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Research Article
Cyclooxygenase, 5-Lipoxygenase and Acetylcholinesterase Inhibitory Effects of Fractions Containing, α-Guaiene and Oil Isolated from the Root of *Xylocarpus moluccensis*

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Abstract

This study aimed to investigate inhibitory effects of methanolic root extract of *Xylocarpus moluccensis* against cyclooxygenase, 5-lipoxygenase and acetyl cholinesterase enzymes using *in vitro* models. Based on bioassay guided, the extract yielded two fractions A and B. The fractions were analyzed using Gas Chromatography-Mass Spectrometry (GC-MS). The major component of fraction A was identified to be α-guaiene (98.54%). For fraction B (oil), 15 components were identified. The α-guaiene fraction showed strong activities against, AchE (IC_{50} 21 μg mL^{-1}), 5-lipox (IC_{50} 27 μg mL^{-1}) and COX-1 (IC_{50} 43 μg mL^{-1}). It was weaker against COX-2 with IC_{50} of 84 μg mL^{-1}. The oil possessed strong activities against 5-lipox and AchE with IC_{50} of 33 and 25 μg mL^{-1}, respectively. However, it was weaker against both the COX enzymes (IC_{50} values ≥125 μg mL^{-1}). Indomethacin, zileuton and galanthamine were used as positive controls. Concentration responses of the α-guaiene and oil against the enzymes were obtained using 5 concentrations (25-125 μg mL^{-1}). Means were plotted in a graph with error bars representing 95% confidence intervals. For the α-guaiene, the inhibition response increased significantly with the increase of concentrations from 25-100 μg mL^{-1} against the four tested enzymes. Both the isolates showed remarkable dual inhibitory effects against 5-lipox and AchE enzymes. The results indicated the potential therapeutic effects of the plant in the treatment of inflammatory related ailments and cognitive disorders. Further study is needed to verify mechanism of actions and effective doses. The isolation and the biological activities observed contribute to the novelty of this study.

Key words: Mangrove plants, α-guaiene, natural products, antiinflammatory agents, anticholinergic agents, *Xylocarpus moluccensis*

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Natural products have long been used as a folk remedy for several ailments including diseases of inflammatory conditions such as fever, pain, migraine and arthritis (Yuan *et al*., 2006).

Inflammatory response is a mechanism of self-defense that must be regulated as excess responses may lead to pathological conditions (De Simone *et al*., 2005). Synthesis of prostaglandins and the enzyme cyclooxygenase (COX) which catalyzes the first two steps in the biosynthesis of prostaglandins are known to be the key factors in the inflammation process (Smith and DeWitt, 1995; Lipsky, 1999; Murias *et al*., 2004; De Simone *et al*., 2005). On the other hand, leukotrienes are pro-inflammatory mediators that could also increase inflammatory responses. They are lipid mediators derived from arachidonic acid via the 5-lipoxygenase pathway. Leukotriene modifiers are a class of drugs used to inhibit activity of 5-lipoxygenase (5-lipox). Development of molecules that inhibit 5-lipox or both COX-2 and 5-lipox would be advantageous due to their ability to target both proteins (De Simone *et al*., 2005).

Non-steroidal anti-inflammatory drugs (NSAIDs) are proven to be effective for treatment of inflammatory symptoms due in most cases to their ability to inhibit prostaglandin synthesis. However, their side effects represent a major concern (Tracey, 2002; De Simone *et al*., 2005). Natural molecules remain a useful source of potential compounds with possible dual Lipox /COX inhibitory effects (Eldeen *et al*., 2015).

Other mechanisms that contribute to the regulation of the inflammatory response are through the effects on the cross talk between the immune and nervous systems. Borovikova *et al*. (2000) reported that acetylcholine (ACh) as the main parasympathetic neurotransmitter had effectively deactivated peripheral macrophages and inhibited the release of proinflammatory mediators, including the cytokine tumor necrosis factor (TNF-α). This findings suggest that inhibition of acetylcholinesterase enzyme may contribute to the enhancement of the ACh-dependent macrophage deactivation which represents an essential step for cholinergic anti-inflammatory pathway (Rao *et al*., 2012).

