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The Measurement of Antioxidant Capacity of *Melicope glabra* by ORAC-$_{FL}$ and DPPH and its Chemical Constituents

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Abstract.. The hexane, ethyl acetate and methanol extracts from bark of *Melicope glabra* were evaluated for their antioxidant capacity by measuring DPPH free radical scaveging activity and their oxygen radical absorbance capacity (ORAC) . Both ethyl acetate and methanol extracts showed strong antioxidant capacity against DPPH and ORAC assay. In DPPH scaveging activity assay, the IC_{50} values of 24.81 µg/mL and 13.01 µg/mL were obtained for ethyl acatate and methanol, respectively. The ORAC values (µmolTE/kg) quantified for ethyl acetate and methanol extracts were 190160 and 272760, respectively. The IC_{50} value recorded for hexane extract was higher than 500 µg/mL, thus no further chemical and biological investigations were performed on hexane extract. The assay guided isolation of ethyl acetate and methanol extracts by various column chromatography techniques revealed that two known coumarins and one lignan present in both extracts. The structural identification by detail spectroscopic methods showed the structures as umbelliferone (**1**), sopoletin (**2**) and sesamin(**3**). These compounds were first time reported to be isolated from this species. All data were compared to the literature values published earlier.

Keywords: Dpph ; Melicope Glabra; Orac; Scopoletin; Sesamin; Umbelliferone

1. Introduction

Damaging of cellular molecules by free radicals and reactive oxygen species (ROS) have long been associated with the onset of many chronic diseases like diabetes [1], atherosclerosis [2] and cancer [3]. The study of plants as source of natural antioxidant compounds with free radical scavenging activity have received great interest of many researchers in the last few years. *Melicope glabra* of the family Rutaceae was phytochemically and biologically investigated for antioxidant activity. Previous studies on other species of *Melicope* showed they possess significant biological activity such as antimicrobes and antioxidant. In this paper the antioxidant potential of *Melicope glabra* bark extracts and the isolates were investigated.

2. Materials and Methods

2.1. Plant Material, Extraction and Isolation.

Melicope glabra bark was collected from Kedah in 2001 and given voucher specimen number 4563 and deposited in the Department of Biology, Universiti Malaya. The dried and ground *Melicope glabra* stem bark (1kg) were sequentially extracted with hexane, ethyl acetate and methanol. The extracts were concentrated by rotary evaporator under reduced pressure to give 39.75, 36.78 and 19.11g dark viscous semi solid respectively. The ethyl acetate and methnol extracts were subjected to flash column chromatography and eluted with n-hexane and ethyl acetate solvent mixture of increasing polarity. The fractions were further

chromatographed on silica gel column and eluting with various mixture of n-hexane and ethyl acetate to yield the following three compounds.

Umbelliferone **(1).** Colourless needles, m.p. 224-227°C (m.p. 223-225°C)[4]; EIMS *m/z* (rel. intensity): 162 $[M]^+$ (77), 134 (100),105 (24), 78 (30) ; UV λ_{max} (MeOH) nm (log ε): 339 (0.50), 294 (0.36), 242 (0.77); IR (KBr) υ_{max} cm^{-1}: 3165, 1702, 1613, 835; ^{1}H-NMR (400MHz, $CDCl_3$) δ: 6.16 (1H, *d*, *J* =10.0 Hz, H-3), 7.87 (1H, *d*, *J* =9.1 Hz, H-4), 7.50 (1H, *d*, *J* =9.1 Hz, H-5), 6.83 (1H, *dd*, *J* =2.7 Hz , 8.2 Hz, H-6), 6.74 (1H, *d*, *J* =2.7 Hz, H-8); ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 160.5 (C-2), 112.0 (C-3), 144.2 (C-4), 111.9 (C-4a), 129.7 (C-5), 113.2 (C-6), 161.6 (C-7), 102.5 (C-8), 156.2 (C-8a).

