International Journal of Pharmacology

ISSN 1811-7775

science alert

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Asian Network for Scientific Information

OPEN ACCESS International Journal of Pharmacology

ISSN 1811-7775
DOI: 10.3923/ijp.2019.916.925

Research Article
Inhibition of Pro-inflammatory Enzymes and Growth of an Induced Rheumatoid Arthritis Synovial Fibroblast by *Bruguiera cylindrica*

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Abstract

Background and Objective: *Bruguiera cylindrica* is a mangrove tree species. It has various medicinal uses including usage for, lower blood pressure, bleeding, hemorrhage and ulcers. This study aimed to investigate the anti-inflammatory and anti-rheumatoid arthritis effects of the plant. **Materials and Methods:** Extracts obtained from the leaves and roots of the plant were evaluated for their inhibitory effects against the enzymes: Cyclooxygenase-2, 5-Lipoxygenase and Acetylcholinesterase using the *in vitro* bioassay models. The GC-MS was used to determine the major active constituents. Synovial fibroblast cells were induced using TNF-α and IL-1 β and MTT cell viability assay was used to determine the cell viability of both, the normal and the induced cells. **Results:** The DCM leave extract showed the highest inhibitory effects against the 3 enzymes tested. Fraction 6 (the main constituent was 1,2-Benzenedicarboxylic acid), possessed the strong activity against the 3 enzymes with inhibition percentage of 96% (IC_{50} = 56), 91% (IC_{50} = 44) and 89% (IC_{50} = 25) against 5-lipox, AChE and COX-2 enzymes, respectively. Fractions 1 and 3 (containing pyrrolidine related compound) showed inhibitory effects against the three enzymes with inhibition percentages ranging between 89 and 75%. The tested materials showed no cytotoxic effects at the highest concentration used (50 μg mL^{-1}) against the normal synovial cells but they possessed activities against the induced RA cell. The results indicated that the activities were dose-dependent. **Conclusion:** The metabolites obtained from *B. cylindrica* in this study possessed inhibitory effects on pro-inflammatory enzymes and cell viability of the induced RA cell line. These results provide scientific validation of the bio-efficacy of *B. cylindrica* as a source of potential bioactive substances for the treatment of inflammatory and arthritis-related diseases. Further study is needed to assess the observed bio-efficacy on an *in vivo* experiment.

Key words: Rheumatoid arthritis, inflammation, natural products, mangrove plants, *Bruguiera* species, COX-2, 5-lipoxygenase, acetylcholinesterase, synovial fibroblast

Citation: Ibrahim M.S. Eldeen, Jochen Ringe and Noraznawati Ismail, 2019. Inhibition of pro-inflammatory enzymes and growth of an induced rheumatoid arthritis synovial fibroblast by *Bruguiera cylindrica*. Int. J. Pharmacol., CC: CC-CC.

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Competing Interest: The authors have declared that no competing interest exists.

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

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory and autoimmune disorder primarily affects the joints and leads to the destruction of the articular cartilage with subsequent severe morbidity and disability. It is characterized by chronic proliferative synovitis, infiltration of inflammatory cells into the synovial tissue and articular cartilage destruction. This damage is mainly driven by lymphocytes, macrophages and fibroblast-like synoviocytes[1]. The growth factors in the inflamed synovium drive the proliferation of resident synovial fibroblast, forming a large cellular mass called a pannus which is known to have an invasive behaviour[2-4].

The inflammatory response is a mechanism of self-defence that must be regulated as excess responses often lead to pathological conditions [5]. The lipoxygenase (Lipox) and cyclooxygenase (COX) pathways are found to be involved in the inflammatory actions[6].

A class of drugs used to inhibit the activity of 5-lipoxygenase (5-lipox) by regulating the formation of interleukins which involve in joint cartilage destruction and related joint inflammatory diseases[7,8]. On the other hand, the non-steroidal anti-inflammatory drugs (NSAIDs) are proven to be effective for the treatment of inflammatory symptoms. However, their side effects represent a major concern[5,9]. Blockage of COX enzymes will also lead to a shift of the arachidonic acid metabolism to the 5-lipox pathway. Development of molecules that inhibit both enzymes (COX-2 and 5-lipox) would be advantageous by targeting both proteins[5,8]. Natural molecules remain a useful source of potential compounds with possible dual Lipox/COX inhibitory effects[10].

