Asian Journal of Chemistry; Vol. 30, No. 5 (2018), 975-978

ASIAN JOURNAL OF CHEMISTRY

https://doi.org/10.14233/ajchem.2018.21045

Phytochemicals from *Calophyllum depressinervosum*

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Received: 15 September 2017; *Accepted*: 6 February 2018; *Published online*: 29 March 2018; AJC-18823

A phytochemical investigation on the stem bark of *Calophyllum depressinervosum* resulted in the isolation of four xanthones, one coumarin and one kavalactone which were elucidated to be caloxanthone B (**1**), caloxanthone I (**2**), caloxanthone J (**3**), xanthochymone B (**4**), calopolynolide B (**5**) and desmethoxyyangonim (**6**). The structures of the compounds were elucidated using spectroscopic analysis such as 1D and 2D NMR together with MS technique. The dichloromethane extract of *Calophyllum depressinervosum* gave good cell viability on RAW246.7 cells for potential antiinflammatory test with an IC_{50} value of 17.19 ± 0.007 µg/mL.

Keywords: *Calophyllum depressinervosum*, Xanthone, Coumarin, Kavalactones, Antiinflammatory.

INTRODUCTION

The use of plants since ancient times by human civilization to treat and cure diseases has helped in the expansion and development of the pharmaceutical industries [1]. Malaysia, a tropical rain forest country is rich in a wide variety of plants. *Calophyllum depressinervosum* is one Calophyllum genus that grows abundantly in Malaysia. These plants are also known as bintagor lekok by local Malaysians [2]. This genus has gained medicinal uses such as antiseptics, astringents, diuretics and purgatives [3]. Besides, it also shows biological activities such as anti-HIV, anticancer, antifungal, antimicrobial, and antimalarial [4-6]. Our ongoing research on the chemical constituents and biological activities of *Calophyllum depressinervosum* has resulted in the isolation of four xanthones, one coumarin and one kavalactone (Fig. 1). They are caloxanthone B (**1**), caloxanthone I (**2**), caloxanthone J (**3**), xanthochymone B (**4**), calopolynolide A (**5**) and desmethoxyyangonin (**6**), respectively. Astonishingly, desmethoxyyangonin (**6**) from the class of kavalactone normally found in the Piperacease family was found in this species. This paper discussed the NO inhibition of the extracts of *Calophyllum depressinervosum*.

EXPERIMENTAL

The stem bark of *Calophyllum depressinervosum* was collected from Sri Aman district in Sarawak, Malaysia and identified by Prof. Dr. Rusea Go, Department of Biology, Faculty of Science, Universiti Putra Malaysia. A voucher specimen (RG5028) was deposited in the Herbarium of Biology Department, Faculty of Science, Universiti Putra Malaysia.

General procedure: 1D and 2D NMR spectra were obtained using a JOEL FT-NMR 500MHz spectrometer and using tetramethylsilane (TMS) as an internal standard. GC-MS were obtained using a shimadzu GCMS-QP5050. The ultraviolet spectra were recorded in ethanol on a Shimadzu UV-160A UV-Visible recording spectrometer. Meanwhile, the infrared spectra were measured using the universal attenuated total reflection (UATR) on a Perkin-Elmer 100 series FTIR spectrometer. The melting point was measured using a Leica Galen III microscope, equipped with a Testo 720 Temperature recorder.

Extraction and isolation: Air-dried stem bark of *Calophyllum depressinervosum* (~ 1.8 kg) was grounded into a fine powder. The powdered stem bark was then extracted three times by soaking in hexane at room temperature for 72 h. This same procedure was repeated for three other extraction solvents which are dichloromethane, ethyl acetate and methanol. All the extracts were evaporated to dryness under reduced pressure to obtain 27 g of hexane, 26 g of dichloromethane, 33 g of ethyl acetate and 87 g of methanol extracts. The extracts were chromatographed in a silica gel glass column under vacuum using a stepwise gradient system (hexane:dichloromethane, dichloromethane:ethyl acetate, ethyl acetate:methanol). Further purification of the hexane extract using silica gel column gravity column chromatography afforded caloxanthone B (**1**). Mean-