Xylocarpus moluccensis (Lam.) M. Roem. (Meliaceae), is a mangrove tree grow up to 22 m high. It has light brown bark, peeling in longitudinal flakes and pencil-like, stout and air-breathing roots (pneumatophores). *Xylocarpus moluccensis* is one of the useful medicinal plants with various reported applications in indigenous systems of medicine. In Bangladesh, the plant is used for the treatment of inflammatory related ailments (used as an astringent and a febrifuge). It is also used for elephantiasis and swelling of the breast (Medicinal Plants Database of Bangladesh http://www.mpbd.info/). In Indonesia the plant is used to cure scabies and kudis (Sarker *et al*., 2007). Bandaranayake (2002), reported the traditional uses of *X. moluccensis* for the treatment of fever, malaria and as an aphrodisiac. In Tonga, the plant is used for scabies, baby rash, stomach pains and constipation (Raja and Ravindranadh, 2014). In Malaya, it is used for treating abdominal problems (Uddin *et al*., 2005; Raja and Ravindranadh, 2014). In Fiji, the plant is used for headaches, fatigue, joint pains, chest pains and relapsing sickness (Raja and Ravindranadh, 2014). Relapsing sickness is a term used to describe diagnosable state of illness related to Central Nervous System (CNS) disorders (Ellin *et al*., 2015). Oil from seeds of *X. moluccensis* is used in the Philippines to treat insect bites (Ghani, 1998). Extracts of the barks and pneumatophores of *X. moluccensis* were also reported to possess significant effects on CNS related *in vivo* test using a mice model (Alamgir *et al*., 2006; Sarker *et al*., 2007).

Phytochemical investigation of Xylocarpus species indicated the presence of diverse classes of chemical agents in the leaves, stem bark and fruits of these plants. These agents including: Alkaloids, flavonoids, monoterpnes, triterpenoids, tetratriterpenoids, limonoids, phenolic acids and steroids (Das *et al*., 2014). Bark of *X. moluccensis* was reported containing catechin, epicatechin and procyanidin (Das *et al*., 2014). The limonoids xyloccensin (A-I), xylocarpin and humilin B were previously isolated from the bark and leave extracts of *X. moluccenesis* (Li *et al*., 2009). An investigation on limonoids from seeds of *X. moluccensis* also led to the identification of several new and known molecules including moluccensins R-Y, 6-hydroxymexicanolide, 2-hydroxyfissinolide and xyloccensins (Li *et al*., 2012). Seven new phragmalins with a C-30 carbonyl moiety, from the seeds of *X. moluccensis* were also reported (Li *et al*., 2009). However, no available details on chemical constituents of roots of the plant.

Based on, the reported properties and the traditional uses of *X. moluccensis* for treatments of ailments related to inflammation and central nervous system disorders, we attempted to investigate anti-inflammatory and cholinergic effects of metabolic agents from the plant.

This study highlights *in vitro* inhibitory effects of cyclooxygenase, 5-lipoxygenase and acetylcholinesterase enzymes by α-guaiene and oil isolated from the methanolic root extract of *X. moluccensis*.

MATERIALS AND METHODS

Plant material and extraction: The name *Xylocarpus moluccensis* (Lam.) M. Roem has been checked and verified with www.theplantlist.org as an accepted name for this plant species with original 3 publication details: Fam. Nat. Syn. Monogr. 1: 124 1846. Root of *X. moluccensis* was collected from Setiu Wetland mangrove plantation in Terengganu-Malaysia (GPS, Latitude 5:40:37.99972; Longitude: 102:42:45.999). A voucher specimen (Eldeen *et al*., 2015) was deposited in the herbarium of the Institute of Marine Biotechnology, University Malaysia Terengganu. The collected material was dried in an oven at 55°C for 7 days and powdered. The powdered root material (915 g) was then extracted sequentially using dichloromethane (residue obtained was 13.9 g), ethyl acetate (residue obtained was 43.8 g) and methanol (residue obtained was 34.7 g). The extracts were evaluated for their inhibitory effects against the enzymes: Cyclooxygenase (COX1 and 2), 5-lipoxygenase (5-lipox) and acetylcholinesterase (AchE) using the *in vitro* bioassay models (described later in this section). The methanolic extract showed the best overall performance against the three tested enzymes and therefore, was selected for further investigation. Fifteen grams of the methanolic extract was subjected to a column chromatography over silica gel (Merck 230-400 meshes, 150 g) using a gradient of hexane: ethyl acetate. Initially, 100% hexane was used and then further reduced to 50% hexane in 5% increments. Then ethyl acetate: methanol gradient was employed with 5% increments till 100% methanol. Twenty three fractions were collected and fractions of the same RF values (fractions: 1-9, 10-16, 17-20, 21-23) were combined and tested for their inhibitory effects against the three tested enzymes. The best overall activity was recorded for combination 1 (combination of fraction 1-9). This fraction (2 g) was subjected to a second column chromatography using gradient of chloroform: butanol: methanol 2:1:1. This separation yielded, fraction A white powdered material (6 mg) and fraction B a yellow color oil.

