

## **Madangones A and B: Two new neolignans from the stem bark of *Beilschmiedia madang* and their bioactivities**

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## ABSTRACT

Two new neolignans, madangones A (**1**) and B (**2**), together with (+)-kunstlerone (**3**), vanillin, vanillic acid, betulin,  $\beta$ -sitosterol and  $\beta$ -sitostenone, were isolated from the stem bark of *Beilschmiedia madang* (**Lauraceae**). The structures of the compounds were determined by spectroscopic means. The compounds were tested for antioxidant, acetylcholinesterase inhibitory and anti-inflammatory activities. Compound (**3**) displayed the strongest DPPH radical-scavenging activity with an  $IC_{50}$  value of 68.7  $\mu$ M. Compound (**2**) exhibited the highest level of activity on the COX-2 model and acetylcholinesterase inhibition assay, with  $IC_{50}$  values of 27.4 and 70.3  $\mu$ M, respectively.

*Keywords:*

*Beilschmiedia madang*

Neolignan

Antioxidant

Acetylcholinesterase

Anti-inflammatory

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## 1. Introduction

The pantropical genus *Beilschmiedia* Nees of the family Lauraceae comprises about 250 species, distributed mainly in tropical regions of Asia and Africa. Most of these species grow in tropical climate, and are widespread in tropical Asia, Africa, Australia, New Zealand, Central America, Caribbean Islands and South America (Nishida, 1999). This genus produces alkaloids, endiandric acid derivatives, essential oils, fatty acids, epoxyfuranoid lignans, flavonoids and terpenoids, and many of these compounds possess antioxidant, antibacterial, antimalarial and anti-tuberculosis activities (Chen et al., 2007; Lenta et al., 2009; Chouna et al., 2010). A few species of this genus are used in traditional medicines for the treatment of various diseases. For example, the stem bark of *B. anacardioides* is used in Cameroon to treat uterine tumours, rubella, rheumatism, bacterial and fungal infections, while the seeds are used as spices (Tchoula, 2001); *B. manii* is used to treat dysentery and headache, and also as an appetite stimulant (Iwu, 1993); the stem bark of *B. pahangensis* is used in a drink usually given after childbirth and also for stomach pains and diarrhoea; the leaves of *B. tonkinensis* are used by Indonesians and Malays to make poultices for application on the broken bones (Wiart, 2006).

*Beilschmiedia madang* is commonly known as 'medang merah' or 'lahu' in Malaysia. It grows in primary, secondary or mixed dipterocarp forest, and on sandy loam soils in Malaysia and Indonesia (Nishida, 2008). Dehatrine, a bisbenzylisoquinoline alkaloid, was isolated previously from *B. madang* and found to have antimalarial activity (Kitagawa et al., 1993). The chemical compositions and biological activities of the leaf and bark oils from *B. madang* have been reported recently (Salleh et al., 2015a). The bark oil showed significant  $\beta$ -carotene/linoleic acid bleaching (90.3%) and DPPH radical-scavenging ( $IC_{50}$  212.0  $\mu$ g/mL) activities, and strong antifungal property against *Aspergillus niger* and *A. fumigatus* (MIC 62.5  $\mu$ g/mL). The leaf oil also displayed significant inhibition of acetylcholinesterase (55.2%), butyrylcholinesterase (60.4%) and antityrosinase (53.1%) activities (Salleh et al., 2014). In

continuation of our phytochemical and bioactivity studies on this genus (Salleh et al., 2015a,b), herein we report the isolation, identification and bioactivities (antioxidant, acetylcholinesterase inhibitory and anti-inflammatory) of compounds from the stem bark of *B. madang* (**Fig. 1**).

