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Article

Anti-hyperglycemic fraction from *Alternanthera sessilis* L. leaves gets elucidated following bioassay-guided isolation and mass spectrometry

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The anecdotal use of *Alternanthera sessilis* L. as a relief for diabetes has been known in the Philippines for generations, and antidiabetic activity of similar varieties in other countries is likewise documented. However, the compounds responsible for this activity remain unclear. This study aims to isolate the anti-hyperglycemic fraction of local *A. sessilis* leaves and identify the compounds in this fraction. Methanol extract of *A. sessilis* leaves and its hexane, ethyl acetate (ASE), and water fractions were administered to alloxan-induced diabetic mice. ASE (250mg/kg) had the highest anti-hyperglycemic activity at 6-h post-treatment (25.81%±12.72%), with almost similar blood glucose reduction rate as metformin (30.13±3.75%, $p=0.767$). Repeated fractionation employing chromatographic separation techniques followed by *in vivo* anti-hyperglycemic assay yielded partially purified subfractions. *A. sessilis* ethyl acetate subfraction 4-2 (100mg/kg) displayed remarkable suppression of blood glucose rise in diabetic mice at 6-h post-treatment (26.45±3.75%, $p<0.0001$), with comparable activity with metformin (100mg/kg, 27.87±5.65%, $p=0.652$). Liquid chromatography/mass spectrometry showed eight distinct peaks, with four peaks annotated via the Traditional Chinese Medicine library and custom library for *A. sessilis*. Among these, luteolin, apigenin, ononin, and sophorabioside were identified as putative compounds responsible for the anti-hyperglycemic activity. This result provided basis for the reported anecdotal claims and potential utility of the local variety of *A. sessilis* leaves as sources of anti-hyperglycemic agents.

Keywords: *Alternanthera sessilis*. Anti-hyperglycemic. Apigenin. Luteolin. Bioassay-guided isolation.

INTRODUCTION

Diabetes mellitus is a chronic disease consisting of an array of dysfunctions characterized primarily by abnormally high blood glucose levels (hyperglycemia) resulting from multiple genetic, lifestyle, and environmental factors. In the Philippines, 6.3% of adults have diabetes in 2019, which is estimated to increase in prevalence to 11.0% by 2030 (International Diabetes Foundation, 2019). This increasing proportion of diabetes cases results in additional healthcare expenditures, not only for treating the disease but also for preventing associated complications. The total cost of expenditures for diabetes management and its complications in the Philippines has reached 428.8 USD per person (International Diabetes Foundation, 2019). Given the disease to be affecting mainly the adult population, this would mean loss of productivity, lower quality of life, and decreased economic growth, imposing a huge socio-economic burden. Moreover, studies revealed that people with diabetes and other

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metabolic diseases were at higher risk for contracting COVID-19 due to their immunocompromised state (Arcellana, Jimeno, 2020). Thus, diabetes necessitates careful management to prevent vulnerability to other debilitating diseases.

Different drug classes are used for the pharmacological treatment of diabetes. Although these commercially available drugs have proven to aid in the management of diabetes by acting through various mechanisms, adverse effects may occur in patients such as gastrointestinal problems, organ toxicity, weight gain, and hypersensitivity reactions, among others (Luna, Feinglos, 2001; Moses, 2010). Cases of diabetes continue to rise despite the availability of treatments, and these have been associated with lifestyle changes including increased food intake, lack of exercise, and stress. Thus, it becomes imperative to search for alternative sources of antidiabetic treatments with advantageous features such as enzyme inhibitor, gene expression inhibitor, modulator of multiple metabolic pathways, transport pathways, or signaling pathways, in combinatorial format.

