

Radical Scavenging and Antibacterial Activity of Phenolic Compounds from *Anacardium occidentale* L. Stem Barks from South East Sulawesi-Indonesia

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Wahyuni, *et al.*: Radical Scavenging and Antibacterial Potencies of *Anacardium occidentale* Stem Barks

Three phenolic compounds, pinostrobin, pinocembrin and 4-hydroxybenzaldehyde have been isolated and identified for the first time from methanol extract of *Anacardium occidentale* L. stem bark. The isolation was carried out using various techniques of chromatography such as thin layer chromatography, vacuum liquid chromatography and radial chromatography. Silica gel as adsorbent and a mixture of solvents as eluent were used during the separation process. Structures of the isolated compounds were determined by Fourier transforms infrared, mass and nuclear magnetic resonance (1- and 2-Dimensi) spectroscopies. Biological properties of the isolated compounds were evaluated against four strains of bacteria (*Shigella dysenteriae* ATCC 13313, *Salmonella typhi* YCTC, methicillin-resistant *Staphylococcus aureus* ATCC33591, and *Escherichia coli* ATCC 35219), as well as the radical scavenging potential in DPPH assay. Results indicated that pinocembrin was the most active isolated compound towards *S. dysenteriae*, *S. typhi*, methicillin-resistant *S. aureus* and *E. coli*, however in comparison to the MIC₅₀ value of the standard, pinocembrin possessed low antibacterial activity. Meanwhile, 4-hydroxybenzaldehyde showed the highest radical scavenger activity with IC₅₀ value of 134.93±0.13 µM, but much lower compared to vitamin C with IC₅₀ value of 63.32±0.22 µM.

Key words: *Anacardium occidentale*, pinocembrin, pinostrobin, 4-hydroxybenzaldehyde, antibacterial, radical scavenging activity

South East Sulawesi province (Indonesia) is located at Wallacea line, so this area has big biodiversity of both plants and animals. Our laboratory was involved in evaluating chemical and pharmacological aspects of traditional medicinal plants and in that process we previously reported our studies on plants of Dipterocarpaceae family^[1-3], *Jatropha*^[4-6], Annonaceae family^[7], *Pongamia*^[8], *Imperata*^[9], *Polygonum*^[10,11] and *Dillenia*^[12]. The present investigation dealt with phytochemical and pharmacological evaluation of *Anacardium* plants available in the South East Sulawesi Province.

Anacardium occidentale L. (cashew) grows widely in this province. This plant is an important plantation crop in contributing to the national economy^[13]. It produces various secondary metabolites, which have interesting biological activities. In Indonesia, the plant is used as a purgative (roots), for aphtha (stems and barks), dermatitis and combustion (leaves), as a

food and for dermatitis (fruits) and seeds for food^[14]. Moreover, an infusion of the barks and leaves is used to relieve a toothache and sore gums, while the young leaves are used for the treatment of dysentery, diarrhoea and piles^[15,16]. In West Africa and South America, people used the leaves infusion in the treatment of gastrointestinal disorders, mouth ulcers and throat problems^[17]. In addition, the cashew stem barks are utilized by Portuguese as an alternative antidiabetic medicine^[18].

Previous pharmacological studies indicated that *A. occidentale* has various activities such as antiinflammatory, immunomodulatory,

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hypocholesterolemic, antioxidant, and antimicrobials^[19]. The plant from Brazil has potency as an antiulcer and inhibits the activity of several enzymes like lipooxygenase and cyclooxygenase^[20]. In addition, extracts of the leaves and barks of *A. occidentale* from Benin is active against *Escherichia coli*, *Staphylococcus aureus*, *S. epidermidis*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Micrococcus luteus*, *P. vulgaris*, *Streptococcus oralis*, *Enterococcus faecalis* and *Candida albicans*^[21]. The stem barks from Portugal and Nigeria, also has good potential as antioxidants^[22,23] and antiinflammatory^[24]. Furthermore, leaves and nuts extract of this plant from India showed cytotoxicity against several tumor cell lines and also provided an analgesic effect^[25,26].

