

## ANTIOXIDANT AND ANTIMICROBIAL ACTIVITIES OF FERULIC ACID ESTERS FROM *Ochrosia oppositifolia*

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**ABSTRACT** Two ferulic acid esters namely (E)-methyl 3-(4'-hydroxy-3',5' dimethoxyphenyl) acrylate **1** and (E)-methyl 18-((E)-3-(4'-hydroxy-3'methoxyphenyl)acryloyloxy)octadec-3-enoate **2**, were isolated from *Ochrosia oppositifolia*. The compound **1**, and **2** and the crude methanol and hexane extracts of *Ochrosia oppositifolia* showed a moderate in vitro antioxidant and antimicrobial activity.

( **Keywords:** *Ochrosia oppositifolia*, Apocynaceae, Ferulic acid esters, antimicrobial and antioxidant)

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### INTRODUCTION

Malaysia is known with its green tropical vegetation and forest [1]. The Malaysians has been reported to practice traditional and herbal remedies as an alternative choice in treatment of diseases [2]. *Ochrosia oppositifolia* belongs to Apocynaceae family [3], plants of this genus find wide use in the traditional system of medicine [4, 5]. It is an evergreen tree that can vary greatly in size, growing to a maximum height of 15 m or more. This species produces five-petalled white flowers with yellow centers. The flowers usually drop to the ground like confetti. The fallen flowers provide a clue to find the tree. The fruit comes in pairs, is elliptical in shape, and is about 5-8 cm long and turns yellow when it is ripe. The seed is about 10-20 cm, apparently growing quite slowly [6].

### MATERIAL AND METHODS

#### General Experimental procedures

CC was run on silica gel 60 (40-63  $\mu$ m). TLC was performed on aluminum and glass plates pre-coated with silica gel 60 F<sub>254</sub> (Merck). <sup>1</sup>H NMR and <sup>13</sup>C NMR spectra were determined in

CDCl<sub>3</sub> (JEOL JNM-FX400), UV spectra were recorded on a Shimadzu UV-160A spectrophotometer using MeOH as solvent. MS was obtained with Agilent 6530.

#### Collecting of plant material

The plant materials (barks) of *Ochrosia oppositifolia* (KL 5349) were collected from Pangkor Islands in 2007. The botanical identification was made by Mr.Teo Leong Eng, Faculty of Science, University of Malaya. Voucher specimens are deposited in the Herbarium of Chemistry Department, University of Malaya.

#### Extraction and Isolation

The extraction of the plant (1.0 kg) was carried out by extracted exhaustively with hexane after moistened with 10% ammonia solution for 48 hours to remove waxes and fats. Then the extract was dried on the rotary evaporator. The plant material was dried and again wetted with 10 % ammonia solution and left for overnight. They were then re-extracted successively with dichloromethane (CH<sub>2</sub>Cl<sub>2</sub>) and methanol (MeOH). After removal of the solvents, the

hexane crude extract (1.2 g), dichloromethane (15 g) and methanol (15 g) were obtained.

#### Total Phenolic Content Evaluation

A protocol based on the Folin-Ciocalteu method described by Slinkard and Singleton [7] was employed to determine the total amount of present phenolic compounds in various crude extracts of *Ochrosia oppositifolia*. Methanol and hexane crude extracts at concentration of 10 mg/mL were added to 100 µL of 2N Folin-Ciocalteu reagent. Sodium carbonate solution (300 µL) at concentration of 0.2 mg/mL was added after the final volume of mixture was made up to 1600 µL using distilled water. The mixture was then incubated at 37 °C for 45 min before the absorbance of solutions was measured at 760 nm using a spectrophotometer. Lastly, the total phenolic contents were expressed as a gallic acid equivalent (GAE) based on Folin-Ciocalteu calibration curve using gallic acid as the standard.

