MJFAS MALAYSIAN JOURNAL OF FUNDAMENTAL AND APPLIED SCIENCES
PRINT ISSN: 2289-5981 | ONLINE ISSN: 2289-599X

RESEARCH ARTICLE

Phytochemical, cytotoxicity and antioxidant activities of the stem bark of *Piper arborescens*

Nicholas Daniel [a,b,*], Fasihuddin Badruddin Ahmad [a], Zaini Assim [a], Chua Hun Pin [b]

[a] *Department of Chemistry, Faculty of ResourceScience and Technology, University Malaysia Sarawak, 94300 Kota Samarahan, Sarawak, Malaysia*
[b] *Food Science & Technology Research Centre, Malaysian Agricultural Research and Development Institute, MARDI Kuching, Lot 411 Block 14, Sultan Tengah Road, Petra Jaya, 93055 Kuching, Sarawak, Malaysia*

* *Corresponding author: nicholas@mardi.gov.my*

Article history
Received 8 June 2017
Accepted 2 October 2017

Abstract

Crude extract from stem bark of *P. arborescens* was fractionated by using column chromatography to isolate and purify its metabolite content. Six secondary metabolites were successfully isolated and their identification was performed by using Gas Chromatography - Mass Spectrometry (GC-MS), Nuclear Magnetic Resonance (NMR) and Fourier Transform Infrared (FTIR) spectoscopy. The isolated metabolites were identified as caryophyllene oxide **(1)**, *α*-bisabolol **(2)**, benzamide 2-(methylamino) **(3)**, 2-ethylpiperidine **(4)**, piperine **(5)** and methyl eugenol **(6)**. Toxicity test on the four crude extracts of *P. arborescens* shows high cytotoxicity against *Artemia salina* brine shrimp with LC_{50} values ranging from 13.12 to 58.70 µg/mL. Greater cytotoxicity of the crude extracts of *P. arborescens* indicated the presence of potent cytotoxic components in this *Piper* spp. Antioxidant assay of *P. arborescens* against 2-diphenyl-1-picrylhydrazyl (DPPH) indicated moderate antioxidant activities of methanol, dichloromethane, chloroform and hexane crude extracts with EC_{50} values of 21.68, 23.82, 32.88 and 36.88 µg/mL, respectively. It is suggested that the six secondary metabolites identified in *P. arborescens* contribute as an active content for the cytotoxicity and antioxidant activities. This study showed that the crude extracts of *P. arborescens* is definitely having potential to be used as a source of natural product of various application.

***Keywords*: *Piper arborescens*, phytochemical, cytotoxicity, antioxidant**

INTRODUCTION

Most of indigenous *Piper* plants in Borneo are future potential herbs but still underutilized and scientific information on its phytochemical and biological activities are very scarce. *Piper* spp. which is widely distributed over the tropical and subtropical regions of the world is used medicinally in various manners. Beside the well studied of the commercial black pepper (*Piper nigrum*) and the abundance of *Piper aduncum* (Liew *et al.*, 2000; Micheal & Douglas, 2014), studies on other wild *Piper* spp. in the state of Sarawak and Sabah of Borneo region, particularly on the phytochemical and biological activity is a potential and interesting field to be explored. This indigenous *Piper* spp. can be introduced as a potential herb and cultivated as future crops, where further research can be conducted to provide sufficient information on the health beneficial properties of the plants and their specific usage.

This paper discusses phytochemical and biological activities of a wild *Piper* spp. that can be found in the forest mostly throughout Sarawak; namely *P. arborescens* or locally known as *lada hutan*. Few studies on this *Piper* spp. have been conducted previously mostly focusing on its leaves and stems, but there are still potential elements to discover. A study by Lee *et al.* (2004) in Taiwan have discovered cyclobutanoid amides from the stem and leaves of *P. arborescens*, while a study by Tsai *et al.* (2005) also in Taiwan have discovered cytotoxic cyclobutanoid amides and furanoid lignan from the stem of *P. arborescens.* Toxicity of the plants does not always indicated its danger or outright toxicity toward human, but may also suggest the presence of cytotoxic component that may contribute to antitumor or anticancer activities (Moshi *et al.*, 2010). There are various useful method available for assessment of cytotoxicity of the plant extract. The most common and widely used is the Brine Shrimp Lethality assay by using *Artemia salina* (Mentor *et al.*, 2014). *Artemia salina* is an invertebrate inhibiting saline aquatic which suitable to be used in laboratory assay for cytotoxicity screening of the plants by estimation of lethality concentration to kill 50% (LC_{50}) of the test organism.

