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Phytochemical study and biological activities of *Scurrula parasitica* L (Loranthaceae) leaves

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ABSTRACT: Chemical constituents of *Scurrula parasitica* leaves from Loranthaceae family and some biological activities have been studied. Cold extractions were carried out using *n*-hexane, ethyl acetate and methanol to obtain the crude extracts. Purification of the extracts led to the isolation of quercetin **1**, quercitrin **2**, kaempferol 3-*O*-α-L-rhamnoside **3**, (+)-catechin **4**, lupeol **5,** lupeol palmitate **6**, β-sitosterol **7**, squalene **8**, octacosane **9**, octadecane **10** and eicosane **11**. The *in vitro* antioxidant activity of the extracts and isolated compounds were evaluated. Compounds **1**, **2**, **3** and **4**, together with ethyl acetate and methanol extracts exhibited effective antioxidant activities against DPPH, ABTS and FRAP assays, while *n*-hexane and other compounds were inactive. The ethyl acetate extract gave the highest tyrosinase percent inhibition value of 66.02%, while quercetin gave the best result with tyrosinase percent inhibition value of 79.09%. All extracts and isolated compounds showed weak activity on antimicrobial inhibition assay with the exception of quercetin which exhibited moderate activity against *Pseudomonas aeruginosa* with MIC and MBC value of 250 µg/mL.

KEYWORDS: Quercetin; *Scurrula parasitica*; loranthaceae; antityrosinase, antioxidant.

1. INTRODUCTION

Loranthaceae is the leading pantropical plant family with 77 genera and about 1000 species spread across the world. They are woody flowering plants generally known as mistletoes which are found growing in Africa, Asia, Europe, Australia, South America and New Zealand [1]. The medicinal application of species from Loranthaceae have been rationalized based on the ethnomedicinal uses, isolation and identification of the bioactive compounds in the classes of flavonoids, phenols, glycosides, alkaloids and triterpenes. Several plants belonging to this family have been widely used in traditional medicine as therapeutic herbs. For instance, the tea made from Loranthaceae plants is believed to cure body pain and bone fracture [2]. In Saudi Arabia, fresh stem of *Tapinanthus globiferus* are administered orally to all kind of livestock for fever treatment and removal of placenta after parturition [3]. In Europe, various preparations of *Viscum album* in the form of injectable extracts are sold as *Abnobaviscum*, *Helixor*, *Iscador*, and as fermented *V. album* and are used to treat arthritis, epilepsy, hypertension and infertility [4]. In Africa, infusion of the Loranthaceae leaves is used in the treatment of abdominal pains, diabetes, fever, and urinary tract infections [2]. The genus *Scurrula* is a prevalent parasitic plant found in Asia and has been used as traditional medicinal herbs for cancer, hypertension, microbial infections and as antioxidant [5]. In addition, species of genus *Scurrula* were used in Indonesia and Java to treat cancer and as infusion for fatigue [6]. The leaves and stems of *Scurrula parasitica* have been reported to be used as antioxidants, cardiotonic and antineoplastic agents in the southern part of China [7].

Previous phytochemical study carried out on the leaves of the Southern China *Scurrula parasitica* growing on *Nerium indicum*, reported the isolation of a new hydroxylated lupeol-based triterpenoid ester (3β,7β)-7-hydroxyl-lup-20(29)-en-3-yl hexadecanoate [8]. In addition, Quan-Yu *et al*. [9] have successfully investigated the methanol extract of *Scurrula parasitica* leaves and reported the isolation of a new triterpenoid ester 7β-hydroxyl-hop-22(29)-en-3β-*O*-palmitate. Our previous study showed that the methanol and ethylacetate extracts of *Globimetula braunii,* a Loranthaceae parasitic plant from Africa have a significant

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antioxidant activity [10]. Meanwhile, two lactone derivatives, (1*R*,5*S*,7*S*)-7-[2-(4-hydroxyphenyl)ethyl]-2,6-dioxabicyclo[3.3.1]nonan-3-one and dodoneine were identified from *Globimetula braunii* [11]. In our continuing chemical investigation of bioactive compounds from Loranthaceae plants, we have isolated quercetin **1**, quercitrin **2**, kaempferol 3-*O*-α-L-rhamnoside **3**, (+)-catechin **4**, lupeol **5**, lupeol palmitate **6**, β-sitosterol **7** squalene **8**, octacosane **9**, octadecane **10** and eicosane **11** (**Figure 1**) from the leaves part of *Scurrula parasitica*. To date, there is no report on the isolation of kaempferol 3-*O*-α-L-rhamnoside **3**, squalene **8**, octacosane **9**, octadecane **10** and eicosane **11**, and evaluation of tyrosinase inhibition activity from *S. parasitica*. We report here from this species, the isolation, structural elucidation and the antioxidant, antityrosinase and antimicrobial activities of the extracts and isolated compounds.