GC-MS analysis: The isolates were subjected to Gas Chromatography-Mass Spectrometry (GC-MS) analysis using Shimadzu QP 2010 equipped with a Elite-I, fused silica capillary column (30×0.25 mm 1D, BP5MS, 0.25 μM). For GC-MS detection, an electron ionization system with ionizing energy of 70 eV was used. Helium gas (99.999%) was used as the carrier gas at constant flow rate of 1 mL min^{-1} and an injection volume of 1 μL was employed. Injection temperature were 100 and 300°C for the powdered material and the oily fraction respectively. Ion-source temperature was 200°C for both. Mass spectra were taken at 70 eV; a scan interval of 0.5 sec and fragments from 50-600 Da. Total GC running time were 10 and 56 min for the powdered material and the oily fraction, respectively. The relative percentage amount of each component was calculated by comparing its average peak area to the total areas, software adopted to handle mass spectra and chromatograms was a Turbo mass.

In vitro bioassay models used

Cyclooxygenase inhibitor screening assay: The anti-inflammatory activity of the isolated compounds was indicated by inhibition of prostaglandins biosynthesis. This was assessed using the COX inhibitor screening assay kit (No. 560131; Cayman Chemical, USA) based on the manufacturer's protocol provided. The assay (includes both ovine COX-1 and human recombinant COX-2) directly measures PGF2α by $SnCl_2$ reduction of COX-derived PGH2 produced in the COX reaction. It is based on the competition between PGs and a PG-acetyl cholinesterase 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 be inversely proportional to the concentration of PG in the well. The plate was washed to remove any unbound reagents and then Ellman's reagent was added to the well and yielded yellow color. The intensity of the color was determined spectrophotometrically using micro plate Reader at 420 nm. The COX 100% initial activity was generated by the addition of 10 μL of heme to 950 μL of the reaction buffer in test tube followed by the addition of 10 μL of COX-1 (for COX-1 100% initial activity) or COX-2 (for COX-2 100% initial activity) enzymes. Background values were generated for the two enzymes by transferring 20 μL of each enzyme to a 500 μL microfuge tube and placed it in boiling water for 3 min. The test samples were re-dissolved in ethanol to a concentration of 100 μg mL^{-1}. Indomethacin (positive control) (Sigma) was dissolved to a concentration of 50 μg mL^{-1}. Pre-incubation time between enzyme and inhibitor was 10 min with 2 min incubation in the presence of AA 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 PGE2 production by the tested compounds and indomethacin was calculated from the standard curve using Graph pad prism version 3.00 for windows. The IC_{50} values were calculated from the concentration response curve by regression analysis using Graph pad prism. The values reported are the means of three separate experiments.

The 5-lipoxygenase inhibitor screening assay: The 5-lipoxygenase (5-lipox) inhibitory effects of the plant extracts were evaluated using the 5-lipox inhibitor screening assay kit (Item No. 760700; Cayman Chemical, USA). The test was performed based on the manufacturer's protocol provided. In this assay, the detection reaction is confirmed to be sensitive to hydroperoxides at various positions within the fatty acid of any carbon length, therefore, the reaction is suitable as a general detection method for 5-lipox and could therefore be used for screening of natural products from different origins with unknown mechanism of actions. Two blank wells were prepared using assay buffer. The 100% initial activity wells were made by adding 10 µL of methanol (the solvent used to dissolve the test samples) to 90 µL of 5-lipox enzyme. Inhibitor wells were generated by adding 10 µL of the tested samples to 90 µL of the enzyme. The test samples were re-dissolved in ethanol to a concentration of 100 µg mL^{-1}. Zileuton (positive control) was dissolved to a concentration of 50 µg mL^{-1}. The reaction was initiated by the addition of 10 µL of the substrate (arachidonic acid) to all wells of the used 96-well plate. The plate was then placed on a shaker for at least 5 min, followed by the addition of chromogen to stop enzyme catalysis. The plate was then covered and placed on a shaker for 5 min. The absorbance was measured at 420 nm using a plate reader after removing the plate cover. Inhibition percentages were calculated by subtracting the average absorbance of the 100% initial activity from the absorbance of inhibitors. The IC_{50} values were calculated from the concentration response curve by regression analysis using Graph pad prism. The values reported are the means of three separate experiments.