Phytochemical studies reported that *A. occidentale* produced both phenolic and non-phenolic compounds^[24]. Cardol, cardanol and anacardic acid are isolated from nut-shells^[27,28]. Catechin, epicatechin, carotene, lutein and tocopherol are extracted from kernels of cashew nuts^[29]. The leaves produce agathistflavone and mixture of quercetine-3-O-rutinoside and quercetine-3-O-rhamnoside^[17], and carotenoids are got from cashew apples^[30]. Stem barks and artificial fruits also contain phenolic compounds, flavonoids, tannins, and alkaloids^[25]. The flavonoids were agathistflavone^[31], mirisetin, miristrin, rhamnetin, quercetin, and kaempferol^[32].

To further explore the stem barks of *A. occidentale*, a study on the secondary metabolites and biological properties of the isolated compounds against some strains of bacteria and 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay would be still very interesting. In this paper, we have reported the isolation and structure determinations of phenolic compounds from stem barks of *A. occidentale* and their potential as antibacterial and radical scavenging agents. Selected bacteria used were *Shigella dysenteriae* ATCC 13313, *Salmonella typhi* YCTC, methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC 33591, and *Escherichia coli* ATCC 35219.

MATERIALS AND METHODS

Isolation of secondary metabolites was carried out at Laboratory of Pharmacy Universitas Halu Oleo by using thin layer chromatography (TLC), vacuum liquids chromatography (VLC) and radial chromatography (RC). VLC and RC were performed by employing silica gel 60 GF as an adsorbent and a mixture of solvents as eluent. Melting points were determined

on a Buchi MP-1665 melting point apparatus and uncorrected. Proton (¹H) and carbon-13 nuclear magnetic resonance (¹³C NMR) spectra were recorded with a JEOL ECP 500 spectrometer, operating at 500 MHz (¹H) and 125 MHz (¹³C), worked at LIPI Serpong, Indonesia. Molecular mass was analysed by MS Waters LCT Premier XE, Detector TOF, solvent: acetone+ 0.1 % formic acid in acetonitrile:water (1:1) in Institute Teknologi Bandung, Indonesia. Chloramphenicol and vitamin C are positive control for antibacterial and radical scavenger activities, respectively.

Collection of plant material and isolation of secondary metabolites:

Samples of the stem barks of *A. occidentale* were collected from Kendari, South East Sulawesi. The plant was identified in School of Life Science, Institut Teknologi Bandung, Indonesia. The stem bark powder of *A. occidentale* (1 kg) was macerated with methanol for 3×24 h. The methanol extract was concentrated by vacuum rotary evaporator at low/reduced pressure, giving a brown gummy solid (50 g). The extract was fractioned twice using VLC (25 g; column diameter, Φ: 10 cm, silica gel: 150 g) by increasing polarity (n-hexane, n-hexane-ethyl acetate, ethyl acetate, methanol) to give 18 fractions, which were grouped into 4 major fractions by combining fractions with similar TLC profiles, F1 (6.1 g), F2 (13.0 g), F3 (12.1 g), and F4 (10.2 g). F1 fractioned by VLC using a column Φ 5 cm, adsorbent: silica gel (80 g) and mixture of ethylacetate:n-hexane (10:100 %, methanol 100 %) as eluent to get 5 fractions i.e F11 (0.3 g), F12 (0.2 g), F13 (1.1 g), F14 (0.8 g) and F15 (1.4 g). Purification of F14 using RC, adsorbent: silica gel and eluent n-hexane-ethylacetate (30:70 %), to give compound 1 (25 mg), a yellow amorphous powder. F2 refraction by conducting VLC with a column Φ 5 cm, adsorbent:silica gel (80 g) and mixture of ethylacetate:n-hexane (20:100 %, methanol 100 %) as eluent, to give 4 fractions, i.e., F21 (1.1 g), F22 (1.9 g), F23 (3.2 g), and F24 (4.2 g). Purification of F21 gave compound 2, a yellow amorphous powder (18 mg). F22 was purified by RC, adsorbent: silica gel and the eluent mixture of ethylacetate:n-hexane (30:100 %, the methanol 100 %), to give compound 3 (22 mg), a pale yellow crystalline.