Plants have been considered valuable resources in the discovery and introduction of new therapeutic agents for diabetes. One of these plants is *Alternanthera sessilis* L., which is used in folkloric medicine in the Philippines, particularly in Western Visayas, as a treatment for diabetes (Monroy, Limsiaco, 2016). The study of Das *et al.* (2015) demonstrated the antidiabetic activity of the ethanol extract of *A. sessilis* where its aerial parts is found in India. This antidiabetic activity reduces the fasting blood glucose in streptozotocin-induced rats. Another variety of this plant in Bangladesh displayed blood glucose reduction in oral glucose tolerance test in mice (Hossain *et al.*, 2014). A Malaysian variety of this plant, *A. sessilis* Red, also showed α-glucosidase inhibitory activity (Chai *et al.*, 2016), and antidiabetic activity in high fat, streptozotocin-induced diabetic rats (Tan, Kim, 2013). The Philippine variety of this plant has green stems and white flowers. Conversely, Malaysia, India, and Bangladesh varieties have traces of red violet shade in their stems and flowers, which are different from our local variety (India Biodiversity Portal, 2018; Flora, Fauna Web, 2020). Based on these literatures, it would be of interest to determine if the local variety of this plant possesses the same blood glucose-lowering activity compared to other variants, including the identification of the putative compounds responsible for this activity. Thus, this study aimed to determine the anti-hyperglycemic fractions of the local *A. sessilis* leaves through bioassay-guided isolation in alloxan-induced diabetic mice.

MATERIAL AND METHODS

Material

Reagents for the extraction and fractionation were of analytical grade. Alloxan monohydrate and Tween 80 used for the *in vivo* anti-hyperglycemic studies were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO) while column chromatography was performed using silica gel (60 – 200 mesh, JT Baker). Chromatography-grade acetonitrile (Duksan), triethylamine (Scharlau), formic acid (Fischer Chemical), and methanol (JT Baker) were used for the ultra-performance liquid chromatography/quadrupole time-of-flight tandem mass spectrometry (UPLC/Q-TOF MS). Glucophage® metformin procured from a local pharmacy was used as the positive control for the *in vivo* anti-hyperglycemic study.

Methods

Collection and preparation of A. sessilis

The plant *A. sessilis* was obtained from San Jose de Buenavista, Antique (10° 45' 7.20" N, 121° 56' 27.60" E) during the mornings of March - September 2016. Fresh leaves (3000 g) were collected, placed in a Styrofoam cooler, garbled, and air-dried. A voucher specimen was authenticated in the National Museum-Philippines with control number: 16-04-427.

Preparation of crude extract and fractions

The air-dried leaves of *A. sessilis* were milled into powder (1255.47 g), macerated with methanol (12 L)

for 24 h, filtered, then macerated again twice for 4 h. The dried crude methanol extract was subjected to fractionation of solvents with increasing polarity in sequential order via Kupchan method of partitioning (Tan, Kim, 2013). A portion of the crude extract (110 g) was dissolved in distilled water (1000 mL) and extracted exhaustively with hexane (940 mL) using a separatory funnel until no color change was observed in the hexane layer. The hexane fraction was separated, and the remaining water fraction was extracted five times with ethyl acetate (700 mL). The extract and fractions were concentrated using a rotary evaporator, except for the lyophilized water fraction.

Experimental animals

ICR mice, ages 6 - 10 weeks old and weighing 23 ± 6 g were purchased and housed in the animal laboratory of the National Institutes of Health, University of the Philippines Manila. The mice were placed in cages and acclimatized one week before the study under ambient temperature and a 12-h dark/light cycle. They were fed on a standard pellet diet and water *ad libitum*. Baseline body weight was measured 7 days after acclimatization. The acute toxicity and *in vivo* anti-hyperglycemic studies were approved by the University of the Philippines Manila Institutional Animal Care and Use Committee (IACUC) prior to the conduct of the animal study with approval number: 2016-018.

Acute toxicity studies

The acute toxicity of the extract/fractions was based on the Organization for Economic Cooperation and Development (OECD) 425 Limit Test (2008). Twenty (20) female ICR mice were fasted for 6 h prior to dosing. They were randomized into four (4) groups: crude methanol extract, hexane, ethyl acetate, and water fractions of *A. sessilis* leaves, with five (5) mice in each group. Crude methanol extract and water fraction were dissolved in 0.1% Tween 80, while ethyl acetate and hexane fractions were dissolved in corn oil for the preparation of doses. A 2000 mg/kg body weight of each treatment was given sequentially to each mouse per group at 48-h intervals. The mice were observed individually for signs of toxicity, as specified in the guidelines, at first 30 min after dosing, at 24 h, and daily thereafter, for a total of 14 days. Body weights were measured before treatment, and after 7 and 14 days.

