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Reveal of Antimicrobial Effect of *Lawsonia inermis* Extract on *Staphylococcus aureus* Using Molecular and Biochemical Techniques

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Abstract

Leaf of *Lawsonia inermis* (*Li*) was examined to their antimicrobial potential. Broth extracts in different concentrations were prepared and bioassayed in vitro for the growth of *Staphylococcus aureus.* It was found that the growth of *Staphylococcus aureus* pathogen was decreased. The protein pattern has one common band of Rf 0.24 and one characteristic band of Rf 0.27 for *Staphylococcus aureus* sample. Small quantitative mutation was observed in the bacteria with 5, 10, 15% of *Lawsonia inermis* compared with the control. Some types of proteins in *Staphylococcus aureus* were completely disappeared after inoculated with 5, 10, 15% *Li*. The catalase pattern showed one common band at R_1 with Rf 0.11 and the characteristic bands observed at R_2 , R_3 , R_4 , R_5 with Rf ranged between 0.22- 0.41 for *Staphylococcus aureus* sample. These results confirmed the antibacterial activity of henna leaves and supported the traditional use of the plant in therapy of bacterial infections. The broth extract of the *Lawsonia inermis* leaves showed obvious antibacterial activity against *Staphylococcus aureus.*

1. Introduction

Plants produce a diverse range of bioactive molecules, making them rich source of different types of medicines. Most of the drugs today were obtained from natural sources or semi-synthetic derivatives of natural products and used in the traditional systems of medicine. Thus it is a logical approach in drug discovery to screen traditional natural products. Approximately 20% of the plants found in the world have been submitted to pharmaceutical or biological test and a sustainable number of new antibiotics introduced on the market were obtained from natural or semi synthetic resources [1].

The antimicrobial activity of the henna sample was generally more evident in the leaves of the plant rather than the seeds, the latter having only demonstrated a limited antibacterial activity and at higher concentrations. The anti Candida albicans activity is self evident as it demonstrated sensitivity to the leaves but not the seeds. It is the presence of quinones in henna which gives that material its dyeing properties. The switch between diphenol (or hydroquinone) and diketone (or quinone) occurs easily through oxidation and reduction reactions. The individual redox potential of the particular quinone hydroquinone pair is very important in many biological systems [2].

Leaves of the henna are strikingly most effective against the spectrum of the tested bacteria comparing to the seeds. This is probably due to the inherent characteristics of the fully grown plant and the maturity of its chemically active constituents such as quinones. Such constituents would not have been found in seeds. Although fresh leaves demonstrated bacteriostatic activity in general, this was less evident when compared with the effect of dry leaves. It is

possible that the drying effect on the plant causes the active ingredients to be more concentrated than those in the green leaves, where water and other constituents are still present.

Staphylococcus aureus ranks second to coagulase-negative staphylococci as the cause of nosocomial blood stream infections that lead to increased morbidity, mortality, length of hospital stay, and costs [3-5].

Quinones are a source of stable free radicals and know to complex irreversibly with nucleophilic amino acids in proteins often lead to inactivation of the protein and loss of function [6-8]. For that reason the potential range of quinone antimicrobial effects is great. Portable targets in the microbial cell are surface exposed adhesions, cell wall polypeptides, and membrane bound enzymes. Quinones may also render substrates unavailable to the microorganism. In addition they show to inhibit cell growth in culture.

Proteins are the chief actors within the cell, say to be carrying out the duties specify by the information encod in genes. With the exception of certain types of RNA, most other biological molecules are relatively inert elements upon which proteins act. Other macromolecules such as DNA and RNA make up only 3% and 20%, respectively.The set of proteins express in a particular cell or cell type is known as its proteome [9, 10].

Polyacrylamide gel electrophoresis has become a standard tool in every laboratory in which proteins are analyzed and purified. Most frequently, the amount and location of the proteins were of interesting and staining were sufficient. However, it may also be important to correlate an activity of a protein with a particular band on the gel. Enzymatic and binding activities can sometimes be detected in situ by letting substrates or ligands diffused into the gel [11, 12]. The range of gel electrophoretic separation systems is limited by the pore size of the gels and diffusion of the antibody. The systems were also dependent on concentration and type of antigen or antibody to give a physically immobile aggregate. Analysis of cloned DNA has been revolutionized [13] by the ability to fractionate the DNA electrophoretically in polyacrylamide/ agarose gels.