## 2. Results and discussion

Compound (**1**) was obtained as a white amorphous solid,  $[\alpha]_D^{20} = +0.29$ . The EIMS spectrum revealed the molecular ion peak  $[M]^+$  at  $m/z$  386 corresponding to the molecular formula  $C_{22}H_{26}O_6$ . The IR spectrum revealed absorption bands at 1699 and 1595  $cm^{-1}$  for carbonyl and olefinic stretching vibrations, respectively (Rossi, 1997). The  $^1H$  NMR spectrum (Table 1) established the presence of three ABX type phenyl protons at  $\delta_H$  6.83 (1H, d,  $J = 8.0$  Hz, H-5'), 6.82 (1H, dd,  $J = 8.0$  and 1.6 Hz, H-6'), and 6.92 (1H, d,  $J = 1.6$  Hz, H-2'), four methoxyl groups at  $\delta_H$  4.13, 3.92, 3.88, and 3.74, and protons of a propenyl group H-10 ( $\delta_H$  2.42, m), H-11 ( $\delta_H$  5.70, m) and H-12 ( $\delta_H$  5.13, d,  $J_{trans} = 16.0$  Hz/ $\delta_H$  5.17, d,  $J_{cis} = 10.4$  Hz). The proton at C-7 of the cyclobutane moiety in compound (**1**) (**Fig. 1**) produced a doublet of doublets at  $\delta_H$  3.30 with coupling constants of  $J = 8.4$  and 7.6 Hz, which indicated that it was *trans* to H-8 ( $\delta_H$  2.92) and H-6 ( $\delta_H$  2.96, d,  $J = 8.4$  Hz). The COSY spectrum (Table 1) displayed coupling between olefinic protons, H-12 with H-11 along with cross peaks of proton H-7 with H-6 and H-8. In addition, protons of H-10 also produced cross peak with the olefinic proton H-11. The  $^{13}C$  NMR spectrum (Table 1) showed the presence of 22 signals and the DEPT spectra characterized them as seven quaternary, eight methines, three methylenes, and four methoxyl carbons. The HMQC spectrum exhibited direct connectivities ( $^1J$  coupling) of all protons with associated carbons, e.g., H-7 to C-7 ( $\delta_C$  44.2), H-8 to C-8 ( $\delta_C$  49.1), and H-9 to C-9 ( $\delta_C$  70.8), and thus confirmed the assignment of all  $^1H$  and  $^{13}C$  NMR signals corresponding to methine, methylene, and methoxyl groups. The HMBC spectrum (Table 1) displayed cross peak between H-10 with C-11/C-6/C-8/C-1, while H-9 was observed to

correlate with C-1/C-6/C-7/C-8. Compound (1) and (3) showed similar  $^1\text{H}$  and  $^{13}\text{C}$  NMR profiles except the methylenedioxy group at C-3 and C-4 in (3) are replaced by two methoxyl groups in (1). These assignments were further confirmed by HMQC which displayed correlations between methoxyl proton signals at  $\delta_{\text{H}}$  3.92 (H-3') and 3.88 (H-4') with carbon signals at  $\delta_{\text{C}}$  56.0 (C-3') and 55.9 (C-4'). The HMBC spectrum (Table 1) displayed, among the key  $^1\text{H}$ - $^{13}\text{C}$  long-range correlations,  $^3J$  correlations from H-10 to C-6 ( $\delta_{\text{C}}$  50.0), C-8 ( $\delta_{\text{C}}$  49.1), and  $^2J$  to C-1 ( $\delta_{\text{C}}$  45.9) and C-11 ( $\delta_{\text{C}}$  132.0), while H-9 was observed to correlate  $^3J$  with C-1 ( $\delta_{\text{C}}$  45.9), C-2 ( $\delta_{\text{C}}$  79.1) and C-7 ( $\delta_{\text{C}}$  44.5), and  $^2J$  with C-8 ( $\delta_{\text{C}}$  49.5), and a *zig-zag*  $^4J$  coupling to C-6 ( $\delta_{\text{C}}$  50.0). In the NOESY spectrum, among the nOes, the key nOe correlations were between H-6/H-8, H-2/3-OCH<sub>3</sub>, H-6/H-10, and H-7/H-9. Thus, based on the NOESY spectrum and comparison of its optical rotation with that of the known compound (3) (Eagler et al., 1994; Mollataghi et al., 2011), the relative configuration of the five stereogenic centers in compound (1) were assigned as 7*R*, 8*R*, 1*R*, 6*S* and 2*R*. On the basis of the spectroscopic evidence discussed above, compound (1) was unequivocally identified as 3,4,3',4'-tetramethoxy-2,9-epoxy-6,7-cyclo-1,8-neolign-11-en-5-one, or (+)-madangone A (1), a new furonolignan, from *B. madang*.

