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Full Length Research Paper

Evaluation of antioxidant potential of medicinal plants from Malaysian *Rubiaceae* (subfamily *Rubioideae*)

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This paper evaluates the antioxidant potential of 22 species of medicinal plants from Malaysian *Rubiaceae*. The ferric thiocyanate (FTC), thiobarbituric acid (TBA), total phenolic content (TPC) and the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assays were employed. The tested extracts showed strong antioxidant potential when compared to butylated hydroxytoluene (BHT), quercetin and vitamin E with percent inhibition of 91 - 99% in the FTC, and 77 - 97% in the TBA assays. The TPC of extracts found varied from 8.55 to 120.63 mg_{GAE}/g_{PE}. *Psychotria griffithii and Hydnophytum formicarum* showed total phenolic content of more than 100 $mg_{GAE/gPE}$ and strong DPPH radical-scavenging activity with IC_{50} values of 14.0 and 22.4 µg/ml, respectively. The standard vitamin C and BHT showed IC_{50} values of 12.8, and 5.6 µg/ml, respectively. A good correlation was observed between total phenolic content and radical-scavenging activities. The medicinal plants in this study tested are expected to be good sources of natural antioxidants.

Key words: Antioxidant, *Rubiaceae*, ferric thiocyanate (FTC), thiobarbituric acid (TBA), 1,1-diphenyl-2-picrylhydrazyl (DPPH), total phenolic content (TPC).

INTRODUCTION

With 10700 species distributed in 637 genera, the *Rubiaceae* represents one of the largest plant family which mainly consists of tropical woody plants. They also grow as trees, shrubs or less often as perennial to annual herbs, as in Rubieae (subfamily *Rubioideae*) which are found in temperate regions. The most recent and complete classification based on molecular, morphological and chemical evidence has subdivided this family into four subfamilies, including Cinchonoideae, Ixoroideae, *Antirheoideae* and *Rubioideae* (Mongrand et al., 2004).

Most of the species in this family have been used widely by various indigenous people and particularly, by traditional practitioners as remedies. Among the common ones are species from the genera *Hedyotis*, *Ophiorrhiza* and *Psychotria*. In Malaysia, reports on Rubiaceous plants include phytochemical and pharmacological studies on *Mitragyna*, *Uncaria* and our own work on *Morinda* and *Hedyotis* species (Ahmad et al., 2005; Houghton et al., 1991; Ismail et al., 1997; Kam et al., 1992; Takayama et al., 2001). Some *Hedyotis* species are used as ingredients in Chinese herbal medicines for the treatment of cancer, and the medicine namely "peh-hue-juwa-chi-cao" contains *Hedyotis diffusa* as one of the active components (Ahmad et al., 2005). Another species, *Hedyotis hedyotideae*, is also a medicinal herb used in the treatment of colds, stomatitis and various inflammations. Pharmacological studies of ethanolic extracts of this plant revealed that it possesses an anti-ulcer effect. A full review on the phytochemical studies and pharmacological activities of plants in genus *Hedyotis* (formerly known as *Oldenlandia*) has been recently documented (Lajis and Ahmad, 2006).

Numerous phytochemical and pharmacological studies

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Abbreviations: FTC, Ferric thiocyanate; **TBA,** thiobarbituric acid; **DPPH,** 1,1-diphenyl-2-picrylhydrazyl; **TPC,** total phenolic content; **OD,** optical density; **AOA,** antioxidant activity; **BHT,** butylated hydroxytoluene; **PTDC,** pyrrolidinedithiocarbamate; **TNF,** tumor necrosis factor-α.

carried out on herbaceous plants have resulted in the development of natural antioxidants (Balunas and Kinghorn, 2005). According to Shahidi (1997), antioxidants are known to act at different levels in the oxidative sequence in the body. They may act by decreasing oxygen concentration, intercepting singlet oxygen, preventing first-chain initiation by scavenging initial radicals such hydroxyl radicals, binding metal ion catalysts, decomposing primary products to non-radical compounds, and chain-reaction breaking to prevent continued hydrogen abstraction from substrates. Various methods have been used to evaluate and compare the antioxidant activity in an attempt to provide a meaningful interpretation relating to health. In this study, we evaluate the antioxidant activity of plants via their inhibition of lipid peroxidation in a biochemical system and investigate the radical-scavenging activities (via the DPPH method) as a further method of evaluating antioxidant activity.