#### Antioxidant activity

##### Radical Scavenging Capacity Assay

DPPH (2,2-diphenyl-1-picrylhydrazil) free radical scavenging capacity assay was obtained from the protocol described by Rafat [8]. DPPH (950 µL) in concentration of 90 µM was mixed with 50 µL of the plant extracts and pure compounds (10 mg/mL) and the volume was adjusted to 4 mL using 95% ethanol before incubation at room temperature in the dark for 120 min. Scavenging of DPPH reduced the colour of the solution and was measured using a spectrophotometer at 515 nm. Comparison of the reduction of the colour by examined samples with the blank (solution without plant crude extract) was used to measure the potential of scavenging capacity of our plant crude extracts using the following equation.

Radical Scavenging Capacity (%) = [(Blank – Sample A) / Blank] x 100

##### Superoxide dismutase (SOD) Determination Assay

SOD determination kit was used to determine the superoxide dismutase (SOD) activity of prepared crude extracts. The procedure was carried out based on the manual descript of the kit. Plant crude extracts and pure compounds (20 µL) of the concentration of 10 mg/mL were added to

200 µL of the kit Working Solution. The mixture after a gentle shaking incubated at 37 °C for 20 min after adding 20 µL the kit Enzyme Working Solution. The absorbance of the mixtures was measured at 450 nm using a microplate reader and the SOD activity was calculated using the following equation.

Percentage of Inhibition (SOD Activity) =  $\frac{[(\text{blank 1} - \text{blank 3}) - (\text{Sample A} - \text{Sample A's blank 2})]}{(\text{blank 1} - \text{blank 3})} \times 100$

Blank 1 was the mixture of Working Solution (200 µL) and Enzyme Working Solution (20 µL) containing 20 µL double distilled water (ddH<sub>2</sub>O). Blank 2 contained the plant extract (20 µL) with Working Solution (200 µL) and (20 µL) Dilution Buffer while ddH<sub>2</sub>O (20 µL) was replaced the plant crude extracts in the blank 3. Ascorbic acid (1 mg/mL) and 1 mg/mL of Tert-butylated hydroxytoluene (BHT) were employed as the positive controls in this study.

##### Antibacterial Activity Assay

The antibacterial potential of our samples was studied using the paper disc diffusion method of Kil [9]. Two Gram-Negative pathogenic bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*) and two Gram-Positive pathogenic bacteria (*Staphylococcus aureus* and *Bacillus cereus*) were obtained from Microbiology Division of Institute of Biological Sciences, University of Malaya and then grown in Nutrient Broth medium to yield a final concentration of 10<sup>7</sup> CFU/mL. The test bacteria (0.1 mL) were streaked on whole Mueller Hinton medium plates using sterile swab cotton. Sterilized filter paper discs were soaked in MeOH, DCM and Hexane crude extracts (100 mg/mL) as well as pure compounds (10 mg/mL) and were then placed in the centre of test bacteria plates. The plates were incubated for 24 h and diameter of the produced inhibition zone was measured. Tetracycline disc (30 µg) and PBS were used as the positive and negative controls respectively.

##### Statistical Analysis

All experiments were carried out in triplicate. One-way analysis of variance (ANOVA) was used to analyze the data using SPSS version 15. The means were compared by Duncan's Multiple Range Test (DMRT) and p<0.05 was considered to indicate statistical significance.

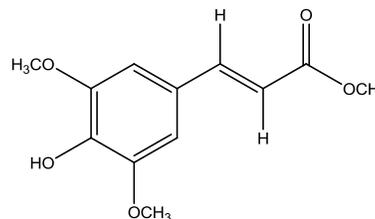
## RESULTS AND DISCUSSION

Repeated column chromatography of the MeOH extract of the bark of *Ochrosia oppositifolia* led to the isolation of two ferulic acid esters. The structure of compound **1**, (*E*)-methyl 3-(4'-hydroxy-3',5'-dimethoxyphenyl)acrylate and the compound **2**, (*E*)-methyl 18-((*E*)-3-(4'-hydroxy-3-methoxyphenyl)acryloyloxy)octadec-3-enoate were deduced from their spectral data and also by comparing their spectral data with those previously reported.

