Efficacy of three mangrove plants against 5-lipoxygenase, acetylcholinesterase enzymes and five pathogenic bacterial strains

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Abstract

Mangroves are highly productive ecosystem with various important economic and environmental functions. They are the key elements in marine environment that produce diverse metabolites to adapt with the requirement of their challenging ecosystem. This make them an interesting source for natural bioactive molecules.

In this study, we investigated inhibitory effects of extracts from Avicenna lanata, Ceriops tagal and Sonneratia alba against 5-lipoxygenase, acetylcholinesterase enzymes and four pathogenic bacterial strains using in vitro models.

Best dual inhibitory effects against the two enzymes was recorded for the methanolic and ethylacetate bark extracts (final concentration used in the assay was 90 µg/ml) of Sonneratia alba and dichloro-root extract of C.tagal (inhibition percentage ranging between 74-91%). Roots of Ceriops tagal showed the highest activity against lipoxygenase (93%), but was slightly weaker against AchE (83%).

Antimicrobial properties of the extracts was determined using the microdilution assay. A. lananta (bark) showed the best antimicrobial effects with the lowest minimum inhibitory concentration (MIC) value of 90 µg/ml against S.aureus, E.coli and K. pneumoniae. Methanolic root and leaf extracts of C.tagal showed the same MIC values against S.aureus. Phytochemical analysis indicated the presence of alkaloids, steroids and tannins in the investigated plant parts. These results support the ethnobotanical uses of these plants. Chemical profiling, isolation and determination of mechanism of actions of the observed bioactivities are currently in progress in our laboratory.

Keywords —Mangroves, Natural products, Inflammation Lipoxygenase
I. INTRODUCTION

Mangroves are highly productive ecosystem associates with diverse economic and environmental functions. The term is used to designate halophytic and salt resistant marine tidal forests comprising of trees, shrubs, palms, epiphytes, ground ferns and grasses (Premanathan et. al., 1999). Apart from the ecological roles, ethnobotanical evidences indicated the utilization of a number of mangrove plant species in traditional medicine against human, animal and plant pathogens. Due to their special growth environment, mangroves produce diverse group of metabolic substances with wide range of biological activities including inflammatory related ailments and infectious diseases. The medicinal properties of mangrove trees therefore, provide a wide domain for biological applications that worth further investigation (Eldeen and Effendy, 2013)

(Simone et.al. 2005) Inflammation is a disorder involving localized increases in the number of leukocytes and a variety of complex mediator molecules. The inhibitions of numerous rate-limiting processes could be important in the successful treatment of an inflammatory disorder (Premanathan et. al., 1999). Prolong inflammation may lead to pathological conditions including immune-mediated diseases such as multiple sclerosis, acute neurodegeneration following ischemia or trauma, and, more recently, chronic neurodegenerative diseases (Simone et.al., 2005).

![Inflammation diagram]

The non-steroidal anti-inflammatory drugs (NSAIDs) are proven to be effective for treatment of inflammation symptoms due in most cases to their ability to inhibit prostaglandin synthesis by binding reversibly and irreversibly to the enzyme. However, their draw back or toxicities are due to their ability to
block synthesis of the housekeeping prostaglandin as a result of inhibition of COX-1 (Portanova et.al., 1996). Leukotriene modifiers are a class of drugs used for the treatment of inflammatory related disorders such as asthma. These drugs have introduced recently into clinical practices (Samaria, 2004). Their mechanism of actions are believed to be due to their effects on leukotrienes by interrupting the 5-lipoxygenase pathway. leukotrienes are pro-inflammatory mediators that could rapidly increase the inflammatory responses. They may also contribute to development of certain types of tumor such as colon tumor (Samaria, 2004; chan et.al. 2012). Development of compounds that inhibit 5-LOX or both COX-2 & Lipox would be advantageous due to their ability to target both proteins, enhancing their individual anti-inflammatory effects and reducing their associated side effects (chan et.al., 2012 ; Rao et.al., 2012;).

