Tyrosinase inhibition, anti-acetylcholinesterase, and antimicrobial activities of the phytochemicals from *Gynotroches axillaris* Blume

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Abstract: The leaves of *Gynotroches axillaris* were chemically and biologically studied. Sequential extraction of the leaves using petroleum ether, chloroform, and methanol afforded three extracts. Purification of pet. ether extract yielded, squalene and β-amyrin palmitate as the major compounds, together with palmitic acid and myristic acid as the minor components. The methanol extract yielded two flavonoids, quercitrin and epicatechin. The isolated compounds were characterized by MS, IR and NMR (1D and 2D). Anti-acetyl cholinesterase screening using TLC bio-autography assay showed that palmitic acid and myristic acid were the strongest inhibition with detection limit 1.14 and 1.28 µg/ 5 µL respectively. Antibacterial against Gram-positive and negative and antifungal activities exhibited that β-amyrin palmitate was the strongest (450-225 µg/mL) against all the tested microbes. The tyrosinase inhibition assay of extracts and the pure compounds were screened against tyrosinase enzyme. The inhibition percentage (*I*%) of methanol extract against tyrosinase enzyme was stronger than the other extracts with value 68.4%. Quercitrin (59%) was found to be the highest in the tyrosinase inhibition activity amongst the pure compounds. To the best of our knowledge, this is first report on the phytochemicals, tyrosinase inhibition, anti-acetycholinesterase and antimicrobial activities of the leaves of *G. axillaris*.

Keywords: *Gynotroches axillaris*, phytochemicals, anti tyrosinase, anti-cholinesterase, antimicrobial.

INTRODUCTION

Gynotroches axillaris is the only species of *Gynotroches* genus, belongs to Rhizophoraceae family, locally called as mata keli (eyes' fish) (Ng, 1992; Kochummen, 1989). Its leaves have been reported to relief fever and headache (Wiart, 2006). Recently the total phenolic content together with several bioactivities including antioxidant, toxicity, and antimicrobial activities of the crude extracts of this plant was published (Abed *et al*., 2013). There have not been many reports on the phytochemistry of *Gynotroches axillaris*, only one report on the alkaloid (+)-hygroline isolated from the bark and detected in the leaves of *G. axillaris* collected from rain forest near Mount Shungol in the Morobe District (Johns *et al*., 1967; Bick, 1996), and anthocyanin from the fruit of *G. axillaris* collected from Malaysia (Lowry, 1976). However, there is no bioactivity reports on the chemical components of this species, therefore we would like to report the isolation, identification of phytochemicals, as well as the tyrosinase inhibition, anti-acetycholinesterase and antimicrobial activities of the leaves of *G. axillaris*, since there has been no literature reported the study on the leaves parts of *G. axillaris.*

MATERIAL AND METHOD

General methods

Melting points were measured using a Kofler hot plate Leica Gallen apparatus and were uncalibrated. Perkin Elmer 1650 FTIR spectrophotometer was used to record Infrared (IR) spectra on KBr disc or NaCl cell. The ^{1}H and ^{13}C-NMR spectra were obtained from Bruker Avance at 400, and 100 MHz respectively, using $CDCl_3$, $(CD_3)_2CO$ and CD_3OD as solvents. Mass spectra were obtained from Kent Mass Spectrometry Services, United Kingdom. Gas chromatography (GC) was carried out using Hewlett-Packard HP6890 Gas Chromatography with FID, and fitted with column Ultra-2 (5% phenyl-95% methyl polysiloxane) and Ultra-1 (100% dimethyl polysiloxane) (25 m × 0.33 µm film thickness × 0.20mm i.d.). Analytical thin layer chromatography (TLC) was carried out on a silica gel aluminum sheets (Merck Kieselgel 60 $F_{254,}$ 0.20 mm). Spots were visualized with UV light (254 nm and 365 nm) and sprayed with vanillin reagent. The vanillin reagent was prepared by mixing vanillin (0.5 g), methanol (85 mL), concentrated acetic acid (10 mL) and concentrated sulphuric acid (5 mL). Vacuum liquid chromatography (VLC) was performed using short sintered column with Merck silica gel 60 (230-400 mesh), and column chromatography (CC) were carried out by using silica gel 60 F_{254} (70-230 mesh) and Sephadex LH-20 from Merck.

Plant materials

Gynotroches axillaris leaves were collected in February 2011. The species was identified by Dr Shamsul Khamis from University Putra Malaysia, and the Voucher specimen (No. 22) has been deposited at Kulliyah of Pharmacy, International Islamic University of Malaysia (IIUM), Kuantan, Pahang.