Antibacterial activity:

The antimicrobial activity of the isolated compounds from *A. occidentale* stem barks was carried out by employing the disc diffusion method outlined by

CLSI Guidelines, 2007. A 20 mg/ml stock solution of each compound was prepared by using dimethyl sulfoxide (DMSO). Blank discs were impregnated with 20 ml of each compound (400 mg/ml) and allowed to dry. The bacterial isolates, grown overnight on brain heart infusion or tryptone soya agar plates, were suspended in sterile distilled water and the turbidity of cell suspensions adjusted equivalent to that of a 0.5 McFarland standard. These were used to inoculate Mueller-Hilton agar plates by spreading swabs over the entire agar surface followed by the application of the respective compounds. Then, plates were incubated for 24 h at 37°. Testing was worked in duplicate and chloramphenicol (Sigma) was used as standard antimicrobial agent controls, while DMSO-impregnated discs were used as negative controls. Zone diameters were determined and averaged. Minimum inhibitory concentration (MIC) of isolated compounds were determined by microdilution methods^[33]. The concentration of which there was no visually detectable bacterial growth was taken as the MIC^[34]. The MIC is the lowest concentration of the compound at which the microorganism tested does not demonstrate visible growth (turbidity).

Radical scavenger activity:

The radical scavenging activity of the isolated compounds was adapted from Sahidin *et al.*^[11] and Ching *et al.*^[35] with slight modification. The reduction of DPPH radical was analysed by using both qualitative and quantitative methods. One millilitre of 500 µM (0.2 mg/ml) DPPH in methanol was mixed with the same volumes as of the tested compounds at various concentrations. They were mixed well and kept in the dark for 30 min. The absorbance at λ 517 nm was monitored in the presence of different concentrations of the samples. The blank experiment, i.e. with only solvent and DPPH (2 ml of 500 µM in methanol), was also carried out to determine the absorbance of DPPH before interacting with the compounds. The amount of sample, compounds and standard (vitamin C) in mg/ml at which the absorbance at 517 nm decreased to half of its initial value was used as the IC₅₀ of compounds. The analysis was done in triplicate for the standard and compounds.

Statistical analysis:

Data were expressed as mean±standard deviation (SD) for three parallel measurements using IBM SPSS Statistics 19 for Windows®, IBM, USA. Statistical

analysis was done by post hoc test and p<0.05 considered as significant.

RESULTS AND DISCUSSION

Pinostrobin (1), a yellow amorphous powder, melting point (MP): 102-103°. Spectrum of UV/Vis (MeOH) λ_{max} (nm): 212 and 288. Fourier transform infrared (FTIR) spectrum (KBr), ν_{max} (cm⁻¹): 3430 (-OH), 2905 (Csp³-H), 1649 (C=O), 1510, 1445, 1401 (aromatic), 1302, 1158 (C-O ether). Spectrum of ¹H-NMR (500 MHz, CDCl₃) δ_H (ppm): 2.68 (1H, *dd*, *J*=14.5, 3.0 Hz), 2.95 (1H, *dd*, *J*=16.5, 2.4 Hz); 3.68 (3H, *s*); 5.26 (1H, *d*, *J*=3.0); 5.97 (1H, *d*, *J*=4.0); 6.02 (1H, *d*, *J*=4.0); 7.33 (2H, *m*), 7.36 (1H, *m*), 7.39 (2H, *m*), 12.04 (1H, *s*, *chelated*). Spectrum of ¹³C-NMR (125 MHz, CDCl₃) δ_C (ppm): 42.8; 55.2; 78.7; 93.9; 94.7; 102.7; 125.8 (2C); 128.4; 128.5 (2C); 138.1; 162.4; 163.7; 167.5; and 195.4. HRMS-ESI-TOF: m/z [M+H]⁺ calcd. for C₁₆H₁₄O₄ 271,0970, found 271,0973.

Pinocembrin (2), a yellow amorphous powder, MP: 188-190°. UV/Vis spectrum (MeOH) λ_{max} (nm): 210, 289. Spectrum of FTIR (KBr), ν_{max} (cm⁻¹): 3512 (OH), 2910 (C-H *sp*³), 1732 (C=O), 1300-1600, 1208 (C=C aromatic), 1105 (C-O ether). Spectrum of ¹H-NMR (500 MHz, acetone-d₆) δ_H (ppm): 2.81 (1H, *dd*, *J*=17.0; 3.0 Hz), 3.16 (1H, *dd*, *J*=17.5; 12.5 Hz); 5.54 (1H, *dd*, *J*=13.0; 3.0 Hz); 6.01 (2H, *dd*, *J*=13.5; 2.0 Hz), 7.37-7.46 (3H, *m*); 7.55 (2H, *d*, *J*=7.5 Hz), 9.67 (1H, *s*), 12.17 (1H, *s*, *chelated*). Spectrum of ¹³C-NMR (125 MHz, acetone-d₆) δ_C (ppm): 43.6; 79.9; 95.9; 97.1; 103.2; 127.2 (2C); 129.4 (2C); 129.5; 140.1; 164.1; 165.2; 167.3 and 196.7. HRMS-ESI-TOF: m/z [M+H]⁺ calcd. for C₁₅H₁₂O₄ 257.0814, found 257.0815.