Acetylcholinesterase enzyme inhibitory activity: Inhibition of acetylcholinesterase biosynthesis by plant extracts was investigated using the microplate assays. The assay is based on Ellman's method (Ellman *et al*., 1961) with modifications. The enzyme activity is measured by observing the increase of a yellow color produced from thiocholine when it reacts with the dithiobisnitrobenzoate ion. Acetylthiocholine iodide (ATCl), acetylcholinesterase (AChE), from electric eels (type VI-S lypophilized powder), 5,5-dithiobis-2-nitrobenzoic acid (DTNB) and galanthamine (standard drug) were obtained from Sigma-Aldrich. The following buffers were used; Buffer A:50 mM tris-HCl, pH 8; Buffer B: 50 mM tris-HCl, pH 8 containing 0.1% Bovine Serum Albumin (BSA); Buffer C: 50 mM tris-HCl, pH 8, containing 0.1 M NaCl and 0.02 M $MgCl_2.6H_2O$. In the 96-well plates, 25 µL of 15 mM ATCl in water, 125 µL of 3 mM DTNB in buffer C, 50 µL of buffer B. The tested materials were dissolved in methanol to a concentration of 100 µg mL^{-1}. From which, 25 µL were added to well A and serially diluted till well H. Galanthamine (positive control) was used with concentration was 50 µg mL^{-1} in well A. The absorbance was measured at 405 nm every 45 sec (five times). Then 25 µL of 0.2 U mL^{-1} of enzyme were added, the absorbance was measured again every 45 sec (eight times). The rate of reaction was calculated. Any increase in absorbance due to the spontaneous hydrolysis of the substrate was corrected by subtracting the ratio of reaction before adding the enzyme from the rate after adding the enzyme. Percentage of inhibition was calculated by comparing the reaction rates for the sample to the blank (methanol in buffer A).

RESULTS

GC-MS analysis and identification: The GC-MS analysis of the fraction A and B isolated from the methanolic root extract of *X. moluccensis* including list of the constituents identified and their peak area percentage, are presented in Table 1. For fraction A, α-guaiene (Fig. 1) was detected as a major components with a peak area of 98.54%, in addition to other 3 minor constituents with area percentage <1%. For fraction B, 15 components were identified. Phthalate ester group was appeared to be the dominant chemical class, representing 96.08% of the total yellow oil fraction. This was mainly due to the existence of bis (2-ethylhexyl) phthalate (32.17%), bis (2-ethylhexyl) isophthalate (25.52%), di (2-octyl) phthalate (15.53%) and 6-ethyloct-3-yl 2-ethylhexyl phthalate (12.46%).

Biological activities observed by the isolated agents: Results of the inhibition (inhibition percentage and IC_{50} values) of cyclooxygenase (COX-1 and 2), 5-lipox and AchE enzymes by, the crude extract (combination 1), the isolated α-guaiene (fraction A), the isolated oil (fraction B) and positive controls used, are presented in Table 2. Combination 1 (combined fraction 1-9) possessed inhibitory effects against, COX-1 (88%), COX-2 (67%), 5-lipox(86%) and AchE (93%), when tested at a concentration of 100 µg mL^{-1}. The subsequent fraction isolated from combination 1 was the fraction containing α-guaiene (fraction A). This fraction showed strong activities against, COX-1 (IC_{50} 43 µg mL^{-1}), 5-lipox (IC_{50} 27 µg mL^{-1}) and AchE (IC_{50} 21 µg mL^{-1}). However, it showed weak activity against COX-2 with IC_{50} value of 84 µg mL^{-1}.