The effectiveness of henna is caused through investigated the antimicrobial activity of leaves of Lawsonia inermis against Staphylococcus aureus.

In the present work the effect of henna leaves extract (Li) on the Staphylococcus aureus bacteria was tested using Vertical slab gel electrophoresis and U.V. / Visible spectrophotometer.

Materials and methods

The study was carried out in the Control labs, Kebab building of the nuclear research center, atomic energy authority, Inshas.

The marker in this study is the broth media free of both the bacteria (Staphylococcus aureus) and henna leaves powder (Lawsonia inermis). It used as a signal for the growth of bacteria (Staphylococcus aureus) after and before inoculated with Lawsonia inermis. Therefore the molecular weight of the marker expressed in the molecular weight of the broth.

1.1. Samples

2.1.1. Plant material:

Henna plant Lawsonia inermis leaves samples used in this study were collected from Aswan city at winter 2006 where the soil is sandy. Fresh leaves were dried in shade then were ground to powder [14].

2.1.2. Preparation of nutrient agar

It was prepared by using [15, 16] technique and steps of [17].

2.1.3. Preparation of nutrient broth

It was prepared by using [18] technique and steps of [17].

2.1.4. Bacterial strain

Bacterial pathogen included in this study was Staphylococcus aureus obtained from clinical isolates obtained at Microbiology department, faculty of Science, Helwan University. The bacteria was cultured on nutrient agar medium, incubated at 37°C for 24h to obtain inoculums for testing [19].

2.1.5. Inoculation of Staphylococcus aureus

Different concentrations of herbal powder (5, 10 and 15gm/100ml nutrient broth) to obtain different concentrations of powdering henna leaves. Constant volumes of nutrient broth were inoculated with 1ml of Staphylococcus aureus (bacterial suspension) under sterile conditions using Laminar flow, sterilr pipette and ethanol 70% then incubated at 37°C for 24h [20].Turbidity in the conicals were checked because the nature of the herbal solution turbidity wasn't clearly visible.

After the beginning of incubation the total soluble solids was measured as function of incubation period using (ATAGO Hand Performance, E-Type Series). The data were shown in tables 1, 2 and growth curves were shown in figures 1, 2.

2.1.6. The homogenate preparation

The bacterial growth on henna nutrient broth was collected by sterile pipette into eppendorf and centrifuged at 15000 rpm for 5 minutes. The supernatant then decanted and ground cell debris with liquid nitrogen, water and 1%SDS in a mortar. The homogenous solution was collected in new eppendorf and centrifuged at 6000 rpm for 5 minutes. The supernatant which obtained contains proteins which will be measured in each bacterial concentration of bacterial pathogen sample [21].

2.1.7. Protein Reagent

Protein Reagent prepared by dissolving 100 mg of Coomassie brilliant blue G-250 in 50 ml 95% ethanol. To this solution 100 ml of 85% (w/v) phosphoric acid was added. The resulting solution was then diluted by deionized water to a final volume of 1 liter.

2.1.8. Protein Assay

One milliliter of protein reagent was added to the test tubes those contained 0.1 ml of each sample as well as the standard solution. The contents of the tubes were mixed by vortexing. The absorbance measured after two minutes at 595 nm [21].

2.1.9. Preparation of the sample

The homogenate sample mixed with the sample buffer which prepared by method mentioned before with different percentages depending on concentration of the total protein in each sample. The protein concentration in each well must be in the range between 60 – 80 µg protein.

2.1.10. Protein Electrophoresis

Methods and procedures were taken from the book Gel electrophoresis of proteins [22, 23].

2.1.11. Resolving Gel (10%)

Gel solution prepared by mixing 12.3 ml distilled water, 9.9ml of Acrylamide/Bis (30% T, 2.67% C) stock solution, and 7.5 ml Tris (1.5M, pH8.8). The total volume of the solution was 30 ml. To this solution 150µl of 10% APS, freshly prepared, and 30 µl of TEMED added prior to pouring into the gel plate assembly. The prepared gel plate assembled to running conditions. At the end of the run the gels were stained overnight through shaker then photographed after destaining.

