ANTIOXIDANT, ANTIDIABETIC AND CYTOTOXIC ACTIVITIES OF *RENNELLIA ELLIPTICA* KORTH.

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Abstract

Rennellia elliptica is a small tropical shrub that commonly known as 'segemuk'. The root decoction is traditionally used for health vitality. We have previously isolated *Rubia*-type anthraquinones from the root extract of *Rennellia elliptica*. The root extract is a potent antiplasmodial and some of the anthraquinones were strong inhibitors with the IC_{50} values of less than 2 µM. In continuation of our investigation on the pharmacological potential of *R. elliptica*, the root extracts and the anthraquinones were screened for antioxidant, antidiabetic and cytotoxic activities. Dichloromethane extract is a strong lipid peroxidation inhibitor but showed no activity against 3T3, MCF7 and 4T1 cancer cell lines and a-glucosidase. The anthraquinones showed weak radical scavenging activities against DPPH radicals. 2-Formyl-3-hydroxy-9,10-anthraquinones, damnacanthal and 2-methyl-3-hydroxy-9,10-anthraquinones showed moderate activity when screened for cytotoxicity using 3T3 cell lines at 30 µg/ml with 74.15, 67.34 and 50.40 %, respectively. When screened using 4T1 and MCF7 cell lines, only 2-formyl-3-hydroxy-9,10-anthraquinone showed moderate cytotoxicity after 72 hours of incubation. 1,2-Dimethoxy-6-methyl-9,10-anthraquinones and damnacanthal showed weak a-glucosidase inhibitory activity with 21.3 and 19.9 %, respectively, when screened at 10 µg/ml.

Keywords: *Rennellia elliptica*, anthraquinones, antioxidant, antidiabetic, cytotoxic

Abstrak

Rennellia elliptica adalah pokok renek tropika yang dikenali sebagai segemuk. Air rebusan akar digunakan di dalam perubatan tradisional untuk kesihatan badan. Kami telah melaporkan pemencilan antrakuinon jenis *Rubia* dari ekstrak akar *Rennellia elliptica*. Ekstrak akar berpotensi sebagai antiplasmodial dan beberapa antrakuinon merupakan agen antimalaria yang kuat dengan nilai IC_{50} lebih rendah daripada 2 µM. Dalam meneruskan penyiasatan terhadap potensi famakologi *R. elliptica*, ekstrak akar dan antrakuinon disaring untuk aktiviti antioksidan, antidiabetik dan sitotoksik. Ekstrak diklorometana merupakan agen penghalang pengoksidaan lipid yang berpotensi tetapi ekstrak ini tidak menunjukan aktiviti sitotoksik terhadap sel kanser 3T3, MCF7 dan 4T1 serta a-glukosidase. Antrakuinon menunjukkan aktiviti memerangkap radikal terhadap radikal DPPH yang lemah. 2-Formil-3-hidroksi-9,10-antrakuinon, damnakantal dan 2-metil-3-hidroksi-9,10-antrakuinon menunjukkan antiviti yang sederhana apabila disaring untuk aktiviti sitotoksik menggunakan sel 3T3 pada kepekatan at 30 µg/ml masing-masing dengan hambatan pada 74.15 %, 67.34 % dan 50.40

%. Apabila disaring menggunakan sel 4T1 dan MCF7, cuma 2-formil-3-hidroksi-9,10-antrakuinon menunjukkan aktiviti sitotoksik yang sederhana selepas proses inkubasi selama 72 jam. 1,2-Dimetoksi-6-metil-9,10-antrakuinon and damnakantal menunjukkan aktiviti yang lemah terhadap enzim a-glukosidase dengan peratus hambatan 21.3 % dan 19.9 % bila disaring pada kepekatan 10 µg/ml.

Kata kunci: *Rennellia elliptica*, antrakuinon, antioksidan, antidiabetik, sitotoksik

1.0 INTRODUCTION

Oxidation is an essential process in all living organisms. Oxidation and reduction reactions which involve radical species represent the basis for numerous biochemical mechanisms. The balance between antioxidation and oxidation is important in maintaining a healthy biological system. Protective defense enzymes, superoxide dismutase, catalase and peroxidase maintain these processes by converting radical oxygen species to harmless species [1]. The imbalance in redox processes due to overproduction of reactive oxygen species can overwhelm protective enzymes and subsequently leads to oxidative stress.

