

Preliminary investigations of *in vitro* antioxidant, antityrosinase, acetylcholinesterase and anti-inflammatory activities of *Actinodaphne* species

Wan Mohd Nuzul Hakimi Wan SALLEH, Farediah AHMAD

ABSTRACT

The purpose of this study was to investigate the biological activities of extracts from two *Actinodaphne* species from Malaysia, which are *Actinodaphne macrophylla* and *Actinodaphne pruinosa*. The *in vitro* activity studies were assessed for their antioxidant, antityrosinase, acetylcholinesterase and anti-inflammatory activities. The methanolic bark extract of *A. pruinosa* have shown the highest activity on DPPH[•] (IC₅₀: 70.2 µg/mL), ABTS^{•+} (IC₅₀: 147.2 µg/mL) and phenolic content (55.1 mg gallic acid/g), while the bark extracts of *A. macrophylla* showed the

best inhibitory activity against mushroom tyrosinase (I: 45.2%) and lipoxygenase (I: 69.2%) assays. All extracts have shown strong AChE inhibitory activity (I: 70.5–88.1%), comparable to galantamine (I: 95.9%). The isolation of specific bioactive compounds as well as evaluating their safety may be necessary in the exploration of these species for potential new therapeutic drugs or drug leads.

Keywords: *Actinodaphne*, antioxidant, antityrosinase, acetylcholinesterase, anti-inflammatory

Wan Mohd Nuzul Hakimi Wan Salleh
Department of Chemistry, Faculty of Science, Universiti Teknologi Malaysia (UTM), 81310 Skudai, Johor, Malaysia.

Farediah Ahmad
Department of Chemistry, Faculty of Science, Universiti Teknologi Malaysia (UTM), 81310 Skudai, Johor, Malaysia.

Corresponding author:

Farediah Ahmad
E-mail: farediah@kimia.fs.utm.my
Tel: +6075534137
Fax: +6075566162

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INTRODUCTION

The genus *Actinodaphne* belongs to the family Lauraceae with about 100 species of evergreen trees and shrubs, occurs mainly in tropical-subtropical Asia and is an important component of tropical forests. They are widely distributed in Malaysia, Indonesia, Eastern Asia and few in North America (1). It is locally known as *wuru* (Indonesia) or *medang kuning* and *medang kunyit* (Malaysia) (2). Some species of the genus are used in traditional medicine for the treatment of several ailments. The decoction of leaves of *A. angustifolia* is used in kidney trouble due to stone. The leaves of *A. hookeri* are used in the treatment of diabetes, urinary disorders, while the bark of *A. obovata* is used to treat fractures (3). The root of *A. lancifolia* is a traditional Chinese medicine used for the treatment of stomachache, arthritis, overexertion, and edema (4). This genus have been reported to produce isoquinoline alkaloids (5), lactones (6), lignans (7), and phenolic amides (8). From the literature survey, *Actinodaphne* species have been reported to have antimicrobial (9), hepatoprotective (10), anti-inflammatory (11), antibacterial (12) and antioxidant (13) activities. Phytochemistry of *A. macrophylla* has been studied and resulted in the isolation of isoquinoline alkaloids and their antimalarial activities (14). In addition,

phytochemical study on *A. pruinosa* were successfully isolated aporphines alkaloids and their cytotoxicity activity (15).

Hence, in the present study, work has been carried out to determine the antioxidant, antityrosinase, acetylcholinesterase and anti-inflammatory activities of the *n*-hexane, ethyl acetate and methanol extracts of *A. macrophylla* and *A. pruinosa*. To the best of our knowledge, this is the first report describing these activities on the species.

MATERIALS AND METHODS

Plant materials

The leaves and bark of *A. macrophylla* (SK2956/16) and *A. pruinosa* (SK2957/16) were collected from Hutan Simpan

Bangi, Selangor in September 2015, and identified by Dr. Shamsul Khamis from Institute of Bioscience (IBS), Universiti Putra Malaysia (UPM). The voucher specimens were deposited at the Herbarium of IBS, UPM.

