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Research Article

IN VITRO ANTIBACTERIAL ACTIVITY OF EXTRACTS OF LAWSONIA INERMIS AND PUNICA GRANATUM AGAINST CLINICALLY ISOLATED ANTIBIOTIC RESISTANT PSEUDOMONAS AERUGINOSA AND STAPHYLOCOCCUS AUREUS

THIYAGARAJAN SANTHANAMARI*; PUTHUKODE RAMANATHAN MEENAKSHI; SREEKALA VELAYUTHAM

Post Graduate and Research Department of Microbiology, Asan Memorial College of Arts and Science, Chennai, India.
E-mail: dresteaar@gmail.com

ABSTRACT

The antibacterial activity of the extracts of two plants mainly *Punica granatum* and *Lawsonia inermis* were evaluated against two wound borne drug resistant bacterial pathogens *Pseudomonas aeruginosa* and *Staphylococcus aureus*. 100 specimens were collected from the patients having open wound infections from the trauma ward of Government General Hospital, Chennai and processed for the isolation and identification of bacteria by standard microbiological procedures. The different bacteria isolated were *Staphylococcus aureus* (31.19%), *Escherichia coli* (27.52%), *Klebsiella sp*. (18.34%), *Proteus sp*. (11.92%) and *Pseudomonas aeruginosa* (11.01%). The bacteria *Pseudomonas aeruginosa* and *Staphylococcus aureus* were subjected to Kirby Bauer method to test their antibiotic resistance pattern. Substantial antibiotic resistance were shown by *Pseudomonas aeruginosa* against erythromycin, methicillin, tetracycline and penicillin, moderate resistance was shown against ampicillin, oflaxacin and low resistance was observed with gentamicin. The isolates of *Staphylococcus aureus* showed moderate resistance to methicillin, ofloxacin and low degree of resistance was shown with erythromycin. All isolates of *Staphylococcus aureus* were found to be sensitive to vancomycin. The antibacterial activity of ethanolic extract of *Lawsonia inermis* and methanolic extract of *Punica granatum* were tested against the resistant isolates of *Pseudomonas aeruginosa* and *Staphylococcus aureus*. The highest antimicrobial potency was observed for the extracts of *Punica granatum* which inhibited 75% of resistant isolates of *Pseudomonas aeruginosa* and for *Lawsonia inermis* which inhibited 68.75% of resistant isolates of *Staphylococcus aureus*.

Key words: Antibacterial activity, plant extracts; antibiotic resistance, *Pseudomonas aeruginosa*, *Staphylococcus aureus, Lawsonia inermis, Punica granatum.*

INTRODUCTION

The development of wound infection chiefly depends on the integrity and protective function of the skin. It has been shown that wound infection is universal and the bacterial type varies with geographical location, resident flora of the skin, clothing at the site of wound and time between wound and examination [2, 35]. As the wound is exposed with the damaged physical barriers, heavy colonization to the extent of 30 or more colony-forming units (CFU) of bacteria can be cultured from a wound [13]. The organisms frequently isolated from different types of wounds include *Staphylococcus aureus* (20%), Coagulase negative *Staphylococci* (14%), *Pseudomonas aeruginosa* (8%), *Escherichia coli* (8%), *Klebsiella pneumoniae* (3%), *Proteus* (3%), and other gram positive aerobes (2%) [26].

One of the major problems worldwide is the increase in antibiotic-resistant strains of bacteria, mainly in hospitals, that poses constrain for their control without considerable resources and expenditure [3, 36, 37]. It has been well documented that most of the clinical isolates of *Staphylococcus aureus* are multiple-drug resistant (resistant to three or more of agents such as methicillin, ciprofloxacin, erythromycin, clindamycin, gentamicin, trimethoprim, linezolid, and vancomycin) [33]. *Pseudomonas aeruginosa* is a notoriously difficult organism to control with antibiotics or disinfectants owing to low permeability of its cell wall and abundant resistance mechanisms [17].

The problem of microbial resistance is increasingly alarming and the outlook for the use of antimicrobial drugs in the future is still uncertain [23]. One of the measures to combat this increasing rate of resistance is to have continuous investigations into new, safe and effective antimicrobials as alternative agents to substitute with less effective ones.