Compound (2) was obtained as a white amorphous solid,  $[\alpha]_D^{20} = -0.16$ . The IR spectrum showed the presence of a hydroxyl group at  $3466\text{ cm}^{-1}$ , and a highly strained carbonyl group at  $1738\text{ cm}^{-1}$ . The  $^1\text{H}$  NMR spectrum of compound (2) (Table 1) displayed similar signal patterns as observed in the  $^1\text{H}$  NMR spectrum of compound (1), e.g., the presence of ABX type phenyl protons (H-5'/H-6'/H-2'), and protons of the cyclobutane group (H-5/H-6/H-7). The spectrum also showed the presence of two equivalent methylene protons H-8 at  $\delta_{\text{H}}$  3.68 (2H, dd,  $J = 13.2, 8.4\text{ Hz}$ ). Clearly, the signals for four methoxyl groups as found in the  $^1\text{H}$  NMR spectrum of compound (1) (Table 1) were absent in the  $^1\text{H}$  NMR spectrum of compound (2). Instead, a 1H singlet signal at  $\delta_{\text{H}}$  6.55 could be attributed to H-3, and a sharp 2H singlet at  $\delta_{\text{H}}$

5.97 was characteristic for a methylenedioxy functionality linked to the phenyl ring in compound (2) (Fig. 1). Cross peaks between methylene protons of H-11 ( $\delta_{\text{H}}$  5.12 and 5.15) and H-10 ( $\delta_{\text{H}}$  5.76) along with H-8 ( $\delta_{\text{H}}$  3.66 and 3.68) with H-7 ( $\delta_{\text{H}}$  2.69) were observed in the COSY spectrum (Table 1). The  $^{13}\text{C}$  NMR, DEPT and HMQC spectra exhibited eighteen carbon, assigned for six quaternary, eight methine, and four methylene carbons (one of them was a characteristic methylenedioxy signal). The HMBC spectrum (Table 1) revealed connectivities of proton H-5 and H-6 with C-1/C-6/C-7/C-8/C-9. Proton H-8 was found to correlate with C-6/C-7/C-9/C-1, but there was no correlation observed between H-8 and C-2 (as it was present in compound 1). The HMBC experiment also showed H-3 ( $\delta_{\text{H}}$  6.55) correlating ( $^2J$ ) with the carbonyl group at C-4 ( $\delta_{\text{C}}$  203.4). Comparing the spectroscopic data and optical rotation data of compound (2) with related compounds published in the literature (Mollataghi et al., 2011), the relative configurations at the stereogenic centres in compound (2) were assigned as 6*R* and 7*S*. The above data were in complete agreement with the EIMS spectrum which exhibited the molecular ion peak at  $m/z$  314 corresponding to the molecular formula  $\text{C}_{18}\text{H}_{18}\text{O}_5$ . Thus, compound (2) was identified as 2,8-dihydroxy-3',4'-methylenedioxy-5,6-cyclo-1,7-neolign-10-en-4-one, or (-)-madangone B (2). All known compounds, vanillin, vanillic acid, betulin (Lin et al., 2010),  $\beta$ -sitosterol (Huang et al., 2011) and  $\beta$ -sitostenone (Lin et al., 2009), were identified by comparison of their spectroscopic data with literature data. The plants of the Lauraceae tend to produce alkaloids and lignans/neolignans, but generally not both groups of compounds in a single plant. However, there are also examples of plants of this family (including *Beilschmiedia madang*) where alkaloids and lignans/neolignans coexist. Because of this, it is somewhat difficult to draw any conclusive chemotaxonomic inference just on the basis of the presence or absence of alkaloids or lignans/neolignans in the species of the genus *Beilschmiedia* or the family Lauraceae.

Several biological activities of lignans and neolignans, e.g., cytotoxic, antitumor, antileukaemia, antiviral, antimicrobial, anti-inflammatory, anti-allergy, as well as antifungal, insecticidal and miscellaneous physiological effects, have been reported to date (MacRae and Towers, 1984; Whiting, 1987). The neolignans (**1-3**), isolated in this study, were tested for their antioxidant, acetylcholinesterase inhibitory and anti-inflammatory activities. The results are showed in **Table 2**. The antioxidant activity was evaluated by using the DPPH free-radical-scavenging with a series of different concentrations. Compounds (**1**) and (**3**) showed a strong antioxidant activity comparable to that of ascorbic acid with  $IC_{50}$  the values of 73.5 and 68.7  $\mu$ M. Previous study on compound (**3**) has showed a potent antioxidant activity (Mollataghi et al., 2011). The compounds were screened for AChE inhibitory activity using the Ellman's colorimetric method in a 96-well plate. Compound (**2**) had better activity than that of compound (**1**) against AChE with an  $IC_{50}$  value of 70.3  $\mu$ M. **It can be noted that in a previous study, the bark oil showed DPPH radical-scavenging ( $IC_{50}$  212.0  $\mu$ g/mL) activity, and the leaf oil inhibited acetylcholinesterase (55.2%) (Salleh et al., 2014). Both these activities, as observed with the purified compounds (**1-3**) in the present study, have further confirmed the potential bioactivities of this plant.**

