

ANTI-QUORUM SENSING AND ANTIBIOTIC ENHANCEMENT OF ALLYLPYROCATECHOL AND METHYL GALLATE

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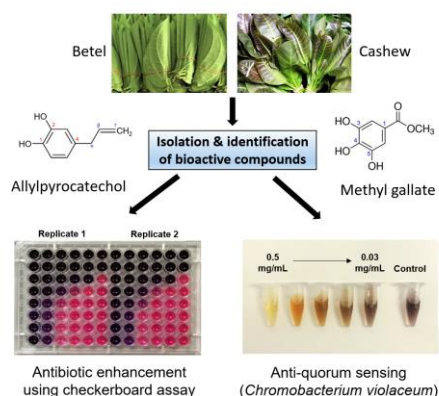
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Graphical abstract



- Allylpyrocatechol (APC) and methyl gallate (MG) were isolated from betel and cashew leaves.
- The antibiotic enhancement effect of APC was additive while that of MG was synergistic.
- Both compounds were equally effective with violacein inhibition observed at 60 µg/mL.
- The purity of APC in the methanol betel leaf extract was increased from 13.2% to 31.5% w/w through solvent partitioning.
- This study is the first to document the anti-QS activity of APC, which is comparable to that of MG.

Abstract

In this study, the antibiotic enhancement and quorum sensing (QS) inhibition of allylpyrocatechol (APC) and methyl gallate (MG) isolated from leaves of *Piper betle* (betel) and leaf shoots of *Anacardium occidentale* (cashew), respectively, were compared. When applied with streptomycin and tested against *Staphylococcus aureus* and *Pseudomonas aeruginosa*, the effect of APC was additive while that of MG was synergistic. In terms of QS inhibition against *Chromobacterium violaceum*, both compounds were equally effective with violacein inhibition observed at concentrations of 60 µg/mL. We successfully increased the purity of APC in the methanol betel leaf extract from 13.2% to 31.5% w/w through solvent partitioning. This study is the first to document the anti-QS activity of APC, which is comparable to that of MG.

Keywords: Allylpyrocatechol, *Piper betle*, methyl gallate, *Anacardium occidentale*, antibiotic enhancement, quorum sensing inhibition

Abstrak

Dalam kajian ini, peningkatan antibiotik dan perencatan penghindaran kuorum (QS) terhadap allylpyrocatechol (APC) dan metil gallate (MG) yang diasingkan dari daun sirih dan gajus dibandingkan. Apabila digunakan dengan streptomisin dan diuji terhadap *Staphylococcus aureus* dan *Pseudomonas aeruginosa*, kesan APC adalah adiitif sementara MG bersifat sinergistik. Dari segi perencatan QS terhadap *Chromobacterium violaceum*, kedua sebatian tersebut sama-sama berkesan dengan perencatan violacein yang diperhatikan pada kepekatan 60 µg/mL. Kami berjaya meningkatkan ketulenan APC dalam ekstrak daun sirih metanol dari 13.2% kepada 31.5% w/w melalui pembahagian pelarut. Kajian ini adalah yang pertama untuk mendokumentasikan aktiviti anti-QS APC, yang setanding dengan MG.

Kata kunci: Allylpyrocatechol, *Piper betle*, metil gallate, *Anacardium occidentale*, peningkatan antibiotik, perencatan penginderaan kuorum

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1.0 INTRODUCTION

Piper betle L. (betel) is a woody vine of the family Piperaceae [1]. The heart-shaped leaves of *P. betle* are best known as a component of betel quid consisting of slices of areca nut wrapped in betel leaves with a spread over of slake lime [2]. Chewing of betel quid may be addictive but is supposed to ameliorate bad breath, harden gums, and prevent indigestion, bronchitis, constipation, congestion, cough and asthma. Pharmacological properties of *P. betle* leaves include antioxidant, antibacterial, anti-quorum sensing (anti-QS), tyrosinase inhibition, anti-malarial, anti-diabetic, anti-ulcerogenic anti-inflammatory, hepatoprotective, neuroprotective and cytotoxic activities [2].

