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Isolation, Identification and Glucose Uptake Activity of Flavonoids from *Bouea macrophylla* Griff.

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Bouea macrophylla Griff. (*B. macrophylla)* is a tropical fruit-bearing tree belonging to the Anacardiaceae family. Various parts of this plant have many uses including in culinary, cultural as well as ethnopharmacology uses. Previous research by our group on a closely related plant from the same genus, *B. oppositofolia*, revealed the presence of various flavonoids. The objective of the current study was to isolate and identify flavonoids from *B. macrophylla*. The isolation process involved purifying the twig extract of *B. macrophylla* using chromatographic techniques, including Vacuum Liquid Chromatography (VLC), Column Chromatography (CC), and preparative-Thin Layer Chromatography (pTLC). The structures of isolated compounds were determined through spectroscopic methods including Nuclear Magnetic Resonance (NMR), infrared (IR), and ultraviolet (UV) spectral data analysis, as well as comparison with previous literature data. Five flavonoids (**1**–**5**) have been successfully isolated, namely fustin (**1**), taxifolin (**2**) and the isomers of astilbin which are neoastilbin (**3**), astilbin (**4**) and isoastilbin (**5**). The results of glucose uptake assay indicated that the astilbin isomers tested has effect on glucose uptake rate of insulin-resistant myoblast cell line as compared to the standard drug, rosiglitazone. This is the first report describing elucidation of the isolated flavonoids from *B. macrophylla* as well as its glucose uptake study.

Keywords: *Bouea macrophylla*; kundang; flavonoids; isolation; elucidation; HPLC; NMR; glucose uptake

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The present study focused on investigating the chemical constituents of the twigs of *B. macrophylla*, a flowering plant native to Southeast Asia and belonging to the Anacardiaceae family. This plant is extensively grown in countries such as Indonesia, Burma, Malaysia, and Thailand [1]. In Malaysia, the plant is known by several names, including *kundang*, *kundang daun besar*, and *setar*. Both the fruit and young leaves of this plant are edible, with claims of health benefits, such as antipyretic activity related to fever-reducing efficacy [2]. Several reports have also validated the bioactivities of this plant, including antidiabetic, antimicrobial, anticancer, antioxidant, anti-photoaging and it also possesses moisturizing effects [3-5]. Flavonoids including flavone, flavonol, flavanol, flavanonol, flavanone, chalcone, anthocyanidin and aurone as well as triterpenoids are two major classes of compounds found in the plants of the same tribes (Anacardieae), followed by tannins, some alkaloids and many other phenolic derivatives [6-8]. The research was specifically focused on the flavonoid class of compounds, which has previously shown significant antidiabetic properties in related plants. Considering the limited literature available on the isolation and bioactivities of compounds from *B. macrophylla*, this study was conducted to isolate and identify flavonoids from this plant. The identified chemical constituents of *B. macrophylla* will provide insights into its potential pharmacological activities, particularly in terms of antidiabetic properties. It contributes valuable information to the limited existing literature on this plant and opens avenues for further research and development of potential medicinal applications.

EXPERIMENTAL

Chemicals and Materials

All chemicals utilized in the study were of analytical grade and obtained from Sigma Chemical Co. (St. Louis, Missouri). The twigs of *B. macrophylla* were collected in Pasir Mas, Kelantan, Malaysia, and their identification was confirmed by Dr. Shamsul bin Khamis, a botanist at Universiti Kebangsaan Malaysia

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(UKM). Voucher specimen (Voucher number UKMB 40432) of the collected twigs were deposited in the Universiti Kebangsaan Malaysia's Herbarium, Department of Biological Sciences and Biotechnology, Faculty of Science and Technology.

