ANTIBACTERIAL ACTIVITY OF *LAWSONIA INERMIS L* .LEAVE EXTRACTS ON *STAPHYLOCOCCUS AUREUS* ISOLATES

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ABSTRACT

The antibacterial activity of the aqueous, ethanolic and methanolic extracts of *Lawsonia inermis* (henna) leaves were tested against 46 isolates of *Staphylococcus aureus* isolated from raw milk, also tested against standard bacteria (*Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853). The highest antibacterial potency was observed for the methanolic extract with zone of inhibition (14.3043 ± 1.8722 mm), followed by ethanolic (12.9565 ± 2.0106 mm) then aqueous (11.6304 ± 2.2446 mm). The effect of methanolic extract against methicillin resistant *S. aureus* (MRSA) isolates was the excellent in comparison to other extracts (14.1± 1.88 mm) zone of inhibition followed by ethanolic (12.91 ± 2.372 mm) then aqueous (12 ± 2.41 mm). The isolates were subjected Kirby Bauer method to test their antibiotic susceptibility pattern, substantial antibiotic resistance were shown by 46 (100%) of isolates for ampicillin. Moderate resistance was shown by 31(67.4%) for oxacillin and low resistance was observed by erythromycin. The preliminary phytochemical analysis of the extracts revealed that the presence of high amount of phenolic compounds in methanolic extract (5.4) mg/ml, ethanolic (4.9) mg/ml and aqueoeus (3) mg/ml.

MRSA provides a prospecting for new compounds which may be particularly effective against infections that are currently difficult to treat (1).

Aims of the conducted study are: 1) an attempt to determine the antibacterial activity of aqueous, ethanolic and methanolic extracts of henna (*Lawsonia inermis* Linn) leaves against *S. aureus* isolates, and Gram negative bacteria. 2) antibiotic susceptibility pattern of the isolates. 3) explore the biochemical constituents of extracts.

MATERIALS AND METHODS

-Plant samples

Leaves of *Lawsonia inermis* (henna) were collected from local markets of Basrah. Dried leaves were powdered using an electric device, powdered henna leaves were stored in refrigerator until analysis.

-Extract preparation

The plant extracts was prepared according to (10). In brief, 50 grams of powdered henna were allowed to soak with 500 ml of sterile distilled water, 75% ethanol or 75% methanol for 48 h. at room temperature. The extracts were filtered through sterile gauze then filtered using Whatman filter paper no. 1. The solvents were distilled under reduced pressure in a rotary evaporator until the extracts became completely dry. The percentage yield for each extract was determined, and the products were stored at 4°C for further use.

-Phytochemical analysis of extracts

A-Qualitative phytochemical analysis

Phytochemical analysis for various phytoconstituents of *Lawsonia inermis* extracts was carried out using standard procedures described by (11).

B-Total phenolic compounds assay

Total phenolic compounds were determined using Folin-Ciocalteu reagent (12). Briefly, 2 ml of freshly prepared 2% (W/V) sodium carbonate was added to 0.1 ml (10mg/ml) of henna extract, the mixture was mixed with vortex for 30 seconds and left for 5 min. While the mixture was mixed with vortex, 0.1 ml of 1:1 dilution of Folin-Ciocalteu (Merck) reagent was added. The mixture was left for 30 min, then, absorbance was measured at 750 nm. Total phenolic compounds were calculated as tannic acid equivalents (13).

C-Thin layer chromatography (TLC) analysis of phenolic compounds

Separation of phenolic compounds were done according to (14). Briefly, 20 µl of *Lawsonia inermis* extract was applied approximately 2 cm from the bottom of TLC plate (Merck, 4×10 cm^2). The plates were developed in n-butanol: acetic acid: water (4:1:5) to separate the phenolic compounds. The developed plates were air dried and 5% $FeCl_3$ in 0.5 N HCl was sprayed on the surface of developed plates, phenolic compounds produce a deep blue to greenish blue color (14).

-Bacterial isolates

The isolates of microorganisms employed were :Forty six *S. aureus* isolates were isolated from cow milk; suspected colonies on mannitol salt agar were identified by Gram's staining, colony morphology and hemolysis. The isolate was confirmed by the tube coagulase test with rabbit plasma and voges-proskauer test (15). Gram negative bacteria were used in this study included *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853. All bacterial strains were pure cultured preserved in nutrient agar slant at 4°C.