Anacardium occidentale L. (cashew) of the family Anacardiaceae [1]. In Southeast Asian countries of Malaysia, Indonesia and Thailand, the pliable and reddish leaf shoots of *A. occidentale* are consumed raw or blanched as ulam or traditional salad [3]. Served with hot and spicy sauce, ulam functions as an appetizer and has health-promoting properties such as antioxidant, antibacterial, antifungal, antiviral, antityrosinase, anti-ulcerogenic, analgesic, anti-inflammatory and anti-QS activities [3].

This study isolated allylpyrocatechol (APC) from leaves of *P. betle* and methyl gallate (MG) from leaf shoots of *A. occidentale*. Antibiotic enhancement and anti-QS properties of these two phenolic compounds were then compared. Finally, solvent partitioning was examined as a way of enriching the content of APC from the methanol *P. betle* extract.

Antibiotic resistance especially among Gram-negative bacteria has become a growing problem in infections [4]. Consequently, the quest for promising phytochemicals with antibiotic enhancing properties is vital. Because of the importance of QS in bacterial pathogenesis, studies have focused on QS inhibition which can result in a significant decrease in the virulence of a bacterial pathogen [5].

2.0 METHODOLOGY

2.1 Extraction and Isolation

APC was isolated from leaves of *P. betle* (1 kg) purchased from a local market in Kuala Lumpur. Leaves were immersed in methanol (4.5 L) and repeatedly extracted three times for 24 h each time. The samples were then filtered through Whatman No. 1 filter paper and the solvent was removed using a rotary evaporator under pressure at 45°C. The extract was dissolved in minimum quantity of methanol together with same amount of packing material and oven-dried at 45°C for 3 h before loaded on the top of the packed column. All chromatographic packing materials were immersed in methanol at least 3 h before they were packed into their respective

columns and equilibrated with the first mobile phase. The crude extract (35 g) was fractionated with 300 g of Diaion® HP-20, using 700 mL H₂O:MeOH 0–100% step-gradient at 20% intervals and 100% acetone into seven fractions. Fractions 4 and 5 having the same bands from thin layer chromatography (TLC) were pooled into one fraction to designate as fractions 1–6. A bioactive compound (2 g), the major constituent of Fraction 4 (8 g), was further isolated with Sephadex LH-20 (320 mL of H₂O:MeOH; 20–100%) and MCI gel CHP20P (80 mL of H₂O:MeOH; 10–100%) using step-gradient at 10% intervals and collected at 15 mL and 1 mL each, respectively, followed by 30 g silica gel 60 (60 mL of CHCl₃:MeOH:H₂O; 10:0:0, 9:1:0.1 and 8:2:0.2). The eluting solvent was then removed using a rotary evaporator under pressure at 45°C. The same spots from TLC were pooled into one fraction and the recovery yield of fractions were determined. NMR spectra were acquired using Bruker DRX-300 in deuterated chloroform (300 MHz for ¹H and 75 MHz for ¹³C). ESI mass spectra were obtained using Agilent 1290 Infinity LC system coupled to Agilent 6520 Accurate-Mass Q-TOF mass spectrometer with dual ESI source.

MG was isolated from leaves of *A. occidentale*. The extraction and isolation of the compound were according to the procedures described in our previous study [6]. Briefly, the compound was dissolved in deuterated MeOH (CD₃OD), and subjected to ¹H and ¹³C NMR analyses using a Varian Unity Inova 500 MHz spectrometer (500 MHz for ¹H and 125.7 MHz for ¹³C). The 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.

2.2 Antibacterial Activity

The minimum inhibitory concentration (MIC) was determined using the serial dilution technique and 96-well microtiter plates [7]. The samples were dissolved in 100% methanol to a concentration of 25 mg/mL and diluted to 1 mg/mL with sterile water. Stock solution (100 µL) was added to the first well and serially diluted two-fold. Diluted bacterial cultures (90 µL) and 10 µL of resazurin indicator solution (10 mg/mL) were then added to each well. The stock solutions of the fractions were therefore subjected to final concentration of 0.5 mg/mL. The subsequent 1:2 dilution brought the final inoculum to 5 x 10⁷ CFU/mL [7]. Methanol (4%) was used as the control solvent. Mueller Hinton Broth (MHB) with inoculation was used as negative control and MHB alone was prepared as blank. Plates were inoculated within 20 min of standardizing the inoculum to maintain viable cell density. Each sample was prepared in triplicate and the plate was incubated at 37°C for 18 h under aerobic conditions. Any change in color from blue to pink was recorded as bacterial growth.