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Extraction and isolation

The leaves of the *G. axillaris* (300 g) were dried, powdered, and extracted successively with pet. ether (60-80°C) using cold method and hot method by soxhlet extraction for 20 h each with chloroform and methanol. The extracts were filtered under reduced pressure and concentrated under vacuum at 50°C to afford pet. ether (GAP), chloroform (GCL) and methanol (GMOH) extracts, 14.0 g, 5.0 g and 45.0 g, respectively. GAP extract (12.0 g) was separated using VLC over silica gel (230-400 mesh) and eluted with increasing polarity of eluent (hexane, petroleum ether, $CHCl_3$, EtOAc, MeOH). The fractions were analyzed by TLC on silica F_{254} plate, and fractions with similar TLC profile were combined to afford 12 fractions (GAPF1- GAPF12). Fraction GAPF2 (200 mg) yielded compound (1), showed one spot on TLC. Fraction GAPF5 yielded a white powder (5.5 g), and a portion (1.5 g) was recrystallized from acetone for two days at room temperature, to afford compound (2) (600.0 mg, 5%). Fraction GAPF9 (1.2 g) was purified by CC using column (40×3) cm with 40 g of silica gel, and hexane as mobile phase. The eluent polarity was gradually increased by using diethyl ether, and then dichloromethane to afford 60 subfractions. Subfractions GAPF 9,12-13 were purified by repeated CC with silica gel and *n*-hexane: diethyl ether as an eluent to yield compound (3) (8.3 mg, 0.07%). Subfraction GAPF 9, 14 was purified by CC to give pure compound (4) (7.2 mg, 0.06%).

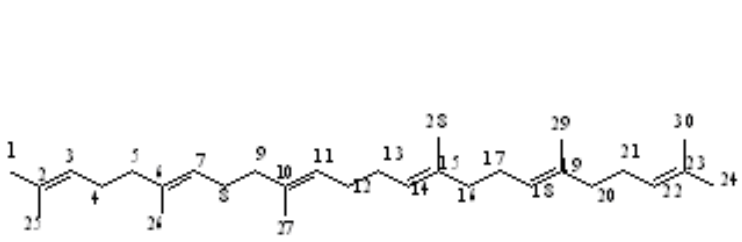
Methanol extract (45.0 g) was dissolved in a mixture of warm water (250mL) and acetone (20mL). The aqueous layer was partitioned with dichloromethane: ethanol (2:1, 3×200mL) to afford organic (2.8g) and aqueous GMAQ (42.0 g) extracts. Aqueous extract GMAQ (5.0 g) was chromatographed on CC (4 x 50 cm) with silica gel (70-230 mesh) (9 cm in height) and eluted with DCM, DCM: EtOH (9:1, 8:2, 6:4, 5:5), followed by acetone: Et_2O (2:3, 3:3), EtOAc: Acetone (1:1), acetone, acetone: MeOH (3:2), and MeOH, to give 160 fractions. Fractions 27-43 showed similar profiles were combined as fraction (GMAQ6), followed by column chromatography (2 x 20 cm) using sephadex LH 20 (10 cm in height) with ethanol, ethanol: chloroform (4:1, 3:2, 2:3, 1:4, 0: 5) and methanol as eluents, to give 30 subfractions. The subfractions 9-11 were further purified in a CC using sephadex LH 20, with ethanol to give compounds (5) (5.0 mg, 0.1%) and (6) (3.3 mg, 0.07%) from sub fractions GMA6f9, 10 and GMA6f9, 20 respectively.

Compound (1)

Colourless oil, R_f 0.83 in *n*-hexane: $CHCl_3$ (4:1). IR (NaCl) cm^{-1}: 2921 (CH_3), 1667 (C=C). GC-MS: one peak at t_R 23.07 min, *m/z* 410 (5%) $[M^+]$ ($C_{30}H_{50}$) and 69 (100%). ^1H NMR ($CDCl_3$) δ_H: 1.61 (6×3H, s, H-25, H-26, H-27, H-28, H-29, H-30), 1.69 (2×3H, s, H-1, H-24), 2.01 (4x2H, dd, *J*=16.0, 17.0 Hz, H-4, H-12, H-13, H-21), 2.02 (4×2H, dd, *J*=16.0, 17.0 Hz, H-5, H-9, H-16, H-20). ^{13}C NMR ($CDCl_3$) δ_C: 15.7 (C-27, C-28), 16.0 (C-26, C-29), 17.6 (C-25, C-30), 25.5 (C-1, C-24), 26.6 (C-8, C-17), 26.7 (C-4, C-21), 28.2 (C-12, C-13), 39.7 (C-9, C-16), 39.7 (C-5, C-20), 124.0 (C-11, C-14), 135.0 (C-10, C-15).

