

Full Length Research Paper

Anti-oxidant and anti-inflammatory activities of leaves of *Barringtonia racemosa*

Mandana Behbahani, Abdul Manaf Ali, Radzali Muse and Noorjahan Banu Mohd

Department of Cell and Molecular Biology, UPM, Malaysia.

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The medicinal plant of *Barringtonia racemosa* (Lecythidaceae family) has been used widely in traditional medicine for anti-inflammation and anticancer in Malaysia. The present investigation was carried out to study of the anti-oxidant and anti-inflammatory effects of fully expanded leaf extracts of *B. racemosa*. Antioxidant activity was measured by using FTC, TBA and DPPH free radical scavenging methods and Griess assay was also used for the measurement of nitric oxide inhibition in lipopolysaccharide (LPS) and interferon- (IFN-)-treated RAW 264.7 cells. Different crude extracts of fully expanded leaf extracts of *B. racemosa* have exhibited a very good level of nitric oxide (NO) inhibitory and antioxidant activities. In the Griess assay, non polar extracts such as chloroform and hexane extracts were found to be strong inhibitors of NO at different concentrations (25, 50, 100 and 200 µg/ml) in comparison with polar extract (ethanol extract). Chloroform extract didn't show cytotoxicity against RAW 264.7 cells at different concentrations contrary to hexane and ethanol extracts. The chloroform and hexane extracts exhibited very strong antioxidant properties when compared to Vitamin E (α-tocopherol) in the FTC and TBA methods, the chloroform and hexane extracts exhibited the radical scavenging activity with an IC₅₀ value of 54.29 and 63 µg/ml respectively. Results demonstrated that chloroform extract of *B. racemosa* leaf may have the potential to be used as anti-inflammation and anti-oxidant agents and it was revealed that the active compound in *B. racemosa* is lycopene.

Key words: *Barringtonia racemosa*, anti-oxidant, anti-inflammatory.

INTRODUCTION

Barringtonia racemosa is a tropical higher plant and is a member of the Lecythidaceae family. *B. racemosa* is a moderate sized evergreen tree found in the West Coast of India, Sundarbans, Assam and Andaman Islands and Malaysia. It is used as a traditional medicine in Malaysia and locally known as putat. Its fruits are used to treat cough, asthma and diarrhea; the seeds are aromatic and useful in treating colics and ophthalmic problems. Pre-vious studies on some *Barringtonia* species including *Barringtonia asiatica*, *Barringtonia acutangul* and *Barringtonia lanceolata* have showed that most of the species possess medicinal properties (Grosvenor et al., 1995; Khan and Omoloso, 2002). Other related genus of *Barringtonia edulis* also was reported to possess definitive sterility and used to induce abortion (Bourdy and Waller, 1992).

Previous investigation on the bioactivity of *B. racemosa* has shown that the ethanol extract of *B. racemosa* barks

has anti-tumor property and toxicity in mice (Khan et al., 2001). whilst the ethanol extract of the plant leaves displayed cytotoxicity against the HeLa (human cervila carcinoma) cell line with a IC₅₀ value of 10 g/ml (Mackeen et al., 1997). The aqueous extract isolated from the stem bark of *B. racemosa* has been displayed to have antinociceptive and toxicological effect on rats (Thomas et al., 2002) while an ethanol extract of the roots of *B. racemosa* provided two novel clerodane diterpenoid nasimalun A and B by NMR and MS analysis (Khan et al., 2000).

B. racemosa well known in Malaysia as a traditional medicine has been shown to exhibit anti-oxidant and anti-inflammatory effects. In the recent past, there has been an increase in the use of plants as sources of natural anti-oxidants for the scavenging of free radicals. The latter are known to initiate a series of (oxygen robbing) chain reactions resulting in oxidative tissue damage and a wide range of degenerative diseases, such as cancer and a host of cardiovascular diseases (Galati and O'Brien, 2004).

Deraniyagala et al. (2003) reported that the aqueous

*Corresponding Author email: ma_behbahani@yahoo.com

Table 1. Percentage of antioxidant activity of leaves of *Barringtonia racemosa* extracted with three different solvents (chloroform, ethanol and hexane).