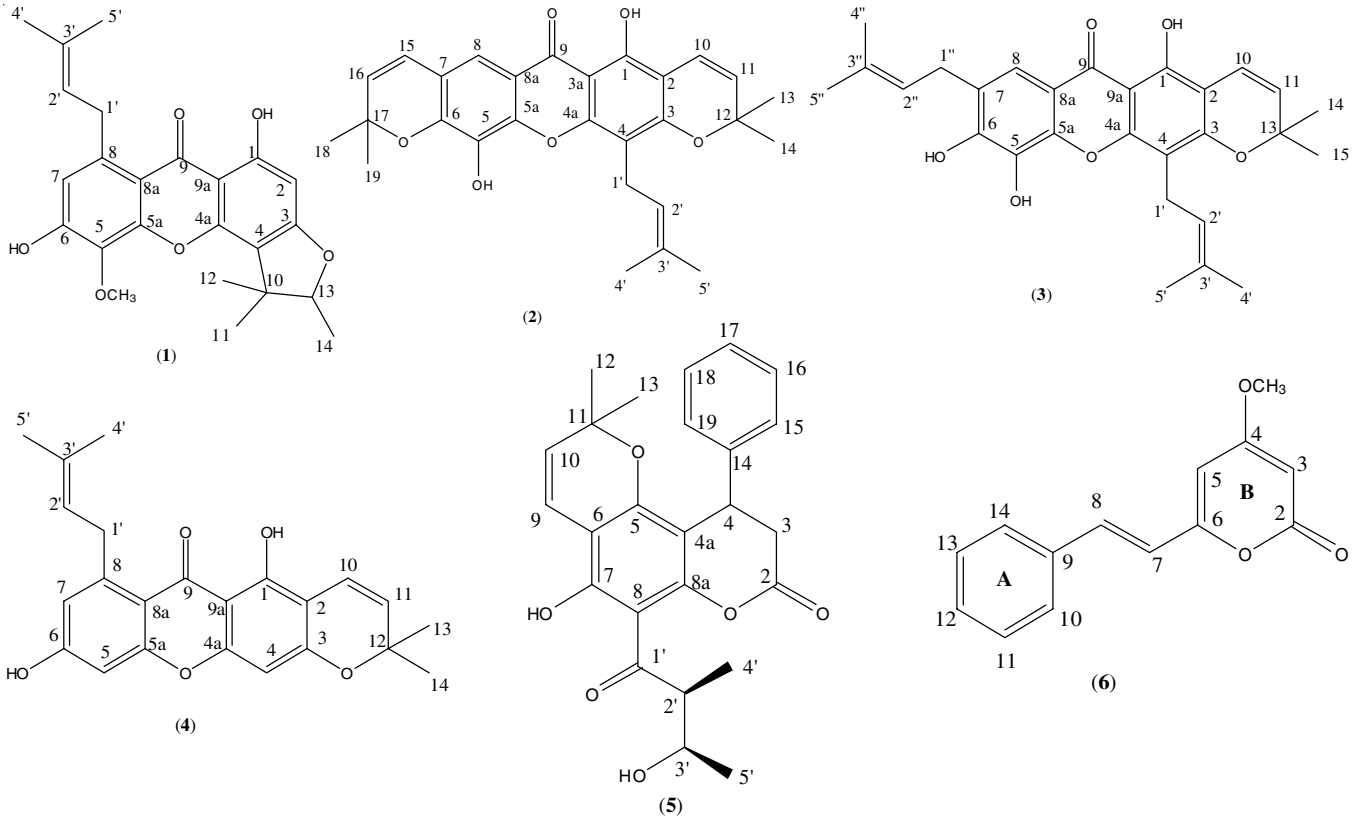

Fig. 1. Structure of caloxanthone B (**1**), caloxanthone I (**2**), caloxanthone J (**3**), xanthochymone B (**4**), calopolynolide A (**5**) and desmethoxyyangonin (**6**)

while, further purification on the dichloromethane extract using the same method gave caloxanthone I (**2**), caloxanthone J (**3**), xanthochymone B (**4**) and desmethoxyyangonin (**6**). Calopolynolide A (**5**) was obtained from a silica gel column gravity purification of the ethyl acetate extract using hexane:ethyl acetate (2:10) as mobile phase followed by further purification using a C-18 reverse phase column and eluting with acetonitrile.