Scopoletin **(2).** White needle, m.p. 202-205°C (m.p. 203-204°C)[5]; EIMS *m/z* $[M]^+$ (rel. intensity): 192 (100), 177 (61), 149 (55), 69 (49); UV $\lambda_{max,}$ (MeOH) nm (log ε): 339 (0.50), 294 (0.36), 242 (0.77); IR (KBr) υ_{max} cm^{-1}: 3341, 1709, 1566, 1288; ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 6.26 (1H, *d*, *J* =10.3 Hz, H-3), 7.58 (^{1}H, *d*, *J* =9.1 Hz, H-4), 6.90 (^{1}H, *s*, H-5), 3.93 (3H, *s*, OMe, H-6), 6.82 (^{1}H, *s*, H-8); ^{13}C NMR data (100MHz, $CDCl_3$) δ: 161.5 (C-2), 113.3 (C-3), 143.3 (C-4), 143.8 (C-4a), 103.2 (C-5), 56.4, OMe (C-6), 149.8 (C-7), 107.4 (C-8), 150.2 (C-8a).

Sesamin **(3).** White needle-shaped crystal, m.p. 122-124 °C (m.p.121-125°C)[6]; EIMS *m/z* (rel. intensity): 354 $[M]^+$ (34), 149 (100); UV λ_{max} (MeOH) nm (log ε): 255 (3.91), 270 (3.95), 285 (3.99); IR (KBr) υ_{max} cm^{-1}: 1497, 1247, 1033, 923; ^{1}H-NMR (400MHz, $CDCl_3$) δ: 3.04 (2H, *m*, H-1/H-5,), 3.83(1H, *dd*, *J* =3.68 Hz, 9.16 Hz, H-4/H-8), 4.20 (1H, *dd*, *J* =6.4 Hz, 9.2 Hz, H-4/H-8), 4.68 (IH, *d*, *J* = 4.6 Hz, H-2/H-6), 5.96 (2H, *s*, OCH_2O), 6.90 (IH, *d*, *J* = 2.1 Hz, H-2'/H-2"), 6.80(1H, *d*, *J* = 8.3 Hz , H-5'/H-5"), 6.87 (1H, *dd*, *J* =1.8 Hz , 8.3 Hz, H-6'/H-6"), ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 54.8 (C- 1, C-5), 71.8 (C-4, C-8), 86.0 (C-2, C-6), 101.8 (OCH_2O), 136.8 (C-1',C-1"), 107.0 (C-2', C-2"), 147.8 (C-3',C-3"), 148.7 (C-4', C-4"), 119.36 (C-5', C-5"), 108.81 (C-6', C-6").

2.2. Determination of DPPH Free Radical Scaveging Activity

Each diluted samples (3µl) of hexane, ethyl acetate and methanol extracts were loaded onto a silica gel F_{254} Merck TLC plate (20cm x 20cm) and allowed to dry. The TLC then was developed with mobile phase of chloroform: acetone (4:1). To screen the antioxidant activity of the extracts, the plate was sprayed with 0.2% 1,1-diphenyl-2-pycrylhydrazyl (DPPH) in methanol, as an indicator. The presence of antioxidant compounds were detected by white spots against a purple background. As for the assay, a DPPH solution was prepared by dissolving 5 mg DPPH in methanol. The diluted sample of the extracts of various concentration (500 µg/mL-7.8 µg/mL) were prepared in 96-well plates in triplicates, followed by the addition of 5 µL of methanolic DPPH solution (final concentration of DPPH 300 µM). The optical density (OD) of the reaction mixture was read at 517 nm using microplate reader (Bio-Tek Instrumentation, USA) after 30 min . The percentage inhibition was calculated by the formula:

$$\% \text{ Inhibition} = [1\text{-OD(DPPH+sample)/OD(DPPH)}] \times 100.$$

The IC_{50} value was determined as the concentration of the sample needed to inhibit the free radical by 50%.

