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Anti Methicillin-resistant *Staphylococcus Aureus* (MRSA) Activities of Compounds Isolated from *Psidium Guajava* Linn. Leaves

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Abstract : Methicillin-resistant *Staphylococcus aureus (*MRSA) is reputedly known as one of the most difficult bacteria to treat in patients and to eradicate in a hospital environment. Limited choice and capability of available drugs necessitates the quest for alternative cure. In this study, four compounds isolated from *Psidium guajava* leaves were evaluated for their potential inhibitory and resistance-modifying abilities against MRSA clinical isolates. Ursolic acid and 2α-hydroxyursolic acid showed inhibitory activities with minimum inhibitory concentration (MIC) values below 1 mg/mL while quercetin exhibited potential efflux inhibitory properties as compared to reserpine, a common plant-based efflux pump inhibitor (EPI). The presence of a hydroxyl group in 2α-hydroxyursolic acid may be responsible for the higher MIC value since ursolic acid did not have the additional functional group. In addition, the presence of an arabinose sugar group in guaijaeverin may lessen its efflux potentials as compared to quercetin, which is more hydrophobic. Both ursolic acid and 2α-hydroxyursolic acid showed good and moderate inhibitory activities, respectively. Additionally, this is the first communication to report quercetin's potential as an EPI against a MRSA isolate. Furthermore, this study shows an example and usability of the simplified EPI assay to evaluate plant compounds against clinical MRSA isolates using compounds that exhibited weak antimicrobial activities.

Keywords: Efflux pump inhibitor (EPI) evaluation assay; MIC assay; methicillin-resistant *Staphylococcus aureus* (MRSA); *Psidium guajava*.

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Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) infection is a serious and costly problem in the healthcare sector. Treatment of nosocomial infection, which held MRSA as the main culprit, has cost up to £1billion/year in Britain and RM2 million/year in one Malaysian hospital [1,2]. In the past decade, efflux mechanism in MRSA has been identified as one the main contributor of its multidrug-resistance towards various structurally-unrelated antibiotics and this discovery has impeded further the worldwide effort to eradicate this dangerous 'superbug' [3,4]. Fortunately, studies have shown that plants have a high potential to be a source of antibacterial compounds due to its ability to produce cytotoxic agents and the fact that antibacterial natural products should be present or synthesized *de novo* following microbial attack to protect plants from invasive and pathogenic microbes in their environment [1,5]. *Psidium guajava* Linn. or guava is an evergreen growing wild plant native to the torrid and subtropic zones. The decoctions of the leaf, root or stem of guava have been used in antidiarrhoeal and antidiabetic therapies in many systems of traditional medicine in tropical countries [6,7]. On the other hand, the water, alcohol and chloroform extracts of guava leaves were reported to be an effective antibacterial agent against *Aeromonas hydrophila*, *Staphylococcus aureus*, *Shigella* spp. and *Vibrio* spp.[8]. Guava has also been reported to possess antimutagenic effect against *Salmonella typhimurium* [9]. However, most studies were only carried out using the plant extracts and the disc diffusion method [6,8].

Result and discussion

In this study, four compounds isolated from *P. guajava* leaves were evaluated for their potential inhibitory and resistance-modifying abilities using minimum inhibitory concentration (MIC) assay and efflux pump inhibitor (EPI) evaluation assay against three MRSA and a methicillin-susceptible *S. aureus* (MSSA) clinical isolates. Four compounds were successfully isolated from the *Psidium guajava* leaves. The identity of each compound was confirmed by comparing their spectroscopic data with literature. The compounds

are quercetin, guaijaverin, ursolic acid and 2α-hydroxyursolic acid [10,11,12,13,14]. Both quercetin and guaijaverin (quercetin-3-*O*-L-arabinoside) are flavonoid compounds while ursolic acid and 2α-hydroxyursolic acid are triterpenoid compounds.

The results of the MIC and EPI assay are summarized in Table 1. Ursolic acid exhibited good inhibitory potential of 15.6 to 31.3 µg/mL while 2α-hydroxyursolic acid showed moderate inhibitory activity with MIC value of 125 to 1000 µg/mL. The remaining two compounds (quercetin and guaijaverin) displayed MIC values of >1000 µg/mL. Both compounds were evaluated further for efflux pump inhibitor potential based on the foundation that antimicrobial producing plants may developed a variety of EPIs against different efflux pumps of plant pathogens [15].

In the EPI assay, quercetin as opposed to guaijaverin, exhibited good efflux potentiation against U949 by a four-fold reduction of EtBr MIC. Besides that, both compounds exhibited weak efflux potentiation against other test strains. Additionally, no EtBr MIC reduction was observed in both ATCC strains.

