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Original Research Article

Evaluation of Biological Activities of Extracts and Chemical Constituents of *Mimusops elengi*

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Abstract

Purpose: *To isolate some compounds from the leaves and bark of Mimusops elengi, and examine them for their antibacterial and anti-inflammatory properties.*
Experimental: *The compounds were isolated from the leaf and bark chloroform extracts using column chromatography, and characterized using physical and spectroscopic methods. The isolated compounds and their respective extracts were tested for antibacterial activity by micro-dilution antibacterial assay, and for anti-inflammatory activity by cyclooxygenase inhibitory assay.*
Results: *of the compounds isolated include spinasterol **(1)**, ursolic acid **(2)** and 3β, 6β, 19α, 23-tetrahydroxyurs-12-en-28-oic acid **(3)** from the leaves; and taraxerol **(4)** and spinasterol β-D-glucopyranoside **(5)** from the bark. A majority of the samples showed good activity against Staphylococcus aureus (9.7 — 78.0 µg/mL), while moderate activity was observed against Gram-negative bacteria (78.0 — 156 µg/mL). Strong COX inhibition was observed for the leaf extract, and **(1)**; selective COX-2 inhibition for **(2)** and **(3)**; and selective COX-1 inhibition for bark extract, **(4)** and **(5)**.*
Conclusion: *This is the first report describing the anti-inflammatory potential of M. elengi on the basis of its isolated constituents. The results of this study support the traditional use of the plant as antibacterial and anti-inflammatory remedy.*

Keywords: *Mimusops elengi, Sapotaceae, Steroids; Triterpenoids, Antibacterial, Anti-inflammatory.*

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INTRODUCTION

The family Sapotaceae comprises 35 – 75 genera and approximately 800 species distributed pantropically with a few species in temperate regions. The genus Mimusops is native to the tropical parts of Africa and Asia. Mimusops elengi L. is an evergreen tree 30 feet tall, with a greyish brown fissured bark, wavy and dull green leaves, oblong berry fruit and creamy fragrant flowers. It is distributed in tropical and subtropical regions [1-3].

M. elengi is known to possess various phytochemicals such as gallic acid esters, flavones, triterpenoids and steroids [2,4-5]. The volatile constituents of the flowers have also been reported [6].

Different parts of the plant are reported to be used in traditional medicine for the treatment of

microbial diseases such as diarrhoea, gum diseases, sore mouth, stomaches, ulcers, wounds and inflammation [1,3,7-9].

Previous studies on *M. elengi* were mainly focused on the isolation and characterization of its phytochemical constituents, mostly from the stem bark, fruit, seeds and roots [10]. Moreover, most of the biological activities reported for *M. elengi* were on the extracts rather than the active constituents which might be responsible for its observed activities [1,9].

This paper reports the isolation and characterization of five constituents of the leaves and stem bark of *M. elengi* as well as the antibacterial and anti-inflammatory activities of the crude extracts and their isolated constituents.

Previous phytochemical investigations of *M. elengi* had revealed the presence of **1** in the bark, heart-wood and seeds [11], **2** in the bark and fruit [12], and **4** in the roots and bark [12] (see Figure 1). Jahan *et al*. [4, 12] had also reported the occurrence of **1** and **2** in *M. elengi* but the plant part they examined was not specified. The present study showed conclusively the occurrence of **1** and **2** in the leaves, and confirmed the presence of **4** in the bark. Interestingly, **3**, previously reported from *Adina rubella*, *Dischidia esquirolii* and *Guettarda grazielae* [13] and found in the leaves of *M. elengi* in the present study, has hitherto not been reported in a member of the Sapotaceae. Compound **5** was found for the first time in the bark of *M. elengi* although the C-3 epimer of **5** has been reported previously for this plant [11].

EXPERIMENTAL

Plant material

The plant material was collected in April 2009 from trees growing in the campus of Universiti Sains Malaysia and identified by one of the authors (F.A.). A voucher specimen (USM 9255) was deposited in the herbarium of the School of Biological Sciences, Universiti Sains Malaysia.

