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Antioxidant Activity of Leaves of *Calophyllum rubiginosum*

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Abstract: Problem statement: *Calophyllum* species have been used in traditional medicine for their therapeutic values for many years. This use was explained by several previous studies where antimicrobial, antifungal, anti-HIV and anti-cancer and antioxidant compounds were isolated from several *Calophyllum* species. **Approach:** The leaves were extracted with n-hexane (hexane), dichloromethane (DCM) and methanol using soxhlet apparatus. Total phenolic contents of each extract were evaluated using Folin Ciocalteau Reagent (FRC) while Ferric Thiocyanate (FTC) and 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) were used for lipid peroxidation and free radical scavenging activity respectively. **Results:** As the results, methanol extracts (5 mg mL^{-1}) has higher phenolic contents of 66.2 μg mg^{-1} Gallic Acid Equivalent (GAE) compared to n-hexane and DCM extracts with phenolic content of 50.5 and 52.5 μg mg^{-1} GAE respectively. In FTC method, methanol had the lower absorbance values which indicate high antioxidant activities. In DPPH radical scavenging test however, DCM extract showed high antioxidant activity with an IC_{50} value of 0.11mg mL^{-1}, followed by methanol and hexane with IC_{50} values of 0.23 and 4.5 mg mL^{-1} respectively. The IC_{50} of standards were 0.74 and 0.56 mg mL^{-1} for ascorbic acid and α-tocopherol, respectively. **Conclusion:** Finally, the positive outcome of the research can be further tested in the future to ascertain the active compound of the leaves of *C. rubiginosum.*

Key words: Antioxidant activity, *Calophyllum rubiginosum*, Folin ciocalteau reagent (FRC), Ferric Thiocyanate (FTC), Total Phenolic Compound (TPC), phytochemical screening

INTRODUCTION

Plants are important to the running of human societies and ecosystem. In the field of ethnobotany, there is an interaction and relationship between plants and human beings. The attention of ethnobotany is more on how plants being used, managed and perceived in human societies; this includes plant materials that are used for food, medicine, divination, cosmetics, dyeing and textiles, for building, tools, currency, clothing, rituals and social life. The benefits of plant materials have been found in studies which include many branches of plant studies such as phytochemistry, ethnomedicine, botany and pharmacology (Velioglu *et al*., 1998).

The protective effect of many plant spices and herbs indicates the presence of antioxidative and antimicrobial constituents in the plants (Hirasa and Takemasa, 1998). Recently, interest has increased considerably in finding naturally occurring antioxidants for use in foods or medicinal materials to replace synthetic antioxidants, which are being restricted due to their carcinogenicity (Velioglu *et al*., 1998). It has been reported that there is an inverse relationship between the antioxidative status occurrences of human diseases (Rice-Evans *et al*., 1997). In addition, antioxidant compounds which are responsible for such antioxidants activity could be isolated and then used as antioxidants for the prevention and treatment of free radical-related disorders (Middleton *et al*., 2000). For that objective, research to identify antioxidative compounds becomes an important issue. Although it remains unclear which of the compounds, of medical plants are the active

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constituent, polyphenols recently have received increasing attention because of some interesting new findings regarding their antioxidant activities. From pharmacological and therapeutic points of view, the antioxidant properties of polyphenols, such as free radical scavenging and inhibition of lipid peroxidation, are the most crucial.

The genus *Calophyllum* (Clusiaceae/Guttiferae) is composed of about 180-200 different species confined to the warm humid tropics of the world (Stevens, 1980). Its members are native to Australasia, Madagascar, Eastern Africa, South and Southeast Asia, the Pacific islands, the West Indies and Latin America. In Malaysia, the plant *Calophyllum* is known as *Bintangor* tree. Extensive chemical investigation of this genus has resulted in the isolation of a wide variety of natural products, including xanthones, polyisoprenylated ketones (Taher *et al*., 2005), coumarins, biflavonoids, chalcones, benzofurans and triterpenes (Da Silva *et al*., 2001; Ito *et al*., 2002; 2003; Oger *et al*., 2003). Some of these species are frequently employed in folk medicine to treat several injuries (Sartori *et al*., 1999). Furthermore, xanthones and coumarins in *Calophyllum* species demonstrate antioxidant properties, specifically by inhibiting lipid peroxidation. The antioxidant activity of *Calophyllum* species helps to protect skin cells from damage by reactive oxygen species and other oxidative antagonists (Mahmud *et al*., 1998).