Extraction method

The dried and powdered leaves and bark of the above mentioned species (150 g each) were extracted firstly with *n*-hexane (hex), followed by ethyl acetate (EtOAc) and methanol (MeOH) (500 mL each). The extracts were filtered and the solvent was removed under vacuum using a rotary evaporator (Eyela, Germany) to obtain the Hex, EtOAc and MeOH extracts. Percentage yields (w/w) of all plant extracts obtained are shown in Table 1.

Table 1. Percentage yield and antioxidant activities of two *Actinodaphne* species

Species	Part	Extracts	Yield	DPPH IC ₅₀ (µg/mL)	ABTS IC ₅₀ (µg/mL)	TPC (mg GA/g)
<i>Actinodaphne macrophylla</i>	Leaves	Hex	0.82 g, 0.54%	224.6	401.5	32.6 ± 0.5
		EtOAc	1.05 g, 0.70%	292.5	425.2	25.4 ± 0.3
		MeOH	2.52 g, 1.68%	196.5	352.8	38.2 ± 0.2
	Bark	Hex	1.05 g, 0.70%	158.2	305.1	39.5 ± 0.1
		EtOAc	2.10 g, 1.40%	122.5	240.2	41.8 ± 0.2
		MeOH	3.21 g, 2.14%	92.5	186.4	54.1 ± 0.1
<i>Actinodaphne pruinosa</i>	Leaves	Hex	0.75 g, 0.50%	251.0	395.2	29.5 ± 0.4
		EtOAc	1.85 g, 1.23%	185.2	225.6	36.5 ± 0.1
		MeOH	2.82 g, 1.88%	176.8	224.5	42.8 ± 0.3
	Bark	Hex	0.92 g, 0.61%	135.2	208.2	48.7 ± 0.2
		EtOAc	1.44 g, 0.96%	95.4	180.3	52.2 ± 0.2
		MeOH	2.42 g, 1.61%	70.2	147.2	55.1 ± 0.1
BHT			18.5	52.2	-	

TPC - Total phenolic content; BHT - Butylated hydroxytoluene; GA - gallic acid; I% - percentage inhibition

Solvents and chemicals

Analytical grade *n*-hexane, ethyl acetate and methanol used for extraction were purchased from Merck (Germany). *Antioxidants*: β-carotene, linoleic acid, 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), potassium persulfate, gallic acid and butylated hydroxytoluene (BHT) were obtained from Sigma-Aldrich (Germany). Analytical grade methanol, ethanol and dimethylsulfoxide (DMSO), HPLC grade chloroform, Folin-Ciocalteu's reagent, anhydrous sodium sulfate, sodium carbonate

and polyoxyethylene sorbitan monopalmitate (Tween-40) were purchased from Merck (Germany). *Antityrosinase*: Mushroom tyrosinase enzyme (EC1.14.18.1), kojic acid and L-dopa were purchased from Sigma-Aldrich (Germany). *Acetylcholinesterase*: Electric eel AChE (Type-VI-S, EC 3.1.1.7), acetylcholine iodide, 5,5'-dithiobis (2-nitrobenzoic acid) 99% (DTNB), and galantamine were purchased from Sigma-Aldrich (Germany). *Anti-inflammatory*: Lipoxigenase inhibitor screening assay kit (Item No. 760700 Cayman Chemicals Co) was purchased from i-DNA Biotechnology (M) Sdn. Bhd. (Malaysia).

Antioxidant activities

Total Phenolic Content (TPC)

Total phenolic contents of the extracts were determined by gallic acid equivalent (16). A sample of stock solution (1.0 mg/mL) was diluted in MeOH to final concentrations of 1000 µg/mL. A 0.1 mL aliquot of sample was pipetted into a test tube containing 0.9 mL of methanol, then 0.05 mL Folin-Ciocalteu's reagent was added, and the flask was thoroughly shaken. After 3 min, 0.5 mL of 5% Na₂CO₃ solution was added and the mixture was allowed to stand for 2 h with intermittent shaking. Then, 2.5 mL of methanol was added and left to stand in the dark for 1 h. The absorbance measurements were recorded at 765 nm. The same procedure was repeated for the standard gallic acid solutions. The concentration of total phenolic compounds in the extracts was expressed as mg of gallic acid equivalent per gram of sample. Tests were carried out in triplicate and the gallic acid equivalent value was reported as mean ± SD of triplicate.