Other mechanisms that contribute to the regulation of inflammatory response are on the cross-talk between the immune and nervous systems. Pavlov *et al.*[11] reported a mechanism for neural inhibition of inflammation which interfaces the brain with the immune system indicating the role of the cholinergic anti-inflammatory pathway in the regulation of inflammation. These findings suggest that inhibition of acetylcholinesterase enzyme may contribute to the enhancement of the enzyme-dependent macrophage deactivation which represents an essential step for the cholinergic anti-inflammatory pathway[12,13]. Suppression of acetylcholinesterase will also inhibit the release of pro-inflammatory factors in locally inflamed cells[14].

Bruguiera cylindrica Blume (Rhizophoraceae), is a mangrove plant grow up to 23 m tall. It occurs across South-East Asia and Australia and reported to have various traditional uses. The bark is used to stop hemorrhage and applied to malignant ulcers. The skin of the fruit is used to stop bleeding and the leaves are used to lower blood pressure. Other *Bruguiera* species are also reported to be used in folk medicine for the treatment of different ailments including wound healing, burns and inflammation[15-20]. To our knowledge, no report made available on anti-inflammatory and rheumatoid arthritis effects of *B. cylindrica*. This article highlights *in vitro* inhibition of, cyclooxygenase, 5-lipoxygenase and acetyl cholinesterase enzymes by leave extracts and fractions from *B. cylindrica*. The report also highlights the effects of the plant materials on cell viability of induced rheumatoid arthritis synovial fibroblast.

MATERIALS AND METHODS

Plant materials and extraction: This study was commenced in the month of July, 2014 and completed in December, 2017, in the Institute of Marine Biotechnology, University Malaysia Terengganu. The name *Bruguiera cylindrica* (L.) Blume was checked and verified with The Plant List[21]. Leave, root and bark of the plant were collected from Setiu Wetland mangrove plantation in Terengganu-Malaysia (GPS, Latitude 5:40:37.99972, Longitude: 102:42:45.999). The plant was identified by Dr. Eldeen who was a Herbarium curator of mangrove plants in the Institute. A voucher specimen (Eldeen 14) was deposited in the Herbarium. The collected materials including leaves and roots were dried in an oven at 55°C for 7 days and powdered. The powdered materials (800 g and 1.2 kg for root and leave, respectively) were extracted sequentially using hexane, dichloromethane, ethyl acetate and methanol. The extracts were evaluated for their inhibitory effects against the enzymes: Cyclooxygenase-2 (COX-2), 5-lipoxygenase (5-lipox) and Acetylcholinesterase (AChE) using the *in vitro* bioassay models as will be described later. The methanolic extracts of the 2 parts showed the best overall performance against the 3 tested enzymes and therefore was subjected to liquid separation using hexane, DCM and chloroform. Best activities were observed with the leave DCM extract, which was then subjected to 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. Fifteen fractions were collected and fractions of the same RF values were combined and tested for their inhibitory effects against the three tested enzymes. The best overall activity was recorded for the combination 3 (combination of fraction 7-9). This fraction (5 g) was loaded on

TLC plate and developed using the solvent system: 1:1 diethyl ether: ethyl acetate. This yielded 6 bands. The bands scratched out and washed with methanol using glass pasture pipettes and received in vials. After dryness, the samples were run for GC-MS to identify their major components.

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 a constant flow rate of 1 mL min^{-1} and an injection volume of 1 μL was employed. Total GC running time was 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. The software adopted to handle mass spectra and chromatograms was a Turbo mass.

5-lipoxygenase inhibitor screening assay: The 5-lipoxygenase (5-lipox) inhibitory effects of the plant extracts and the isolated fractions 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. The test samples were re-dissolved in ethanol to give a final concentration of 100 μg mL^{-1} in the assay. 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.

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 test samples were re-dissolved in ethanol to a concentration of 100 μg mL^{-1}. Celecoxib (positive control) (Sigma) was dissolved to a concentration of 50 μg mL^{-1}. The pre-incubation time between the enzyme and inhibitors was 10 min with 2 min incubation in the presence of AA at 37°C. Inhibition of PGE2 production by the tested compounds and the celecoxib was calculated from the standard curve using Graph pad prism version 3.00 for windows. The values reported are the means of 3 separate experiments.