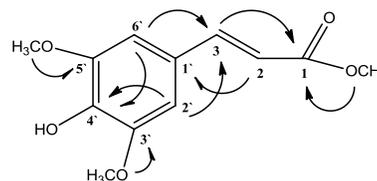
Compound **1** was isolated as a white amorphous solid. The mass spectrum revealed a molecular ion peak at  $m/z$  238.09  $[M]^+$  corresponding to the molecular formula of  $C_{12}H_{14}O_5$ . The UV spectrum revealed maximum at 196, 273 and 328 nm, indicating the presence of highly conjugated double bonds. The IR spectrum showed an absorption peak at  $3401\text{ cm}^{-1}$  which characterised for hydroxyl group. In addition, an absorption peak was observed at  $1704\text{ cm}^{-1}$  which indicated the presence of the conjugated carbonyl group.

The  $^1\text{H}$  NMR showed the presence of two  $\text{sp}^2$  methine groups resonated at  $\delta$  6.29 and 7.59 as a doublet with coupling constant 16 Hz which were attributable to methine protons at C-2 and C-3 of acrylate group, respectively. The two aromatic protons resonated at  $\delta$  6.77 and 6.77 as a siglet belongs to proton at C-2' and C-6' respectively. It also exhibited three singlet peaks with three protons at  $\delta$  3.92, 3.91 and 3.79 which were attributable to methoxyl groups at C-3', C-5' and C-1, respectively. The hydroxyl group was resonated at  $\delta$  5.80 as a broad singlet. In the  $^{13}\text{C}$  NMR spectrum, There are twelve carbon signals consist of three methoxyl groups, one carbonyl group, four  $\text{sp}^2$  methine carbons and four  $\text{sp}^2$  quaternary carbons. The carbonyl carbon resonated at  $\delta$  167.6 whereas two  $\text{sp}^2$  carbons C-2 and C-3 appeared at  $\delta$  115.6 and 145.2. The equivalent carbons, C-3' and C-5' bearing methoxyl group resonated at  $\delta$  147.2 Both methoxyl carbon at C-3' and C-5' appeared at  $\delta$  56.4 and the methoxyl ester group resonated at  $\delta$  51.7. The C-4' bearing hydroxyl group appeared at  $\delta$  137.1. The quaternary aromatic carbon resonated at  $\delta$  125.9 was attributable to C-1'. By comparing  $^1\text{H}$  NMR and  $^{13}\text{C}$  NMR spectral data

with those that have been previously reported, it is confirm that compound **1** was (*E*)-methyl 3-(4'-hydroxy-3',5'-dimethoxyphenyl)acrylate.



**1**

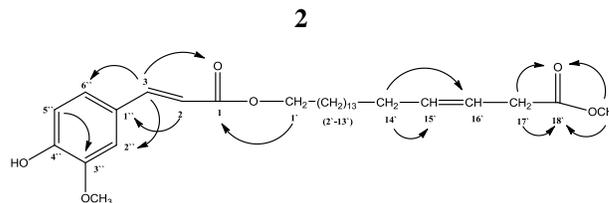


### HMBC correlation of compound 1

Compound **2** was isolated as a white amorphous solid. The mass spectrum revealed a molecular ion peak at  $m/z$  488.3  $[M]^+$  corresponding to the molecular formula  $C_{29}H_{44}O_6$ . The UV spectrum revealed maximum at 196, 273 and 328 nm and the IR spectrum showed an absorption band at  $3431\text{ cm}^{-1}$  indicating the presence of OH group. In addition, two peaks were observed at  $1711$  and  $1738\text{ cm}^{-1}$  which implied the presence of two carbonyl groups.