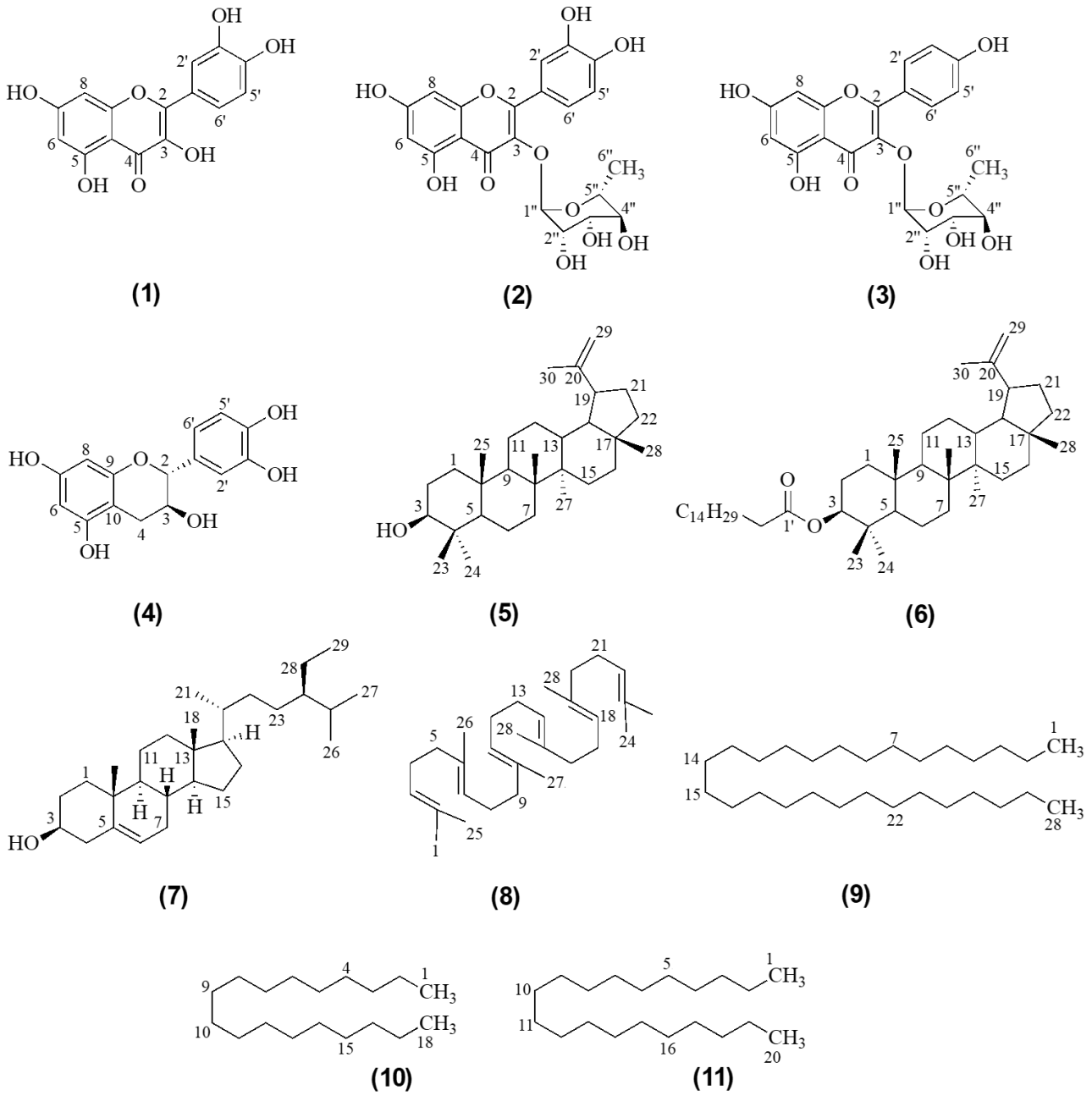

Figure 1. Chemical structures of compounds **1-11** isolated from the leaves of *S. parasitica*.