Acetylcholinesterase enzyme inhibitory activity: Inhibition of acetylcholinesterase biosynthesis by the plant extracts and isolated fractions was investigated using the microplate assays. The assay is based on Ellman's method[22], with modifications[23]. 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).

Preparation of the synovial cell line and MTT assay for cell viability test: Human synovial fibroblast (408-05A SFB) with the following characteristic: Tissue Normal healthy human synovial tissue; cryovial: 500,000 HFLS (2nd passage) frozen in Basal Medium w/10% FBS, 10% DMSO; kit cryovial frozen HFLS (408-05a); growth medium (415-500), Subculture Regent Kit (090K), proliferating: Shipped in Gr Med, 3rd passage (flasks or plates); doublings at least 5, was purchased from cell Applications Inc. The cells were prepared and cultured following the manufacturer instructions. The MTT cell viability assay was used to analyze the effect of the extracts and fractions of *B. cylindrica* leaves on the cell viability and determination of toxicity threshold of the test samples on both normal and induced synovial fibroblast cell line. Cells were seeded in 96-well plates at a density of 1×10^4 cells per well. After 24 h culturing, the original culture medium was collected and replaced by culture medium with the test samples at a concentration of 200, 100, 50 and 25 μg mL^{-1}. Methotrexate was used as a positive control. The plate was then kept for another 24 h. Then, 10 mL of MTT (5 mg mL^{-1}) was added to each well and incubated for an additional 4 h at 37°C. Finally, the medium was removed and 100 mL of dimethyl sulfoxide was added to dissolve dye crystals. The absorbance was read on a microplate reader using a 570 nm filter. Percentage of cell viability was calculated. The plates were read using the plate reader at 570 nm with reference at 630 nm wavelengths (Infinite M200, TECAN, Switzerland).

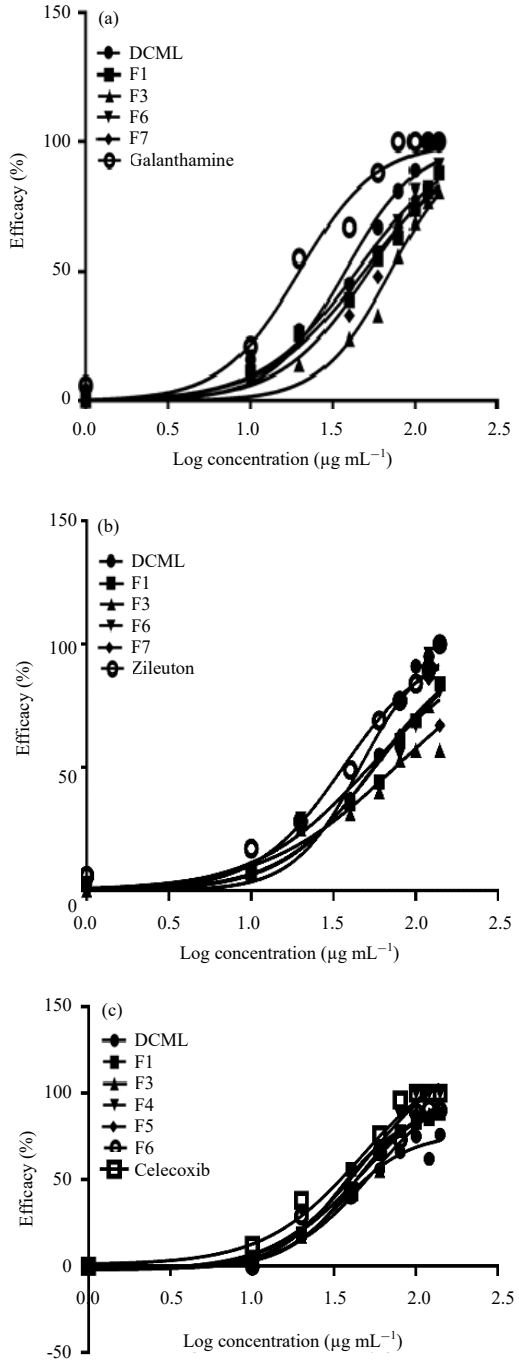

Fig. 1(a-c): Concentration response (µg mL^{-1}) of the DCM leave extract and the most active fractions obtained from *Bruguiera cylindrica* against, (a) Acetylcholinesterase enzyme, (b) 5-lipoxygenase enzyme and (c) Cyclooxygenase enzyme and positive controls used