The checkerboard assay was used to study antimicrobial synergy [8]. Stock solutions and serial

two-fold dilutions of fraction and streptomycin to at least double the MIC were prepared. Streptomycin (50 μ L) was diluted along the row, while 150 μ L of sample was serially diluted along the column. Serially diluted samples (50 μ L) were then transferred into each well containing streptomycin. They were inoculated with 90 μ L of inoculum and 10 μ L of resazurin indicator solution (10 mg/mL). The plates were then incubated at 37°C for 18 h under aerobic conditions. Changes in colour from blue to pink were recorded as bacterial growth.

Antibiotic enhancement was determined by observing synergistic effects between sample and the antibiotic. Evaluation was based on the fractional inhibitory concentration (FIC) index, calculated as the MIC of streptomycin and sample combined divided by the MIC of streptomycin or sample alone. The formula used was: FIC index = antibiotic FIC (combined MIC/MIC of antibiotic) + sample FIC (combined MIC/MIC of sample). The antibacterial properties of sample were then classified according to its FIC i.e. synergistic ≤ 0.5 , additive > 0.5 to ≤ 1.0 , indifferent > 1.0 to ≤ 2.0 and antagonistic > 2.0 [9].

2.3 QS Inhibition

The violacein inhibition assay was carried out in accordance to earlier described procedures [6, 10]. Samples were dissolved in methanol to produce stock solution of 5 mg/mL. The stock solution (0.5 mL) was transferred into sterile petri dishes with 4.5 mL of nutrient broth inoculated with *Chromobacterium violaceum*. Optical density at 720 nm was 0.100 A, which corresponded to 1.5×10^7 CFU/mL. Transferring the stock solution would dilute the samples to a final concentration of 0.5 mg/mL. The petri dishes were incubated for 24 h at 26°C with gentle swirling at 100 rpm before cell density was measured at OD₇₂₀. A streak plate of each petri dish was done to ensure no contamination and that the optical density was a valid representation of cell density. To measure violacein production, 1 mL of broth culture were centrifuged at 13,000 rpm for 15 min to recover the *C. violaceum* cells. Violacein was then extracted using 1 mL of butanol and centrifuged again before absorbance was measured at 577 nm (OD₅₇₇) using a blank solution of butanol. Violacein unit (VU) was expressed as the ratio of A_{577}/A_{720} . Violacein reduction (%) was calculated as $100 - (VU_{\text{sample}}/VU_{\text{control}} \times 100)$. Lower ratio values indicate stronger QS inhibition.

2.4 Solvent Partitioning

Solvent partitioning of the crude extract of *P. betle* (20 g) was carried out using three different solvents (aqueous methanol, hexane and chloroform) with different polarity indices. To separate the polar compounds, the crude extract was dissolved in 400 mL of 90% v/v aqueous methanol and then in 400 mL of hexane using a separatory funnel. Hexane was

used to separate the non-polar compounds. The funnel was shaken for 1 min before being allowed to settle for 1 h at room temperature. The hexane fraction was filtered through Whatman No. 1 filter paper and the solvent was removed using a rotary evaporator under pressure at 45°C. The hexane extract was stored at 4°C for analysis. The lower phase which was aqueous methanol fraction was withdrawn and further partitioned using chloroform.

Chloroform and water were added into aqueous methanol fraction with 1:1:1 ratio v/v/v into a separatory funnel. The funnel was shaken for 1 min and allowed to settle for 1 h at room temperature. Both chloroform and aqueous methanol fractions were collected separately and filtered through Whatman No. 1 filter paper. The solvents were removed using a rotary evaporator under pressure at 45°C and stored at 4°C for further analysis.