Antioxidant activity of <i>Barringtonia racemosa</i> (%)							
Tocopherolic extract		Chloroform extract		Hexane extract		Ethanol extract	
FTC	TBA	FTC	TBA	FTC	TBA	FTC	TBA
67.08±1.32	46.4±1.54	79.13±1.75	58±1.42	76.52±1.64	54.4±1.14	51.39±0.30	33±0.54

Derived leaves were separately extracted using chloroform, hexane and ethanol at room temperature. Each value represents the mean ± SD (n = 3).

bark extract of *B. racemosa* have several bioactivities such as antinociceptive effect and this effect was mediated through opioid mechanisms.

The literature survey revealed that there are no scientific studies carried out regarding antioxidant and anti-inflammatory activity of the leaves of *B. racemosa*. Hence, the present study is focused to evaluate the antioxidant and anti-inflammatory potentials of different extracts of leaves of *B. racemosa*.

MATERIAL AND METHOD

Plant material

Ten kg of the fresh fully expanded leaves of *B. racemosa* were collected from Herbal unit, at University Putra Malaysia, in December 2005. Young leaves (10 – 15 cm long) as well as older leaves (15 – 25 cm long) were chosen. The petioles were removed. The leaves without petioles were washed under running water, air dried and ground. Out of 10 kg of the fresh leaves of *B. racemosa*, 1.8 kg of the dried powder was obtained. The dried plants were stored in plastic bags and kept in a dark at room temperature (28 – 30°C).

Extraction of compound using different organic solvents

The dried plant sample (60 g) was placed in a stopped conical flask and macerated with 500 ml of 98% chloroform (Merck, Germany) at room temperature (28 – 30°C) for 3 days with occasional stirring. This experiment has done three times. The solvent was then filtered and evaporated in a rotary evaporator (Somatco, Germany) under vacuum at 45°C. The residue was placed in oven (Napco, USA) at 40°C until dry. The crude extract was stored in a well-closed container and protected from light and kept in a refrigerator at –4°C. The extraction was carried out on three separate occasions (n = 3). The above procedure was repeated using 95% ethanol and 98% hexane (Merck, Germany) as solvent instead of chloroform, again replicated on three occasions (n = 3). Out of 60 g of the dried plant sample of *B. racemosa* could be able to produce 15 g ethanol, 14 g hexane, 18 g chloroform extracts.

Anti-inflammatory activity

Inflammation is characterized by the over production of nitric oxide (NO). The inducible isoform of NO synthase (iNOS) is directly responsible for the generation of NO. Selective inhibition of iNOS and its biosynthetic product NO, has been shown to suppress inflammation in a variety of inflammation states. Griess assay has been used here to determine the inhibition of nitric oxide formation in RAW 264.7 cells induced by lipo polysaccharide (LPS) and

interferon – (Heras et al., 2001).

Culture of RAW 264.7 Cell

The murine monocytic macrophage cell line RAW 264.7 was purchased from American Type Culture Collection (ATCC, USA). This macrophage cell line was initially established from a tumor induced by Abelson murine leukemia virus in Balb/c mice (Raschke et al., 1978). The RAW 264.7 cells were cultured in Dulbecco's Modified Eagle Media (DMEM) (containing 4 mM L-glutamine, 4.5 g/L glucose, 1.5 g/L (w/v) sodium bicarbonate, 50 U/ml (w/v) penicillin, 50 Ag/ml (w/v) streptomycin) with 10% foetal calf serum (FCS). The cells were cultured at 37°C with 5% CO₂ and were split twice a week. The RAW 264.7 cells were semi-adherent cells, where some cells grew in suspension, some were loosely attached to the surface and others flatted out and attached to the flask. The subcultures were prepared by scraping and the sub cultivation ratio was between 1:3 to 1:6 (culture: media).