2.3. Determination of Oxgen Radical Absorbance Capacity (ORAC)

The FLUOstar OPTIMA microplate fluorescence reader (BMG LABTECH, Offenburg, Germany) was used with an excitation wavelength of 485 nm and an emission wavelength of 520 nm. 2,2'-Azobis(2-amidino-propane) dihydrochloride (AAPH), fluorescein, and 6-hydroxy-2,5,7,8-tetra-methylchroman-2-carboxylic acid (Trolox) were prepared in 75 mM phosphate buffer (pH 7.4). AAPH (153 mM) and Trolox (2 mM) were prepared fresh while fluorescein (10 nM) was prepared earlier and kept at 4°C in dark condition. Trolox standard was diluted in the phosphate buffer to give 1.5 µM to 50µM working solutions. To the 96-well plates (Nunc, Thermo Scientific) 150 µL of fluorescein was added followed by 25 µL of trolox, buffer(blank), or sample. AAPH (25 µL) was injected via injector. ORAC values were calculated based on net area under the curve (AUC) obtained by subtracting the AUC of the blank from that of a sample and compared to Trolox standards curve. The antioxidant capacity (ORAC) related to trolox is calculated as:

$$\text{ORAC value} = [(AUC_{sample}\text{-}AUC_{blank})/(AUC_{Trolox}\text{-}AUC_{blank})\ [\text{trolox}]\ \text{dilution factor}.$$

3. Results and discussion

3.1. DPPH free radical scaveging activity

This is the most widely used chemical assay method which involved the scavenging reaction of free radical by the antioxidant compounds [7]. The rapid and simple method for analysis of antioxidant activity by using the stable free radical 1,1-diphenyl-2-pycrylhydrazyl (DPPH) with the addition of scavenging compound. The addition of antioxidant results in discoloration of DPPH and decrease the absorbance proportional to the concentration and the activity of the antioxidant compound. From the dot blot DPPH staining, the ethyl acetate and methanol extracts produced white spots but hexane extract was inactive. The IC_{50} of the methanol extract displayed the strongest activity (13.01µg/ml). The ethyl acetate extract also showed good activity with IC_{50} of 24.81 µg/ml. However, hexane extract gave the weakest activity with IC_{50} higher than 500 µg/ml (Table 1)

3.2. Oxgen Radical Absorbance Capacity (ORAC)

The ORAC method measures the inhibition of the peroxyl-radical-induced oxidation initiated by thermal decomposition of azo-compounds such as [2,2'-azobis(2-amidino-propane) dihydrochloride (AAPH)] [8]. In principle, the ORAC-$_{FL}$ assay uses fluorescein as the probe and the AAPH as the radical generator [9,10,11]. Over time, the free radical generated from the thermal decomposition of AAPH, will quench the signal from the fluorescent probe fluorescein. The subsequent addition of an antioxidant produces a more stable fluorescence signal due to the inhibition of fluorescein decay by single antioxidant or/and complex mixture. Rate of decay of fluorescene measures the antioxidant's capacity. Figure 1 illustrates that both ethyl acetate and methanol extract were able to stabilize the fluorescein intensity. Hexane extract was not used due to its initial low activity. The two extracts inhibited the consumption of fluorescein with the presence of AAPH. The ORAC value calculated presence in methanol extract was 272,760 µmol of Trolox equiv/kg which is slightly higher than of the ethyl acetate extract, 190,160 µmol of Trolox equiv/kg as shown in Table 1. The graph in Figure 1 indicated the ability of the ethyl acetate and methanol extracts to inhibit the fluorosence decay compared to the Trolox as a standard. Green tea catechins belong to the flavonoid family and are known to have fairly strong antioxidant activity with ORAC values of 81,000-220,000 µmol of Trolox equiv/g [12]. Previous studies of ORAC on *Coleonema album*, also from Rutacaea family showed the ethanol and acetone extracts exhibited high ORAC value and good DPPH activity [13]

Table 1: Evaluation of antioxidant capacity of *Melicope glabra extacts* through the DPPH and ORAC assays.

Sample extract	DPPH(µg/mL) IC_{50}	ORAC µmolTE/kg
Hexane	>500	
Ethyl acetate	24.81	190,160
Methanol	13.01	272,760
Vit C	27.04	-