Caloxanthone B (1): Yellow needle crystals; m.p: 159-161 ºC (literature 160.5 ºC, [Ref. 7]). IR (ν_{max} cm^{-1}): 3422, 2928, 1586, 1419, 1129. UV(EtOH) λ_{max} nm: 364, 318, 284 and 250; EIMS *m/z*: 410, 395, 367, 352, 325, 176. ^{1}H NMR (500 MHz, $CDCl_3$, δ): 13.75 (1H, s, OH-1), 6.21(1H, s, H-7), 6.21(1H, s, H-2), 5.35 (1H, t, J = 8.02, H-1′), 4.51 (1H, q, *J* = 5.73, H-13), 3.99 (3H, s, 5-OCH_3), 3.96 (2H, d, *J* = 8.02, H-1′), 1.74 (3H, s, H-5′), 1.70 (3H, s, H-4′), 1.58 (3H, s, H-12), 1.40 (3H, d, *J* = 5.73, H-14), 1.29 (3H, s, H-11). ^{13}C NMR (125 MHz, $CDCl_3$, δ): 182.2 (C-9), 165.6 (C-3), 164.5 (C-1), 153.6 (C-6), 151.7 (C-4a), 151.1 (C-5a), 142.0 (C-8), 133.4 (C-3′), 132.3 (C-5), 122.3 (C-2′), 113.5 (C-7), 112.7 (C-8a), 112.2 (C-4), 103.9 (C-9a), 94.0 (C-2), 90.7 (C-13), 61.9 (5-OCH_3), 43.7 (C-10), 33.6 (C-1′), 25.9 (C-4′), 25.6 (C-12), 21.6 (C-11), 18.0 (C-5′), 14.2 (C-14).

Caloxanthone I (2): Yellow amorphous powder. IR (ν_{max} cm^{-1}): 3398, 2918, 1570, 1436 and 1126; UV(EtOH) λ_{max} nm: 361, 294 and 228; EIMS *m/z*: 460, 445, 417, 405, 215, 187. ^{1}H NMR (500 MHz, $CDCl_3$, δ): 7.44 (1H, s, H-8), 6.74 (1H, d, *J* = 10.3, H-10), 6.43 (1H, d, *J* = 10.3, H-15), 5.71 (1H, d, *J* = 10.3, H-16), 5.59 (1H, d, *J* = 10.3, H-11), 5.49 (1H, s, 5-OH), 5.30 (1H, t, *J* = 8.02, H-2′), 3.51 (2H, d, *J* = 8.02, H-1′), 1.86 (3H, s, H-5′), 1.68 (3H, s, H-4′), 1.52 (6H, s, H-18 & H-19), 1.47 (6H, s, H-13 & H-14). ^{13}C NMR (125 MHz, $CDCl_3$, δ): 180.7 (C-9), 157.9 (C-3), 155.8 (C-1), 154.1 (C-4a), 145.3 (C-5a), 144.7 (C-6), 132.4 (C-5), 131.6 (C-3′), 130.9 (C-16), 127.3 (C-11), 122.4 (C-2′), 121.5 (C-15), 117.8 (C-7), 115.9 (C-10), 114.5 (C-8a), 113.3 (C-8), 107.6 (C-4), 104.5 (C-2), 103.1 (C-9a), 78.8 (C-17), 78.0 (C-12), 28.5 (C-18 & C-19), 28.4 (C-13 & C-14), 25.9 (C-4′), 21.6 (C-1′), 17.9 (C-5′).