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2. RESULTS AND DISCUSSION

Phytochemical investigation of the leaves extract of *Scurrula parasitica* growing on *P. pinnata* yielded 11 known compounds. The chemical structures of these compounds have been identified as quercetin **1** [12], quercitrin **2** [13], kaempferol 3-*O*-α-L-rhamnoside **3** [14], (+)-catechin **4** [15], lupeol **5** [16], lupeol palmitate **6** [17], β-sitosterol **7** [18], squalene **8** [19], octacosane **9** [20], octadecane **10** [21] and eicosane **11** [22] on the basis of spectral analysis (1D, 2D NMR and MS) and comparison with reported data. **Table 1** summarized the complete analysis of ^{1}H and ^{13}C NMR of compounds **1**, **2**, **3** and **4**. The *in vitro* assays on the crude extracts (*n*-hexane (SPPPH), EtOAc (SPPPE) and MeOH (SPPPM)) and isolated compounds from the leaves of *Scurrula parasitica* were performed for antioxidant, antityrosinase and antimicrobial activities. The results are compiled in **Table 2**. It is important to point out that, this work represents the first report on the antityrosinase activity from the chemical point of view of this mistletoe species. The antioxidant activity was determined using DPPH, ABTS and FRAP assays. Our obtained data showed that SPPPE and SPPPM possessed potent antioxidant effect. Quercetin **1** exhibited strong antioxidant activity comparable to the positive controls used. The higher reducing value obtained for quercetin was previously suggested by Strube *et al*. [23]. The result demonstrated the higher oxidation potential of quercetin than quercitrin and kaempferol-3-*O*-α-L-rhamnoside. This is due to the importance of the catechol structure in the B ring which significantly enhances the antiradical activity as well as reducing the 3-hydroxyl group on the saturated C ring adjacent to a carbonyl group. The carbonyl group (4 oxo function) and 2,3 double bond in C ring support electron delocalization between the A and B ring and stabilize the aryloxyl radical after hydroxy donation. This implies that the presence of the 2,3 double bond in combination with carbonyl group (4 oxo function) in the C-ring could be beneficial for radical stabilization and strengthen the activity of the flavonols [24]. The result is in accordance with the previous work, in which a good antioxidant activity was shown by the crude extract of *S. parasitica* growing on different hosts plants [6]. In contrast, the weak antioxidant activity observed for SPPPH extract and the tested triterpenes could be described by the presence of non-polar compounds which have no ability to act as reducing agent [25].

SPPPE exhibited a significant antityrosinase percent inhibition of 66.03% (IC_{50} = 0.21±0.03 mM). However, SPPPM and SPPPH inhibited the L-DOPA oxidation at a percent inhibition less than 50.00%. Quercetin **1**, lupeol **5** and β-sitosterol **7** showed the highest inhibition activities with percent inhibition of 79.09%, 52.77% and 50.00% (IC_{50} values of 0.13±0.12 mM, 0.44±0.30 mM and 0.53±0.70 mM) respectively. Other isolated compounds were found to have weak to very low percent inhibition. The tyrosinase percent inhibition of the isolated compounds ranged from 19.60 - 79.09% lower than that of the positive control, kojic acid (83.81%, IC_{50} = 0.12±0.31 mM). Comparing the percent inhibition trends of all the tested compounds, it can be deduced that the presence of 3-hydroxy-4-keto moiety of quercetin played an important role in eliciting activity for the competetive inhibition of L-DOPA by tyrosinase enzymes through their power to chelate the copper in the active position, resulting to irreversible inactivation of tyrosinase [26]. This is in conformity with the previous finding of the tyrosinase inhibition activity carried out on some naturally and synthetic tyrosinase inhibitors [26]. The result showed that quercetin exhibited a significant tyrosinase inhibition activity with IC_{50} value of 0.07 mM (Kojic acid, IC_{50} = 0.014 mM) [26]. However, the flavonol glycosides, were found to exhibit weak or no inhibitory activity due to lack of free hydroxyl group at C-3 position which act as a significant part in copper chelation [26]. Previous work also reported dose-dependent tyrosinase inhibitory effect of triterpenoids such as betulin (IC_{50} = 1.44±0.03 mM) and lupeol (IC_{50} = 2.24±0.12 mM), which is comparable to that of the standard used kojic acid (IC_{50} = 0.68±0.11 mM) [27]. Therefore, the activity observed in lupeol **5** and β-sitosterol **7** suggested that triterpenoids can be considered as potential agents for treatment of pigmentation disorders. The antimicrobial activity of all the tested crude extracts and isolated compounds displayed weak to inactive inhibition against all the tested *Gram*-positive, *Gram*-negative and fungal strain tested with MIC values ranging from 500 – >1000 µg/mL. Only quercetin **1** showed moderate inhibition against *Pseudomonas aeruginosa* (MIC and MBC value 250 µg/mL). The weak antimicrobial activity of the extracts is in justification with the antibacterial assay carried out on acetone/water (8:2 v/v) extract of Malaysian *S. ferruginea* against *Gram*-positive and *Gram*-negative bacteria. The results showed that all the tested extracts including stem, leaf and flower possessed weak antimicrobial activities against *Staphylococcus aureus*, *Bacillus subtilis* and *Escherichia coli*. The moderate inhibition (225 µg/mL) observed in the study was against *Pseudomonas putida* which was not tested in this study [28]. The significance of the mistletoes in ethnopharmacology gave them the name "cure all" and this is supported by the scientific informations recorded in the biological studies of some of the crudes and purified extracts [2].