Free radicals can be defined as molecules or molecular fragments containing one or more unpaired electrons. Reactive oxygen species (ROS) can be produced from endogenous source such as mitochondria and exogenous source such as non-genotoxic carcinogens. It has been estimated that one human cell is exposed to approximately 1.5×10^5 oxidative hits a day from hydroxyl radicals and other reactive species [2]. The burden of oxidation defense lies on nonenzymatic antioxidants such as vitamins C and E and many other natural source antioxidants.

Free radicals can be beneficial at optimum concentration. They play physiological roles in cellular response to noxia, for example in defense against infectious diseases and in the function of a number of cellular signalling systems. In contrast, at high concentration, ROS cause damage to cell structures including lipids membranes, proteins and nucleic acids [2]. ROS are known not only to attach DNA but additional cellular components such as proteins and lipids, leaving behind reactive species that can couple with DNA. The radicals may form adducts with DNA bases or the deoxyribosyl backbone of DNA to produce damaged bases and breaks as well as initiate lipid peroxidation [3]. In stress conditions, these species can initiate deleterious effects on biomolecules and therefore cellular damage. Permanent modification of genetic material resulting in oxidative damage incidents represents the first step involved in mutagenesis, carcinogenesis and various disorders such as Alzheimer, Hungtinton's disease, diabetes, and Parkinson [4, 5].

Rennellia elliptica is a local tropical shrub that can be found in the lowlands of the tropical rainforests. The decoctions of the root are taken by the locals for health vitality. The plant is popularly dubbed as Malaysian ginseng probably due to the appearance of its yellow root as well as its many medicinal uses [6]. We have previously isolated *Rubia*-type anthraquinones from the root extract of *R. elliptica*. The root extract is a potent antiplasmodial and some of the anthraquinones were strong inhibitors with the IC_{50} values of less than 2 µM [7]. 2-Formyl-3-hydroxy-9,10-anthraquinones **1**, nordamna-canthal **2** and damnacanthal **3** were isolated as major compounds and these compounds are widely reported as anticancer [8, 9]. In continuation of our investigation on the pharmacological potential of *R. elliptica*, the root extracts and the anthraquinones were screened for antioxidant, antidiabetic and cytotoxic activities. This paper reports the antioxidant, antidiabetic and cytotoxic activities of root extracts of *R. elliptica* and its anthraquinones.

2.0 METHODOLOGY

2.1 Plant Material

The roots of *Rennellia elliptica* Korth. were collected from Endau Rompin State Park, Pahang, Malaysia at an altitude of 165 m above sea level and were identified by Dr Shamsul Khamis of Universiti Putra Malaysia. The voucher specimen (SK1512/08) was deposited at Herbarium of the Institute of Bioscience, Universiti Putra Malaysia and Universiti Teknologi MARA. The roots were air dried, cut into small pieces and ground to powder of about 1 mm mesh size using a grinder.

2.2 Extraction and Isolation

The air dried ground root (1 kg) was extracted successively with hexane, dichloromethane and methanol for 72 hours. The combined filtrates were evaporated to dryness to give 27 g of brown coloured crude dichloromethane extract and 3 g methanol extract. The purification of pure anthraquinones was accomplished using multiple column chromatography and preparative thin layer chromatography as described previously [7].

2.3 Biological Assays

2.3.1 Ferric thiocyanate (FTC) Antioxidant Assay

The primary lipid peroxidation inhibition of the root extract was assessed according to the method described by Ismail *et al.* with slight modification [10]. Sample (2 mg) in 4 ml 99.5 % ethanol, 4.1 ml of 2.51 % of linoleic acid in 99.5 % ethanol, 8 ml of 0.05 M (pH 7) of phosphate buffer and 3.9 ml of water were placed in a screw cap vial (ø=38 mm, h=75mm) and mixed thoroughly. The reaction mixture was incubated at 40°C in the dark. To 0.1 ml of this solution, 9.7 ml of 75 % ethanol, 0.1 ml of 30 % ammonium thiocyanate were added and precisely 3 minutes after the addition of 0.1 ml of 20 mM ferrous chloride in 3.5 % hydrochloric acid to the reaction mixture, the amount of peroxide was determined by reading the absorbance at 500 nm each. The absorbance was taken at 24 hour intervals until the absorbance of the control reached a maximum. Ethanol (99.5 %) and butylated-hydroxytoluene, vitamin E and quercetin were used as negative and positive controls.