Caloxanthone J (3): Yellow amorphous powder. IR (ν_{max} cm^{-1}): 3217, 2928, 1610, 1461, 1298 and 1151. UV(EtOH) λ_{max} nm: 382, 274 and 233. EIMS *m/z*: 462, 447, 419, 391, 215, 188. ^{1}H NMR (500 MHz, $CDCl_3$, δ): 13.27 (1H, s, 1-OH), 7.58 (1H, s, H-8), 6.74 (1H, d, *J* = 10.35, H-10), 6.05 (1H, s, 6-OH), 5.60 (1H, d, *J* = 10.35, H-11), 5.50 (1H, s, 5-OH), 5.35 (1H, t, *J* = 6.62, H-2′), 5.24 (1H, t, *J* = 8.02, H-2′), 3.49 (2H, d, J = 8.02, H-1′), 3.42 (2H, d, *J* = 6.62, H-1′), 1.86 (3H, s, H-4′), 1.76 (3H, s, H-5′), 1.74 (3H, s, H-4′), 1.70 (3H, s, H-5′), 1.48 (6H, s, H-13 & H-14). ^{13}C NMR (125 MHz, $CDCl_3$, δ): 180.7 (C-9), 157.7(C-3), 156.1 (C-1), 153.9 (C-4a), 147.6 (C-6), 143.6 (C-5a), 134.4 (C-3′), 131.5 (C-3′), 130.2 (C-5), 127.3 (C-11), 125.4 (C-7), 122.9 (C-2′), 121.1 (C-2′), 117.1 (C-8), 115.9 (C-10), 113.5 (C-8a), 106.9 (C-4), 104.7 (C-2), 103.0 (C-9a), 78.1 (C-12), 28.5 (C-1′), 28.4 (C-13 & C-14), 25.7 (C-5′), 25.9 (C-5′), 21.7 (C-1′), 18.0 (C-4′), 17.9 (C-4′).

Xanthochymone B (4): Yellow gum. IR (ν_{max} cm^{-1}): 2978, 1611, 1455, 1295, 1145. UV(EtOH) λ_{max} nm: 338, 264, 218. EIMS *m/z*: 378, 363, 335, 279. ^{1}H NMR (500 MHz, $CDCl_3$, δ): 13.54 (1H, s, 1-OH), 7.20 (2H, s, H-5 & H-7), 6.73 (1H, d, *J* = 10.31, H-10), 6.25 (1H, s, H-4), 5.58 (1H, d, *J* = 10.31, H-11), 5.41 (1H, s, 6-OH), 5.27 (1H, t, *J* = 6.87, H-2′), 2.28 (2H, d, *J* = 6.87, H-1′), 1.87 (3H, s, H-4′), 1.75 (3H, s, H-5′), 1.46 (6H, s, H-13 & H-14). ^{13}C NMR (125 MHz, $CDCl_3$, δ): 183.4 (C-9),

160.4 (C-3), 158.0 (C-1), 156.6 (C-5a), 152.0 (C-4a), 151.2 (C-6), 135.0 (C-3′), 127.2 (C-11), 127.0 (C-8a), 123.6 (C-5), 121.5 (C-2′), 118.5 (C-8), 116.8 (C-7), 115.7 (C-10), 104.3 (C-2), 104.2 (C-9a), 94.1 (C-4), 78.1 (C-12), 28.4 (C-13 & C-14), 25.7 (C-1′), 25.1 (C-5′), 18.17 (C-4′).

Calopolynolide A (5): Yellowish oil; IR (ν_{max} cm^{-1}): 2977, 1624, 1447 and 1128. UV(EtOH) λ_{max} nm: 313, 271 and 228. EI-MS *m/z*: 422, 407, 363, 291, 203, 107. ^{1}H NMR (500 MHz, $CDCl_3$, δ): 12.36 (1H, s, 7-OH), 7.27 (2H, m, H-15 & H-19), 7.23 (2H, m, H-16 & H-18), 7.14 (1H, m, H-17), 6.56 (1H, d, *J* = 10.31, H-9), 5.42 (1H, d, *J* = 10.31, H-10), 5.02 (1H, m, H-4), 4.55 (1H, m, H-3′), 3.23 (2H, m, H-3), 2.59 (1H, m, H-2′), 1.39 (3H, s, H-12), 1.29 (3H, d, *J* = 5.73, H-5′), 1.10 (3H, s, H-13), 1.08 (3H, d, *J* = 8.02, H-4′). ^{13}C NMR (125 MHz, $CDCl_3$, δ): 200.9 (C-1′), 178.5(C-2), 159.5 (C-5), 159.1 (C-8a), 157.6 (C-7), 143.3 (C-14), 127.9 (C-16 & C-18), 127.5 (C-15 & C-19), 125.9 (C-10 & C-17), 115.6 (C-9), 109.9 (C-4a), 102.9 (C-9), 101.4 (C-8), 78.4 (C-11), 76.4 (C-3′), 44.2 (C-2′), 36.9 (C-3), 35.1 (C-4), 28.4 (C-12), 27.6 (C-13), 19.5 (C-5′), 9.36 (C-4′).

