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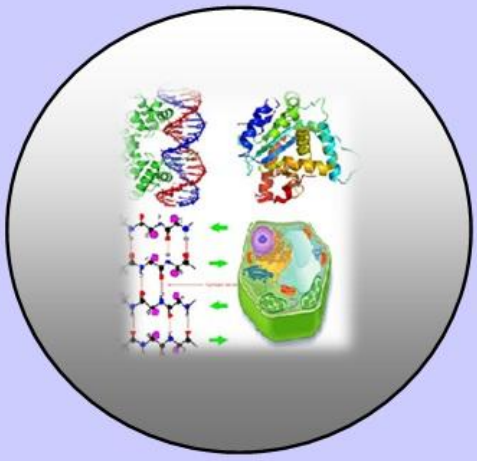

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RESEARCH PAPER

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Isolation of Bactericidal and Fungicidal Saponin from *Lawsonia alba* L. Fruit Extract

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ABSTRACT

The aqueous-methanol extract obtained from the fruits of Lawsonia alba L. was investigated for their antifungal and antibacterial activities. The fruits of plant were extracted with aqueous (60%)-methanol (40%). The saponin isolated from extract was screened against Escherichia coli, Pseudomonas aeruginosa and Candida albicans by disc diffusion and microdilution methods. Some antibacterial and antifungal antibiotics were used as reference standard to determine the sensitivity of the strains. The saponin showed strong antimicrobial activity against E. Coli and Candida albicans with inhibition zones of 19.0 and 16.0 mm, with MIC's and MBC's and MFC's 32(64) and 64(128) µg/mL, respectively. Our findings support the use of fruits of Lawsonia alba L. for the treatment against the above microbes.

***Keywords: Antifungal and Antibacterial Activity, Lawsonia alba* L., Saponin and TLC.**

INTRODUCTION

Saponins are generally non volatile surface active compounds that are widely distributed in plant kingdom (Hostettmann and Marston, 2005). The name saponin means 'soap' is derived from Latin word because this compound for soap like foams when shaken with water. Chemically referred as triterpene and steroidal glycosides. Saponins are earlier isolated as secondary metabolites from *chlorophytum borivilianum* plant and used in indigenous medicinal system and in pharmaceutical industry as steroidal drug as health tonic, aphrodisiac (Oudhia, 2001).

Saponin have a diverse range of medicinal activity like hemolytic, antimicrobial activity, antiviral activity etc. (Oda et al., 2000, Sparg et al., 2004, Sundaram et al., 2011 and Rohit et al., 2014).

Plants produce a good deal of secondary metabolites which have benefited mankind in various ways including treatment of diseases (Elaine et al., 2012). These secondary

metabolites have variously been shown to exhibit interesting biological and pharmacological activities and are important as prophylactics, chemotherapeutics or have served as the starting points in the development of modern medicines (Verpoorte, 1998).

Lawsonia alba L. commonly known as Mehndi belongs to family Lythraceae. The methanol aqueous extracts obtained from the fruits of *L. alba* L. has been applied for spilling over the larvae from eye, so the name of plant is locally "shedhelminth". So, the aim of this work was to evaluate the antimicrobial activity of saponin isolated from *L. alba* L. fruits that have been shown to have antimicrobial activity against the pathogens.

Plant Material

Lawsonia alba L. fruits were collected and plant washed thoroughly with distilled water followed by drying in hot air oven at 70°C for 4–5 days. On complete drying, the plant material was grinded uniformly with the help of mortar-pestle and stored in an air tight container.

Chemicals used

Chemicals used for isolation purposes were 95% Ethanol, Methanol, Petroleum ether, Ethyl acetate, Chloroform, Methanol, Acetone, Distilled water. Quality of isolated saponin was confirmed on TLC plates Silica gel 60 (Merck) with Sulphuric acid (Rankem) as spraying agent.

Saponin Extraction Procedure

The extraction process was carried out by soaking the dried fruit powder in methanol-aqueous mixture overnight. The extraction was done with Petroleum ether, Ethyl acetate, Chloroform, Methanol and Acetone. Petroleum ether was used for defatting and chloroform for deproteinization of dried powder. On extraction of crude saponin, methanol was used followed by drop wise addition into acetone solution leading to precipitation. The precipitated material was extracted and dried in hot air oven leading to formation of whitish brown crystals (Lakshmi *et al.,* 2012).

Saponin Confirmatory Test

Froth test: 0.5 gm of the alcoholic extract was dissolved in 10 ml of distilled water in a test tube. The test tube was shaken vigorously for about 60 seconds. The test tube was allowed to stand in vertical position and was observed over a 30 min period of time. Thick persistent froth was observed on the surface of the liquid indicating presence on saponin.