The  $^1\text{H}$  NMR spectrum indicated the presence of three aromatic protons at  $\delta$  7.10, 7.01 and 6.89 attached to C-6'', C-2'' and C-5'', respectively. There were two singlet peaks appeared at  $\delta$  3.91 and 3.64 attributed to two methoxyl groups attached to C-3'' and C-18' respectively. The cross peak of COSY showed one coupling set (H-3 to H-2). This was confirmed by the existence of two sets of doublet ( $J = 16\text{ Hz}$ ) in the  $^1\text{H}$  NMR at  $\delta$  7.50 and 6.27 which corresponded to the resonances of H-3 and H-2, respectively. The HMBC correlation between H-14' to C-15', C-16' established the position of double bond in the long chain. The  $^{13}\text{C}$

NMR and DEPT showed the presence of 29 carbon atoms in the molecule which consists of three quaternary aromatic carbons, three aromatic protons, four  $sp^2$  methines, fourteen methylenes, two methoxyl groups and two carbonyl esters. The two carbonyls appeared at  $\delta$  174.4 and 167.4 which were attributable to C-18' and C-1 respectively. The  $sp^2$  methines appeared at  $\delta$  6.27, 7.50, 5.30, 5.31 belongs to the proton at C-2,3,15' and 16', respectively. From the spectral data, it is confirmed that compound **2** was (E)-methyl 18-((E)-3-(4'-hydroxy-3'-methoxyphenyl)acryloyloxy octadec-3-enoate).



HMBC correlation of compound **2**

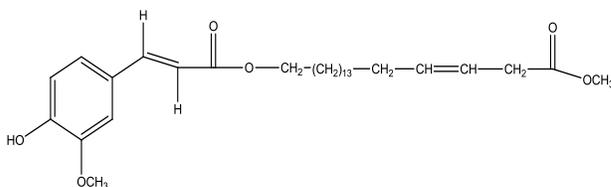


Table 1.  $^1\text{H}$  NMR [400 MHz,  $^1\text{H}$  ( $J$ , Hz)] and  $^{13}\text{C}$  NMR [100 MHz,  $\delta_{\text{C}}$ ] of (**1**) in  $\text{CDCl}_3$

Position	$^1\text{H}$ , $\delta_{\text{H}}$ , $J$ in Hz	$^{13}\text{C}$ , $\delta_{\text{C}}$
1	-	167.6
2	6.29,d,16	115.6
3	7.59,d, 16	145.2
1'	-	125.9
2'	6.77,s	105.1
3'	-	147.2
4'	-	137.1
5'	-	147.2
6'	6.77,s	105.1
1- $\text{OCH}_3$	3.79,s	51.7
3'- $\text{OCH}_3$	3.92,s	56.4
5' $\text{OCH}_3$	3.91,s	56.4
4'-OH	5.80,bs	-

Table 2. <sup>1</sup>H NMR [400 MHz, <sup>1</sup>H (J,Hz)] and <sup>13</sup>C NMR [ 100 MHz, δ<sub>C</sub>] of (2) in CDCl<sub>3</sub>

Position	<sup>1</sup> H, δ <sub>H</sub> , J in Hz	<sup>13</sup> C, δ <sub>C</sub>
1	-	167.4
2	6.27,d,16	115.7
3	7.50,d,16	144.7
1''	-	127.1
2''	7.01,s	109.3
3''	-	146.8
4''	-	147.9
5''	6.90,d,8.2	114.7
6''	7.10,d,8.2	123.1
1'	4.10,t,6.8	64.6
2'-13'	1.28-1.58	29.2-29.7
14'	2.1,d, 5.4	29.8
15'	5.3,m	129.9
16'	5.3,m	130.1
17'	2.3,m	34.02
18'	-	174.4
C18'-OCH <sub>3</sub>	3.64,s	51.5
C3''-OCH <sub>3</sub>	5.90,s	56.01
OH	5.90,bs	-

### Total Phenolic Content

Table 3. Total phenolic contents of extracts

Crude extract	Gallic Acid Equivalents (g/100g)
Methanol	5.08 ± 0.164 <sup>b</sup>
Hexane	16.09 ± 0.180 <sup>a</sup>
Dichloromethane	4.15 ± 0.163 <sup>c</sup>

Table 3 showed that the phenolic compounds are higher in the hexane extract compared to methanol and dichloromethane extracts.

