The extracted secondary metabolites yield varied depending on the solvent used and the portion of the *L. inermis* seeds used. At large, seed coat extracts had a higher yield than cotyledon extracts. Specifically, ethanolic extracts for both seeds' cotyledon yielded 16.6%, and seed coat yielded 22% product, whereas acetonic extracts yielded 13.6 and 14.8%, respectively. The extraction yield was beneficial in calculating the total antimicrobial activity (TAA) after performing the microbial sensitivity test and the subsequent identification of the minimum inhibitory concentration (MIC) [17].

In three independent experiments, the minimum inhibitory concentration (MIC) of crude seeds' cotyledon and coats extracts against 3 pathogenic bacterial (2 gram positive: *S. aureus* and *B. subtilis and* one gram negative*: K. pneumonia*)in addition to a fungal strain (*C. albicans*) was determined in triplicate using a globally accepted, simple, reproducible, cheap, and sensitive serial dilution microplate method [18]. The sensitivity of the various extracts (e.g., ethanolic or acetonic, and cotyledon or seed coat) to the different microbial strains varies (Table 1, Figure 1). Although the seeds cotyledon ethanolic or acetonic extract has no inhibitory effects on the *Klebsiella pneumonia* ATCC 53657 strain. A 0.32 mg/mL concentration of the seed coat extract could inhibit its activity. While *Staphylococcus aureus* ATCC 25923 was unaffected by the acetonic seed coat extract, *Bacillus subtilis* NCTC 8236 was only inhibited by the ethanolic seeds cotyledon extract (MIC = 1.25 mg/mL).

Generally speaking; while the sensitivity of the gram-positive and gram-negative bacteria varies between the various extract types, the fungal *Candida albicans* ATCC 7596 has a constant MIC of 0.63 mg/mL across all extracts. The mean difference between the inhibitory zones for all extracts is significant at $P_{0.05}$ compared to the other microbes. However, unlike other microbes against the various extracts, *S. aureus* ethanolic seed coat extract has significantly higher total antimicrobial activity (TAA = 687.5 mL/g, $P_{0.05}$) (Table 2). When tested against resistant strains of S. aureus (R4, R10, R11, and R14) and *Pseudomonas aeruginosa* (R5), the 15 mg/mL ethanolic and acetonic seeds cotyledon and seed coat extract significantly inhibited all resistant strains with the highest zones. All strains of *S. aureus* were unaffected by the acetonic seed coat extract at the used concentration, and no inhibition was observed except for strain R10 (Table 3, Figure 1).

The effect of *L. inermis* protein crude extracts

The amount of total protein in the seed coat extract (FrcA-SC) is 1.4 times greater than that in the seed cotyledon extract (FrcA-S). Comparatively to the content recovered after using a 3 kDa cutoff dialysis tube, nearly half of the protein content of FrcA-S and FrcA-SC is removed during the dialysis procedure. Before and after the dialysis, the microbial inhibition zones in tests using a fixed concentration (8.3 µg/100 µl) of each extract on gram-positive and gram-negative bacterial species as well as *C. albicans* revealed significant differences (Supplementary file 1). All the tested microbial strains' inhibitory activity of FrcA-SC was lost when the protein content with a molecular weight 10 kDa was removed, except *B. subtilis*. FrcA-S, on the other hand, retained antimicrobial activity against *C. albicans*, *E. coli*, and *B. subtilis* with an inhibition zone of 11±1.2 mm after being exhaustively dialyzed in a 10 kDa tube (Figure 1).