Griess assay for nitric oxide inhibitory activity

Briefly, cells were seeded in 96 -well tissue culture plates (1 × 10⁶ cells/100 µl) and incubated for 24 h at 37°C with 5% CO₂. One hundred µl of chloroform, hexane and ethanol extracts in 0.1% DMSO was then added separately and serially diluted to give final concentrations of 25, 50, 100 and 200 µg/ml (Table 1) . Cells were then stimulated with 200 U/ml (w/v) of IFN-γ and 10 µg/ml (w/v) LPS for another 17 h. The presence of nitrite, a stable oxidized product of NO, was determined in cell culture media by Griess reagent (1%, w/v) of sulfanamide and 0.1% (w/v) N-(1-naphthyl) ethylenediamine dihydrochloride in 2.5% (v/v, H₃PO₄). One hundred µl of cell culture supernatant was removed and combined with 100 µl of Griess reagent in a 96-well plate followed by spectrophotometric measurement at 550 nm using a microplate reader. Nitrite concentration in the supernatants was determined by comparison with a sodium nitrite standard curve. The amount of live cells was detected by 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) cytotoxicity assay and values given in percent cell cytotoxicity.

Measurement of nitrite

For determination of nitric oxide concentration, the stable conversion product of nitric oxide, nitrite (NO₂⁻) was measured using the Griess reagent (Chi et al., 2001). After 24 h incubation 50µl of supernatant from each well of cell culture plates was transferred into 96-well microplates and equal volume of Griess reagent (1% sulfanilamide, 0.1% (w/v) of N-(1-naphthyl)-ethylene diamine hydrochloride, 2.5% (v/v, H₃PO₄) was then added to the supernatant at room temperature. The absorbance at 550 nm was determined using a Spectramax Plus (Molecular Devices) UV-Vis microplate reader after 10 min. The concentrations of nitrite were

derived from regression analysis using serial dilutions of sodium nitrite as a standard. Percentage inhibition was calculated based on the ability of extracts to inhibit nitric oxide formation by cells compared with the control (cells in media without extracts containing triggering agents and DMSO), which was considered as 0% inhibition.

Ferric thiocyanate (FTC) method

The ferric thiocyanate was performed by using Osawa and Namiki method (1981). FTC method was used to determine the amount of peroxide at the initial stage of lipid peroxidation. The peroxide reacts with ferrous chloride (FeCl_2) to form a reddish ferric chloride (FeCl_3) pigment. In this method, the concentration of peroxide decreases as the antioxidant activity increases. A mixture of 4 mg of sample was placed in 4 ml of absolute ethanol (Merck, Germany), 4.1 ml of 2.52 % (v/v) of linoleic acid (Sigma, USA) in absolute ethanol, 8 ml of 0.05 M phosphate buffer (pH 7.0) and 3.9 ml of water was placed in a vial with a screw cap and then placed in an oven at 40°C in the dark.

To 0.1 ml of this solution, 9.7 ml of 75% (v/v) of ethanol and 0.1 ml 30% (w/v) of ammonium thiocyanate (Sigma, USA) was added. Exactly 3 min after the addition of 0.1 ml of 0.02 M ferrous chloride in 3.5% (v/v) of hydrochloric acid (HCl) to the reaction mixture. The absorbance was measured at 500 nm every 48 h until the absorbance of the control reached maximum.

The control and standard were subjected to the same procedures as the sample, except that for the control, only the solvent was added, and for the standard, 4 mg of sample was replaced with 4 mg of vitamin E (Endrini et al., 2002).

DPPH free radical scavenging activity

The method of DPPH free radical scavenging was measured based on a method of Yen and Hsieh (1997). The reaction mixture of 1 ml of 0.3 mM diphenyl-p-picrylhydrazyl radical (DPPH) in ethanol and 0.5 ml of different concentrations of leaf extracts of *B. racemosa* (500, 250, 125, 63, 32, 16 and 8 $\mu\text{g}/\text{ml}$) was left in the dark at room temperature for 30 min. The remaining DPPH was measured by colorimetry at 517 nm. This method was used to determine DPPH free radical scavenging activity of compound isolated from *B. racemosa* leaves

Thiobarbituric acid (TBA) method

The method of Ottolenghi (1959) was used to determine the TBA values of the samples. The formation of malonaldehyde is the basis for the well-known TBA method used for evaluating the extent of lipid peroxidation. At low pH and high temperature (100°C), malonaldehyde binds TBA to form a red complex that can be measured at 532 nm. The increase amount of the red pigment formed correlates with the oxidative rancidity of the lipid.