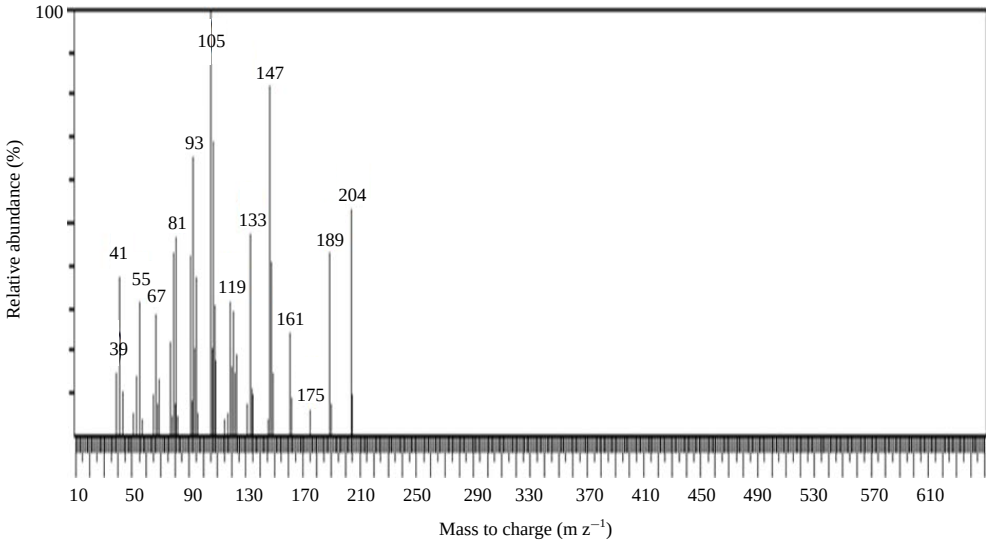

Fig. 1: Mass spectrum of the peak with retention time of 6.553 and peak area of 98.54%. The spectrum was searched against the library and the compound was identified as α-guaiene

Table 1: Chemical composition of fraction A and B, isolated from the methanol roots extract of *X. moluccesis*

Analyzed material	No.	Retention time	Compounds	Peak area (%)
Fraction A (powdered material)	1	0.263	1H-cycloprop(e)azulen-7-ol,	0.06
	2	0.411	2-(1-methyl-4-methylene)- cycloheptane	0.93
	3	5.046	Kaur-16-ene	0.34
	4	6.553	α-guaiene	98.54
Fraction B (oily fraction)	1	15.028	Methoxytriethylene glycol	0.67
	2	22.061	Butoxytriethylene glycol	1.77
	3	22.748	Pentaethylene glycol monomethyl ether	0.27
	4	26.658	4-(3-hydroxy-1-butenyl)-3,5,5-trimethyl-2-cyclohexen-1-one	0.27
	5	28.218	4-(2-methyl-cyclohex-1-enyl)-but-3-en-2-one	0.28
	6	28.747	3,6,9,12,15-pentaoxanonadecan-1-ol	0.27
	7	29.600	2,5,5,8a-tetramethyl-6,7,8,8a-tetrahydro-5H-chromen-3-one	0.38
	8	32.818	Methyl hexadecanoate	0.30
	9	36.789	Methyl octadecanoate	0.77
	10	41.663	Diisooctyl phthalate	0.19
	11	44.083	Bis(2-ethylhexyl) phthalate	32.17
	12	44.157	Di(2-octyl) phthalate	15.53
	13	44.258	Bis(2-ethylhexyl) isophthalate	25.52
	14	44.329	L-alanine, N-(2-chloroethoxycarbonyl) and pentadecyl ester	9.14
	15	44.373	6-ethyloct-3-yl 2-ethylhexyl phthalate	12.46

Table 2: Inhibition of Cyclooxygenase 1 and 2, 5-lipoxygenase and Acetylcholinesterase enzymes by, the combination1, the α-guaiene and oil isolated from the root of *Xylocarpus moluccensis* as detected using *in vitro* biological models

