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Antioxidant and Cytotoxic Activities of *Calophyllum rubiginosum*

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Abstract

Clusiaceae / Guttiferae an evergreen shrubs or trees, it is considered as a family of 27 genera and 1090 species. *Calophyllum rubiginosum* one of these species and belongs to this family. In Malaysia they use it as folk remedies, where they believe its' activity. Problem: Evaluation of antioxidant and cytotoxic activities of three fractions. n-hexane, dichloromethane (DCM) and methanol (MeOH) of *Calophyllum rubiginosum* species. Approach: The three main fractions were tested to find out the antioxidant capacity using three different methods, DPPH radical scavenging, reducing power and chelating iron ions. The fractions were tested against lung cancer A-549 cell line to assess the anti-proliferation activity. Results: The MeOH fraction showed no effect against lung cancer cell line but achieved a significant result in antioxidant testing. The DCM and n-hexane fractions considered as moderate antioxidant agent, they showed a significant result against lung cancer cell line. Non and semi polar fractions were able to inhibit the proliferation of A-549 cell line at low concentration. The results were statistically significant ($P< 0.05$). Conclusions: *C. rubiginosum* fractions have showed significant activity. DCM and n-hexane fractions proved to be effective against A-549 lung cancer cell line, where they were able to disturb the cell proliferation. These results can benefit the local community, and to guide how to use this plant.

Keywords: *Calophyllum rubiginosum*; Antioxidant; DPPH; Reducing power; Chelating iron ions; Cytotoxicity.

Introduction

The plant kingdom constitutes a source of new chemical compounds which may be important due to their potential use in medicine or for their other biological properties. In the group of secondary plant metabolites, phenolic compounds are considering as the strong antioxidants and anti-proliferation [1, 2]. Antioxidant possess the ability to protect the cellular organelles from damage caused by free radicals induced oxidative stress [3, 4], as oxidative stress is an important factor in cell damage and it has been implicated in the development of certain cancers [5]. Many studies have demonstrated the potential of plant products as antioxidants against various diseases induced by free radicals, It has been suggested that natural antioxidants are more safe and healthy than synthetic one, where it is functioning as free-radical scavengers and chain breakers, complexes of pro-oxidant metal ions and quenchers of singlet-oxygen formation. It has been determined that the antioxidant effect of plants is mainly attributed to phenolic compounds [1, 6, 7].

As it was mentioned previously, oxidation can lead to cancer disease which is the largest single cause of death in man kind. Recently, resistance to anticancer drugs has been observed. Therefore, the researchers carried on to develop more effective and less toxic drugs. It is well known that plants have been a useful source of clinically relevant anti-proliferation compounds, many substances derived from medicinal plants are known to be effective chemo-preventive and anti-proliferation agents. Indeed there have been worldwide efforts to discover new anticancer agents from plants. Many phytochemicals with various bioactivities, including antioxidant and anticancer activities were isolated from plants. Therefore, many plants have been examined to identify the new and effective antioxidant and anticancer compounds [4, 8, 9].

Calophyllum rubiginosum belongs to Guttiferae family which distributed in the warm humid tropics of the world. Wide phytochemical studies have reported that Calophyllum genus rich in xanthones, coumarins, biflavonoids, chalcones, benzofurans, and triterpenes [10, 11]. Some of these species are commonly employed in folk medicine, however it is used to treat bronchitis, hepatic disturbances, inflammation, [10], preventing wound infections [12, 13]. studies have reported that triterpenoids, coumarins which have isolated from C. inophyllum, C. dispar and C. brasiliens, have shown a cytotoxicity effect against human leukemia HL-60, Nasopharynx carcinoma KB and K562 (lymphoma), U251 (central nervous system), and PC3 (prostata) cell lines, respectively [14, 15]. There is no enough data relative to biological activity of the *C. rubiginosum* species had reported. Therefore, the investigation carried on by using in vitro model of relevance. DPPH radical scavenging, reducing power and iron chelating activity carried out to examine the antioxidant activity. The MTT assay was curried out to evaluate the cytotoxicity.

Materials and Methods.

Chemicals

All solvents used were of analytical grade. n-hexane, dichloromethane (DCM) and methanol, also silica gel 60 (230-400 mesh), sodium acetate, and aluminium chloride was obtained from MERCK. Trichloroacetic acids, potassium hexacyano ferrate, potassium phosphate, ferrous chloride, ferric chloride, ascorbic acid, BHT (butylated hydroxytoluene) were purchased from R & M chemicals.1,1-Diphenyl-2-picrylhydrazyl (DPPH), and 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-p, p'-disulfonic acid, 3 -(4, 5-dimethylthiazol-2-yl) -2, 5 -diphenyltetrazolium bromide (MTT); Quercetin were purchased from Sigma-Aldrich Chemical.