For this target, natural products containing chemical entities with a wide structural diversity serve as a useful source of potential compounds with possible dual LOX/COX inhibitors. Among the other endogenous mechanisms that regulate the inflammatory response are the cross-action between the immune and nervous systems (Rao et.al. 2012). An In vivo study indicated that electric stimulation of the vagus nerve attenuates the inflammation during endotoxemia in rats, and that acetylcholine (ACh), the main parasympathetic neurotransmitter, effectively deactivates peripheral macrophages and inhibits the release of pro inflammatory mediators. Inhibition of acetylcholinesterase enzyme therefore, is also relevant with its possible actions on some of the pro inflammatory mediators (Rao et.al. 2012).

This reports aimed to evaluate inhibitory effects of 5-liopoxygenase, acetylcholinesterase enzymes, and eradication of microbial growth by three mangrove plants Avicennia lanata, Ceriops tagal and Sonneratia alba.

II. MATERIAL AND METHODS

Plant material and extraction

Plant materials including leaves, root and/or bark of the three mangroves were collected from Setiu Wetland, Terengganu. A voucher specimen (Eldeen 9,10,11) was deposited in the Herbarium of The Institute of Marine Biotechnology, University Malaysia Terengganu.
The collected materials were dried in an oven at 55°C for 7 days, powdered and extracted sequentially using dichloromethane, ethyl acetate and methanol. Residue obtained were concentrated to dryness and kept in room temperature for further bioassay tests.

- **Phytochemical Screening**

The plant extracts were subjected to phytochemical screening test to determine the presence of carbohydrates, phenols tannins, saponins, glycosides, steroids, terpenoids and alkaloids.

- **Bioactivity Screening**

  - Lipoxygenase inhibitor screening assay

    The 5-lipoxygenase (5-Lipox) inhibitory effects of the plant extracts were evaluated using the Lipox inhibitor screening assay kit (Item No. 760700; Cayman Chemical, USA). The assay was performed based on the supplier's provided protocol. In this assay, the detection reaction was confirmed to be sensitive to hydroperoxides at various positions within the fatty acid of any carbon length, therefore, the reaction is seen to be suitable as a general detection method for Lipox, and can be used for screening of natural products from different origins with unknown mechanism of actions. Inhibition percentages were calculated by subtracting the average absorbance of the 100% initial activity from the absorbance of inhibitors.

  - Acetylcholinesterase enzyme inhibitory activity

    Inhibition of acetylcholinesterase biosynthesis by the plant extracts was investigated using the microplate assays based on Ellman's method with modifications as described before (Eldeen et al., 2005). The enzyme activity was measured by observing the increase of a yellow color produced from thiocholine when it reacts with the dithiobis-nitrobenzoate ion. The bioassay was carried out using the 96-well microplate. One 96-well microplate was used for three samples. 25µl of 15mM ATCI in water, 125µl of 3mM DTNB in buffer C, 50µl of buffer B, 25µl of sample, methanol and galanthamine, and 25µl of AchE
was loaded into each well of the 96-well microplate. 25µl of each sample (resuspended to a concentration of 100 µg/ml using methanol to give final concentration of 10 µg/ml in the assay) was added the first row (row A) and two fold serially diluted. The galanthamine was used as the positive control while methanol as the negative control in the test. The absorbance was measured at 405 nm immediately after the addition of the enzyme. The rate of reaction was calculated. Three replicate of the test was done.

- **Micro-dilution antibacterial assay**

To determine the minimum inhibitory concentration (MIC) of the extracts, 96-well microplates was used following the serial dilution technique methods (Eloff, 1998). Five bacterial strains–two Gram-positive: *Bacillus cereus* and *Staphylococcus aureus*, and three Gram-negative: *Escherichia coli*, *Salmonella typhimurium* and *Klebsiella pneumoniae* was subcultured overnight in the Mueller Hinton broth. On the next day, 100 µl of each subcultured bacteria was inoculated into the new vials containing 10 ml of new MH broth. The plant extracts were re-dissolved in sterile water to a concentration of 5 mg/ml (final concentration of 1.25 mg /ml in the assay) was used. The assay was performed as described before (Eldeen et.al., 2010 ; Eldeen, 2014).

To indicate the bacterial growth, 50µl of Resazurine blue (1 mg/5ml millipore water) was added into each of the well. Bacterial growth in the wells was indicated by a purple/pink color, whereas clear blue wells indicated inhibition by the tested substances (Eldeen, 2014).

