

Original article:

**COX-2 INHIBITORS FROM STEM BARK OF
BAUHINIA RUFESCENS LAM. (FABACEAE)**

Aminu Muhammad and Hasnah Mohd Sirat*

Department of Chemistry, Faculty of Science,
Universiti Teknologi Malaysia, 81310 Skudai, Johor Bahru, Malaysia

*Corresponding author: Dr. Hasnah Mohd Sirat

Department of Chemistry, Faculty of Science, Universiti Teknologi Malaysia,
81310 Skudai, Johor Bahru, Malaysia, E-mail: hasnah@kimia.fs.utm.my

ABSTRACT

Chemical investigation of the stem bark of *Bauhinia rufescens* resulted in the isolation of a new cyanoglucoside and menisdaurin from methanol extract and oxepin from petroleum ether extract. The isolated compounds were tested for their anti-inflammatory potentials based on the cyclooxygenase-2 enzyme (COX-2) model. Cyanoglucoside exhibited the highest activity among the compounds with an inhibition activity of 49.34 % at 100 μ M (IC_{50} 0.46 μ M) compared to the positive control, indomethacin (79.20 %, IC_{50} 0.24 μ M).

Keywords: COX-2, *Bauhinia rufescens*, phytochemicals

INTRODUCTION

Prostaglandins are involved in diverse functions in a human body system, including blood clotting, ovulation, bone metabolism, nerve growth and development, wound healing, kidney function and immune responses. Cyclooxygenase (COX) is an enzyme that catalyses cyclisation of arachidonic acid to prostaglandins. Two isoforms of the enzyme have been identified as COX-1 and COX-2 (Dubois et al., 1998; Huff et al., 2005). COX-1 is constitutively expressed in a variety of cell types and is involved in regulating normal physiological functions (Bertanha et al., 2012). Whereas, COX-2 isoform is induced by a variety of stimuli, such as lipopolysaccharides and cytokines that are responsible for the biosynthesis of prostaglandins under inflammatory conditions (Blobaum and Marnett, 2007). Therefore, COX-2 is the target enzyme for the screening of anti-inflammatory potential of a drug.

Bauhinia rufescens Lam. (Fabaceae) known in northern part of Nigeria as ‘Matsatsagi’ is a tropical forage plant that grows up to 8 meters high (Balogun et al., 1998). It is used for the establishment of hedges, as well as an ornamental tree (Asiedu et al., 2012). In folk medicine, the plant is used in the treatment of gout, gingivitis, diarrhea, dysentery, diabetes, leprosy and malaria (Compaoré et al., 2011; Tapsoba and Deschamps, 2006; Jansen et al., 2010; Inngjerdigen et al., 2004; Maillard et al., 1991). Previous phytochemical and bioactivity studies in the genus *Bauhinia* have resulted in the isolation of anti-inflammatory compounds including triterpenes from *B. tarapotensis* (Sosa et al., 2002), dihydrodibenzooxepins from *B. purpurea* (Boonphong et al., 2007), and kaempferol and a triterpene caffeate from *B. variegata* (Rao et al., 2008). Compaoré et al. (2011) reported the inhibition activities of the leaves and stem bark extracts of *B. rufescens* against

