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Antibacterial and anti-hemolytic activity of tannins from *Pimenta dioica* against methicillin resistant *Staphylococcus aureus*

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Abstract

High rate of resistance among *Staphylococcus* infection initiates scientists to discover new antibiotics. The objective of this study is to determine the effect of tannins isolated from the *Pimenta dioica* leaves on *Staphylococcus aureus* and methicillin resistant *S. aureus* as well as to evaluate their effect on hemolysin production. The antimicrobial activity of 4,6-(*S*)-hexahydroxydiphenoyl-(α/β)-D-glucopyranose and casuarinin, pedunculagin and nilocitin tannins from *P. dioica* was examined using agar diffusion method. Moreover, minimum inhibitory concentrations were evaluated by microtiter plate assay method. Pedunculagin and nilocitin exhibited antibacterial and anti-hemolytic effect against *S. aureus*. This will open the era for *in vivo* assessment of such compounds for clinical applications.

Introduction

Antimicrobial resistant among pathogenic bacteria has been increased during the past decades due to the misuse and the extended use of antimicrobials. Gram positive bacterial infection especially with *Staphylococcus aureus* and methicillin resistant *S. aureus* (MRSA) is categorized as one of the main causes of nosocomial infection (Lyer et al., 2014).

S. aureus are opportunistic pathogens as they can invade human body and cause a wide variety of acute and chronic infections. It is the main cause of skin and soft tissue infections as furuncles, carbuncles, boil, abscesses and wounds infection (Brackman et al., 2015). Mild infection of *S. aureus* may disseminate through the body leading to severe infections. The severity of *S. aureus* infection depends mainly on the exposure to virulence factors as protease, lipase, hemolysin, and toxins. *S. aureus* has developed resistance over the past few decades to many antimicrobial drugs (McCaig et al., 2006). The elevated levels of *S. aureus* resistance encouraged the search for new therapeutic alternatives derived from various sources to manage *S. aureus* infection.

Plants constituents represent an important source for antibacterial legends as tannins, flavonoids and volatile oil. Tannins are water soluble polyphenolic compounds with high molecular weight as well as they are widely distributed in a large number of higher plants and human diet. They have the ability to form complexes with proteins (Ozdal et al., 2013). Tannins get an intense focus of research interest due to their health-beneficial effects especially in the treatment and prevention of several infectious diseases (Scalbert, 1991).

Pimenta dioica (L.) Merr, syn. *P. officinalis* (L.) Berg belonging to the family Myrtaceae and is communally known as allspice, pimenta, pimento, clove pepper and

Jamaica pepper. The plant is the native to the Southern Mexico and Central America (Riffle, 1998) but it is cultivated in many warm parts of the world. *P. dioica* is traditionally used as a spice and condiment, flavoring agent as well as in tanning purposes. Moreover, different plant parts have been used to relieve dental and muscle aches, bronchitis, menstrual cramps, flatulence, diabetes, viral infections, depression, arthritis and fatigue (Kikiuzaki et al., 1994). *P. dioica* is a precious source of different metabolites such as phenylpropanoids, galloylglucosides (Kikiuzaki et al., 1994; Marzouk et al., 2007), flavonoids and tannins (Marzouk et al., 2007).

The aim of this study is the evaluation of antimicrobial activity of the pure tannin compounds isolated from *P. dioica* leaves against *S. aureus*, and MRSA isolates as well as estimation of their effect on hemolysin production as one of the main virulence factors of *S. aureus.*

Materials and Methods

Tested compounds

Tannins compound namely 4,6-(*S*)-hexahydroxydiphenoyl-(α/β)-D-glucopyranose, casuarinin, pedunculagin and nilocitin were isolated and identified from the leaves of *P. dioica* (Marzouk et al., 2007) (Figure 1). Samples were kindly provided by one of the authors (FAM) and authentic were kept in the Pharmacognosy and Pharmaceutical Chemistry Department, Faculty of Pharmacy, Taibah University.