DCML: Dichloromethane leave extract, F: Fraction

Induction of rheumatoid arthritis using TNF and Interleukin (TNF-α and IL-1β): The frozen and subsequently thawed SFB were cultured to 80% confluence in SFB-medium (Dulbecco's modified eagle media (DMEM) containing 100 µg mL^{-1} gentamicin, 100 µg mL^{-1} penicillin/streptomycin, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid and 10% FCS). For embedding of the SFB cells, a total of 450 µL hot, liquid, 2% agarose (normal melting point, Invitrogen) was filled into the wells of a 48-well plate according to the method described by Pretzel *et al.*[4], with some modification for a monolayer test (Fig. 1). Cylinders of defined size were created by inserting a self-manufactured metal-pin plate into the hot agarose. The cell suspension was then embedded in the preformed cylinders. Afterwards, the wells were filled with 300 µL SFB medium and kept in an incubator of 37°C, 5% carbon dioxide for 48 h. The use of agarose, on the other hand, allowed sufficient diffusion of nutrients. The media was then removed and replaced with a 25 µL of the trypsin-treated SFB suspensions (n = 5 separate RA-SFB cultures, 2×10^4 cells each) to analyze the effect of the tested samples and positive control on the induced arthritis synovial fibroblast cell line in monolayer. After 3 h, 550 µL of SFB media with/without a combination of TNF-α (10 ng mL^{-1}) and IL-1 β (5 ng mL^{-1}) (PeproTech, Hamburg, Germany) were added to the wells. These cytokine concentrations represent the dose of each cytokine with the maximum effect in monocultures of stimulated SFB[4]. The culture was then continued for 7 days at 37°C and 5% (v/v) carbon dioxide after which, a different concentrations of the test samples and methotrexate were added with fresh media, the cell was incubated for 72 h and then analyzed using the MTT assay (Fig. 1).

RESULTS

GC-MS analysis and identification of major constituents: The GC-MS analysis of the 6 fractions isolated from the dichloromethane leave extract of *B. cylindrica* including a list of the major constituents identified and their peak area percentage is presented in Table 1. For fraction 1, 2 and 3, pyrrolidine was appeared to be the major components with a peak area of 78, 55 and 57%, respectively. Fraction 6 was dominated by 1,2-Benzenedicarboxylic acid, diisooctyl with the peak area of 99.81%. However, fraction 4, 5, 7 and 9 were a mixture of different components. Based on the peak area obtained, the main compounds for fraction 4 was 2-propenoic acid and pentadecyl ester. Cyclohexen-1-one appeared to be the main constituents in fraction 5 whereas, Di-n-octyl phthalate and alpha-d-Mannofuranoside was observed in fraction 7 and 9, respectively.

Table 1: Chemical composition of fractions 1-9, isolated from the DCM leave extract of *B. cylindrica*

Analyzed material	No	Retention time	Main constituents	Peak area (%)
Fraction 1	10	30.975	2,3-Bis(1-methylallyl) pyrrolidine	78.03
Fraction 2	8	30.953	2,3-Bis(1-methylallyl)pyrrolidine	55.54
Fraction 3	3	29.889	2(1H)-Naphthalenone	24.51
	4	30.944	2,3-Bis(1-methylallyl)pyrrolidine	57.66
Fraction 4	1	18.626	Cyclohexasiloxane	15.79
	2	23.243	3-Isopropoxy-1	20.09
	3	27.289	Octamethyl-3,5-bis(trimethylsiloxy)tetrasiloxane	18.89
	4	28.564	2-Propenoic acid, pentadecyl ester	25.54
	5	31.912	5,6,11,12-Tetrahydrodibenz(b,f)azocine	19.69
Fraction 5	1	23.247	3-Butoxy-1,1,1,7,7,7-hexamethyl-3,	12.25
	2	27.290	1,1,1,3,5,7,7,7-Octamethyl-3,5-bis(trim	8.31
	3	28.570	2-Propenoic acid, tridecyl ester	10.78
	4	30.868	2-Cyclohexen-1-one, 4-hydroxy-3,5,5	31.59
	5	31.906	5,6,11,12-Tetrahydrodibenz(b,f)azocine	14.57
	5	33.633	Hexadecanoic acid, methyl ester	7.77
	7	34.305	1,2-Benzenedicarboxylic acid, butyl 2	14.74
Fraction 6	2	43.122	Diisooctyl ester	99.81
Fraction 7	3	28.562	2-Propenoic acid, pentadecyl ester	30.17
	5	44.665	Di-n-octyl phthalate	40.13
Fraction 9	5	29.386	alpha-d-Mannofuranoside	62.09