An antioxidant is defined chemically as an agent that prevents oxidation. Many attempts have been made to define an antioxidant, but the most useful definition is any substance that is present at low concentration compared to that of an oxidisable substrate, significantly delays or inhibits oxidation of the substrate (Williamson *et al*., 1999). Normally, there is a balance between the quantity of free radicals generated in the body and the antioxidant defense systems which scavenge/quench these free radicals preventing them from causing deleterious effects in the body (Nose, 2000).

This study was conducted to investigate the antioxidant activities of extracts from the leaves of *C. rubiginosum.* The antioxidant tests in this study were conducted using DPPH method for free radicals scavenging activity and ferric thiocyanate method for lipid peroxidation. Total phenolic and phytochemicals contents were also investigated.

MATERIALS AND METHODS

Plant material: The leaves of the plant of *C. rubiginosum* were collected in Melaka Botanical Garden in June 2009. The leaves were left to dry at room temperature for five days and grinded. After grinding, the powder form of the sample was then stored in cold room until extraction process.

Extraction of samples: The powdered sample (300 g) was weighted and transferred into a thimble. The thimble was placed in the soxhlet extractor and the solvents, hexane, DCM and methanol were used in a sequence of increasing polarity for extraction for 18-24 h. Upon obtaining the crude extract, the extract was evaporated using vacuum rotary evaporator at 60°C. The extract was transferred into a beaker and left to air dry. After that, the extracts were stored in a refrigerator for further use.

DPPH radical scavenging activity: The free radical scavenging effects of the extracts and α-tocopherol and ascorbic acid as positive controls on DPPH was determined by using the method describe by Susanti *et al.* (2007) with slight modifications. 100 μL of each sample and controls in methanol at various concentrations (5, 2.5, 1.25, 0.625, 0.3125, 0.1563, 0.0781, 0.0390, 0.0195 and 0.0095 mg mL^{-1}) was added to 3.9 mL of freshly prepared DPPH solution (0.004%) in methanol. For hexane extract the dilution was from 5.00-0.3125 mg mL^{-1}. After 30 min, the absorbance was measured at 515 nm. The absorbance was measured using a UV-VIS^{-1} spectrophotometer. All experiments were performed in triplicates. The color changes were observed from high concentration to low concentration. The capability to scavenge the DPPH radical was calculated using the following equation:

$$\text{Percent inhibition} = [(A_{DPPH}-A_{sample})/A_{DPPH}] \times 100$$

Where:
A_{DPPH} = The absorbance of DPPH
A_{sample} = The absorbance of the sample

Ferric Thiocyanate (FTC) test: The FTC method was adapted from Osawa and Namiki (1981). Samples (4 mg) in ethanol 99.5% (4.0 mL) were mixed with 2.51% linoleic acid in ethanol 99.5% (4.1 mL), 0.05 M phosphate buffer, pH 7.0 (8 mL) and distilled water (3.9 mL) and kept in screw cap containers under dark conditions at 40°C. To 0.1 mL of this solution was added 9.7 mL of ethanol 75% and 0.1 mL of 30% ammonium thiocyanate. Precisely 3 min after addition of 0.1 mL of 2×10^{-3} M ferrous chloride in 3.5% hydrochloric acid to the reaction mixture, the absorbance of the red color was measured at 500 nm each 24 h for 8 days. The positive control and standard

were subjected to the same procedure as the sample except for the control, where there was no addition of sample. For the standard, 4 mg of sample were replaced with 4 mg of α-tocopherol.

Phytochemical screening: Phytochemical screening was done as described by (Dohou *et al*., 2003) with slight modifications. The phytochemicals analyzed were alkaloids, trepenoid, steroids, flavonoids, saponins and phenols.