Plant material

The stembark of *C. rubiginosum* was collected from Malaca botanical garden, Malaysia in June, 2009. Plant species was identified by Dr Shamsul Khamis, botanist from Universiti Putra Malaysia.

Extraction and fractionation.

Air-dried and powdered stembark (1kg) was macerated in (2.5 L) 98% Ethanol for 72 h. The EtOH extract was filtrated and evaporated under reduce pressure to yield the dark brown gummy (200g) EtOH crude. A part of EtOH crude (100 g) was chromatographed by VLC (silica gel 230-400 mesh,1:30 ratio) to obtain three main fractions n-hexane, DCM and MeOH.

Antioxidant activity.

DPPH assay.

The scavenging effect of *C. rubiginosum* fractions was assessed using the method, as it was described by R Amarowicz et al, 2010, M Taher et al, 2010 [21, 22] with slight modifications. A 100 µL of methanolic solution containing between 0.031-1 mg of the fractions was mixed with methanolic solution of DPPH (1 mM, 200 µL) in a 96-microwells plate. The content was mixed and left in a dark area at room temperature for 20 min, and then the absorbance of the mixture was measured at 517 nm by using multi-detection microplate reader (INFINITE M 200

Nanoquant). The methanol was used as a control. The blank was 100 µL of fraction with 200 µL of methanol. Radical scavenging activity was expressed as the inhibition percentage and was calculated using the Eq.1:

[% Radical scavenging =((Control OD-Sample OD)/Control OD)*100] **(1)**

The required fraction's concentration (µg/mL) for scavenging of 50% of DPPH radical (IC_{50}) was determined. All measurements were carried out in triplicate.

Reducing power assay.

The reducing power of *C. rubiginosum* fractions and two standards ascorbic acid and BHT was determined according to the M Ozturk et al, 2007 [19] method with slight modification. A 100 µL of three different concentrations 1, 5 and 10 mg/mL of each fraction were mixed with 50 µL of 0.2 M phosphate buffer (pH 6.6) and 250 µL of potassium ferricyanide (1%). After the mixture was incubated at 50 °C for 20 min, 250 µL of (10%) trichloroacetic acid were added and the mixture was centrifuged at 1000 g for 10 min. 250 µL supernatant was moved to 48-microwells plate and mixed with 250 µL distilled water and 50 µL of ferric chloride (0.1%). The mixture was measured at 700 nm by using multi-detection micro-plate reader (INFINITE M 200 Nanoquant). All the measurements were carried out in triplicates, and higher absorbance of the reaction mixture indicates greater reducing power.

Iron (II) chelating activity.

The chelating of iron (II) ions by different fractions' concentrations was carried out by the method described by I Hinneburg et al, 2006 [16]. 100 µL of four different concentrations 1, 2.5, 5, and 10 mg/mL of each fraction, which dissolved in methanol, was mixed with 10 µL of (2.0 mM) aqueous $FeCl_2$. After 5 min incubation at room temperature, the reaction was initiated by 20 µL of (5.0 mM) ferrozine. After 10 min the absorbance was measured at 562 nm by using the multi-detection micro-plate reader (INFINITE M 200 Nanoquant). The controls contained all the reaction reagents except the samples which were replaced by methanol. The iron chelating activities were calculated from the Eq. (2).

[% Chelating = $((A_c - A_s)/A_c)*100$] **(2)**

Where (Ac) is represented the absorbance for control, and (As) is represented the absorbance for sample. The values were presented as the means of triplicate analyses.

The cytotoxicity assay (MTT assay)

Cytotoxic effect of C.rubiginosum fractions against A549 cells was investigated by using 3-(4, 5- dimethylthiazol- 2- yl)- 2, 5- diphenyltetrazolium bromide (MTT) assay described by D Susanti et al, 2007 [23]. For this purpose, A549 cells were cultured in a completed media in a T- flask until the cells were confluent. Then, the cells were seeded in a 96-microwells plat at a density of 0.5 x 10^5 cells/ well and incubated at 37 °C in 5% CO_2 humidified incubator. After 24 hours, a fresh media was added and the cells were treated with different concentrations of samples obtained by double fold serial dilution. The (95% ethanol) was used as a control. After 24 hours incubation, the supernatants were discarded, and the adherent cells were washed twice with phosphate buffer saline (PBS). 20 µL of (5 mg/mL) MTT stock solution was added to each well and the plate was further incubated overnight at 37 °C. DMSO (100 µL) was added to each well to solubilise the water-insoluble purple formazan crystals produced by viable cells. After complete dissolving of formazan blue, 100 µl of the solution was transferred to a new 96-microwells plate and the absorbance was measured at 570 and 690 nm, as reference wavelength, using a multi-detection microplate reader (INFINITE M 200 Nanoquant). All samples were assayed in triplicate. The percentage of cell viability was calculated and the concentrations required for inhibition of 50% of cell viability (IC_{50}) were determined according to Eq. 3.