DPPH Radical scavenging

The free radical scavenging activity was measured by the DPPH method with minor modifications (17). Briefly, 0.1 mM DPPH• (1 mL) dissolved in EtOH was added to an EtOH solution (3 mL) of the tested samples and standard (BHT) at different concentrations (200, 150, 100, 50, 25 µg/mL). An equal volume of EtOH was added in the control test. The mixture was shaken vigorously and allowed to stand at room temperature for 30 min. Then the absorbance at 517 nm was measured with a UV-vis spectrophotometer. The percent inhibitions (I%) of DPPH radical were calculated as follow:

$$I\% = [A_{\text{blank}} - A_{\text{sample}} / A_{\text{blank}}] \times 100$$

where A_{blank} is the absorbance value of the control reaction (containing all reagents except the test extracts) and A_{sample} is the absorbance values of the test extracts/standard. The sample concentration providing 50% inhibition (IC_{50}) was calculated by plotting inhibition percentages against concentrations of the sample. All tests were carried out in triplicate and IC_{50} values were reported as means ± SD of triplicates.

ABTS radical scavenging

The antioxidant capacity was estimated in terms of the ABTS•+ radical scavenging activity following the previously method (18). Briefly, ABTS was obtained by reacting 14 mM ABTS•+ stock solution (5 mL) with 4.9 mM potassium persulfate (5 mL) and the mixture was left to stand in the dark at room temperature for 12–16 h before use. The

ABTS solution (stable for 2 days) was diluted with distilled water to an absorbance at 734 nm of 0.70 ± 0.02 by UV-vis spectrophotometer. After the addition of 15 µL of extracts (concentration of 200, 150, 100, 50, 25 µg/mL) to 285 µL of diluted ABTS•+ solution, the absorbance was measured after 30 min. The same procedure was repeated for the standard gallic acid solutions. The percentage inhibition (I%) of ABTS•+ radical-scavenging activity of the extracts was expressed as:

$$I\% = [A_{\text{control}} - A_{\text{sample}} / A_{\text{control}}] \times 100$$

where A_{control} is the absorbance of the blank control (ABTS solution without extracts) and A_{sample} is the absorbance of the extracts. All tests were carried out in triplicate and IC_{50} values are reported as means ± SD of triplicates.

Antityrosinase activity

Tyrosinase inhibition assay was performed according to the previously method with slight modifications (19). Briefly, the extracts and kojic acid were dissolved in DMSO prepared as 1 mg/mL. The reaction was carried out using 96-well microplate and microplate reader (Epoch Micro-Volume Spectrophotometer, USA) was used to measure the absorbance at 475 nm. 40 µL of extracts dissolved in DMSO with 80 µL of phosphate buffer (pH 6.8), 40 µL of tyrosinase enzyme and 40 µL of L-dopa were put in each well. Each sample was accompanied by a blank that had all the components except for L-dopa. Kojic acid was used as reference standard inhibitor for comparison. The percentage of tyrosinase inhibition (I%) was calculated as follows:

$$I\% = [A_{\text{control}} - A_{\text{sample}} / A_{\text{control}}] \times 100$$

where A_{control} is the absorbance of the control reaction and A_{sample} is the absorbance of the extracts/reference. Analyses were expressed as means ± SD of triplicates.

Acetylcholinesterase (AChE) activity

AChE inhibitory activity of the extracts was measured by slightly modifying the spectrophotometric method (20-21). Electric eel AChE was used, while acetylthiocholine iodide (AChI) was employed as the substrate of the reaction. DTNB acid was used for the measurement of the AChE activity. Briefly, 140 µL of sodium phosphate buffer (pH 8.0), 20 µL of DTNB, 20 µL of test compounds and 20 µL of AChE solution were added by multichannel automatic pipette in a 96-well microplate and incubated for 15 min at 25°C. The reaction was then initiated with the addition of 10 µL of AChI. Hydrolysis of AChI was monitored by the formation of the yellow 5-thio-2-nitrobenzoate anion as a result of the reaction of DTNB with thiocholines, catalyzed by enzymes at 412 nm utilizing a 96-well microplate reader (Epoch Micro-Volume Spectrophotometer, USA). Percentage of inhibition (I%) of

AChE was determined by comparison of rates of reaction of samples relative to blank sample (EtOH in phosphate buffer pH = 8) using the formula:

$$I\% = [E - S / E] \times 100;$$

where E is the activity of enzyme without test sample and S is the activity of enzyme with test sample. The experiments were done in triplicate. Galantamine was used as the positive controls.