	Biological activities obtained as inhibition (%) and IC_{50} values (μg mL^{-1})							
	Cyclooxygenase							
	COX-1		COX-2		5-lipoxygenase		Acetylcholinesterase	
Isolates and positive controls tested	Inhib (%)	IC_{50}	Inhib (%)	IC_{50}	Inhib (%)	IC_{50}	Inhib (%)	IC_{50}
Combination1 (fraction1-9)	88±2.7	-	67±1.6	-	86±3.1	-	93±2.8	-
α-guaiene	76±2.5	43	55±1.4	84	79±2.0	27	88±1.7	21
Oil	44±3.3	120	26±4.2	nt	88±1.6	33	79±1.3	25
Indomethacin	94±2.1	17	73±5.1	44	-	-	-	-
Zileuton	-			-	92±4.5	22.16	-	-
Galanthamine	-	-	-	-	-	-	93±2.2	14

Inhib: Inhibition. Inhibition (%) was obtained at a concentration of 100 μg mL^{-1} for the tested plant materials. Inhibition (%) of the standard drugs, indomethacin, zileuton and galanthamine were obtained at a concentration of 50 μg mL^{-1}. nt: Not active at the highest concentration used (125 μg mL^{-1})

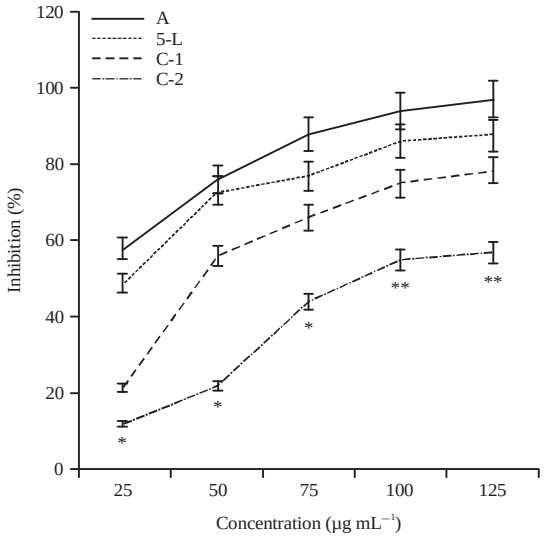

Fig. 2: Concentration response of α-guaiene (fraction A) against COX-1 (C-1), COX-2 (C-2), 5-lipox(5-L) and AchE (A). Concentration used of α-guaeine (µg mL^{-1}). Points represent means with 95% confidence intervals (n = 8, p~05). *: Significant difference and **: No significant difference

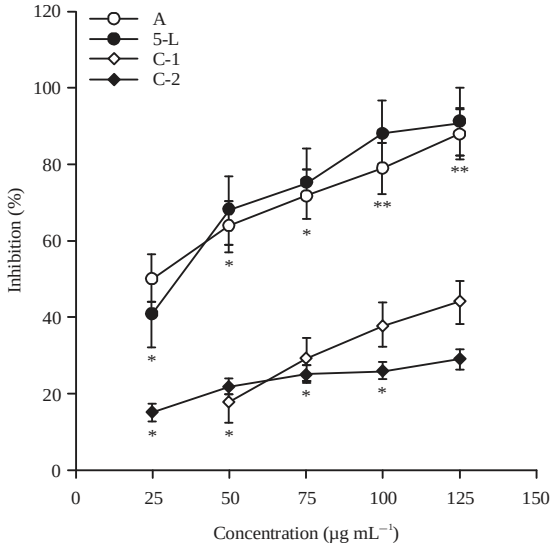

Fig. 3: Concentration response of the oil (fraction B) against COX-1 (C-1), COX-2 (C-2), 5-lipox(5-L) and AchE (A). Concentration used of α-guaeine (µg mL^{-1}). Points represent means with 95% confidence intervals (n = 8, p~05). *: Significant difference and **: No significant difference

The oil (fraction B), possessed strong activities against 5-lipox and AchE with IC_{50} values of 33 and 25 µg mL^{-1}, respectively. However, it appeared weaker against both the cyclooxygenase enzymes (IC_{50} values ≥125 µg mL^{-1}). Indomethacin (the positive control) inhibited COX enzymes with IC_{50} values of, 17 and 44 µg mL^{-1} against COX-1 and 2, respectively. Inhibition of 5-lipoxygenase by the standard drug Zileuton was recorded with IC_{50} value of 22.16 µg mL^{-1}. Galanthamine inhibited activity of acetylcholinesterase with IC_{50} value of 14 µg mL^{-1}.