One ml sample solution was prepared and incubated as in the FTC method. 2 ml of 20% (w/v) of trichloroacetic acid (CCl_3COOH) and 2 ml TBA aqueous solution were added to 1 ml of sample solution prepared as in the FTC procedure, and incubated in a similar manner. The mixture was then placed in a boiling water bath for 10 min. After cooling, it was centrifuged at 3,000 rpm for 20 min and the absorbance of the supernatant was measured at 532 nm. Antioxidant activity was determined based on the absorbance on the final day.

Sample preparation

Standard solution was prepared by dissolving pure individual compound including lycopene in chloroform containing BHT (1%,

w/v), which is as the antioxidant. The concentration of standard solution of lycopene was 1 mg/ml.

The concentration of chloroform extract of *B. racemosa* was also 1 mg/ml. All solutions were stored in brown flasks at -20°C.

Analysis of lycopene by high performance liquid chromatography

Determination of lycopene was done on chloroform extract of *B. racemosa* leaves by the modified method of Xu et al. (2006).

Isocratic HPLC analysis was performed using a Jasco Series HPLC (Jasco, USA) system equipped with 1580 pump and a UV-1570 detector. Peaks were separated on a Diamonsil C18 column using the mobile phase such as acetonitrile, dichloromethane (25:75, v/v).

To 250 ml of dichloromethane was mixed with 750 ml of acetonitrile. The mobile phase solution was sieved through 0.45 μm Millipore (Millipore, USA) filter then degassed in an ultra sonicator (Sonicator, USA) for 25 min (Xu et al., 2006) to prevent from the bubbles.

The flow-rate of the mobile phase was 1.5 ml/min. The absorption of analyses was done at 450 nm. Samples were injected manually, and the injection volume was 10 μl . In this case, T 2000 software was used for peak integration and calculation.

To identify the peaks, the spectral patterns and retention time of the samples were compared with standard (lycopene).

Statistical analysis

Each experiment was performed in triplicate and repeated two times. The experiments were performed using complete randomized design (CRD) design and results were analyzed using one way ANOVA. Experiment was a factorial. Statistical analysis was performed by using Software SAS, Version 6.12. Probability $P < 0.05$ were considered significant.

RESULTS

Antioxidant activity

Ferric thiocyanate method

Figure 1 shows absorbance values obtained using FTC method, for *B. racemosa* leaves extracted using absolute ethanol, chloroform and hexane. Absorbance values of the control as well as -tocopherol and *Baringtonia* leaves increased until day 6, and then decreased on day 8. In comparing the total antioxidant activity of leaf extracted using 3 different solvents with vitamin E (-tocopherol), the result showed that nonpolar extract (chloroform and hexane extracts) was significantly higher ($p < 0.05$) in total antioxidant activity content than -tocopherol. The chloroform extract of *B. racemosa* had the highest activity ($79.13 \pm 1.75\%$), followed by hexane extract ($76.52 \pm 1.64\%$), tocopherol ($76.52 \pm 1.64\%$) and ethanol extract ($51.39 \pm 0.30\%$) by using FTC method. The FTC method was used to measure the amount of peroxide at the primary stage in linoleic acid peroxidation. The control showed increasing in the absorbance value from day 1 to day 4, but the levels reached maximum on day 6 and finally dropped on day 8 due to the MDA compounds from linoleic acid oxidation, where the pero-