Antibacterial activity

Bacterial isolates growth conditions and inoculum preparation

The clinical isolates of *S. aureus* were collected from the Al-Madina Al-Munawarrah Hospitals and Taibah University. The isolates were purified from different clinical sources; four from wound, three from nasal swap, two from sputum, one from blood, one from tonsils and one from urine. Standard *S. aureus* (ATCC 29213) strains were kindly provided by the Ohod Hospital, Al-Madina Al-Munawarrah, Saudi Arabia. All isolates were confirmed according to the clinical laboratory standards (Cheesbrough, 1989).

All cultures of *S. aureus* were propagated using nutrient broth medium and incubated at 37°C for 24 hours. The harvested microorganisms were preserved in 10% glycerol stocks (Simione and Brown, 1991).

Antimicrobial susceptibility of *S. aureus* clinical isolates

The susceptibility of *S. aureus* to different antimicrobial agents was examined according to Clinical Laboratory Standard Institute method (CLSI, 2013). The antimicrobial agents examined were amoxicillin/clavulanic acid (30 µg), ampicillin (10 µg), imepenem (10 µg), cephalothin (30 µg), cefoxitin (30 µg), ceftazidime (30 µg), erythromycin (15 µg), ciprofloxacin (5 µg) and trimethoprim/sulfamethoxazole (2 µg) (Bioanalyse, Turkey).

Determination of MIC of cloxacillin

The minimum inhibitory concentration (MIC) of cloxacillin was determined against different clinical isolates. MIC was measured using microtitre plate-dilution method (CLSI, 2013). Cloxacillin was diluted 1:1 in 100 µL Muller Hinton broth to have concentrations from (125-7.8 µg/mL). The plates were incubated at 37°C for 24 hours. MIC was determined as the lowest concentration of cloxacillin that inhibited microbial growth.

Antimicrobial assay of tannins

Antimicrobial susceptibility test

The effect of tannin compounds on the tested *S. aureus* was evaluated using agar well diffusion method. Muller Hinton agar (20 mL) at 45°C was inoculated with 20 µL inoculums of each tested isolate diluted at 0.5 McFarland, mixed well and poured into sterile petri-dish and left till complete solidification. Wells of 10 mm were made in the plates using a cork borer. The wells were filled with 100 µL of the each compound 2 mg/mL for *S. aureus* isolates and with 5 mg/mL for MRSA

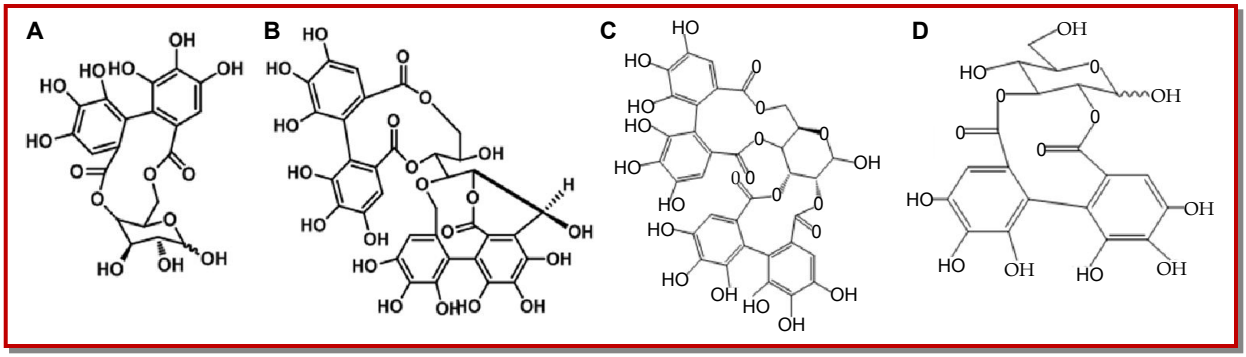

Figure 1: Structure of the tannins isolated from *P. dioica* leaves. A = 4, 6-(*S*) hexahydroxydiphenoyl-(α/β)-D-glucopyranose; B = casuarinin; C = pedunculagin; D = nilocitin

isolates. Amoxicillin/clavulanic acid was used as a positive control. Antibacterial activity of the applied compounds was determined by measuring the diameter of the zone of inhibition around the wells (Devi et al., 2011).