4-hydroxybenzaldehyde (3), a pale yellow crystalline, MP: 120.1-120.4°. Spectrum of FTIR (KBr), ν_{max} (cm⁻¹): 3612 (OH), 1749 (C=O), 1590, 1505, 1451 (C=C aromatic), 1158 (C-O), para-disubstituted benzene (835 cm⁻¹). Spectra ¹H-NMR (acetone-d₆, 500 MHz): 9.85 (1H, *s*, H-6), 9.37 (1H, *brs*, 4-OH), 7.81 (2H, *d*, *J*=10 Hz, H-2/4), 7.02 (2H, *d*, *J*=5 Hz, H-3/5). Spectra ¹³C-NMR (acetone-d₆, 125 MHz): 191.1 (C-6), 163.9 (C-4), 132.9 (C-2/4), 130.5 (C-1), 116.7 (C-3/5). HRMS-ESI-TOF: m/z [M+H]⁺ calcd. for C₇H₄O₂ 121.0290, found 121,0287.

Three known compounds have been isolated and identified from *A. occidentale* stem barks that are pinostrobin, pinocembrin and 4-hydroxybenzaldehyde. Previously, the compound 1 and 2 were obtained jointly from *Boesenbergia rotunda*^[36], *Kaempferia*

pandurata^[37], and *Piper ecuadorensense*^[38]. While some plant only produces pinostrobin namely *Polygonum lapathifolium*^[39], *Cajanus cajan*^[40], and *Renealmia alpinia*^[41]. The plant, which produces the only pinocembrin is *Teloxys graveolens*^[15]. In addition, 4-hydroxybenzaldehyde was produced by *Mimusops elengi*^[42]. According to the above information, the presence of pinostrobin, pinocembrin and 4-hydroxybenzaldehyde from the stem barks of *A. occidentale* is the first research report. The compound structures are showed in fig. 1.

Compound 1 was isolated as a yellow amorphous powder with MP: 102-103°. The spectrum of UV/Vis (methanol) λ_{max} 288 nm showed that the compound has chromophore of the conjugated unsaturated carbonyl. The presence of carbonyl group was supported by FTIR spectra, which have a strong stretching band of C=O at 1649 cm^{-1} . FTIR spectra also displayed the presence hydroxyl unit that revealed a broad absorbance band at 3430 cm^{-1} and the vibration band at 1158 cm^{-1} indicated the C-O bending. Moreover, the presence of aromatic ring was indicated by peaks at 1510, 1445, 1401 cm^{-1} . According to FTIR data, compound 1 comprises hydroxyl, carbonyl, and aromatic units. This prediction was verified by NMR (¹H and ¹³C) data. The ¹³C-NMR spectrum analysis exhibited that the compound comprised fourteen signals for sixteen carbon atoms, two pairs of them are symmetrical carbons. The sixteen carbons include twelve aromatic carbons i.e. at δ_{C} (ppm) 102.7 (C-4a), 163.7 (C-5), 93.9 (C-6), 167.5 (C-7), 94.7 (C-8), 162.4 (C-8a), 138.1 (C-1'), 125.8, 128.5, (C-2'/6', symmetry), 128.4, 128.5 (C-3'/5', symmetry), three aliphatic carbons at δ_{C} (ppm) 78.7 (C-2) and 42.8 (C-3) and also a carbonyl carbon at δ_{C} 195.4 ppm. The attendance two pair symmetrical carbons were confirmed by ¹H NMR data at δ_{H} (ppm) 7.33 (2H, *m*) and 7.39 (2H, *m*). One shielded proton has a highly chemical shift at δ_{H} 12.04 ppm. It is caused by chelating proton of hydroxyl unit (C-5) with

the oxygen atom of the carbonyl group. This spectral data are characteristic for chelated 5-OH flavones and flavanones^[38].

