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Full Length Research Paper

Evaluation of anti-inflammatory, antioxidant and anti-nociceptive activities of six Malaysian medicinal plants

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The anti-inflammatory, antioxidant and anti-nociceptive properties of six Malaysian medicinal plants, including *Carica papaya, Musa acuminata, Oenanthe javanica, Piper sarmentosum, Psophocarpus tetragonolobus* and *Sauropus androgynus* were investigated. The anti-inflammatory effects of the methanolic plant extracts were evaluated by using Griess assay on nitric oxide inhibitory activity upon IFN-γ/LPS stimulated RAW 264.7 cells. The antioxidant property and total phenolic content of the plant extracts were determined by using DPPH radical scavenging assay and Folin-Ciocalteu's assay, respectively. Anti-nociceptive activity of the plant extracts were evaluated by measuring the number of writhing response of mice upon acetic acid induction. All plant species showed significant nitric oxide (NO) inhibitory activity (IC_{50} <61 µg/ml) without causing cytotoxicity to RAW 264.7 cells. Besides, all six plants exhibited different degree of antioxidant activities (IC_{50} value, 86.74 ± 2.92 to 192.92 ± 2.60 µg/ml). The antioxidant activity might be due to the present of phenolic compounds (34.20 to 50.01 mg GA/g DW samples). Moreover, all plant species suppress the writhing response of mice at different degree of inhibition (10.65 to 43.12% inhibition) at concentration tested of 200 mg/kg. Thus, this study validates the traditional medicinal uses of the evaluated plant species in management of inflammation and free radical related disorders.

Key words: Malaysian medicinal plants, anti-inflammatory, antioxidant, anti-nociceptive.

INTRODUCTION

Nitric oxide (NO) is a short-live free radical synthesized by nitric oxide synthase (NOS) and plays a vital role in nervous and immunological system. However, many studies have been reported that excessive production of NO is associated with various diseases such as rheumatoid arthritis, diabetes, hypertension and septic shock (Pacher et al., 2007). The pathogenesis of these inflammatory diseases is mainly due to NO generation via up-regulation of the inflammatory enzyme, inducible nitric oxide synthase (iNOS) (Heller et al., 1997). The expression of iNOS are regulated by variety of signaling molecules such as mitogen activated protein kinases (MAPKs), nuclear factor-kappaB (NF-κB), activator protein-1 (AP-1), and signal transducer and activator of transcription (STAT) (Kleinert et al., 2004). Therefore, pharmacological interference in these signaling pathways can leads to the suppression of iNOS expression and NO synthesis. Studies have shown that overproduction of free radical molecules such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) are linked with a number of inflammatory disorders (Seifried et al., 2007). ROS and RNS can mediate the cells damages, including membrane fluidity disruption, protein denaturation, lipid peroxidation, DNA oxidation and platelet functions alteration (Arouma, 1993).

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Table 1. List of six plants species tested for anti-inflammatory, antioxidant and anti-nociceptive activities. Their local name, herbarium number, traditional uses, part tested and percentage of yield % (w/w) from the methanolic crude extraction were stated.

Plant	Herbarium number	Local name	Traditonal usage	Part tested	Yield (%) (w/w)
Carica papaya	SK 1753/10	Betik (papaya)	Treatment of dysuria, gastric problems, paediatric burns, eczema and psoriasis (Anuar et al., 2008)	Leaf	13.93
Musa acuminata	SK 1754/10	Pisang abu nipah (banana)	Treat ulceration (Perez-Perez et al., 2006)	Flowering stalk	6.26
Oenanthe javanica	SK 1752/10	Selom	Epidemic influenza, jaundice, hypertension, haematuria, and fever (Wang et al., 2005)	Whole plant	7.83
Piper sarmentosum	ACP0155	Kaduk	Treatment of toothace, fungoid dermatitis on the feet, coughing asthma and pleurisy; lowering blood glucose level (Rukachaisirikul et al., 2004)	Leaf	6.89
Psophocarpus tetragonolobus	SK 1750/10	Kacang botol (winged bean)	Treat vertigo, cure boils and ulcer (Sasidharan et al., 2008)	Pod	8.53
Sauropus androgynus	SK 1751/10	Cekur manis (sweet shoot)	Relieve internal fever and treat urinary problems (Benjapak et al., 2008)	Leaf	5.20