Determination of MIC of tannins

The MIC of pedunculagin and nilocitin with the largest inhibition zone was performed. Amoxicillin/clavulanic acid was used also as positive control. MIC was measured using microtitre plate-dilution method against *S. aureus* and MRSA (CLSI, 2013). 2-fold serial dilutions of the tested compounds were performed in 100 µL nutrient broth to obtain concentrations from 1,000 to 4.7 µg/mL. The plates were incubated at 37°C for 24 hours. MIC was determined as the lowest concentration of compounds with no visible growth. Triphenyltetrazolium chloride (40 µL of 0.5%) (Sigma–Aldrich, USA) was added to each well to visualize the microbial growth by reducing the yellow dye to red color (Vogel et al., 2011).

Effect of tested compounds on hemolysin production

The effect of the compounds on hemolysin released by *S. aureus* was determined by adding tannins in a sub-MIC concentration (1/2 MIC) to *S. aureus* cultures. The mixture was incubated at 37°C for 48 hours. The incubation of the tested isolate without compound was performed under the same conditions. The supernatants were centrifuged at 3,000 rpm for 20 min. The ability of *S. aureus* to produce hemolysin was examined using a technique (Dacheux et al., 2001). Sheep blood erythrocytes were washed three times in sterile physiological saline and centrifuge at 3,000 rpm for 5 min. The washed erythrocytes were resuspended in Tris buffered saline (50 mM Tris HCL and 150 mM NaCl, pH 7.4) with 2% final concentration. Mixture of erythrocytes suspension with supernatant was prepared (1:1 concentration) and incubated at 37°C for 2.5 hours. The suspension was centrifuged at 3,000 rpm for 5 min. The release of hemoglobin was evaluated by reading the absorbance at 540 nm. The incubation of RBCs in sterile Luria-Bertani containing 0.1% sodium dodecyl sulfate was used as a positive control (T), negative control (B) was prepared by incubating RBCs with equal volume of the Tris buffer. The percentage of cell lysis was calculated using the following formula:

$$\%\text{Hemolysis} = [(X-B)/(T-B)] \times 100$$

where X is the absorbance value for the sample analyzed (Dacheux et al., 2001)

Results

Antimicrobial susceptibility tests

The antimicrobial susceptibility test of nine antimicrobial agents was performed against *S. aureus* clinical isolates. All isolates were resistant to ampicillin and cefoxitin except isolate number 3 (Table I). All isolates were susceptible to cephalothin and imipenem except isolates 33 and 212. It was found that six isolates were resistant to cefoxitin, ampicillin and amoxicillin/clavulanic acid but sensitive to other antimicrobials. Among the tested isolates, 50% were resistant to erythromycin. Most isolates were susceptible to ciprofloxacin except isolate number 33. Isolate 33 showed multidrug resistant against all tested antimicrobial agents.

Table I

Antimicrobial susceptibility of *S. aureus*

Isolate	AP (10 µg)	AUG (30 µg)	CEF (30 µg)	FOX (30 µg)	CAZ (30 µg)	IMI (10 µg)	TS (2 µg)	ER (15 µg)	CIP (5 µg)
1	R	S	S	R	S	S	R	R	I
2	R	R	S	R	R	S	R	I	S
3	S	S	S	S	S	S	S	R	S
33	R	R	R	R	R	R	R	R	R
48	R	R	S	R	S	S	R	S	S
56	R	R	S	R	S	S	S	R	S
61	R	R	S	R	I	S	R	I	S
87	R	S	S	R	R	S	S	R	S
97	R	R	S	R	I	S	S	I	S
212	R	R	R	R	R	R	R	I	S
372	R	S	S	R	R	S	S	R	S
724	R	R	S	R	S	S	S	I	S