Extraction and isolation

Fresh leaves (3 kg) were macerated in 30 L of methanol-water (room temprature) (4:1 v/v). The extract was filtered and concentrated under reduced pressure at < 40 ºC to 1/10 of its original volume in a rotary evaporator, acidified with aqueous 2M H_2SO_4 to a pH of 2 — 3, and each 500 mL portions of it extracted with chloroform (200 mL × 2, then 100 mL × 1) to give an extract (31 g). This extract was subjected to silica gel column chromatography, yielding ten fractions (F1 - 10) on elution with hexane/ethyl acetate/methanol (1/0/0 to 0/0/1 v/v/v). Fractions F2 (yellow oil, 1.3 g), F3 (green solid, 1.1 g) and F7 (green solid, 1.6 g) were rechromatographed to yield sub-fractions F2A-C, F3A-E and F7A-E, respectively. F2B (0.1 g), which eluted with hexane-ethyl acetate (8/2 v/v), yielded **1** (20 mg, 6.6×10^{-4} %) upon recrystallization from diethyl ether. F3C (0.2 g), which eluted with hexane-EtOAc (7/3 v/v), afforded **2** (25 mg, 8.3×10^{-4} %) upon recrystallization from methanol. F7C (0.34 g), which eluted with EtOAc, gave **3** (32 mg, 1.0×10^{-3} %) upon recrystallization from methanol.

Fresh bark (4 kg) was air-dried for two weeks at room temperature (28 ºC) to a weight of 1 kg, powdered, and 150 g each of the powdered material was first defatted for 4 h with hexane in a Soxhlet extractor, and then extracted for 8 h with $CHCl_3$, yielding a combined $CHCl_3$ extract of 14 g. This extract was fractionated on a silica gel column using hexane/EtOAc/MeOH (1/0/0 to 0/0/1 v/v/v) to yield six fractions (FB1 - 5). FB1 and FB3 were rechromatographed on a silica gel column using a hexane/EtOAc gradient to afford sub-fractions FB1A and FB3C, respectively. FB1A, which eluted with hexane/EtOAc (9:1 v/v), yielded **4** (25 mg, 6.2×10^{-3} %) after recrystallization from $CHCl_3$. FB3C, which eluted with hexane/EtOAc (7:3 v/v), gave **5** (35 mg, 8.7×10^{-3} %) after recrystallization in pyridine.

Identification of isolated compounds

Infrared spectra were recorded on a Perkin-Elmer 1330 spectrophotometer. Nuclear Magnetic Resonance spectra were obtained with a Bruker Avance 400 MHz spectrometer. Electron Impact and Fast Atom Bombardment mass spectra were recorded using an Agilent 5975C MSD and a Thermo Finnigan MAT95XL mass spectrometer, respectively.

Micro-dilution antibacterial assay

Serial dilution technique [14] using a 96-well micro-plate, was employed to determine minimum inhibitory concentration (MIC) as a measure of antibacterial activity. Two-millitre cultures of two Gram-positive bacteria, namely, *Bacillus subtilis* (ATCC6633) and *Staphylococcus aureus* (ATCC12600), and three Gram-negative bacteria, namely, *Escherichia coli* (ATCC25922), *Klebsiella pneumoniae* (ATCC13883) and *Pseudomonas stutzeri* (ATCC17588), were separately prepared and placed in an incubator overnight at 37 °C. The overnight-cultures were diluted with sterile nutrient broth (Merck) (500 µL bacteria/50 mL

broth) to yield density of bacterial cells range of 10^5 - 10^6 cell mL^{-1}. The isolated compound and crude extract samples under investigation were re-suspended to a concentration of 5 mg mL^{-1} with ethanol to yield a final concentration of 1.25 mg mL^{-1} in the assay for the first well. For each of the five bacteria used, 100 µL of the tested samples were serially diluted two-fold with 100 µL sterile distilled water in a sterile 96-well micro-plate. A similar two-fold serial dilution of gentamicin sulphate (1 mg mL^{-1}, Sigma) was used as positive control against each bacterium. One hundred µL of each bacterial culture were added to each well of the test samples, gentamicin sulphate (positive control), ethanol, water and nutrient broth (negative controls). The plates were covered and incubated overnight at 37 °C. To indicate bacterial growth, 50 µL of 0.2 mg mL^{-1} *p*-iodonitrotetrazolium violet (INT, Sigma) was added to each well and the plates incubated at 37 °C for 30 min. Bacterial growth in the wells was indicated by red colour, while clear wells indicate inhibition by the test substances. This assay was repeated three times.