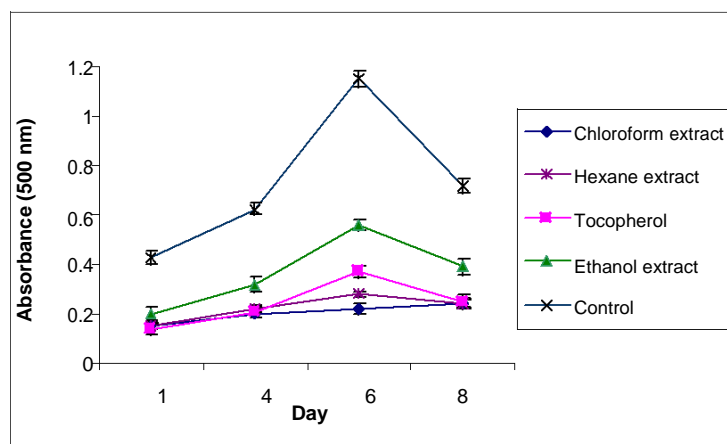


Figure 1. Absorbance value of *B. racemosa* leaf at 0.02% concentration using FTC method. The values are compared with the absorbance values of the control and -tocopherol at 500 nm. Each value represents the mean of three replications \pm SD.

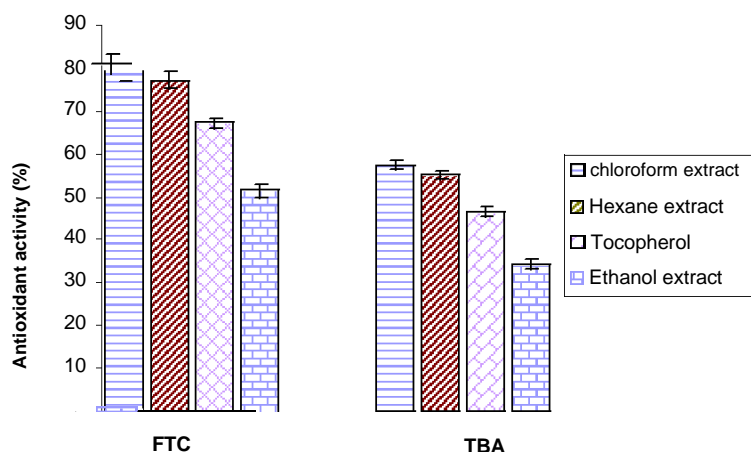


Figure 2. The total antioxidant activity of chloroform and Ethanol extracts of *B. racemosa* by using FTC and TBA method. Values are means \pm SD (vertical lines).

FTC: Ferric thiocyanate, TBA: Thiobarbituric acid

xide reacts with ferrous chloride to form a reddish ferric chloride pigment. Since the concentration of the peroxide decreased as the antioxidant activity increase, the intensity of the reddish pigment will reduce, leading to lower absorbance value. As such, the absorbance values of the control were the highest since it did not contain antioxidants.

Thiobarbituric acid method

Figure 2 shows absorbance values obtained using TBA

method, for *B. racemosa* using three different solvents chloroform, hexane and ethanol. The TBA method was used to measure the secondary stage of linoleic acid peroxidation. The absorbance values of the chloroform and hexane extracts were lower than the control and -tocopherol, on day 8 of the FTC method due to the formation of MDA. Malonaldehyde binds TBA to form a red complex that can be measured at 532 nm. As shown in Table 1, result obtained from the TBA method showed that total antioxidant activity of chloroform and hexane extracts were significantly ($p < 0.05$) higher compared to

