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Antimicrobial Activity of Methyl Gallate isolated from the Leaves of *Glochidion superbum* Against Hospital Isolates of Methicillin Resistant *Staphylococcus aureus*

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Abstract – An antimicrobial compound has been isolated from the leaves of *Glochidion superbum.* The compound was determined as methyl 3, 4, 5-trihydroxybenzoate (methyl gallate), based on ultraviolet (UV), infrared (IR), nuclear magnetic resonance (NMR) and mass spectroscopy (MS) analysis. The isolated compound exhibited potent antimicrobial activity against three clinical isolates of methicillin resistant *Staphylococcus aureus* (MRSA) by qualitative agar disc diffusion method and quantitative broth dilution method. Agar disc diffusion was done in a dose-dependent manner for each bacterial isolate at disc potencies of 25, 50, 100, and 150 μg/disc. The zones of inhibition were on average equal to 12.27, 14.20, 15.43, and 24.17 mm respectively. The inhibition zones were compared with that of vancomycin disc at 30 μg as a reference standard. The MIC and MBC values were 50 μg/mL and 100 μg/mL respectively. The results of anti MRSA activity were analyzed using one-way ANOVA with Turkey's HSD and Duncan test. In conclusion, methyl gallate which was isolated from *G. superbum* showed the inhibition activity against methicillin resistant *S. aureus*.
Keywords – *Glochidion superbum*, antimicrobial activity, isolation, methyl gallate

Introduction

Glochidion superbum is one of 250 species of sub-canopy trees in the family *Euphorbiaceae*.[1] Geographical distribution of this plant includes the Pacific Islands, tropical Asia and Malaysia.[2] Various parts of the tree, especially the leaves were in use for medicinal purposes, e.g. to treat skin infections.[3] The literature search revealed that members of the genus *Glochidion* were known to contain many bioactive compounds, mainly terpenoids, flavonoids, phenolic acids, alkaloids and to a lesser extent cyanogenic glycosides and glucosinolates.[4] But reported studies on *Glochidion superbum* are more limited than those on other species. Methyl gallate is widely found in many families and species of plants especially in the Acaraceae family.[5]

The compound also serves as a major component of *Galla rhois* that shows high antimicrobial activity.[6] It is also present in the methanolic leave extract of the Chinese plant, *Toona sinensis.*[7] Previous research revealed that methyl gallate isolated from *Rhus glabra* possessed anti-MRSA activity.[8] In addition, methyl gallate recently isolated from *Toona sureni* (Blume) also possessed antimicrobial activity.[9]

In this work, we report the identification and structural elucidation of the phenolic acid methyl gallate, isolated from the leave extract of *Glochidion superbum* and its antimicrobial activity against MRSA in-vitro, by agar disc diffusion and broth dilution methods.

Experimental

General experimental procedures – NMR spectra were recorded on BRUKER 400 MHz NMR spectrophotometer at the SIRIM Ber had, National Metrology Laboratory, Selangor, Malaysia. ATR-IR spectra were recorded on a Perkin Elmer infrared spectrophotometer. LTQ-FT mass spectra were obtained by Thermo LTQ-FT

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mass spectrometer at the National University Singapore. Ultraviolet spectrum was recorded on a ShimadzuUV-1800 UV/VIS spectrophotometer. The mobile phase used for column chromatography was methanol (MeOH) and ethyl acetate (EtOAc), 70 - 230 mesh ASTM silica gel. TLC was prepared using Merck precoated silica gel plates. Acetone and chloroform were from Merck Germany. Bacterial growth media and reagents that were used throughout the project were Mueller-Hinton agar (MHA), Mueller-Hinton broth (MHB), and Mannitol Salt agar (MSA) were purchased from Oxoid (Engl and, UK). and Columbia blood agar base.

Plant materials – Plant materials were collected from Kuantan, Pahang in East Malaysia during November 2013, and identified at the Institute of Bioscience, University Putra Malaysia by Dr. Shamsul Khamis. A voucher specimen (MT-1301) of the plant was deposited at the Kulliyyah of Pharmacy, International Islamic University, Kuantan Campus, Malaysia.

Bacterial isolates – The isolates were initially collected from clinical samples processed at the diagnostic microbiology laboratory in Hospital Tungku Ampuan Afzan (HTAA), Kuantan, Pahang, Malaysia. Samples were molecularly and phenotypically characterized as MRSA at the Microbiology Laboratory, Basic Medical Sciences Department, Faculty of Medicine, IIUM, Kuantan Campus. All samples were labeled according to collection number and stored in cryobeads at –40 ºC for further use. Three MRSA isolates were randomly selected from a large number of stored MRSA on cryobeads designated as blood (B450), pus (P5116) and tissue (T175). All selected samples were subcultured on sheep blood agar before being used for antimicrobial activity assays.