Phenolic phytochemicals are secondary metabolites of plant origin which constitute one of the most abundant groups of natural metabolites and are synthesized by plants in order to protect themselves from biological and environmental stresses. Recent studies have shown that phenolic compounds possess high antioxidant activity and certain therapeutic properties, including anti-diabetic and anti-hypertension activity (Apostolidis et al., 2007). Thus, the determination of total phenolic content may be used as an indirect evaluation of the antioxidant potential of the extracts.

In the first phase of our study, we reported the antioxidant and other biological activities of seven *Hedyotis* species and other Rubiaceous plants (Ahmad et al., 2005; Saha et al., 2004). However, towards a chemotaxonomic study of the antioxidant potential of *Rubiaceae* family, screening of more plants is necessary. Thus, the second phase of our study is the antioxidant screening of 22 species of Malaysian *Rubiaceae* with a focus on the subfamily *Rubioideae*. The 22 species from 10 genera and their local name in parenthesis include *Argostemma* sp., *Argostemma yappii*, *Chasalia minor*, *Chasalia pubescens*, *Hedyotis corymbosa*, *Hedyotis philippinensis* (lidah jin), *Hedyotis havilandi*, *Hydnophytum formicarum* (kelapa berok/naga stong), *Lasianthus cyanocarpus*, *Lasianthus maingayi* (telinga rusa), *Lasianthus pilosus*, *Ophiorrhiza major (sebueh bukit)*, *Ophiorrhiza discolor*, *Psychotria griffithii* (bunga mempitit), *Psychotria lasiocaphala*, *Psychotria ophirensis*, *Rennellia elliptica*, *Spermacoce exilis*, *Spermacoce latifolia*, *Urophyllum ferrugineum*, *Urophyllum glabrum* and *Urophyllum griffithianum*.

MATERIALS AND METHODS

Plant material

Plant samples were collected from various parts of Peninsular Malaysia. Voucher specimens were deposited at the herbarium of the Laboratory of Natural Product, Institute of Bioscience, Universiti Putra Malaysia, Serdang, Selangor, Malaysia.

Sample preparation and extraction

Leaves, stems or whole samples were cut into small pieces, air-dried in the shade and ground into fine powder before being extracted with methanol. Extracts were evaporated to dryness under reduced pressure before being subjected to the antioxidant assays.

Antioxidant activity

Antioxidant activities were evaluated by the inhibition of lipid peroxidation in the ferric thiocyanate (FTC) and thiobarbituric acid (TBA) assays. FTC and TBA assays were carried out as described previously (Ahmad et al., 2005). Total phenolic content (TPC) of extract was estimated by the Folin-Ciocalteu assay (Kumaran and Karunakaran, 2007), while the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay is as described in Saha et al. (2004) with slight modification. The strength of radical scavenging property was evaluated via the reduction in the optical density (OD) of DPPH at 517 nm. As cited by Abas et al. (2006), extract with an IC_{50} value of less than 30 µg/ml, between 30 - 100 µg/ml and more than 100 µg/ml is considered to possess strong, moderate, and weak free radical-scavenging activity, respectively.

RESULTS AND DISCUSSION

In the search of new therapeutic agents from natural sources, especially from medicinal plants, various direct and indirect approaches have been applied to determine their antioxidant activity (AOA). In this study, the antioxidant potential of methanolic extracts of Malaysian *Rubiaceae*, particularly the subfamily *Rubioideae*, was evaluated using the complementary FTC and TBA assays. These methods are based on the effect of extracts on the inhibition of lipid peroxidation in a biochemical system. Since antioxidant activity is often correlated to the total phenolic contents (Jayaprakasha et al., 2008; Kaur et al., 2008), the Folin-Ciocalteu assay was used to obtain an estimation of the total phenolic content. To further evaluate the antioxidant potential, we also employed the DPPH method. The DPPH assay measures the ability of antioxidants to scavenge free radicals which is not directly associated with the real oxidative degradation or effects of transient metals (Roginsky and Lissi, 2005).