Cyclooxygenase inhibitor screening assay

Evaluation of the anti-inflammatory activity of the isolated compounds was based on the inhibition of prostaglandin biosynthesis. This was assessed using COX inhibitor screening assay kit (no. 560131; Cayman Chemicals, USA). The assay directly measures $PGF_{2\alpha}$ by $SnCl_2$ reduction of COX-derived PGH_2 produced in the COX reaction. This assay is based on the competition between PGs and a PG-acetylcholinesterase conjugate (a PG tracer) for a limited amount of PG antiserum. Because the concentration of the PG tracer is held constant while the concentration of PG varies, the amount of PG tracer that is able to bind to the PG antiserum will inversely be proportional to the concentration of PG in the well.

The plate was washed to remove any unbound reagents and then Ellman's reagent (Sigma) was added to the well to yield a yellow colour. The intensity of the colour was determined spectrophotometrically at 420 nm using Micro Plate Reader. The assay for obtaining 100 % COX activity was performed with ethanol as solvent control. The test samples and indomethacin (positive control) (Sigma) were redissolved in ethanol at a concentration of 10 mg mL^{-1} to yield final concentrations of 5, 2.5 and 1.25 µg mL^{-1}. The pre-incubation time between enzyme and inhibitor was 10 min with 2-min incubation in the presence of arachidonic acid at 37 °C. Enzyme control was performed with COX-1 and 2 that had been inactivated by placing them in boiling water for 3 min. Inhibition of PGE_2 production by the test compounds and indomethacin was calculated from the standard curve using Graph Pad Prism software, version 3.00 for Windows. IC_{50} values were calculated from the concentration-inhibition response curve by regression analysis using Graph Pad prism software. The values reported are the mean of triplicate experiments

Statistical analysis

The data obtained from all experiments were expressed as mean ± standard error (SEM). Statistical difference between treatments and control were evaluated by one-way analysis (ANOVA) followed by Tukey's multiple comparison test. $p < 0.5$ was considered to be significant.

RESULTS

Isolated compounds

Spinasterol (stigmasta-7, 22(E)-dien-3β-ol) **(1)**: m.p. 152-154ºC; $[\alpha]_D^{25}$: +12.4º (*c* 0.012, $CHCl_3$); IR (KBr): 3426, 2956, 2869, 1637, 1456, 1382, 1040, 970 cm^{-1}; MS (FAB, 70 eV): *m/z* (%) = 413 [M + H^+] (15), 395 (22), 271 (26), 255 (10), 83 (100), 69 (60); ^{1}H NMR (400 MHz, $CDCl_3$): 0.55 (3H, s, H-18); 0.81 (3H, m, H-26), 0.84 (3H, d, *J* = 6.4 Hz, H-27), 1.00 (3H, d, *J* = 7.0, H-21), 3.60 (1H, m, H-3), 5.04 (1H, dd, *J* = 15.5, 8.0 Hz, H-23), 5.17 (2H, dd, *J* = 15.5, 8.0 Hz, H-7, H-22); ^{13}C NMR (100 MHz, $CDCl_3$): 37.5 (C-1), 32.2 (C-2), 71.4 (C-3), 38.4 (C-4), 40.6 (C-5), 30.0 (C-6), 117.8 (C-7), 139.9 (C-8), 49.8 (C-9), 34.6 (C-10), 21.9 (C-11), 39.8 (C-12), 43.6 (C-13), 55.5 (C-14), 23.4 (C-15), 28.9 (C-16), 56.3 (C-17), 12.6 (C-18), 13.4 (C-19), 41.2 (C-20), 21.7 (C-21), 138.5 (C-22), 129.8 (C-23), 51.6 (C-24), 31.8 (C-25), 19.2 (C-26), 21.5 (C-27), 25.8 (C-28), 12.4 (C-29) [4].

Ursolic acid (3β-hydroxyurs-12-en-28-oic acid) **(2)**: m.p 239-242 ºC; $[\alpha]_D^{25}$: +70.3º (*c* 0.025, MeOH); IR (KBr): 3428, 2927, 2870, 1692, 1457, 1030 cm^{-1}; MS (EI, 70 eV): *m/z* (%) = 456 M^+ (3), 248 (100), 219 (8), 203 (44), 133 (31); ^{1}H NMR (400 MHz, C_5D_5N): 0.88 (3H, s, H-25), 0.95 (3H, d, *J* = 6.5 Hz, H-29), 1.00 (3H, d, *J* = 6.5 Hz, H-30), 1.04 (3H, s, H-26), 1.11 (3H, s, H-27), 1.24 (3H, s, H-27), 3.44 (1H, dd, *J* = 10.0, 6.0 Hz, H-3), 5.48 (1H, t, *J* = 5.0 Hz, H-12); ^{13}C NMR (100 MHz, C_5D_5N): 38.9 (C-1), 28.1 (C-2), 78.6 (C-3), 39.4 (C-4), 55.7 (C-5), 18.4 (C-6), 33.3 (C-7), 39.7 (C-8), 48.6 (C-9), 37.0 (C-10), 23.3 (C- 11), 125.8 (C-12), 138.6 (C-13), 42.2 (C-14), 28.1 (C-15), 24.3 (C-16), 48.6 (C-17), 53.3 (C- 18), 39.4