Anti-inflammatory activity

Lipoxygenase (LOX) inhibition was determined using an enzyme immuno assay (EIA) kit (Catalog No. 760700, Cayman Chemical, USA) according to the manufacturer's instructions and previous study (22). The Cayman Chemical lipoxygenase inhibitor screening assay detects and measures the hydroperoxides produced in the lipoxygenation reaction using a purified lipoxygenase. Stock solutions of the extracts were dissolved in a minimum volume of DMSO and were diluted using the supplied buffer solution (0.1 M, Tris-HCl, pH 7.4). To a 90 μ L solution of 5-LOX enzyme in 0.1 M, Tris-HCl, and pH 7.4 buffer, 10 μ L of various concentrations of test samples (final volume of 210 μ L) were added and the lipoxygenase reaction was initiated by the addition of 10 μ L (100 μ M) of arachidonic acid. After maintaining the 96-well plates on a shaker for 5 min, 100 μ L of chromogen was added and the plate was retained on a shaker for 5 min. The lipoxygenase activity was determined after measuring absorbance at a wavelength of 500 nm. The percentage inhibition (I%) of the extracts was calculated as follows:

$$I\% = [A_{\text{initial activity}} - A_{\text{inhibitor}} / A_{\text{initial activity}}] \times 100$$

where $A_{\text{initial activity}}$ is the absorbance of 100% initial activity wells without sample and $A_{\text{inhibitor}}$ is the absorbance of extracts/reference. All tests were carried out in triplicate and expressed as means \pm SD.

Statistical analysis

Data obtained from biological activities were expressed as mean values. The statistical analyses were carried out by employing one way ANOVA ($p < 0.05$). A statistical package (SPSS version 11.0) was used for the data analysis.

RESULTS AND DISCUSSION

A number of methods and variations have been developed and applied for the assessment of antioxidant capacity. Thus, we applied several antioxidant assays that would provide a better insight into the true antioxidant potential of the

extracts. They were DPPH radical scavenging, ABTS radical scavenging and total phenolic content. The antioxidant activity of *Actinodaphne* species is summarised in Table 1. In the DPPH \cdot assay, the MeOH bark extracts of *A. macrophylla* and *A. pruinosa* were found to be good radical scavengers with IC_{50} values of 70.2 and 92.5 μ g/mL, respectively. However, these values were lower than the positive control, BHT which showed IC_{50} value of 18.5 μ g/mL. Both extracts were also found to have the highest TPC (*A. pruinosa* 55.1 mg GA/g; *A. macrophylla* 54.1 mg GA/g) among others. The MeOH and EtOAc bark extracts of *A. pruinosa* have showed the highest radical scavenging activity in the ABTS assay with IC_{50} values 147.2 and 180.3 μ g/mL, respectively, followed by the MeOH bark extracts of *A. macrophylla* with IC_{50} of 186.4 μ g/mL. Over all, in the present analysis, a strong correlation was established among the TPC, DPPH \cdot and ABTS $^{+}$ assays. The MeOH extracts showed the highest antioxidant activity in all assays compared to other extracts. These results are in line with the previous finding (23) which reported that extracts with higher TPC also showed strong activity against DPPH \cdot . Most of the antioxidant potential of medicinal plants is due to the redox properties of phenolic compounds, which enable them to act as reducing agents, hydrogen donors and singlet oxygen scavengers (24). Prajapati *et al* (13), have reported that the leaves extract *A. hookeri* displayed potent antioxidant properties against β -carotene-linoleic acid (IC_{50} of 194.16 μ g/mL), lipid peroxidation (IC_{50} of 51.71 μ g/mL) and total antioxidant capacity (290 μ g ascorbic acid/mg). Akki *et al* (25), reported that the alcoholic and chloroform leaves extracts of *A. hookeri* showed significant antioxidant activity by increasing the decreased levels of antioxidant enzymes such as superoxide dismutase, catalase, and reduced glutathione and decreasing the lipid peroxidation state.

