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Contributed Paper

Potent Quorum Sensing Inhibition by Methyl Gallate Isolated from Leaves of *Anacardium occidentale* L. (cashew)

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ABSTRACT

Our earlier work has shown that the methanol leaf extract of *Anacardium occidentale* (cashew) displayed antibacterial activity. In this study, the leaf extract was assessed for quorum sensing (QS) inhibition using the disc-diffusion assay with *Chromobacterium violaceum* (wild-type ATCC 12472) as the test organism. The extract exhibited strong anti-QS activity based on the diameter of inhibition zone (DIZ) and minimum inhibition dose (MID) of violacein production and *C. violaceum* growth. From the extract, methyl gallate (methyl ester of gallic acid) was isolated by column chromatography, identified by NMR and MS analyses, and quantified using reversed-phase HPLC. Using the disc-diffusion assay and broth dilution method, methyl gallate (MG) at 500 μg/disc, inhibited violacein production with DIZ of 14.5 ± 0.1 mm, MID of 6 μg/disc, and minimum inhibition concentration of 100 μg/ml. The closely related gallic acid did not display any anti-QS activity suggesting that the methyl moiety in MG may be responsible for QS inhibition. The content of MG was quantified as 1830 ± 180 μg/g of leaves. This is the first report on the anti-QS activity of cashew leaf extract, and on the systematic isolation and quantification of MG, a potent QS inhibitor.

Keywords: cashew, methyl gallate, anti-quorum sensing, *Chromobacterium violaceum*

1. INTRODUCTION

Anacardium occidentale L. (cashew in English, himmaphan in Thai and gajus in Malay), of the family Anacardiaceae, is a small-sized tree with low and spreading branches, and greyish brown bark with longitudinal fissures. Leaves are simple, alternate, narrowly to broadly obovate with a rounded apex. In Southeast Asian countries of Malaysia, Indonesia and Thailand, the pliable and reddish leaf shoots of the culinary herb are consumed raw as *ulam*. Cashew leaves have been used in folk medicine to treat rheumatic disorders, hypertension, dysentery, diarrhoea and piles. Phenolic content and antioxidant activity of cashew leaves were outstanding, surpassing those of temperate culinary herbs such as rosemary, thyme and marjoram [1]. Antityrosinase activity of cashew leaves was comparable to *Psidium guajava* and *Hibiscus tiliaceus*, which have strong skin-whitening properties.

Quorum sensing (QS) is a mechanism used by some bacteria to regulate gene expression in response to fluctuations in cell density [2]. QS bacteria produce and release chemical signal molecules or auto-inducers that increase in concentration as cell density increases. In general, Gram-negative bacteria use acylated homoserine lactones as auto-inducers while Gram-positive bacteria use processed oligo-peptides to communicate. *Chromobacterium violaceum* (CV) is a Gram-negative bacterial species that synthesises a purple pigment called violacein from L-tryptophan and oxygen [3]. Production of violacein is induced by N-acyl homoserine lactone (AHL).

Because of the importance of QS in bacterial pathogenesis, studies have focused on QS inhibition, which inactivates AHL molecule biosynthesis, degrades AHL molecules by bacterial lactonase and blocks the activation of AHL receptor protein [4]. It has been reported that QS inhibition of a bacterial pathogen can result in a significant decrease in its virulence. Currently, there is an active interest in developing new therapeutic remedies for pathogenic bacteria. In particular, the alternative modes of action against opportunistic bacteria that use QS to express virulence have received special attention.

Using the disc-diffusion technique, our earlier work has shown that the leaf extract of cashew inhibited both Gram-positive bacteria of *Brevibacillus brevis*, *Micrococcus luteus* and *Staphylococcus cohnii*, and Gram-negative bacteria of *Escherichia coli*, *Pseudomonas aeruginosa* and *Salmonella enterica* with minimum inhibition doses ranging from 130−500 μg/disc [1]. In this study, screening of the leaf extract of cashew showed strong QS inhibition. A compound, isolated by column chromatography, identified by NMR and MS analyses, and quantified using reversed-phase HPLC, was found to be a potent QS inhibitor.

2. MATERIALS AND METHODS

2.1 Extraction of Plant Materials

Fresh leaves of cashew were purchased from the Chow Kit market in Kuala Lumpur. The leaves (10 g) were powdered with liquid nitrogen in a mortar and extracted with 100 ml of MeOH, three times for 1 h each time. After swirling continuously at 120 rpm in an orbital shaker, the extracts were filtered under suction and stored at 4°C for further analysis.