Extraction and Isolation

4 kg of powdered *B. macrophylla* twigs were subjected to extraction using three different solvents - hexane, ethyl acetate (EA), and methanol. Each extraction was performed by macerating the plant material in 10 L of the respective solvent for 24 hours. The solvents were then collected and evaporated using a rotary evaporator under reduced atmospheric pressure, resulting in dark brown sticky residues. The hexane extraction was performed three times, yielding a total of 32 g of crude extract. Similarly, the EA extraction was repeated, resulting in 25 g of crude extract, while the methanol extraction produced 142 g of crude extract. Thin-layer chromatography (TLC) was used to profile all the extracts. Among the three extracts, the EA crude extract showed the most promising profile and was selected for further purification. Conventional methods such as vacuum liquid chromatography (VLC), column chromatography (CC), and preparative TLC (pTLC) were used for this purpose. The EA crude extract was subjected to VLC using silica gel as the stationary phase. It was eluted with mixtures of hexane and EA in increasing order of polarity (ranging from 100:0 to 0:100), followed by an EA and methanol gradient (ranging from 90:10 to 80:20). This process resulted six fractions 1-6. Further fractionation of fraction 5 (2.22 g) by CC using the same solvent ratios as in VLC produced nine subfractions (51-59). Subsequently, fraction 56 (55.7 g) was subjected to pTLC with a 9:1 ratio of chloroform : methanol, resulting in the isolation of fustin (**1**) (2.6 mg). Further fractionation was done on fraction 72 (40.7 mg) by using pTLC with solvent system chloroform : methanol (ratio 8:2) to yield a pure compound namely taxifolin (**2**) (5.4 mg). The CC on fraction 7 (2.53 g) yielded seven subfractions, 71-77. Directly from this step, a mixture of isomers namely neoisoastilbin (**3**), astilbin (**4**) and isoastilbin (**5**) (7.7 mg) were isolated.

Purification and Structure Elucidation

The structural elucidation of the isolated compound was done by using several spectroscopic methods. IR spectra were performed on Bruker Tensor II FT-IR while UV spectra were recorded on Gen-5 Microplate Reader (Synergy HT). The ^{1}H NMR and ^{13}C NMR were recorded in methanol-d_4 on Bruker 600 Ultra-shield NMR spectrometer measured at 600 *MHz* for ^{1}H NMR and 151 *MHz* for ^{13}C NMR. Peak multiplicities were presented in *Hz* and chemical values were shown in ppm (δ). Various chromatographic techniques have been used to purify the chemical constituents. For fractionation, liquid chromatography (VLC) was applied by using silica gel 60, 70 – 230 mesh ASTM (Merck 1.07747). Further fractionation was done by using conventional method including VLC, CC and pTLC.

Glucose Uptake Assay

Glucose uptake assay was performed to determine the rate of uptake of radioactively tagged 2-deoxy glucose in differentiated C2C12 myoblast cells [9]. Initially, cells were plated in 96 well plate (black and clear bottom) at a cell density of 8×10^4 cells/mL and incubated in an incubator supplemented with 5% (v/v) CO_2 at 37°C for overnight to allow cell attached. Cells were then allowed for differentiation process from myoblasts to myotubes cells. The differentiated myotube cells were treated with various concentration of sample, from 100 – 1.562 µM for isolated compound **1** – **5**; 100 – 1.562 µg/ml for crude extracts and 200 µM insulin as positive control [10]. The plate was read for relative fluorescence units (RFU) using microplate reader at 465/540 nm (Tecan, USA). The fold increase of glucose uptake activity was calculated.

Cytotoxicity Assay

Cytotoxicity assay was performed on C2C12 cells. Briefly, 150,000 cell/mL were plated into the 96 well plate (clear bottom). The plates were incubated in an incubator supplemented with 5% (v/v) CO_2 at 37°C for overnight to allow cells to be attached. Cells were exposed to compounds with various concentrations from 1000 – 1.5625 µM and 1000 – 1.562 µg/ml for crude extracts and incubated for another 24 hours. The ranges of concentrations were chosen to access the highest test concentrations caused a dose dependent cytotoxic effects on cells [11]. The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reagent was applied to access the metabolic activity of living cells. The assay plate was analysed by micro-plate reader (Tecan, USA) measured at a wavelength of 570 nm. Measurements were performed, and the concentration required for a 50% inhibition of viability (IC_{50}) was determined.

RESULTS AND DISCUSSION

Characterization

Phytochemical studies on the twigs of *B. macrophylla* have resulted on the isolation of five flavonoid compounds. These included fustin (**1**), taxifolin (**2**) and the isomers of astilbin which are neoastilbin (**3**), astilbin (**4**) and isoastilbin (**5**). The identification of these compounds was achieved through ^{1}H and ^{13}C NMR spectroscopy and by comparing their spectroscopic data with values obtained from existing literature.