Furthermore, ROS is potent activator of NF-κB signaling pathway; indirectly promote the iNOS expression and NO synthesis (Geoffrey et al., 2006). Thus, inhibition of free radical scavenging property also serves as an important therapeutic consideration in development of anti-inflammatory agents. Inflammation is usually coupled with a sensation of pain as a secondary reaction due to the release of algesic mediators such as prostaglandin molecules (Millan, 1999). Prostaglandins (PGE_2 and PGI_2) bind to the specific nociceptor, which localized on nerve fibre and caused membrane depolization. Subsequently, an action potential is generated, where pain impulses will be transmitted from the peripheral terminal of nociceptor to the central nervous system and result in nociceptive pain (David, 2007).

In Malaysia, several medicinal plants are either consumed raw as salad or cooked. These plants are popular in Malay ethnic because of their taste and benefits to the balance diet and health (Ismail, 2000). Nutritional studies have shown that many of these plants are nutrient rich and possess potential bioactive compounds in controlling various inflammation disorders or age-related diseases (Abas et al., 2006; Saha et al., 2004). The traditional used of the plant species investigated were illustrated in Table 1. In view, lack of investigation of the pharmacological

properties of these medicinal plants, herein, anti-inflammatory, antioxidant and anti-nociceptive activities of selected methanolic extracts of Malaysian Medicinal plants were investigated.

MATERIALS AND METHODS

Chemicals

The following reagents were obtained commercially, antibiotic (5000 U/ml penicillin and 5000 µg/ml streptomycin) and dulbecco's modified eagle's medium (DMEM) from Flowlab™, Australia; fetal bovine serum (FBS) from iDNA technologies Inc., recombinant mounse IFN-γ from eBioscience Inc., USA; lipopolysaccharide from *Escherichia coli* (strain 055:B5), Sulphanilamide, naphtyethyenediamine and diphenylpicrylhydrazine (DPPH) from SIGMA Chemical Co., USA. Dimethyl sulfoxide (DMSO), acetic acid and absolute ethanol were purchased from Fisher Scientific, USA; 3-(4,5–dimethylthiazol–2-yl)-2,5-diphenyl tetratzolium bromide (MTT) from Fluka Chemie GmbH, Switzerland; Folin - Ciocalteu reagent from Merck, Germany.

Plants material

Plants samples were collected from Agricultural Park, University Putra Malaysia (UPM) and identified by botanist Mr. Shamsul Khamis, Laboratory of Natural Product, Institute of Bioscience, UPM.

Experimental animals

For animal test, male Balb/c albino mice (20 to 30 g) of four-week old were used for this study. They were fed with standard food and water *ad libitum* as well as housed in 22 ± 2°C under a 12 h light/ 12 h dark cycle. All mice were maintained in accordance with the current guidelines of the care of laboratory animal and the ethical guidelines (Ref: UPM/FPSK/PADS/BR-UUH/00262).

Sample preparation and extraction

Plants samples were cut into small pieces and dried in hot air-blowing oven at 50ºC until constant weight was obtained. Then, the dried samples were pulverized to powder form and soaked in 100% methanol. The methanolic plants extract were evaporated at 40°C to dryness under reduced pressure before being subjected to the bioassay. For animal experiment, plant extracts were dissolved in 5% Tween -20 in 0.9% normal saline. For other experiments, plant extracts were dissolved in 100% DMSO.