Ursolic acid from other plants has also been reported to displayed inhibitory action against MRSA isolates [16, 17] as seen in this study. The presence of a hydroxyl group at position C2 of the chemical structure on 2α-hydroxyursolic acid may be responsible for the higher MIC value since ursolic acid did not have the additional hydroxyl group as seen in Figure 1. On the other hand, the MICs of quercetin and guaijaverin were higher against a susceptible strain (ATCC 25923) than the other three MRSA strains. Similar result was observed recently where ursolic acid from *Salvia officinalis* (sage) exhibited higher MIC value against hyper-susceptible mutants of *P. aeruginosa* and *E. coli* [17]. These results may suggest that MRSA isolates are more susceptible towards both triterpenoid compounds.

Quercetin's weak inhibitory potential against MRSA as observed in this study supports previous findings [16]. Similar studies too have shown that flavonols (of which quercetin belongs to) without a free hydroxyl group at position C3 were effective as *S. aureus* EPIs [18,19] as reflected in Figure 1. The lower susceptibility of U949 against EtBr (250 µg/mL) as compared to the other three isolates (15.6 –31.3 µg/mL) suggested the presence of a wider array of unidentified efflux pumps and/or other mechanism to extrude EtBr.

On the other hand, the almost absence of potentiation activity of guaijaverin against U949 may be due to the presence of an arabinose sugar group on position C3 of its structure (Figure 1). Additional hydroxyl groups on the arabinose structure too, renders guaijavarine to be less hydrophobic than quercetin hence, lessen the efflux potentiation. Studies have suggested that efflux

Table 1 : MIC value of the pure compounds and in combination with ethidium bromide (EtBr).

Compounds	MIC (µg/mL)			
	N441	U949	ATCC33591	ATCC25923
Quercetin	>1000	>1000	>1000	>1000
Guijavarine	>1000	>1000	>1000	>1000
Ursolic acid	15.6	15.6	15.6	31.3
2α-hydroxyursolic acid	125	500	125	1000
Ethidium bromide (EtBr)	31.3	250	15.6	15.6
* Reserpine	7.8(4)[a]	125(2)	7.8(2)	7.8(2)
* Quercetine	15.6(2)	62.5(4)	15.6(NC)	15.6(NC)
* Guaijavarine	15.6(2)	250(NC)	15.6(NC)	15.6(NC)

+ Reserpinea 7.8 (4)b 125 (2) 7.8 (2) 7.8 (2)
+ Quercetin 15.6 (2) 62.5 (4) 15.6 (NC) 15.6 (NC)
+ Guaijaverin 15.6 (2) 250 (NC) 15.6 (NC) 15.6 (NC)
aReserpine at 50 µg/mL was used as a positive control efflux inhibitor.
bFold reduction are given in parentheses.
NC = No change in EtBr MIC value after combination with respective compounds.

pumps recognized hydrophobic compounds that could bind directly to its hydrophobic region thus preventing drug transport [20,21].

In summary, both ursolic acid and 2α-hydroxyursolic acid showed good and moderate inhibitory activity, respectively. To the authors' knowledge, this is the first communication to report quercetin's potential as an EPI against an MRSA isolate. Furthermore, this study shows an example and usability of the simplified EPI assay to evaluate plant compounds against clinical MRSA isolates using compounds that exhibited weak antimicrobial activities.

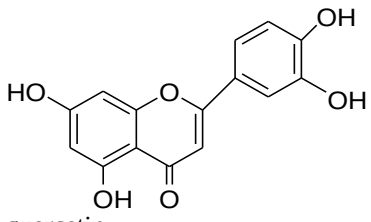
Experimental

1. Plant material

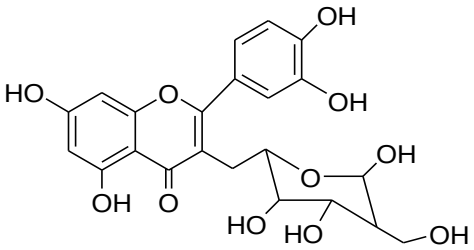
Leaves of *Psidium guajava* Linn.(Myrtaceae) were collected from Bidor, Perak, Malaysia. A voucher specimen (FRI 48906) was deposited at the FRIMs' Herbarium, Malaysia. The plant was identified by our resident botanist, Madam Zainon Abu Samah from the Bioresources Branch, Medicinal Plants Division.