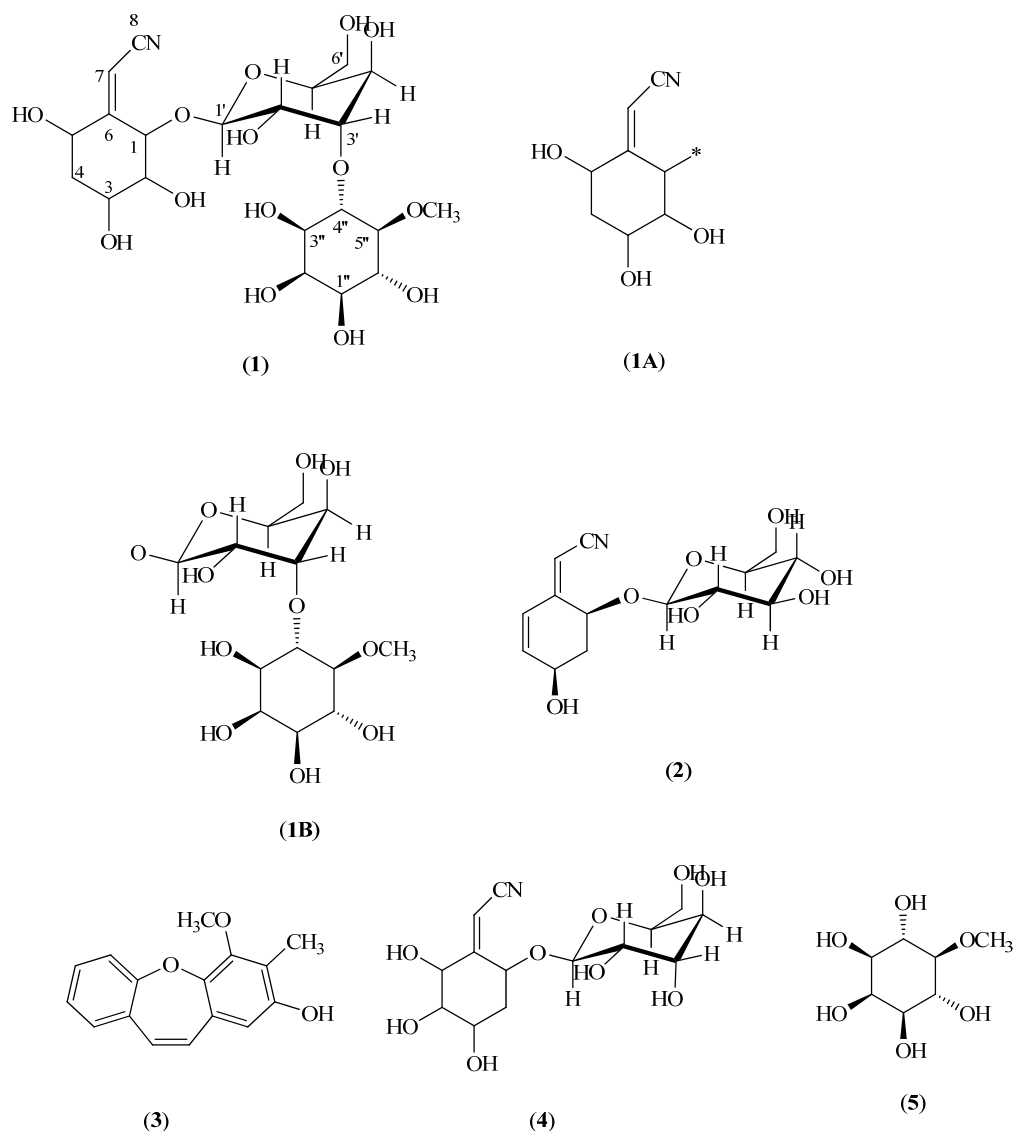


Figure 1: Structures of compounds 1-5

xanthine oxidase and lipoxygenase enzymes. In the present study, a new cyanoglucoside (**1**), menisdaurin (**2**) and oxepin (**3**) were isolated from the stem bark of *B. rufescens* (Figure 1). Therefore, we would like to report structure elucidation of cyanoglucoside (**1**) and anti-inflammatory potential of the isolated compounds against COX-2 enzyme.

MATERIALS AND METHODS

General

Melting points (uncorrected) were determined using a Leica Gallen III Kofler micro

melting point apparatus. UV spectra were measured with a Shimadzu UV 1601PC spectrophotometer, and IR spectra were recorded on a Perkin-Elmer 1600 FTIR spectrophotometer. The ^1H - and ^{13}C -NMR spectra were recorded on Bruker Avance 400 MHz spectrometer. Chemical shifts were recorded in parts per million (δ) in acetone- d_6 and methanol- d_4 . Mass spectra were obtained from NUS Mass Spectrometry Service, Singapore. Column chromatography was carried out on silica gel 70-230 mesh (Merck). Silica gel 60 F₂₅₄ precoated aluminium plates (0.2 mm, Merck) were used for TLC analysis; detection was per-

formed by spraying with 5 % H₂SO₄ in methanol and 1 % vanillin in methanol, followed by heating at 120 °C for 5 min.