2.3.2 Thiobarbituric Acid (TBA) Antioxidant Assay

The quantification of malondialdehyde as secondary lipid peroxidation product was carried out using method described by Kikuzaki and Nakatani (1993) with some modification [11]. Reaction mixture (1 ml) from the FTC method was mixed with 2 ml of 20 % trichloroacetic acid and 2 ml of 20 % thiobarbituric acid solutions. The reaction mixture was heated in boiling water at 100°C for 10 minutes. After cooling to room temperature, the solution was centrifuged at 3000 rpm for 20 minutes. The absorbance of the supernatant was measured at 532 nm. The antioxidant activity was determined based on the absorbance on the final day.

2.3.3 2,2-Diphenyl-1-picrylhydrazyl (DPPH) Method

The assay was performed in a 96-well microtitre plate. Methanol (180 µl) was added to each well. Sample stock solution (20 µl, 1 mg sample in 0.8 ml MeOH) was added to the well. A series of dilutions was carried out to give final concentrations of 100, 10, 1, 0.1 and 0.01 µg/ml. The last row was filled with 160 µl methanol as control. The solution mixture (40 µl) was discarded from each well. DPPH solution (5 µl, 600µg/ml) was added to each well and mixed thoroughly. This will give a DPPH final concentration of 317 µM. The plate was incubated in the dark for 30 minutes and the absorbance was measured using an Elisa UV reader at 517 nm. The IC_{50} value was expressed as the concentration of each sample required to give a 50 % inhibition shown by the control. All tests and analyses were run in triplicates and an average reading was calculated.

2.3.4 a-Glucosidase Inhibitory Activity

The a-glucosidase enzyme inhibitory activity assay was adopted from Lee *et al.* with slight modifications [12]. Stock solutions of plant extracts were prepared in DMSO at concentration of 100 µg/ml. The sample solution (10 µl), a-glucosidase enzyme, type 1 from Baker's yeast (20 µl), phosphate buffer saline (40 µl, pH 6.5) and deionised water (20 µl) were mixed in 96-well plate. The mixture was incubated at 37 °C. After 10 min, 10 µl of 20 mM p-nitrophenyl-a-*D*-glucopyranoside solution were added into the mixture to give a final concentration of 10.0 µg/ml plant extract. The absorbance of the reaction mixture was measured immediately using spectrophotometer (λ = 405 nm). The reaction mixture was incubated at 37 °C. After 30 min, the absorbance was measured again. The percentage inhibition was calculated by dividing the difference of absorbance between control and sample with the absorbance of control.

2.3.5 Cytotoxicity against 3T3 Cell Line

The cells were seeded at a density of 5 x 10^4 cells/ml (100 µl/well) in Dulbecco's Modified Eagle Medium, supplemented with 5 % of foetal bovine serum (FBS), 100 1U/ml of penicillin and 100 µg/ml of streptomycin and kept at 37°C in 5 % CO_2 incubator. Next day, the medium was removed and 200 µl of fresh medium was added with different concentrations of compounds. After 48 hours incubation, 200 µl of MTT (0.5 mg/ml) was added to each well and incubated further for four hours at same conditions. Subsequently, 100 µl of DMSO was added to each well. Finally, the extent of MTT reduction to formazan within the cells was calculated by measuring the absorbance at 540 nm using a micro plate reader.

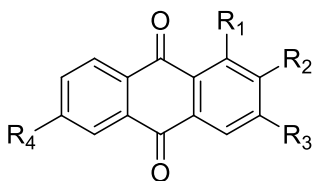#### 2.3.6 Cytotoxicity against 4T1 and MCF7 Cell Lines

The extract and compounds were dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich) and stored at -20˚C as a 10-mg/ml stock solution. The cytotoxocity assay was carried out as prescribed previously [13].

3.0 RESULTS AND DISCUSSION

We have previously reported the isolation of eleven anthraquinones from the root extract. The root extract is a potent antiplasmodial and many of the anthraquinones were strong inhibitors [7]. Anthraquinones are known chromophore for anticancer. They act mainly *via* DNA intercalation [3] and induce lipid peroxidation *via* free radical chain reaction and consequently induce oxidative stress on cancerous cells [14]. Oxidative stress can cause permanent modification of genetic material [2] which represents the first step involved in

mutagenesis, carcinogenesis and various disorders such as Alzheimer, Hungtinton's disease, diabetes, and Parkinson [4, 5].