2. Extraction and isolation

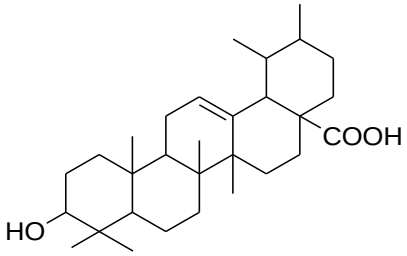
Fresh leaves were cut into smaller pieces and dried in the oven at 40 ^{o}C for three days. Next, the dried leaves were then ground and extracted using methanol at room temperature for three days. The resultant methanol solution was then filtered through Whatman filter paper No. 4 and the obtained filtrate was concentrated and dried under reduced pressure using a rotary evaporator. The extraction process was repeated twice and the yield of extract was 10% based on sample dry weight. The methanol extract (150 g) was suspended in water and subjected to successive solvent-solvent partitioning using petroleum ether, chloroform and ethyl acetate. The resulting ethyl acetate fraction (15.6 g) was further fractionated by column chromatography (1.0 x 15 cm) employing Sephadex LH-20 (sigma) with methanol 100% (2 L) as the eluent. Fractions of 20 ml were collected and monitored by using TLC. The developed TLC plate was sprayed with 10% H_2SO_4 and heated on the hot plate until full color developed. Fractions 60-71 were combined and was further separated on MCI gel CHP20P column (2.5 x 24 cm) using a step gradient of H_2O: CH_3OH (100: 0 until 0: 100, 100 ml, 10% increment of methanol) to give 60 fractions (20 ml each). Combination of the fractions based on TLC profile gave fractions 1-3. Fraction 3 was further chromatographed on the silica gel column (1 x 15 cm, 2 g) using gradient system of $CHCl_3$: CH_3OH (95:5, 90:10, 85:15, 50:50; 100 ml). Fractions 10-17 and 20-25 were each combined to give quercetin (6.0 mg) and guaijaverin (8.9 mg), respectively. The chloroform fraction (15 g) was subjected to Diaion HP-20 column chromatography in order to remove the chlorophyll. Further fractionation with silica gel (250 g, 4.5 x 24 cm) using step gradient of $CHCl_3$: CH_3OH led to the isolation of compound ursolic acid (13.0 mg) (fractions 27-33) and 2α-hydroxyursolic acid (128.0 mg) (fractions 135-153).



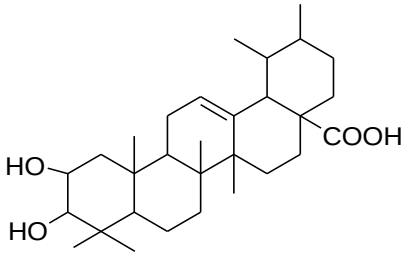
quercetin



guaijavarine



ursolic acid



2α-hydroxyursolic acid

Figure 1 : Structure of compounds present in *Psidium guajava* Linn leaves

a. ***Bacterial strains used***

Two MRSA clinical isolates from Hospital Universiti Kebangsaan Malaysia (HUKM) and two ATCC standard strains were used in this experiment as listed in Table 1. All of these isolates have been previously identified to show active efflux activity and possesses two efflux genes (*norA* and *mdeA*) except for ATCC 25923 which did not harbour *norA* gene [23]. Isolates were maintained on Protect Bacterial Preservers (Technical Service Consultants Limited, Heywood, Lancashire, England) and cultured in tryptic soy agar (TSA) at 4^{o}C. Prior use, isolates were sub-cultured overnight at 35^{o}C in Mueller-Hinton broth (MHB), adjusted to obtain a turbidity comparable to that of McFarland standard tube No. 0.5. Detailed identification, characterization, antibiogram and active efflux properties of the *S.aureus* strains used in this study were as reported in previous publications [22,23].

b. Minimum inhibitory concentration (MIC) value determination assay

MIC assay were carried as described previously [23]. All compounds were dissolved in dimethyl sulfoxide (DMSO) and MIC was determined in triplicate using double-broth microdillution method involving 96-well titre-plates. Additionally, the growth of the microorganisms was determined by adding 20 µL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) at 1 mg/mL prior further 20 minutes incubation. Clear-yellow wells indicated inhibition of the growth of organism, whilst dark-bluish colouredwells indicated absence of inhibition. The MIC was defined as the lowest concentration producing no visible growth.

c. ***Efflux pump inhibitor (EPI) evaluation assay***

The EPI assay was done as described [23] with the following modifications; upon completion of ethidium bromide (EtBr) serial dilutions, each test compound was added into the mixture at concentration of at least one fourth (1/4) of their respective MIC result or at 50 µg/mL (for compounds with MIC values of > 250 µg/mL). Beside enabling comparison to reserpine's EPI, 50 µg/mL concentration was also used to ensure that any decrease in MIC value noted was due to the efflux pump inactivation by the tested compound and not its' antibacterial properties. Potential EPIs were compounds that showed lower MIC value when added with EtBr than the MIC value of EtBr alone.

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