Table 1. ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectral data for compounds **1 - 4** in (δ in ppm, *J* in Hz)

Position	Compound 1 (CD_3COCD_3)		Compound 2 (CD_3OD)		Compound 3 (CD_3OD)		Compound 4 (CD_3OD)	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
2	-	147.5	-	157.8	-	157.9	δ 4.57 (1H, *J* = 7.4 Hz)	81.5
3	-	135.9	-	134.7	-	134.1	4.00 (1H, ddd = *J* = 8.0, 7.4 and 5.6 Hz)	67.4
4	-	175.7	-	178.2	-	177.2	2.53 (1H, dd, *J* = 16.8 and 7.0 Hz)	27.1
							2.88 (1H, dd, *J* = 16.8 and 7.0 Hz)	
5	12.19 (1H, s)	161.3	-	165.3	-	161.2	-	156.4
6	6.28 (1H, d, *J* = 2.0 Hz)	98.3	6.19 (1H, d, *J*= 2.0 Hz)	98.7	6.04 (1H, d, *J* = 2.0 Hz)	101.4	5.87 (1H, d, *J* = 2.4 Hz Hz)	94.9
7	-	164.3	-	161.7	-	161.3	-	156.2
8	6.54 (1H, d, *J* = 2.0 Hz)	93.6	6.35 (1H, d, *J*= 2.0 Hz)	93.5	6.16 (1H, d, *J* = 2.0 Hz)	95.5	5.94 (1H, d, *J* = 2.4 Hz)	94.1
9	-	103.2	-	157.2	-	156.8	-	155.5
10	-	156.9	-	104.3	-	102.1	-	99.4
1'	-	122.8	-	121.6	-	121.2	-	130.8
2'	7.84 (1H, d, *J* = 2.4 Hz)	114.8	7.35 (1H, d, *J*= 2.0 Hz)	115.6	7.73 (2H, d, *J* = 8.8 Hz)	130.3	6.85 (1H, d, *J* = 2.0 Hz)	113.8
3'	-	145.1	-	145.0	6.93 (2H, d, *J* = 8.8 Hz)	115.4	-	144.9
4'	-	146.2	-	148.5	-	160.9	-	144.8
5'	7.02 (1H, d, *J* = 8.4 Hz)	115.2	6.93 (1H, d, *J* = 8.4 Hz)	115.0	6.93 (2H, d, *J* = 8.8 Hz)	115.3	6.77 (1H, d, *J* = 8.2 Hz)	114.7
6'	7.72 (1H, dd, *J* = 8.4 and 2.4 Hz)	120.6	7.31 (1H, dd, *J* = 8.4 and 2.0 Hz)	121.4	7.73 (2H, d, *J* = 8.8 Hz)	130.3	6.74 (1H, dd, *J* = 8.2 and 2.0 Hz)	118.6
1''	-	-	5.36 (1H, d, *J* = 1.6 Hz)	102.3	5.35 (1H, d, *J* = 1.5 Hz)	101.9	-	s
2''	-	-	4.24 (1H, dd, *J* = 3.4 and 1.6 Hz)	71.9	4.24 (1H, t, *J* = 1.6 Hz)	70.5	-	-
3''	-	-	3.78 (1H, dd, *J* = 3.4 and 1.6 Hz)	70.5	3.75 (1H, m)	70.8	-	-
4''	-	-	3.40 (1H, m)	70.7	3.66 (1H, m)	71.9	-	-
5''	-	-	3.46 (1H, m)	70.6	3.49 (1H, m)	70.5	-	-
6''	-	-	0.96 (3H, d, *J* = 6.0 Hz)	16.3	0.93 (3H, d, *J* = 5.6 Hz)	16.2	-	-