(C-19), 39.7 (C-20), 30.7 (C-21), 37.1 (C-22), 27.7 (C-23), 15.3 (C-24), 15.0 (C- 25), 16.7 (C-26), 23.0 (C-27), 180.6 (C-28), 16.0 (C-29), 20.5 (C-30) [15].

3β, 6β, 19α, 23-Tetrahydroxyurs-12-en-28-oic acid **(3)**: m.p. 250-252 ºC; $[\alpha]_D^{25}$: +13.2º (*c* 0.025, MeOH); IR (KBr): 3440, 2927, 2870, 1687, 1461, 1032 cm^{-1}; MS (EI, 70 eV): *m/z* (%) = 505 [M + H^+] (3), 248 (10), 203 (15); ^{1}H NMR (400 MHz, C_5D_5N): 1.11 (3H, d, *J* = 6.3 Hz, H-30), 1.47 (3H, s, H-29), 1.69 (3H, s, H-25), 1.71 (3H, s, H-24), 4.03 (1H d, *J* = 10.4 Hz, H-23a), 4.25 (1H, dd, *J* = 11.5, 4.3 Hz, H-3), 4.37 (1H, d, *J* = 10.4 Hz, H-23b), 5.06 (1H, br s, H-6), 5.67 (1H, br s, H-12); ^{13}C NMR (100 MHz, C_5D_5N): 41.3 (C-1), 27.1 (C-2), 73.6 (C-3), 44.1 (C-4), 49.5 (C-5), 67.9 (C-6), 41.5 (C-7), 39.9 (C-8), 48.4 (C-9), 37.0 (C-10), 24.2 (C-11), 128.6 (C-12), 139.4 (C-13), 42.7 (C-14), 29.4 (C-15), 26.5 (C-16), 48.4 (C-17), 54.8 (C-18), 72.9 (C-19), 42.5 (C-20), 27.7 (C-21), 38.6 (C-22), 67.3 (C-23), 14.8 (C-24), 17.6 (C-25), 18.4 (C-26), 24.9 (C-27), 180.9 (C-28), 27.3 (C-29), 16.9 (C-30) [16].

Taraxerol (13α-methyl-27-norolean-14-en-3β-ol) **(4)**: m.p. 276-278 ºC; $[\alpha]_D^{25}$: +7.4º (*c* 0.012, $CHCl_3$); IR (KBr): 3484, 2933, 2865, 1641, 1473, 1036 cm^{-1}; MS (EI, 70 eV): *m/z* (%) = 426 M^+ (26), 411 (20), 393 (5), 302 (55), 287 (50), 204 (100), 135 (40), 69 (30); ^{1}H NMR (400 MHz, $CDCl_3$): 0.84 (3H, s, H-27), 0.95 (3H, s, H-29), 0.97 (3H, s, H-18), 1.11 (3H, s, H-27), 3.20 (1H, dd, *J* = 9.7 Hz, H-3), 5.55 (1H, dd, *J* = 8.1, 4.0 Hz, H-15); ^{13}C NMR (100 MHz, $CDCl_3$): 38.1 (C-1), 27.5 (C-2), 79.4 (C-3), 39.3 (C-4), 55.9 (C-5), 19.1 (C-6), 35.5 (C-7), 39.1 (C-8), 49.1 (C-9), 36.1 (C-10), 17.9 (C-11), 37.0 (C-12), 37.9 (C-13), 158.4 (C-14), 117.2 (C-15), 38.1 (C-16), 38.3 (C-17), 49.6 (C-18), 41.7 (C-19), 29.1 (C-20), 34.0 (C-21), 33.4 (C-22), 28.3 (C-23), 15.8 (C-24), 15.8 (C-25), 30.2 (C-26), 26.3 (C-27), 30.3 (C-28), 33.3 (C-29), 21.7 (C-30) [17].