In the current study, antityrosinase, acetylcholinesterase and 15-LOX inhibiting activity were analysed using mushroom tyrosinase, AChE enzyme and 15-lipoxygenase enzyme, respectively. Table 2 summarises the results of inhibition percentage at the concentration of 1 mg/mL. Tyrosinase inhibitors are chemical agents capable of reducing enzymatic reactions, such as food browning and melanisation of human skin. Therefore, these agents have good commercial potential in both food processing and cosmetic industries. All extracts showed weak inhibitory activity with the percentage inhibition in the range of 30.8 - 45.2%. The MeOH bark extract of *A. macrophylla* displayed the highest tyrosinase inhibition activity which gave 45.2% inhibition, followed by the leaves of *A. pruinosa* extracts with 44.6%. These results

are lower than that of kojic acid which revealed inhibition of 97.1%. The inhibition of tyrosinase might depend on the hydroxyl groups of phenolic compounds of the mushroom extracts that could form a hydrogen bond to active site of the enzyme, leading to a lower enzymatic activity. Some

tyrosinase inhibitors act through hydroxyl groups that bind to the active site on tyrosinase, resulting in steric hindrance or change in conformation. Phenolic acids and flavonoids proved to be effective inhibitors of tyrosinase activity such as gallic acid and (-)-epicatechin (26-27).

Table 2. Antityrosinase, acetylcholinesterase and anti-inflammatory activities of two *Actinodaphne* species

Species	Part	Extracts	Antityrosinase (I%)	Acetylcholinesterase (I%)	Anti-inflammatory (I%)
<i>Actinodaphne macrophylla</i>	Leaves	Hex	35.2 ± 1.3	78.5 ± 1.2	35.2 ± 1.2
		EtOAc	33.5 ± 1.5	75.2 ± 1.1	42.8 ± 1.4
		MeOH	32.5 ± 1.1	80.2 ± 1.1	40.5 ± 1.2
	Bark	Hex	34.2 ± 0.2	85.2 ± 0.2	52.2 ± 0.1
		EtOAc	35.4 ± 0.2	83.8 ± 0.2	56.5 ± 0.3
		MeOH	45.2 ± 0.1	88.1 ± 0.1	69.2 ± 0.2
<i>Actinodaphne pruinosa</i>	Leaves	Hex	42.6 ± 0.4	72.5 ± 0.3	32.5 ± 0.1
		EtOAc	43.2 ± 0.3	70.5 ± 0.5	30.2 ± 0.1
		MeOH	44.6 ± 0.2	75.8 ± 0.2	40.8 ± 0.4
	Bark	Hex	35.4 ± 1.3	84.1 ± 0.1	50.5 ± 0.1
		EtOAc	30.8 ± 1.3	83.5 ± 0.2	52.5 ± 0.4
		MeOH	36.2 ± 1.2	85.1 ± 0.2	60.2 ± 0.2
Kojic acid		97.1 ± 0.1	-	-	
Galantamine		-	95.9 ± 0.2	-	
Quercetin		-	-	89.1 ± 0.2	

Data represent as mean ± SD of three independent experiments; I% - percentage inhibition

In acetylcholinesterase activity, all extracts have found to have strong activity, in the range of 70.5 – 88.1%, comparable to galantamine, 95.9%. The MeOH bark and leaves extract of *A. macrophylla* exerted the highest AChE activity with inhibition of 88.1% and 85.2%, respectively. In addition, for *A. pruinosa*, the MeOH bark extract found to have significant inhibition, 85.1% followed by their hexane (84.1%) and EtOAc (83.5%) extracts. Inhibition of AChE, the key enzyme in the breakdown of acetylcholine, is considered as one of the treatment strategies against several neurological disorders such as Alzheimer disease (28). Since the most strong synthetic or natural product driven AChE inhibitors are known to contain nitrogen, the promising activity of both *Actinodaphne* extracts could be due to their high alkaloidal contents (29-30). These results were in accordance with the previous phytochemical studies of both species, which found to have alkaloids as their major compounds (14-15).