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Table 2. Free radical scavenging (DPPH, $ABTS^{\bullet+}$ and FRAP) and tyrosinase inhibitory activities of leaf extracts and isolated compounds of *S. parasitica* L.

Extracts/ Compounds	DPPH IC_{50} (µg/ml)	ABTS IC_{50} (µg/ml)	mM FRAP equivalent to $FeSO_4.7H_2O$ (1.0 Mm)	TIA Inhibition (I%) at 100 µg/mL	TIA IC_{50} (mM)
Extracts					
n-Hexane	383.10	>5000	ND	44.60	ND
Ethyl Acetate	30.15	392.30	3.56±0.07	66.03	0.21±0.03
Methanol	30.71	386.30	4.80±0.10	48.44	ND
Pure Compounds					
(1)	0.82	312.01	3.94±0.49	79.09	0.13±0.12
(2)	22.48	410.90	3.74±0.07	49.82	ND
(3)	6.35	504.60	2.70±0.09	47.94	ND
(4)	8.49	513.50	3.44±0.02	31.53	ND
(5)	>1000	NT	NT	52.77	0.44±0.30
(6)	>1000	NT	NT	19.60	ND
(7)	>1000	NT	NT	50.00	0.53±0.70
(8)	>1000	NT	NT	NT	NT
(9)	>1000	NT	NT	NT	NT
(10)	>1000	NT	NT	NT	NT
(11)	>1000	NT	NT	NT	NT
Standard					
BHA	11.30	317.70	5.69±0.42	NA	NA
Trolox	0.90	354.01	NT	NA	NA
AA	NT	NT	5.06±0.07	NA	NA
Kojic Acid	NA	NA	NA	83.81	0.12±0.31

All values are expressed as mean ± SD for three replicates experiment. IC_{50} is defined as the concentration sufficient to obtain 50% of the maximum scavenging capacity; ND = Not Determined, NT =Not tested, NA =Not applicable, TIA = tyrosinase inhibitory activities

3. CONCLUSION

The information in this studies has demonstrated the antioxidant, antityrosinase and antimicrobial potential of *Scurrula parasitica* and thus provide some logical evidence supporting the traditional uses and medicinal beliefs by different ethnic groups in Asia and other parts of the world for alleviating skin disorders and as antioxidants based on the information in the literatures. To the best of our knowledge, this is the first report on the isolation of kaempferol 3-*O*-α-L-rhamnoside **3**, squalene **8**, octacosane **9**, octadecane **10** and eicosane **11**, and evaluation of tyrosinase inhibition activity from *S. parasitica*.

4. MATERIALS AND METHODS

4.1. General experimental procedures

The 1D and 2D NMR spectra were recorded on a Brüker Avance 400 MHz spectrometer. Chemical shifts were reported in ppm relative to tetramethylsilane (TMS) in deuterated solvents; chloroform ($CDCl_3$), acetone (CD_3COCD_3) or methanol (CD_3OD). The IR spectra were recorded on Perkins Elmer series 1600 spectrophotometer (KBr pellet for solid and NaCl discs for liquid samples). The ultraviolet (UV) spectra were measured on Shimadzu UV 1601PC spectrophotometer. Gas chromatography (GC) analysis was carried out on Hewlett Packard HP6890 and equipped with an Ultra-1 capillary column. The Gas Chromatography-Mass spectrometry (GCMS) analysis were recorded using Wiley Library Software in a similar capillary condition with GC except for the oven temperature program in which the temperature was programmed at 50°C (5 min), then 5°C/min to 300°C (5 min). Mass spectra were on a Finnigan-MAT-95 mass spectrometer from the Chemistry Department, National University of Singapore. Thin layer chromatography (TLC) was performed on 0.20 mm precoated silica gel aluminum sheets (Merck Kieselgel 60 F_{254}). The spots on TLC were detected by ultraviolet (UV) illumination at 254 nm and 365 nm and sprayed with vanillin sulphuric acid, before being heated on a hot plate. Vacuum liquid chromatography (VLC) was performed using Merck SiO_2 (230-400 Mesh). Gravity column chromatography (CC) was carried out on Merck SiO_2 (70-230 Mesh) and Sephadex LH-20, while preparative thin layer chromatography (PTLC) was conducted using 1 mm thin glass plate of Merck SiO_2 60 F_{254} (20 × 20 cm). All solvents and reagents used were of general purpose and analytical grade.