Desmethoxyyangonin (6): White needle crystals; m.p: 139-142 ºC (literature 138-140 ºC [Ref. 8]. IR (ν_{max} cm^{-1}): 3077, 2939, 1714, 1638, 1406, 1253 and 1152. UV(EtOH) λ_{max} nm: 321, 282 and 175. EI-MS *m/z*: 228, 200, 157, 129, 69. ^{1}H NMR (500 MHz, $CDCl_3$, δ): 7.51 (3H, m, H-8, H-11 & H-13), 7.48 (3H, m, H-10, H-12 & H-14), 6.59 (1H, d, *J* = 16.05, H-7), 5.94 (1H, d, *J* = 3.4, H-5), 5.49 (1H, d, *J* = 3.4, H-3), 3.82 (3H, s, 4-OCH_3). ^{13}C NMR (125 MHz, $CDCl_3$, δ): 171.1(C-4), 164.1 (C-2), 158.7 (C-6), 135.9 (C-8), 135.3 (C-9), 129.5 (C-12), 128.9 (C-10 & C-14), 127.5 (C-11 & C-13), 118.7 (C-7), 101.4 (C-5), 88.9 (C-3), 56.0 (C-4OCH_3).

NO inhibition assay: Raw cells (2×10^6 cells/mL) were seeded in a 96-well plate and incubated for 24 h. These cells were then treated and induced with 10 µg/mL lipopolysaccharide (LPS) in the presence of ethyl acetate extract and made up to a final volume of 100 µL and further incubated for 24 h. Griess reagent (50 µL) was then added to react with 50 µL of cell-free culture supernatant and incubation was then carried out for 10 min at room temperature. These cells were introduced into the microplate reader and readings were taken at 550 nm. A fresh culture medium was used as blank. The result was expressed as a mean ± SEM.

RESULTS AND DISCUSSION

The purification of fraction 3 of dichloromethane extract using hexane and chloroform as mobile phase yielded xanthochymone B (**4**). Compound **4** was obtained as a yellow gum. The molecular formula $C_{23}H_{22}O_5$ was deduced from the EIMS spectrum where M^+ was at *m/z* 378. The FTIR spectrum exhibited absorption bands at 2978 cm^{-1} (C-H stretch), 1611 cm^{-1} (C=O), 1455 cm^{-1} (C=C aromatic), 1295 cm^{-1} (CH_3) and 1145 cm^{-1} (C-O stretch) due to functional groups present in compound **4**. The UV spectrum gave maxima absorption peaks at 338, 264 and 218 indicating a xanthone derivative. The ^{13}C and DEPT spectra for compound **4** showed resonances for twelve quaternary carbons, six methines, one methylene and four methyls. ^{1}H NMR spectrum displayed a characteristics resonance for a chelated phenolic hydroxyl group at δ13.54 (1-OH). The HMBC spectrum gave correlation peaks between δ13.54 (1-OH) with the aromatic carbons at δ158 (C-1), δ104.3 (C-2) and δ104.2 (C-9a). Three aromatic carbons were present in compound **4** which are δ6.25 (H-4) and δ7.20 (H-5 and H-7). The aromatic proton at δ6.25 (H-4) was assigned at position C-4 due to its correlation with the aromatic carbon at δ104.2 (C-9a) in the HMBC spectrum. The ^{1}H NMR spectrum showed signals of two methyl groups at δ1.46 (H-13 and H-14) and two *cis*-olefinic protons at δ6.73 (H-10) and δ5.58 (H-11) indicative of a pyrano moiety in compound **4**. Both protons were adjacent as shown by a coupling between these two protons at δ6.73 (H-10) and δ5.58 (H-11) in the COSY spectrum. The HMBC correlation between the two olefinic protons with the aromatic carbon determined the position of the pyrano group to be at C-2 and C-3. The HMBC spectrum showed ^3J correlations between the proton at δ6.73 (H-10) with the carbon at δ160.4 (C-3) and the proton at δ5.58 (H-11) with the carbon at δ104.3 (C-2). The presence of the two methyl groups attached to C-13 was confirmed by cross peaks in the HMBC spectrum between methyl proton at δ1.46 (H-13 and H-14) with the carbons at δ78.16 (C-12) and δ127.28 (C-11). The ^{1}H NMR spectrum displayed characteristic resonances for one prenyl unit. The prenyl moiety consist of one methylene group (δ4.28, H-1′), one methine group (δ5.27, H-2′) and two methyl groups (δ1.87, H-4′ and δ1.75, H-5′). The 1H-1H COSY showed a coupling between the methylene proton at δ4.28 (H-1′) with the olefinic methyl proton at δ5.27 (H-2′) implying these protons were adjacent to each other. Meanwhile, the attachment of the two methyls (δ1.8, H-4′ and δ1.75, H-5′) at position C-3 was confirmed by the HMBC correlations between these two methyl protons with the carbons at δ135.0 (C-3′) and δ121.5 (C-2′). The prenyl was located at peri position (C-8) relative to the carbonyl group based on the HMBC correlation contours. Correlation peaks between δ2.28 (H-1′) with the carbons at δ127.0 (C-8), δ118.5 (C-8a) and δ151.2 (C-6) were shown in the HMBC spectrum. From the information obtained, compound **4** was elucidated as xanthochymone B (**4**) previously isolated from twigs of *Garcinia xanthochymus* [9].