### III. RESULTS AND DISCUSSION

**Phytochemical Constituents**

The phytochemical tests indicated the presence of alkaloids, carbohydrates, glycosides, saponins, steroids, tannins and terpenoids in the leaf and bark of *S.alba*. With the exception of *A. Lanata*, tannins and terpenes were also detected in almost all the plant part tested. Glycoside and steroids were not detected in the leaf and root of *C.tagal* and leaf of *A.lanata* (Table 1).

Phenols, tannins, glycosides, steroids, terpenoids, alkaloids and flavonoids are secondary metabolites exist in higher plants. These secondary metabolites are needed for the plants to interact with their environment for the protection against biotic or abiotic stresses such as infections, wounding, UV irradiation, exposure to ozone, pollutants, and herbivores (Oksman-caldentey and Barz, 2002). Some of these classes are
parent molecules for biosynthesis of numerous structurally and functionally diverse plant-derived end product molecules which play essential roles in plant physiology (Oksman-caldentey & Barz, 2002; Korkina et al., 2011).

The spectrum of secondary metabolites produced by plants increase with the environment and harsh growth conditions. Due to the harsh condition of mangrove environment, such metabolic agents are expected to be diverse in quantity and quality including their wide range of biological activities (Oksman-caldentey and Barz, 2002). Plant-derived phenylpropanoids represent the largest group of secondary metabolites produced by higher plants. Their medicinal roles as antioxidants, anti-inflammatory, wound healing, and antibacterial agents were previously highlighted. However, these molecules also reported to possess limiting factors indicated by their potential toxicity and difficulties with sensitization (Oksman-caldentey and Barz, 2002). Therefore, biological evaluation and assessment of their efficacy is urgently needed.

Table 1 Phytochemical screening of extracts of the three mangrove plants A. Lanata, C.tagal and S.alba

| Plant name | Plant part analyzed | alk | car | gly | sap | Ster | tann | terp |
|------------|---------------------|-----|-----|-----|-----|------|------|------|
| Avicennia lanata | leaf | + | + | - | - | - | - | - |
| | bark | - | + | + | - | + | + | - |
| | root | - | + | + | + | + | + | - |
| Ceriops tagal | leaf | + | + | - | + | - | + | + |
| | bark | nt | nt | nt | nt | Nt | nt | nt |
| | root | + | + | - | + | - | + | + |
| Sonneratia alba | leaf | + | + | + | + | + | + | + |
| | bark | + | + | + | + | + | + | + |
| | root | nt | nt | nt | nt | nt | nt | nt |

Al=alkaloids; car= carbohydrates; gly= glycosides; sap= saponins; ster= Steroids; tann= tannins; terp= terpenoids. nt= not tested.

- 5- lipoxygenase enzyme inhibitory activity

Results of 5- lipoxygenase enzyme inhibitory effects by the three mangrove plant extracts are given in Table 2. Extracts showed inhibitory effects with percentage of inhibition >80% are considered highly
active when tested at final concentration of 90 µg/ml in the assay. Both leaf and root extracts of C.tagal showed strong activities against the enzyme (exception was ethyl acetate and methanolic root extracts).

Table 2
Lipoxygenase and acetylcholinesterase enzymes inhibitory effects by plant extracts (10 mg/ml) of three mangrove plants: A.lananta, C.tagal and S.alba when tested using in vitro models.