Cell culture and stimulation

The murine monocytic macrophages cell line (RAW 264.7) was purchased from European Collection of Cell Cultures (Porton Down, UK) and maintained in DMEM supplemented with 10% FBS, 4.5 g/L glucose, sodium pyruvate (1 mM), L-glutamine (2 mM), streptomycin (50 µg/ml) and penicillin (50 U/ml) at 37°C and 5% CO_2. The cells (4×10^5 cells/well) were seeded into a tissue culture grade 96-well plate and incubate for 2 h at 37°C, 5% CO_2 to attach the cells. Then, the attached cells in the well were triggered with stimuli (100 U/ml of IFN-γ and 5 µg/ml of LPS) with or without the presence of plant sample tested at final concentration range from 1.56 to 100 µg/ml. DMSO was used as vehicle to facilitate the plant sample tested into the culture medium and the final concentration of DMSO was 0.1% in all cultures. Cells were then incubated at 37°C, 5% CO_2 for 17 - 20 h. The culture supernatant was subjected to griess assay for nitrite determination and the cells remaining in the well were tested for cell viability assay by using 3-(4,5–dimethylthiazol–2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reagent.

Nitrite determination

Griess assay was used to determine the concentration of nitrite (NO_2^-), which is the stable metabolite of NO in culture medium. Briefly, an equal volume of griess reagent (1% sulphanilamide and 0.1% N - (l - naphthyl) - ethylene diamine dihydrochloride, dissolved in 2.5% H_3PO_4) was mixed with culture supernatant and colour development was measured at 550 nm using a microplate reader (SpectraMax Plus,

Molecular Devices Inc., Sunnyvale, CA, USA). The amount of nitrite in the culture supernatant was calculated from a standard curves (0 - 100 µM) of a sodium nitrite freshly prepared in deionized water. Percentage of the NO inhibition was calculated by using this formula where control is the nitrate level of IFN - γ/LPS - induced group.

$$\text{NO inhibitory (\%)} = \frac{[NO_2^-]_{control} - [NO_2^-]_{sample}}{[NO_2^-]_{control}} \times 100\%$$

Cell Viability

The cytotoxicity of the sample tested on cultured cells was determined by assaying the reduction of MTT reagents to formazan salts. After removing of supernatant, the MTT reagents (0.05 mg/ml dissolved in sterile PBS, pH 7.0) were added into each well. The cells remaining were incubated at 37°C for 4 h and the formazan salts formed were dissolved by adding 100 µl of 100% DMSO in each well. The absorbance was then measured at 570 nm using microplate reader (SpectraMax Plus, Molecular Devices). The percentage of cell viability calculated as below, where control is the cell viability of IFN - γ/LPS - induced group.

$$\text{Cell Viability (\%)} = \frac{OD_{control} - OD_{sample}}{OD_{control}} \times 100\%$$

DPPH free radical scavenging assay

The free radical scavenging activity assay was carried out to evaluate the antioxidant properties of the plant extracts. Briefly, stock solution of plant extracts was prepared at 100 mg/ml concentration in 100% methanol. Then, the samples were two-fold serially diluted to seven different concentrations (3.13 to 200 µg/ml). Then 5 µl of diphenylpicrylhydrazine radical (DPPH) solution (5 mg/ml dissolved in methanol) was then added to each well. The plate was shaken gently and incubated in the dark for 30 min. The absorbance was measured at 517 nm and percentage of total radical scavenging activity was calculated based on the formula below:

$$\text{DPPH radical scavenging activity (\%)} = \frac{OD_{blank} - OD_{sample}}{OD_{blank}} \times 100\%$$

Total phenolic content

The amount of total phenolic content in the plant samples were determined by using Folin-Ciocalteu's assay. One hundred microliter (100 µl) of each plants sample (1 mg/ml) were transferred into tube, followed by 500 µl of Folin - Ciocalteu reagent (1 N) and 400 µl of Na_2CO_3 [5% (w/v). The mixtures were incubated for 90 min at room temperature in the dark. After incubation, the absorbance was measured at 765 nm by using microplate reader (SpectraMax Plus, Molecular Devices) and the total phenolic content was expressed as mg Gallic acid equivalent (GAE) / g dry weight (DW) samples. The GAE present in the plant samples were calculated from a standard curve of GA ranging from 4.69 - 300 µg/ml.