Compound **5** was isolated as yellowish oil from ethyl acetate extract. A molecular formula of $C_{25}H_{26}O_6$ was determined for compound **5** from its mass spectrum (*m/z* 422). The FTIR gave absorptions at 2977 (sp^3 C-H), 1624 (C=O), 1447 (aromatics C=C) and 1128 (C-O-C) while the UV spectrum showed absorptions at 313, 271 and 228 nm. The ^{1}H NMR spectrum showed a singlet peak at δ12.36 (7-OH) for a chelated hydroxyl group attached to the coumarin ring carbon at δ157.6 (C-7). This position was confirmed by HMBC experiment from ^2J and ^3J correlations between the hydroxyl proton at δ12.36 (7-OH) with the carbons at δ157.6 (C-7) and δ101.4 (C-8), respectively. Meanwhile, two proton signals for the protons attached to the coumarin skeleton at the right ring were seen at δ3.23 (H-3) and δ5.02 (H-4). The HMBC gave ^2J and ^3J correlations between both signals at δ5.02 (H-4) and δ3.26 (H-3) and the carbons at δ109.9 (C-4a) and δ178.54 (C-2) indicating their position. Moreover, these two proton signals showed couplings in ^{1}H-^{1}H COSY implying they are adjacent to each other. Three triplet signals at δ7.27 (H-15 & H-19), δ7.23 (H-16 & H-18) and δ7.14 (H-17) which integrated for 5 protons indicated a phenyl ring to be present in the molecule. The location of the phenyl

group at the carbon at δ35.1 (C-4) is accounted for a cross peak between the proton at δ5.02 (H-4) with the carbons at δ143.3 (C-14) and δ127.5 (C-15 & C-19) together with cross peak signals between the protons at δ3.23 (H-3) with the carbon at δ143.3 (C-14). The presence of two doublet proton signals at δ5.42 (H-10) and δ6.56 (H-9) show a pyrano group to be present in compound **5**. The attachment of this pyrano group at carbon δ102.9 (C-6) and δ159.5 (C-5) was confirmed through ^3J proton-carbon correlation between δ6.56 (H-9) with δ159.5 (C-5) and δ5.42 (H-10) with δ102.9 (C-6), respectively. Hence, the final substituent which consists of $CH_3CHOHCHCH_3CO$ chain was duly assigned to δ101.4 (C-8). This substituent moiety gave proton signals which are a doublet at δ1.08 (H-4′) for the methyl attached to δ44.2 (C-2′), another doublet at δ1.29 (H-5′) for the methyl attached to δ76.4 (C-3′). Another two proton signal which is multiplet at δ2.59 (H-2′) and δ4.57 (H-3′) were represented as methines moiety. As a result, the structure was characterized as calopolynolide A (**5**) as previously isolated from *Calophyllum polyanthum* [10].