Consistent with data of FTIR, NMR 1-D (¹³C, ¹H), and comparison NMR 1-D data of isolated compound to the same data from the reference such as displayed in Table 1^[35] showed that compound 1 has high similarity parameters with pinostrobin (fig. 1). It can be concluded that compound 1 is pinostrobin. The conclusion was confirmed by NMR 2-D (HMBC) and mass spectroscopy (MS) data. The MS (HRMS-ESI-TOF) spectrum of compound 1 showed molecular ion [M+H]⁺ at m/z 271,0970 corresponding to molecular formula C₁₆H₁₄O₄. It was further indicated that compound 1 is pinostrobin.

Structure elucidation stages of compounds 2 and 3 are the same as structure elucidation of compound 1, which refers to the spectroscopy data including spectroscopy UV, FTIR, NMR 1-D, NMR 2-D and mass spectroscopy. Consistent with all spectroscopy data, compound 2 is pinocembrin^[35] and compound 3 is 4-hydroxybenzaldehyde^[42].

Leaves and stem barks extracts of *A. occidentale* were active materials against *E. coli*, *S. aureus*, *S. epidermidis*, *P. aeruginosa*, *P. mirabilis*, *M. luteus*, *P. vulgaris*, *S. oralis*, *E. faecalis*, and *C. albicans*^[19]. Besides that, the stem barks also have potency as an antioxidant^[20,21]. To support the potency of the plant tissue extracts, three compounds, which were isolated from methanol extract, were evaluated for their biological activities against some bacteria and DPPH (radical scavenger).

Pinostrobin and pinocembrin are flavonoid derivatives. A number of biological activities of the compounds have been reported. As antibacterial, pinocembrin is an active compound towards *Bothrops asper*^[41], while pinostrobin has potency for antibacterial against *E. coli*, *B. subtilis*, *S. dysenteriae*^[43]. To continue antibacterial

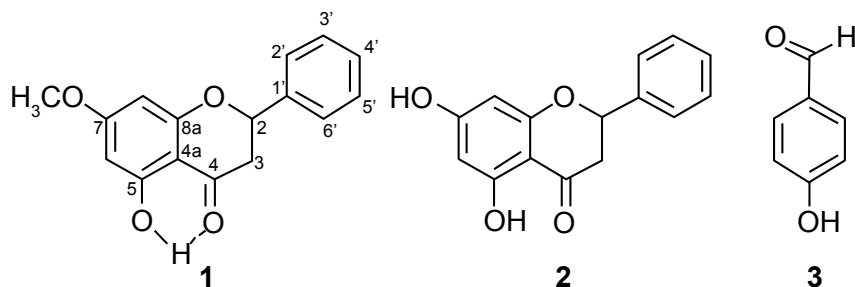


Fig. 1: Structure of isolated compounds from stem barks of *A. occidentale*
1: Pinostrobin, 2: pinocembrin, 3: 4-hydroxybenzaldehyde

properties study of pinostrobin, pinocembrin and 4-hydroxybenzaldehyde, we evaluated their activities against *S. dysenteriae*, *S. typhi*, MRSA and *E. coli* such as presented in Table 2. Generally, all isolated compounds are active towards all the tested bacteria. It supports the activity of methanol extracts of *A. occidentale* stem barks against some bacteria, which reported by Rout^[19]. However, those isolated compounds are less active than chloramphenicol (positive control). Of the three isolated compounds, pinocembrin is the most active compound toward all selected bacteria. To further study on antiinfective

potency, the MIC₅₀ data of MeOH extracts and isolated compounds of *A. occidentale* stem barks were displayed in Table 3. According to Cos *et al.*^[44], for all antiinfective bioassays, the IC₅₀ of pure compounds should be below 25 µM and for mixtures less than 100 µg/ml (Table 4). Based on the reference, the methanol extracts included in a prospective antiinfective materials, while pinocembrin not included in the grade. Considering to the MIC value, can be concluded that strong antibacterial activity of the methanol extracts is not caused by these isolated compounds. An extensive investigation of all secondary metabolites present in

TABLE 1: COMPARISON OF NMR DATA BETWEEN ISOLATED COMPOUND AND PINOSTROBIN^[35]