- Determination of antibacterial activity

A- Determination of the antibiotic susceptibility of isolates

All the isolates that were identified as *S. aureus* were tested for antibiotic susceptibility using Kirby-Bauer disc diffusion assay (16). The antibiotic tested were from (Bioanalyse/ Turkey), including; Oxacillin (1 µg), Erythromycin (15

µg), Gentamycin (10 µg), Clindamycin (2 µg), Vancomycin (30 µg), Ampicillin (25µg) and Chloramphenicol (30 µg).

B- Determination of the minimum inhibitory concentration (MIC) and zone of inhibition

The minimum inhibitory concentration (MIC) and zone of inhibition of henna extracts were determined using disc diffusion procedure according to (17) with little modification. Briefly, sterilized filter paper disc (Whatman no.1, 6 mm diameter) impregnated with different concentrations of henna extracts (1.5, 3, 6, 9, 12, 15 and 18 mg/disc) were prepared before 24 h and keep at 4°C, however, discs impregnated with ethanol 70% and methanol 70% were used as control. Mueller Hinton agar media were seeded with 0.1 ml of bacterial cultures were adjusted to 0.5 McFarland turbidity standard, after 15 min prepared discs were placed on the surface of seeded agars.

For MIC, ten isolates randomly selected from *S. aureus* isolates were chose, *Escherichia coli* ATCC 25922, and *Pseudomonas aeruginosa* ATCC 27853 (for *E. coli*, and *P. aeruginosa* the procedure was repeated 3 times). However, discs impregnated with 9 mg/disc were used for testing the susceptibility of all bacterial isolates. Zones of inhibition were read after incubation for 24 h at 37°C. Antibacterial activity was determined by measuring the diameter of zones of inhibition (mm).

-Statistical analysis

All statistical calculation were carried out with the statistical package SPSS V.13.

Results and discussion

-Phytochemical analysis of extracts

A-Qualitative phytochemical analysis

The phytochemical screening of *Lawsonia inermis* extracts reveal that the presence of tannins, flavonoids, phenolic compounds and glycosides. Table (1) shows the results of qualitative analysis of *Lawsonia inermis* extracts, these results are in agreement with (18), except for alkaloids.

B-Total phenolic compounds assay

Table (1) shows the results of total phenolic compounds of different *Lawsonia inermis* extracts. The results are slightly higher than that recorded by (13). The variation may be due to different types of soil used for culture.

C-Thin layer chromatography analysis of phenolic compounds

The extracts of *Lawsonia inermis* were fractionated by silica gel TLC plate by using n-butanol: acetic acid: water (4:1:5). The relative flow (R_F) of aqueous extract was 0.1, this result disagree with result reported by (6), whom noted that, R_F of aqueous extract was 0.66. This different result may be due to different fractionation liquid that used by (6), this liquid contain n-butanol: acetic acid: water (4:2:1).

Table (1) and figure (1) show the results of TLC of ethanolic and methanolic extracts. The R_F is 0.66 for both, results are in agreement with (6).

Table (1): Results of phytochemical analysis of *Lawsonia inermis* extracts.

Phytochemical test	Aqueous extract of *Lawsonia inermis*	Ethanolic extract of *Lawsonia inermis*	Methanolic extract of *Lawsonia inermis*
Alkaloids	-	-	-
Tannins	+	+	+
Flavonoids	-	+	+
Phenolic compounds	+	+	+
Saponins	-	-	-
Glycosides	+	+	+
Peptides	-	-	-
Total phenolic compounds concentration	3 mg/ml	4.9mg/ml	5.4 mg/ml
Relative flow (R_F)	0.1	0.66	0.66
Yield %	10.1%	20.4%	20.9%

(+,-) indicate presence or absence of chemical compound detected by qualitative analysis.

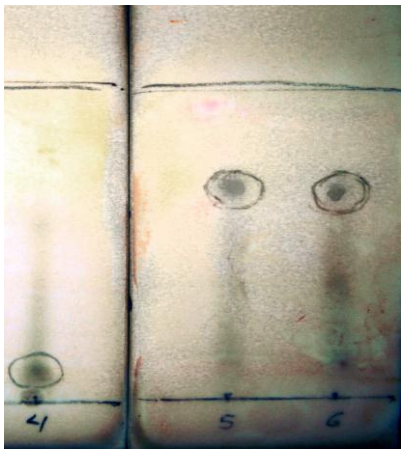

Figure (1): Chromatogram of phenolic compounds of *Lawsonia inermis* extracts, from left to right, aqueous extract, ethanolic extract and methanolic extract. The greenish blue color indicate presence of phenolic compounds.