Induction of hyperglycemia

The induction of hyperglycemia was based on the method of Zhao *et al.,* (2013) with minor modifications in the rest period. Mice of different sexes were fasted for at least 12 h, then injected intraperitoneally with a freshly prepared alloxan in normal saline at a dose of 200 mg/kg body weight. The mice were given a standard pellet diet and water *ad libitum.* A rest period of 48 h, with free access to food and water, was allowed for stabilization. Blood glucose levels were measured before induction (baseline) via a blood glucose meter. The precision of the blood glucose meter used was determined using a control solution.

Mice with baseline blood glucose levels of ≥250 mg/dl were considered diabetic and were selected for the study. Negative control (nondiabetic) mice were injected with 10 mL/kg body weight of normal saline solution and were given the same care and management as diabetic mice.

In vivo anti-hyperglycemic study of crude extract and fractions

Crude methanol extract, water fraction, and metformin were dissolved in 0.1% Tween 80, while ethyl acetate and hexane fractions were dissolved in corn oil. The mice were then fasted for at least 6 h and randomized into groups (n = 5 mice per group): ND – normal control, NC CO – corn oil (negative control for hexane and ethyl acetate fractions), NC T80 – Tween 80 (negative control for crude methanol extract and water fraction), MET – metformin 250 mg/kg (positive control), ASM – *A. sessilis* crude methanol extract 250 mg/kg, ASH - *A. sessilis* hexane fraction 250 mg/kg, ASE – *A. sessilis* ethyl acetate fraction 250 mg/kg, and ASW – *A. sessilis* water fraction 250 mg/kg. The 250 mg/kg dosing was based on the effect of two-

week administration of the same dose of *Alternanthera sessilis* red fraction in diabetic rats (Tan and Kim, 2013). The treatments were administered orally via a gastric feeding tube. Blood glucose levels from the tail vein were then measured at 0.5 h, 1 h, 3 h, and 6 h after treatment administration. To compare the anti-hyperglycemic activity among groups, % blood glucose reduction was computed using the equation below. The fraction with the highest % blood glucose reduction was used as the basis for determining the most active anti-hyperglycemic fraction.

$$\%\ Blood\ glucose\ reduction = 1 - \frac{Blood\ glucose\ levels_{x-h}}{Blood\ glucose\ levels_{0-h}} \text{ x } 100$$

where,

Blood glucose levels$_{x\text{-}h}$ – blood glucose levels collected at specified time point

Blood glucose levels$_{0\text{-}h}$ – blood glucose levels collected at baseline

Bioassay-guided partial purification of anti-hyperglycemic fractions

The dried *A. sessilis* ethyl acetate (ASE) obtained from the solvent-solvent partitioning step was further fractionated using a glass column packed with silica gel. The fraction was loaded in the packed column and successively eluted with gradient mixtures of hexane and ethyl acetate (100:0%, 95:5%, 93:7%, 91:9%, 89:11%, 87:13%, 85:15%, 83:17%, 81:19%, 79:21%, 77:23%, 75:25%, 50:50%, 30:70%, and 0:100%), followed by gradient mixtures of ethyl acetate, methanol and 0.1% formic acid (90:10:0.1%, 70:30:0.1%, 30:70:0.1%, and 0:100:0.1%). The subfractions were pooled based on their thin layer chromatographic (TLC) profiles and subjected to *in vivo* anti-hyperglycemic study (n = 5) at a 250 mg/kg dose using the above method (ND – normal control, NC T80 – Tween 80 (negative control), MET – metformin 250 mg/kg (positive control), ASEF1 to ASEF4 – *A. sessilis* ethyl acetate subfractions 1-4, 250 mg/kg).

Fraction 4 (5 g) was selected for further purification using repeated reversed-phase HPLC chromatographic separation. The subfraction was diluted with methanol to 20 mg/mL and separated on Symmetry® C18 5.0 µm 3.9 x 150 mm HPLC column, connected to a Phenomenex® C18 3 x 4 mm pre-column. The mobile phase consisted of acetonitrile with 0.01% triethylamine, pH 10 (solvent A), and water with 0.01% triethylamine, pH 10 (solvent B). The gradient conditions of the mobile phase were as follows: 0 → 7 min – 5.5% A; 7 → 14 min – 7% A; 14 → 15 min – 90% A; 15 → 20 min – 100% A. The applied flow rate for the analysis was 0.3 mL/min and the injection volume was 20 µL. Samples were filtered using a 0.45 µm PTFE syringe filter prior to injection. The detection wavelength used were 366 and 254 nm. The subfractions were dried and subjected to the same *in vivo* anti-hyperglycemic study above using a 100 mg/kg dose, with the following groups (n = 5): ND – normal control, NC T80 – Tween 80 (negative control), MET – metformin 100 mg/kg (positive control), ASEF4-1 to ASEF4-5 – *A. sessilis* ethyl acetate 4 subfractions 1-5, 100 mg/kg. The dose of the subfractions given was lowered due to the limited amount of samples collected from the second fractionation.