Extraction and isolation of plant products – Dried and powdered leaves (270 g) of *Glochidion superbum* was extracted by maceration using methanol as solvent. The concentrated methanol extract (48 g) was then successfully partitioned by ethy lacetate: methanol (1:1). The ethy lacetate-methanol fraction was subjected to column chromatography (CC) for fractionation over 120 g packed silica gel (70 - 230 mesh) with a mobile phase of ethylacetate: methanol (100 ml for each) of 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, 10:90 and 0:100 (v/v). The fractions with the same TLC retention factor (R_f) were combined and re-chromatographed. It was further purified and crystalized in 100% acetone to give an off white powder (56.1 mg).

Methyl gallate was characterized as follows: ^{1}H NMR (Acetone-D_6, 400 MHz), δ 3.80 (3H, s, OCH_3), δ 7.12 (2H, s, H-2 and H-6); ^{13}C NMR (Acetone-D_6, 100 MHz), δ 51.2 (OCH_3) δ 109.1 (C-2 and C-6), δ 129.79 (C-4), δ 138.79 (C-1), δ 145.4 (C-3 and C-5), δ 166.5 (C=O); ESI-MS m/z : 184 $[M]^+$.

Antimicrobial sensitivity test – Agar disc diffusion test was carried out according to previously described procedure[10], in line with the National Committee on Clinical Laboratory Standard (NCCLS) regulations. The test compound was used at 25 μg, 50 μg, 100 μg and 150 μg/disc concentrations to test for activity against MRSA. The discs were evenly distributed on the agar surface 2 cm apart. While, 30 μg vancomycin disc was used as a positive control. The prepared plates were incubated for 18 - 24 hours at 37 ºC.[10] Antimicrobial activity was determined by measuring the diameter of inhibition zone in mm using transparent meter ruler.

Minimum inhibitory concentration (MIC) – The MIC was determined by microbroth dilution method according to Raeisi *et al*.[11] with slight modification using sterile 96-well microtiter plate. The plate was partitioned into three parts for the three MRSA isolates (MRSAI) from pus, blood, and tissue samples. Selected wells were filled with 100 μL of Mueller-Hinton broth (MHB). Subsequently, 100 μL of methyl gallate solution was added to the first well containing MHB. Subsequently, serial doubling dilution was done to create a concentration of 100, 50, 25, 12.5, 6.25 and 3.125 μg/mL.100 μL of the mixture was discharged from the last well. The total volume of each well was 100 μL. 50 μL of inoculum adjusted to 0.5 McFarland standard was introduced into each well. The microtiter plates were shaken for 1 min prior to incubation for 24 hours at 37 ºC. The MIC was determined as the minimum concentration of test compound that inhibits the bacterial growth (showing no visible turbidity).

Minimum bactericidal concentration (MBC) – The MBC was performed according to the method suggested by NCCLS. All wells that showed no visible growth, were sub-cultured on MHA, followed by incubation at 37 ºC for 18 - 24 hours. The lowest concentration of the sample compound at which inoculated bacteria were unable to grow was considered as MBC.

Statistical analysis – SPSS software (version 21, IBM) for windows was used to run statistical analysis. One-way analysis of variance (ANOVA) alongside Turkey's honestly significant difference (HSD) and Duncan's multiple range test (MRT) were applied to multiple comparisons of the means of the susceptibility of MRSA strains to methyl gallate. Statistical significance was expressed as ($p < 0.05$ and $p < 0.01$). All results were expressed as mean ± standard deviation (SD) of the mean of triplicate values

Table 1. Zones of inhibition of *methyl gallate* against three Hospital isolates of MRSA

MRSA Isolates	25 µg/mL	50 µg/mL	100 µg/mL	150 µg/mL	Vanco-30 µg
P5116	12.27 ± 0.25	14.20 ± 0.10^{b}	15.43 ± 0.12^{a}	24.17 ± 0.15^{b}	18.30 ± 0.10^{a}
B460 isolate	12.63 ± 0.15	14.77 ± 0.15^{c}	17.50 ± 0.30^{b}	24.63 ± 0.15^{c}	18.53 ± 0.15^{a}
T175	12.20 ± 0.10	13.47 ± 0.15^{a}	15.63 ± 0.15^{a}	20.60 ± 0.20^{a}	18.37 ± 0.15^{a}

^{a,b,c}value of the various samples with different superscript letter (a, b, c etc.) in the same column are significantly different ($p < 0.01$) as measured by Turkey's hsd and Duncan test. ***Key***: **MRSA**-methicillin resistant *S. aureus*, **B**-Blood, **P**-Pus, **T**-Tissue.

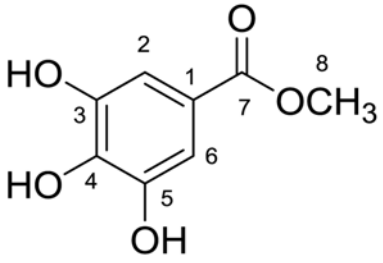

Fig. 1. The structure of methyl 3, 4, 5 trihydroxybenzoate (methyl gallate) isolated from *G. superbum*.