Compound (2)

Colourless needles, Mp: 71-73°C. R_f 0.66 in n-hexane: $CHCl_3$ (4:1). IR (KBr) cm^{-1}: 2921, 1728. EIMS: *m/z* 665 (2%) $[M + 1]^+$ ($C_{46}H_{80}O_2$), 408 (4%), 203 (17%), 218 (100%). ^1H NMR ($CDCl_3$): δ 1.31 (1H, m, 1H ax.), δ 1.60 (1H, m, 1H eq.), 1.65 (1H-2a,), 1.85 (1H, H-2b), 4.51



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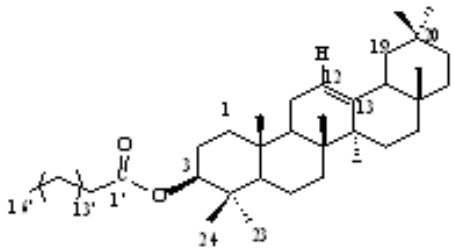

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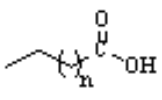
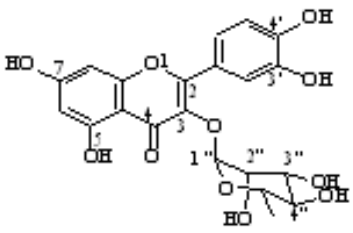


(3) n = 11
(4) n = 13



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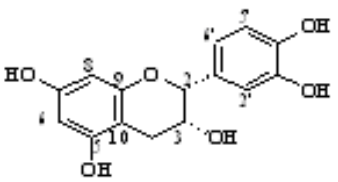

(6)

(1H, dd, *J*=8.0, 7.5 Hz, H-3), 0.89 (1H, H-5), 1.58 (1H, m, H-6a), 1.54 (1H, m, H-6b), 1.25 (1H, m, H-7a), 1.15 (1H, m, H-7b), 1.63 (1H, m, H-11a), 1.95 (1H, m, H-11b), 5.2 (1H, t, *J*=3.3 Hz, H-12), 1.13 (2H, m, *J*=1.3 Hz, H-15), 1.15 (1H, m, H-16a), 1.25 (1H, m, H-16b), 1.95 (1H, m, H-18), 1.63 (1H, m, H-19a), 1.97 (1H, m, H-19b), 1.45 (1H, m, H-21a), 1.25 (1H, m, H-21b), 1.63 (1H, m, H-22a), 1.25 (1H, m, H-22b), 0.89 (4×3H,s, 3H-30, 3H-29, 3H-25, 3H-23), 0.85 (2×3H, s, 3H-24, 3H-28), 0.98 (3H, s, H-26), 2.3 (2H, dd, *J*=7.6, 7.2 Hz, 2H-2′), 1.6 (2H, m, H-2′), 1.25 (10×2H, H4′ -13′), 0.85 (3H-14′). ^{13}C NMR ($CDCl_3$): δ 14.14 (C-14′), 15.5 (C-26), 16.7 (C-25), 16.8 (C-24), 18.2 (C-6), 22.7 (C-13′), 23.5 (C-11), 23.6 (C-2), 23.7 (C-30), 25.1 (C-3′), 25.9 (C-27), 26.1 (C-16), 26.9 (C-15), 28.4 (C-23), 28.9 (C-28), 29.1-29.6 (C-4′ -C-11′), 31.1 (C-20), 31.9 (C-12′), 32.5 (C-7), 33.3 (C-29), 34.7 (C-21), 34.8 (C-2′), 36.8 (C-10), 37.1 (C-22), 37.7 (C-4), 38.2 (C-1), 39.8 (C-8), 41.7 (C-14), 46.7 (C-19), 47.2 (C-18), 47.5 (C-9), 80.5 (C-3), 121.6 (C-12), 145.2 (C-13), 173.6 (C=O).

Compound (3)

White wax, Mp: 54-56°C. R_f 0.73 in CH_2Cl_2: EtOAc (4.5: 0.5). IR (KBr) cm^{-1}: 2917, 3400-2500 (br), 1700.6. ^{1}H NMR ($CDCl_3$): δ_H 0.89 (3H, t, *J*=7.0 Hz, H-14), 1.26 (10×2H, m, CH_2), 1.64 (2H, m, H-3), 2.35 (2H, t, *J*=7.6 Hz, H-2). ^{13}C NMR ($CDCl_3$): δ_C 14.14 (C-14), 22.7-31.9 (10 × CH_2), 24.6 (C-3), 33.94 (C-2), 179.4 (C=O).