Metabolite profiling by UPLC/Q-TOF MS

Subfraction 2, which was prioritized for UPLC/Q-TOF MS analysis, was dissolved in acetonitrile. A 5-µL subfraction was filtered in a 0.2 µm syringe filter and injected on an Acquity HSS T3 1.8 µm 2.1 x 100 mm column using Waters UPLC I-Class system. A binary mobile phase was employed consisting of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B) at a flow rate of 0.2 mL/min. The gradient conditions of the mobile phase were as follows: 0 → 0.01 min – 95% A; 0.01 → 3 min – 60% A; 3 → 10 min – 56% A; 10 → 13.5 min – 5% A; 13.5 → 17 min – 95% A.

Mass spectrometry was performed on Xevo G2-XS QTOF and operated in MSE small molecules screening acquisition mode. The capillary voltage was set to 2 kV, source temperature at 120°C, desolvation temperature at 500°C, desolvation gas flow at 950 L/h, cone voltage at 40V, cone gas flow at 50 L/h, and collision energy at 6 – 50 eV. A triplicate run of MS data was performed in positive ESI with an m/z range of 100 – 1000. Leucine

enkephalin was used as a reference lock mass calibrant for mass correction. Sodium formate was used for the mass range calibration and Catechin Mix standards for the quality control.

The MS data were acquired and processed using the incorporated UNIFI Scientific Information System v18 software (Waters Corp., USA) which enables the identification of parent mass by matching from the in-house libraries. The base peak chromatogram (BPC) was used for the analysis, with ≥ 30,000 detector counts as criteria to discriminate prominent peaks. The BPC of the subfraction and blank (acetonitrile) were compared, and the peaks present in the blank injection were excluded from the analysis.

The distinct peaks of the subfraction were annotated through an accurate mass screening of metabolites from the Waters Traditional Chinese Medicine Library and constructed the library of known compounds of *A. sessilis* from literatures. The identified putative compounds from the libraries were manually verified using METLIN and Mass Bank based on their MS data.

Statistical analysis

Quantitative results were presented as mean ± standard deviation (SD). Analysis of variance (ANOVA) followed by Tukey's post hoc test using SPSS software version 23.0 was used to compare the % blood glucose reduction of controls and treatment. A 0.05 level of probability ($p \leq 0.05$) was used as a criterion for significance.

RESULTS AND DISCUSSION

Acute toxicity studies of crude extract and fractions

In Western Visayas, the *A. sessilis* plant is used as a vegetable and relish in local cuisines. Although there has been no incident of reported poisoning/toxicity from the consumption of this plant, it is still important to determine the safety profile of the fractionated components to check for their individual toxicity.

The mice treated with *A. sessilis* leaf crude extract and its fractions did not display any signs of toxicity (distress, convulsions, coma, or death) at 2000 mg/kg dose during the short- (first 24 h) and long-term (14 days) observations. There was no statistically significant difference ($p>0.302$) in the weights of the mice of the crude extract and fractions before treatment, after 7 and 14 days.

The median lethal doses (LD50) of the extract and fractions were greater than 2000 mg/kg, indicating their wide safety profile.

In vivo anti-hyperglycemic study of crude extract and fractions

The air-dried leaves of *A. sessilis* were extracted with methanol to yield ASM (154.05 g). Solvent-solvent partitioning of a portion of ASM yielded ASH (26.74 g), ASE (16.14 g), and ASW (14.65 g). The extract and fractions were then subjected to *in vivo* anti-hyperglycemic study to identify the potent fraction for further purification.