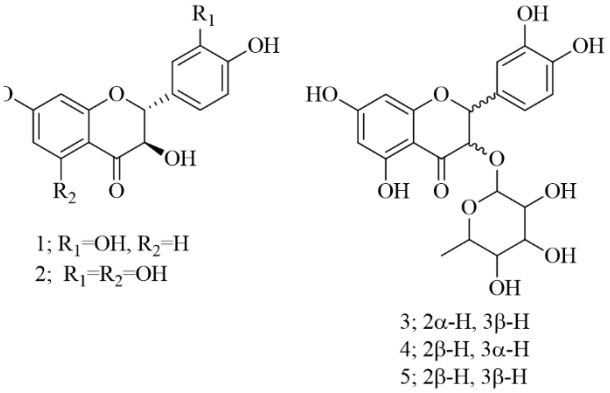

Figure 1. Structure of compound **1** – **5**

Compound **1** (2.6 mg) was isolated as yellow amorphous powder and has a molecular formula of $C_{15}H_{12}O_6$, consistent with the pseudo molecular ion $[M+H]^+$ peak shown at *m/z* 287.05 in HR-ESI-TOF-MS (Figure 4). Due to the limited amount of the isolated compound, further characterisation was made based on the ^{1}H NMR only. The ^{1}H NMR signals which resonates at δ_H 5.00 (*d*, *J* = 11.8 Hz, 1H, H-2) and 4.54 (*d*, *J* = 11.9 Hz, 1H, H-3) are the identity of a flavanonol moiety. The large coupling constant between H-2 and H-3 indicated a *trans* relationship. Signals forming an ABD spin system can be observed in the spectrum which resonates at δ_H 7.74 (*d*, *J* = 8.7 Hz, 1H, H-5), 6.63 (*dd*, *J* = 8.6, 2.2 Hz, 1H, H-6) and 6.42 (*d*, *J* = 2.3 Hz, 1H, H-8), which is an indication of 7-hydroxyl substitution in ring A. However, the arrangement of the substituents in ring B differs in which another ABD spin system appears at δ_H 7.10 – 6.88, confirming the attachment of two substituents at C-3' and 4'. Based on the data discussed above as well as comparison with literature [7], the compound is elucidated as fustin (**1**). It was previously isolated from the trunk of *Horsfieldia pandurifolia* [12] and shows cytotoxicity against HeLa cells [13].

Compound **2** (5.4 mg) was purified as yellow amorphous powder. The pseudo molecular ion $[M+H]^+$ peak at *m/z* 303.05 (Figure 5) corresponding to the molecular formula of $C_{15}H_{12}O_7$. The ^{13}C APT NMR spectrum showed a total 15 signals. A conjugated ketone signal can be seen at downfield region at δ_C196.7 (C-4). The signals at δ_C 83.68 (C-2) and 72.26 (C-3) which belongs to the aliphatic oxygenated methine carbons indicate the flavanonol skeleton. Other than that, four aromatic oxyaryl carbons (δ_C 144.84-163.94), five aromatic methine carbons (δ_C 95.20-119.49) and two quartenary carbons (δ_C 100.17 and 128.56) is shown in the spectrum. The presumed flavononol skeleton is further supported by the resonances of two oxygenated aliphatic methine protons at δ_H 4.90-4.80 (overlapped with solvent signal, 1H, H-2) and 4.45 (*d*, *J* = 11.3 Hz, 1H, H-3) in ^{1}H NMR. The coupling constant indicated a *trans*-configuration at H-2 and H-3. Other than that, two doublets at δ_H 5.81 (*d*, *J* = 2.1 Hz, 1H, H-6), 5.78 (*d*, *J* = 2.0 Hz, 1H, H-8) can be seen which corresponded to two *meta*-coupled aromatic methine protons in ring A. Furthermore, an ABD spin system appears at δ_H 6.99 (d, *J* = 2.0 Hz, 1H, H-2'), 6.86 (*dd*, *J* = 8.2, 2.1 Hz, 1H, H-6') and 6.82 (*d*, *J* = 8.2 Hz, 1H, H-5') which indicative to trisubstitution in ring A. Through the analysis of aforementioned spectral data and comparison with the literature, this compound is identified as taxifolin (**2**) [7]. The occurrence of taxifolin has been previously reported from *Oryzae sativa* [14]. This compound has been proven to gives antigenotoxic effects to H_2O_2-induced DNA damage [15], and have the ability to cause tumor regression [16].