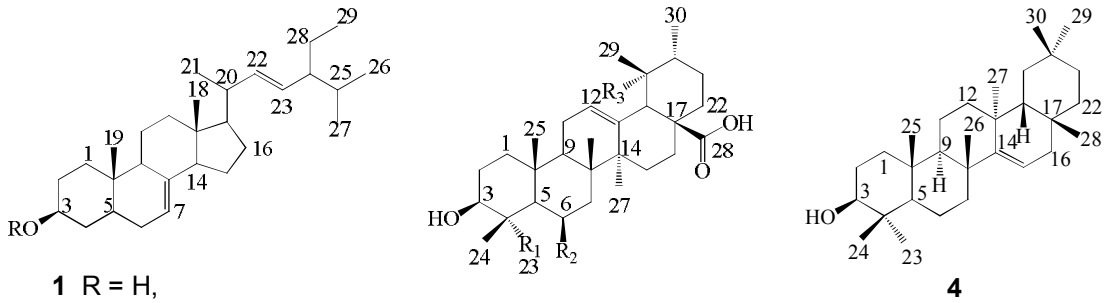
Spinasterol β-D-glucopyranoside **(5)**: MP: 282-285 ºC; $[\alpha]_D^{25}$: 16.4º (*c* 0.015, MeOH/$CHCl_3$); IR (KBr): 3406, 2955, 2871, 1644, 1444, 1028 cm^{-1}; MS (EI, 70 eV): *m/z* (%) = 574 M^+ (3), 412 (10), 397 (30), 273 (20), 255 (40), 229 (19), 83 (85), 55 (100); ^{1}H NMR (400 MHz, C_5D_5N): 0.58 (3H, s, H-18), 1.09 (3H, d, *J* = 6.5), 4.06 (1H, m, H-3), 4.42 (1H, d, *J* = 9.5, H-6'a), 4.60 (1H, d, *J* = 9.5, H-6'b), 5.04 (1H, m, H-23), 5.04 (1H, m, H-1'), 5.19 (2H, dd, *J* = 15.0, 8.0 Hz, H-7, H-22); ^{13}C NMR (100 MHz, C_5D_5N): 37.4 (C-1), 30.1 (C-2), 77.2 (C-3), 34.6 (C-4), 40.3 (C-5), 30.1 (C-6), 118.0 (C-7), 139.7 (C-8), 49.7 (C-9), 34.8 (C-10), 21.9 (C-11), 39.7 (C-12), 43.6 (C-13), 55.4 (C-14), 23.5 (C-15), 29.0 (C-16), 56.1 (C-17), 12.4 (C-18), 13.2 (C-19), 41.3 (C-20), 21.8 (C-21), 138.8 (C-22), 129.7 (C-23), 51.6 (C-24), 32.3 (C-25), 19.3 (C-26), 21.4 (C-27), 25.8 (C-28), 12.7 (C-29), 102.4 (C-1'), 75.5 (C-2'), 78.8 (C-3'), 71.9 (C-4'), 78.6 (C-5'), 63.0 (C-6') [18].

Antibacterial activity

The antibacterial activity of the leaf and stem bark extracts, and isolated compounds **1** – **5**, based on minimum inhibitory concentration (MIC), is shown in Table 1. Varying levels of activities were observed for the test samples against the five bacterial strains employed (Table 1).

Cyclooxygenase inhibitor screening activity:

The inhibition of prostaglandin biosynthesis are indicated in Table 2. The highest concentration used was 5 µg/mL. Therefore, all IC_{50} values exceeding this value are expressed in the Table as > 5 µg/mL.



1 R = H,
5 R = β-D-glucopyranosyl

2 $R_1 = CH_3$, $R_2 = R_3 = H$
3 $R_1 = CH_2OH$, $R_2 = R_3 = OH$

4

Figure 1: Structures of compounds **1-5**

Table 1: Minimum inhibitory concentration (MIC, µg/mL) of the extracts, isolated compounds and gentamicin (control)