Compound (4)

White wax, Mp: 63-66°C. R_f 0.75 in CH_2Cl_2: EtOAc (4.5: 0.5). IR (KBr) cm^{-1}: 2917, 3400-2500 (br), 1700.6. EIMS at *m/z* : 256 [M^+] ($C_{16}H_{32}O_2$). ^{1}H NMR ($CDCl_3$): δ_H 0.89 (3H, t, *J*=6.8 Hz, H-14), 1.26 (12×2H, m, CH_2), 1.64 (2H, m, H-3), 2.35 (2H, t, *J*=7.6 Hz, H-2). ^{13}C NMR ($CDCl_3$): δ_C 14.1 (C-14), 22.7-31.9 (12×CH_2), 24.6 (C-3), 33.9 (C-2), 179.4 (C=O).

Compound (5)

Yellow solid (5), reddish-yellow under UV short wavelength (254 nm), and yellow-orange after sprayed with vanillin reagent. R_f 0.63 in diethyl ether: acetone (3:2). IR (NaCl) cm^{-1}: 3369, 2925, 1654, 1607, 1505, 1202, 1088, 1065. ^{1}H NMR (CD_3OD): δ_H 7.36 (1H, d, *J*= 2.0 Hz, H-2′), 7.33 (1H, dd, *J*=2.0, 8.0 Hz, H-6′), 6.94 (1H, d, *J*=8.0 Hz, H-5′), 6.39 (1H, d, *J*=2.0 Hz, H-8′), 6.22 (1H, d, *J*=2.0 Hz, H-6′), 5.36 (1H, d, *J*=1.6 Hz, H-1″), 4.2 (1H, dd, *J*=1.6, 3.2 Hz, H-2″), 3.76 (1H, dd, *J*= 3.2, 9.2 Hz, H-3″), 3.4 (1H, m, H-4″ overlapped with H-5″), 3.35 (1H, m, H-5″ overlapped with H-4″), 0.96 (3H, d, *J* =6.0 Hz, H-6″). ^{13}C NMR (CD_3OD): δ_C 178.2 (C-4), 164.4 (C-7), 161.7(C-5), 157.9 (C-2), 157.1 (C-9), 148.2 (C-4′), 145.0 (C-3′), 134.8 (C-3) 121.6 (C-1′), 121.4 (C-6′), 115.8 (C-2′) 115.2 (C-5′), 104.5 (C-10), 102.1 (C-1″), 98.4 (C-6), 93.3 (C-8), 71.8 (C-4″), 70.1 (C-2″), 70.6 (C-3″), 70.4 (C-5″) 16.2 (C-6″). EIMS: *m/z* 448 (0.05%) [M^+] ($C_{21}H_{20}O_{11}$), 302 (100%) [M + 1-rhamnose unit]$^+$.

Compound (6)

Yellow solid **(6)**, appeared as light red under UV short wavelength (254 nm), dark-red after spraying with vanillin reagent. R_f 0.69 in diethyl ether:acetone (3:2). IR ν_{max} (NaCl) cm^{-1}: 3306 (OH), 2925 (C-H), 1606, 1518 and 1449 (C=C), 1095, 1062 (C-O) cm^{-1}.^{1}H NMR ((CD_3)$_2$CO): δ_H7.06 (1H, d *J*=2.0 Hz, H-2′), 6.84 (H, dd, *J*=2.0, 8.3 Hz, H-6′), 6.78 (H, d, *J*=8.3 Hz, H-5′), 6.03 (1H, d, *J*=2.0 Hz, H-6), 5.93 (1H, d, *J*=2.0 Hz, H-8), 4.89 (1H, s, H-2), 4.21 (1H, m, H-3), 2.80 (1H, dd, *J*=4.4, 16.8 Hz, H-4a), 2.75 (1H, dd, *J*=4.4, 16.8 Hz, H-4b).^{13}C NMR ((CD_3)$_2$CO): δ_C 156.4 (C-7), 156.0 (C-5), 155.8 (C-9), 144.4 (C-4′), 144.3 (C-3′), 130.8 (C-1′), 118.2 (C-6′), 114.8 (C-5′), 113.9 (C-2′), 98.8 (C-10), 95.3 (C-6), 94.8 (C-8), 78.4 (C-2), 66.0 (C-3), 27.8 (C-4). EIMS: *m/z* 290 (28%) [M^+] ($C_{15}H_{14}O_6$), 152 (43%), 123 (28%), 139 (100%).

Bioactivity studies

Chemicals and materials

The mushroom enzyme tyrosinase, the substrate L-DOPA, kojic acid and dimethyl sulfoxide DMSO were purchased from Sigma Chemical Co (St. Louis. MO). Disodium hydrogen phosphate dihydrate and sodium dihydrogen phosphate anhydrous of AR grade were purchased from QReC. 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) reagent and control positive galanthamine hydrobromide were purchased from Merck. Acetylthiocholine iodide (ATCI), the buffer Tris–hydrochloride (pH 8) and electric eel acetyl cholinesterase (type VI-s; lyophilized powder; 518 U/mg solid; 844 U/mg protein) were purchased from Sigma.