In this study, the focus is on the stem bark of *P. arborescens*, which the parts are believed associated to medicinal purposes, yet the related scientific literature are very limited. The stem bark of the *P. arborescens* was extracted using several solvents to obtain the crude extracts, followed by various series of chromatographic method such as column chromatography and thin layer chromatography for separation and purification of the secondary metabolites. The isolated metabolite was further analyzed using Gas Chromatography - Mass Spectrometry (GC-MS), Nuclear Magnetic Resonance (NMR) and Fourier Transformed Infrared (FTIR) for identification and confirmation of the compound. The crude extracts of *P. arborescens* were analyzed for their biological activity, which involved cytotoxicity and antioxidant assays.

EXPERIMENTAL

Plant material

The sample of *P. arborescens* was collected from Betong, Sarawak. The stem bark of *P. arborescens* was air-dried, cut into pieces and ground prior to analysis.

Extraction and isolation

The stem bark of *P. arborescens* was extracted by conventional solvent extraction method described by Fasihuddin *et al*. (2010). This was achieved by soaking the ground plant material in various solvents namely hexane (C_6H_{14}), dichloromethane (CH_2Cl_2), chloroform ($CHCl_3$) and methanol (MeOH), in the order of increasing polarity. The sample was first soaked in hexane for 72 hours. The resulting solution was filtered using filter paper and the residue was re-extracted with fresh hexane for another 72 hours and filtered. Both extracts were combined and concentrated with a rotary evaporator under reduced pressure to obtain the hexane crude extract. The residues were re-extracted using similar procedure with dichloromethane, followed by chloroform and methanol to obtain the respective crude extracts.

Secondary metabolite from the crude extracts of *P. arborescens* was isolated and purified by using chromatographic method namely column chromatography (CC), with thin layer chromatography (TLC) as a medium for visual identification. The crude extract was fractionated by CC over silica gel 60 (Merck 70-230 Mesh @ 0.063-0.200 mm) eluted with various solvent system to obtain a semi-pure and pure metabolite.

Identification

Identification of the isolated metabolite was made by various spectrometric methods namely Gas Chromatography - Mass Spectrometry (GC-MS), Nuclear Magnetic Resonance (NMR) and Fourier Transform Infrared spectrometry (FTIR) as described by previous study [3]. Analysis by GC-MS was performed on a Shimadzu model QP 2010 Plus to obtain molecular mass of the compounds according to mass-to-charge (M/z) ratio, based on the method described by Kalaiselvan *et al.* (2012). Nuclear Magnetic Resonance (NMR) was performed by using JEOL JNM-ECA 500 spectrometer, based on the method as described by Efdi *et al.* (2010). The sample was dissolved in chloroform D1 ($CDCl_3$), and ^1H and ^{13}C spectra were measured at 500 and 125 MHz, respectively. FTIR analysis was performed by using spectrometer model Nicolet iS10 SMART iTR of Thermo Scientific brand. The range of the scan for the FTIR was started from 400 to 4000 cm^{-1} with a resolution of 4 cm^{-1}, according to the method described by Shalini & Sampathkumar (2012).

Cytotoxicity assay

Cytotoxicity test against brine shrimp (*Artemia salina*) developed by McLaughlin (1991) was used in this study. Leached brine shrimp eggs were hatched in seawater and incubated for 48 hours at 25 ºC. Exactly 3 mg of sample was dissolved in 3 mL methanol, and the mixture was sonicated to ensure the homogeneity of the extract. Four different volumes of 500, 250, 50 and 5 µL each of the stock solution were transferred into Nunc multidish in triplicate. The solvent was allowed to evaporate under a running fume hood for overnight, and followed by the addition of 0.2 mL dimethylsulphoxide (DMSO) and 4.8 mL seawater to give a final concentration of 100, 50, 10 and 1 µg/mL, respectively. Ten brine shrimp nauplii were transferred into each concentration in the multidish, and was observed every 6 hours for 24 hours. The numbers of dead nauplii were observed. Thymol was used as positive control, whereas 0.2 mL dimethylsulphoxide (DMSO) and 4.8 mL seawater was used as negative control. The data was analyzed to determine the concentration of the samples that kill 50% of brine shrimp at 24 hours or known as LC_{50}. LC_{50} was calculated and determined by performing Probit analysis in IBM SPSS Statistic software of version 21.

Antioxidant assay

The free radical scavenging assay of compound 2,2-diphenyl-1-pycryl-hydrazyl (DPPH) was used to evaluate the antioxidant properties of the crude extracts. The measurement was based on the method as described by Wang *et al.* (2008). Four concentration of sample were prepared at 10, 50, 100 and 1000 µg/mL from each crude extract, by diluting the crude extract with a methanol.