In the COX-2 assay, compound (**1**) furnished strong activity with an  $IC_{50}$  value of 59.4  $\mu$ M. The  $IC_{50}$  value for indomethacin, used as a positive control, was 21.3  $\mu$ M. The chemistry of the Lauraceae family is recognized by the presence of neolignans, which are known to possess anti-inflammatory activity through suppression of tumour necrosis factor (TNF)- $\alpha$  and nitric oxide (NO) production (Cho et al., 2000) and inhibitory activity against the two isozymes of COX- 2 (Su et al, 2002). These results imply that the neolignans isolated from *B. madang* might be beneficial in the treatment of Alzheimer and vascular diseases, predominantly caused by oxidative cell damages.

### **3. Experimental**

### 3.1. General experimental procedures

The optical rotations were recorded on a Perkin Elmer 341 Polarimeter equipped with a sodium lamp and chloroform as solvent. The mass spectra were recorded on Bruker Mass Spectrometry, obtained from the National University of Singapore (NUS). The UV spectra were obtained in methanol on a Shimadzu UV 1601PC spectrophotometer. The IR spectra were obtained on a Perkin-Elmer 1600 FTIR spectrophotometer. The 1D and 2D NMR spectra were recorded in deuterated chloroform on a Bruker Avance 400 MHz spectrometer, chemical shifts ( $\delta$ ) are reported in ppm on  $\delta$  scale, and the coupling constants ( $J$ ) are given in Hz. Column chromatography was carried out on silica gel 70-230 mesh (Merck). Silica gel 60 F<sub>254</sub> precoated aluminium plates (0.2 mm, Merck) were used for TLC analysis. The TLC and PTLC spots were visualized under UV light (254 and 366 nm) followed by spraying with 5% H<sub>2</sub>SO<sub>4</sub> and 1% vanillin in MeOH and heating at 120°C for 5 min. All solvents were AR grade.

### 3.2. Plant material

The stem bark of *Beilschmiedia madang* Blume was collected from Hutan Simpan Bangi, Selangor, Malaysia, in September 2012. The species was identified by Dr. Shamsul Khamis from Institute of Bioscience, Universiti Putra Malaysia and a voucher specimen, SK1984/2012 was deposited at the Herbarium of IBS, UPM.

### 3.3. Extraction and isolation

Cold extraction of the powdered stem bark (3 kg) of *B. Madang*, sequentially, with *n*-hexane, EtOAc and MeOH yielded the crude extracts, *n*-hexane (BMBH: 14.5 g, 0.7%), EtOAc (BMBE: 26.2 g, 1.3%) and MeOH (BMBM: 28.1 g, 1.4%). The EtOAc extract (10.0 g) was fractionated by VLC on silica gel, eluting with *n*-hexane:CHCl<sub>3</sub>:EtOAc to afford 15 major fractions (BMBE1-15). Fractions BMBE2-6 and BMBE10-15 were purified by CC on silica gel to obtain compounds (**1**) (344.0 mg) and (**2**) (34.4 mg), respectively. Similar VLC (on silica gel) purification of the *n*-hexane extract (10.0 g) eluting with *n*-hexane:CHCl<sub>3</sub>:EtOAc afforded



20 major fractions (BMBH1-20). The combined fractions BMBH15-20 was subjected to multiple CC on silica gel to yield compound (**3**) (462.9 mg). The MeOH extract (BMBM) (10.0 g) was subjected to VLC on silica gel eluting with *n*-hexane:CHCl<sub>3</sub>:EtOAc to afford 20 major fractions. Fraction BMBM7-10 was purified by multiple CC on silica gel to yield vanillin (25.0 mg) and vanillic acid (15.0 mg). CC (on silica gel) purification of the fractions BMBM2-4 and BMBM5-6 followed by washing with cold *n*-hexane produced betulin (10.2 mg), β-sitostenone (12.8 mg) and β-sitosterol (40.5 mg), respectively.