4.2. Plant material

Fresh leaves of *S. parasitica* parasitizing on *P. pinnata* were collected from Universiti Teknologi Malaysia (UTM), Southern Malaysia (Latitude N 1° 33' 54.9", Longitude E 7° 103', 29.2") in August 2016. A voucher specimen was authenticated by Dr. Shamsul Khamis, a plant botanist and deposited at the Department of Landscape Architect, Faculty of Design and Architecture, Universiti Putra Malaysia, (UPM) under the No SK2800/17 and SK28001/17 for *S. parasitica* and *P. pinnata*, respectively.

4.3. Extraction and isolation

The powdered leaves (1.45 kg) of *S. parasitica* was extracted using cold extraction method sequentially with solvents of different polarity starting with *n*-hexane, EtOAc and MeOH for three days each with shaking at room temperature to yield the crude extracts, *n*-hexane (SPPPH: 27 g, 1.87%), EtOAc (SPPPE: 32 g, 2.20%) and MeOH (SPPM: 45 g, 3.10%). Quercetin **1** (8 mg 0.03%) was isolated as yellow powder with R_f 0.56 (*n*-Hexane: EtOAc, 2:3) and m.p 300-302°C from SPPPE by repeated CC over SiO2 with *n*-hexane: EtOAc as eluents. Purification process of SPPPM using VLC (SiO_2 600 g, 10.0 cm × 10.0 cm) with $CHCl_3$:EtOAc:MeOH as eluents in stepwise gradient followed by CC over SiO_2 resulted in the isolation of quercitrin **2** (7 mg, 0.02%) as a yellow solid; R_f 0.62 ($CHCl_3$: MeOH, 4.2:0.8) and m.p 176-178°C, kaempferol 3-*O*-α-L-rhamnoside **3** (7.5 mg, 0.02%) as a yellow powder; R_f 0.65 ($CHCl_3$: MeOH, 4.2:0.8) and m.p 171-174°C, and (+)-catechin **4** (9.2 mg, 0.02%) as a pale brown powder with m.p. 174-176°C and R_f value of 0.45 in *n*-hexane: EtOAc (1:4). Purification of the *n*-hexane extract (SPPPH, 27 g) by VLC over SiO_2 (600 g, 10.0 cm × 10.0 cm) with *n*-hexane: $CHCl_3$: EtOAc as eluents in stepwise gradient followed by CC over SiO_2 *n*-hexane: EtOAc yielded lupeol **5** (28 mg, 0.10%) as a white powder; R_f 0.67 (*n*-Hexane: EtOAc, 4:1) and m.p. 214-216°C, lupeol palmitate **6** (243 mg, 0.9%) as white waxy solid; R_f 0.23 (*n*-Hexane:Et_2O, 4.6:0.4) and m.p 79-81°C, β-sitosterol **7** (174 mg, 0.64%) as white crystalline needles; R_f 0.45 (*n*-Hexane: EtOAc, 4.2:0.8) and m.p 132-134°C, squalene **8** and octacosane **9**, (151 mg, 0.56%) as white waxy powder with R_f 0.78 (*n*-Hexane: EtOAc, 4.9:0.1), and octadecane **10** and eicosane **11** (34 mg, 0.13%) as white waxy powder with R_f 0.78 (*n*-Hexane: EtOAc, 4.9:0.1).