Approximately 3 mL of 0.1 mM methanol solution of 2,2 -diphenyl-1-pycrylhydrazyl (DPPH) was each added into the 1mL each of four series of prepared sample concentrations, making a final concentration of 2, 12, 25 and 250 µg/mL, respectively. The analysis was done in triplicate. The solution was mixed vigorously and left to stand at room temperature for 30 min in the darkness after which its absorbance was measured spectrophotometrically at 517 nm, performed by ultraviolet spectrophotometer. Methanol was used as a blank sample and negative control (1 mL methanol: 3 mL DPPH), while ascorbic acid (vitamin C) as the standard. The value of EC_{50} was determined using log dose inhibition curve (Tailor & Goyal, 2014) which performed by using PRISM version 3.02 software, based on the calculated values of the DPPH scavenging activity (%) of the sample.

RESULTS AND DISCUSSION

Six secondary metabolites have been isolated from the stem bark of *P. arborescens*, which reveal the presence of two terpenes namely caryophyllene oxide ($C_{15}H_{24}O$) **(1)** and *α*-bisabolol ($C_{15}H_{26}O$) **(2)**, along with three alkaloids namely benzamide 2-(methylamino) ($C_8H_{10}N_2O$) **(3)**, 2-ethylpiperidine ($C_7H_{15}N$) **(4)** and piperine ($C_{17}H_{19}NO_3$) **(5)**, and one phenylpropanoid identified as methyl eugenol ($C_{11}H_{14}O_2$) **(6)**.

Caryophellene oxide

Compound caryophyllene oxide **(1)** was isolated as white yellowish crystalline, isolated from the methanol crude extract of *P. arborescens.* The crude extract was fractionated by column chromatography eluted with hexane : ethyl acetate (4:1), (9:1). Analysis of compound **(1)** in TLC plate shows the R_f value of 0.66 in hexane : ethyl acetate (9:1) as mobile phase, viewed after staining with vanillin dipping reagent. Melting point 60-63 °C. GC-MS [$C_{15}H_{24}O$] (S.I. 93%); IR Vmax cm^{-1}: 2926, 1630, 1453, 861; MS *m/z* (% rel. int): 220 (8), 205 (10), 187 (10), 177 (10), 161 (10), 149 (12), 135 (15), 121 (35), 109 (55), 93 (95), 79 (100), 69 (75), 43 (95), 41 (75); ^1H-NMR (500 MHz, $CDCl_3$) δ (ppm): 4.96 (s, 1H, H-15a), 4.85 (s, 1H, H-15b), 2.87 (t, J = 5.4 Hz, 1H, H-1), 2.61 (q, J = 9.4 Hz, 1H, H-8), 2.37-2.21 (m, 2H, H-10), 2.13-2.06 (q, 1H, H-5), 1.75 (t, J = 9.9 Hz, 2H, H-3), 1.68-1.57 (q, 4H, H-2, H-9), 1.36 (d, J = 59.6 Hz, 2H, H-6), 1.19 (s, 3H, H-12), 1.00 (s, 3H, H-13), 0.97 (s, 3H, H-14); ^{13}C-NMR (125 MHz, $CDCl_3$) δ (ppm): 151.91 (C-4), 112.88 (C-15), 63.89 (C-1), 59.99 (C-11), 50.72 (C-8), 48.83 (C-5), 39.20 (C-6), 39.81 (C-10), 34.13 (C-7), 30.31 (C-2), 29.98 (C-3), 29.80 (C-9), 27.28 (C-14), 21.70 (C-13), 17.10 (C-12).

Both ^1H-NMR and ^{13}C-NMR signals of compound **(1)** were associated with the one produced in the NMR signal of caryophyllene oxide reported by Morten & Sparling (2007), while the IR spectrum indicated similarity with caryophyllene oxide reported by Gohari *et al*. (2005). Caryophyllene oxide **(1)** is a natural product grouped in terpene, derivatives of caryophyllene, which usually used as food and flavour ingredients, apart from its wide application in the perfumery industry for aromatic purposes. Caryophyllene oxide **(1)** has been approved by the Food and Drug Administration (FDA) and European Food Safety Autority (EFSA) as a natural flavoring. According to the information published by www.terpenes.info.com, caryophyllene oxide **(1)** can give benefit as anti-fungal and as an anticoagulant. A study by Chavan *et al.* (2010) reported that caryophyllene oxide **(1)** was comparable to standard aspirin, which showed a significant inhibition of inflammatory adema. Besides, caryophyllene oxide **(1)** was reported to possess significant anticancer activities as reported by Fidyt *et al.* (2016). Beside been identified in the crude extract of *P. arborescens* in this study, the occurrence of caryophyllene oxide **(1)** also reported in the essential oil of various *Piper* spp. namely the *P. nigrum*, *P. porphyrophyllum*, *P. aduncum*, *P. arborescens*, *P. erecticaule* and *P.betle* as reported by Irna *et al.* (2012) and Chieng *et al.* (2003).