[% of cell viability

=(OD of treated cells/OD of control cells)×100] (3)

Results.

Antioxidant activity

DPPH assay

The result display in Table 1 shows the IC_{50} of free radical scavenging activity, which was carried out by using the DPPH assay. The MeOH fraction shows a lowest IC_{50} 15.62 ± 3.135 µg/mL value as an antioxidant, while n-hexane and DCM fractions show a moderate activity. The MeOH value is close to Quercetin standard.

Table (1) IC_{50} free radical scavenging activity DPPH assay.

Fractions	IC_{50} (µg/mL)
n-hexane	49.66 ± 3.786
DCM	71.33 ± 3.055
MeOH	15.62 ± 3.135
Quercetin	10.23 ± 2.403

Reducing power

The reducing power agents are concentrating in the MeOH fraction, where it is achieving a result closer to BHT the moderate reducing power agent. Fig.1 depicts the reducing power value of MeOH fraction and other substances. The n-hexane and DCM fractions show low reducing power activity, where they are less than BHT. The higher reducing power agent is ascorbic acid as it illustrates in the figure that MeOH fraction very close to ascorbic acid.

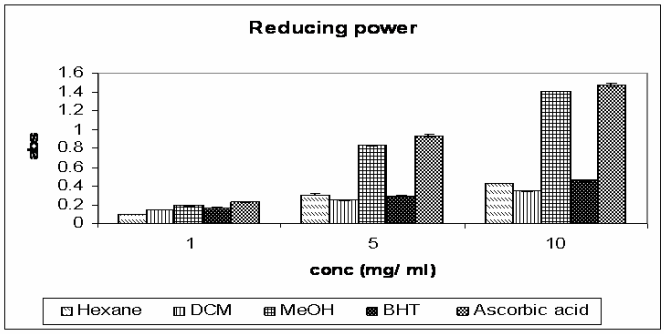

Figure 1 The Reducing power activity of three C.rubiginosum fractions (N-hexane, DCM, MeOH) standard BHT and Ascorbic acid at 700nm.

Iron (II) chelating activity

The chelation of ferrous ions by the C .rubiginosum fractions and standards were examined by (I Hinneburg et al, 2006) method. Fig. 2 shows two standard Quercetin moderate and EDTA high chelating agent. All fractions were comparing with standards; the n-hexane and DCM show activity less than activity of Quercetin at all concentrations, while the MeOH fraction shows activity quite higher than activity of Quercetin, it chelates the iron at concentration ≥ 2.5 mg/mL, comparing with Quercetin and MeOH fraction activity the MeOH fraction was a stronger. The EDTA chelating agent shows the highest activity.

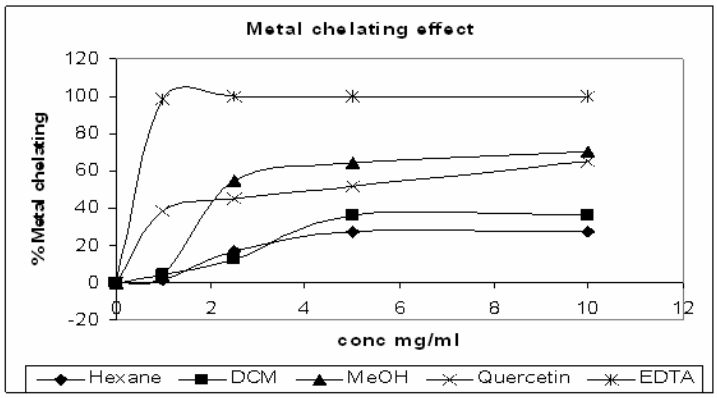

Figure 2. The % of Iron chelating activity for three C.rubiginosum fractions at four concentrations (0, 1, 2.5, 5, 10 mg/mL).

Cytotoxicity (MTT assay)

The cytotoxicity activity of the C .rubiginosum fractions was investigated using MTT assay on human lung cancer A549 cell line. Fig.3 shows the % of viability where it display the activity of n-hexane, DCM and MeOH fractions. The n-hexane and DCM fractions show a good activity, where it is inhibiting the cell growth at low concentration. But the n-hexane fraction shows a strong activity. The MeOH fraction shows no activity where the growth is almost 100%.