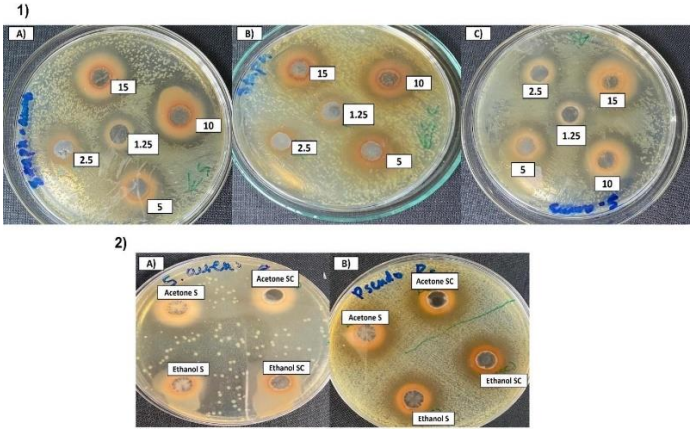

Figure 1: Bacterial inhibition using well-diffusion method. 1) Secondary metabolites inhibition against *S. aureus* (Conc. 1.25 -15 mg/mL), A) ethanolic seed extract (cotyledon), B) ethanolic seed coat extract, and C) acetonic seed extract (cotyledon). 2) seed cotyledon and seed coat of ethanolic and acetonic extracts against resistant bacterial strains (conc. 15 mg/mL). A) *S. aureus* R4 strain, and B) *P. aeruginosa* R5 strain.

Source material		Inhibition zone of ethanol extracts (mm)				Inhibition zone of acetone extracts (mm)			
-	Conc. (mg/mL)	*S. aureus*	*B. subtilis*	*K. pneumonia*	*C. albicans*	*S. aureus*	*B. subtilis*	*K. pneumonia*	*C. albicans*
Cotyledon	15	24.00±2.6	18.33±1.5	0.00	26.33±2.3	25.67±0.6	0.00	0.00	23.00±0.0
	10	23.33±2.9	17.67±0.6	0.00	24.33±0.6	25.67±0.6	0.00	0.00	22.00±0.0
	5	22.67±2.5	15.67±0.6	0.00	21.33±1.56	23.00±1.0	0.00	0.00	21.00±1.0
	2.5	20.67±3.1	15.33±1.5	0.00	20.33±1.5	20.33±0.6	0.00	0.00	18.67±2.3
	1.25	17.33±2.5	15.67±2.3	0.00	18.33±1.5	18.00±1.0	0.00	0.00	16.33±2.1
	0.63	13.00±0.0	0.00	0.00	11.00±0.0	13.33±0.6	0.00	0.00	12.00±0.0
	0.32	11.00±0.0	0.00	0.00	0.00	11.67±1.2	0.00	0.00	0.00
	0.16	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Seed coat	15	24.33±2.1	0.00	21.33±1.5	23.33±0.6	0.00	0.00	18.00±0.0	24.00±1.0
	10	23.00±1.7	0.00	20.33±3.1	22.33±0.6	0.00	0.00	18.00±0.0	22.00±0.0
	5	22.00±2.0	0.00	18.33±4.2	20.00±1.7	0.00	0.00	17.00±1.7	20.33±0.6
	2.5	20.00±2.6	0.00	16.33±1.5	18.33±1.5	0.00	0.00	15.33±0.6	18.67±1.2
	1.25	16.67±0.6	0.00	13.67±1.2	16.67±0.6	0.00	0.00	13.00±0.0	17.00±1.7
	0.63	15.00±2.6	0.00	12.67±0.6	11.33±0.6	0.00	0.00	13.00±1.0	12.00±0.0
	0.32	13.33±1.5	0.00	11.67±0.6	0.00	0.00	0.00	0.00	0.00
	0.16	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Table 1: Antimicrobial activity of examined *Lawsonia inermis* seed cotyledon and seed coat different concentrations of ethanolic and acetonic extracts against different microbial strains to determine the minimum inhibitory concentration (MIC).