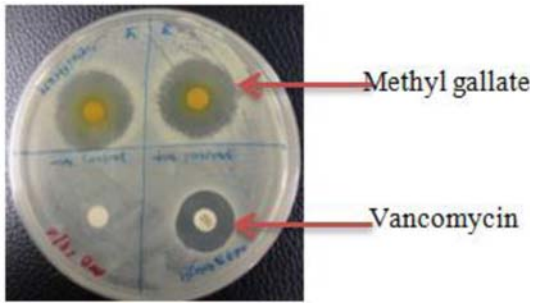

Fig. 2. Zone of inhibition of methyl gallate against the hospital isolates of MRSA. Zone of inhibition of methyl gallate against a hospital isolate of MRSA at a concentration of 150 µg/disc and Vancomycin zone of inhibition at 30 µg/disc, on Mueller-Hinton agar plate.

for the tested pure isolate.

Result and Discussion

The ^{1}H NMR spectrum showed the presence of a methoxyl group by a singlet signal δ 3.80. The aromatic proton signal appeared as a singlet signal at δ 7.12 with two integrations, indicating that the two aromatic protons were present within the molecule. Hydroxyl proton appeared as a broad signal at δ 8.21. ^{13}C NMR spectrum displayed the existence of 6 carbon signals, where two of them are at same chemical environment (overlapped) indicated by high peak integration at δ 109.1 and 145.4.

The DEPT 45 showed 2 signals, carbon at δ 51.2 and 109.1. DEPT 90 showed one signal at δ 109.1 for methine proton. DEPT 135 showed two signals at δ 51.2 and 109.1. The signals were assigned with the aid of correlation spectroscopy (COSY experiment), which was a homonuclear 2D technique that explains which pairs of ^{1}H nuclei in a molecule were coupled to one another. ^{1}H-^{1}H COSY spectrum of methyl gallate shown no correlation, indicated that there was no neighboring proton adjacent. Heteronuclear multiple quantum coherence or the 2D HQMC NMR spectrum were conducted to determine which hydrogens were connected to which carbons. The spectrum showed that the proton signals at δ 3.80 correlated with the corresponding carbon at δ 51.2. Meanwhile the signals at δ 7.12 correlated with signal at δ 109.1. HMBC spectrum of methyl gallate showed long range coupling of proton with carbon in the molecule. The proton signal at δ 7.12 was coupled with the carbon at δ 109.1. A proton signal at δ 3.8 was coupled with carbon signal at δ 166.5. ESI-MS yielded a nominal mass of *m/z* 184.2 in positive ionization mode which suggested the molecular formula of $C_8H_8O_5$. The structural formula is shown in Fig. 1., This finding was in line with previously reviewed work on isolation of methyl gallate.[12-13]

Table 1 depicts the effect of methyl gallate on MRSA growth in a dose-dependent manner presented as the diameter of inhibition zones to the nearest millimeter against the standard positive control drug used i.e. vancomycin. Purified methyl gallate at a concentration 150 µg/disc gave a larger bacterial growth inhibition zone as compared to vancomycinin (Fig. 2). Dose-dependent activity was demonstrated for each of the tested bacterial isolates. But at 25 µg/disc concentration, methyl gallate exerted more than 60% of vancomycin activity at 30 µg/disc. MIC and MBC values of methyl gallate were 50 and 100 µg/mL respectively as shown in Tables 2 and 3. This result is in line with a previous study on antimicrobial activity of methyl gallate isolated from *Toona sureni*[9] and *Anacardium accidentale L.*[14]

In conclusion, methyl 3,4,5-trihydroxybenzoate (methyl gallate) has been isolated from the leaves of *Glochidium superbum,* This is the first report giving detailed description of the structural properties and antimicrobial activity of this chemical constituent from this plant. The

Table 2. Minimum Inhibitory Concentration of methyl gallate against MRSA Isolates

Test MRSA	Concentrations in μg/mL						Controls	
	100	50	25	12.5	6.25	3.125	MHB	MHB+Bact.
P5116	*	*	–	–	–	–	–	–
B460	*	–	–	–	–	–	–	–
T175	*	–	–	–	–	–	–	–

*clear wells, indicate growth inhibition (MIC)at 100 μg/mL for blood and tissue isolates and 50 μg/mL for pus isolate of MRSA.
¯turbid wells, indicate that no growth inhibition is observed.

Table 3. Minimum bactericidal concentration of methyl gallate against MRSA Isolates

Test MRSA	Concentrations in μg/mL					
	100	50	25	12.5	6.25	3.125
P5116	*	*	n/t	n/t	n/t	n/t
B460	*	n/t	n/t	n/t	n/t	n/t
T175	*	n/t	n/t	n/t	n/t	n/t

*= no bacterial growth on agar plates, n/t= not tested.

results presented here for the anti-MRSA activity of methyl gallate provide supportive ground for the use of leaves of this plant as antimicrobial preparation for traditional medicinal purposes. Further study is needed to test the activity of methyl gallate, on other resistant forms of bacteria such as those producing extended spectrum beta lactamases and carbapenemases and to check for the presence of methyl gallatein extracts from other parts of this plant.

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