2.1.12. Catalase enzyme

Native protein gel stained by staining solution of catalase enzyme pattern used certain stain prepared according to [24-26].The stained gel was stained by mixing 1 gm Potassium Iodide, 10 ml glacial acetic acid and 100 ml distilled water then washed by mixing 5 ml H_2O_2 completed to 150 ml with distilled water.

1.2. Data analysis

Gel plate was photographed, scanned and then analyzed by using a gel pro Analyzer (Version 3.1 Media Cybernetics USA) for the analysis of tested samples. This program is a comprehensive computer software application designed to determine the relative fragmentation, the molecular weights and the amounts of peptide chains as well as scanned graphical presentation of the fractionated bands of each lane.

The similarity index (S.I.) compared patterns within different concentrations of Lawsonia inermis inoculated with Staphylococcus aureus as well as Staphylococcus aureus sample used the formula [27]:

S.I. = (2 Nab/Na + Nb)

Where Na and Nb are the number of bands in individuals a and b and Nab is the number of shared bands between a and b. The similarity values were converted into genetic distance (D) using the formula: D = 1 – S.

2. Results

2.1. Protein pattern:

The protein profile pattern of Staphylococcus aureus and Staphylococcus aureus inoculated with different concentrations of Lawsonia inermis (5, 10 and 15%) showed in figure (3) and the data were presented in table (3). Inspection of figure (3) and table (3) reveal that five types of protein fractions produced from Staphylococcus aureus at R1, R2, R5, R6, R7 with Rf ranged between 0.24- 0.41. When Staphylococcus aureus injected with 5, 10, 15% L1 produced three types of protein fractions at R1, R3, R5 with Rf ranged between 0.24- 0.32.

Comparing the effect of 5, 10, 15% LI, it was observed that the 2nd band at R2 with Rf 0.27, 4th band at R6 with Rf 0.36 and 5th band at R7 with Rf 0.41 were disappeared from Staphylococcus aureus(Qualitative mutation) and appearing of new band at R3 with Rf 0.28 for 5, 10, 15% LI injected with Staphylococcus aureus.

In case of broth, it was produced four bands at R1, R2, R5 and R7 with Rf ranged between 0.2- 0.59. so the broth showed disappearing of three bands at R2 with Rf 0.27, at R5 with Rf 0.32, at R6 with Rf 0.36 from Staphylococcus aureus and appearing of two new bands at R4 with Rf 0.29 and at R10 with Rf 0.58 for broth.

In case of broth injected with Staphylococcus aureus, it was produced six types of proteins or bands at R1, R5, R6, R7, R8, R9 with Rf ranged between 0.24- 0.53. So the broth injected with Staphylococcus aureus showed disappearing of one band at R2 with Rf 0.27 from Staphylococcus aureus and appearing of two new bands at R8 with Rf 0.49 and at R9 with Rf 0.53 for broth injected with Staphylococcus aureus.

From this data, it was observed the presence of one common band at R1 with Rf 0.24, one characteristic band observed at R2 with Rf 0.27 for Staphylococcus aureus sample, one characteristic band observed at R3 with Rf 0.28 for 5, 10, 15% LI injected with Staphylococcus aureus, two characteristic bands observed at R4 with Rf 0.29 and at R10 with Rf 0.58 for broth and two characteristic bands observed at R8 with Rf 0.49 and at R9 with Rf 0.53 for broth inoculated with Staphylococcus aureus.

The quantitative mutation observed at R1 for 5, 10, 15% recorded amount percent ranged between 27.3- 34.7 compared with Staphylococcus aureus recorded amount percent 21.5 from the total proteins secreted so Staphylococcus aureus injected with 5, 10, 15% Li recorded small quantitative mutation compared by Staphylococcus aureus.