Inhibition of acetylcholinesterase enzyme (AChE) on thin layer chromatography (TLC)

Leaves extracts and isolated compounds (1-6) were screened to inhibit acetyl cholinesterase enzyme (AChE) on silica gel TLC bio-autography according to Ellman's method (Rhee, 2003) with minor modification. Each sample was dissolved individually in a proper solvent (ethanol, ethyl acetate) and diluted to obtain a various concentrations, 2.0-10.0 mg/mL of extracts and 1.0-100.0 mM of compounds. Aliquots (5 µL) of each sample were spotted on silica gel plate. Extracts samples were chromatographed on silica gel using a proper solvent system, pet. ether and chloroform extracts were in diethyl ether: chloroform (4:1), while methanol extract was in diethyl ether: acetone (3:2). The plates were sprayed with 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB)/ acetylthiocholine iodide (ATCI) reagent [1 mM DTNB (Ellman's reagent) and 1 mM ATCI in buffer 50 mM Tris–HCl] until the layer was just saturated with the reagent, then the plate was allowed to dry using cool win for 3-5 min after that 1 unit/mL of enzyme solution in buffer (pH 8.0) was uniformly sprayed. A white spots with yellow background of Ellman's reagent were showed within 3-5 min due to the samples inhibition. These white spots were observed and reported within 15 min as they vanished in

20-30 min. Galanthamine was used as reference inhibitor. The lowest concentration showed white spot was recorded as the detection limit for every sample.

Antityrosinase assay

Inhibitory of tyrosinase enzyme was screened using the procedure described in literature (Kubo *et al*., 2000) and was slightly modified. Stock solution of each extracts, pure compounds and standard kojic acid were dissolved in different solvents at 1 mg/mL concentration according to their solubility. Final concentration of each extracts (0.2 mg/mL) and pure compounds (0.1 mg/mL) were prepared by dilution of their stock solution. Each diluted sample (40 µL) was mixed with 80 µL of 0.1 M sodium phosphate buffer (pH 6.8) in a 96-well plate and then 40 µL of L-DOPA (0.0025 M) solution was added and incubated for 10 min. Mushroom tyrosinase (100 unit/mL, 40 µL) was added, next the test mixture (200 µL) was well mixed and incubated at 37ºC for 10 min. The solvent instead of the extract was used as control. Blank sample and blank control were prepared by adding 40 µL of sodium phosphate buffer instead of tyrosinase enzyme. The absorbance level was obtained with Elisa plate reader at 515 nm with reference 665 nm. Inhibition potency % of tyrosinase was obtained by using the equation below:

Inhibition (I%) = [1-((Abs$_{sample}$ - Abs$_{blank\ sample}$)/ (Abs$_{control}$ - Abs$_{blank\ control}$))] x100

where, Abs is the absorbance at 515 nm.

Antibacterial Assay

Compounds (2), (5) and (6) were screened to determine the antibacterial activity using minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) according to the methods reported in literature [Abed *et al*., 2013]. Gram-negative bacteria (*Escherichia coli* (ATCC 10536) and *Pseudomonas aeruginosa* (ATCC 9027)), Gram-positive bacteria (*Bacillus subtilis* (ATCC 6633) and *Staphylococcus aureus* (ATCC 29737)), yeasts (*Candida glebreti* (ATCC 2001)) and fungi, *Aspergillus niger* (ATCC 16888) were selected, and obtained from the American Type Culture Collection (ATCC).

RESULTS

Extraction of leaves of *Gynotroches axillaris* in pet. ether by shaking, followed by chloroform and methanol in a soxhlet afforded pet. ether (GAP) (4.6%), $CHCl_3$ (GCL) (1.6%) and MeOH (GMOH) (15.0%) extracts respectively. Pet. ether and $CHCl_3$ extracts showed similar profile on TLC, therefore only GAP and GMOH extracts were submitted to phytochemical investigations. Fractionation of pet. ether extract using vacuum liquid column chromatography (VLC) and gravity column chromatography (CC) yielded two triterpens 1-2 and two fatty acids 3-4, whereas purification of MeOH extract by column chromatography (CC) on silica gel and sephadex LH-20 afforded falvonoids 5 and 6.