### Antioxidant Assays

#### DPPH Radical Scavenging Activity Assay

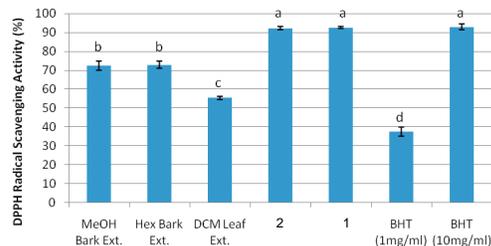


Figure 1. Antioxidant activity of compounds 1, 2 and crude extracts isolated from *Ochrosia oppositifolia*

**Figure 1** showed the antioxidant activity of crude extracts and isolated compounds measured using DPPH scavenging activity assay and it is presented as percentage value. BHT (1 and 10 mg/mL) were applied as the positive controls. The data were analyzed by one-way ANOVA and the DPPH scavenging activity percentage means of samples were compare by Duncan's Multiple Comparison Test (DMCT). Samples labeled with different letters are significantly different ( $p < 0.05$ ).

The results showed that the methanol and hexane extracts have equal activity when antioxidant assay was performed by DPPH Radical Scavenging method. The antioxidant activity of the crude extracts and compound **1** and **2** are slightly lower compared with positive control (10mg/mL).

#### SOD Assay

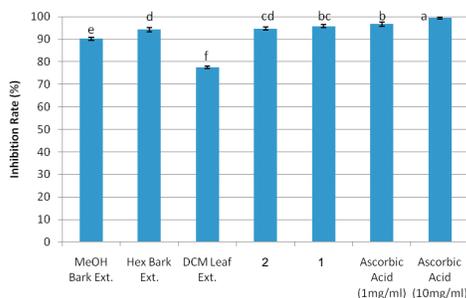


Figure 2. Antioxidant activity using SOD assay

**Figure 2** showed the antioxidant activity of crude extracts and isolated compounds measured using superoxide dismutase (SOD) assay and the results are presented as percentage of inhibition rate. Ascorbic acid (1 and 10 mg/mL) were used as the positive control. The data were analyzed by one-way ANOVA and the inhibition rate means of samples were compare by Duncan's Multiple Comparison Test (DMCT). Samples labeled with different letters are significantly different ( $p < 0.05$ ).

The results showed that higher activity has been observed in methanol and hexane extracts and compounds **1** and **2** have the same activity with ascorbic acid.

#### Antibacterial Activity Assay

**Table 4** showed the Inhibition effect of 100 mg/mL of plant extracts and 1.0 mg/mL of isolated compounds against the growth of four pathogenic

bacteria. The results indicated that there were no significant antibacterial activity shown by the crude extracts and isolated compounds.

Table 4. Inhibition effect of 100 mg/mL of plant extracts and 1mg/ml of isolated compounds against the growth of four pathogenic bacteria

Bacteria	Inhibition Zone (mm)					
	MeOH Bark Ext.	Hex Bark Ext.	DCM Leaf Ext.	Compound 2	Compound 1	Tetracycline (30 µg)
<i>Escherichia coli</i>	9	11	8	8	8	21 mm
<i>Staphylococcus aureus</i>	10	11	10	-	-	35 mm
<i>Pseudomonas aeruginosa</i>	-	-	-	8	8	18 mm
<i>Bacillus cereus</i>	10	10	-	-	-	22 mm

## CONCLUSION

Two ferulic acid esters have been isolated from the bark of *Ochrosia oppositifolia*. The crude extracts and the esters **1** and **2** have shown moderate antioxidant activity but there were no antibacterial activity was observed.

## ACKNOWLEDGEMENT

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