Acetic acid-induced abdominal writhing response

The acetic acid induced abdominal constriction test was performed to determine the anti-nociceptive activities of the plant extracts. Briefly, mice (n = 6) was administered with 200 mg/kg of methanolic extract of plant sample (i.p.) 30 min prior to injection of 0.6% acetic acid (10 ml/kg, i.p.). Control animals received a similar volume of 5% Tween-20 as vehicle control (10 ml/kg, i.p). After five minutes of lag period, post-administration of acetic acid, abdominal constrictions (full extension of both hind limbs) were observed cumulatively for 30 min. Aspirin as positive control drug (100 mg/kg, i.p.) was administrated 30 min before the nociceptive agent. The percentage inhibition of constrictions was calculated using the formula below, where the control is the constriction number of the acetic acid-treated group.

$$\text{Inhibition of constriction (\%)} = \frac{Mean_{control} - Mean_{sample}}{Mean_{control}} \times 100\%$$

Statistical analysis

All experiments was conducted 3 times except for the animal study. The results were expressed as mean ± S.E.M and the data obtained were statistically analyzed by using one–way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. Significance of differences between groups was accepted at *P<0.05, **P<0.01 and ***P<0.001.

RESULTS

Anti-inflammatory activity

As illustrated in Table 2, six plant species tested have showed significant NO inhibitory activity upon IFN-γ/LPS-treated macrophages in a concentration-dependent manner. Most of the methanolic plant extracts reduced the NO production of 60 to 70% inhibition at the highest concentration tested (100 µg/ml), except *Psophocarpus tetragonolobus* which showed relatively low NO inhibition of 39.28 ± 2.01%. MTT assay was conducted to determine the cell viability in order to exclude the possibility that inhibitory activity of plant extracts were due to cytotoxicity. In this study, the NO suppressive action of the plant extract tested was not caused by the cytotoxicity effect; where the cell viability of RAW 264.7 cells upon treatments (25, 50 and 100 µg/ml) were exceeded 90%. On the other hand, all six plants showed moderate anti-inflammatory activity with IC_{50} values of > 40 µg/ml as compared to L-NAME (0.008 ± 0.1 µg/ml) (Table 3).

Antioxidant activity

Antioxidant activities of six plants were performed in the scavenging system of the DPPH radical. All six plants tested showed the capacity to scavenge DPPH radical in a concentration-dependent manner (Figure 1). Among six plants investigated, *Carica papaya, Oenanthe javanica* and *Sauropus androgynus* showed the greatest antioxidant capacity with IC_{50} values of 89.43 ± 2.02, 87.42 ± 0.64 and 86.74 ± 2.92 µg/ml, respectively (Table 3). Ascorbic acid was used as positive control and significantly inhibits radical scavenging activity with IC_{50} value of 4.15 ± 0.50 µg/ml.

Total phenolic content

Total phenols were expressed in term of gallic acid (GA) equivalent per gram of the dry weight sample and the results were summarized in Table 3. Six plants studied showed the present of phenolic content with the mean value of the phenols range from 31.61 to 50.01 mg GA/g DW samples.

Anti-nociceptive activity

Results on the effect of six plant extracts on anti-nociceptive activity were shown in Figure 2. All plant extracts significantly reduced the number of abdominal constriction in acetic acid-induced mice at different degree of inhibition at concentration tested of 200 mg/kg. The non-steroidal anti-inflammatory drug (NSAID), aspirin, manifested significant diminished number of writhes (constriction inhibition of 60.00 ± 1.37%) at concentration tested of 100 mg/kg.

DISCUSSION

Over production of NO is harmful and interference in NO

Table 2. Effect of six methanolic plant extracts tested at three different concentrations (25, 50 and 100 µg/ml) on nitric oxide (NO) production and RAW 264.7 cell viability. $P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ were significantly different from IFN-γ/LPS- treated group.