α-bisabolol

Compound *α*-bisabolol **(2)** was obtained also from the methanol crude extract of *P. arborescens*. It was obtained as an amorphous form with yellowish colour. The crude extract was fractionated by column chromatography eluted with hexane : ethyl acetate (4:1), (9:1). TLC test on compound **(2)** shows R_f value of 0.34 in hexane : ethyl acetate (9:1), viewed after staining with vanillin dipping reagent. GC-MS [$C_{15}H_{26}O$] (S.I. 96%); IR Vmax cm^{-1}: 3445, 2921, 1719, 1375, 913; MS *m/z* (% rel. int): 204 (20), 189 (10), 161 (15), 148 (10), 134 (15), 119 (100), 109 (95), 93 (48), 71 (28), 69 (90), 43 (80), 41 (40); ^{1}H-NMR (500 MHz, $CDCl_3$) δ (ppm): 5.39 (t, J = 2.0 Hz, 1H, H-10), 5.12 (t, J = 6.3 Hz, 1H, H-6), 2.39-2.14 (t, 2H, H-2), 2.07-1.96 (t, 2H, H-5), 1.83 (dd, J = 41.0, 12.9 Hz, 2H, H-9), 1.68 (s, 1H, O-H), 1.61 (s, 3H, H-15), 1.56-1.45 (m, 1H, H-4), 1.33-1.23 (m, 8H, H-3, H-12, H-13), 1.13 (s, 3H, H-14), 0.87 (t, J = 6.9 Hz, 2H, H-8); ^{13}C-NMR (125 MHz, $CDCl_3$) δ (ppm): 133.98 (C-1), 131.90 (C-11), 124.65 (C-10), 120.86 (C-6), 74.48 (C-7), 43.39 (C-4), 39.39 (C-8), 31.07 (C-2), 29.80 (C-5), 26.17 (C-3), 25.88 (C-9), 24.07 (C-12), 23.47 (C-13), 22.38 (C-14), 17.79 (C-15).

^{1}H-NMR and ^{13}C-NMR signal of compound **(2)** was identical with the NMR signals of *α*-bisabolol from the previous study by Luiz Gustavo *et al.* (2015). Besides, IR spectrum of compound **(2)** shows similarity to IR stretching and bending vibrations of *α*-bisabolol reported by Santos Nara *et al*. (2013). *α*-bisabolol **(2)** is a natural monocyclic sesquiterpene, a metabolite in a group of terpenes. Previous study done by Chieng *et al.* (2003) had detected *α*-bisabolol **(2)** in the essential oils of four *Piper* spp. namely the *P. arborescens*, *P. sarmentosum*, *P. erecticaule*, and *P. betle*, with *P. erecticaule* contained the highest percentage of 0.67%, while 0.21% was found in *P. arborescens.* In natural product application, *α*-bisabolol **(2)** usually associated as an analgesic, anti-bacterial, anti-inflammatory, antimutagenic, antioxidant, anticancer and as a neuroprotectant, as mentioned in www.terpene.info and reported by Santos Nara *et al.* (2013). Hence, *α*-bisabolol has been widely used in dermatological and cosmetic industries as body lotions, after-shave cream, deodorants, lips sticks, and in sun-care, baby-care and sports products. The combination of a natural product of *α*-bisabolol with the conventional drug demonstrated an enhance in antibacterial activity against resistant bacteria pathogens (Santos Nara *et al.*, 2013)

Benzamide 2-(methylamino)

Compound benzamide 2-(methylamino) **(3)** was isolated as crystalline form with pale brown colour from methanol crude extract of *P. arborescens*. The crude extract was fractionated by column chromatography eluted with hexane : ethyl acetate (4:1), (2:1), (1:1), followed by re-column with dichloromethane 100%, dichloromethane : ethyl acetate (5:1). Analysis of compound **(3)** in TLC plate shows R_f value of 0.42 (uv) in hexane : ethyl acetate (3:1) as mobile phase. Melting point 160-162 °C. GC-MS [$C_8H_{10}N_2O$] (S.I. 78%); IR Vmax cm^{-1}: 3324, 2975, 1604, 1516, 1107, 909; MS *m/z* (% rel. int): 150 (100), 123 (48), 103 (50), 91 (10), 77 (50), 65 (10), 51 (32), 40 (8); ^{1}H-NMR (500 MHz, $CDCl_3$) δ (ppm): 6.77 (d, J = 8.0 Hz, 1H, H-5), 6.69 (d, J = 1.7 Hz, 1H, H-3), 6.60 (d, J = 8.0 Hz, 1H, H-2), 5.91 (s, 2H, N-H_2), 5.04 (t, J = 10.3 Hz, 1H, H-4), 3.25 (s, 3H, H-8), 2.50 (s, 1H, N-H); ^{13}C-NMR (125 MHz, $CDCl_3$) δ (ppm): 143.58 (C-7), 141.78 (C-4), 137.77 (C-3), 133.34 (C-2), 121.12 (C-5), 115.92 (C-1), 115.60 (C-6), 39.59 (C-8).