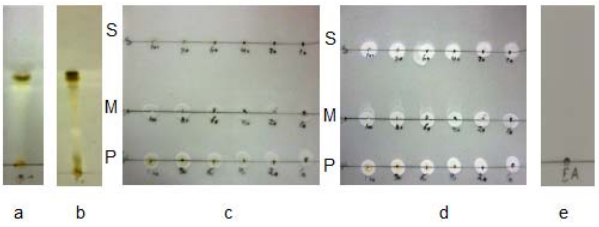

Fig. 1: White spots on a yellow background represent the AChE inhibition using chemical components from the leaves of *G. axillaris* (Rhizophoraceae): (a) pet. ether extract, (b) $CHCl_3$ extract, (c) inhibition zones of the components from pet. ether extract, S=squalene, M = myristic acid and P=palmitic acid after 4.0 min of spraying with enzyme (d) inhibition zones components, S, M and P after 8.0 min of spraying with enzyme. (e) Back ground: spot of solvent ethyl acetate was sprayed with (DTNB / ATCI) reagent followed by enzyme

In the field of the bioactivity, the extracts and pure compounds from the leaves of *G. axillaris* were screened on AChE inhibition. The results of acetyl cholinesterase inhibition were expressed in detection limit, as the lowest active concentration (table 1). The best inhibition was recorded for the Pet. ether and $CHCl_3$ extracts with detection limit 10 µg/5 µL as shown in fig. 1a. The isolated compounds, squalene (1), myrstic acid (3) and palmitic acid (4) showed inhibition with detection limit at concentrations 2.0, 1.14 and 1.28 µg/5 µL respectively, β-amyrine palmitate (2) exhibited inhibition with detection limits 332.5 µg/5 µL, whereas quercitrin and epicatechin were inactive.

$$\text{Acetylcholine} + H_2O \underset{\text{Extract}}{\overset{\text{AChE}}{\rightleftharpoons}} \text{Choline} + \text{Acetic Acid}$$

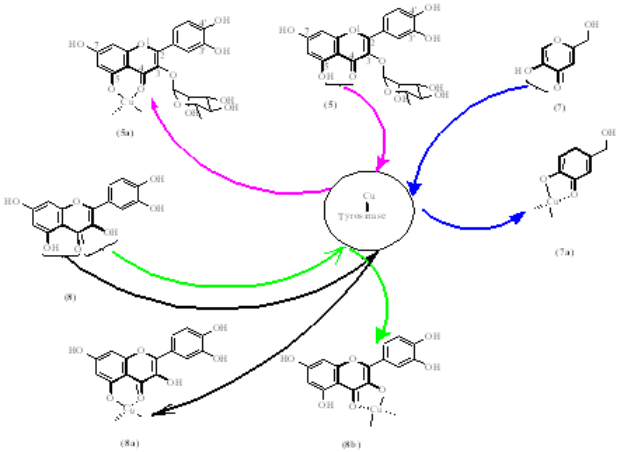

Fig. 2: Chemical structures of quercitrin (5), quercetin (8) and kojic acid (7) showing copper chelation by quercitrin (5a), kojic acid (7a) and quercetin (8a) and (8b).

The extracts and isolated compounds were evaluated for tyrosinase enzyme inhibition and the results were listed in

Table 1: Acetyl cholinesterase and tyrosinase inhibition of crude extracts and isolated compounds from the leaves of *Gynotroches axillaris*.

Samples	Anti-acetylcholinesterase	Anti-tyrosinase	
	Detection limits[a] µg/5 µL	Solvent	Inhibition (*I*%)
Pet. ether extract	10.0	DMSO	>0.0
$CHCl_3$ extract	10.0	DMSO	11.3
Methanol extract	ND[b]	Methanol	68.11
β-Amyrin palmitate	332.5	Ethyl acetate	>0.0
Squalene	2.0	DMSO	8.5
Myristic acid	1.14	-	-
Palmitic acid	1.28	-	-
Quercitrin	ND[b]	Methanol	57.7
Epicatechine	ND	Methanol	8.5
Galanthamine	1.84	-	-
Kojic acid	-	Methanol	83.5

[a]Detection limits expressed in weight (µg) in one spot (5 µL). [b]Not detected

table 1. Kojic acid (7) was used as standard in this activity. Methanol extract exhibited the highest percentage of inhibition (68.1%) followed by quercitrin (5) with *I*% (57.7%).

Antimicrobial activity of compounds, (2), (5) and (6) were tested against selected Gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*), Gram-positive bacteria (*Bacillus subtilis* and *Staphylococcus aureus)*, yeast (*Candida glebreti*) and fungi (*Aspergillus niger*), obtained from the American Type Culture Collection (ATCC). The antibacterial test was carried out using minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) assays. The *in vitro* antibacterial activities of MIC and MBC results were presented in table 2. β-amyrin palmitate (2) was found most active to inhibit both Gram-positive and Gram-negative bacteria, as well as fungi with MIC (450-225 µg/mL).