Plants	Concentration (µg/ml)	Inhibition of no production (%)	RAW 264.7 cell viability (%)
	100	72.63 ± 4.95***	99.79 ± 6.41
C. papaya	50	37.86 ± 2.24*	101.72 ± 11.19
	25	30.29 ± 2.96	108.91 ± 9.12
	100	71.06 ± 2.51***	100.67 ± 13.89
M. acuminata	50	49.39 ± 1.15*	101.08 ± 9.01
	25	36.10 ± 4.01	114.11 ± 3.38
	100	75.64 ± 4.45***	91.28 ± 9.70
O. javanica	50	45.16 ± 4.23*	105.66 ± 10.45
	25	32.61 ± 4.25	107.54 ± 10.43
	100	62.82 ± 1.53***	90.01 ± 1.78
P. sarmentosum	50	46.53 ± 1.15*	95.09 ± 0.99
	25	26.83 ± 1.73	100.57 ± 1.20
	100	39.28 ± 2.01**	111.53 ± 6.78
P. tetragonolobus	50	25.13 ± 1.56	113.98 ± 2.15
	25	27.44 ± 2.90	117.88 ± 1.62
	100	68.28 ± 7.05***	92.26 ± 2.16
S. androgynus	50	44.46 ± 2.36**	106.93 ± 5.86
	25	28.75 ± 2.32*	107.24 ±9.35

Table 3. The IC_{50} values of DPPH radical scavenging and nitric oxide (NO) inhibitory activities as well as writhing inhibitory activity and total phenolic content of six methanolic plant extracts. NT; not tested,[a] Positive control used for DPPH assay,[b] Positive control used for Griess assay ,[c] Positive control used for writhing test.

Samples	IC_{50} (µg/ml)		Writhing inhibition (%)	Total phenolic (mg GAE/g DW)
	DPPH scavenging activity	NO inhibitory activity		
C. papaya	89.43 ± 2.02	60.18 ± 3.30	43.12 ± 1.05	34.20
M. acuminata	192.92 ± 2.60	42.24 ± 3.90	40.00 ± 1.83	34.20
O. javanica	87.24 ± 0.64	54.12 ± 7.93	22.86 ± 1.54	44.70
P. sarmentosum	129.65 ± 1.04	60.24 ± 2.39	10.65 ± 0.87	50.01
P. tetragonolobus	94.64 ± 2.59	> 100	21.82 ± 1.10	31.61
S. androgynus	86.74 ± 2.92	58.34 ± 1.11	38.96 ± 1.36	44.93
A. acid[a]	4.15 ± 0.50	NT	NT	NT
L-NAME[b]	NT	0.008 ± 0.01	NT	NT
Aspirin[c]	NT	NT	60.00 ± 1.37	NT

production cascade is one of the main targets in inflammation research. Thus, in the present study we evaluate the effect of six Malaysian Medicinal plants on NO production in murine macrophages cell line (RAW 264.7). Interestingly, all six plant species showed inhibitory activity on NO production upon IFN-γ / LPS-activated macrophages. Few reports have shown that plant extracts suppressed NO production and/or iNOS