| Plant species       | Plant part analyzed | 5-Lipox inhibition (%) | AchE Inhibition (%) |  |  |  |  |  |  |  |  |  |  |
|---------------------|---------------------|-------------------------|---------------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|
|                     |                     | Extracts tested         |                     |                          |                          |                          |                          |                          |                          |                          |                          |                          |                          |
|                     |                     | Dichloromethane         | Ethyl acetate       | Methanol                 | Dichloromethane         | Ethyl acetate         | Methanol                 |                          |                          |                          |                          |                          |                          |
| Avicennia lanata    | Leaf                | 66±3.1                  | 69±1.9              | 55±3.1                   | 61±2.9                   | 61±2.9                   | 67±2.7                   |                          |                          |                          |                          |                          |                          |
|                     | Bark                | 71±3.0                  | 70±1.7              | 64±5.5                   | 63±3.1                   | 66±4.6                   | 57±1.3                   |                          |                          |                          |                          |                          |                          |
|                     | Root                | 67±2.8                  | 65±2.6              | 58±5.2                   | 55±4.6                   | 71±3.8                   | 73±5.5                   |                          |                          |                          |                          |                          |                          |
| Ceriops tagal       | Leaf                | 80±0.8                  | 83±3.9              | 93±2.4                   | 72.4±1.1                 | 54.3±1.4                 | 43.7±1.8                 |                          |                          |                          |                          |                          |                          |
|                     | Root                | 91±4.0                  | 58±3.0              | 30±2.5                   | 83±2.3                   | 40.6±2.4                 | 38.73±2.7                |                          |                          |                          |                          |                          |                          |
| Sonneratia alba     | Leaf                | 77±3.4                  | 78±3.3              | 81±2.4                   | 68.7±4.4                 | 62.4±1.4                 | 68.2±4.0                 |                          |                          |                          |                          |                          |                          |
|                     | Bark                | 81.2±5.9                | 74.6±3.7            | 85±3.1                   | 76.2±3.2                 | 74.6±3.4                 | 81.3±2.9                 |                          |                          |                          |                          |                          |                          |

Inhibition obtained (%) is expressed as mean ± S.D. Percentage Inhibition of prostaglandin synthesis by indomethacin (standard) was 80±1.9% for COX-1 and 69±2.4% for COX-2. Inhibition (%) of acetylcholinesterase enzyme by galanthamine (20µM) was 93±3.2%

All the extracts from S.alba also showed strong to moderate activities against the enzyme. Percentage of inhibition ranging between 85 and 74%.

It is well known that one of the traditional method to reduce inflammation is through inhibition of enzymes associated with the arachidonic acids including lipoxygenase and cyclooxygenase. Epidemiological and clinical studies on the other hands suggest that COX-2 inhibitors have a better GI toxicity profile than indiscriminative NSAID. However, others also raised concerns regarding effects of of leukotrienes in GI
toxicity (Gale et.al. 2007). Therefore, it is relevant to look for both COX and 5- Lipox inhibitors which may provide better anti-inflammatory effects (Gale et.al. 2007). This was also supported by data from clinical trials which confirmed that, interrupting the leukotriene pathway offers a new opportunity for treating inflammatory related ailments such as asthma (Samaria, 2004). The inhibitory effects observed by both C.tagal and S.alba therefore are in line with this concept of dual inhibitory actions as these extracts also possessed inhibitory effects against cyclooxygenase (data not published yet). The potential anti-inflammatory properties of C.tagal is in agreement with previously reported biological activities of some mangrove plants (Simlai and Roy, 2013). Indomethacin was used as positive control. It was previously reported to possess dual inhibitory effects against COX and 5-Lipox [17].

- Acetylcholinesterase enzyme inhibitory activity

The cholinesterase inhibitory activity of the plant extracts obtained by using the microplate assay are presented in Table 2. Different extracts of the three plants showed activities against acetylcholinesterase enzyme. Best inhibition percentage (83%) was obtained by dichloromethane root extract of C.tagal followed by, all bark extract of S.alba (74-81%), methanolic root extract of A.lanata (73%), dichloromomethane leaf of C.tagal (72%) and ethyl acetate root of A.lanata (71%).

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 (Zangara, 2003; Eldeen et.al. 2008 ;). Inhibition of acetylcholinesterase activity may indicate potential for therapeutic use 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 plant as indicated by the inhibition of 5-lipox. Since inhibition of AchE also contributed or accounted for anti-inflammatory effects (Rao et.al. 2012), we have correlate the activities against the two enzymes (Fig 1). C.tagal (root) and S.alb (bark) appeared to be the best in terms of dual inhibitory effects. This is also in line with the concept that some of anti-inflammatory drugs may lead to a protective effect reducing the incidence of eurodegenerative disorders (McGeer et.al. 1996; Howes and Houghton, 2003 ;). Galanthamine was used as the positive control. It is an alkaloid with mechanism of actions believed to be on both peripheral and central nervous system (Heinrich and Lee, 2004). Both the active parts of the investigated plant confirmed the presence of alkaloids which may suggest similar mechanism of action with the Galanthamine.
Antimicrobial activities of the plant extracts obtained by microdilution assay