The protein similarity index between Staphylococcus aureus and Staphylococcus aureus inoculated with 5, 10 and 15% Li recorded a low value (0.5), between Staphylococcus aureus and broth recorded a low value (0.44) and between Staphylococcus aureus and broth injected with Staphylococcus aureus recorded a high value (0.72) as shown in table(4). By comparing all three concentrations of Li inoculated with Staphylococcus aureus and Staphylococcus aureus indicating that there was good effect of Li on protein fractions of Staphylococcus aureus and showed high difference among the usage of Li with protein fraction of Staphylococcus aureus.

From this data indicating that the broth has no effect on protein fractions of Staphylococcus aureus but used only as a signal for growth of bacteria on it.

2.2. Catalase enzyme:

The catalase pattern of Staphylococcus aureus and Staphylococcus aureus inoculated with 15% Lawsonia inermis showed in figure (4) and the data were presented in table (5). Inspection of figure (4) and table (5) reveal that 5 types of catalase fractions produced from Staphylococcus aureus at R1, R2, R3, R4 and R5 with Rf ranged between 0.11 - 0.41. When Staphylococcus aureus injected with 15% Li produced only one type of catalase fraction at R1 with Rf 0.11.
Comparing the effect of 15% LI, it was observed that the 2nd band at R2, 3rd band at R3, 4th band at R4 and 5th band at R5 were disappeared from Staphylococcus aureus (Qualitative mutation).

From this data, it was observed the presence of one common band at R1 with Rf 0.11 and 4 characteristic bands observed at R2 , R3 , R4 , R5 with Rf ranged between 0.22- 0.41 for Staphylococcus aureus sample.
The obvious quantitative mutation observed at R1 for 15% Li recorded the highest value of amount % 100 due to it is the only band which still after Staphylococcus aureus inoculated with 15% Li compared with Staphylococcus aureus recorded amount % 20.8 from the total proteins secreted. So, Staphylococcus aureus injected with 15% Li recorded has five duplicate amount compared by Staphylococcus aureus.

The catalase similarity index between Staphylococcus aureus and Staphylococcus aureus injected with 15% Li recorded a low value (0.33) as shown in table (6).

By comparing 15% Li inoculated with Staphylococcus aureus and Staphylococcus aureus indicating that the effect of Li on catalase fractions of Staphylococcus aureus and showed difference among the usage of Li with catalase fractions o Staphylococcus aureus.

3. Discussion

3.1. Protein profile of Staphylococcus aureus

The profile gel electrophoresis f Staphylococcus aureus revealed six to ten reactive bands [28]. Naphthoquinones blocked oxygen uptake and induced oxygen consumption. 1,4-naphthoquinones effectively act as electron acceptors and induce an increase in reactive oxygen species that are toxic to S. aureus cells.

In Staphylococcus aureus, menaquinone (2-methyl-1,4-naphthoquinone) has an important role in the respiratory chain, i.e., it accepts electrons from several dehydrogenases (e.g., NADH , L-lactate, and succinate)and passing them to the cytochromes or eventually to molecular oxygen through menaquinol oxidase [29, 30]. Furthermore, S. aureus is a facultative anaerobic organism. Strains that are auxotrophic for menaquinone undergo hindrance of the electron transport in the absence of this compound, indicating its central role in nitrate respiration [31]. In addition, the synthesis of cytochromes, protoheme, menaquinone, and membrane-bound proteins dropped when menadione (a menaquinone precursor) was deprived from S. aureus that was auxotrophic to this precursor [32].

The oxygen uptake of S. aureus in the presence of different concentrations of NQs was evaluated. After 10 min, the oxygen uptake of S. aureus was significantly inhibited by NQ. This result reflects the effect of NQs on blocking the

respiratory activity of S. aureus. The effects of different concentrations of NQ added to exponentially growing cells of S. aureus. Growth inhibition was consistently observed at all concentrations tested [33]. Clinical isolates of Staphylococcus aureus was treated with the leaves of L. inermis for antimicrobial activity using the henna leaves were able to inhibit the growth pattern of S. aureus. Inhibition of the microorganisms growth suggests that henna may be valuable in the management of burnt wound infections [34].