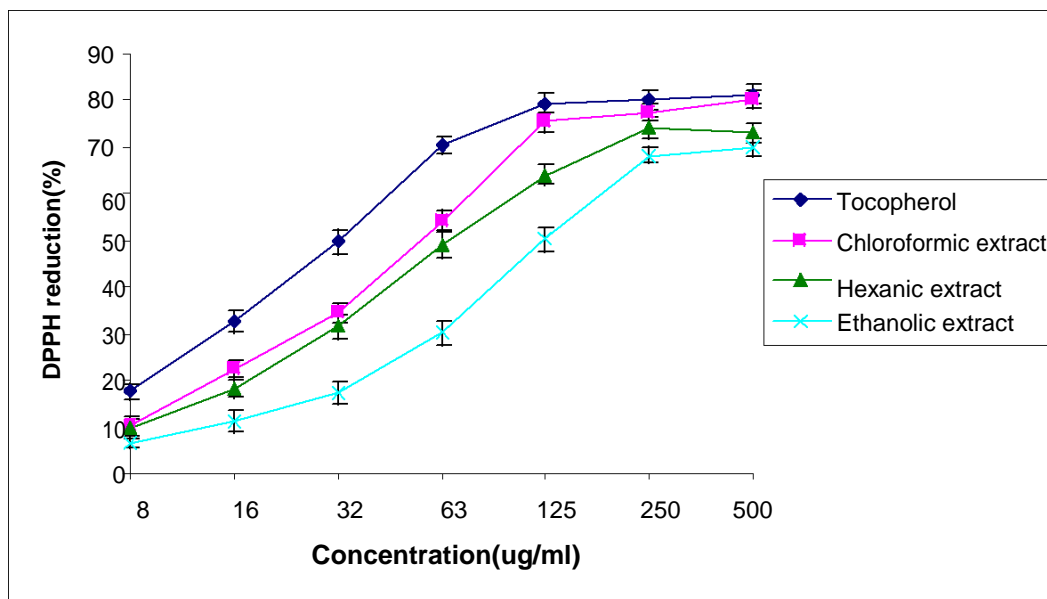


Figure 3. Free radical scavenging activity of leaf extracts of *Barringtonia racemosa* determined by DPPH free radical. Each value represents the mean of three replications \pm SD.

Table 2. Anti-inflammatory activities of leaves of *Barringtonia racemosa* extracted with hexane, chloroform and ethanol.

Conc. (µg/ml)	NO inhibition (%)	cell viability
Chloroform extract		
25	23.40 \pm 1.5	131
50	49.62 \pm 2.4	125
100	57.70 \pm 2.3	109
200	73.85 \pm 2.5	100
Ethanol extract		
25	16.14 \pm 2.0	84
50	22.65 \pm 1.5	93
100	29.80 \pm 2.2	91
200	45.60 \pm 2.1	95
Hexane extract		
25	25.80 \pm 3.1	111
50	37.79 \pm 2.9	85
100	42.39 \pm 2.0	55
200	60.60 \pm 1.6	41

vitamin E. Among nonpolar and polar solvents, there was significantly difference ($p < 0.05$) in total antioxidant activity. As in FTC method, the lower absorbance value indicated higher antioxidant activity. Since peroxide production was inhibited by the antioxidant, similarly to the FTC method findings, chloroform extract showed to have the highest content of total antioxidants activity compared to hexane and ethanol extracts.

DPPH radical scavenging activity

The model of scavenging the stable DPPH radical is a widely used method to evaluate antioxidant activities in a relatively short time compared with other methods. Figure 3 shows the free radical scavenging activity of chloroform, Hexane and ethanol extracts of *B. racemosa* and one reference compound (-tocopherol). -tocopherol was the most potent scavenger. IC₅₀ values of -atocopherol, chloroform, hexane and ethanol extracts were found to be 32, 54, 63 and 125 µg/ml respectively. IC₅₀ values of plant extracts tested showed lower activity than -atocopherol. The result showed that non-polar extract (chloroform and hexane extracts) was significantly higher ($p < 0.05$) in DPPH activity compared to polar extract (ethanol extract). Among two kind of non polar extracts, chloroform extract was significantly higher ($p < 0.05$) in DPPH activity compared to hexane extract.

The Effect of different leave extracts on NO production in RAW 264.7 Cells

The overall efficacy of all extracts on nitrite production in IFN /LPS activated macrophage is shown in Table 2. Hexane, chloroform and ethanol extracts of *B. raceomosa* were examined for their inhibitory effect on NO release in RAW264.7 cells. Each sample was as-sayed at concentration of 25, 50, 100 and 200 µg/ml with 0.2% DMSO. All samples were compared with control; the non-treated LPS activated macrophage in culture media. The extracts were considered as having a strong, moderate, or week activity if inhibition of NO release was