Table 2: Inhibition of 5-lipoxygenase, cyclooxygenase-2 and acetylcholinesterase enzymes, by the extracts and fractions isolated from the leave and root of *B. cylindrica* as detected using *in vitro* biological models

	Biological activities obtained as inhibition (%) and IC_{50} values (μg mL^{-1})					
	5-lipoxygenase		Cyclooxygenase-2		Acetylcholinesterase	
Isolates and positive controls tested	Inhib (%)	IC_{50}	Inhib (%)	IC_{50}	Inhib (%)	IC_{50}
DCM leave extract	91±3.1	45.7±1.6	75.0±2.1	25±1.3	100±1.6	37.60±0.7
MeOH root extract	66±4.0	71.0±2.4	nt		70±2.3	55.90±1.0
DCM root extract	76±2.8	64.0±2.4	nt		75±2.6	68.30±2.8
Fraction 1	89±2.0	55.4±2.0	83.0±3.1	23±1.6	88±1.7	39.16±2.4
Fraction 2	54±1.3	90.0±1.0	62.0±4.2	35±2.9	71±2.1	60.20±3.7
Fraction 3	75±1.6	74.3±2.0	87.0±1.7	20±1.4	81±1.3	43.40±2.0
Fraction 4	75±2.1	58.0±3.3	100.0±1.0	15±2.0	66±1.8	80.70±0.8
Fraction 5	58±4.0	90.0±3.2	88.0±2.6	20±1.6	85±1.0	46.70±2.5
Fraction 6	96±2.7	56.4±1.2	89.0±3.2	25±1.1	91±2.0	44.10±0.8
Fraction 7	86±3.8	52.3±2.9	0	Not detected	88±1.6	52.6±3.8
Fraction 9	49±2.0	100±1.4	54.0±3.0	30±3.4	58±2.3	88.5±3.2
Zileuton	92 ±4.5	22±1.6	-	-	-	-
Celecoxib	-	-	88.0±0.8	10±0.2	-	-
Galanthamine					93±2.2	14±0.3

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})

Biological activities observed by the extracts and the isolated fractions

Inhibition of the pro-inflammatory enzymes by the test materials from *B. cylindrica*: Inhibition of 5-lipox, COX-2 and AChE enzymes by the crude extracts and fractions from the leave and root of *B. cylindrica* and positive controls (Zileuton, Celecoxib and Galanthamine) were obtained using the 5-lipoxygenase, COX-2 inhibitory screening assays and the micro-dilution assay, respectively. The obtained results (inhibition percentage and IC_{50} values) were presented in Table 2. The DCM leaves extract showed the highest inhibitory effects against the three enzymes tested with inhibition percentage of, 91% (against 5-lipox), 100% (against AChE) and 75% (against COX-2). The methanol and DCM root extracts appeared weaker in comparison with the DCM leaves extract.

For the fractions obtained from the active DCM extract, fraction 6 (the main constituent was 1,2-Benzenedicarboxylic acid), possessed the strongest activity against the 3 enzymes with inhibition percentage of 96% (IC_{50} = 56), 91% (IC_{50} = 44) and 89% (IC_{50} = 25) against 5-lipox, AChE and COX-2 enzymes, respectively. Fractions 1 and 3 (containing pyrrolidine related compound) showed inhibitory effects against the three enzymes with inhibition percentages ranging between 89 and 75%. Fraction 7 (containing mixture)

was active against 5-lipox and AChE enzymes with inhibitory effects >80%, but it showed no activity against COX-2. Fraction 4 (the main constituent was 2-Propenoic acid, pentadecyl ester) possessed strong selective inhibition against COX-2 followed by fraction 5 and 3. Inhibition percentages recorded for the positive controls used were 92% For zileuton against 5-Lipox (IC_{50} 22 μL mL^{-1}), 93% for the galanthamine against AChE (IC_{50} 14 μL mL^{-1}) and 88% for the celecoxib against COX-2 (IC50 20 μL mL^{-1}).