The blood glucose levels of ASM and its fractions against alloxan-induced diabetic ICR mice are shown in Figure 1. These treatments showed an increase in blood glucose levels at 0.5-h to 3-h post-treatment, followed by a slow decline. This blood glucose pattern of ASM and its fractions are consistent with previous literature in which blood glucose levels tend to rise upon meal administration in fasted mice. Subsequent glucose uptake by different tissues results in a gradual drop in blood glucose levels (Benedé-Ubieto *et al.*, 2020). Metformin was also observed to have a similar anti-hyperglycemic activity to the study of Khatun *et al.,* (2011), wherein treatment of 200 mg/kg of metformin decreased the blood glucose levels from 32.0 to 14.9 mmol/L within 4 h in alloxan-induced diabetic rats.

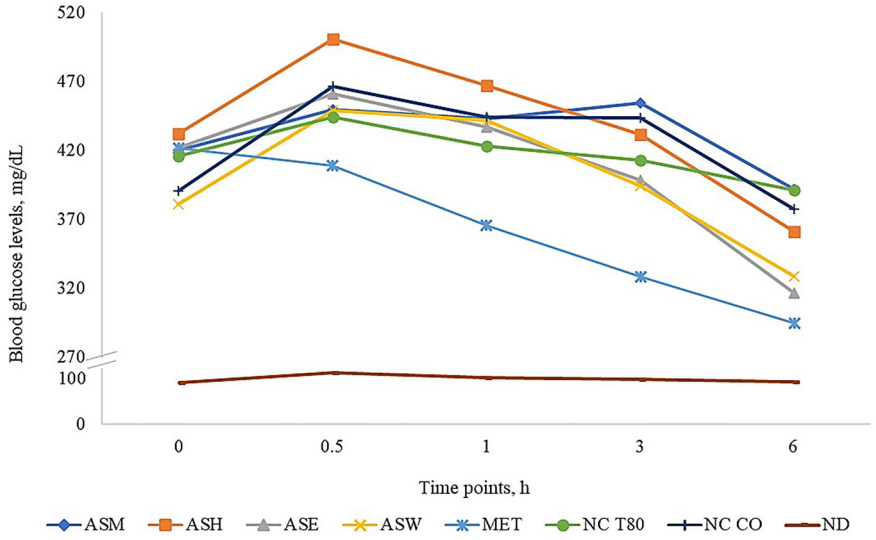

FIGURE 1 - Blood glucose levels of diabetic mice treated with 250 mg/kg *A. sessilis* crude extract and fractions measured at different time points. ASM – *A. sessilis* crude methanol extract, ASH – *A. sessilis* hexane fraction, ASE – *A. sessilis* ethyl acetate fraction, ASW – *A. sessilis* water fraction, MET – metformin (250 mg/kg), NC T80 – negative control 0.1% Tween 80 (10 mL/kg), NC CO – corn oil (10 mL/kg), ND – nondiabetic control (10 mL/kg saline).

To determine the most active anti-hyperglycemic fraction, % blood glucose reduction was computed. A noticeable increase (>5%) in the blood glucose lowering from baseline (0 point) to 6-h post-treatment was observed in MET > ASE > ASH > ASW > ASM (Figure 2). However, nondiabetic mice (ND) and negative controls (NC T80 and NC CO) did not show prominent lowering of blood glucose levels below baseline (≤5%). Only ASH *($p=0.026$)* and ASE *($p<0.0001$)* at 6-h post-treatment demonstrated statistically significant differences with their negative control, NC CO. Interestingly, only ASE (25.81±12.72%) had the potent anti-hyperglycemic activity and presented almost similar activity with MET at 6-h after treatment *($p=0.944$)*. ASE was then selected for partial purification using open-column chromatography.