Resistant strains	Inhibition zone of ethanol extract (mm)		Inhibition zone of acetone extract (mm)	
	Seeds cotyledon	Seed coat	Seeds cotyledon	Seed coat
R4	19.3±1.5	22.7±0.6	20.3±0.6	0.0
R10	22.3±0.6	24.3±0.6	23.0±1.7	0.0
R11	19.0±1.0	21.0±2.0	19.0±2.0	0.0
R14	16.0±1.0	13.0±1.0	14.0±1.0	0.0
R5	14.0±1.0	NA	12.0±10	NA

NA: Not Applicable.

Table 2: The bactericidal activity of *Lawsonia inermis* seed cotyledon and seed coat of ethanolic and acetonic extracts against resistant bacterial strains using the well-diffusion method.

Source material			*S. aureus*		*B. subtilis*		*K. pneumonia*		*C. albicans*	
		Yield (%)	MIC (mg/mL)	TAA* (mL/g)	MIC (mg/mL)	TAA (mL/g)	MIC (mg/mL)	TAA (mL/g)	MIC (mg/mL)	TAA (mL/g)
Ethanol	Seed cotyledon extract	16.4	0.32	512.5	1.25	131.2	NA	NA	0.63	260.3
	Seed coat extract	22.0	0.32	687.5	NA	NA	0.32	687.5	0.63	349.2
Acetone	Seed cotyledon extract	13.6	0.32	425	NA	NA	NA	NA	0.63	215.9
	Seed coat extract	14.8	NA	NA	NA	NA	0.63	234.9	0.63	234.9

*TAA: Total antimicrobial activity (mL/g) is a function of the extraction yield (1mL/g) of plant material and the minimal inhibitory concentration (MIC); NA: Not Applicable

Table 3: The Minimal Inhibitory Concentration (MIC), related inhibitory zones (mm), and Total Antibacterial Activity (TAA) of the different extracts of *L. inermis* against the tested microbes.

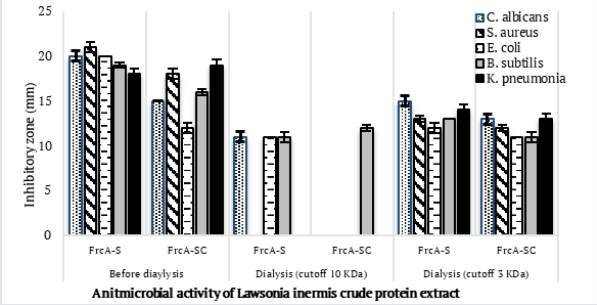

Figure 2: The antimicrobial activity of Lawsonia inermis Tri-HCl protein crude extracts (FrcA) from both seeds' cotyledon (FrcA-S) and seed coat (Frc-SC) before and after dialysis using dialysis tubes with different cutoff (3 and 10 KDa). A total protein concentration of 8.3 µg/100 µL was used.

Discussion

Although the extraction of seeds cotyledon and seeds coat was done with three different solvents, i.e., acetone, ethanol, and hexane. Acetone and ethanol extracts had appreciable detectable antimicrobial activity, while hexane did not show any activity against the tested microbes (not shown). Therefore, only acetonic and ethanolic extracts were used in this study. This study's findings are very similar to those of Mastanaiah *et al.*, who used *Lawsonia inermis* leaf hexane extract and found that only when they used concentrations of 300 mg/mL did they see inhibition of *Klebsiella pneumonia* and *Streptococcus aureus* [19]. Our findings concur with those of henna leaves collected in Saudi Arabia, where no inhibition zone for either bacterial strains or *Candida albicans* was noted [20]. The same author reported that the MIC for an ethanolic extract of L. inermis leaves against *C. albicans* was 3.1 mg/mL, while it was 1.5 mg/mL and 6.2 mg/mL for *S. aureus* and *K. pneumonia*, respectively [20].