In the FTC assay, all tested extracts (Figure 1) showed lower absorbance values when compared to the control with high AOA and percent inhibition of 91.7 - 98.5% (Table 1) which is comparable to those of butylated hydroxytoluene (BHT), vitamin E and quercetin. The absorbance values for *H. formicarum* and *P. griffithii* is further shown as an inset in Figure 1 along with the absorbance values for BHT, vitamin E and quercetin. In the TBA assay, a similar trend was observed with all tested extracts exhibiting low absorbance values correlating to a percent inhibition of 77.3 - 97.3%. The

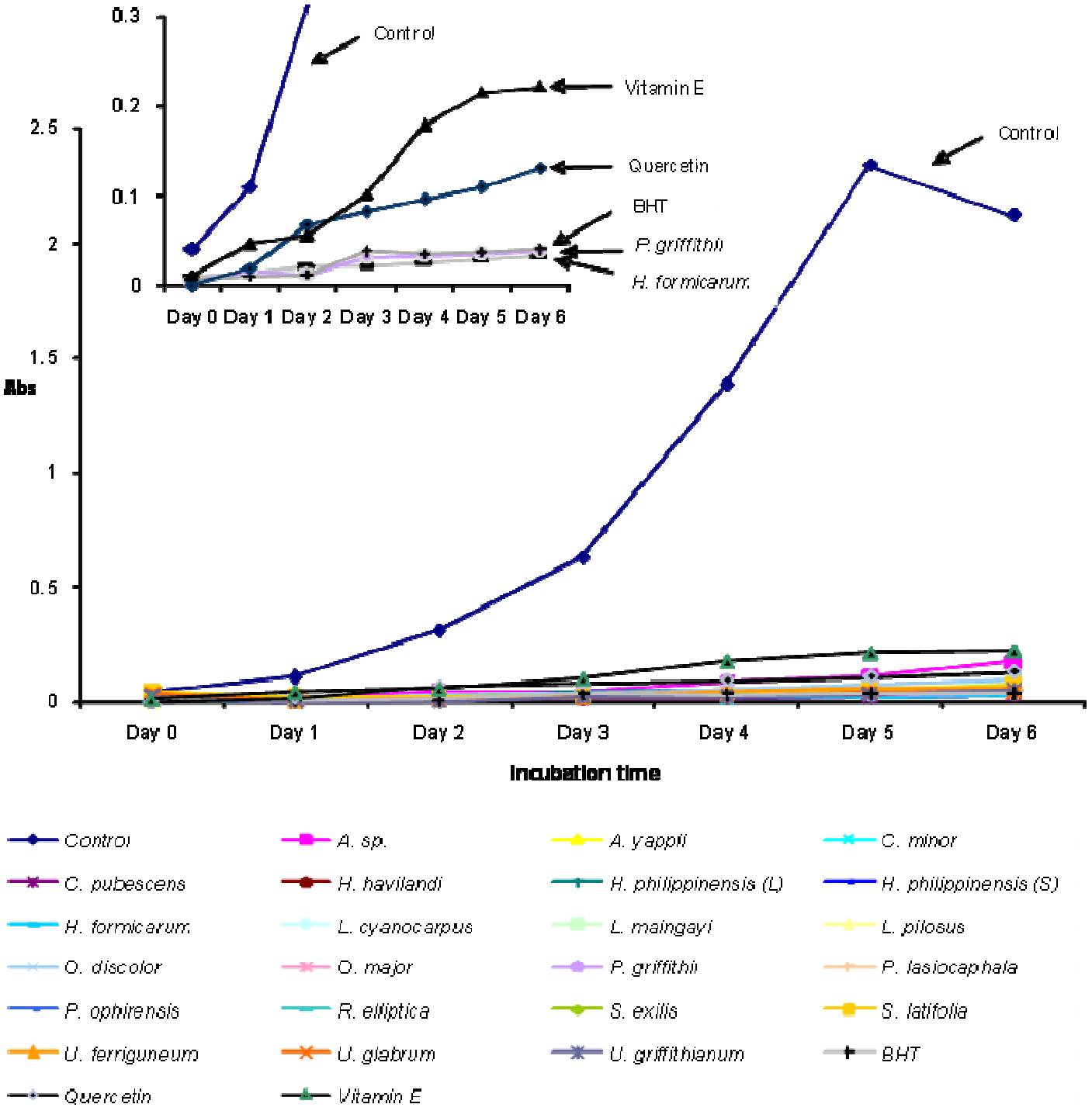

Figure 1. Antioxidant activity of crude methanolic extracts of 22 Rubiaceous species as measured by the FTC method.

standards, BHT, vitamin E and quercetin showed a slightly lower inhibition of 93.5, 85.6 and 91.4%, respectively, when compared to the values in the FTC. Almost all samples and standards in TBA showed slightly lower percent inhibitions than those obtained in FTC. The difference could indicate that the amount of peroxide in the initial stages of lipid peroxidation (as measured by FTC) is relatively higher than the amount of secondary products in the later stages (as measured by TBA). The comparison of the AOA observed for the plant samples and the three standards between the two methods is shown in Table 1.