D-Extracts yield

Dry residue of *Lawsonia inermis* extracts in terms of dry starting materials. Table (1) shows the yield of methanolic extract was (20.9%), ethanolic extract (20.4%) and aqueous extract (10.1%), these results indicated that yields of methanolic and ethanolic

extract were high in comparison to aqueous extract. Results of this study is in agreement with (19).

- Determination of antibacterial activity

A- Determination of the antibiotic susceptibility of isolates

The *S. aureus* isolates were screened by Kerby-Bauer method to test their antibiotic resistance pattern, table (2) shows results of antibiotic susceptibility. Substantial antibiotic resistance were shown against Ampicillin (100%) and Oxicillin (67.4%). Moderate resistance was shown against Clindamycin (4.4%) and Erythromycin (2.2%). The results disagree with (20), whom recorded that (26.47%) and (11.76%) of *S. aureus* isolates were resistant to Ampicillin and Erythromycin respectively. Resistant isolates to Methicillin (Oxacillin) a count for (67.4%), this is in agreement with (21).
All isolates were found to be sensitive to Vancomycin and Gentamycin and (97.8%) of isolates were sensitive to Chloramphenicol. These results are nearly similar to that reported by (17), also were in accordance with (22) for Vancomycin.

Table **(2): Antibiotic susceptibility of *S. aureus* isolates screened with disc diffusion**

Antibiotics	Sensitive		Intermediate		Resistance		Total
	No.	Percent	No.	Percent	No.	Percent	
Oxacillin	9	19.6%	6	13%	31	67.4%	46
Erythromycin	36	78.2%	9	19.6%	1	2.2%	46
Gentamycin	46	100%	0	0%	0	0%	46
Clindamycin	38	82.6%	6	13%	2	4.4%	46
Vancomycin	46	100%	0	0%	0	0%	46
Ampicillin	0	0%	0	0%	46	100%	46
Chlormphenicol	45	97.8%	1	2.2%	0	0%	46

method.

[Antibiotic discs =Oxacillin (1µg), Erythromycin (15µg), Gentamycin (10µg), Clindamycin (2µg), Vancomycin (30 µg), Ampicillin (25µg) and Chloramphenicol (30 µg)]

B-Determination of the minimum inhibitory concentration (MIC)

The observations of MIC have been tabulated in table (3). The MICs of *Lawsonia inermis* aqueous, ethanolic and methanolic extracts were (3,3,1.5) mg/disc respectively with respect of the tested *S. aureus* isolates. Also, the recorded MICs of *Lawsonia inermis* extracts against *P. aeruginosa* were (3,3,1.5) mg/disc respectively. However, MICs of *E. coli* were (12,6,3)mg/disc respectively.

In present study *S. aureus* isolates and *P. aeruginosa* were found to be the most sensitive bacteria to *Lawsonia inermis* extracts, on the other hand, *E. coli* was more resistant to these extracts. Results of this study are in agreement with (8), whom noted that, variations in bacterial sensitivity to antibacterial agents may be due to intrinsic tolerance of microorganisms and the nature and combinations of phytocompounds present in the crude extract. Results of MIC are in agreement with (23).

Table (3): Minimum inhibitory concentration (MIC) of *Lawsonia inermis* extracts against *S. aureus* isolates, *E. coli* and *P. aeruginosa* .

Type of extract	*S. aureus* isolates (mg/disc)	*E. coli* ATCC 25922 (mg/disc)	*P. aeruginosa* ATCC 27853 (mg/disc)
Aqueous extract	3	12	3
Ethanolic extract	3	6	3
Methanolic extract	1.5	3	1.5

*The lowest concentration of extract showing a zone of inhibition was considered as MIC.

C-Susceptibility of all *S. aureus* isolates tested against *Lawsonia inermis* extracts.

The antibacterial activity of aqueous, ethanolic and methanolic extracts of *Lawsonia inermis* were tested against 46 isolates of *S. aureus* isolated from raw milk. Table (4) and figure (2) show the results of antibacterial activity of *Lawsonia inermis* extracts.

The highest antibacterial potency was observed for methanolic extract with zone of inhibition (mean ± S.D.= 14.3043 ± 1.8722 mm), the data nearly distributed normally around the mean (0.7811) and lowest relative standard deviation (C.V.= 13.09%). The observed antibacterial potency of ethanolic extract was (12.9565 ± 2.0106 mm), followed by aqueous extract with zone of inhibition (11.6304 ± 2.2446 mm).

The difference in potency between aqueous and ethanolic (d.f.=45, t= 4.892, p= 0.001), aqueous and methanolic (d.f.=45, t= 8.09, p= 0.001), ethanolic and methanolic (d.f.=45, t= 4.697, p= 0.001) extracts were statistically high significant.