Compound benzamide 2-(methylamino) **(3)** is less explored and related research publication is very limited. Benzamide 2-(methylamino) **(3)** is categorized in alkaloid group, a compound derived from benzamide. Benzamide derivatives have been extracted from various natural sources (Anonymous, 2016a) with several of it have been synthesized from natural anacardic acid, a major constituent of cashew nut shell as reported in Vittal Mallya's Scientific Research Foundation of Natural Product Derivatives. According to Anonymous (2016a), several benzamide derivatives have been biologically evaluated which the result indicated the compounds were with great potential as an agent for antimicrobial, antifungal, anti-enterovirus, antihypertensive and antioxidant.

2-ethylpiperidine

Compound 2-ethylpiperidine **(4)** was isolated from the chloroform crude extract of *P. arborescens*. The compound was isolated as pale white brown powder. The crude extract was fractionated in a three series of column chromatography started with hexane 100%, hexane : dichloromethane (1:1), dichloromethane 100%, dichloromethane : chloroform (1:1), chloroform 100%, chloroform : ethyl acetate (1:1), ethyl acetate 100%, ethyl acetate : methanol (1:1). Re-column of the fractions was performed with chloroform : ethyl acetate (9:1), (1:1), (1:10),(1:15), ethyl acetate 100%, ethyl acetate : methanol (1:1), followed by the third re-column by using ethyl acetate : methanol (10:1), (1:1). TLC test on compound **(4)** shows the R_f value of 0.48 (uv) in the ethyl acetate : methanol (12:1) as mobile phase. Melting point 172-176 °C. GC-MS; [$C_7H_{15}N$] (S.I. 86%); IR Vmax cm^{-1}: 3372, 2924, 1249, 1017; MS *m/z* (% rel. int): 112 (10), 84 (100), 69 (10), 40 (10); ^{1}H-NMR (500 MHz, $CDCl_3$) δ (ppm): 3.89 (s, 1H, N-H), 3.64-3.41 (m, 3H, H-5, H-4), 1.79-1.63 (q, 2H, H-1), 1.63-1.50 (m, 2H, H-6), 1.38-1.20 (m, 4H, H-2, H-3), 0.87 (t, J = 6.9 Hz, 3H, H-7); ^{13}C-NMR (125 MHz, $CDCl_3$) δ (ppm): 47.62 (C-4), 43.22 (C-5), 29.85 (C-1), 26.41 (C-2), 25.64 (C-3), 24.53 (C-6), 14.21 (C-7).

IR spectrum of compound **(4)** shows similarity with the published IR data on 2-ethylpiperidine reported by Anonymous (2002). Comparison with proton NMR signals reported by Abraham (2008) showed that compound **(4)** was similar to 2-ethylpiperidine. Besides, the predicted ^{1}H-NMR and ^{13}C-NMR spectrum of 2-ethylpiperidine provided by www.nmrdb.org showed a high similarity with NMR spectrum of compound **(4)** in term of chemical shift and pattern for every signal. Compound 2-ethylpiperidine **(4)** is categorized in the alkaloid group, a derivative from piperidine. It is an organic compound that usually found in black pepper, also being reported found in various *Piper* spp. (Reshmi *et al.*, 2010) and the presence of 2-ethylpiperidine **(4)** in *P. arborescens* was further discovered in this study. Piperidine and 2-ethylpiperidine **(4)** were extracted and mainly used in pharmaceutical and agro-industry (Scherer, 2003).