Data obtained in the present study indicated that some protein bands in Staphylococcus aureus at high concentrations of lawsonia inermis disappeared completely. The disappearance of protein bands may be due to the effect of lawsonia inermis which inhibits the synthesis and expression process of Staphylococcus aureus proteins. The similarity index between Staphylococcus aureus and Staphylococcus aureus inoculated with different a high concentrations of Lawsonia inermis recorded a low value (0.5) and genetic distance (0.5). Indicated the impact of Lawsonia inermis effective in eliminating protein bands (Qualitative mutation).

The quantitative mutation observed at R1 for 5, 10, 15% recorded small quantitative mutation compared by Staphylococcus aureus.

3.2. Catalase of Staphylococcus aureus

The shape and configuration of the Gram-positive cocci helps to distinguish staphylococci. The catalase test is important in distinguishing streptococci (catalase-negative) from staphylococci, which are vigorous catalase-producers. Catalase-positive cultures produce O2 and bubble at once. This means that they can produce coagulase. However, while the majority of S. aureus are coagulase-positive, some may be atypical in that they do not produce coagulase. S aureus is also catalase-positive (meaning that it can produce the enzyme catalase) and able to convert hydrogen peroxide (H2O2) to water and oxygen, which makes the catalase test useful to distinguish Enterococci from Staphylococci and Streptococci [35, 36].Bacterial catalase and SOD combat reactive oxygen species enabling S. aureus to persist within macrophages, inducing local inflammation, causing greater induction [37].

Molecular oxygen accepts electrons easily to form the reduced derivatives superoxide radical and hydrogen peroxide, which damage living cells. These reactive oxygen species and their products can modify nucleotide bases, cleave the phosphate backbone of DNA, crosslink proteins and lipids by free-radical driven chain reactions, and damage the active sites of critical enzymes. Organisms that thrive within or tolerate an oxygen-rich environment mount two critical lines of enzymatic defense against these reactive oxygen species. Superoxide dismutase converts superoxide to molecular oxygen and hydrogen peroxide [38, 39] whereas catalases (and catalases/peroxidases) convert hydrogen peroxide to oxygen and water [40].

Oxygen is known to form highly reactive free radicals (reactive oxygen species: ROS) such as superoxide ions, hydroxyl radicals and hydrogen peroxide in prokaryotic and eukaryotic cells. Free radicals, by the possession of unpaired electrons, make them very reactive as they urgently seek to gain or lose electrons in order to reach a more stable configuration [41].Oxygen is converted into reactive oxygen (RO) by radiation, light, the electron transport system in mitochondria, or by other enzymes and is regulated by the action of antioxidative enzymes which convert RO into an inactive state [42]. It has been suggested that bacterial carotenoids such as those expressed by S. aureus could serve a protective function against these defense molecules [43-45].

In the catalase pattern, the similarity index between Staphylococcus aureus and Staphylococcus aureus inoculated with 15% of Lawsonia inermis recorded a low value (0.33) and genetic distance (0.67) Indicated the strong effect of Lawsonia inermis to disappear of catalase bands at high concentrations (Qualitative mutation). Lawsonia inermis has active role in the disappearance of catalase bands.

The obvious quantitative mutation observed at R1 for 15% Li recorded 5 duplicate amount compared by Staphylococcus aureus.

4. Conclusions

Medical plants commonly used by local inhabitants for its invitro antibacterial activity. Used of simple method for extraction of Lawsonia inermis leaves had proven to be successful in the estimation of antimicrobial activity against Staphylococcus aureus which responsible for nosocomial blood stream infections.

The data confirmed the effective role of Lawsonia inermis to cause high disturbances for protein and catalase patterns of Staphylococcus aureus and a high effect of Lawsonia inermis on bacterial growth at higher concentrations of Lawsonia inermis.

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Table 1: The total soluble solids measurement for different concentrations of *Lawsonia inermis* (5, 10, 15%) with and without *Staphylococcus aureus*.

Concentration of henna in nutrient broth(*Staphylocooccus aureus*)	With bacteria	Without bacteria
5%	3	3.2
10%	4.8	4.8
15%	6.2	6.4
N.B	1.2	1

Table 2. The total soluble solids measurement for different concentrations of *Lawsonia inermis* (5, 10, 15%) with and without *Staphylococcus aureus*.