4.4. Solvent and chemicals

Antioxidant: Analytical grade butylated hydroxyanisole (BHA), 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) were purchased from Sigma Aldrich. Ascorbic acid (AA) was purchased from Goodrich Chemical Enterprice (GCE), while sodium acetate trihydrate, ferric sulphate, 2,4,6-tripyridyl-s-triazine (TPTZ) and potassium persulfate were purchased from Fluka. All absorbances were measured using microplate reader EPOCH (Bio Tek). *Antimicrobial*: Nutrient agar (NA), sabouraud dextrose agar (SDA), Nutrient broth (NB), sabouraud dextrose broth (SDB), streptomycin sulphate and nystatin were obtained from Oxoid (Italy). All bacterial and fungi strains were purchased from Mutiara Saintifik Sdn. Bhd. (Malaysia). The bacterial and fungi strains were cultured and preserved in LB glycerol stock at -20°C prior to use. *Antityrosinase*: Kojic acid, mushroom tyrosinase, L-tyrosinase-3,4-dihydroxyl-L-phenyla- lanine (L-DOPA) were purchased from Sigma Aldrich.

4.5. Antioxidant activities

4.5.1. DPPH radical scavenging assay

The effect of the samples on DPPH radical scavenging activity were measured according to technique shown by Najihah *et al*. [29] with slight modification. DPPH reagents (100 µL) were added to the samples (100 µL) with concentration ranging from 1000-10 µg/mL in methanol acquired from serial dilution. The DPPH methanol solution was kept for each microplate and used as control while the radical scavenging effect was observed and compared with butylated hydroxyanisole (BHA) and trolox as the references. The reaction was allowed to incubate for 30 minutes in the dark at room temperature, the absorbance of the resulting solutions were measured at 517 nm. DPPH free radical scavenging activity in percentage (%) antioxidant was calculated using the formula:

$$\% \text{ DPPH Scavenging} = \frac{A_{\text{blank DPPH}} - A_{\text{sample}}}{A_{\text{blank DPPH}}} \times 100 \quad \text{(Eq. 1)}$$

$$*A_{\text{Sample}} = A_{\text{sample}} - A_{\text{bank DPPH}}$$

Where $A_{\text{blank DPPH}}$ is the absorbance of DPPH reagent with methanol and A_{sample} is the absorbance of the test compound with DPPH reagent. The SC_{50} value was determined as the concentration of each sample required to give 50% of the absorbance revealed by the control.

4.5.2. ABTS radical scavenging assay

ABTS assay was determined by method described by Zou *et al*. [30] with slight modification. Concentrations of 7 and 4.9 mM were respectively obtained by dissolving the ABTS and potassium persulfate in distilled water. The two solutions were equally mixed and kept for 12 to 16 hours in dark at room temperature. Distilled water was added repeatedly to the ABTS solution to obtained an absorbance of 0.7 at 734 nm. The ABTS solutions (190 µL) and sample solutions (10 µL) were added to 96-well plates. The mixture was allowed to react in dark at room temperature for 30 minutes and the absorbance was recorded at 734 nm. Percentage inhibition of ABTS radical was calculated as follows.

$$(\%) \text{ ABTS Scavenging} = \frac{A_{\text{blank ABTS}} - A_{\text{sample}}}{A_{\text{blank ABTS}}} \times 100 \quad \text{(Eq. 2)}$$

$$*A_{\text{Sample}} = A_{\text{ABTS}} + A_{\text{Sample}}$$

Where $A_{\text{blank ABTS}}$ is the absorbance of ABTS solution with methanol and A_{Sample} is the absorbance of the test samples with ABTS solution.

4.5.3. Ferric reducing antioxidant potential (FRAP) Assay

FRAP assay was measured according to technique described by Channarong *et al*. [31] and Shahwar *et al*. [32] with slight modification. Acetate buffer (300 mM), TPTZ (2,4,6-tripyridyl-s-triazine) 10 mM in HCl (40 mM) and $FeCl_3 \cdot 6H_2O$ (20 mM) were mixed in the ratio of 10: 1: 1 to obtained the FRAP reagent. The FRAP reagent (150 µL), methanol (15 µL) and test sample (0.5 µL) were added to the 96-well plates. The reduction of ferric-tripyridyltriazine (Fe^{3+}-TPTZ) complex to ferrous (Fe^{2+}) resulted in the development of absorption maximum with an intense blue coloration. The absorbance was then measured at 593 nm after water bath incubation at 37°C [33]. The solutions of the $FeSO_4 \cdot 7H_2O$ ranging from 0.1 to 1.0 mM in methanol was used to acquire the known Fe^{2+} concentration calibration curve. Antioxidant concentration of ferric-TPTZ reducing capacity corresponding to (0.1 to 1.0 mM) $FeSO_4 \cdot 7H_2O$ was considered as the parameter equivalent concentration.