Piperine

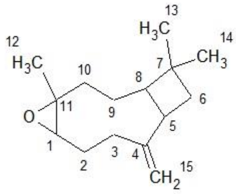
Compound piperine **(5)** was isolated as white powder from the dichloromethane crude extract of *P. arborescens*. The crude extract was initially dissolved with some dichloromethane and cooled in an ice-bath. Diethyl ether was added into the solution. The solution was stirred gently until precipitation occured. The solidified residue was filtered, and then dissolved in ethanol and 10% potassium hydroxide. The solution was concentrated using rotary evaporator to obtain the crude extract. The crude extract was then fractioned by column chromatography, eluted with dichloromethane : ethyl acetate (6:4), (4:6). Analysis of compound **(5)** in TLC plate shows R_f value of 0.46 (uv) in dichloromethane as mobile phase. Melting point 126-128 °C. GC-MS [$C_{17}H_{19}NO_3$] (S.I. 96%); IR Vmax cm^{-1}: 2934, 1624, 1578, 1437, 1245, 1023; MS *m/z* (% rel. int): 285 (70), 201 (95), 173 (48), 159 (10), 143 (30), 137 (15), 115 (100), 100 (10), 84 (40), 63 (10), 55 (10), 40 (10); ^{1}H-NMR (500 MHz, $CDCl_3$) δ (ppm): 7.39 (q, J = 7.9 Hz, 1H, H-5), 6.97 (d, J = 1.5 Hz, 1H, H-10), 6.88 (dd, J = 8.0, 1.9 Hz, 1H, H-6), 6.77 (s, 1H, H-8), 6.76 (s, 1H, H-9), 6.73 (d, J = 9.2 Hz, 1H, H-4), 6.43 (d, J = 14.5 Hz, 1H, H-11), 5.96 (s, 2H, H-1), 3.57 (d, J = 55.8 Hz, 4H, H-13, H-17), 1.65 (m, J = 5.9 Hz, 2H, H-15), 1.58 (m, J = 5.7 Hz, 4H, H-14, H-16); ^{13}C-NMR (125 MHz, $CDCl_3$) δ (ppm): 165.51 (C-12), 148.27 (C-2), 148.19 (C-3), 142.57 (C-10), 138.30 (C-8), 131.10 (C-7), 125.43 (C-9), 122.60 (C-5), 120.14 (C-11), 108.58 (C-4), 105.75 (C-6), 101.36 (C-1), 47.00 (C-13), 43.33 (C-17), 26.82 (C-14), 25.71 (C-16), 24.76 (C-15).

Comparison of IR spectrum of compound **(5)** showed that it was identical to IR data of piperine reported by Saha *et al.* (2013), while comparison of ^{1}H-NMR and ^{13}C-NMR signals of compound **(5)** with NMR data of piperine reported by Gottumukkala *et al.* (2012) indicated similarity. Piperine **(5)** is a type of amide alkaloid that commonly found in various *Piper* spp. This compound has always been referred as a contributor for the pungency's smell and taste of the black pepper (*P. nigrum*). Malaysian Agricultural Research and Development Institute (MARDI) has developed food product known as a botanical cube and drink from *P. arborescens*. Panelist's feedback from the sensory test has mentioned a smell and taste of the product that resembles black pepper (Chua *et al*., 2016). This 'taste of black pepper' in *P. arborescens* most probably contributed by the major presence of piperine **(5)** as discovered and isolated in this study.

Methyl eugenol

Compound methyl eugenol **(6)** was isolated as yellowish amorphous from dichloromethane crude extract of *P. arborescens*. The crude extract was fractioned by column chromatography, eluted with dichloromethane 100%, followed by re-column using hexane : dichloromethane (1:1) and dichloromethane 100%. TLC test on compound **(6)** showed R_f value of 0.54 in hexane : dichloromethane (1:1) as mobile phase. GCMS [$C_{11}H_{14}O_2$] (S.I. 86%); IR Vmax cm^{-1}: 2924, 1739, 1258, 1030; MS *m/z* (% rel. int): 178 (68), 163 (25), 147 (30), 135 (20), 115 (30), 103 (70), 91 (100), 77 (50), 65 (48), 51 (45), 45 (10); ^{1}H-NMR (500 MHz, $CDCl_3$) δ (ppm): 6.80 (d, J = 7.6 Hz, 2H, H-6, H-9), 6.72 (d, J = 10.7 Hz, 1H, H-5), 6.00-5.91 (m, 1H, H-2), 5.08 (d, J = 15.3 Hz, 2H, H-1), 3.87 (s, 6H, H-10, H-11), 3.33 (d, J = 6.1 Hz, 2H, H-3); ^{13}C-NMR (125 MHz, $CDCl_3$) δ (ppm): 148.94 (C-7), 147.43 (C-8), 137.78 (C-2), 132.70 (C-4), 120.46 (C-5), 115.70 (C-1), 111.89 (C-6), 111.28 (C-9), 56.00 (C-11), 55.86 (C-10), 39.89 (C-3).