Plants have been traditionally proved to be a rich source of novel drug compounds, as the herbal mixtures have made large contributions to human health and well-being [12]. A wide variety of secondary metabolites, such as tannins, terpenoids, alkaloids, quinones and flavonoids are endowed with antimicrobial properties [8, 18]. Currently the research is being carried out to investigate ethnobotanical uses of plants prevailing among native people [22, 31]. There are numerous reports evidencing the antibacterial activity of plants against microorganisms [4, 9, 10, 11, 15, 19, 20, 22, 23, 26, 28, 34]. Thus, it is very much necessary to analyze the potential of the plants in combating the antibiotic resistant organisms. The henna plant and pomegranate fruit are suitable alternatives, and is now a subject of intense scientific study.

Henna (*Lawsonia inermis*) contains lawsone (2-hydroxy-1,4-naphthoquinone), as the major bio-active ingredient. The biological activity of lawsone has long been reported to include antibacterial effects on several species of both aerobic and anaerobic organisms [9]. Henna has been used as a therapeutic agent in medical treatment for wide range of ailments to cure almost anything from headache to leprosy and other skin disorders. Inhibitory action of henna has been shown against both gram negative and gram positive microbes. The invitro inhibitory action of henna against *Bacillus anthracis* and other tested bacteria has been well reported [20].

The Pomegranate (*Punica granatum*) plant is an erect shrub and its fruit is known to be a rich source of bioactive ellagitannins. Pomegrante's use has been mentioned in the ancient literature, including Ayurvedic texts, Ebers papyrus and Greek, Unani and Egyptian documents. In Greek mythology, pomegranate is known as the "fruit of the dead". It has been used as a vermifuge, bacteriocide, astringent, refrigerant, stimulant, stomachic, styptic, hair dye, and to alleviate the adverse effects of asthma, bronchitis, cough, cardiac problems, dysentery, diarrhea, dyspepsia, fever, inflammation, bleeding disorders, piles, wounds, ulcers, bruises, sores, mouth lesions, stomatitis, vaginitis, respiratory and urinary tract infections, and as a febrifuge to ameliorate malaria and seasonal fevers [28]. Methanolic extract from the fruit pericarp of pomegranate has been proved to be active against *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli* [7].

The current study is an attempt to determine the antibacterial activity of the extracts of *Lawsonia inermis* and *Punica granatum* against the resistant clinical isolates of *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

MATERIALS AND METHODS

Wound samples

100 specimens were collected for a period of 3 months between January and March 2009 from the patients having open wound infections from the general out patients section of trauma ward of

Government General Hospital, Chennai. Samples were collected aseptically with sterile cotton swabs. The specimens were then transferred to the Microbiology Department of Asan Memorial College of Arts and Science for processing.

Isolation, identification and antibiotic susceptibility testing

The obtained samples were plated on Cetrimide agar, Mannitol Salt agar, 5% blood agar and MacConkey agar and incubated at 37°C for 48 hours for the isolation of bacteria. The colonies with characteristic growth were subjected to routine biochemical tests. All the isolates that were identified as *Pseudomonas aeruginosa* and *Staphylococcus aureus* were tested for antimicrobial susceptibility using Kirby Bauer disk diffusion assay. *Pseudomonas aeruginosa* ATCC 25668 and *Staphylococcus aureus* ATCC 12598 were used as the control strains. The antibiotics tested were gentamicin (10 mcg), erythromycin (10 mcg), vancomycin (30 mcg), methicillin (5 mcg), penicillin (10 mcg), tetracycline (30 mcg), ampicillin (25 mcg) and oflaxacin (5 mcg).

Preparation of extracts

The plant extracts were prepared as per standardized procedures [10, 11, 22]. The parts of the plants (leaves of henna and peel of pomegranate) were washed with sterile distilled water to remove dirt, dried under shade and were ground to powder using a household electric blender. 30g and 50g of the dry powdered henna and pomegranate peel respectively were weighed. They were then transferred separately to conical flasks containing 100ml of 85% ethanol and 100ml of 75% methanol respectively and allowed to soak at ambient temperature for 72 hours. The extracts were then filtered using Whatman no.1 filter paper and the filtrates were concentrated in vacuo at 40°C using a rotary evaporator. Residues of the extracts were made into suspensions using sterile distilled water and sterile dimethyl sulphoxide at concentrations of 100, 200, 300, 400 and 500 mg/ml for henna and pomegranate extracts respectively.