Test sample	Bacterial MIC (µg/mL)				
	Bs	***Ec***	***Kp***	***Ps***	***Sa***
Leaf extract	156±0.0	156±0.0	78±0.0*	156±0.0	102±0.0
1	58±0.1*	312±0.0	78±0.0*	195±0.2	9.7±0.0*
2	468±0.2	468±0.2	312±0.0	468±0.3	243±0.1
3	312±0.0	312±0.0	312±0.0	156±0.0	78±0.0*
Bark extract	234±0.1	312±0.0	165±0.2	312±0.0	9.7±0.0*
4	234±0.1	312±0.0	312±0.0	312±0.0	78±0.0*
5	312±0.0	234±0.2	175±0.2	312±0.0	234±0.2
Gentamicin (control)	0.3±0.1**	0.1±0.0**	1.7±0.1**	0.1±0.0**	0.01±0.0**

Bs = Bacillus subtilis, Ec = Escherichia coli, Kp = Klebsiella pneuomoniae, Ps=Pseudomonas stutzeri, Sa=Staphylococcus aureus; values are expressed as mean ± S.E.M (n = 3); * $p < 0.5$; ** $p < 0.1$

Table 2: Inhibition of prostaglandin biosynthesis by plant extracts, isolated compounds and indomethacin (positive control)

Test sample	% Inhibition (5 µg/mL)		IC_{50} (µg/mL)	
	COX-1	***COX-2***	***COX-1***	***COX-2***
Leaf extract	91**	63*	3.3±0.4	3.6±0.7
1	93**	70*	4.4±0.8	3.7±0.2
2	20	41*	>5	>5
3	12	47*	>5	>5
Bark extract	52*	23	4.5±0.3	>5
4	50*	49*	>5	>5
5	78*	42*	3.2±0.7	>5
Indomethacin (control)	95**	85**	3.1±0.3	4.6±0.6

*Values are expressed as mean ± S.E.M (n = 3); * $p < 0.5$; ** $p < 0.1$*

DISCUSSION

The leaf extract indicated strong inhibitory effects against the Gram-positive *Staphylococcus aureus* and the Gram-negative *Klebsiella pneuomoniae*, with MIC values of 102 and 78 µg/mL, respectively, whereas moderate activity (156 µg/mL) was observed against the other strains. *Staphylococcus aureus* and *Klebsiella pneumoniae*. Compound **1** was the most active and showed strong inhibitory effects against *Staphylococcus aureus*, *Bacillus subtilis* and *Klebsiella pneuomoniae*, with MIC values of 9.7, 58 and 78 µg/mL, respectively. Compound **3** indicated strong activity (78 µg/mL) against *Staphylococcus aureus* and moderate activity (156 µg/mL) against *Pseudomonas stutzeri*.

The bark extract showed strong activity (9.7 µg/mL) against *Staphylococcus aureus* and moderate activity (165 µg/mL) against *Klebsiella pneuomoniae*. Compound **4** showed strong activity (78 µg/ml) against *Staphylococcus aureus* while compound **5** showed moderate activity (175 µg/mL) against *Klebsiella pneuomoniae*. All the isolated compounds were in accordance with the crude extracts in their antibacterial activity; all samples assayed showing reasonable activity against the tested strains. However, the observed activities of the leaf extract against *Escherichia coli* (156 µg/mL) and the bark extract against *Staphylococcus aureus* (9.7 µg/mL) were higher than the activity observed for the isolated compounds. This may be due to the synergistic effects of the bioactive agents in the crude extract.

Both the leaf extract and compound 1 possessed strong inhibitory effects against prostaglandin biosynthesis produced by both COX-1 and COX-2 enzymes. IC_{50} µg/mL values obtained for the leaf extract were 3.3 µg/mL and 3.6 µg/mL against COX-1 and COX-2, respectively, while IC_{50} for t compound 1 was 4.4 µg/mL (COX-1) and 3.7 µg/mL (COX-2). Compounds 2 and 3 showed selective COX-2 inhibition, but slightly higher inhibition was shown by compound 3. Bark extract showed moderate anti-inflammatory activity with selective COX-1 inhibition. Compound 4 showed moderate cyclooxygenase inhibitory activity, while compound 5 indicated the strongest COX-1 inhibitory activity, with IC_{50} value of 3.2 µg/mL. IC_{50} for indomethacin (positive control) was 3.1 µg/mL (COX-1) and 4.6 µg/mL (COX-2).

CONCLUSION

The results obtained justify the reported anti-inflammatory and antibacterial use of the plant in traditional medicine also and support its Ayurvedic medicinal use for ailments such as cephalalgia, diarrhoea, gum diseases, ulcers, sore teeth, stomach ache and wounds. Further

study is needed to determine its mechanisms of action and structure-activity relationship.

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