Thin layer Chromatography

TLC technique was used for purification of saponins isolated from *L. alba* fruits. Samples (crude saponin) and the reference standards (Saponin, Sigma) were loaded on the pre-coated TLC plates silica gel 60 plates. Mobile phase chloroform: methanol: water (65:35:10 v/v/v) was used for the separation. Two drops of standard and sample were loaded upon TLC plates with the help of a micropipette. The loaded plates were placed in the TLC jar which contained the solvent system. After the completion of the run the plates were taken out and kept at room temperature to get dried for 10 minutes. The plates were developed with the spraying reagent (5%, H_2SO_4). After spraying the reagents, the plates were kept at 100 ^{O}C for 10–15 minutes in hot air oven and results were observed.

Mass Precipitation and Isolation of Saponins

In this step, ethyl acetate-chloroform was evaporated by heating the mixture at 45–55^{o}C in hot water bath and leading to formation of a crude residue.

The residue was again dissolved in methanol and heated at 45–55^{O}C. The remaining warm residue was dropped in acetone solution drop by drop. White colored powder was obtained as precipitate in acetone. The precipitate was filtered and oven dried to obtain white crystals. Saponin in form of small crystals was collected on filter paper and preserved in air tight container for further testing.

Preparation of the extracts

The fruits of plant were extracted with 60% aqueous -40% methanol. 10 g amounts of fruits materials were extracted in flasks placed in an ultrasonic bath first with 50 mL aqueous solvent for 120 min. The methanol was removed vacuum rotary at 40^{O}C until dryness. The resulting dried extract was stored in sterile screw-capped bottles at -20^{O}C. The extract was dissolved in 0.1 mL of DMSO (5 ml/g) (dimethyl sulfoxide) before testing.

Microorganisms

Pathogens (*Escherichia coli, P. aeruginosa* and *Candida albicans*) were isolated from the urine of patients diagnosed with urinary infections.

Disc diffusion method

The paper disc diffusion method was employed (Collins and Lyne, 1987). Sterile 6 mm disc filter paper disc (Schleicher and Schul, No. 2668, Dassel, Germany) were impregnated with 50 µL of the plant extract. The bacterial cultures were inoculated on Nutrient Broth (Oxoid) and incubated for 24 h at 37±0.1 ^{O}C, while the yeast cultures were inoculated on Malt Extract Broth (Oxoid) and incubated for 48 h at 28.0±0.1 ^{O}C. Adequate amounts of Mueller Hilton Agar (Oxoid) were dispensed into sterile plates and allowed to solidify under aseptic conditions. The counts of bacterial and yeast cultures were adjusted to yield 107-108 CFU mL-1 and 105-106 CFU mL-1, respectively, using the standard McFarland counting method. The test microorganisms (0.1 mL) were inoculated with a sterile swab on the surface of appropriate solid medium in plates. The agar plates inoculated with the test microorganisms were incubated for 1 h before placing the extract impregnated paper disc on the plates. The bacterial plates were incubated at 37±0.1 ^{O}C for 24 h while yeast plates were incubated at 28±0.1 ^{O}C for 48 h. After incubation, all plates were observed for zones of growth inhibition and the diameter of these zones was measured in millimeters. All tests were performed under sterile conditions in duplicate and repeated three times. Penicillin (10 µg/disc), tobramycin discs (10 µg/disc), ampicillin (20 µg/disc), nystatin (30 µg/disc), clotrimazole (30 µg/disc) and ketoconazole (20 µg/disc) discs were used as positive controls.

Microdilution method

Determination of the minimum inhibitory concentration (MIC) was carried out according to the method described by Zgoda and Porter, with some modifications [Zgoda and Porter, 2001]. A dilution series of the extract, ranging from 10 to 0.5 mg/mL, were prepared and then transferred to the broth in 96 well microtitre plates. The final concentrations were in the range 1000 to 50 µg/mL in the medium. Before inoculation of the test organisms, the bacterial and yeast strains were adjusted to 0.5 McFarland and diluted 1:1000 in Mueller Hinton Broth (Oxoid) and Malt Extract Broth (Oxoid), respectively. The plates were incubated at 35 ^{O}C for 18-24 h for bacteria and 30 ^{O}C for 48 h for the yeast cultures. All the tests were performed in broth and repeated twice. While the MIC values of the extracts were defined as the lowest concentration that showed no growth, minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) were determined by

plating samples from clear wells onto Mueller Hinton Agar and Malt Extract Agar, respectively. MBC and MFC were defined as the lowest concentration yielding negative subculture. Ampicillin and streptomycin were used as the standard antibacterial agents, while nystatin was used as the standard antifungal agent. Their dilutions ranged from 128.0 to 0.25 µg/mL concentrations in microtitre plates.