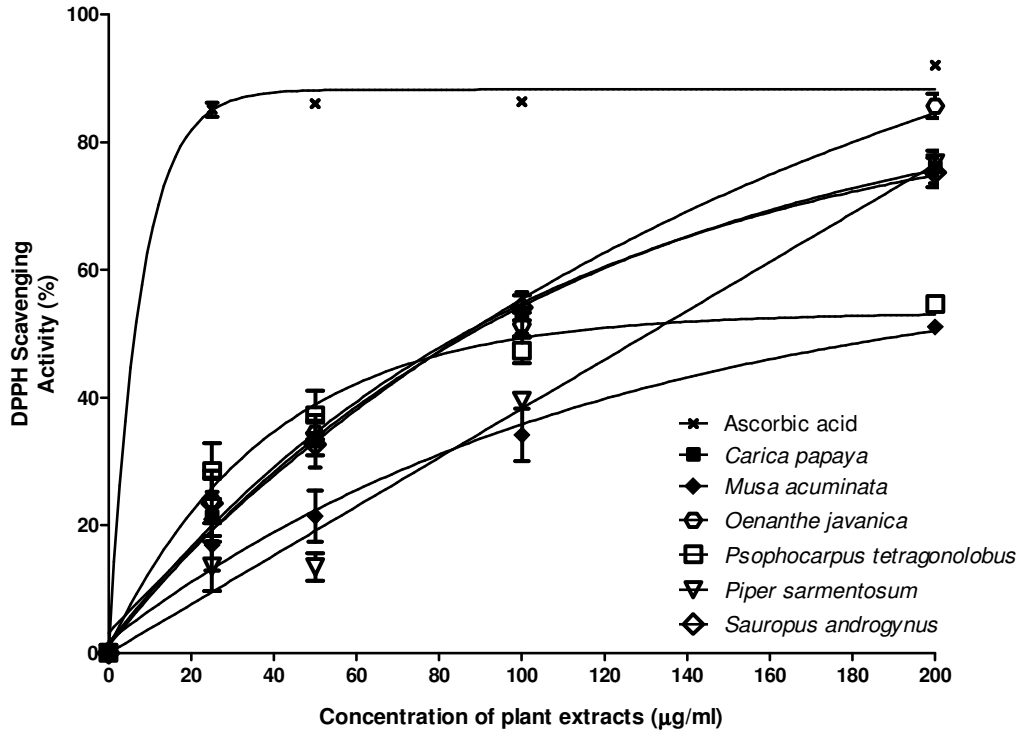

Figure 1. Antioxidant activities of six methanolic plant extracts tested at seven concentrations (3.13 to 200 µg/ml) by using DPPH assay.

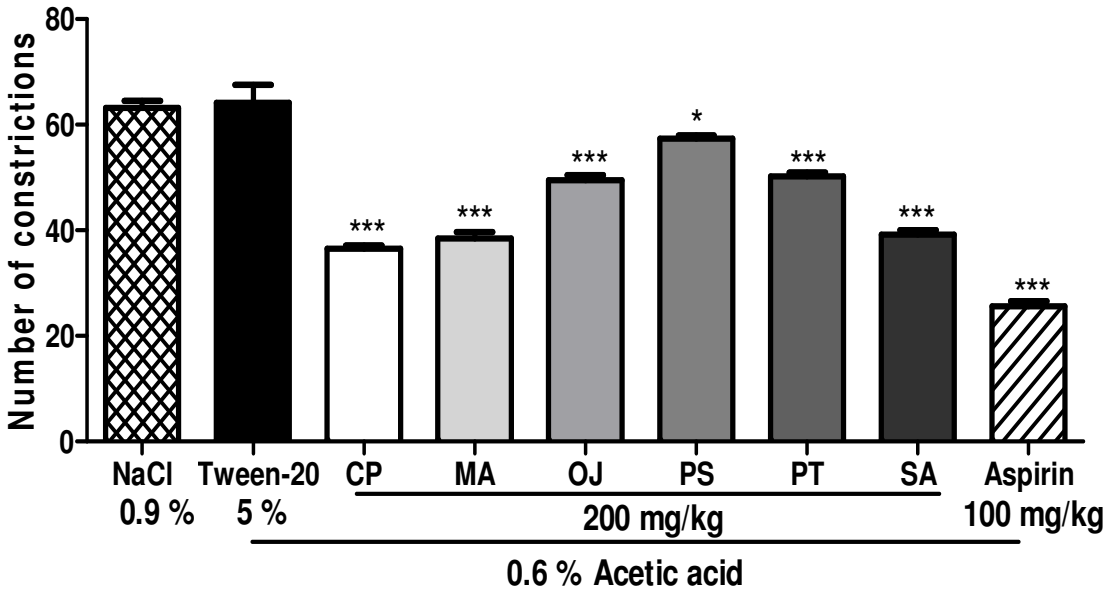

Figure 2. Effect of the plant extracts on 0.6% acetic acid-induced writhing response in mice. Each column represents number of abdominal constrictions observed within 30 minutes upon treatments from six different animals. *P<0.05, ***P<0.001, significantly different from vehicle treated group (5 % Tween-20). (CP, MA, OJ, PS, PT and SA represent methanolic extracts of *C. papaya, M. acuminata, O. javanica, P. sarmentosum, P. tetragonolobus* and *S. androgynus,* respectively).