In the Folin-Ciocalteu assay, the content of total phenolics in Rubiaceous plant extracts determined using

Table 1. A comparison between antioxidant activity (AOA) of crude methanolic extracts of 22 Rubiaceous species by FTC and TBA methods.

Sample*	Absorbance on final day FTC	AOA (% inhibition) FTC	Absorbance on final day TBA	AOA (% inhibition) TBA
Argostemma sp.	0.1757	91.7	0.5713	78.1
Argostemma yappii	0.0792	96.3	0.2121	91.9
Chasalia minor	0.0815	96.2	0.1217	95.3
Chasalia pubescens	0.0313	98.5	0.1629	93.8
Hedyotis havilandi	0.0647	96.9	0.1265	95.2
Hedyotis philippinensis (leaves)	0.0865	95.9	0.1391	94.7
Hedyotis philippinensis (stems)	0.0430	97.9	0.2877	88.9
Hydnophytum formicarum	0.0321	98.5	0.2084	92.0
Lasianthus cyanocarpus	0.7950	96.3	0.3228	87.7
Lasianthus maingayi	0.0835	96.1	0.2145	91.8
Lasianthus pilosus	0.0672	96.8	0.2934	88.3
Ophiorrhiza discolor	0.0971	95.4	0.3828	85.4
Ophiorrhiza major	0.0470	97.8	0.1220	95.3
Psychotria griffithii	0.0389	98.2	0.1630	93.8
Psychotria lasiocaphala	0.0647	96.9	0.1879	92.8
Psychotria ophirensis	0.0501	97.6	0.3243	87.6
Rennellia elliptica	0.0456	97.8	0.0828	96.8
Spermacoce exilis	0.0378	98.2	0.1164	95.5
Spermacoce latifolia	0.0759	96.4	0.5935	77.3
Urophyllum ferrugineum	0.0576	97.3	0.1784	93.2
Urophyllum glabrum	0.0423	98.0	0.1195	95.1
Urophyllum griffithianum	0.0550	97.4	0.0714	97.3
BHT	0.0397	98.1	0.1702	93.5
Quercetin	0.1302	93.9	0.2245	91.4
Vitamin E	0.2213	89.6	0.2462	85.6

*Final concentration of 0.02% (w/v).

the linear regression equation varied from 8.55 to 120.63 mg_{GAE}/g_{PE}. The results (Table 2) revealed that *H. formicarum* possessed the highest amount of total phenolics with 120.63 ± 3.09 mg_{GAE}/g_{PE} followed by *P. griffithii* with 102.46 ± 0.96 mg_{GAE}/g_{PE}, respectively. The lowest phenolic content was found in *L. pilosus* with 8.55 ± 2.50 mg_{GAE}/g_{PE}. Based on the FTC and TBA assays, high percentage of inhibition observed towards lipid oxidation indicated strong antioxidant potential and may be correlated to the total phenolics in the plant extracts which may be in the form of flavonoids, simple phenolics, terpenes or tannins.

While the FTC and TBA assays are non-specific, the DPPH radical-scavenging are mechanism-based. In the DPPH method, the antioxidant potential of the plant extracts was assessed on the basis of their scavenging activity of the stable DPPH free radical. Among the samples tested (Table 2), the extracts of *P. griffithii* and *H. formicarum* were found to be strong free radical scavengers with IC_{50} values of 14.0 and 22.4 µg/ml, respectively in comparison with vitamin C (5.6 µg/ml) and BHT (12.8 µg/ml). As shown in Table 2, the activity found correlates well with the results of the TPC assay in which both extracts were found to contain high amounts of total phenolics which has been correlated with the number of hydrogen donating groups in the phenolic compounds (Kaur et al., 2008). However, moderate activity was found for *U. griffithianum* and weak activities were found for *P. ophirensis*, *H. philippinensis* (leaves and stems) and *R. elliptica* with IC_{50} values of 43 - 227 µg/ml. The high AOA of the extracts could justify well their use as traditional remedies. Generally, all of the tested plants have been used by the Malays as well as Chinese community for medicinal purposes, either internally or externally. Some are used as poultice to treat snakebites, broken bones, bruises, rheumatism, lumbago, ague and skin disorders such as eczema. Internally, a decoction of some plants is given for constipation, indigestion, heartburn, gastric verigo, dysentery, gonorrhoea and also as a tonic (Burkill, 1966) and taken after childbirth.