Determination of Total Phenolic Content (TPC): The amount of total phenolics in extracts was determined according to the Folin-Ciocalteu procedure (Singleton and Rossi, 1965) with slight modifications. About 5 mg of each sample was diluted in 1 mL of distilled water. About 1 mL of each sample was then transferred into test tubes and 5 mL of Folin-Ciocalteu were added to the samples. After 5 min, 4 mL of sodium carbonate were added to the solution and then vortex. The mixture was incubated for two h in the dark at room temperature. The absorbance was measured at 725 nm using a spectrophotometer.

Statistical analysis: Data were generated for each assay from three separate extracts of *C. rubiginosum* in triplicate. A one-way ANOVA test was performed on the antioxidant activity results to investigate significant differences between the extracts. Post Hoc test was conducted for multiple comparisons between the extracts.

RESULTS

Radical scavenging activities: All of the extracts and standards showed antioxidant activities with different IC_{50}. Here, IC_{50} means the 50% inhibitory concentration of the standards and extracts to scavenge free radicals and reduce the concentration to 50% of the original effect. IC_{50} values were directly calculated from linear regression equation of the extracts and standards. Figure 1 shows the calculated IC_{50} values of the standards and extracts. DCM extract showed higher scavenging activity with an IC_{50} of 0.11 mg mL^{-1} followed by methanol then hexane with IC_{50} values of 0.23 and 4.5 mg mL^{-1}, respectively. The ability of DCM extract to scavenge free radical was dose dependent manner (Table 1 and 2).

Lipid peroxidation activities: Table 3 indicated the percent inhibition of extracts and positive control on day 6. The methanolic extract showed lower absorbance in FTC method, which indicates that methanol extract has high antioxidant activity.

Phytochemical screening: The phytochemical screening revealed the presence of terpenoids, steroids and phenolic in all the extracts (Table 4).

Total phenolic contents: Methanol extract possessed the highest concentration of total phenolic content followed by DCM and hexane extracts (Table 5).

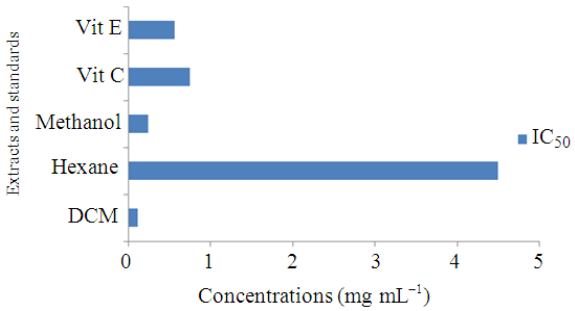

Fig. 1: IC_{50} of extracts and positive controls against DPPH

Table 1: Absorbance of DCM extract at 515 nm

	Before (sample + methanol)			After (sample + DPPH)				
Conc. (mg mL^{-1})	1st trial	2nd trial	3rd trial	1st trial	2nd trial	3rd trial	Mean	SD
5	0.0856	0.0802	0.0834	0.1364	0.1309	0.1276	0.1316	0.004
2.5	0.0538	0.0448	0.0444	0.1469	0.1408	0.1354	0.1410	0.005
1.25	0.0204	0.0232	0.0261	0.1524	0.1466	0.1462	0.1484	0.003
0.625	0.0210	0.0085	0.0180	0.2397	0.2063	0.2007	0.2156	0.020
0.3125	0.0209	0.0075	0.0090	0.2874	0.2266	0.2281	0.2473	0.001
0.1563	0.0040	0.0066	0.0086	0.3101	0.2893	0.2117	0.2704	0.002
0.0781	0.0035	0.0018	0.0075	0.3306	0.3048	0.3250	0.3201	0.010
0.039	0.0027	0.0017	0.0020	0.3898	0.3357	0.3386	0.3547	0.030
0.0195	-0.0030	-00000	-0.0020	0.4545	0.4483	0.4452	0.4493	0.003
0.0095	-0.0050	-0.0020	-0.0030	0.5824	0.5504	0.5498	0.5608	0.010

Table 2: Mean percent inhibition of DCM extract

Concentrations (mg mL^{-1})	Inhibition (%)
5.0000	93.3
2.5000	87.3
1.2500	82.9
0.6250	72.6
0.3125	67.9
0.1563	63.9
0.0781	56.9
0.0390	51.9
0.0195	38.9
0.0095	23.5