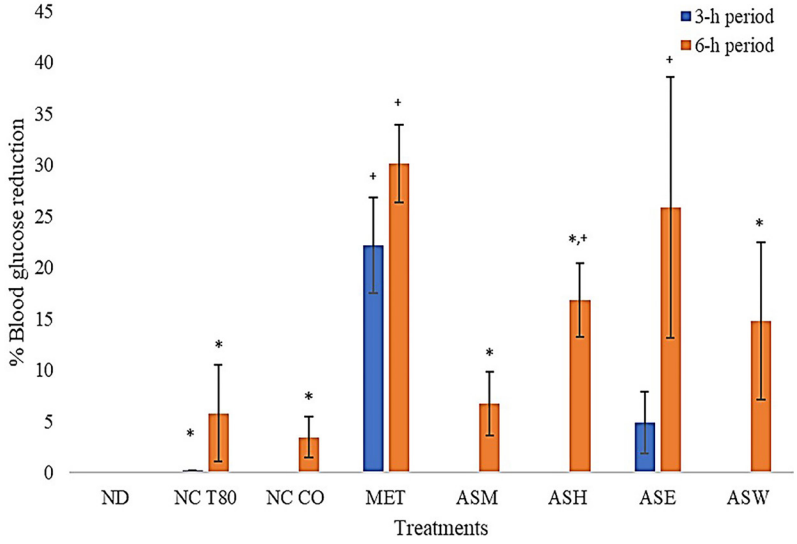

FIGURE 2 - Blood glucose reduction values of diabetic mice treated with 250 mg/kg *A. sessilis* crude extract and fractions at 3-h and 6-h period (less than 0 values not shown). ASM – *A. sessilis* crude methanol extract, ASH – *A. sessilis* hexane fraction, ASE – *A. sessilis* ethyl acetate fraction, ASW – *A. sessilis* water fraction, MET – metformin (250 mg/kg), NC T80 – negative control 0.1% Tween 80 (10 mL/kg), NC CO – corn oil (10 mL/kg), ND – nondiabetic control (10 mL/kg saline). $^{*,+}$ - Significantly different compared to positive (MET) and negative controls, respectively.

ASE (15 g) was subjected to column chromatography with silica gel as the stationary phase, which resulted in four fractions: ASEF1 (1.58 g), ASEF2 (1.17 g), ASEF3 (0.52 g), and ASEF4 (6.14 g). Consistent with the results of the *in vivo* anti-hyperglycemic study above, the subfractions manifested blood glucose lowering of more than 5% at 0.5- to 3-h post-treatment relative to their baseline values (Figure 3). Conversely, NC T80 and ND displayed no significant changes in their blood glucose levels compared to baseline.

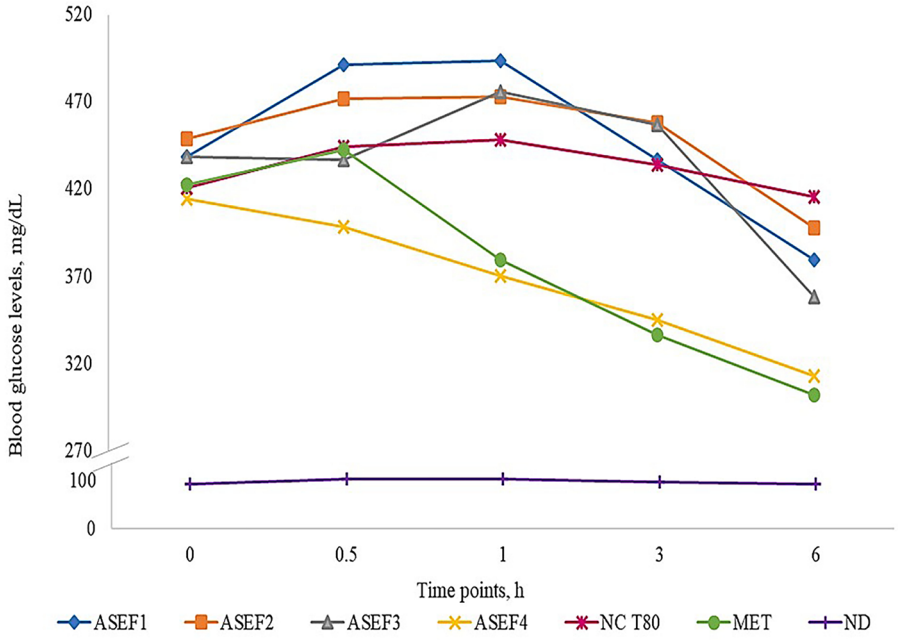

FIGURE 3 - Blood glucose levels of diabetic mice treated with 250 mg/kg *A. sessilis* ethyl acetate (ASE) subfractions measured at different time points. ASEF1 to 4 – ASE subfractions 1 to 4, MET – metformin (250 mg/kg), NC T80 – negative control 0.1% Tween 80 (10 mL/kg), ND – nondiabetic control (10 mL/kg saline).