These is similar between IR spectrum of compound (6) and IR spectrum of methyl eugenol reported by Riyanto *et al.* (2016). Proton NMR of compound **(6)** was comparable with ^{1}H-NMR data of methyl eugenol reported by Raquel *et al.* (2011), whereas carbon NMR was similar to ^{13}C-NMR data of the same compound reported by Anonymous (2016b). Methyl eugenol **(6)** is a phenylpropanoid chemical, which is derived from eugenol, a product from phenylalanine or known as essential amino acid. Methyl eugenol **(6)** can be found in many plant species especially in spices and medicinal plants. The presence of methyl eugenol **(6)** in *P. arborescens* is further supported in this study by the isolation of the compound from the dichloromethane crude extract. A study by Raquel *et al.* (2011) reported that methyl eugenol **(6)** showed antifungal activity against *Candida* spp. and *Microsporum canis*, and suggesting for more studies in the future as its parent compound known as eugenol is skin irritants.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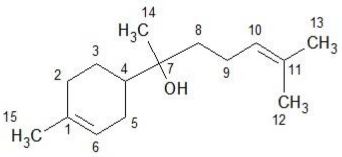
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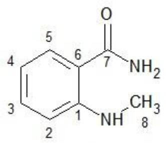
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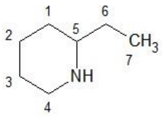
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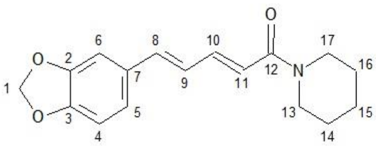
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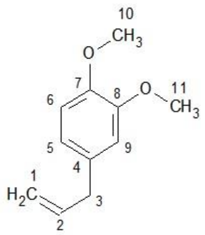
(6)

Fig. 1 Chemical structure of six secondary metabolites isolated from *P. arborescens.*

Cytotoxicity activity

Hexane and dichloromethane crude extracts of *P. arborescens* showed strong cytotoxic activity against the brine shrimp with LC_{50} values of 13.12 and 16.51 µg/mL, respectively. Average death of the brine shrimp in chloroform crude extract was slightly lower with LC_{50} value at 26.56 µg/mL. Methanol crude extract showed the lowest cytotoxic activity against the brine shrimp with LC_{50} of 58.70 µg/mL. At higher concentration of 100 µg/mL, methanol crude extract caused a number of 8 ± 0.57 death of the brine shrimp (Table 1) or an average of 75%, whereas in hexane, dichloromethane and chloroform crude extracts the average death were up to 95-100% at the similar concentration, as illustrated in Fig. 2.

Based on the information reported by Moshi *et al.* (2010), the test sample that showed LC_{50} between 30-100 µg/mL is categorized as mildly toxic, whereas those with LC_{50} more than 100 µg/mL are considered as being practically low or non-toxic. With this guideline, the result from this study showed that the four crude extracts from *P. arborescens* were categorized as toxic for having LC_{50} values ranging from 13.12 to 58.70 µg/mL. Hexane (LC_{50} 13.12 µg/mL), dichloromethane (LC_{50} 16.51 µg/mL) and chlorofom (LC_{50} 26.56 µg/mL) crude extracts of *P. arborescens* can be considered as highly toxic because the LC_{50} are lower than 30-100 µg/mL (mildly toxic) as suggested by Moshi *et al.* (2010).

Greater cytotoxic activity of the four crude extacts of *P. arborescens* towards brine shrimp indicated the presence of potent cytotoxic components in this *Piper* spp. Studies by Moshi *et al.* (2010) suggested that some of the plant extracts with LC_{50} below 100 µg/mL which are categorized as toxic, does not always indicated its danger or outright toxicity toward human, but may also suggest a potential antitumor or anticancer activities.

Table 1. Average death of *Artemia salina* at different concentrations of crude extracts of *P. arborescens.*

Crude extracts	**Average death of *Artemia salina***				**LC_{50} (µg/mL)**
	Concentration (µg/mL)				
	1	**10**	**50**	**100**	
Hexane extract	0	2 ± 1.15	10 ± 0.00	10 ± 0.00	13.12
Dichloromethane extract	0	2 ± 1.00	10 ± 0.57	10 ± 0.00	16.51
Chloroform extract	0	1 ± 0.57	7 ± 0.57	10 ± 0.57	26.56
Methanol extract	0	0	4 ± 1.15	8 ± 0.57	58.70
(-ve Control)	0	0	0	0	-
(+ve Control) Thymol	5 ± 0.57	7 ± 0.57	10 ± 0.00	10 ± 0.00	1.15

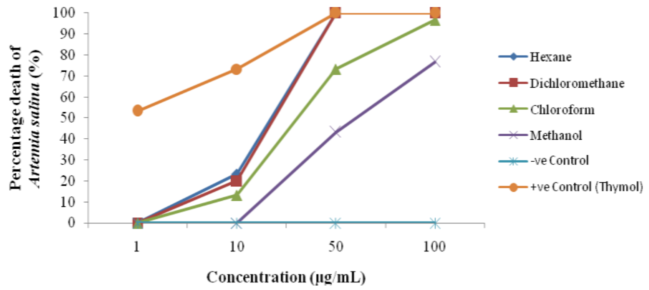

Fig. 2 Average death of *Artemia salina* (%) as a function of concentration of various crude extracts of *P. arborescens.*