Lipoxygenases are the key enzymes in the biosynthesis of leukotrienes that play an important role in several inflammatory diseases. Inhibition of LOX may influence

the inflammation processes and thus be of interest for modulation of the lipoxygenase pathway. Therefore, inhibitors of oxidative stress and LOX have been considered as therapeutically useful in the treatment of many related diseases such as liver disease, arthritis and cancer (31). According to Table 2, a standard lipoxygenase inhibitory chemical, quercetin, inhibited lipoxygenase activity by 89.1% at 1 mg/mL. The MeOH bark extracts of *A. macrophylla* and *A. pruinosa* exhibited moderate lipoxygenase inhibitory effect of 69.2% and 60.2%, respectively. It is noteworthy that, the MeOH bark extracts of *A. macrophylla* and *A. pruinosa* had the highest TPC with good antioxidant activity, a finding which is consistent with a previous study which found a relationship between the anti-inflammatory activity and the presence of polyphenols (32). Antioxidants are also known to inhibit plant lipoxygenases. Studies have implicated that oxygen free radicals in the process of inflammation and phenolic compounds may block the cascade process of arachidonic acid metabolism by inhibiting lipoxygenase activity and serve as a scavenger of reactive free radicals that are produced during arachidonic acid metabolism (33-34).

CONCLUSION

We have herein screened the leaves and bark extracts of *A. macrophylla* and *A. pruinosa* of Malaysian origin for their enzyme (AChE, tyrosinase, 15-LOX) inhibitory potentials as well as antioxidant activity of *n*-hexane, ethyl acetate and methanol extracts. The results revealed that all extracts from both *A. macrophylla* and *A. pruinosa* have significant activity as AChE inhibitors but inactive towards antityrosinase and weak activity against 15-LOX. In addition, all extracts were also found to have low antioxidant capacity except the methanol extract of bark of *A. pruinosa* in the assay used.

Although, phytochemical investigation has been carried out by other researchers before, but this is the first report on *in vitro* bioactivity studies on the extracts of *A. macrophylla* and *A. pruinosa*. In addition, further studies also needed to evaluate the *in vivo* activity in animal model.

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Actinodaphne türlerinin in vitro antioksidan, antitirozinaz, antikolinesteraz ve antienflamatuvar etkilerinin araştırılması

ÖZET

Bu çalışmanın amacı Malezya'da yetişen iki *Actinodaphne türünün*; *Actinodaphne macrophylla* ve *Actinodaphne pruinosa*'nın biyolojik etkilerinin araştırılmasıdır. *In vitro* deneysel yöntemler kullanılarak *Actinodaphne macrophylla* ve *Actinodaphne pruinosa*'nın antioksidan, antitirozinaz, antikolinesteraz ve antienflamatuvar etki potansiyelleri araştırılmıştır. *A. pruinosa*'nın metanolik kabuk ekstresi DPPH (İK₅₀: 70.2 µg/mL), ABTS (İK₅₀:

147.2 µg/mL) analizlerinde en yüksek etkiyi gösterirken fenolik içeriği 55.1 mg gallik asit/g olarak bulunmuştur. *A. macrophylla*'nın kabuk ekstreleri ise mantar tirozinaz (% 45.2) ve lipooksijenaz (% 69.2) analizlerinde en yüksek etkiyi göstermiştir. Bütün ekstrelerin, galantamin'le (%95.9) karşılaştırıldığında yüksek AChE inhibitörü etki gösterdiği (%70.5–88.1) bulunmuştur. Çalışmaya dahil edilen *Actinodaphne* türlerinin biyoaktif bileşenlerinin izole edilmesi ve bu bileşenlerin güvenilirliğinin gösterilmesi bahsi geçen *Actinodaphne* türlerinin potansiyel ilaç öncü bileşikler olarak gösterilebilmesi için gereklidir.

Anahtar kelimeler: *Actinodaphne*, antioksidan, antitirozinaz, asetilkolinesteraz, antienflamatuvar

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