Concentration responses of the DCM leave extract and the most active fractions obtained against the 3 enzymes were assessed using the relevant *in vitro* bioassay model for each test as described earlier. Five concentrations (25, 50, 75, 100 and 150 μg mL^{-1}) were used in 3 replicates. Results were analyzed using Graph pad prism to obtain the response curves and the maximum efficacy (E_{max}) for each sample. For the DCM leaves extract, the E_{max} obtained was 100 at a concentration of 140 μg mL^{-1} against 5-lipox. The E max recorded for fraction 1, 6 and 7 at a concentration of 120 μg mL^{-1} were 89, 75, 96 and 86, respectively, against the enzyme. These activities were dropped down when higher concentration was used (140 μg mL^{-1}). Zileuton showed an E_{max} of 100% at a concentration of 140 μg mL^{-1} (Fig. 1).

For AChE enzymes, both the DCM leave extract and the Galanthamine (positive control) showed E_{max} of 100 % at a concentration of 120 and 80 μg mL^{-1}, respectively. The E_{max} recorded for fraction, 1 (88%), 3 (81%), 6 (91%) and 7 (88%) were at concentration of 140 μg mL^{-1}.

For COX-2 enzyme, the best concentration-response performance was observed with fraction 4 which showed E_{max} of 100 at a concentration of 100 μg mL^{-1}. This was similar to the performance of celecoxib (the positive control).

Effects of the test materials against the growth of the fibroblast cell: The effect of the tested plant materials and control on both normal and induced RA synovial cell line was determined as described earlier. Assessment of toxicity threshold against the normal synovial cell line indicated that all the tested extracts and fractions obtained from *B. cylindrica* showed no cytotoxic effects at the highest concentration used (50 μg mL^{-1}) when tested using the MTT assay (Fig. 2a). The test materials were then used to test their effects on cell viability of the induced synovial cells (induced by TNF-α (10 ng mL^{-1}) and IL-1 β (5 ng mL^{-1}). As shown in Fig. 2b, the extracts and fractions showed different growth inhibitory effects on the induced synovial cells at a concentration of 50 μg mL^{-1}. The lowest cell viability was observed with fraction 7 (25%) and 6 (27%) followed by the crude DCM leave extract (34%) and fraction 1 and 3 (36 and 37%, respectively. Methotrexate (positive control) showed a cell viability of 23% at the concentration of 50 μg mL^{-1}. These fractions and the extracts showed lesser effects when tested at a concentration of 25 μg mL^{-1} (Fig. 2c). However, at the concentration of 12.5 μg mL^{-1}, fraction 6 appeared relatively active against the induced cell with cell viability of just 40% followed by 47 % cell viability recorded for the crude DCM leave extract (Fig. 2d). These results indicated that the observed activities of the tested plant material against the induced synovial cell line were dose-dependent. No different effects recorded for methotrexate on cell viability when tested at the two concentration of 25 and 12.5 μg mL^{-1}.

DISCUSSION

In this study, we investigated effects of extracts and fractions from *B. cylindrica* against, 3 enzymes associated with rheumatoid arthritis and cell viability of induced synovial fibroblast cell. A number of clinical studies suggest that COX-2 and leukotrienes inhibitors perform with a better efficiency when compared to other drugs and therefore, it is relevant to look for both COX and 5-lipox inhibitors for more anti-inflammatory effects[10]. 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, as 5-lipoxygenase reported to be present in RA synovium and mostly expressed in macrophages, neutrophils and mast cells in the sub lining layers[24]. The 5-lipox and leukotriene, therefore, confirmed to be implicated in the pathogenesis of a chronic inflammatory diseases such as rheumatoid arthritis[25,26]. Lin *et al.*[24] revealed another mechanism for 5-lipox in immune inflammation. They reported that, some of the investigated 5-lipox inhibitors, had significantly antagonized TNF-α induced IL-6 in a concentration-dependent manner in human synovial fibroblast. The authors drawn an inference that 5-lipox pathway plays a crucial role in TNF-α-induced cytokine and chemokine up regulation in human synovial fibroblast.