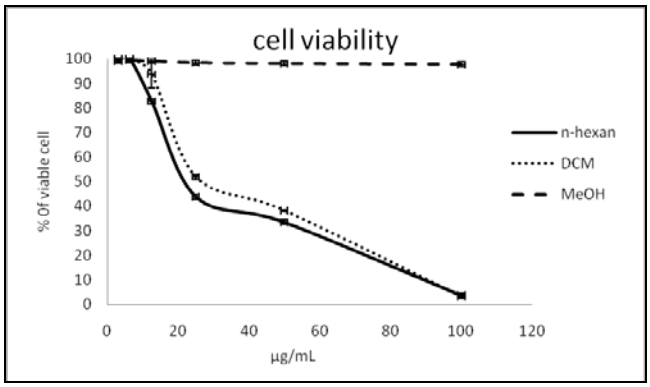

Figure 3. The % of viability activity for *C. rubiginosum* fractions against A549 cell line.

Table 2 shows the IC_{50} values for n-hexane, DCM, MeOH fractions. The IC_{50} of n-hexane and DCM are 24.166 and 26.5 µg/mL respectively. The % of viable cell for n-hexane and DCM fractions at recommended concentration 30 µg /mL is 35 and 39.8 % respectively

Table (2) The IC_{50} and % of A549 cell viability of *C. rubiginosum* fractions.

Fractions	IC_{50} µg/mL	% of viability*
n-hexane	24.166 ± 2.25	35 ± 5.0
DCM	26.5 ± 0.1	39.8 ± 0.1
MeOH	N.d	N.d
N.d: Not determined		
*:% of viability at 30 µg/mL		

Discussion

Cellular mechanisms and external factors involved in the production of oxidative stress, which is possibly leading to the permanent cellular damage [8]. The role of oxidative developments in disease is a subject of intense research interest. Such developments have been concerned in, among other ailments, cardiovascular diseases, some forms of cancer and inflammatory diseases [17]. The free radical chain reaction is widely accepted as a common mechanism of lipid peroxidation. Radical scavengers may directly react with and quench peroxide radicals to terminate the peroxidation chain reaction and improve the quality and stability of food products [18], the excess of free radicals are known to be a major factor in biological damages [4]. The diphenylpicryhydrazyl (DPPH) radical, a commonly used to evaluate the free radical-scavenging activity of natural antioxidants, which are ease, convenience and validated against several other assays for antioxidant activity [4, 17, 18]. DPPH is a stable free radical, can react with antioxidant agent to convert colour from purple to yellow when reduce electron or hydrogen radical [3, 4]. A dose response relationship is found in DPPH scavenging activity of MeOH fraction and increases in concentration are synonymous of an increase in scavenging capacity [7]. Many studies have demonstrated that reducing power in plant extracts was significant indictor and highly correlated with their antioxidant activities [6, 18].The reducing properties are generally associated with the presence of reductones (i.e. antioxidants) in the herbal extracts, causes the reduction of the Fe^{3+}/ferricyanide complex to the ferrous form, the yellow colour of the test solution changes to various shades of green and blue [1, 6, 7]. Among the transition metals, metal chelating activity was significant since it reduced the concentration of the catalyzing transition metal in lipid peroxidation, iron is known as lipid oxidation pro-oxidant. The ferrous state of iron accelerates lipid oxidation by breaking down hydrogen and lipid peroxides to reactive free radicals. It has been reported that chelating agents, which form σ bonds with a metal are effective as secondary antioxidants because they reduce the redox potential, thereby stabilizing the oxidized form of the metal ion [18, 19]. The MeOH fraction showed a significant results in all anti-oxidant tests, where it was able to scavenge the DPPH, reduce the Fe^{3+} to Fe^{2+} form and it chelat the Fe^{2+} ions. The results provide us information about MeOH fraction, which can be worked as a natural antioxidant. In general the antioxidant compounds should be rich in hydroxyl groups as phenolic compounds. These finding are consistent with the logic, because MeOH able to extract the phenolic compounds. However, the other fraction showed low activity

as antioxidant. A mitochondrial enzyme in living cells, succinate dehydrogenase, cleaves the tetrazolium ring and converts the MTT to an insoluble purple formazan and the amount of formazan produced is directly proportional to the number of viable cells [3, 4]. Therefore, Cytotoxicity measured by MTT assay to evaluate the viable cells after treated. The American National Cancer Institute (NCI) considered crude extract materials cytotoxic if they reach the IC_{50} value less than 30 µg/mL in preliminary assay [20]. In this study the finding that n-hexane and DCM fractions had IC_{50} 24.16 and 26.5 µg/mL respectively. Regarding to NCI we can consider these fractions are anti-proliferating agent.

Conclusion

In a conclusion this plant has the biological effectiveness, and as the results showed it is possible to isolate anti-oxidant and anti-proliferation compounds. The results provided beneficial information can help to search for alternative drugs to be used in pharmacotherapy, and will contribute to establish safe and effective use of phytomedicines in the treatment of diseases.

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