*The results in the table were interpreted according to CLSI, 2013; Amoxicillin/clavulanic acid (AUG), ampicillin (AP), imipenem (IMI), cephalothin (CEF), cefoxitin (FOX), ceftazidime (CAZ), erythromycin (ER), ciprofloxacin (CIP) and trimethoprim/sulfamethoxazole (TS), S (sensitive), R (resistant), I (intermediate)

Table II

Antimicrobial activity of tannins compounds against *S. aureus*

	Inhibition zone diameter (mm)				
Staphylococcus aureus ATCC 29213	Amoxicillin/ clavulanic acid	Nilocitin	Pedunculagin	Casuarinin	4,6-(*S*)-hexahydroxydiphenoyl-(α/β)-D-glucopyranose
Sample name/isolate No.	50	22	20	19	21
1	41.5	8	15	8	9
2	46	30	30	23	25
3	40	17	17	19	18
48	23	20	21	16	18
61	25	20	19	18	20
87	40	15	16	16	20
97	25	17	17	15	16
212	30	13	12	12	12
372	32	16	16	16	14
33 MRSA	23.5	22	18	20	10
56 MRSA	28	17	16	15	12
724 MRSA	20	17	13	16	16

MRSA = Methicillin resistant *Staphylococcus aureus;* ATCC =American type culture collection

Table III

Minimum inhibitory concentration of pedunculagin and nilocitin

Sample name	Minimal inhibitory concentration (µg/mL)		
	Pedunculagin	Nilocitin	Amoxicillin/clavulanic acid
Staphylococcus aureus ATCC 29213	625 ± 0	187 ± 0.1	39 ± 0
1	1250 ± 0	78 ± 0	310 ± 0
2	312 ± 0	94 ± 0.1	125 ± 0
48	625 ± 0	125 ± 0	125 ± 0
61	1250 ± 0	1875 ± 0.8	1250 ± 0
81	625 ± 0	125 ± 0	93 ± 0.1
97	625 ± 0	125 ± 0	125 ± 0
212	1250 ± 0	1250 ± 0	156 ± 0
372	1250 ± 0	1250 ± 0	321 ± 0
33 MRSA	2500 ± 0	2500 ± 0	2500 ± 0
56 MRSA	2500 ± 0	2500 ± 0	>2.5
724 MRSA	2500 ± 0	2500 ± 0	>2.5

Data represented as mean of two replicates ± SD

Characterization of MRSA isolates

The MIC of cloxacillin against tested *Staphylococcus* isolates was determined. According to Clinical Laboratory Standard Institute, (CLSI, 2013), MRSA isolates were assigned at MICs >2 µg/mL. All the collected isolates had MIC<2 µg/mL except isolates 33, 56 and 724 which were categorized as MRSA with MIC >125 µg/mL.

Antimicrobial activity of tested compounds on the recovered isolates

Antimicrobial activities were determined based on the diameter of inhibition zone (mm). It was observed that (4,6-(*S*)-hexahydroxydiphenoyl-(α/β)-D-gluco-pyranose, casuarinin, pedunculagin and nilocitin were effective against most *S. aureus* and MRSA isolates with variable degree (Table II). The inhibition zone diameter of pedunculagin and nilocitin was more than that of 4,6 -(*S*)-hexahydroxydiphenoyl-(α/β)-D-gluco-pyranose and casuarinin. The highest zone of inhibition 30 mm was observed against *S. aureus* isolate number 2. On the other hand, the zone of inhibition of peduncula-gin against MRSA isolates was 13-18 mm. Furthermore, nilocitin was effective against MRSA with inhibition

zone diameter range 17-22 mm. Both pedun-culagin and nilocitin were effective against MRSA33 which were resistant to all assessed antimicrobials (Figure 2).