(+)-Madangone A (**1**): white amorphous solid,  $[\alpha]_D^{20} = +0.29$  (*c* 1.2, CHCl<sub>3</sub>); UV<sub>max</sub> (MeOH) 231 (1.48) and 285 (1.24) nm; IR (KBr): 3075, 2923, 1699, 1595, 1515, 1463, 1272, 1116 cm<sup>-1</sup>; HREIMS *m/z* 386.1736 (calculated 386.1729 for C<sub>22</sub>H<sub>26</sub>O<sub>6</sub>); <sup>1</sup>H-NMR (CDCl<sub>3</sub>, 400 MHz), <sup>13</sup>C-NMR (CDCl<sub>3</sub>, 100 MHz), COSY, HMBC and NOESY data see **Table 1**.

(-)-Madangone B (**2**): white amorphous solid,  $[\alpha]_D^{20} = -0.16$  (*c* 0.18, CHCl<sub>3</sub>); UV<sub>max</sub> (MeOH) 230 (1.30) and 275 (1.54) nm; IR (KBr): 3466, 3066, 2927, 1738, 1654, 1614, 1490, 1249, 1038 cm<sup>-1</sup>; HREIMS *m/z* 314.1147 (calculated 314.1154 for C<sub>18</sub>H<sub>18</sub>O<sub>5</sub>); <sup>1</sup>H-NMR (CDCl<sub>3</sub>, 400 MHz), <sup>13</sup>C-NMR (CDCl<sub>3</sub>, 100 MHz), COSY and HMBC data see **Table 1**.

(+)-Kunstlerone (**3**): white amorphous solid; IR (KBr): 3071, 2927, 1727, 1491, 1442, 1231; <sup>1</sup>H-NMR (CDCl<sub>3</sub>, 400 MHz): δ 2.42 (m, 2H, H-10), 2.86 (dq, 1H, *J* = 10.8, 6.8 Hz, H-8), 2.93 (d, 1H, *J* = 8.0 Hz, H-6), 3.25 (dd, 1H, *J* = 7.6, 7.6 Hz, H-7), 3.77 (dd, 1H, *J* = 9.6, 3.6 Hz, H-9), 3.95 (d, 1H, *J* = 9.6 Hz, H-9), 4.36 (s, 1H, H-2), 5.14 (d, 1H, *J*<sub>trans</sub> = 16.0 Hz, H-12), 5.18 (d, 1H, *J*<sub>cis</sub> = 10.4 Hz, H-12), 5.69 (m, 1H, H-11), 6.75 (d, 1H, *J* = 1.6 Hz, H-6'), 6.77 (d, 1H, *J* = 1.6 Hz, H-5'), 6.84 (s, 1H, H-2'); <sup>13</sup>C NMR (CDCl<sub>3</sub>, 100 MHz): δ 41.6 (C-10), 44.5 (C-7), 45.9 (C-1), 49.5 (C-8), 50.0 (C-6), 58.3 (3-OCH<sub>3</sub>), 60.4 (4-OCH<sub>3</sub>), 70.7 (C-9), 79.1 (C-2), 100.9 (OCH<sub>2</sub>O), 107.1 (C-2'), 108.2 (C-5'), 119.2 (C-12), 119.5 (C-6'), 132.0 (C-11), 136.4 (C-1'), 138.4 (C-4), 146.3 (C-4'), 147.9 (C-3'), 159.4 (C-3), 193.0 (C-5); EIMS *m/z* 370 (M<sup>+</sup>, C<sub>21</sub>H<sub>22</sub>O<sub>6</sub>).

### 3.4. Biological activities

#### 3.4.1. Solvents and chemicals

*Antioxidant:* 1,1-Diphenyl-2-picrylhydrazyl (DPPH), and butylated hydroxytoluene (BHT) were obtained from Sigma-Aldrich (Germany). Analytical grade methanol, ethanol and dimethylsulfoxide (DMSO) were purchased from Merck (Germany). *Acetylcholinesterase:* AChE enzyme (Type-VI-S, EC3.1.1.7), acetylthiocholine iodide, 5,5'-dithio-bis(2-nitrobenzoic) acid (DTNB), and galantamine were purchased from Sigma-Aldrich (Germany). *Anti-inflammatory:* Indomethacin and COX Inhibitor Screening Kit (Catalog No 560131) were purchased from Cayman Chemical Co. (Ann Arbor, Michigan USA).