Cyclooxygenase (COX)-2 is known to be up regulated in the rheumatoid arthritis (RA) synovium. One of the suggested mechanism of action was believed to be its role on mediation of angiogenesis[5,27]. The COX-2 inhibitors have been shown in clinical trials to relieve pain and RA associated inflammation, with significantly less adverse side effects[28] and therefore, may provide an additional rationale for the treatment of RA related diseases[27].

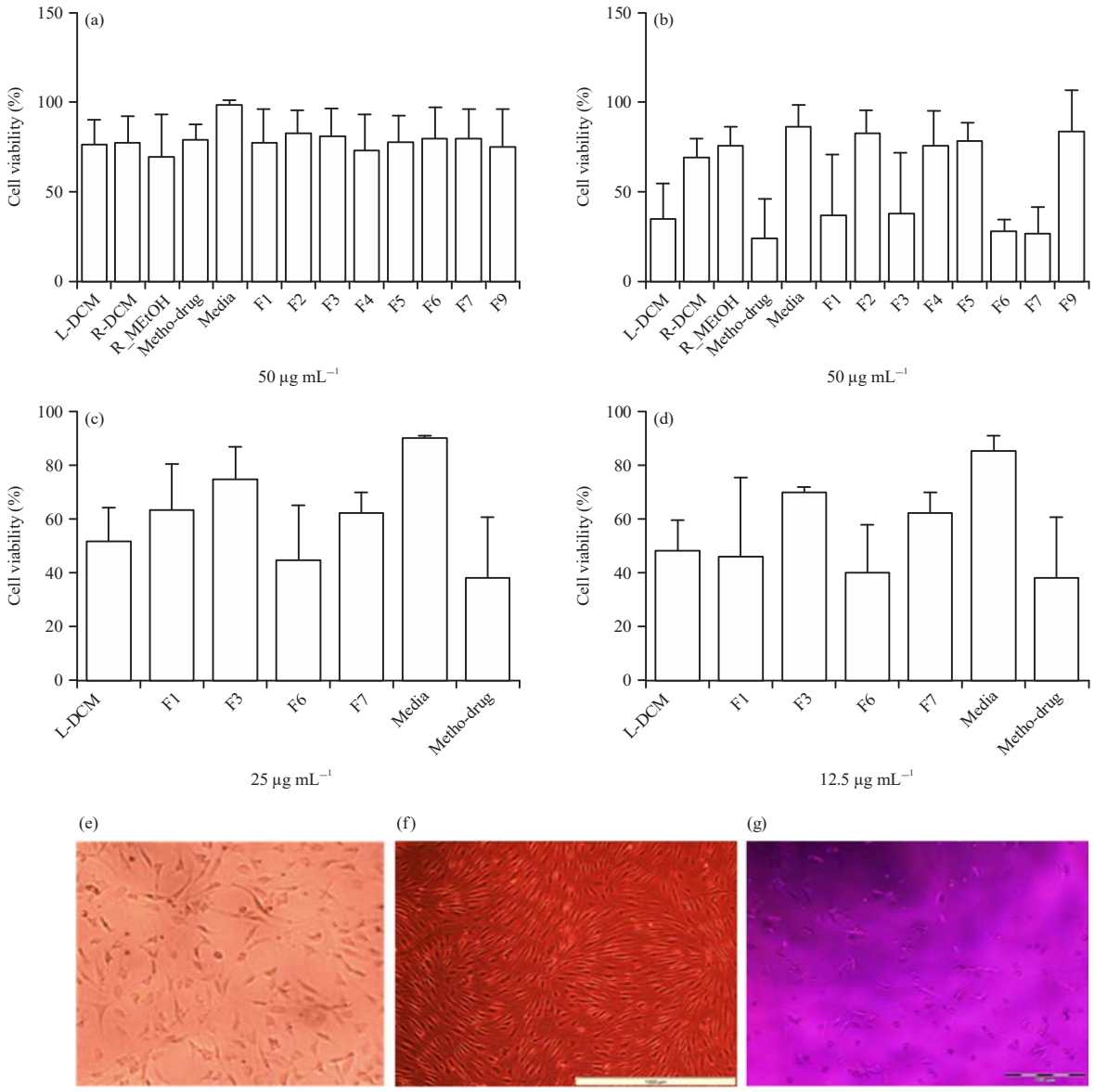

Fig. 2(a-g): Effects of extract, fractions and methotrexate (the positive control) on cell viability using MTT assay. (a): Normal synovial cell line, concentration used was 50 µg mL^{-1}, (b) RA induced cell, concentration used was 50 µg mL^{-1}, (c) RA induced cell, concentration used was 25 µg mL^{-1}, (d) RA induced cell, concentration used was 12.5 µg mL^{-1}. Synovial growth media was used as negative control, (e) Normal synovial cell, (f) RA induced synovial cell and (g) RA induced cell treated with methotrexate