The role of flavonoids as antioxidants has been well-established and there have been numerous reports on structure-activity relationships in the last decade (Abas et al., 2006; Rastija and Medic-Saric, 2008). Among the

Table 2. DPPH free radical-scavenging activity vs. total phenolic contents of 22 Rubiaceous species (in the order of increasing radical scavenging activity).

Sample	IC_{50} value (µg/ml (DPPH)	Radical-scavenging property (DPPH)	Total phenolics (mg_{GAE}/g_{PE})[a] (Folin Ciocalteu)
Lasianthus cyanocarpus	Na	-	8.61 ± 1.02
Lasianthus pilosus	Na	-	8.55 ± 2.50
Hedyotis corymbosa	Na	-	10.98 ± 0.16
Argostemma yappii	Na	-	11.39 ± 0.93
Ophiorrhiza discolor	Na	-	13.11 ± 0.17
Hedyotis havilandi	Na	-	12.26 ± 1.18
Chassalia pubescens	Na	-	12.54 ± 0.92
Psychotria lasiocaphala	Na	-	13.90 ± 2.36
Lasianthus maingayi	Na	-	14.03 ± 2.77
Spermacoce latifolia	Na	-	17.00 ± 0.35
Urophyllum glabrum	Na	-	15.60 ± 2.44
Argostemma cf. viscidum	Na	-	18.54 ± 0.66
Ophiorrhiza major	Na	-	20.41 ± 0.23
Chassalia minor	Na	-	15.77 ± 4.98
Spermacoce exilis	Na	-	27.57 ± 1.71
Urophyllum ferrugineum	Na	-	27.59 ± 2.49
Rennellia elliptica	227.24±4.31	weak	19.48 ± 0.93
Hedyotis philippinensis (stems)	130.78±4.35	weak	9.76 ± 0.81
Psychotria ophirensis	116.22±2.89	weak	21.76 ± 0.45
Hedyotis philippinensis (leaves)	114.19±0.34	weak	9.27 ± 0.02
Urophyllum griffithianum	43.24±2.60	moderate	25.23 ± 1.85
Hydnophytum formicarum	22.40±1.73	strong	120.63 ± 3.09
Psychotria griffithii	13.99±3.25	strong	102.46 ± 0.96
BHT	12.81±2.31	strong	-
Vitamin C	5.59±0.64	strong	-
Quercetin	Nd	strong	-

The data represent the means±standard deviation (SD) of triplicates of three independent experiments. Significance analysis was performed by ANOVA ($P < 0.05$). Na, Not active; Nd, not detectable in experimental concentration range (250 - 3.90 µg/ml); strong radical-scavenging activity, $IC_{50} < 30$ µg/ml; moderate radical-scavenging activity, 30 µg/ml ≤ IC_{50} ≤ 100 µg/ml; weak radical-scavenging activity, $IC_{50} > 100$ µg/ml (Abas et al., 2006); [a]data presented are means of two replicates from two independent experiments.

extracts tested, flavonoids have only been reported in the genus *Hedyotis* and *H. formicarum*, while none has been documented for the other genera (Lajis and Ahmad, 2006; Prachayasittikul et al., 2008). However, since other natural products such as iridoid glycosides and indole alkaloids are also abundant in this subfamily, it is yet to be ascertained whether the antioxidant potential reported can be attributed to the flavonoids alone, or to any of the other natural products, or to a synergistic effect of the compounds present.