In the present study, the extracts and the isolated anthraquinones were screened for antioxidant, antidiabetic and cytotoxic activities. The chemical structures of anthraquinones screened for the bioactivities are illustrated in Figure 1. The root extracts were tested for lipid peroxidation inhibition activity using FTC and TBA methods. FTC measures the primary product of lipid peroxidation while TBA method quantifies malondialdehyde (MDA), the secondary product of lipid peroxidation that is commonly found as marker in oxidative stress related diseases [1]. The dichloromethane root extract of *R. elliptica* showed stronger antioxidant activity than quercetin in both FTC and TBA assays with 93.4 % and 90.6 %, respectively. The percent inhibitions of lipid peroxidation were calculated based on the final day of FTC assay when the absorbance of the control drops. The daily absorbance of FTC experiment is plotted in Figure 2. The major anthraquinones from *R. elliptica* such as nordamnacanthal **2**, damnacanthal **3**, rubiadin **4**, rubiadin-1-methyl ether **5** and lucidin-ω-methyl ether **6** were not tested in this study because their antioxidant activities were widely reported [10, 15].

Figure 1 Chemical Structures of Anthraquinones from *R. elliptica* screened for Antioxidant, Antidiabetic and Cytotoxic Activities

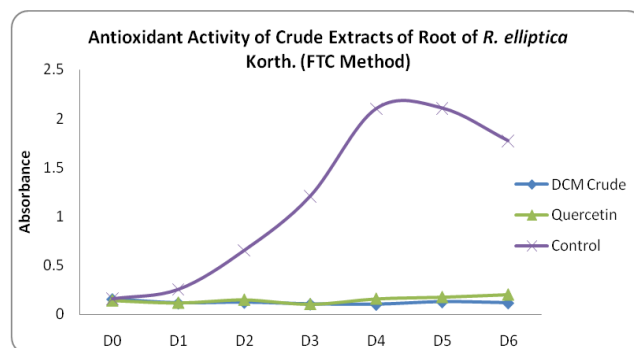|	R₁	R₂	R₃	R₄
1	H	CHO	OH	H
2	OH	CHO	OH	H
3	OCH₃	CHO	OH	H
4	OH	CH₃	OH	H
5	OCH₃	CH₃	OH	H
6	OH	CH₂OCH₃	OH	H
7	H	CH₂OH	OH	H
8	H	CH₃	OH	H
9	OH	OCH₃	H	CH₃
10	OCH₃	OCH₃	H	CH₃
11	H	OH	OCH₃	CH₃

Figure 2 Daily UV absorbance of *R. ellptica* extracts in FTC assay.

The radical scavenging assay was performed using DPPH radicals. The method was modified from reported literature [10, 16]. The absorbance values of samples were compared to quercetin as positive standard. The IC₅₀ values of quercetin (~ 10-20 μM) were comparable with those reported in literature at the same DPPH final concentration of 300 μM. [17,18]. DPPH radical was purple in colour and upon reduction *via* hydrogen acceptance; the purple colour is bleached to yellow and pale yellow [19]. However, DPPH assay is often affected by colour of sample solution which leads to underestimation of actual radical scavenging activity [20].

When tested for the radical scavenging activity against DPPH radicals, the methanol root extract of *R. elliptica* showed stronger activity than dichloromethane extract with IC₅₀ values of 39.0 μg/ml and 250 μg/ml, respectively. Based on these observations, the dichloromethane extract showed antioxidative role by inhibiting lipid peroxidation and has potential as a preventive antioxidant. The lipid peroxidation inhibition could be due to the presence of nordamnacanthal **2** [15] and damnacanthal **3** [10] as major compounds in the root extract. On the other hand, the methanol extract may play antioxidative role by competitive reaction in which antioxidant and substrate compete for radicals in biological system.

Several anthraquinones were screened for DPPH radical scavenging activity at 100 μg/ml (Table 1). All anthraquinones isolated from the root extract were generally weak radical scavengers against DPPH radicals. These observations were consistent with the DPPH radical scavenging data reported by several authors [10, 15]. Anthraquinones showed weak radical scavenging activity probably due to the stability of anthraquinones radicals which could not form uncharged ions with other radicals [21, 22].