Assay for antibacterial activity using agar well diffusion method

The screening of antibacterial activity of plant extracts was carried out using the agar well diffusion method as described by Lino and Deogracious [19]. The resistant isolates were inoculated into tubes of nutrient broth and incubated at 37°C for 12-24 h. Each of the cultures were then adjusted to 0.5 McFarland turbidity standard and inoculated onto Muller Hinton agar (MHA) plates. A sterile cork borer was then used to make wells (6mm diameter) for different concentrations of the extracts on each of the plates containing cultures of the different resistant isolates. 500µl of the varying concentrations (100, 200, 300, 400 and 500 mg/ml) of the extracts were introduced into the wells with the help of micropipettes. 500µl of sterile distilled water, dimethyl sulphoxide (DMSO), 85% ethanol and 75% methanol were introduced into respective wells to serve as negative control. The culture plates were allowed to stand on the working bench for 30 min for pre-diffusion and were then incubated in upright position at 37°C for 24 h. After 24 hrs, antibacterial activity was determined by measurement of diameter of zones of inhibition (mm).

RESULT AND DISCUSSION

Isolation, characterization and antibiotic sensitivity testing of isolates

Subsequent to the isolation procedures bacteria of different types were identified. There has been a high incidence of *Staphylococcus aureus* (31.19%) followed by *Escherichia coli* (27.52%). The other bacteria isolated were *Klebsiella sp* (18.34%), *Proteus sp* (11.92%) and *Pseudomonas aeruginosa* (11.01%). The incidence of *Pseudomonas aeruginosa* has been lower compared to that of other bacteria (Table 1). Though the incidence of *Pseudomonas aeruginosa* has been comparatively lower its implication with complicated wound infection makes its presence significant.

As the bacteria *Pseudomonas aeruginosa* and *Staphylococcus aureus* were of interest for the present study, the same were subjected to antibiogram test with 8 commonly used antibiotics.

The results on antibiotic resistance pattern of *Pseudomonas aeruginosa* isolates inferred that there has been complete resistance to erythromycin (100%) and methicillin (100%). Their resistance to gentamicin has been comparatively lower (16.67%). The pattern of resistance to other antibiotics is presented in Table 2.

Majority of the isolates of *Staphylococcus aureus* were observed to resist methicillin (47.05%) and oflaxacin (41.17%). Other antibiotics resisted were penicillin (38.23%), tetracycline (35.29%), gentamicin (29.41%) and ampicillin (26.47%). There has been a lower degree of resistance to erythromycin (11.76%). All the isolates of *Staphylococcus aureus* seemed to be sensitive to vancomycin (Table 3). Similar observations have been made by Adegoke *et al*. [1], where isolates of *Staphylococcus aureus* exhibited multiple drug resistance to ten frequently prescribed antibiotics.

From the results of antibiotic sensitivity test it has been observed that both the clinical isolates of *Pseudomonas aeruginosa* and *Staphylococcus aureus* were showing resistance to almost all the antibiotics tested except vancomycin (for *S. aureus*). The extensive upsurge of antibiotic resistance is in accordance with an earlier report by Obseiki ebor *et al*. [24] where it was reported that antibiotic abuse and high prevalence of self medication with antibiotics are responsible for the emergence of antibiotic resistant bacterial strains.

***In vitro* antibacterial activity of plant extracts on the resistant isolates**

The extracts of *Punica granatum* and *Lawsonia inermis*, were tested for their antibacterial activity against the isolates of *Staphylococcus aureus* and *Pseudomonas aeruginosa* that were showing higher degree of resistance to the antibiotics.

Antibacterial activity of the extracts was recorded when the zone of inhibition was greater than 6mm [8]. The methanolic extract of peel of *Punica granatum* inhibited *Staphylococcus aureus* and *Pseudomonas aeruginosa* at different concentrations. The extract was more effective for *Staphylococcus aureus* (9mm at 100 mg/ml and 16mm at 500 mg/ml) than to *Pseudomonas aeruginosa* (8mm at 100 mg/ml and 14mm at 500mg/ml) (Table 4). Prashanth *et al.* [26] have tested the antibacterial activity of petroleum ether, chloroform, methanol and water extracts of pomegranate rinds, and reported that the methanol extract was the most effective against the tested organisms such as *Staphylococcus aureus*, *Escherichia coli, Klebsiella pneumoniae, Proteus vulgaris, Bacillus subtilis* and *Salmonella typhi*.

The ethanolic extracts of leaves of *Lawsonia inermis* inhibited *Staphylococcus aureus* and *Pseudomonas aeruginosa* at different concentrations but the latter was inhibited only at higher concentrations of extract. The extract was marginally more effective against *Staphylococcus aureus* (8mm at 100 mg/ml and 18mm at 500 mg/ml) than towards *Pseudomonas aeruginosa* (9mm at 400 mg/ml and 11mm at 500mg/ml) (Table 5). Many reports cite the inhibitory activity of henna against gram negative and gram positive organisms [4, 9, 22, 25, 34].