Among the subfractions, ASEF4 demonstrated a statistically significant difference in its blood glucose reduction values at 3-h *($p=0.005$)*, and 6-h *($p<0.0001$)* post-treatment compared to the negative control, NC T80 (Figure 4). Alternatively, ASEF1 and ASEF3 exhibited marked blood glucose lowering relative to negative control at 6 h after treatment *($p=0.013$* and *0.001,* respectively*)*. For MET, a prominent reduction of blood glucose levels in comparison to negative control was observed at 3-h *($p=0.001$)* and 6-h *($p<0.0001$)* post-treatment.

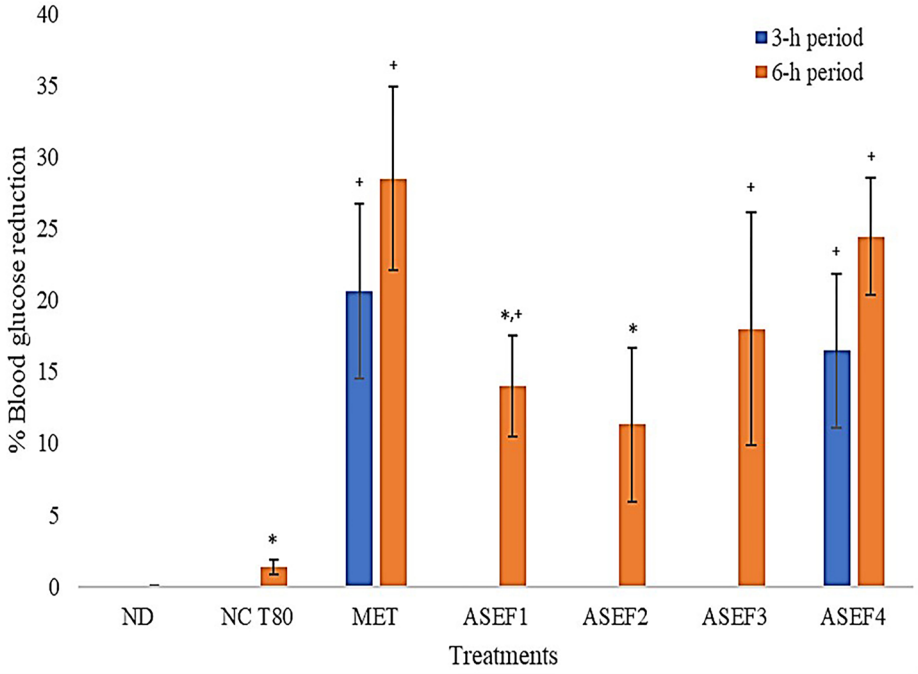

FIGURE 4 - Blood glucose reduction values of diabetic mice treated with 250 mg/kg *A. sessilis* ethyl acetate (ASE) subfractions at 3-h and 6-h period (less than 0 values not shown). ASEF1 to 4 – ASE subfractions 1 to 4, MET – metformin (250 mg/kg), NC T80 – negative control 0.1% Tween 80 (10 mL/kg), ND – nondiabetic control (10 mL/kg saline). *, + - Significantly different compared to positive (MET) and negative controls, respectively.

Comparison of blood glucose reduction values of MET (3 h: 20.59±6.11%, 6-h: 28.50±6.41%) with the subfractions showed that only ASEF4 manifested almost similar blood glucose reduction at 3-h (16.45±5.34%, *p=0.983*) and 6-h (24.44±4.06%, *p=0.888*) post-treatment. The highest anti-hyperglycemic activity based on the 6-h blood glucose reduction values was displayed by ASEF4, followed by ASEF3, ASEF1, and ASEF2. ASEF4 also retained the >20% blood glucose reduction of ASE and this fraction was further purified using HPLC.

ASEF4 yielded five fractions namely ASEF4-1 (0.55 g), ASEF4-2 (0.43 g), ASEF4-3 (0.47 g), ASEF4-4 (0.54 g) and ASEF4-5 (0.80 g) (Figure 5). Due to the limited amount of fractions collected, the subfractions were subjected to *in vivo* anti-hyperglycemic study at a lower dose of 100 mg/kg dose compared to the *in vivo* anti-hyperglycemic studies of the previous fractions. All the subfractions, except ASEF4-2, demonstrated the same blood glucose pattern as the *in vivo* anti-hyperglycemic studies shown above (Figure 6). This is characterized by an increase in the blood glucose levels from 0.5-h to 1-h, followed by a continuous decline after 3-h post-treatment. Interestingly, ASEF4-2 demonstrated a gradual linear decrease in blood glucose levels up to 6-h post-treatment, similar to that of ASEF4. Moreover, only ASEF4-2 exhibited statistically significant suppression of blood glucose rise at 3-h *(p=0.011)*, and 6-h *(p<0.0001)* post-treatment relative to baseline. MET also exhibited a statistically significant decrease in blood glucose levels between baseline and 6-h post-treatment *(p=0.015)*.