Concentration of henna in nutrient broth(*Staphylocooccus aureus*)	With bacteria	Without bacteria
5%	2.8	3.2
10%	4.6	4.8
15%	6	6.5
N.B	1.4	1

Table 3: The protein profile pattern of *Staphylococcus aureus* and *Staphylococcus aureus* inoculated with *Lawsonia inermis* at different concentrations.

Rows	5%		10%		15%		Broth+*St.coccus*		*St.cocccus*		Broth	
	Am%	Rf	Am%	Rf	Am%	Rf	Am%	Rf	Am%	Rf	Am%	Rf
R1	27.3 (5.32)	0.24	34.7 (6.96)	0.24	31.45 (6.07)	0.24	20.72 (8.65)	0.24	21.5 (7.83)	0.24	24.9 (7.52)	0.24
R2	–	–	–	–	–	–	–	–	22.02 (8.02)	0.27	–	–
R3	36.5 (7.1)	0.28	29.2 (5.86)	0.28	36.11 (6.97)	0.28	–	–	–	–	–	–
R4	–	–	–	–	–	–	–		–	–	22 (6.66)	0.29
R5	36.2 (7.05)	0.32	36.1 (7.23)	0.32	32.44 (6.26)	0.32	18.54 (7.74)	0.31	17.44 (6.35)	0.32	–	–
R6	–	–	–	–	–	–	15.7 (6.55)	0.37	18.53 (6.75)	0.36		
R7	–	–	–	–	–	–	17.32 (7.23)	0.41	20.51 (7.47)	0.41	28.7 (8.67)	0.42
R8	–	–	–	–	–	–	13.22 (5.52)	0.49	–	–	–	–
R9	–	–	–	–	–	–	14.5 (6.05)	0.53	–	–		
R10	–	–	–	–	–	–	–	–	–	–	24.4 (7.37)	0.58

Rf: Rate of flow
Am: Amount
Note: The number between brackets expressed the real mass of protein.

Table 4: The protein simiralrity index (SI) and Genetic distance (Gd) between *Staphylococcus aureus* and *Staphylococcus aureus* inoculated with *Lawsonia inermis* at different concentrations , Broth.

S.I

G.d	*Staphylococcus aureus*	Concentrations of inoculated henna powder with *St.coccus.*				
		5%	10%	15%	Broth	Broth+ *St.coccus.*
St.coccus.	–	0.5	0.5	0.5	0.44	0.72
5%	0.5	–	1	1	0.28	0.44
10%	0.5	0	–	1	0.28	0.44
15%	0.5	0	0	–	0.28	0.44
Broth	0.56	0.72	0.72	0.72	–	0.4
Broth+ *St.coccus.*	0.28	0.56	0.56	0.56	0.6	–

Table 5: The Catalase pattern of *Staphylococcus aureus* and *Staphylococcus aureus* inoculated with *Lawsonia inermis* (15%).

Rows	15%		*St.coccus*	
	Am%	Rf	Am%	Rf
R1	100 (24.4)	0.11	23.96 (14.2)	0.11
R2	–	–	25.65 (15.2)	0.22
R3	–	–	21.76 (12.9)	0.29
R4	–	–	15.98 (9.47)	0.34
R5	–	–	12.65 (7.5)	0.41

Rf: Rate of flow **Am: Amount** **Note:** The number between brackets expressed the real mass of protein.

Table 6: The catalase simiralrity index (SI) and Genetic distance (Gd) between *Staphylococcus aureus* and *Staphylococcus aureus* inoculated with *Lawsonia inermis* (15%).