Furthermore, a mixture of compound **3-5** (7.7 mg) was isolated as yellow amorphous powder. The molecular formula of $C_{21}H_{22}O_{11}$ was deduced from the pseudo-molecular ion peak [M+H]+ at m/z 449.11 (Figure 6). The ^{13}C APT spectrum showed three sets of signals clustered at certain chemical shifts with different ratio, an indication of three isomers with different proportion. The presence of the flavanonol core is readily apparent with ^{13}C chemical shifts in the region of δ_C 80-83(C-2), 74-77 (C-3) and 192-196 (C-4). The flavanonol possess two asymmetric centres therefore four stereoisomeric forms are theoretically possible, however, in this mixture only three compounds are detected. Additionally, the signals showed similar pattern as **2**, but with additional sugar moiety. The methyl peaks at δ_C 16.48, 16.43, 16.36 (C-6'') gave an indication of rhamnoside sugars. Analysis on the long-range correlation showed correlation between anomeric proton (C-1'') which resonates at δ_C 101. 44, 100.76 and 98.81 with C-3 of the aglycone. Hence, it can be concluded this isolate comprises of three isomers (which will be referred as **a, b** and **c**) and has the structure of taxifolin with the attachment of rhamnose at C-3. The ^{1}H NMR signals for H-2 and H-3 were compared for the determination of its relative stereochemistry. For **a** and **b**, the large coupling constant (*J* = ~11) were observed indicating *trans* orientation.

In contrast, compound **c** exhibited small coupling (J = ~2) showing the *cis*-isomer. Since the isomers presented in mixtures and ^{1}H NMR coupling constants did not permit unequivocal differentiation for the assignment of the absolute configuration, the structure of the compounds was deduced from previous reports based on the elution order of these isomers on reverse phase C18 column [17]. Thus, based on the spectroscopic evidence and comparison with reported data, compound **a** is identified as neoastilbin (2S,3S)-Taxifolin-3-O-α-L-rhamnoside (**3**), compound **b** as astilbin (2R,3R)-Taxifolin-3-O-α-L-rhamnoside (**4**) and compound **c** as isoastilbin (2R,3S)-Taxifolin-3-O-α-L-rhamnoside (**5**). All of the isomers have been previously reported from *Bafodeya benna* [17] and exhibited antimicrobial property by suppressing the growth of *Streptococcus sobrinus* 18]. On another note, **3** has been reported to has a unique immuno-suppressive activity, a selective inhibition against activated T-lymphocytes [17]. Furthermore, **4** exhibits anti-acne and tyrosinase inhibition properties [6], while **5** shows potent antioxidant and anti-inflammatory activities [19]. Table 1 shows the summary of NMR data obtained for isolated compounds.