3.3. Structural Identification of Isolated Compounds

After repeated separation and purification procedures on ethyl acetate and methanol extracts, two known coumarins (umbelliferone, scopoletin) together with a lignan (sesamin) were obtained. The structure elucidation of the compouds were carried out by detail spectroscopic methods (UV, MS, IR, ^{1}H and ^{13}C NMR). All spectral data were compared to the literatures data [6, 14, 15]. Compound **1**, **2** and **3** were identified as umbelliferone, scopoletin, and sesamin. Coumarins are one of the large groups of phenolic compounds found in plants besides flavonoids. They have been reported to be widespread in the Rutaceae family. Coumarins and flavonoids are phenolic compounds that have been demonstrated to be good antioxidant due to the presence of phenolic hydroxyl group and possess ideal structural features for free radical scavenging activity [16,17, 18]. The antioxidant activity of the chemical constituents isolated from prunes (*Prunus domestica L.*) evaluated by ORAC assay indicated that the phenolic compounds including coumarins were responsible for the antioxidant activity of the prunes. The ORAC value of scopoletin (20µM) presence in the plant was previously reported as 2.72 unit/ µmol [19]

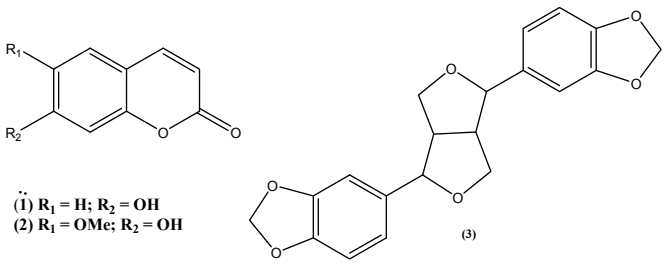

Fig. 1: Structures of isolated *Melicope glabra* compounds.

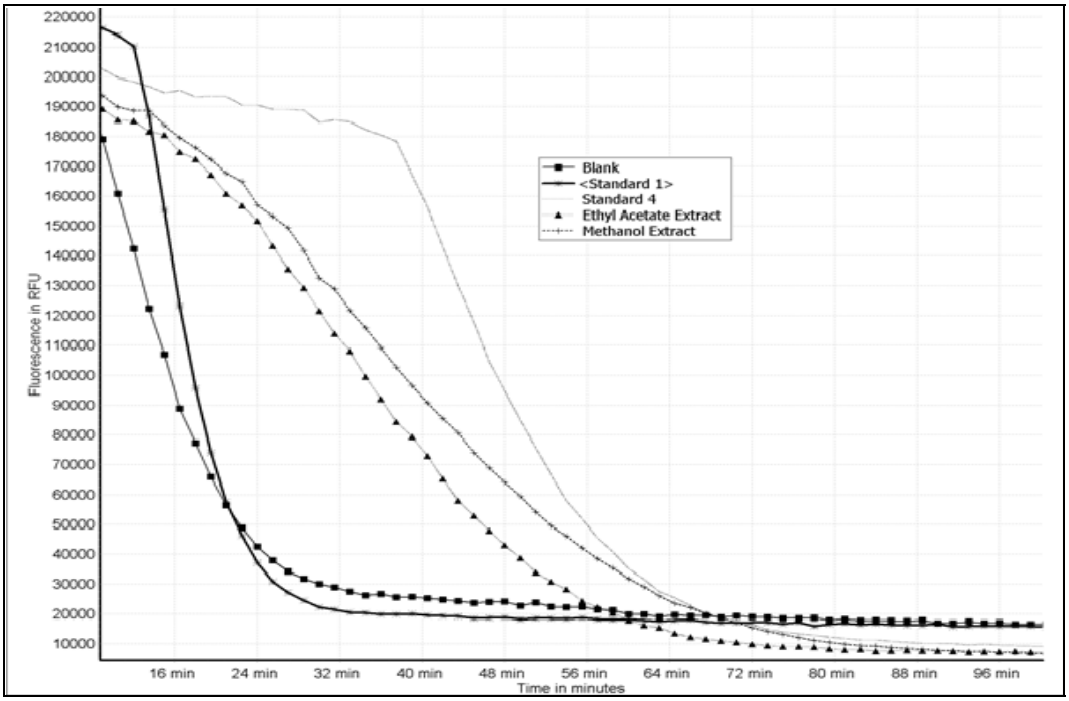

Fig. 2: Fluorescence decay curves of fluorescein induced by AAPH in the presence of phosphate buffer (blank), trolox (standards 1= 1.56 μM and standards 4=12.5 μM), and sample (ethyl acetate and methanol extracts)

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