Desmethoxyyangonim (**6**), kavalactoned, was successfully isolated from the dichloromethane extract of *Calophyllum depressinervosum*. Desmethoxyyangonim (**6**) is a major compound in piper species. Compound **6** was obtained as white needle crystals with a melting point of 139-142 ºC (lit. m.p. 138-140 ºC [8]). The M^+ peak was observed at *m/z* 228 indicating a molecular formula $C_{14}H_{12}O_3$. The FTIR spectrum showed absorption band at 3077 cm^{-1} (OH stretch), 1714 cm^{-1} (C=O), 1638 cm^{-1} (*trans* RCH=CHR), 1406 cm^{-1} (C=C aromatics), 1253 cm^{-1} (CH_3) and 1152 cm^{-1} (C-O stretch). The UV spectrum showed absorption maxima at 321, 282 and 175 indicative of the existence of a kavalactone skeleton. The ^{13}C and DEPT spectra of compound **6** revealed the presence of 14 carbons which consist of one methyl, ten methines and three quaternary carbons. The presence of a carbonyl carbon in compound **6** was shown in the ^{13}C NMR spectrum at low field region, δ164.1 (C-2). The protonated carbons presences in compound **6** were correlated with their protons based on ^1J correlation between protons and carbons in the HMQC spectrum. The ^1H NMR spectrum of compound **6** showed one phenyl moiety (δ7.48, H-10, H-12 and H-14 and δ7.51, H-11 and H-13), one pair of trans olefinic protons (δ7.51, H-8 and δ6.59, H-7), one methoxy moiety (δ3.87, 4-OCH_3) and two protons in *meta*-position (δ5.94, H-5 and δ5.49, H-3). Ring A (phenyl group) and ring B were connected *via* a pair of *trans*-olefinic protons. The coupling between the two *trans*-olefinic protons was determined with a coupling constant value of 16.05 Hz from the COSY spectrum. The arrangements of all substituent moieties were determined through long range correlation in the HMBC spectrum. The phenyl moiety attached to C-8 based on ^2J and ^3J correlations between δ7.51 (H-8) and δ135.3 (C-9), between δ7.48 (H-14 and H-10) and δ135.9 (C-8) and also between δ6.59 (H-7) and δ135.3 as observed in the HMBC spectrum. Meanwhile, the assignment of ring B attached to C-7 position was confirmed *via* ^2J and ^3J correlations between δ6.59 (H-7) and δ158.7 (C-6) and δ101.4 (C-5), between δ5.94 (H-5) and δ118.7 (C-7) and between δ7.51 (H-8) and δ158.7 (C-6). The methoxy hydrogen (δ3.82, 4-OCH_3) gave a ^3J correlation with the carbon at δ171.1 (C-4), thus suggesting this methoxy moiety was substituted at ring B which is attached to C-4. Therefore, compound **6** was identified as desmethoxyyangonim (**6**) previously isolated from *Piper methysticum* by Daharmaratne *et al*. [8].

The hexane and dichloromethane extracts showed good NO inhibition in stimulated RAW 264 cells. This showed that both extracts could have potential antiinflammatory properties. Table-1 summarizes the result for the NO inhibition in lipopolysaccharide (LPS) stimulated RAW 264.7 cell treated with each extract of *Calophyllum depressinervosum*.

TABLE-1
NITRIC OXIDE INHIBITION IN LIPOPOLYSACCHARIDE (LPS) STIMULATED RAW 264.7 CELL TREATED WITH CRUDE EXTRACTS

Sample	IC_{50} (μg/mL)
Methanol extract	> 100
Ethyl acetate extract	> 100
Dichloromethane extract	17.19 ± 0.007
Hexane extract	25.41 ± 0.0187

Note: Each value of IC_{50} represented mean ± S.E.M

Conclusion

In conclusion, the isolation work on stem bark of *Calophyllum depressinervosum* furnished six compounds, caloxanthone B (**1**), caloxanthone I (**2**), caloxanthone J (**3**), xanthochymone B (**4**), calopolynolide B (**5**) and desmethoxyyangonim (**6**). Meanwhile, dichloromethane and hexane extracts gave good no inhibition in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells.

ACKNOWLEDGEMENTS

The authors acknowledge the financial support from Universiti Putra Malaysia under the RUGS research fund. The Sarawak Biodiversity Centre (SBC) is also acknowledged.

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