Results of this study indicate that alcoholic extracts were more active against bacteria, this result is in agreement with (19), whom noted that, alcohols are most superior solvent for extraction of antimicrobial substances as compared to water.

Table (4): Activity of *Lawsonia inermis* extracts (9mg/disc) against *S. aureus* isolates.

Type of extract	Zone of inhibition (mm) Mean ± SD	C.V.	Normal distribution
Aqueous extract	11.6304 ± 2.2446	19.30%	0.0039
Ethanolic extract	12.9565 ± 2.0106	15.52%	0.0037
Methanolic	14.3043 ± 1.8722	13.09%	0.7811

extract			

S.D.= standard deviation, C.V. = relative standard deviation.

Zone of inhibition: is the zone that does not show any bacterial growth.

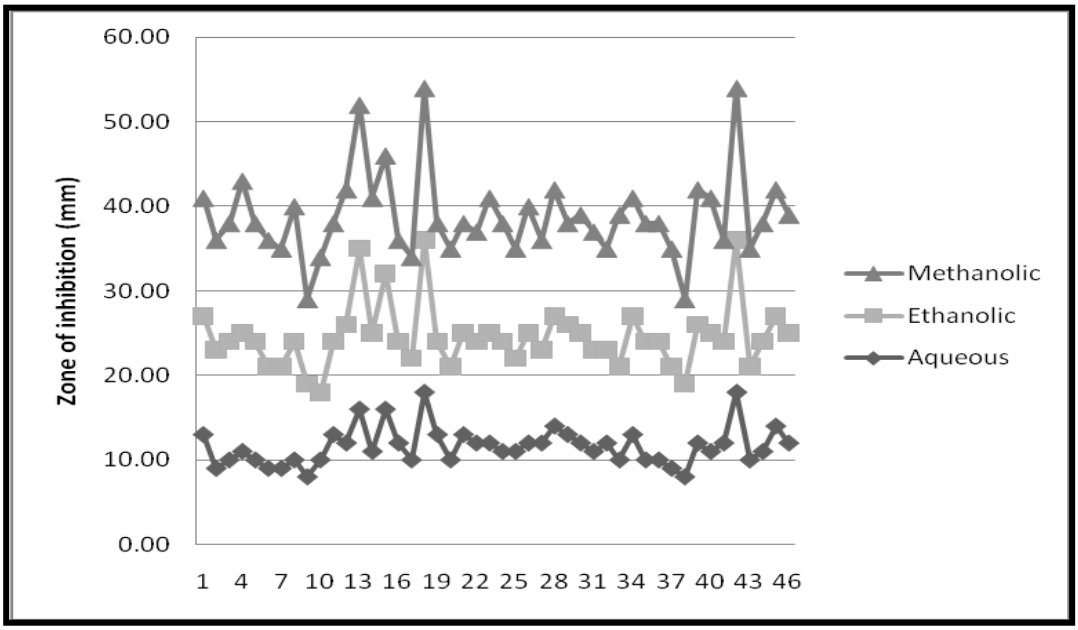

Figure (2): Comparison between activity of different *Lawsonia inermis* extracts (9mg/disc) against *S. aureus* isolates. The number in the base of the figure represent isolates.

D-Susceptibility of methicillin resistant *S. aureus* isolates tested against *Lawsonia inermis* extracts.

Table (5) shows the effect of *Lawsonia inermis* extracts against MRSA, methicillin sensitive and intermediate isolates. Methanolic extract (9mg/disc) was found to be effective against tested MRSA isolates with zone of inhibition (14.1 ± 1.88) mm and (C.V. = 13.33%). Ethanolic extract showed good results (12.91 ± 2.372) mm against MRSA followed by aqueous extract (12 ± 2.41) mm. Intermediate isolates are less sensitive to aqueous and ethanolic extract, whereas, these isolates were more sensitive to methanolic extract (15 ± 2.16) mm. Methicillin resistant *S. aureus* isolates are also more sensitive to methanolic extract (14.833 ± 1.83) mm.

Table (5): Effect of *Lawsonia inermis* extracts (9mg/disc) against *S. aureus* isolates (divided according to susceptibility to methicillin).