For the antimicrobial properties of the investigated plant extracts, 66% of all the extracts tested against the pathogenic bacterial strains showed MIC values ≤ 0.97 mg/ml (Table 3). The leaf and bark extracts of *S.alba* showed activities against all the tested strains with MIC values ranging between 0.97-0.19 mg/ml. Methanolic bark and root extracts of *A.lanata* showed the lowest MIC values (90 µg/ml) against *E.coli*, *S.aureus* and *K.pneumoniae*. Similar effects also observed with the methanolic leaf and bark extracts of *C.tagal* against *S.aureus*.

We reported previously antimicrobial properties of some mangroves species and eendophytes (Eldeen, 2014). The activities observed in this study by *S.alba* are in line with previous findings on the same or closely related species (Saad et.al. 2012). In a previous comparative antimicrobial activities studies on different extracts of aerial part of of *Ceriops decandra*, the methanol extract possessed the best activity (Vadlapudi and Naidu, 2009). This could be comparable to our current finding on the closely related species *C.tagal* using different bioassay test.

IV. CONCLUSION
In this study different extracts from three mangrove plants: *A. lanata*, *C. tagal* and *S. alba* were evaluated for inhibitory effects against 5-Lipox and AchE enzymes beside antimicrobial properties. Phytochemical screening was also carried out to determine the major class of constituents. Methanolic bark and dichloro root extracts of *S. alba* and *C. tagal* respectively were found to possess the best dual inhibitory effects against both the enzyme tested (Fig1). The biological activities observed by leaf extracts in this study are interesting as it can lead to substitution of leaves for roots and bark during utilization of the plants. Harvesting of leaves for medicinal purposes is more sustainable compared to other plant parts such as roots and stem bark.

To our knowledge, this report is the first to highlight the determination of inhibitory effects of these mangrove plants against 5-liopx and AchE enzymes using these bioassay models. Our efforts are now focused on determination of mechanism of actions of these molecules using cell lines based bioassays.

**V. ACKNOWLEDGMENT**

This work was funded by the Fundamental Research Grants (FRGS), managed by Research and Innovation Affairs University Malaysia Terengganu, and arranged by The Research Management Centre (RMC).

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Table 3

Antimicrobial properties of extracts obtained from the mangrove plants *A.lanata, C.taga* and *S.alba* as determined by the micro-dilution assay. Results are minimum inhibitory concentrations (MIC) values in µg/ml.

| Plant species       | Plant part | Dichloromethane | Ethylacetate | Methanol |
|---------------------|------------|-----------------|--------------|----------|
|                     |            | Bacteria tested | Bacteria tested | Bacteria tested |
|                     |            | Bc Sa Ec Kp S.t | Bc Sa Ec Kp S.t | Bc Sa Ec Kp S.t |
| **Avicennia lanata**| Leaf       | 312 320 320 na  na | na 220 550 90 260 | na na na na na |
|                     | Bark       | na 220 na 320 na | na 320 na 320 na | na 90 90 90 na |
|                     | Root       | na na 650 na 520 | na na 550 na na | na 90 90 320 na |
| **Ceriops tagal**   | Leaf       | na 450 580 520 na | na 160 290 220 290 | 260 90 190 130 na |
|                     | Root       | na 320 580 420 na | na 160 290 260 na0.29 | 260 0.26 901 160 130 na 0.26 0.09 |
| **Sonneratia alba**| Leaf       | 870 580 510 580 na | 410 430 210 310 970 | 680 210 430 390 680 |
|                     | Bark       | 730 430 610 580 530 | 580 780 510 530 na | 210 210 190 210 680 |

Gentamicin sulphate | Bc=0.488±0.41; Sa=1.587±2.18; Ec=0.208±0.26; Kp=0.392±0.55; St=1.563±0.00

Bacteria: Bc = *Bacillus cereus*; Ec = *Escherichia coli*; Kp = *Klebsiella pneumoniae*; Sa = *Staphylococcus aureus*; St. *Salmonella typhimurium*. Na = not active at the highest concentration uses (1.25 mg/ml).