Each value represents means±SD of 3 replicate in 2 independent experiments

The concept of cholinergic anti-inflammatory pathway has provided new grounds for understanding and treating inflammatory diseases including autoimmune diseases such as rheumatoid arthritis[29]. The research outcomes support the underlying interdependency of the nervous and immune systems and suggesting that acetylcholinesterase inhibitors may contribute to the suppression of inflammation symptoms[30,31]. Borovikova *et al.*[32], reported that acetylcholine as the main parasympathetic neurotransmitter had effectively deactivated peripheral macrophages and inhibited the release of pro-inflammatory mediators, including the tumor necrosis factor (TNF-α) which can cause synovitis and hyperplasia appearance. Anti-TNF-based therapeutic approaches were previously reported as successful in the treatment of inflammatory diseases, including rheumatoid arthritis[33]. It is therefore, logical to accept that medicinal agents which can decrease the expression of pro-inflammatory cytokines, can also be used for the treatment of RA related symptoms[34,35].

All the 3 fractions containing pyrrolidine in the current study, possessed strong activities against the 3 enzymes and on RA induced cell line. Pyrrole containing analogs are considered as a potential source of biologically active compounds with a significant effects. Tolmetin 147 and Zomepirac 148 are 2 pyrrole acetic acid derivatives that have gained a degree of success in treatment of rheumatoid arthritis and pain[36]. The 3 fraction containing pyrrolidine in this study also showed remarkable activities against the growth of the induced RA cell line. Pyrrolidine related compounds such as dithiocarbamate was reported to induce cytotoxicity in some tumor cell lines in addition to its properties as a pro-oxidant agent, which has been reported to induce growth inhibition and cell death in both *in vivo* and *in vitro* experiments[37]. The observed activities of the extract and the pyrrolidine containing fractions in this study may be due in part to its role as a cytotoxic and growth inhibitor agent and could be accounted for the biological properties of the *B. cylindrica*.

Previous reports indicated biological activities of some of the mangrove plants including *Bruguiera* species[38-40]. Some of these reported findings correlate well with the obtained results in the present study. This implying that the observed activities may be due to the presence of a similar or closely related class of chemical compounds which act on different biological targets. This situation support the movement towards implementation of polypharmacology approach as an emerging paradigm for drug discovery based on the new philosophy of drug design which has been transformed from one drug one target to one drug multiple targets. This phenomena fits very well with these current findings on biological effects of *B. cylindrica* which act on multiple targets pertaining to multiple disease pathways[41]. Applying this method to herbal drugs used in alternative medicine may open up the possibility to understand the explicit targets of active ingredients and their interactions in the context of molecular networks[42].

CONCLUSION

The extract and fractions obtained from *B. cylindrica* in this study possessed a different level of activities on pro-inflammatory enzymes and cell viability of the induced RA cell line. These activities may be due to the synergetic effects of the different bioactive compounds in the active fractions and /or extracts. This implies that the test material may act through different mechanisms against the diseases which required further investigation through a polypharmacology approach.

SIGNIFICANCE STATEMENT

This study discovered some of the medicinal properties of *B. cylindrica*, a mangrove plant with various applications in traditional medicine. To our knowledge, the biological activities observed in this study using the bioassay models are unprecedented and contributing therefore to the novelty of this study. These findings contribute to the phytochemical study of *B. cylindrica* and suggest future research direction for the cross-talk between inflammatory mediators and nervous systems and the possibility of using these plant materials for the treatment of RA related diseases.

ACKNOWLEDGMENTS

The authors would like to thank Mr. Julius, Miss Murni and Mr. Zulhilmi for their assistance in the GC-Ms analysis and the biological bioassays.

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