When screened for a-glucosidase inhibitory activity at 10 μg/ml, the dichloromethane root extract did not show any activity. The anthraquinones showed weak activity and the moderate activity was shown by 1,2-dimethoxy-6-methyl-9,10-anthraquinone **10** and damnacanthal **3** with 21.3 % and 19.9 %, respectively (Table 1).

In our previous report, nordamnacanthal **2** was found to enhance cytotoxic effect of tamoxifen in treating human breast cancer MCF7 [13]. Damnacanthal **3** enhanced the expression of p21 and caspase-7 subsequently increased apoptosis in human breast cancer MCF7 cell [23]. In the present study, only three other major compounds screened for cytotoxic activities using MCF7 and 4T1 cell lines (Table 2).

The dichloromethane root extract did not show cytotoxic effect against the MCF7 and 4T1 cancer cell lines. The presence of known anticancer against MCF7 and 4T1 cell lines, nordamnacanthal **2** and damnacanthal **3** as major compounds in the root extract of *R. elliptica* does not contribute to its cytotoxicity. 2-Formyl-3-hydroxy-9,10 anthraquinone **1** and 2-methyl-3-hydroxy-9,10-anthraquinone **8** showed moderate cytotoxic activity against human breast cancer MCF7 cell line. When tested against 4T1 cancer cell, only 2-formyl-3-hydroxy-9,10-anthraquinone **1** showed moderate activity.

Table 1 DPPH Radical scavenging and antidiabetic activites of anthraquinones from *R. elliptica*

Compounds	DPPH Radical Scavenging Activity at 100 μg/ml Percent Inhibition (%)	a-Glucosidase Inhibitory Activity at 10 μg/ml Percent Inhibition (%)
1	3.26	6.6
2	2.87	9.4
3	9.18	19.9
4	4.87	3.6
5	nt	6.4
6	3.91	na
7	nt	na
8	3.99	6.8
9	nt	na
10	1.30	21.3
11	nt	na
Quercetin	15[a]	-

Each sample was tested in triplicate; The data was recorded as average percent inhibition at 100μg/ml and 10 μg/ml. nt - not tested; na-no activity. [a] unit in μM

The major compounds from *R. elliptica* were screened for cytotoxic activity against 3T3 cell line at 30 μg/ml. 2-Formyl-3-hydroxy-9,10-anthraquinones **1** and damnacanthal **3** showed moderate activity with percent inhibition of 74.15 % and 67.34%, respectively. Other compounds showed weak cytotoxicity. The cytotoxic activity of the selected anthraquinones was tabulated in Table 2.

There is no correlation observed between antioxidant and antidiabetic activities of anthraquinones from *R. elliptica*. Even though the extract is a good antioxidant, the result does not echo in antidiabetic assay. Nordamnacanthal **2** and damnacanthal **3** are widely reported as anticancer agents and antioxidants, however their abundance presence in the root extract of *R. elliptica* do not contribute to the activity of the extract. The activity of the extract could be a result of synergism between matrices of other components and not on the major components alone [24].

4.0 CONCLUSION

The root extract of *Rennellia elliptica* is a good antioxidant which could be due to the abundant presence of nordamnacanthal **2** and damnacanthal **3**. Nevertheless, the presence of these cytotoxic compounds does not contribute to the cytotoxicity of the root. The root of *Rennellia elliptica* did not show significant a-glucosidase activity.

Acknowledgement

Table 2 Cytotoxicity of *R. elliptica* using 3T3, 4T1 and MCF7 cell lines

Compounds	3T3 % Inhibition at 30 μg/ml	4T1 IC$_{50}$ (μg/ml) 24hrs	48 hrs	72hrs	MCF IC$_{50}$ (μg/ml) 24hrs	48hrs	72hrs
1	74.15	3.87	31.21	42.13	33.01	26.31	26.31
2	28.18		nt			nt	
3	67.34		nt			nt	
8	50.40	46.41	45.89	46.29	50.13	na	
10	42.48	50.34	na	44.44	38.70	na	
Dichloromethane extract	nt		na			na	

Each sample was tested in triplicate; na – no activity, nt – not tested

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