	S.I	
	Staphylococcus aureus	Concentration of inoculated henna powder with *St.coccus* 15%
G.d	–	0.33
Staphylococcus aureus 15%	0.67	–

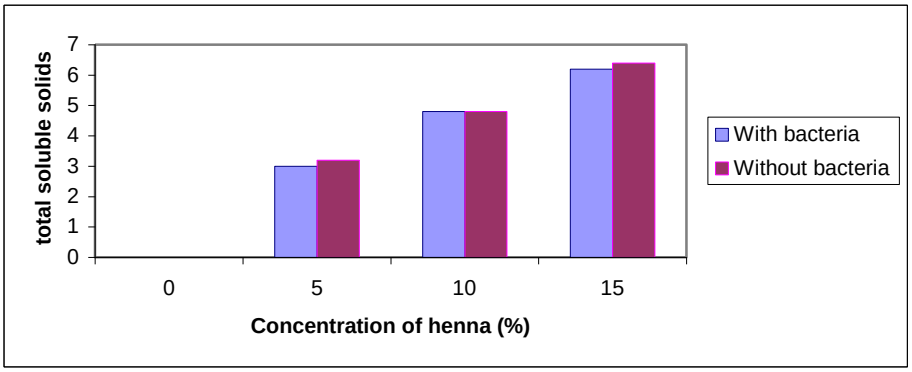

Fig. 1: The diagram of total soluble solids measurement for different concentrations of *Lawsonia inermis* (5, 10, 15%) with and without *Staphylococcus aureus*.

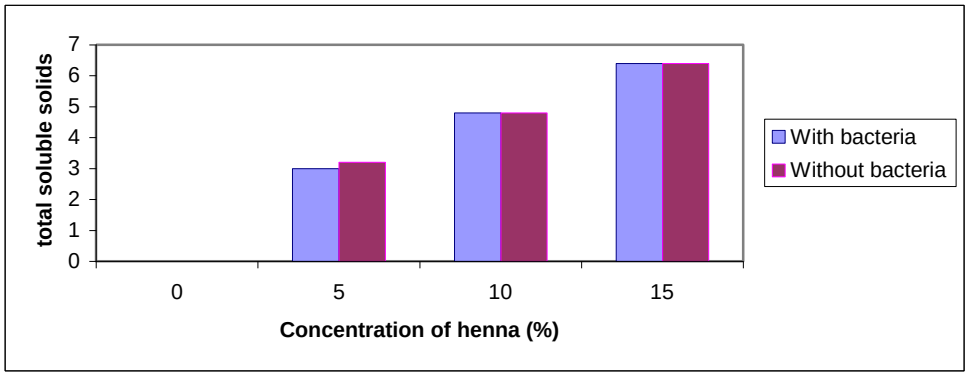

Fig. 2: The diagram of total soluble solids measurement for different concentrations of *Lawsonia inermis* (5, 10, 15%) with and without *Staphylococcus aureus*.

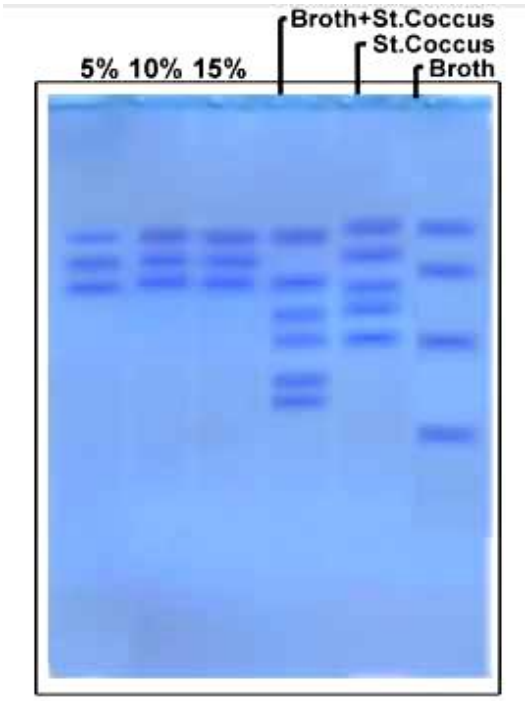

Fig. 3: Photograph and diagrammatic illustration of electrophoretic protein pattern of *Staphylococcus aureus* and *Staphylococcus aureus* inoculated with 5, 10, 15% of *Lawsonia inermis.*

Fig. 4: Photograph and diagrammatic illustration of electrophoretic catalase pattern of *Staphylococcus aureus* and *Staphylococcus aureus* inoculated with 15% of *Lawsonia inermis*.