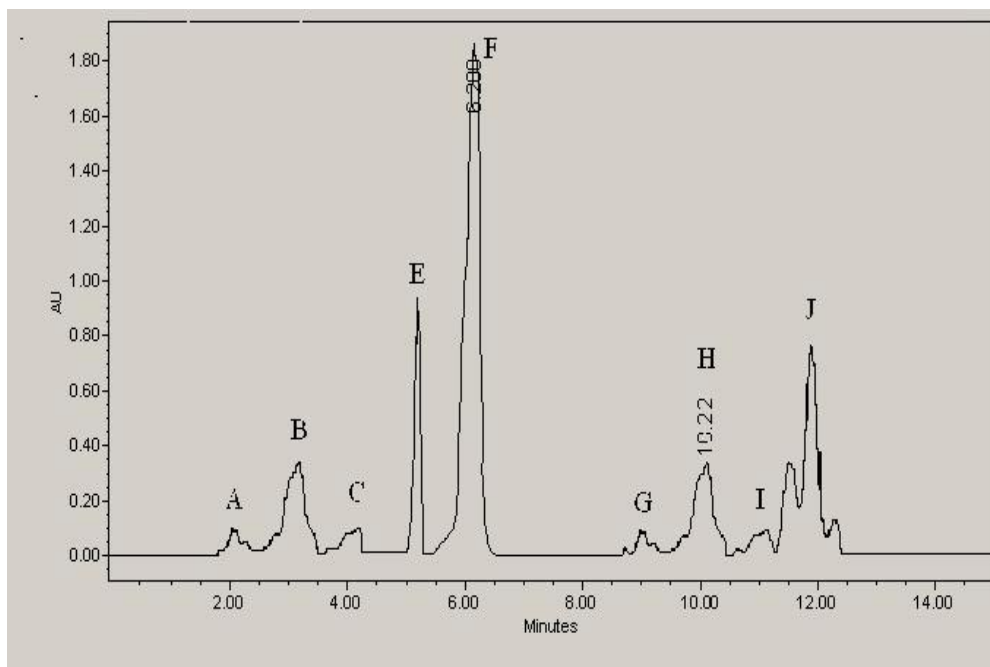


Figure 4. HPLC chromatogram of fully expanded leaf extract of *Barringtonia racemosa* spectra detected by UV-1570 detector at 450 nm using acetonitrile: dichloromethane (25: 75, v/v) solvent system at the flow rate of 1 ml/min. A, C, E, G, I, J and H indicate the unknown peaks. F indicate standard lycopene peak.

was more than 90%, between 50 and 90%, or less than 50% compared to the induced (with 0.1% DMSO) treatment, respectively. In addition, the viability of RAW 264.7 cells was assessed by MTT method (Mossmann, 1983) and must be above 85% indicating that decreased NO were not a result of cell death but rather due to the inhibitory effect of the compound, where applicable. Among the three extracts (Table 2), only chloroform extract showed higher than 50% inhibition of NO production at the concentration of 200 and 100 mg/ml without cytotoxic effect. On the other hand, chloroform extract showed an enhanced effect rather than an inhibitory effect on percentage of cell viability. Hexane extract showed higher than 50% inhibition of NO production at the highest concentration 200 μ g. This extract did not indicate cytotoxicity at concentration 25 μ g/ml in cultures as determined by MTT assay but was highly cytotoxic at higher concentrations (50, 100 and 200 μ g/ml). Ethanol extract showed less than 50% inhibition of NO production at different concentrations and showed low degree of cytotoxicity against RAW 264.7 cells.

As a result only Chloroform extract compared to the other two extracts didn't show cytotoxicity against RAW 264.7 cells at different concentrations and was found to be strong inhibitors of NO at different concentrations. It could be concluded that chloroform is the best Solvent for extraction of active compound from leaves of *B. racemosa*.

HPLC chromatogram of lycopene and leaf extract of *B. racemosa*

The result showed that the retention time of standard lycopene was 6.28. The leaf extract of *B. racemosa* showed ten absorption peaks at 450 nm (Figure 4). The peak areas of lycopene are shown in Figure 5 (F). Repeated experiments showed that, the retention time of lycopene and leaf extract of *B. racemosa* were almost the same. The other peaks that observed in fully expanded leaf extract of *B. racemosa* as shown in Figure 4 (A, B, C, D, E, I, J and H) were unknown compounds.