From the results of antibacterial activity of the extracts against the resistant isolates it has been observed that *Staphylococcus aureus* is more susceptible to the employed plant extracts than *Pseudomonas aeruginosa*. This finding is in agreement with earlier reports where the antibacterial activity of the phytoconstituents of *Lawsonia inermis* were active against gram positive bacteria such as *Staphylococcus aureus*, *Enterococcus faecium* and *Bacillus subtilis*, but were inactive against gram negative bacteria [25]. The lack of susceptibility of *Pseudomonas aeruginosa* to the ethanolic extract of *Lawsonia inermis* could be attributed to the fact that this bacterium is naturally resistant to many antibiotics due to the permeability barrier afforded by its outer membrane. Also its tendency to colonise in a biofilm form makes the cells impervious to therapeutic concentrations of antibiotics. The feature of *Pseudomonas aeruginosa* in exhibiting resistance to a variety of naturally occurring antibiotics may be attributed to its soil occurent nature in association with *Actinomycetes*, molds and bacilli [17].

Staphylococcus aureus and *Pseudomonas aeruginosa* are commonly encountered antibiotic resistant organisms that can be commonly

isolated from sites of wound infection. The resistance mechanism evolved by these organisms enable them to withstand most of the antibiotics that may complicate and cause life-threatening diseases. There are several reports supporting the prevalence of antibiotic resistant *Pseudomonas aeruginosa* and *Staphylococcus aureus* [1, 5, 6, 12, 14, 21, 27, 29, 30, 32, 33]. Therefore, it has become a major necessity to look out for alternative antimicrobial agents to combat such organisms. The current demonstration of antibacterial activity of the plant extracts against the resistant isolates of *Staphylococcus aureus* and *Pseudomonas aeruginosa* is a substantial indication that paves a way for the development of newer and better antibacterial agents for the control of emerging antibiotic resistant organisms.

Further investigations on the efficacy of these plant extracts against a broader range of organisms, isolation and characterization of the phytoconstituents, and *in vivo* toxicological studies must be carried out with an outlook of developing more novel drugs for human welfare.

Table -1: Frequency and percentage of organisms isolated from wounds of patient undergoing treatment at trauma ward

Microorganism	No. Samples	No. Isolates	(%)
S. aureus	28	68	31.19
E. coli	22	60	27.52
Klebsiella species	18	40	18.34
Proteus species	16	26	11.92
P. aeruginosa	12	24	11.01
No growth	4	0	0.02
Total	100	218	100

Table -2: Resistance pattern of *P. aeruginosa* isolated from wounds of traumatic patients (n=24).

Antibiotics	No. *P. aeruginosa*	Resistance (%)
Erythromycin	24	100
Methicillin	24	100
Tetracycline	22	91.66
Penicillin	20	83.33
Ampicillin	16	66.66
Oflaxacin	12	50
Gentamicin	4	16.67

Table -3: Resistance pattern of *S. aureus* isolated from wounds of traumatic patients (n=68).

Antibiotics	No. *S. aureus*	Resistance (%)
Methicillin	32	47.05
Oflaxacin	28	41.17
Penicillin	26	38.23
Tetracycline	24	35.29
Gentamicin	20	29.41
Ampicillin	18	26.47
Erythromycin	8	11.76
Vancomycin	0	0

Table -4: Effect of methanolic extract of peel of *Punica granatum* against *S. aureus* and *P. aeruginosa*

Test isolates (%)	Concentration	Zone of inhibition (mm, diameter)
S. aureus (62.5%)	100 mg/ml	9
	200 mg/ml	13
	300 mg/ml	14
	400 mg/ml	15
	500 mg/ml	16
P. aeruginosa (75%)	100 mg/ml	8
	200 mg/ml	11
	300 mg/ml	12
	400 mg/ml	13
	500 mg/ml	14

Table -5: Effect of ethanolic extract of leaves of *Lawsonia inermis* against *S. aureus* and *P. aeruginosa*

Test isolate (%)	Concentration	Zone of inhibition (mm, diameter)
S. aureus (68.75%)	100 mg/ml	8
	200 mg/ml	12
	300 mg/ml	14
	400 mg/ml	15
	500 mg/ml	18
P. aeruginosa (25%)	100 mg/ml	0
	200 mg/ml	0
	300 mg/ml	0
	400 mg/ml	9
	500 mg/ml	11

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