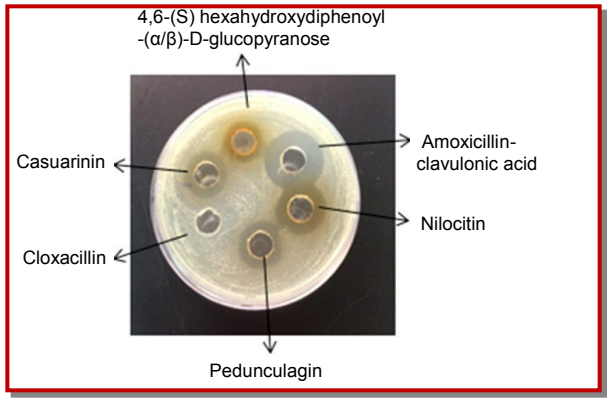

Figure 2: Antimicrobial activity of tannins against *Staphylococcus aureus* MRSA 33

Minimum inhibitory concentration

The MICs of pedunculagin and nilocitin against *S. aureus* and MRSA isolates were evaluated (Table III). *S. aureus* 2 showed the lowest MIC of 312 ± 0 µg/mL and 94 ± 0.1 µg/mL for pedunculagin and nilocitin respectively, and 125 µg/mL for amoxicillin/clavulanic acid. It means that *S. aureus* 2 was strongly inhibited by those compounds.

Effect of tested compounds on hemolysis production

The effect of pedunculagin and nilocitin on hemolysin production by *S. aureus* was tested (Table IV). Peduncu -lagin and nilocitin decreased the percentage of cell lysis. It was also noticed that nilocitin was more effective than pedunculagin.

Table IV

Effect of pedunculagin and nilocitin tannins on cell lysis

Bacteria	% Cell lysis
Staphylococcus aureus No. 1 (control)	97.4
Staphylococcus aureus No. 1 + Pedunculagin	36.3
Staphylococcus aureus No. 1 + Nilocitin	6.5

Discussion

In the present study, the tested tannin compounds from *P. dioica* had an antimicrobial activity against all *S. aureus* clinical isolates and against MRSA (Tables III and IV). Similarly, Doss et al. (2009) observed that all tannin compounds isolated from leaves of *Solanum trilobatum* possess antibacterial activity against *S. aureus* at 2.5 mg/mL. Disintegration of bacterial colonies with tannin compounds may be attributed to their interference with the bacterial cell wall thus inhibiting the microbial growth (Akiyama et al., 2001; Caelli et al., 2000; Erasto et al., 2004). Viljoen et al. (2003) reported that tannins isolated from *Punica granatum* can be used as body wash or nasal ointments for MRSA.

In this research, it was also found that the presence of tannins with *S. aureus* decreased the ability of *S. aureus* to cause blood hemolysis. Choi and colleagues (2007) assumed that both condensed and hydrolysable tannins may form aggregates with α-toxin inhibiting its action on erythrocytes. The structure of tannins may be responsiple for their antimicrobial action. There are many mechanism underlining this activity. One of which, tannins in pure or extract form have great ability to inactivate enzymes due to strong anti-oxidant activity, which could be explained mainly due to the presence of a large number of hydroxyl groups in a huge extended π-electron conjugation system in galloyl. Also, HHDP groups present in the tested compounds are responsible for the stabilization of phenoxide radicals and hence enhance its scavenging affinity in the oxidation reaction (Marzouk et al., 2007). Furthermore, the oxidized phenols cause enzymatic inactivation of the microorganism through reaction with sulfhydryl groups of the enzymes and form covalent linkage. One other point, antimicrobial potential of tannins could be through its effect on membrane via complex formation with the proteins and polysaccharides constituents of the cell membrane (Scalbert, 1991).

Conclusion

Pedunculagin and nilocitin exhibit antibacterial activity against *S. aureus* and MRSA. Moreover, they reduced the hemolytic activity of *S. aureus*.

Conflict of Interest

All authors have completed the ICMJE uniform disclosure form and declare no support from any organization for the submitted work.

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