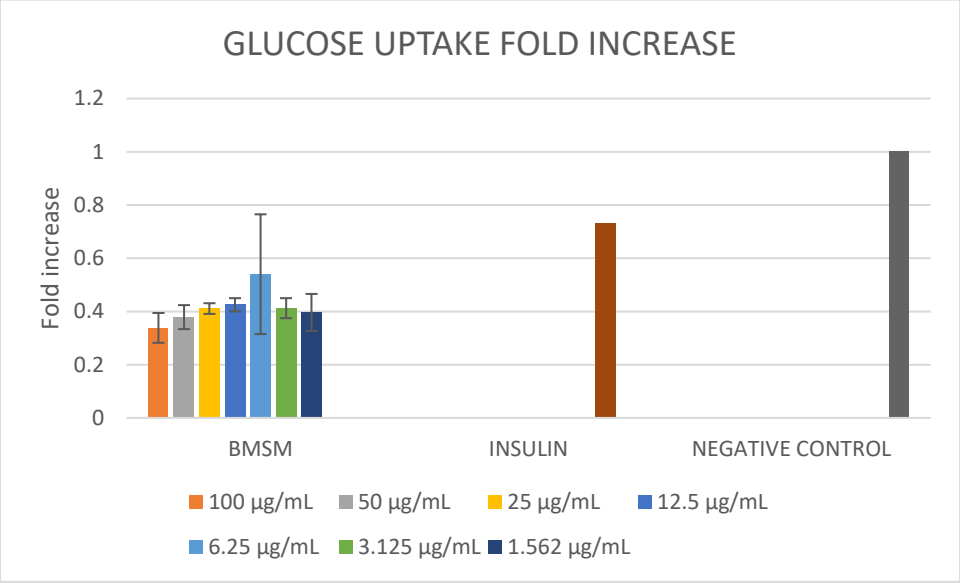

Figure 2. Glucose uptake assay of BMSM on insulin-resistant C2C12 cell line. Glucose uptake activity in untreated control (1% DMSO) was assigned as 1-fold.

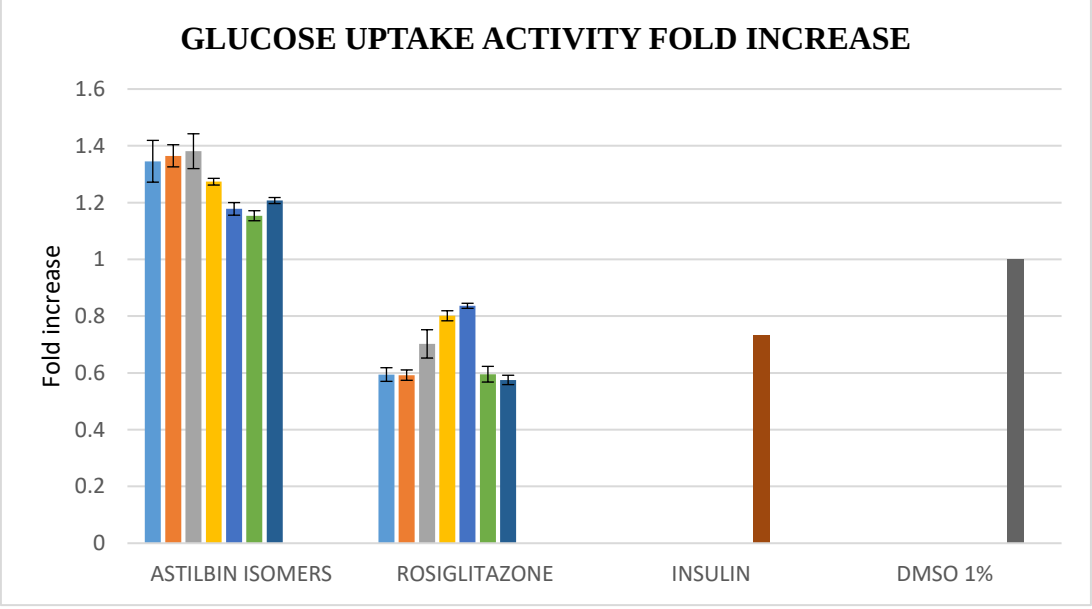

Figure 3. Glucose uptake assay of astilbin isomers, rosiglitazone (standard drug) and insulin (positive control) on insulin-resistant C2C12 cell. Glucose uptake activity in untreated control (1% DMSO) was assigned as 1-fold.

Table 1. The glucose uptake rate and cytotoxicity activity.

Extract/Compound	Folds increase of glucose uptake at the indicated concentration	Cytotoxicity (IC_{50})
BMSM	0.56 at 6.25 ug/ml	> 1000 µg/ml
Astilbin isomers	1.28 at 25 µg/ml	> 100 µM
Rosiglitazone	0.53 at 6.25 µg/ml	> 100 µM

Table 2. NMR data of isolated compound **1** – **5**.