DISCUSSION

The structures of the purified compounds were elucidated by comparison of their physical properties and spectroscopic data (IR, EIMS, ^{1}H NMR, ^{13}C and 2D NMR) with literature data as, squalene (1) (Alam *et al.,* 2000), β-Amyrin palmitate (2) (Wahlberg *et al*., 1972; Wang, 2007; Chvez *et al*., 1996), myristic acid (3) (Bear-Roger *et al*., 2001), palmitic acid (4) (Joshi *et al*., 2009) quercetin-3-*O*-L-rhamnoside (5) (Ma *et al*., 2005) and epicatechin (6) (Ban *et al*., 2006). This is the first report of the isolation of triterpene, pentacyclic triterpene ester and flavonoids from this plant.

Inhibition of acetyl cholinesterase

Acetylcholine (ACh) is a neurotransmitter in humans and many organisms and the hydrolysis of acetylcholine into choline and acetic acid by acetyl cholinesterase (AChE) leads to terminate the nerve impulse transmission through cholinergic synapses. This result in cognitive disorder and memory loss associated to Alzheimer's disease (AD), senile dementia and Parkinson's disease (Mukherjee *et al*., 2007). Several inhibitors of AChE such as galanthamine and huperzine were originally purified from plants (Yang *et al*., 2012) and some meroterpenoids from fungi (Houghton *et al*., 2006). In the hope to search for AChE inhibitors, the extracts and pure compounds from the leaves of *G. axillaris* were screened against AChE using silica gel TLC plate according to Ellman's method (Rhee, 2003). The results of acetyl cholinesterase inhibition were tabulated in table 1. Pet. ether and $CHCl_3$ extracts showed white spots with detection limit 10 µg/ 5 µL, but the white spots for pet. ether extract was more intense than chloroform extract as shown in fig. 1a. However, the methanol extract was inactive on this TLC bio-autography assay. The isolated compounds were screened and compared with galanthamine as positive control. Squalene (1), myrstic acid (3) and palmitic acid (4) showed strong inhibition with detection limit at concentrations 2.0, 1.14 and 1.28 µg/ 5 µL respectively, compared to the galanthamine (1.84 µg/ 5 µL). β-amyrine palmitate (2) exhibited weak inhibition with detection limits 332.5 µg/ 5 µL, whereas quercitrin and epicatechin were inactive. The detection limit values of pet. ether and chloroform extracts indicated to high capacity to inhibit acetyl cholinesterase enzyme, may be by blocking the AChE or by catalyzing the reverse reaction of hydrolysis of acetylcholine as illustrated in the following equation:

The activity of pet. ether extract may have resulted from synergistic effect of the components in this extract including fatty acids and the triterpenes which have been reported to possess AChE inhibitory activity (Houghton *et al*, 2006). The purified components of pet. ether extract, squalene (1), myristic acid (3) and palmitic acid (4) exhibited different rates of inhibition against AChE on TLC assay as shown in fig. 1c. After 4.0 min of spraying with enzyme, the palmitic acid (P) showed white spots faster than myristic acid (M), while the spots of squalene

(S) were found inactive as shown in fig. 1c. However, as shown in fig. 1d, squalene (1), myrstic acid (3) and palmitic acid (4) gave white spots after 8.0 min of spraying with enzyme. Thus, conclude that the fatty acids are more influence in synergistic effect of the inhibition of pet. ether extracts.

Table 2: Antibacterial activity of compounds (2), (5) and (6) from the leaves of *Gynotroches axillaris*

Species	β-Amyrin palmitate (2)		Quercitrin (5)		Epicatechin (6)		Streptomycin sulfate		Nystatin	
	MIC (µg/mL)	MBC (µg/mL)	MIC (µg/mL)	MBC (µg/mL)	MIC (µg/mL)	MBC (µg/mL)	MIC (µg/mL)	MBC (µg/mL)	MIC (µg/mL)	MBC (µg/mL)
G+ bacteria										
B.s	225	225	900	1800	900	1800	225	Nd	nd	nd
S.a	225	225	900	1800	900	1800	28.13	Nd	nd	nd
G- bacteria										
E.c.	450	450	450	900	450	900	112.5	Nd	nd	nd
P.a.	225	225	900	1800	900	1800	225	Nd	nd	nd
Fungi										
A.n.	225	225	900	1800	900	1800	nd	Nd	56.2	nd
C.g.	450	450	450	900	450	900	nd	Nd	28.13	nd

nd = not determined; B.s.=Bacillus subtilis, S.a = Staphylococcus aureus, E.c.= Escherichia coli, P.a.= Pseudomonas aeruginosa, A.n= Aspergillus niger, C.g.= Candida glebreti.

Tyrosinase inhibition assay

Hyper pigmentation is characterized with high accumulation of melanins (Briganti *et al*., 2003). As the skin is exposed to UV radiation or inflammation, it leads to melanogenesis of melanin by tyrosinase enzyme resulting in skin disorder by darkening the skin (Balakrishnan *et al*., 2011). In this study, the efficacy of the extracts and isolated compounds to inhibit tyrosinase enzyme on oxidation of L-Dopa was tested, and the results are listed in table 1.