Antioxidant activity

Studies on antioxidant activities of the crude extracts of *P. arborescens* showed that the EC_{50} values of methanol, dichloromethane, chloroform and hexane crude extracts were 21.68, 23.82, 32.88 and 36.54 µg/mL, respectively (Table 2), and their radical scavenging activities is shown in Fig. 3. This shows that the crude extracts of *P. arborescens* inhibited good DPPH radical scavenging activity, which indicated good antioxidant properties. As a comparison, EC_{50} for ascorbic acid (standard) was 2.40 µg/mL. Among the four crude extracts, methanol crude extract gave the best activity towards the DPPH free-radical scavenging with EC_{50} value at 21.68 µg/mL. Polar extract (methanol) was reported high in phenolic content as compared with less-polar extract. The presence of polar phenolic is important in the evaluation of free radical-scavenging activity. In comparison, antioxidant activity based on EC_{50} value of the crude extracts from *P. arborescens* in this study were slightly greater as compared to antioxidant activities of *P. colubrinum* and *P. nigrum*, as reported by Sruthi & John Zachariah (2017). Findings from this study suggested that the four crude extracts of *P. arborescens* potential to be used as antioxidant agents.

Table 2. Free radical scavenging activity against DPPH radical of the crude extracts of *P. arborescens.*

Crude extract of *P. arborescens*	Calibration equation		EC_{50} (µg/mL)
Hexane	$y = 4.084x + 0.5457$	$R^2 = 0.9991$	36.54
Dichloromethane	$y = 1.807x + 14.000$	$R^2 = 0.9993$	23.82
Chloroform	$y = 1.895x - 0.5374$	$R^2 = 0.9999$	32.88
Methanol	$y = 1.675x - 0.1976$	$R^2 = 0.9997$	21.68
Ascorbic acid	$y = 2.346x - 279.80$	$R^2 = 0.9957$	1.47

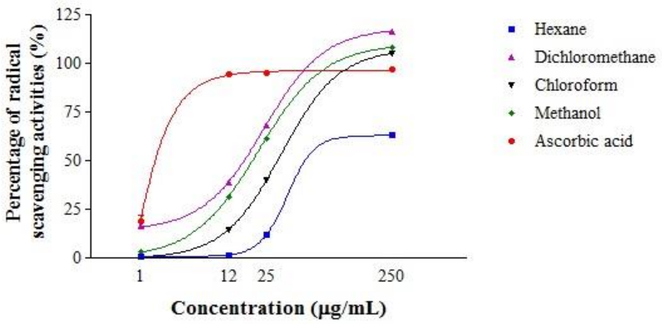

Fig. 3 Radical scavenging activity of various crude extracts of *P. arborescens.*

CONCLUSION

Extraction and purification of the stem bark of *P. arborescens* have isolated six secondary metabolites identified as caryophyllene oxide **(1)**, *α*-bisabolol **(2)**, benzamide 2-(methylamino) **(3)**, 2-ethylpiperidine **(4)**, piperine **(5)** and methyl eugenol **(6)**. Toxicity test was performed against *Artemia salina* brine shrimp and the result showed that hexane, dichloromethane, chloroform and methanol crude extracts from *P. arborescens* showed a high cytotoxic activity. Greater cytotoxic activity of the four crude extrats of *P. arborescens* indicated the presence of potent cytotoxic components in this *Piper* spp. as suggested by the literature. Antioxidant assay on the crude extracts of *P. arborescens* indicated moderate antioxidant properties of the crude extracts of methanol, dichloromethane, and chloroform with EC_{50} values ranging from 21.68 to 36.88 µg/mL. It is suggested that the six secondary metabolites [**(1)** to **(6)**] identified in *P. arborescens* contribute as an active content for the cytotoxicity and antioxidant activities. This study showed that the crude extracts from *P. arborescens* is definitely having potential to be used as a source of natural product.

ACKNOWLEDGEMENT

The Grant GL(F07)/MARDI/2014(12) and FRGS/1/2014/SG03 /MOA/02/1 from Universiti Malaysia Sarawak and Ministry of Education of Malaysia are gratefully acknowledged. The support received through the collaborative work of Universiti Malaysia Sarawak and Malaysian Agricultural Research and Development Institute (MARDI) in this research project is acknowledged and perhaps there will be more collaboration in the future.

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