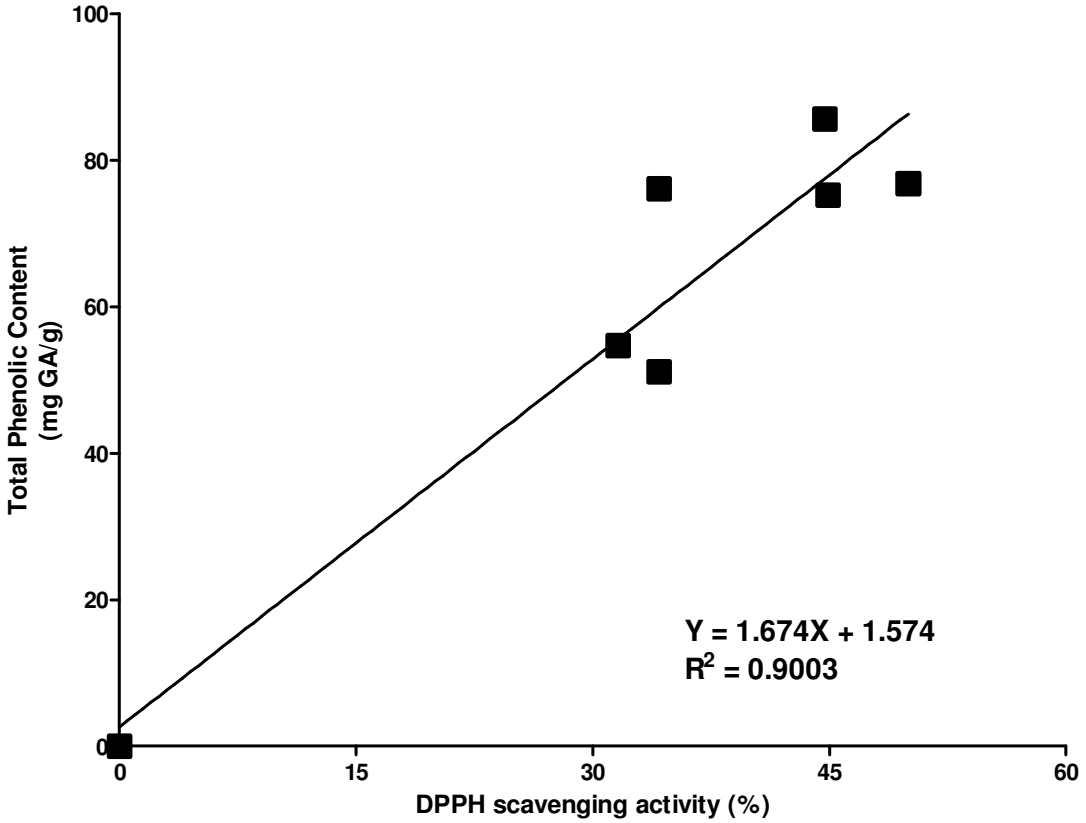

Figure 3. Correlation between the total phenolic content and DPPH scavenging activity (represent the highest activity at 200 µg/ml) of six plant species tested.

expression by attenuating NF-κB and MAPK signalling pathway (Santos et al., 2010; Burk et al., 2009). Thus, we believed that all plant species tested inhibited NO production through the similar way.