Type of extract	Zone of inhibition (mm) of methicillin resistant		Zone of inhibition (mm) of methicillin intermediate		Zone of inhibition (mm) of methicillin sensitive	
	Mean ± SD	C.V.	Mean ± SD	C.V.	Mean ± SD	C.V.
Aqueous extract	12 ± 2.41	20.1 %	11.25 ± 0.95	8.44 %	10.66 ± 1.366	12.81 %
Ethanolic extract	12.91 ± 2.372	18.4%	12 ± 0.816	6.8 %	13 ± 0.632	4.9 %
Methanolic extract	14.1 ± 1.88	13.33%	15 ± 2.16	14.4 %	14.833 ± 1.83	12.34 %

S.D.= standard deviation, C.V. = relative standard deviation.

In conclusion the activity of metahnolic extract of *Lawsonia inermis* against *S. aureus* isolates (MRSA, intermediate and MSSA), *E. coli* and *P. aeruginosa* was the most effective followed by ethanolic and aqueous extracts. The phenolic compounds concentration was high in methanolic and ethanolic extracts compared with aqueous extract, may be due to the effect of solvent. In our believe this study to be the first one deal with using of *Lawsonia inermis* extracts against MRSA.

Further studies are needed to identify compound/ compounds responsible for antibacterial activity of these extracts.

INTRODUCTION

Finding healing powers in plants is an ancient idea (1). Plants are rich in a wide variety of secondary metabolites, such as tannins, terpenoids, alkaloids, and flavonoids, which have been found *in vitro* to have antimicrobial properties (2). Also, (3) noted that the interest in drugs derived from plants primarily stems from the belief that green medicine is safe and dependable, compared with costly synthetic drugs that have adverse effects. Moreover, the use of traditional medicinal plants for primary health care has steadily increased worldwide in recent years (2).

Henna (*Lawsonia inermis* Linn) is a worldwide known cosmetic agent used to stain hair, skin and nails (4). The coloring matter in the leaves of henna is lawsone and identified as 2-hydroxy-1, 4- naphthoquinone which is present in dried leaves in a concentration of 1.0-1.4 % (5). However, henna leaves are used as a prophylactic against skin diseases and used for the treatment of boils, burns, bruises and skin inflammations

(5). On the other hand, (6) reported that, antimicrobial activity of the phenolic compounds present in henna were inhibited the bacterial growth of both Gram positive and Gram negative bacteria. The phenolic compound (Gallic acid) is present in henna leaves to the extent of 5-6 % (5).

The problem of microbial resistance is increasingly alarming and the outlook for the use of antimicrobial drugs in the future is still uncertain (7). Hence, the effective life span of any antibiotic is limited (1).Moreover, development of multi-drug resistance in pathogenic microbes necessitates a search for new antimicrobial substances from other sources, including plants (8).

Methicillin-resistant *S. aureus* strains (MRSA) represent 15-45% of all *S. aureus* isolates (9). The investigation of plant extracts effective against

التأثير الضد بكتيري لمستخلصات أوراق نبات الحناء على عزلات المكورات العنقودية الذهبية

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الخلاصة

تم اختبار الفعالية الضد بكترية الناتجة من المستخلصات المائية والايثانولية والميثانولية لاوراق الحناء على 46 عزلة *Staphylococcus aureus* و التي تم عزلها من الحليب الخام كذلك تم اختبارها على *Escherichia coli* ATCC 25922 وعلى *Pseudomonas aeruginosa* ATCC 27853. تم ملاحظة ان المستخلص الميثانولي اظهر فعالية كبيرة و اعطى نطاق تثبيط (1.8722 ± 14.3043 ملم) ، ثم تلاه المستخلص الايثانولي (2.0106 ± 12.9565 ملم) واخيرا المائي (2.2446 ± 11.6304 ملم).تاثيرالمستخلص الميثانولي ضد عزلات المكورات العنقودية الذهبية المقاومة للمثيسيلين MRSA كان كبيرا" بالمقارنة مع المستخلصات الاخرى واعطى نطاق تثبيط (1.88 ±14.1 ملم) ثم يليه المستخلص الايثانولي (2.372 ± 12.91 ملم) واخيرا المائي ± 12 (2.41 ملم). استخدمت طريقة كيربي – باور لاختبار نمط الحساسية للمضادات الحيوية، حيث تم ملاحظة وجود مقاومة كبيرة ، (100%) 46 من العزلات كانت مقاومة للامبيسيلين وكانت هناك مقاومة معتدلة الاوكسيسيلين (67.4%)31 من العزلات كذلك تم ملاحظة مقاومة قليلة للارثرومايسين. اوضح التحليل الكيميائي التمهيدي للمستخلصات النباتية وجود كمية كبيرة من المركبات الفينولية في المستخلص الميثانولي (5.4) ملغم/ مل ثم الايثانولي (4.9) ملغم/ مل واخيرا المائي (3) ملغم/ مل.

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