No.	Compound 1			Pinostrobin	
	δ H (multiplicity, J in Hz)	δ C (ppm)	HMBC correlations	δ H (multiplicity, J in Hz)	δ C (ppm)
2	5.26 (1H, d, J=3.0)	78.7	C-2'/6', C-1', C-4	5.39 (dd, J=12.84, 2.76 Hz, 1H)	79.0
3	1.68 (1H, dd, J=14.50, 3.02 Hz)	42.8	C-4	2.79 (dd, J=14.68, 2.76 Hz, 1H)	43.2
	2.95 (1H, dd, J=16.53, 2.40 Hz)			3.06 (dd, J=15.18, 12.81 Hz, 1H)	
4	-	195.4			195.7
4a	-	102.7			103.0
5	-	163.7			162.7
6	5.97 (1H, d, J=4.0)	93.9	C-8, C-4a	6.05 (d, J=2.72 Hz, 1H)	94.1
7	-	167.5			167.8
8	6.02 (1H, d, J=4.0)	94.7	C-6, C-4a	6.05 (d, J=2.72 Hz, 1H)	95.0
8a	-	162.4			164.0
1'	-	138.1			138.3
2'/6'	7.33 (2H, m)	125.8 (2C)	C-2'/6'	7.41 (m, 2H)	126.0
3'/5'	7.39 (2H, m)	128.5 (2C)	C-2, C-2'/6', C-3'/5', C-4'	7.41 (d, J=7.63, 2H)	126.0
4'	7.36 (1H, m)	128.4	C-3'/5', C-4', C-1'	7.41 (d, 7.27 Hz, 1H)	128.8
O-CH ₃	3.68 (3H, s)	55.2	C-7	3.79 (s, 3H)	55.6
-OH _{chelated}	12.04 (1H, s)	-	C-6, C-4a, C-5	12.00 (1H, s, 5-OH)	

δ H is chemical shift of proton, δ C is chemical shift of carbon, J is coupling constant, ppm is part per million

TABLE 2: INHIBITION ZONE OF METHANOL EXTRACTS AND ISOLATED COMPOUNDS

Compounds	Diameter of inhibition zone (mm)			
	<i>S. dysenteriae</i>	<i>S. typhi</i>	MRSA	<i>E. coli</i>
MeOH extracts	8.77±0.87	6.43±0.67	6.45±0.49	7.32±0.57
Pinostrobin	10.13±0.51	9.50±0.48	7.61±0.35	8.12±0.46
Pinocembrin	12.56±0.56	11.50±0.76	11.22±0.54	12.34±0.68
4-hydroxybenzaldehyde	9.32±0.44	7.21±0.54	NA	9.12±0.52
Chloramphenicol	21.50±0.78	19.58±0.66	18.71±0.94	19.69±0.88

NA=not active

TABLE 3: MIC₅₀ VALUES OF METHANOL EXTRACT AND ISOLATED COMPOUNDS

Compounds	MIC ₅₀ (ppm)			
	<i>S. dysenteriae</i>	<i>S. typhi</i>	MRSA	<i>E. coli</i>
MeOH extract	62.5	62.5	62.5	62.5
Pinostrobin	250 (925 µM)	250 (925 µM)	500 (1953 µM)	250 (925 µM)
Pinocembrin	125 (488 µM)	125 (488 µM)	250 (976 µM)	125 (488 µM)
4-hydroxybenzaldehyde	250 (2049 µM)	500 (4098 µM)	NA	500 (4098 µM)

NA=not active

TABLE 4: IC₅₀ VALUES OF ISOLATED COMPOUNDS

Compounds	IC ₅₀ (µM)
Pinostrobin	149.72±0.18
Pinocembrin	138.80±0.25
4-hydroxybenzaldehyde	134.93±0.13
Vitamin C (standard)	63.32±0.22

the MeOH extracts should be carried out to determine all possible active compounds.

In evaluating radical scavenger potency, qualitative experiments showed that all isolated compounds bleached the purple DPPH colour to pale yellow when the TLC plate on which they were sprayed by 0.2 % of DPPH in methanol. It is indicated that those samples has potency as radical scavengers. In the quantitative DPPH radical scavenger assay, all isolated compounds were able to neutralize the DPPH free radicals but less active than positive control (vitamin C). DPPH radical scavenging ability was increased in a concentration dependent manner compared to ascorbic acid that used as the positive control of antioxidant. There is a positive correlation between antioxidant activity and isolated compound concentrations ($p < 0.05$). Radical scavenger potency of isolated compounds showed that 4-hydroxybenzaldehyde is more active than pinostrobin and pinocembrin. Meanwhile, pinocembrin was slightly more active than pinostrobin^[37].

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Conflict of interest:

No conflict of interest.

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