In contrast, we report 0.32 mg/mL for the seed's cotyledon and seed coat in the current study, which is very low compared to Kouadri's findings. The seeds' cotyledon ethanolic extract was reactive against *B. subtilis* up to 1.5 mg/mL, but when the seeds' coat ethanolic extract was used, no results were obtained against the same bacteria. The outcome was reversed when *K. pneumonia* was challenged with the same two extracts. The seeds' coat extract was effective up to

0.32 mg/mL, while the cotyledon extract had no effect. These astounding results highlight the different phytochemical compositions of the various henna seed parts. The acetone extract had a weaker inhibitory effect on the four pathogenic microbes under test than the ethanol extract. Medicinal plants are a rich source of many chemicals, including tannins, terpenoids, alkaloids, flavonoids, proteins, and peptides, which have been demonstrated in vitro to have antimicrobial properties. This has attracted the attention of many researchers, who are now concentrating on these plants. This is brought on by increased bacterial resistance to medications that were once intended to kill them [21]. In this study, trials of the ethanolic and acetonic extracts of the seeds cotyledons and seeds coat against antibiotics resistant strain *S. aureus* (R4, R10, R11, and R14) and a *Pseudomonas aeruginosa* (R5) have resulted in interesting, however variable results. Almost all investigated resistant strains were susceptible to ethanolic extracts of both seeds' coats and cotyledons. On the other hand, the acetonic extract had a weaker impact on the tested strain than the ethanolic extract did. The *Pseudomonas aeruginosa* (R5) strain either had no effect or a very slight effect.

The aqueous fraction A (FrcA) extract of the cotyledon (FrcA-S) and seeds' coat (Frc-SC) was effective in inhibiting all of the tested bacterial strains. When the FrcA extracts from both seed parts were dialyzed using a dialysis tube with a 10 kDa cutoff, a significant loss in antibacterial activity was noticed except for *B. subtilis*, which suggests that proteins with molecular weights greater than 10 kDa rather than small peptides are responsible for the inhibition. However, using a 3 kDa cutoff dialysis tube, despite showing reduced extract potency, maintained significant effectiveness compared to the extracts dialyzed with a 10 kDa cutoff tube. In addition to secondary metabolites, plant cells produce antimicrobial peptides (AMPs) through metabolic pathways descended from primary metabolic pathways to produce an effective defense system. AMPs are cationic peptides that can interact with microbial DNA and RNA, inhibit protein transport, ion channels, and or enzymes [22]. The reported antimicrobial activity of FrcA-S and Frc-SC extracts may be caused by proteins/peptides acting alone or in combination with other soluble secondary metabolites with antibacterial potential. Since the reported activity persisted even after dialysis using various dialysis tubes with variable cutoffs (3 and 10 kDa), the latter possibility becomes more likely (Figure 1).

Despite the significant advancements in the synthesis and production of antibiotics, the irrational use of antibiotics ultimately leads to the evolution of microbes and, ultimately, resistance. We investigated the potential antimicrobial properties of the various parts of *Lawsonia inermis* (henna) seeds in the current study. We concluded that *Lawsonia inermis* seed extracts may be a potential candidate as an alternative antibiotic due to their strong antimicrobial activity, broad spectrum that includes resistant bacteria, and long-established safety. Different efficacies against pathogenic microorganisms, notably those with antibiotic resistance, have been demonstrated by the seed coat and cotyledon parts. Therefore, may serves as a stepping stone for additional in-depth research studies in our global continuous struggle against bacterial resistance. The findings of this study may also contribute another element to our understanding of *Lawsonia inermis's* antibacterial characteristics, particularly in relation to those parts of the seeds that have not before been studied.

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Competing Interest

The authors declare no competing interests.

Author Contributions

Sami F. Abdalla: Data analysis and writing the first draft of the manuscript.

Lujayne Yassir Elnour: Doing part of the work and data analysis

Nehad M. Abdulaziz, Ahmed Hassan Idries, and Makarim Elfadil M. Osman: Drafting and analysis of data

Emadeldin H. Konozy: Work supervision and manuscript editing

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