Position	1	2		3		4		5	
	δ_H	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
2	5.00 (d)	4.86 (overlapped)	83.68	5.01 (d,)	82.30	5.10 (d)	82.56	5.44 (d)	80.7
3	4.54 (d)	4.45 (d)	72.26	4.66 (d,)	75.51	4.60 (d)	77.20	4.21 (d)	74.2
4			196.67		196.22		194.54		194.6
5	7.74 (d)		163.94		164.08		164.12		164.8
6	6.63 (dd)	5.81 (d)	96.20	5.95 (d)	96.08	6.00 (d)	96.02	5.99 (d)	96.0
7			175.90		167.48		167.27		168.7
8	6.42 (d)	5.78 (d)	95.20	5.91 (d)	94.90	5.92 (d)	94.92	5.95 (d)	96.8
9			163.09		162.92		162.70		163.1
10			100.17		100.68		101.12		98.8
1'			128.56		128.60		127.82		127.3
2'	7.10 (d)	6.99 (d)	114.70	7.02 (d)	114.97	6.98 (d)	114.95	6.97 (d)	113.8
3'			144.84		145.22		145.15		145.0
4'			145.72		146.04		145.98		145.3
5'	6.88 (d)	6.82 (d)	114.50	6.82-6.87 (m, overlap)	114. 80	6.82-6.87 (m, overlap)	114.14	6.82-6.87 (m, overlap)	115.0
6'	6.94 (dd)	6.86 (dd)	119.49	6.82-6.87 (m, overlap)	119.55	6.82-6.87 (m, overlap)	119.10	6.82-6.87 (m, overlap)	118.0
1''				5.18 (d)	101.44	4.09 (d)	100.76	4.98 (d)	100.4
2''				4.03 (dd)	70.60	3.57 (dd)	70.60	3.68 (dd)	70.5
3''				3.41 (dd)	70.82	3.68 (dd)	70.66	3.48 (dd)	70.6
4''				3.24 (d)	72.08	3.31 (overlapped)	72.45	3.21 (d)	71.9
5''				2.34 (dq)	68.91	4.27 (dq)	69.13	2.50 (dq)	69.1
6''				0.93 (d)	16.48	1.21 (d)	16.43	0.96 (d)	16.4

Glucose Uptake and Cytotoxicity

Type 2 Diabetes mellitus (T2DM) is commonly linked to factors such as obesity, genetics, and physical inactivity, and it may lead to various complications like high blood pressure, heart disease, and kidney failure. To help T2DM patients maintain appropriate blood glucose levels, one effective approach is to enhance glucose uptake in organs and tissues. In this study, a 2-deoxyglucose uptake assay was conducted to assess the insulin-like and insulin-sensitizing activities of isolated compounds from *B.macrophylla*. The C2C12 myoblast cell line was chosen as the *in vitro* model for examining metabolic disease progression, and it has been extensively used for this purpose in previous research [20]. EA crude extract (BMSM) which was chosen for the isolation of flavonoids and the mixture of astilbin isomers (**3-5**) were tested for glucose uptake activity. Compound **1** and **2**, however, were not isolated in sufficient amount for further study. BMSM showed an increase in glucose uptake activity and the highest uptake was produced at concentration of 6.25 µg/mL with 0.56-fold (Figure 2). Additionally, the astilbin isomers exhibited an interesting activity in glucose uptake assessment when compared with rosiglitazone, standard drug and insulin that act as positive control. These isomers significantly increased glucose transport into C2C12 cells, in a dose-dependent manner from 50 µM to 3.125 µM with the maximal glucose uptake of

1.284 ± 0.04-fold increase at 25 µM. In comparison, rosiglitazone yielded a maximal effect at a dose of 6.25 µM of 0.836 ± 0.009-fold increase. Even at the same concentration, the isomers showed higher glucose uptake compared to the standard with 1.129 ± 0.009-fold increase. It showed that the isomers possess a better activity than a standard drug (Figure 3). No toxicity effect was noticed for both compounds when tested on differentiated myotubes C2C12 cell line, with IC_{50} value of more than 1000 µM. Table 1 represents the fold increase of glucose uptake activity and cytotoxicity activity.