2.2 QS Inhibition of Leaf Extract

QS inhibition of the MeOH leaf extract of cashew was assessed using the disc-diffusion assay [5]. CV (wild-type ATCC 12472) grown in LB broth (OD_{720} 1.00 A) was inoculated onto 75% semi-solid LB agar plates. Paper discs (6 mm diameter) impregnated with 2000 μg/disc of leaf extracts were transferred onto the inoculated agar. After incubation overnight at 28°C, the plates were observed and measured for the diameter of inhibition zone (DIZ) in mm of violacein production and CV growth. The inhibition zone of violacein production was visible as an opaque halo around the impregnated disc against a purple violacein background, which indicated uninhibited growth of bacterial cells but an absence of violacein pigment. The inhibition zone of CV growth appeared as a clear halo around the impregnated disc. The amount used to impregnate per disc was then reduced two-fold sequentially until no inhibition zone was visible. The least amount of extract per disc required (μg/disc) was recorded as the minimum inhibition dose (MID) [6] of violacein production and CV growth.

2.3 Isolation and Identification of Compound

The MeOH cashew leaf extract (40 g) was fractionated with 300 g of MCI gel CHP 20P, using a water:MeOH; 0−100% step-gradient,

into 19 fractions designated as Fractions 1−19. Fraction 7 was found to have the highest anti-QS activity. A compound (0.182 mg), the major constituent of Fraction 7 (0.5 g), was isolated with 20 g Sephadex LH-20 (H_2O:MeOH; 0−100%) followed by 20 g silica gel 60 ($CHCl_3$:MeOH:H_2O; 7:3:0.5−8:2:0.7), 20 g MCI gel CHP 20P (H_2O:MeOH; 0−45%) and 10 g C-18 (H_2O:MeOH; 0−85%).

The compound was dissolved in deuterated MeOH (CD_3OD), and subjected to ^1H and ^{13}C NMR analyses using a Varian Unity Inova 500 MHz spectrometer (500 MHz for ^1H and 125.7 MHz for ^{13}C). Using tetramethylsilane as the internal standard, their chemical shifts were recorded in ppm (δ).

The compound was further analysed with ESI-MS using a Perkin Elmer Flexar SQ 300 quadrupole mass spectrometer to determine its molecular weight. Sample components were separated with a C-18 column (2.1 mm x 150 mm x 3.5 μm) at 40°C. Sample elution was conducted using a 10-min linear gradient from 10−100% MeOH acidified with 1% trifluoroacetic acid at 0.2 ml/min. The ESI-MS mass spectrum was acquired in negative ion mode with mass ranging up to 3000 m/z measured.

2.4 QS Inhibition and Quantification of Compound

The compound was assayed for QS inhibition using the broth dilution method to determine the minimum inhibition concentration (MIC) of violacein production [5]. Different amounts were dissolved in 1 ml of MeOH and transferred into sterile Petri dishes and allowed to dry inside a biosafety cabinet. Subsequently, 5 ml of fresh LB broth inoculated with CV (OD_{720} 0.10 A, which corresponded to 1.50 x 10^7 cfu/ml) was aseptically transferred into the Petri dishes. Inoculated Petri dishes were incubated overnight at 28°C before the violacein concentration and CV cell density were measured at OD_{577} and OD_{720}, respectively, using a Secoman UniLive 9400 UV-vis spectrophotometer. Violacein production represented in terms of violacein units was calculated as the ratio of OD_{577} against OD_{720}. The concentration of the compound (starting from 100 μg/ml) was reduced by two-fold serial dilution until the MIC was reached.

To quantify the content of compound, fresh cashew leaves (1 g) were ground in a mortar using liquid nitrogen and extracted using 50 ml of 70% aqueous MeOH with continuous shaking 120 rpm for 1 h at room temperature. The extract was filtered and stored at 4°C for further analysis.

The extract was subjected to reversed-phase HPLC analysis. A 30-min linear gradient ranging from 10−100% MeOH was used to resolve the peaks. A smaller injection volume of 5 μl was found to improve resolution. For quantification, calibration curves were constructed using standard compounds as described for phenolic acids in fruit extracts [7]. In this study, the compound was relatively pure for use as the standard in constructing the calibration curve for quantification.

3. RESULTS AND DISCUSSION

3.1 QS Inhibition of Leaf Extracts

The cashew leaf extract at 2000 μg/disc exhibited strong anti-QS activity. The DIZ of violacein production and CV growth were 22 ± 0.6 mm and 15 ± 0.6 mm, respectively. The MID was 13 μg/disc for violacein production and 250 μg/disc for CV growth.

3.2 Identification of Methyl Gallate

The compound isolated from the MeOH leaf extract of cashew was identified as methyl gallate (IUPAC name: methyl 3,4,5-trihydroxybenzoate), a methyl ester of gallic acid. Its appearance with ESI-MS, and ^1H and ^{13}C NMR spectral data are as follows:

Table 1. ^{1}H and ^{13}C NMR spectral data of methyl gallate.