Table 3: Percent inhibition values of extracts and positive control on day 6 for FTC test

Sample	Absorbance	Inhibition (%)
DCM	0.0563	95.9
Hexane	0.0485	96.5
Methanol	0.0684	95.1
Vitamin E	0.2058	85.1

Table 4: Results of phytochemical screening tests

Samples	Alkaloids	Terpenoids	Steroids	Flavonoids	Saponin	Phenolic
DCM	-	+	+	-	-	+
Hexane	-	+	+	-	-	+
Methanol	-	+	+	-	-	+

Table 5: TPC of samples by µg GAE mg^{-1} extracts

Concentrations (mg mL^{-1})	TPC methanol extract (µg mg^{-1})	TPC DCM extracts (µg mg^{-1})	TPC hexane extracts (µg mg^{-1})
5	66.2	52.5	50.5
2.5	56.3	36.4	42.0
1.25	44.9	21.8	35.7
0.625	33.9	19.7	32.6
0.3125	29.6	6.9	30.8

DISCUSSION

The purpose of this research was to determine the antioxidant activity of a Malaysian plant *C. rubiginosum*. The study was done by screening the leaves extracts for their ability to scavenge DPPH free radicals as well as their lipid peroxidation activity with FTC. The results for both tests were positive indicating the presence of antioxidant molecules in extracts. Since the results of DPPH test and FTC test were positive, additional tests which are phytochemical screening and Total Phenolic Compound (TPC) were conducted.

The DPPH test is a largely used method in plant or food chemistry to evaluate the free radical scavenging effect of the specific compound or extract. In its radical form, DPPH has a broad absorption band in the visible region at 515 nm, however if it is protonated by anti-radical compound, it loses this property (Lo Scalzo, 2008). The test mainly is based on the color change observed when there is a reaction between DPPH free radicals and antioxidant molecules. The degree of discoloration indicates the scavenging potential of the extract.

The DPPH radical scavenging method is based on the reduction of methanolic DPPH solution in the presence of antioxidant resulting in the formation of non-radical DPPH-H by the reaction. The extracts in this study were able to reduce the stable DPPH, thus changing the color from purple to yellow. According to Susanti *et al*. (2007), ascorbic acid, vitamin E, polyhydroxy aromatic compounds, have the potency to reduce DPPH.

The FTC method was used to measure the amount of peroxide formed at the primary stage of linoleic acid peroxidation. The peroxide reacts with ferrous chloride to form a reddish ferric chloride pigment. In this method the concentration of peroxide decreases as the antioxidant activity increases. The controls and extracts showed increasing of the absorbance values from day 0 to day 5 and then all the values dropped on day 6. This reduction is due to the increasing level of melonaldehyde compounds from linoleic acid oxidation, which is not stable. The strong antioxidant activity of these extracts of *C. rubiginosum* may be useful in the treatment of several diseases such as malaria, acquired immunodeficiency syndrome, heart disease, stroke, arteriosclerosis, diabetes and probably cancer.

This study showed that phenolic content in extracts varies with the solvent used according to the polarity of the solvents. Although the methanol extract has higher content of phenol than DCM and hexane extracts (Table 5), it does not show higher activity for every assay assuming not only the content but also properties of phenolic compounds contribute to different activities in different extraction solvents. This is because the nature and concentration of phenolic compounds in plants are highly sensitive to the climatic and environmental conditions and geographical isolation may contribute to increase genetic differentiation among several taxonomic entities (Houghton and Raman, 1998).

CONCLUSION

Based on the results, it can be concluded that the leaves of *C. rubiginosum* contain phenolic that contributed in the determination of total phenolic contents. There is an increase interest for identifying alternative natural and safe sources of antioxidants and the search for natural antioxidants especially of form plant origin. The extracts of *C. rubiginosum* had the ability of scavenging free radicals and inhibiting lipid peroxidation. The findings also showed that the antioxidant activity of extracts varied according to the testing method used. However, the phytochemical findings indicated the presence of the some antioxidant

molecules in all extracts. Thus, further investigations should be conducted for identification of the active constituent and to give more information and benefits of the plant.

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