Plant material

The stem bark of *B. rufescens* was collected at Kiru, Kano State, Nigeria in August 2011. A voucher specimen (Acc. 99) was deposited in the herbarium of the Department of Biological Sciences, Bayero University, Kano, Nigeria.

Extraction and isolation

The dried stem bark of *B. rufescens* (800 g) was ground and extracted successively with petroleum ether (4.0 L), ethyl acetate (4.0 L) and methanol (4.0 L) in a soxhlet extractor each for 18 h. The samples were concentrated using rotary evaporator to give sticky brown substances of petroleum ether (5.82 g, 0.73 %), ethyl acetate (12.51 g, 1.56 %) and methanol (44.93 g, 5.62 %).

The methanol extract (10.0 g) was fractionated using VLC with chloroform-methanol, ethyl acetate-methanol, acetone-methanol and methanol gradient as eluents to afford 22 fractions. Fraction 10 was subjected to further purification using column chromatography with n-hexane-chloroform, chloroform-methanol, acetone-methanol, methanol polarity gradient to afford compounds (**1**, 74.2 mg, 13.98 %) and (**2**, 39.7 mg, 7.48 %) as a brownish oil and flake substances respectively.

The petroleum ether extract (5.0 g) of stem bark was purified over silica gel column using petroleum ether - diethyl ether - chloroform - ethyl acetate - methanol step gradient to afford 230 fractions. Further purification of fraction 62 with petroleum ether, diethyl ether and ethyl acetate in polarity gradient over a silica gel column yielded compound (**3**, 23.7 mg, 0.47 %).

Cyanoglucoside 1

R_f: 0.36 (CHCl₃-MeOH, 3:2); IR (KBr): 3283, 2220, 1629, 1022 cm⁻¹; ¹H NMR (400 MHz, methanol-*d*₄): Table 1; ¹³C

NMR (100 MHz methanol-*d*₄): Table 1; ESI-MS: *m/z* (%) = 498.11 [M + H⁺] (19), 339.6 (5), 197.9 (28), 169.0 (100), 144.9 (18).

COX-2 inhibitory assay

The activity of the test compounds (final concentrations of 100, 10, 1, 0.1 and 0.01 μM) 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 (1.15 mL) containing the reaction buffer (950 μL), heme (10 μL), COX-2 (10 μL) and sample/control (20 μL) were incubated at 37 °C in a water bath for 15 min. The reaction was initiated by addition of arachidonic acid (10 μL) at a final concentration of 100 μM. After 2 min incubation, the reactions were stopped by addition of 1 M HCl (50 μL), followed by saturated solution of stannous chloride (100 μL). Then, prostaglandins (PGs) 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 anti-rabbit 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-nitro-benzoic acid known as Ellman's reagent (200 μL) 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 duplicate. Regression analysis (probit analysis, SPSS 16.0) was employed for the calculation of IC₅₀ values.

RESULTS AND DISCUSSION

Isolation and characterization

Compound **1** was purified from methanol extract by chromatographic techniques as brownish oil. Its structure was elucidated by the analysis of the spectroscopic data comprises of IR, ¹H NMR, ¹³C NMR, 2D-NMR and MS. The absorption band at 3283 cm⁻¹ and 2220 cm⁻¹ were attributed to the presence of hydroxyl and nitrile group respectively. A band for olefinic carbon atoms stretching was also observed at 1629 cm⁻¹. The ¹H NMR data showed the presence of two singlets at δ 5.69 and 3.63 which were assigned to olefinic and methoxyl protons respectively. Two non-equivalent methylene protons at δ 2.00 and 1.89 were also observed. A number of peaks between δ 3.32 and 4.75 were attributed to the presence of oxy-methylene and oxy-methine protons in the molecule.