Table 1 shows the inhibition percentage (*I*%) against the tyrosinase enzyme for each sample tested in this assay. Methanol extract exhibited the highest percentage of inhibition (68.1%) followed by quercitrin (5) with *I*% (57.7%) compared to kojic acid (7) as a positive control with 83.5%. While other samples, pet. ether and $CHCl_3$ extracts, squalene (1), β-amyrin palmitate (2) and epicatechin (6) were not active in inhibiting oxidation of L-Dopa by tyrosinase enzyme.

The finding of this assay indicated that the major ability of methanol extract to inhibit tyrosinase enzyme may be due to the presence of quercitrin (5), which was isolated from the methanol extract, as quercitrin gave reasonably good tyrosinase inhibition activity with 57.7%. Quercitrin (5) contains a part from free hydroxyl at C-5 and carbonyl group at C-4 positions, similar to that part of kojic acid (7), play an important role to capture tyrosinase enzyme by chelating with copper ion of tyrosinase enzyme (Briganti *et al*., 2003), as shown by the bold line of quercitrin (5a) and kojic acid (7a) in fig. 2.

The influence of sugar moiety on reducing the inhibition percentage of quercetin-α-L-rhamnoside (5) was compared to the free aglycone, quercetin (8), which is a strong inhibitor against oxidation of L-Dopa by tyrosinase enzyme (Sirat *et al*., 2010; Hong *et al*., 2013; Jeong and Shim, 2004). Since quercetin (8) has two free hydroxyl groups at C-3 and C-5 positions at both sides of the carbonyl group (C-4), there are two similarities with kojic acid (7) to chelate copper of tyrosinase enzyme as shown in structures (8a) and (8b) in fig. 1. On the other hand, quercitrin (5) has rhamnoside moiety at C-3, which causes a hindrance effect, gives only one similarity (4-keto-5-hydroxy moiety) with kojic acid to chelate the copper ion of tyrosinase enzyme, with the bold lines of quercitrin (5a). As a result, the percentage inhibition against tyrosinase enzyme on L-Dopa oxidation of quercetin-α-L-rhamnoside (5) is lower than its free aglycone, quercetin (8) as compared to standard kojic acid (7). This was in agreement with the other previous studies, which reported that quercetin-α-L-rhamnoside (5) inhibited the tyrosinase enzyme on L-Dopa oxidation by being dependent on concentration, and the percentage inhibition was less than quercetin (8) (Hong *et al*., 2013; Jeong and Shim, 2004).

Antimicrobial activity

The antimicrobial activity results of compounds, (2), (5) and (6) were listed in table 2 and classified according to MIC results as follows: Strong inhibitors, if MIC is less than 500 µg/mL; Moderate inhibitors, if MIC is in between 600 and 1500 µg/mL; and Weak inhibitors, if MIC is more than 1600 µg/mL (Magina *et al*., 2009). The results showed that β-amyrin palmitate (2) was strong inhibitor towards both Gram-positive and Gram-negative bacteria, as well as fungi with MIC (450-225 µg/mL), whereas quercitrin (5) and epichatechin (6) showed varying degree of inhibition ranged from strong to weak towards Gram-positive and negative bacteria besides fungi.

CONCLUSION

Squalene (1), β-amyrin palmitate (2), myristic acid (3), palmitic acid (4), quercetin-3-*O*-L-rhamnoside (5) and epicatechin (6) were yielded and identified for the first time from *G. axillaris*. β-Amyrin palmitate (2) was obtained in a large quantity from this plant using very simple soxhlet extraction method and purification using VLC followed by recrystallization techniques. Hence, *G. axillaris* can be used as a source of β-amyrin palmitate (2), which is useful as antidyslipidemic (Maurya *et al*., 2012) and antidepressant (Subarnas *et al*., 1993) compound. The bioassay study revealed that the methanol extract and quercetin-3-*O*-L-rhamnoside (5) were potential natural inhibitor for tyrosinase enzyme, which causes skin hyperpigmentation. The results of anti-acetyl cholinestrase screening revealed that pet. ether and its components, squalene (1), myristic acid (3), palmitic acid (4), as well as chloroform extracts have high potential to inhibit acetyl cholinesterase which causes Alzheimer and other neurological diseases. In addition, β-amyrin palmitate (2) showed more significant antimicrobial activity than the other phytochemicals.

ACKNOWLEDGMENTS

The authors thank Research University Grant (GUP) from the Ministry of Higher Education (MOHE) for financial support under vote QJ130000.7126.01H01 and the Faculty of Science, University Teknologi Malaysia for the NMR and bioactivity facilities. The authors would also like to thank Kerbala University in Iraq for partial financial support.

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