DISCUSSION AND CONCLUSION

Results obtained from this study revealed that the active compound in *B. racemosa* is lycopene. Lycopene is a major antioxidant present in a range of fresh fruits and vegetables. This compound is known to take part in protecting animals against damage from free radicals and singlet oxygen reactive species. In a study with mice, lycopene has been shown to possess excellent anti-inflammatory/antioxidant properties (Zhao et al., 2003). Lycopene has been shown to suppress the growth of cancer cells in laboratory and animal studies. Additional recent studies in humans have bolstered these findings, showing significant benefits from elevated lycopene levels both in the diet and in the body (Welsch et al., 2001).

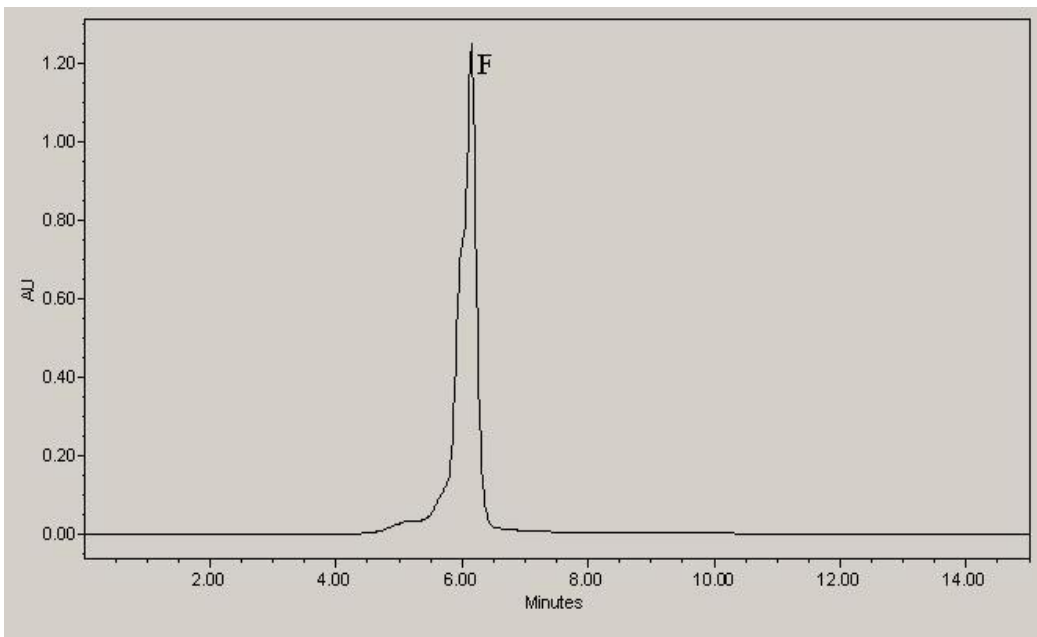


Figure 5. HPLC chromatogram of standard lycopene spectra detected by UV-1570 detector at 450 nm using acetonitrile, dichloromethane (25:75) solvent system at the flow rate of 1 ml/min. F indicates standard lycopene peak.

In the present study, the crude organic extracts from plants were checked for their inhibitory effect on nitric oxide production from macrophages (RAW 264.7 cells) induced by LPS and IFN different concentrations of the crude samples were used. Results demonstrated that Non polar extracts (chloroform and hexane extracts) to be strong inhibitors of NO at different concentrations in comparison with polar extract (ethanol extract).

It has shown good correlation between the anti-inflammation effect of this plant and its antioxidant activity. Antioxidants play a major role in helping to protect our body from the formation of free radicals and prevent or delay the occurrence of lipid peroxidation. Peroxide is gradually decomposed to lower molecular compounds during the oxidation process and these compounds here measured by FTC and TBA methods (Figure 2). The amount of peroxide at the primary stage of linoleic acid peroxidation was measured by FTC method, whereas TBA method measures at the secondary stage (Kikuzaki and Nakatani, 1993). The non polar extracts (chloroform and hexane extracts) exhibited very strong antioxidant properties when compared to Vitamin E (a-tocopherol).

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