#### 3.4.2. Antioxidant activity

The antioxidant activity was determined using DPPH free radical scavenging as described previously with minor modifications (Shimada et al., 1992). Briefly, 0.1 mM DPPH (1 mL) dissolved in EtOH was added to an EtOH solution (3 mL) of the tested samples at different concentrations. An equal volume of EtOH was added in the control test. The mixture was shaken vigorously and allowed to stand at room temperature for 30 min. Then the absorbance at 517 nm was measured with a UV-vis spectrophotometer. The percentage of scavenging of DPPH was calculated using the following equation:

$$I\% = [ A_{\text{blank}} - A_{\text{sample}} / A_{\text{blank}} ] \times 100;$$

where  $A_{\text{blank}}$  is the absorbance value of the control reaction (containing all reagents except the test compound) and  $A_{\text{sample}}$  is the absorbance value of the test compound. The sample concentration providing 50% inhibition ( $IC_{50}$ ) was calculated by plotting inhibition percentages against concentrations of the sample. All tests were carried out in triplicate and inhibition values were reported as means  $\pm$  SD of triplicates.

#### 3.4.3 Acetylcholinesterase (AChE) inhibitory activity

AChE inhibitory activity of the compounds was measured by slightly modifying the spectrophotometric method (Ellman et al., 1961; Salleh et al., 2015). Electric eel AChE was used, while acetylthiocholine iodide (AChI) was employed as the substrate of the reaction. DTNB acid was used for the measurement of the AChE activity. Briefly, 140  $\mu$ L of sodium phosphate buffer (pH 8.0), 20  $\mu$ L of DTNB, 20  $\mu$ L of test compounds and 20  $\mu$ L of AChE solution were added by multichannel automatic pipette in a 96-well microplate and incubated for 15 min at 25°C. The reaction was then initiated with the addition of 10  $\mu$ L of AChI. Hydrolysis of AChI was monitored by the formation of the yellow 5-thio-2-nitrobenzoate anion as a result of the reaction of DTNB with thiocholines, catalyzed by enzymes at 412 nm utilizing a 96-well microplate reader (Epoch Micro-Volume Spectrophotometer, USA). Percentage of inhibition (I%) of AChE was determined by comparison of rates of reaction of samples relative to blank sample (EtOH in phosphate buffer pH = 8) using the formula:

$$I\% = [ E - S / E ] \times 100;$$

where E is the activity of enzyme without test sample and S is the activity of enzyme with test sample. The experiments were done in triplicate. Galantamine was used as the positive controls.

#### *3.4.4 Anti-inflammatory activity*

The anti-inflammatory activity of the compounds on COX-2 was determined by measuring prostaglandin E2 (PGE2) using a COX Inhibitor Screening Kit (Catalog No 560131, Cayman Chemicals, Ann Arbor Michigan USA) as well as the reported method (Bertanha et al., 2012). Reaction mixtures containing the reaction buffer (950  $\mu$ L), heme (10  $\mu$ L), COX-2 (10  $\mu$ L) and sample/control (20  $\mu$ L) were incubated at 37°C in a water bath for 15 min. The reaction was initiated by addition of arachidonic acid (10  $\mu$ L) at a final concentration of 100  $\mu$ M. After 2 min incubation, the reactions were stopped by addition of 1 M HCl (50  $\mu$ L), followed by saturated solution of stannous chloride (100  $\mu$ L). Then, prostaglandins (PG) were

quantified by means of the ELISA method. The contents of the reaction tubes were diluted and transferred to a 96-well plate coated with a mouse antirabbit IgG, followed by addition of the PG screening acetylcholinesterase tracer and the PG screening antiserum. Plates were incubated in an orbital shaker for 18 h, at room temperature. The reaction mixtures were removed, and the wells were washed five times with a buffer containing 0.05% Tween 20. Acetylthiocholine and 5,5'-dithio-bis-2-nitrobenzoic acid known as Ellman's reagent (200  $\mu$ L) was then added to each well, and the plate was incubated in an orbital shaker for 60 min, at room temperature, until the control wells yielded an optical density lying between 0.3-0.8 at 415 nm. A standard curve with PG was generated from the same plate, which was used to quantify the PG levels produced in the presence of the samples. The compound DuP697 (Cayman Chemicals) was used to standardize the assay for COX-2 and indomethacin was employed as positive control. Results were expressed as a percentage relative to a control (100% initial activity, solvent treated samples). All determinations were performed in triplicate. Regression analysis was employed for the calculation of IC<sub>50</sub> values.

### 3.5 *Statistical analysis*

Data obtained from biological activities were expressed as mean values. The statistical analyses were carried out by employing one way ANOVA ( $p > 0.05$ ). A statistical package (SPSS version 16.0) was used for the data analysis.

## **Appendix A. Supplementary data**

Supplementary data including 1D/2D NMR, IR, UV and MS for compound (**1-2**) are available as supporting information.

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