RESULTS

The saponins from dry powder of *Lawsonia alba* L. fruits were isolated by efficient conventional method were isolated. The developed protocol is economic and less time consumable which includes soaking, delipidization and deprotienization by using organic solvent the final quantity obtained from the protocol was tested on froth confirmatory test and on thin layer chromatography against the standard saponin.

The antifungal and antibacterial activity of purified saponins isolated from *L. alba* L. fruits against the pathogens, bacteria *E. coli*, *P. aeruginosa* and fungus *Candida albicans* in this study where qualitatively and quantitatively assed by the presence of inhibition zone, MIC, MBC and MFC (Table 1 and 2).

Table 1. Summary of antimicrobial activity of *H. niger* and some standard antibiotics.

	Inhibition zones (mm)[a]						
Microorganisms	**Plant Extracts (µg/mL)**	**Standard antibiotics**					
		P 10	**AMP 20**	**TOB 10**	**NYS 30**	**KETO 20**	**CLT 30**
Escherichia coli	9.6	16.0	14.0	10.0	Nt	Nt	Nt
Pseudomonas aeruginosa	11.2	8.0	10.0	12.0	Nt	Nt	Nt
Candida albicans	16.2	Nt	Nt	Nt	18.0	22.0	16.0

Table 2. Minimum inhibitory concentration (MIC) of the extracts of *H. niger.*

Microorganisms	**MIC (MBC or MFC)**			
		Standards		
	Extract (µg/mL)	**ST**	**AMP**	**NYS**
Escherichia coli	1000 (1000)	4.0 (4.0)	16 (32)	Nt
Pseudomonas aeruginosa	500 (1000)	4.0 (4.0)	32 (64)	Nt
Candida albicans	64 (128)	Nt	Nt	8.0 (16)

The isolated saponin showed strong antifungal and antibacterial activity against the pathogens, with inhibition zone of 9.0 – 26.0 mm in *E. coli*, thus more susceptible as compared to all standard antibacterial antibiotic such as penicillin, ampicillin and tobramycin (inhibition zone is 26 mm) however *P. aeruginosa* was more resistant to purified saponin.

The *C. albicans* is equal susceptible as the standard antifungal agent clotrimazole, the sample saponin was further tested was microdilution to determine the MICs and MBCs or

MFCs, the lowest MICs and MBCs or MFCs were 4.0 (8.0) µg / ml against *P. aeruginosa*. In case of *C. albicans* (MICs value 32 (64) and 64 (128) µg / ml respectively) the saponin sample showed weak antimicrobial effect against bacteria *P. aeruginosa* with MICs and MBCs ranged from 1000 (1000)-500 (1000 µg/ml)) these values are far below than standard antibiotic in brief we can say on *P. aeruginosa* had insignificant antibacterial activity (table 2).

DISCUSSION

Some studies concerning the isolation method highlighted that methanol / ethanol extraction showed antimicrobial activity than the other solvent (Rosell and Srivastava 1987, Moreau et al., 1988, Sastry and Rao, 1994).

According to the present result methanol extract showed stronger broad spectrum antifungal and antibacterial activity this information confirmed that the methanol has highly effective solvent for isolation of antimicrobial saponin from *L. alba* fruit there are some reports available that antiviral activity from saponin (Khan et al., 1996) earlier antibacterial and antifungal activity from *Hyocsyamus niger* L. (Henbane) was reported by Dulger and Dulger (2015). They reported the strong biological activities against pathogens, *C. parapsilosis, C. neoformans, C. albicans, Bacillus subtilis, Bacillus cereus, Staphylococcus aureus, Escherichia coli, Proteus vulgaris, Salmonella typhimurium, Pseudomonas aeruginosa, S. aureus.*

Similarly extract from 4 species of stachys plant also showed antimicrobial activity (Saeedi et al., 2008).

Some studies concerning the effectiveness of extraction methods highlight that methanol and ethanol extraction yields higher antimicrobial activity than other solvents.

According to present results aqueous-methanol suspended saponins have stronger and broader spectrum antimicrobial activity.

This information confirmed that methanol and ethanol is higher effective solvent for extraction of L. alba L. saponins which showed antibacterial and antifungal activity (Rosell and Srivastava, 2011, Moreau et al., 1988, Sastry and Rao, 1994).

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