Concentration responses of the α-guaiene and oil (fraction A and B) against COX (1 and 2), 5-lipox and AchE enzymes were obtained using the *in vitro* bioassays as described earlier. Five concentrations (25, 50, 75, 100 and 125 µg mL^{-1}) were used with three replicates for each concentration. Means were plotted in a graph with error bars representing 95% confidence intervals. For the α-guaiene (fraction A), the inhibition response increased significantly with the increase of concentrations from 25-100 µg mL^{-1} against the four tested enzymes (Fig. 2). It also showed remarkable dual inhibitory effects against 5-lipox and AchE enzymes. The oil (fraction B) on the other hand showed increased inhibitory effects against 5-lipox and AchE with the increase of concentration from 25-100 µg mL^{-1} (Fig. 3). The overall significant concentration response by both the α-guaiene and oil were observed against 5-lipox and AchE enzymes.

DISCUSSION

Guaienes are a series of closely related natural chemical compounds that have been isolated from a variety of plant sources. The guaienes are sesquiterpenes with the molecular formula $C_{15}H_{24}$. The α-guaiene was first isolated from guaiac wood oil from *Bulnesia sarmientoi*. The guaienes are used in the fragrance and flavoring industries (Yin *et al*., 2013; Huang *et al*., 2014, 2015).

The GC-MS analysis of fraction B indicated the presence of bis (2-ethylhexyl) phthalate, Alanine N-(2-chloroethoxycarbonyl)-pentadecyl ester among other constituents. The bis (2-ethylhexyl) phthalate, is a member of phthalic acid esters (Sui *et al*., 2014). It has been widely used in the production of polyvinyl chloride as plasticizers and non-plasticizers consumer products (Sui *et al*., 2014; Ye *et al*., 2014). Plasticizes are oily liquids and the existence of this agent in the obtained fractionated oil in this study may be due to the effects of polluted environmental reaction rather than being a natural end product of the plant metabolic pathway. This assumption is supported by the detection of compounds such as bis (2-ethylhexyl) isophthalate, di (2-octyl) phthalate and 6-ethyloct-3-yl 2-ethylhexyl phthalate. Moreover, bis (2-ethylhexyl) was found to be an important pollutant in some contaminated soils (Barnabe *et al*., 2008; Ma *et al*., 2015).

Sesquiterpenes, are secondary metabolites produced mainly in higher plants but also in fungi and invertebrates. They could become a rich reservoir of candidate compounds as anti-inflammatory, antiparasitic and anti-carcinogenic agents (Bartikova *et al*., 2014). Systemic treatment with sesquiterpene-related molecules, such as α-humulene and trans-caryophyllene was proven to be effective for eradication of inflammatory symptoms due to their effects on the expression of COX enzymes, beside other mechanism on activation and/or release of inflammatory mediators like bradykinin, platelet activating factor, histamine, IL, IL-1b, TNF and PGE2 (Fernandes *et al*., 2007). Similar to the effects of the carboxylic acids class of NSAIDs, some sesquiterpene molecules, such as patchouli alcohol were found to interact with COX-1, forming a salt bridge of hydrophobic channel, which put the aromatic portion of these molecules in the direction of the tyrosyl radical in the cyclooxygenase active site (Raharjo *et al*., 2014). This may also explain the observed strong activity of the α-guaine against COX-1 in comparison with COX-2 in this study. Mendes *et al*. (2013) reported isolation of two new guaiane-type sesquiterpene glycosides from the methanolic extract of *Pittosporum undulatum* fruits, along with six known guaiane isomers. These isolates possessed good anti-inflammatory effects in an *in vitro* anti-inflammatory models. This is in line with our current findings on the anti-inflammaory effects of the fraction containing α-guaiene isolated from the root of *X. moluccensis* as indicated by the inhibitory effects against the three tested inflammatory related enzymes.

Oil from other parts of *X. moluccensis* were previously analyzed and reported containing different types of linoleic acids among other components (Gunawan *et al*., 2013). Butovich and Lukyanova (2008) studied effects of linoleyl hydroxamic acid-a derivative of linoleic acid -on several enzymes involved in arachidonic acid metabolism in mammals including 5-lipox and COX. The authors indicated that this derivative of linoleic acid showed strong activity against 5-lipox in comparison with its activity against COX. This is similar to the findings on the effects of the oil from the root of *X. moluccensis* against 5-lipox in this study. This activity may be due to the presence of linoleic acid derivatives or other related constituents with similar mechanism of action against 5-lipox as there are some compounds for which GC-MS is not sufficiently sensitive (gas chromatography-mass spectrometry https://en.wikipedia.org/wiki/Gas_chromatography).