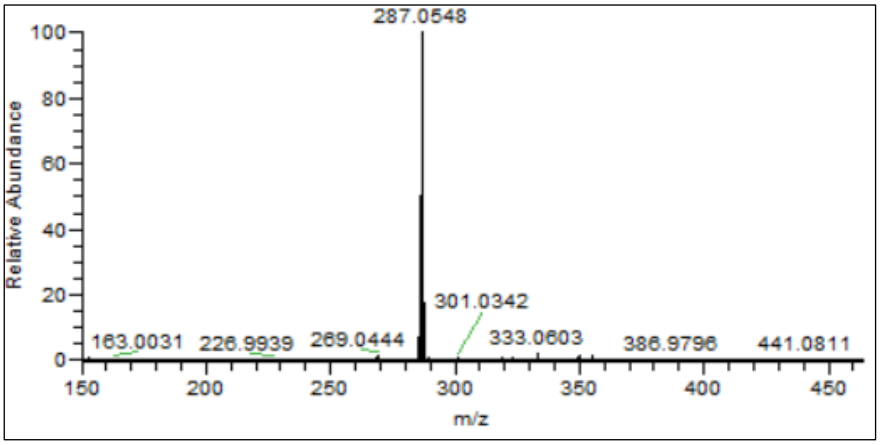

Figure 4. Mass spectrum of **1**.

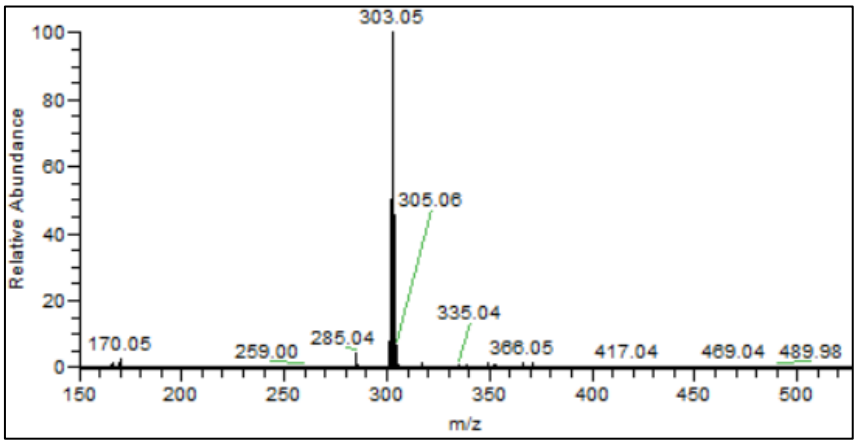

Figure 5. Mass spectrum of **2**.

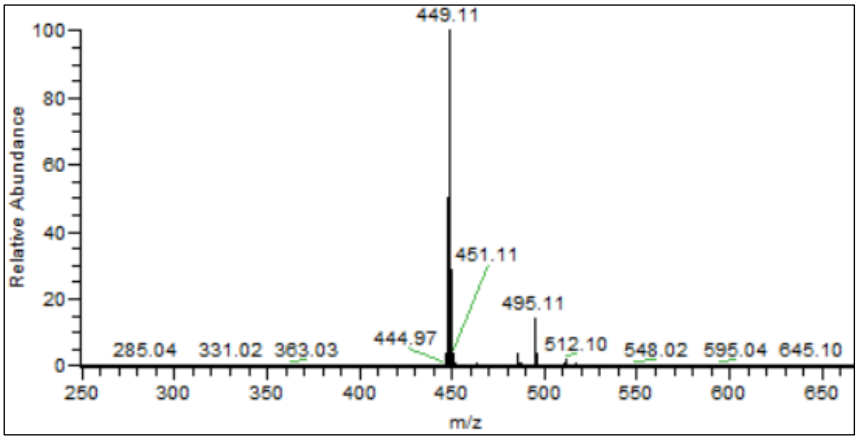

Figure 6. Mass spectrum of **3-5**.

CONCLUSION

Five flavonoids were isolated and identified: fustin (**1**), taxifolin (**2**) and the isomers of astilbin which are neoastilbin (**3**), astilbin (**4**) and isoastilbin (**5**), all of which were reported for the first time in *B. macrophylla*. The glucose uptake results indicated that the isolated isomers (**3** – **5**) showed significant activity in enhancing the glucose uptake rate of insulin-resistant C2C12 cell line compared to the standard drug rosiglitazone. Additionally, no toxicity effects were observed when tested using the MTT assay. Given that previous reports have demonstrated diverse pharmacological properties of similar phenolic compounds, further in-depth investigations into the biological activities of these flavonoids are strongly recommended. The isomers also should be purified and tested individually to validate the bioactivities. By delving deeper into their potential health benefits and mechanisms of action, these newly identified compounds from *B. macrophylla* could hold promise for various therapeutic applications.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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