Atom	^{13}C	^{1}H	^{13}C	^{1}H
	PSD		[Hwang et al., 2005]	
1	120.0		120.5	
2	108.6	7.04	109.1	7.07
3	145.1		145.5	
4	138.3		138.7	
5	145.1		145.5	
6	108.6	7.04	109.1	7.07
C=O	167.6		168.0	
$COOCH_3$	50.9	3.83	51.3	3.82

Values of ^{1}H (500 MHz) and ^{13}C (125.7 MHz) NMR data are in ppm (δ). Spectra from the present study data (PSD) matched those of MG isolated from leaves of *Cedrela sinensis* [8].

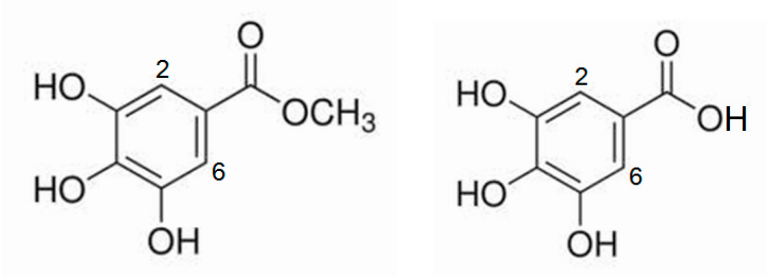

Figure 1. Molecular structures of methyl gallate with quorum sensing inhibition (left) and gallic acid without such activity (right).

White crystalline powder; ESI-MS m/z 182.9 $[M-H]^{-}$; ^{1}H NMR (300 MHz, CD_3OD) δ: 7.04 (H-2, H-6, *s*, 2H); 3.83 (OCH_3, *s*, 3H); ^{13}C NMR (125.7 MHz, CD_3OD) δ; 167.6 (C=O); 145.1 (C-3, C-5), 138.3 (C-4), 120.0 (C-1), 108.6 (C-2, C-6), 50.9 (OCH_3).

The presence of only one 2H aromatic signal indicated that the phenolic compound should be symmetrical and have four constituent groups. Furthermore, the compound has to be a relatively small molecule because the compound had only six carbon signals. The chemical shifts of ^{1}H and ^{13}C NMR spectra of MG obtained in this study (Table 1) matched the data of a previous report on MG isolated from leaves of *Cedrela sinensis* [8]. MG with a molecular weight of 182.9 and molecular formula of $C_8H_8O_5$ has three hydroxyl groups (-OH) attached to carbons 3, 4 and 5. The -OH groups, oriented in the same direction around the benzene ring, function as hydrogen-bond donors and acceptors. A methyl carboxylic acid is attached to carbon 1 (Figure 1).

3.3 QS Inhibition and Content of MG

The QS inhibition of MG was tested using the disc-diffusion assay and the broth dilution method. A lower dose of 500 μg/disc was used as the compound showed a large CV growth inhibition zone at 2000 μg/disc. The violacein production DIZ and MID of MG were 14.5 ± 0.1 mm and 6 μg/disc, respectively. As the

Table 2. Inhibition of different concentrations of methyl gallate from *Anacardium occidentale* against *Chromobacterium violaceum* growth and violacein production based on optical density (OD).

Concentration of MG (μg/ml)	OD_{577} (violacein concentration)	OD_{720} (CV cell density)	Violacein unit (OD_{577}/OD_{720})
100	2.87 ± 0.14	2.14 ± 0.13	1.33 ± 0.01^{c}
50	3.67 ± 0.11	1.86 ± 0.08	1.97 ± 0.03^{b}
25	3.13 ± 0.19	1.68 ± 0.07	1.86 ± 0.18^{b}
13	4.00 ± 0.12	1.97 ± 0.07	2.03 ± 0.01^{a}
6	4.42 ± 0.14	2.24 ± 0.04	1.97 ± 0.03^{b}
Control	4.55 ± 0.20	2.31 ± 0.16	1.97 ± 0.05^{b}

Values are means ± standard deviations (n = 3). Violacein concentration and *Chromobacterium violaceum* (CV) cell density were measured at OD_{577} and OD_{720}, respectively. Violacein production, represented in terms of violacein units, was calculated as the ratio of OD_{577} against OD_{720}. The viable cell count for the control was 2.79 x 10^9 cfu/ml. Within the column, values with different superscripts (a–c) are significantly different at $p < 0.05$, as measured by the Tukey HSD test. By comparing the violacein units of the treatments with the control, the violacein production minimum inhibition concentration (MIC) of methyl gallate (MG) was 100 μg/ml.

violacein unit of 1.33 ± 0.01 was significantly lower at $p < 0.05$ than the control of 1.97 ± 0.05, the violacein production MIC was found to be 100 μg/ml of MG (Table 2). The closely related gallic acid (Figure 1), also tested for anti-QS activity using the disc-diffusion assay and the broth dilution method, did not display any activity, suggesting that the methyl moiety of MG may be responsible for QS inhibition.