The ¹³C NMR data revealed the presence of 21 carbon atoms, which were classified as one methyl, two methylene, sixteen methine and two quaternary carbon atoms. The HMQC experiment shows the correlation between proton atom and its corresponding carbon atom. Methoxyl group in the molecule showed a connection between 3.63 and 59.3, while each of the carbon atoms of the methylene group were connected to a two non-equivalent protons. The prominent correlation observed in the case of methine carbon atoms appeared in the connections of 5.69 and 94.2, 4.53 and 103.9 were assigned to olefinic and anomeric carbon atoms respectively. The NMR data (Table 1) of the cyanomethylenecyclohexylglucosyl and cyclitol moiety in the molecule were reported based on the data of

cyanomethylenecyclohexylglucoside (**4**) and sequoyitol (**5**) compounds isolated from *Simmondsia chinensis* (Van Boven et al., 1994) and *Ceratonia siliqua* (Baumgartner et al., 1986, Binder and Haddon, 1984) respectively. The two parts were bonded to one another through a glycosidic bond between C-3' and C-4'' of cyanomethylenecyclohexylglucosyl and cyclitol parts respectively. The correlation of the connected bonds was observed in HMBC experiment (Figure 2).

Furthermore, the mass spectrum of compound **1** showed [M+1] ion at *m/z* 498 corresponding to the molecular formula of C₂₁H₃₃O₁₄N with a loss of a nitrile group (CN). The fragment ion at 169 corresponds to the cyanomethylenecyclohexyl part of the molecule (**1A**), however, the glucosyl-cyclitol (**1B**) part was observed at *m/z* 339. Combination of the IR, NMR and MS data led to the conclusive identification of a new cyanoglucoside **1** as (Z)-2-(2-((2*S*,3*S*,4*S*,5*R*,6*S*)-3,5-dihydroxy-6-(hydroxyl-methyl)-4-((1*S*,2*R*,3*R*,4*S*,5*R*,6*S*)-2,3,4,5-tetrahydroxy-6-methoxycyclohexyloxy)tetrahydro-2H-pyran-2-yloxy)-3,4,6-trihydroxycyclohexylidene)acetonitrile.

Compound **2** was also isolated from the methanol extract as a white flake substance, identified as menisdaurin which was previously isolated from *B. sirindhorniae*

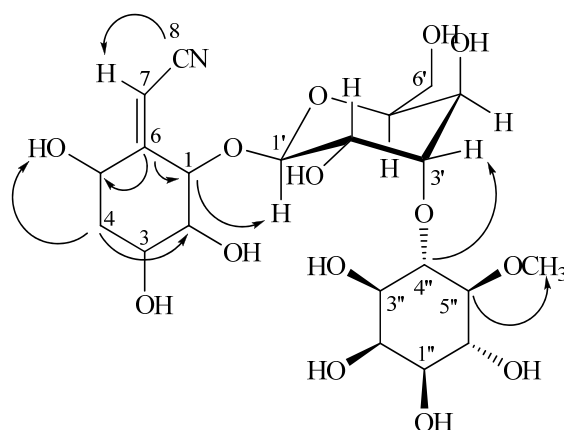


Figure 2: Selected HMBC of compound **1**

Table 1: ^1H - and ^{13}C -NMR (400 and 100 MHz, resp.) spectral data for compound **1**. δ in ppm, J in Hz