4.6. Tyrosinase inhibition assay

The *in vitro* tyrosinase inhibitory activity was determined according to method described by Likhitwitayawuid and Sritularak [34] and Promden *et al*. [35], with slight modification. All the tested samples were dissolve in DMSO and tested at concentraion of 100 µg/mL, 50 µg/mL, 25 µg/mL, 12.5 µg/mL and 6.25 µg/mL. The test reaction mixture comprised of each sample (0.1 mg/mL, 40 µL), mushroom tyrosinase enzyme (100 units/mL, 40 µL) and L-DOPA solution (2.5 mM, 40 µL) in sodium phosphate buffer (100 mM, 80 µL, pH 6.8) all added to a 96-well plate. The reaction mixture (200 µL) was incubated at 37°C for 10 min, and the absorbance was monitored at 475 nm. The absorbance of the same mixture with DMSO instead of sample was used as negetive control, while kojic acid, a known tyrosinase inhibitor was employed as positive control. The percentage inhibition was calculated using the formula:

$$\text{Inhibition (\%)} = \left(\frac{\text{Absorbance (blank)-Absorbance (tyrosinase+sample}}{\text{Absorbance (blank)}} \right) \times 100 \quad \text{(Eq. 3)}$$

4.7. Antimicrobial activity

The *in vitro* antimicrobial activity was carried out against two *Gram*-positive bacteria (*Staphylococcus aureus* ATCC29737 and *Bacillus subtilis* ATCC6633), two *Gram*-negative bacteria (*Escherichia coli* ATCC10536 and *Pseudomonas aeruginosa* ATCC9027) and a fungi (*Aspergillus niger* ATCC16888). The microbes were melted from the glycerol stock and cultured in NB and SDB for bacterial and fungal, respectively. The broth media were pre-incubated to promote the growth of microorganisms. NB (8 g/L) and SDB (8 g/L) were dissolved in distilled water. *Broth Microdilution Assay*: Minimum inhibition concentration (MIC) and minimum bacterial/fungal concentration (MBC/MFC) values were measured by the broth microdilution techniques according to method described by Gulluce *et al*. [36] and Murray *et al*. [37]. The MIC test were determined in a sterile 96-well microliter plates. Each crude extract (14.5 mg) and pure compounds (3.6 mg) were separately dissolved in MeOH (2.0 mL) to obtain a sample stock solution with concentration 1800 µg/mL. A few wells were reserved in each plate for positive and negative controls. Sterile NB/SDB (100 µL) which were used as diluents were added to each microliter plate well from B to H followed by the addition of samples stock (100 µL) in the first two row (A and B). Twofold serial dilution of the mixture of sterile broth and stock samples (100 µL) in row B was transferred to each well in order to obtain a concentration range from 1800 to 14.13 µg/mL. Each bacterium (100 µL) and fungi (100 µL) were added to each well such that the final volume in each plate was 200 µg/mL. Streptomycin sulphate and nystatin were employed as positive controls for bacteria and fungi, respectively. The microplates of different samples were then incubated at 37˚C for 24 hrs for bacteria and 30˚C for 48 hrs for fungi. Microbial growth was indicated by the appearance of turbidity and a pellet at the base of the well. The last clear well was determined as MIC value which is the lowest concentration of the tested samples without visible signs of microorganism growth. In determining the (MBC/MFC) values, 10 µL of the clear well containing mixture of tested samples and inoculums were pipetted and spread gently on the surface of the NA (5 mL) for bacteria and SDA (5 mL) for fungi in a petri dish (90 × 15 mm) using glass rod. The plates were sealed and incubated upside down at 37˚C for 24 hrs (bacteria) and 30˚C for 48 hrs (fungi). The MBC/MFC was recorded as the lowest concentration of the sample at which 99% of the bacteria/fungi colony were killed after the incubation period.

4.8. Statistical analysis

Three replicates for each tested sample were used with data presented as mean ± standard deviation. The statistical analysis was performed using SPSS for windows (version 21). Data obtained from biological analysis were analysed using one way ANOVA.

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