2.5 Quantification of APC

Different solvent extracts and the pure compound were subjected to HPLC analysis. The extract was dissolved in 1 mL of methanol and filtered before analysis. The extract (20 μ L) was injected and a 40-min linear gradient from 10–100% methanol was used to elute the sample at 1 mL/min. Mobile phases were acidified with 0.1% acetic acid to prevent ionization of analytes. Elution was monitored at three different wavelengths of 254, 280 and 365 nm. A standard curve ranging from 0.125–1.000 mg/mL was constructed using APC from *P. betle*.

3.0 RESULTS AND DISCUSSION

3.1 Structural Elucidation of Compounds

APC (1,2-dihydroxy-4-allylbenzene) was obtained as a reddish-brown wax from *P. betle* leaves. Its identity was confirmed by analysing ¹H NMR and ¹³C NMR spectra, and by comparing with published spectral data [11, 12].

Its molecular formula C₉H₁₀O₂ was determined by ESI-MS spectrometry that exhibited a mass of 150 with a molecular ion peak [M+H]⁺ at 151. The molecule has two hydroxyl groups at C1 and C2 and an allyl group at C4 (Figure 1). APC or hydroxychavicol is a phenolic compound that has been reported to possess antimicrobial, antioxidant and anti-inflammatory properties [13]. Other activities include anti-cancer, gastroprotective, hepatoprotective, cardioprotective and radioprotective [14].

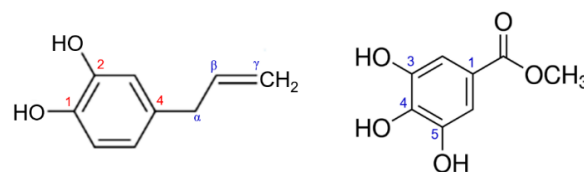


Figure 1 Chemical structures of allylpyrocatechol (left) and methyl gallate (right)

MG (methyl 3,4,5-trihydroxybenzoate) is a methyl ester of gallic acid. It was obtained as a white crystalline powder from *A. occidentale* leaves identified in our previous study [4]. MG with a molecular weight of 183 and molecular formula of $C_8H_8O_5$ has three –OH groups attached to C3, C4 and C5 of the phenyl ring (Figure 1). A methyl carboxylic acid is attached to C1. MG is a phenolic compound with biological activities such as antioxidant, anti-tyrosinase and antibacterial properties [15]. Isolated from cashew leaves, MG is a potent QS inhibitor of *C. violaceum* [4].

3.2 Antibacterial Activity

MIC values of APC and MG against six bacterial species ranged from 125–500 $\mu\text{g/mL}$. *Bacillus cereus* (125 $\mu\text{g/mL}$) was most susceptible to APC, followed by *Staphylococcus aureus*, *Micrococcus luteus* and *Escherichia coli* (250 $\mu\text{g/mL}$). The most resistant towards APC with high MIC value of 500 $\mu\text{g/mL}$ were *Pseudomonas aeruginosa* and *Salmonella enterica*. MG was most effective against *B. cereus* (125 $\mu\text{g/mL}$), and effective against *E. coli* and *S. enterica* (250 $\mu\text{g/mL}$). Despite the trend that most bioactive compounds from plants are more effective against Gram-positive bacteria [16], APC and MG also inhibited Gram-negative bacteria, notably, *E. coli* and *S. enterica*. This is an interesting finding, as these bacterial species are pathogenic and difficult to eradicate [17].

3.3 Antimicrobial Synergy

The FIC index of streptomycin combined with APC or MG ranged from 0.38–0.75 against *S. aureus* and *P. aeruginosa* (Table 1). A synergistic effect ($FI \leq 0.5$) was observed when MG was combined with streptomycin. Conversely, the effect of combining APC and streptomycin was only additive ($FI > 0.5$ to ≤ 1.0). Overall, both compounds induced an increase in the activity of streptomycin with reduction in MIC values by 2-fold to 8-fold. Noteworthy, no indifferent ($FI > 1.0$ to ≤ 2.0) nor antagonistic ($FI > 2.0$) interactions were observed in this study.