Position	$\delta(\text{H})$	$\delta(\text{C})$	COSY	HMBC
1	4.75 (d, $J = 5.2$)	80.4	H-2	H-1'
2	3.83 (t, $J = 5.2$)	74.0	H-1, H-3	
3	4.00 (m)	69.7	H-2	
4	2.00 (m), 1.89 (m)	38.6		H-2
5	4.69 (dd, $J = 8.4, 3.6$)	66.8		
6	-	165.2		
7	5.69 (s)	94.2		
8	-	116.3		H-7
1'	4.53 (d, $J = 7.6$)	103.9	H-2'	C1
2'	3.57 (dd, $J = 9.6, 7.6$)	76.8	H-1', H-3'	
3'	3.40 (t, $J = 9.6$)	73.3	H-2', H-4'	C4''
4'	3.33 (t, $J = 9.6$)	71.1		
5'	3.30 (m)	76.5		
6'	3.90 (dd, $J = 12.0, 2.0$), 3.70 (dd, $J = 12.0, 5.2$)	61.3		
1''	3.33 (dd, $J = 3.2, 2.0$)	72.8	H-2'', H-6''	
2''	3.87 (d, $J = 2.0$)	72.3	H-1''	
3''	3.33 (dd, $J = 3.2, 2.0$)	72.0		
4''	3.73 (dd, $J = 9.6, 2.0$)	70.5		H-3'
5''	3.61 (t, $J = 9.6$)	83.4		
6''	3.73 (dd, $J = 9.6, 2.0$)	70.0	H-1''	
OCH₃	3.63 (s)	59.3		

(Athikomkulchai et al., 2003). Purification of petroleum ether extract over silica gel column chromatography yielded compound **3** as a white solid identified as 6-methoxy-7-methyl-8-hydroxydibenz[b,f]oxepin. The spectral data of compounds **2** and **3** were consistent with the reported data (Mu et al., 2007; Athikomkulchai et al., 2003).

Inhibition of COX-2

Inhibition of COX-2 by the isolated compounds was analyzed in a cell-free immunoassay system, in which the enzyme was sourced from human recombinant enzyme. The degree of COX-2 inhibition by the isolated compounds was recorded as percentage inhibition of prostaglandin syn-

Table 2: Inhibitory activity on COX-2 of the compounds

Compounds	Inhibition at 100 μM (%)	IC ₅₀ (μM)
1	49.39 \pm 2.17	0.46
2	36.35 \pm 8.70	72.28
3	41.48 \pm 3.71	101.16
Indomethacin^a	79.20 \pm 1.82	0.24

1 = cyanoglucoside; 2 = menisdaurin; 3 = oxepin;
 a = positive control

thesis. The results were expressed as mean \pm SEM as shown in Table 2. Each compound was tested at final concentration of 100, 10, 1, 0.1, 0.01 μM and a regression analysis was employed for the calculation of IC₅₀. The incubation of COX-2 with

menisdaurin (**2**) and oxepin (**3**) at 100 μ M yielded inhibition of 36.35 ± 8.70 % and 41.48 ± 3.71 % respectively, whereas, cyanoglucoside **1** furnished the best value among the isolated compounds inhibiting the enzyme at 49.34 ± 2.17 %. However, the concentration of inhibition at 50 % was evaluated for each compound in which compounds **2** and **3** inhibited PG production at a concentration greater than 70.00 μ M, while compound **1** showed inhibition at 0.46 μ M. The percentage inhibition and IC₅₀ values of indomethacin used as a positive control is higher than that of the isolated compounds (79.20 ± 1.82 %, 0.24 μ M).

Compound **1** and **2** are structurally quite similar, both have cyanomethylenecyclohexylglucosyl moiety and differ in the presence of cyclitoyl substituent in compound **1**. This resulted in the higher number of hydroxyl groups in compound **1** compared to compound **2**. This could explain the disparity in the percentage inhibition of the two compounds. Regarding the IC₅₀ values of the isolated compounds, compound **1** has a value 2-fold lower than that of indomethacin. On the other hand compounds **2** and **3** exhibited IC₅₀ value of 300-fold and 400-fold lower than that of the positive control indomethacin. This discrepancy could be related to the concentration independence of the compounds towards inhibition activity of COX-2 enzyme (Ángeles et al., 2012).

CONCLUSIONS

In conclusion the COX-2 inhibitors isolated from *B. rufescens* could explain the use of its decoction in the treatment of gingivitis and other inflammations in West Africa. This study reports the isolation and structure elucidation of cyanoglucoside **1** for the first time and COX-2 inhibition activity of the isolated compounds from the stem bark of *B. rufescens*.

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