On the other hand, we have demonstrated that all plant extracts tested showed significant anti-nociceptive activity concomitant with the NO inhibitory activity. According to Aley et al. (1998), NO contribute to the maintenance or induction of hyperalgesia through secondary messenger either cAMP-dependent or cGMP-dependent by promoting PGE_2 production. Therefore, it was believed that nociceptic inhibitory action of plant extracts might mediated by the suppression of NO production; subsequently reduces the sensitization of the peritoneal nociceptor in mice.

This study justifies the traditional anti-inflammatory uses of the plants studied by its antioxidant properties. Several studies have reported the relationship between antioxidant property of plants and their total phenolic contents (Kouakou-Siransy et al., 2010; Lizcano et al., 2010; Shahidi and Marian, 2003). Our present results have shown a good correlation (R^2 = 0.9003) between antioxidant capacity and total phenolic content (Figure 3), which in agreement with other previous reports (Kouakou-Siransy et al., 2010; Lizcano et al., 2010).

Many phenolic compounds proved to have great pharmacological values and perceived beneficial effects for health due to their potential anti-inflammatory, antioxidant and anti-tumors properties (Soobrattee et al., 2005). Other reports have shown that flavonoids, condensed tannin and gallotannin suppressed the expression of pro-inflammatory targets in pain and inflammatory diseases (Iwalewa et al., 2007). Overall, we believed that amount of phenolic compounds present in the plants studied might be the main significant factors contributing to their anti-inflammatory and antioxidant activities.

To date, several chemical constituents from the plant species investigated have been reported. Naproxene, a well known anti-inflammatory drug was isolated from *Musa acuminate* (Abad et al., 2000), which could be responsible for the NO inhibitory and anti-nociceptive activity of this plant. On the other hand, coumarin was isolated from the leaves of *Carica papaya* and has

exhibited anti-inflammatory by inhibiting TNF-α production (Bissonnette et al., 2009; Pendzhiev, 2002). Therefore, it was suggested that *C. papaya* leaves inhibit NO production and showed anti-nociceptive activity through the suppression of up-stream level of the cytokine, TNF-α. Furthermore, phenolic compounds such as phenylpropanoid derivatives and naringenin were isolated from *S. androgynus* and *P. sarmentosum*, respectively; which displayed powerful antioxidant properties (Kanchanapoom et al., 2003; Subramania et al., 2003). Thus, it strongly supports our present results which showed that both plants exhibit scavenging activity through the present of phenolic compounds.

As far as we can ascertain, the anti-inflammatory, antioxidant and anti-nociceptive activities of the Malay plants investigated have not been reported, yet they are extensively used in traditional medicine. Thus, the preliminary study revealed the traditional medicinal uses of the evaluated plant species (*C. papaya, Musa acuminata, O. javanica, P. sarmentosum, P. tetragonolobus* and *Sauropus androgynus*) in controlling pain and inflammatory diseases.

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Abbreviations: **°C,** Degree celcius; **AP-1,** activator protein-1; **CO_2,** carbon dioxide; **DMEM,** Dulbecco's; modified eagle's medium; **DMSO,** dimethyl sulfoxide; **DNA,** deoxyribonucleic acid; **DPPH,** diphenylpicrylhydrazine radical; **DW,** dry weight; **GAE,** gallic acid equivalent; **h,** hour; **H_3PO_4,** phosphoric acid; **IC50,** inhibitory concentration at 50%; **IFN-γ,** interferon-gamma; **iNOS,** inducible nitric oxide synthase; **L-NAME,** NG-nitro-L-arginine methyl ester; **LPS,** lipopolysaccharide; **MAPK,** mitogen activated protein kinase; **min,** Minute; **MTT,** 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide; **N,** normality; **Na_2CO_3,** sodium bicarbonate; **NF-κB**, nuclear factor kappa-B; **NO,** nitric oxide; **NO_2-,** nitrite; **OD,** optical density; **RAW 264.7,** murine monocytic macrophages cell line; **RNS,** reactive nitrogen species; **ROS,** reactive oxygen species; **S.E.M.,** standard error of mean; **STAT,** signal transducer and activator of transcription; **(w/v),** weight per volume.

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