Iridoid glycosides have long been known to be of chemotaxonomic significance in the subfamily *Rubioideae* (Inouye et al., 1988; Lopes et al., 2004). These iridoids have shown various activities such as antitumoral, hemodynamic, chloretic and hepatoprotective. Loganin, for example, isolated from *Palicourea rigida* from the tribe *Psychotrieae* exhibited high efficacy as an anti-inflammatory agent. In other studies, preliminary evaluation in treating rheumatoid arthritis showed that the iridoids asperuloside and deacetyl asperulosidic acid from *Lasianthus acuminatissimus* exhibited an inhibitory effect on the release of tumor necrosis factor-α (TNF-α) from cultured mouse peritoneal macrophages with IC_{50} values of 0.52 and 1 µg/ml, respectively (Recio et al., 1993).

Indole alkaloids represent another class of compounds found in abundance in this family. In *A. yappii*, an indole alkaloid (+)-isochimonanthine has been isolated from the leaves of the plant (Hibino and Choshi, 2002). Similarly, in *Psychotria* and *Ophiorrhiza* species, the isolation of indole alkaloids such as psychollatine from *Psychotria umbellata* and ophiorrhizine from *O. major* has been reported (Arbain et al., 1992; Fragoso et al., 2008). Although the antioxidant activity of indole alkaloids isolated from these genera are not known, Saha et al. (2004) have reported the cytotoxic effects of *Psychotria rostrata* against RAW 264.7 macrophage cells at a concentration of 250 µg/ml. In a separate study, schischkiniin, an indole alkaloid isolated from the seeds

of *Centaurea schischkinii* (Asteraceae) has shown significant antioxidant activity against the DPPH free radical with an IC_{50} value of 3.8 µg/ml (Shoeb et al., 2005). The bioactive indole alkaloid camptothecin (found in *Ophiorrhiza pumila*) has been reported to act as stimuli in inducing the metal chelator pyrrolidinedithiocarbamate (PTDC) to protect UV-irradiated human leukemia cell line (HL-60 cells) from apoptosis. In this respect, camptothecin may be regarded as an antioxidant since apoptosis has been associated with oxidative stress and antioxidant pathways have been suggested to play a central role in the protection against it (Verhaegen et al., 1995). The compound has also been found to show inhibitory activity against tumor cells by blocking the eukaryotic topoisomerase as well as against HIV-1 (Kitajima et al., 2003).

To the best of our knowledge, no phytochemical study has been done on 17 out of 22 species tested. Phytochemical studies have only been reported for *A. yappii*, *H. formicarum*, *H. corymbosa*, *O. discolor* and *O. major*. Phytochemical study of *H. formicarum* (which has been found to be active in the four assays) has yielded bioactive flavonoid and phenolic compounds: isoliquiritigenin, butin, butein, stigmasterol and protocatechualdehyde. It is conceivable that the potent antioxidative and radical-scavenging activities of *H. formicarum* may result from these constituents and could explain its reported effect on cancer cells (Itharat and Ooraiku, 2007; Prachayasittikul et al., 2008). Although no phytochemical study has been reported for *P. griffithii*, based on previous chemotaxonomic studies, it is expected that flavonoids, iridoids and indole alkaloids are present. For *R. elliptica*, *P. ophirensis* and *H. philippinensis* (leaves and stems), the weak activities found should not be a deterrent in investigating the plant in consideration of the kinetics of DPPH reaction (Bondet et al., 1997). This study, therefore, warrants phytochemical investigations on the six plants in order to investigate the bioactive constituents responsible for its antioxidant activity.

In conclusion, based on the inhibition of lipid peroxidation via the FTC and TBA assays, the methanolic extracts of all plant species tested were found to possess strong antioxidant potential. Taking into account the results of all four assays including the TPC and the DPPH assays, six plants namely, *H. formicarum*, *P. griffithii*, *U. griffithianum*, *P. ophirensis*, *H. philippinensis* (leaves and stems) and *R. elliptica* are expected to be good sources of natural antioxidants. Fractionation of extracts based on polarity and subsequent screening of the fractions would be the next logical step. Since most plants tested are used to treat rheumatism and anti-inflammatory-related diseases, screening of extracts for anti-inflammatory properties such as inhibitory effect on the release of TNF-α or inhibition of nitric oxide production will be a part of our future studies. The findings will hopefully help to explain the mechanism of antioxidant action and lead to the characterisation of new and interesting antioxidant principle which could combat oxidative stress leading to diseases like diabetes, hypertension, inflammation and cancer. It is anticipated that a correlation between the antioxidant activities of plants in the *Rubiaceae* family (particularly the subfamily *Rubioideae*) and its active principles could be established.

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