Table 1 Synergy between compounds and streptomycin against *S. aureus* and *P. aeruginosa*

Compd + STR	MIC alone ($\mu\text{g/mL}$)		MIC combined ($\mu\text{g/mL}$)		FIC
	Compd	STR	Compd	STR	
<i>Staphylococcus aureus</i>					
APC + STR	250	62.5	125	7.81	0.63 (A)
MG + STR	500	62.5	125	7.81	0.38 (S)
<i>Pseudomonas aeruginosa</i>					
APC + STR	500	125	125	62.5	0.75 (A)

Compd + STR	MIC alone ($\mu\text{g/mL}$)		MIC combined ($\mu\text{g/mL}$)		FIC
	Compd	STR	Compd	STR	
MG + STR	500	125	125	31.3	0.50 (S)

Abbreviations: APC = allylpyrocatechol, FIC = fractional inhibitory concentration, MG = methyl gallate, MIC = minimum inhibitory concentration and STR = streptomycin. FIC index was interpreted as S = synergistic at ≤ 0.5 and A = additive at > 0.5 to ≤ 1.0 .

A possible mode of synergistic action between compounds and antibiotics is by inducing alterations in cell membrane permeability and by inhibiting bacterial efflux pumps to increase susceptibility [18]. A study on the anticariogenic effects of the aqueous extract of *P. betle* towards *Streptococcus mutans* postulated that the methyl moiety of APC is responsible for the hydrophobic interaction with the lipids of the bacterial cell membrane, causing cell deformation [19]. When the bacterial cell membrane becomes more permeable, it is more susceptible to streptomycin which inhibits protein synthesis and eventually causing cell death. The synergistic interactions shown in this study suggest that both APC and MG could be useful in treating infections by drug-resistant bacteria.

3.4 QS Inhibition

Violacein production of *C. violaceum* in the presence of APC and MG at 10–500 $\mu\text{g/mL}$ after 24 h is shown in Table 2. Results showed that at 250 and 500 $\mu\text{g/mL}$, both compounds have a bacteriostatic effect on *C. violaceum* with very low values of cell density. Nevertheless, both compounds showed excellent anti-QS with inhibition of violacein production ($> 50\%$) at 60 $\mu\text{g/mL}$. Inhibition was 55.8% and 55.2% for APC and MG, respectively.

Table 2 Violacein production of *Chromobacterium violaceum* based on optical density at different concentrations of allylpyrocatechol (APC) and methyl gallate (MG)

Compd	Conc ($\mu\text{g/mL}$)	OD ₅₇₇	OD ₇₂₀	VU	VR (%)
APC	500	0.00	0.20	0.01	99.0
	250	0.00	0.10	0.04	94.8
	130	0.10	1.00	0.10	86.1
	60	0.44	1.38	0.32	55.8
	30	0.74	1.64	0.45	37.7
MG	10	0.82	1.66	0.62	14.7
	500	0.00	0.14	0.03	96.4
	250	0.00	0.18	0.02	96.7
	130	0.33	1.25	0.26	63.6
	60	0.48	1.47	0.33	55.2
30	0.66	1.54	0.43	40.5	

Compd	Conc (µg/mL)	OD ₅₇₇	OD ₇₂₀	VU	VR (%)
	10	0.92	1.63	0.56	22.5
Control		1.10	1.52	0.73	0.00

Abbreviation: OD = optical density at 577 and 720 nm, VU = violacein unit (OD₅₇₇/OD₇₂₀) and VR = violacein reduction. Lower ratio values indicate stronger QS inhibition.

Our study in 2015 was the first to report that MG from *A. occidentale* is a potent QS inhibitor [6]. They found that the closely related gallic acid did not display any anti-QS activity, suggesting that the methyl moiety in MG may be responsible for the QS inhibition. The strong anti-QS activity of MG may be attributed to the three hydroxyl groups and the methyl carboxylic acid attached to the phenyl ring as shown in Figure 1. Recently, it was reported that MG had stronger QS inhibition than phenolic compounds such as pyrogallol, pyrocatechol, resorcinol and phloroglucinol [20]. In addition, MG decreased the production of QS-regulated virulence factors in *P. aeruginosa*.