Cholinesterase inhibitors increase the amount of acetylcholine at the neuronal synaptic cleft by inhibiting the enzyme responsible for the hydrolysis of acetylcholine and consequently improve neuronal transmission (Huston *et al*., 2006; Pavlov *et al*., 2006). Inhibition of acetylcholinesterase activity by the isolated α-guaiene and oil in this study indicated their potential for therapeutic uses in treatment of cognitive disorders. On the other hand, these biological effects could be evaluated together with the potential anti-inflammatory effects possessed by the inhibition of 5-lipox. Since inhibition of AchE also contributed or accounted for anti-inflammatory properties (Rosas-Ballina and Tracey, 2009), the investigated plant material could be a good candidate as crude anti-inflammatory agent with potential effects on both inflammatory related enzymes 5-lipox and AchE. Sarker *et al*. (2007) reported assessment of the methanolic extracts of the barks and pneumatophores of *X. moluccensis* on the Central Nervous System (CNS) using a series of established pharmacological tests including pentobarbitone-induced sleeping time, open field, hole cross, hole-board and evasions tests in mice model. These extracts produced a dose-dependent reduction of the onset and duration of pentobarbitone-induced hypnosis. The authors concluded that both the bark and pneumatophore extracts of *X. moluccensis* possess CNS depressant activity and the pneumatophore extract being more potent than the bark extract tested. Similar results on methanolic extracts of *X. moluccensis* and *X. granatum* were also reported previously, indicating significant biological effects against symptoms related to central nervous system disorders (Alamgir *et al*., 2006). Characterization of the cholinergic anti-inflammatory pathway has provided new grounds for understanding and treating inflammatory diseases. Nicotine has been used to treat ulcerative colitis, a disease characterized by inflammation in the large intestine. The effect of the drug confirmed its potentiality in clinical trials against conditions related to acute or chronic inflammation including, autoimmune diseases such as rheumatoid arthritis (Rosas-Ballina and Tracey, 2009). These findings support the concept of interdependency of the nervous and immune systems, suggesting that acetylcholinesterase inhibitors may contribute to the suppression of inflammation symptoms (Pollak *et al*., 2005; Yuan *et al*., 2006; Rosas-Ballina and Tracey, 2009). These concepts justify the attempt to correlate between the inhibition of 5-lipox and AchE by the isolated substances from *X. moluccensis*. This study may suggest future research direction for possible reduction of inflammatory symptoms via the cross talk between inflammatory mediators and nervous systems.

Although, the two isolated fractions from the root of *X. moluccensis* in this study showed weak or no selective inhibition against COX-2, the dual inhibitory effects of COX-1 and 5-lipox observed by α-guaiene may still contribute to the

elimination of inflammatory symptoms. Combined inhibition of COX and 5-lipox may provide treatments for inflammatory related diseases with less adverse effects.

Naturally occurring bioactive agents such as terpenes in most cases occur as multi-component and may therefore not, be compatible with the so-called "One-molecule-one-target" dogma of classical pharmacotherapy. However, their remarkable biological activities enable them to be major target in the domain of complementary and alternative medicinal therapies (Buchbauer and Ilic, 2013). This may also lead to the development of nutraceutical agents. Nutraceuticals have recently become essential for the development of nontoxic, inexpensive and easily available drugs to protect different diseases (Ghia *et al*., 2006; Ofek *et al*., 2007).

CONCLUSION

This study is part of the ongoing investigation on ethnopharmacology of Malaysian mangrove plants. The article deals with the medicinal properties of *X. moluccensis*, a reputable mangrove medicinal plant with various applications in traditional medicine for several ethnic groups. These findings contribute to the phytochemical study of *X. moluccensis* and to the scientific validation of the anti-inflammatory and anticholinergic effects of its root. The study also contributes to the verification of claims made on the traditional medicinal properties of the tree. Isolation of α-guaiene and the biological activities observed using these bioassay models are unprecedented and contributing therefore, to the novelty of the work. The efforts are now focused on determination of mechanism of actions of these molecules using gene expression analysis and cell lines based bioassays.

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