The content of MG present in cashew leaves was quantified using reversed-phase HPLC. The compound, eluted at 8.2 min using a 30-min gradient (Figure 2), was relatively pure and was used as the standard to construct a calibration curve for quantification. The calibration equation of peak area (mAU*s) against concentration of MG (μg/l) was y = 12206x (R^2 = 0.9987). From the standard curve, the content of MG was found to be 1830 ± 180 μg/g of leaves.

From the literature, MG has been reported in 14 genera of plants [9]. It is a potent compound with an array of biological activities including antioxidant, antityrosinase and antibacterial properties [10]. It is an active ingredient of many pharmaceutical preparations used for treating enteritis, dysentery and itching. Known to be a major antioxidant, MG has been reported to show pronounced free radical scavenging and reducing power [9]. The effect was however lower than gallic acid, possibly due to substitution of the -OH group with the $-CH_3$ group. The radical scavenging activity of MG

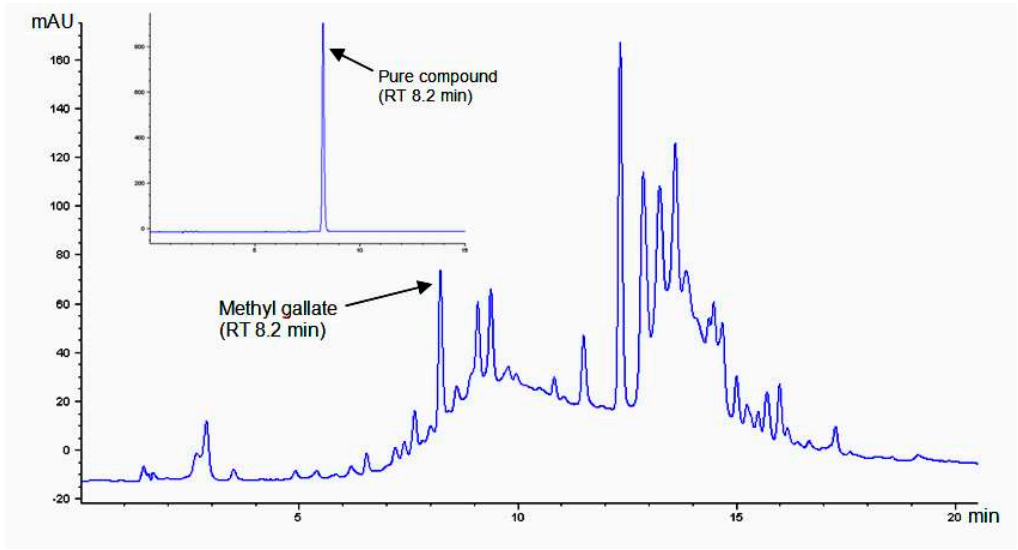

Figure 2. Chromatograms of the pure compound (methyl gallate) and MeOH cashew leaf extract detected at 280 nm.

has been reported to be stronger than BHA and α-tocopherol [8]. Isolated from leaves of *C. sinensis*, MG had stronger tyrosinase inhibition than α-tocopherol and arbutin.

This is the first report on the anti-QS properties of MG against CV. Other compounds with QS inhibition included epigallocatechin gallate, ellagic acid and tannic acid against *Burkholderia cenocepacia* and *P. aeruginosa* [11]. It was postulated that the galloyl moieties in these compounds were responsible for the QS inhibition. A study on the QS inhibition of cinnamaldehyde analogs against *Vibrio* species has been studied [12]. Cinnamaldehyde analogs, like MG, are small molecules with a multi-substituted moiety. The shape and charge of substituent groups determined their effectiveness in QS inhibition.

4. Conclusions

The QS inhibition of MeOH cashew leaf extract has been reported for the first time. Systematically isolated from cashew leaves, MG possessed potent anti-QS properties. The closely related gallic acid, however, did not display any anti-QS activity, suggesting that the methyl moiety in MG may be responsible for QS inhibition. Unlike polyphenols, which are more complex in structure, MG is a phenolic compound with a simple structure. Its anti-QS properties, toxicity and potential to be developed into an anti-pathogenic drug warrant further research.

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