The QS inhibition of APC may also be attributed to its chemical structure and the functional groups attached. It was reported that β-keto esters are competitive antagonists of N-acyl-homoserine lactones (AHL) and that their anti-QS activity may be attributed to the aryl functionality [21]. Pyrocatechol (APC without the allyl moiety) displayed very weak QS inhibition [20]. In this study, APC has strong anti-QS activity and is much less polar than pyrocatechol due to the allyl group.

A study on dietary phytochemicals as QS inhibitors involved mechanisms of modulation of AHL synthesis and interference of AHL activity [22]. The other mechanisms of QS disruption include reducing the activity of AHL synthase, inhibiting the production of QS signal molecules and degradation of the AHL [23]. It can be inferred that both APC and MG can inhibit the QS system either by accelerating degradation of signal molecule or reducing the ability of *C. violaceum* to synthesize signal molecules. The evidence of their mechanisms of action would require further research.

The anti-QS properties of *P. betle* leaves have been reported earlier [24,25]. APC has been isolated from leaves of *P. betle* [26] and has been identified as the dominant component (39%) in the aqueous betel leaf extract [19]. However, this study is the first to document the anti-QS activity of APC, which is comparable to that of MG.

3.5 Enrichment of APC

While column chromatography is an effective method for isolating compounds, it can be very time-consuming. Therefore, we examined solvent partitioning as a way of enriching the APC content of the methanol leaf extract of *P. betle*. Table 3 shows that the chloroform fraction had the highest APC

content of 315 mg/g with purity of 31.5% w/w when compared to other fractions.

Table 3 Allylpyrocatechol (APC) content in different solvent fractions from the crude extract of *Piper betle*

Fraction	Fraction (g)	APC content (mg/g)	Purity (% w/w)	APC yield (mg)	Recovery (% w/w)
Hexane	3.7	19.6	2.0	73	3
Chloroform	5.1	315	31.5	1597	62
Methanol	10.6	84.7	8.5	898	35
Extract	19.4	132	13.2	2568	100

With regard to recovery yield, chloroform solvent recovered 62% from the crude extract and yielded 1597 mg of APC. In contrast, the APC content, purity and recovery yield of the hexane fraction were only 19.6 mg/g, 2.0% and 73 mg, respectively. In spite of having the highest fraction weight of 10.6 g, the methanol fraction yielded relatively low APC content (84.7 mg/g) and purity (8.5% w/w). The recovery yield of the methanol fraction (35% w/w) was 1.8 times lower than the chloroform fraction (62% w/w). The results affirm the efficiency of solvent partitioning as the purity of APC increased by 2.4 times i.e., from 13.2% in the crude extract to 31.5% in the chloroform fraction.

The basis of selection of solvents is based on their polarity. The results obtained suggest that the major phytochemicals in *P. betle* leaves are mostly polar and soluble in methanol. Since APC is relatively non-polar, the highest recovery was observed in the chloroform fraction. It can be deduced that the two hydroxyl groups and one allyl group of APC contribute to the reduced polarity. Solvent partitioning into chloroform, methanol and hexane fractions is therefore a good way to enrich APC from *P. betle* leaves. Chloroform purified APC, methanol removed polar impurities and hexane removed less polar lipids.

4.0 CONCLUSION

Against *S. aureus*, 125 µg/mL of APC and MG, when combined with streptomycin, reduced the MIC of the antibiotic by 8-fold. Against *P. aeruginosa*, 125 µg/mL of APC and MG reduced the MIC of streptomycin by 2-fold and 4-fold, respectively. In terms of QS inhibition, both compounds were shown to be equally effective against *C. violaceum* with violacein inhibition observed at concentrations as low as 60 µg/mL. APC and MG can breathe new life into older antibiotics such as streptomycin given their ability to reduce the MIC. Furthermore, because they exhibit anti-QS against a AHL sensitive Gram-negative

bacteria, they could potentially reduce the virulence of *C. violaceum*, a Gram-negative pathogen, with a much-reduced risk of resistance. We have also successfully increased the purity of APC in the methanol *P. betle* leaf extract from 13.2% to 31.5% w/w using simple solvent partitioning. Solvent partitioning can thus be used to expedite purification of the compound.

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