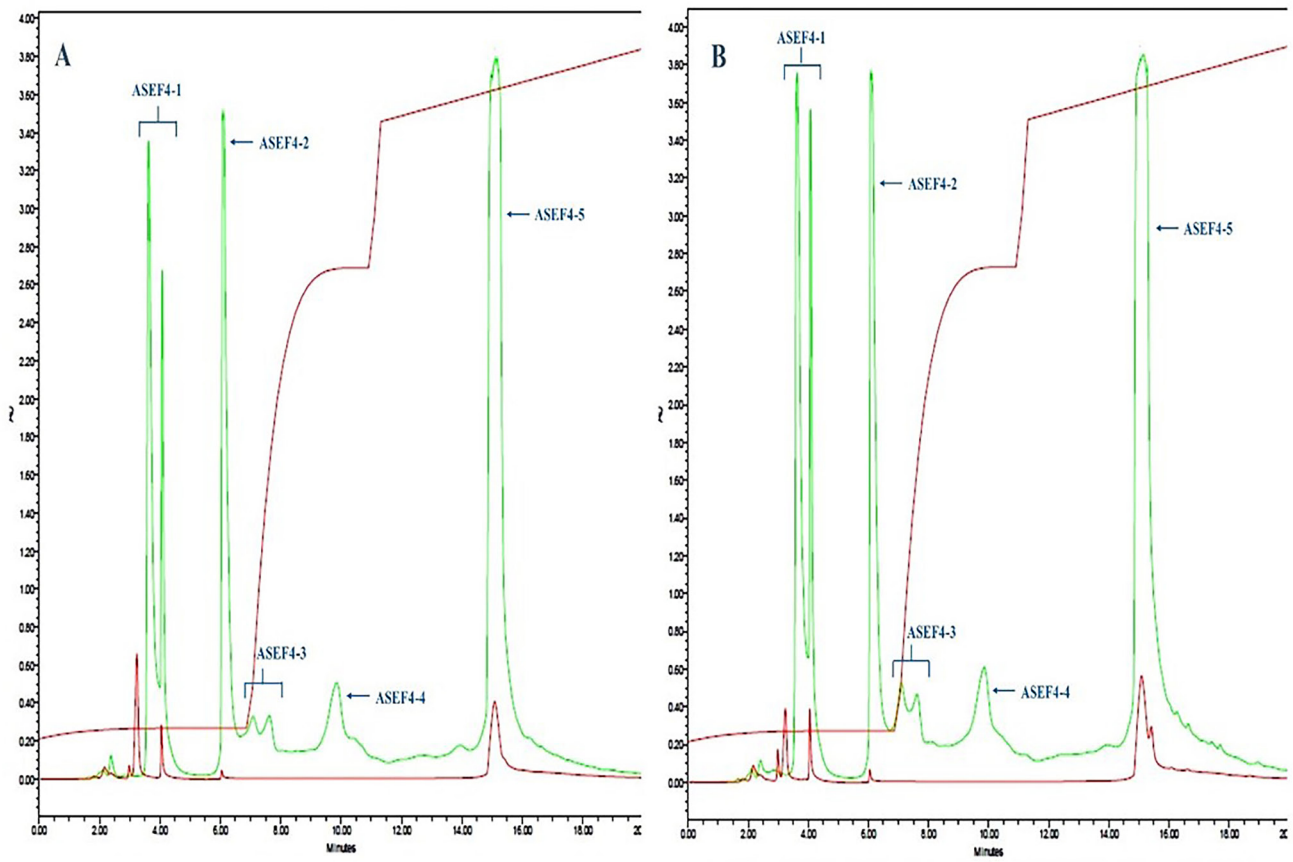

FIGURE 5 - Chromatographic profile of *A. sessilis* ethyl acetate subfraction 4 (green) and blank/diluent (red) at (A) 366 and (B) 254 nm. The red line corresponds to the time program of the mobile phase.

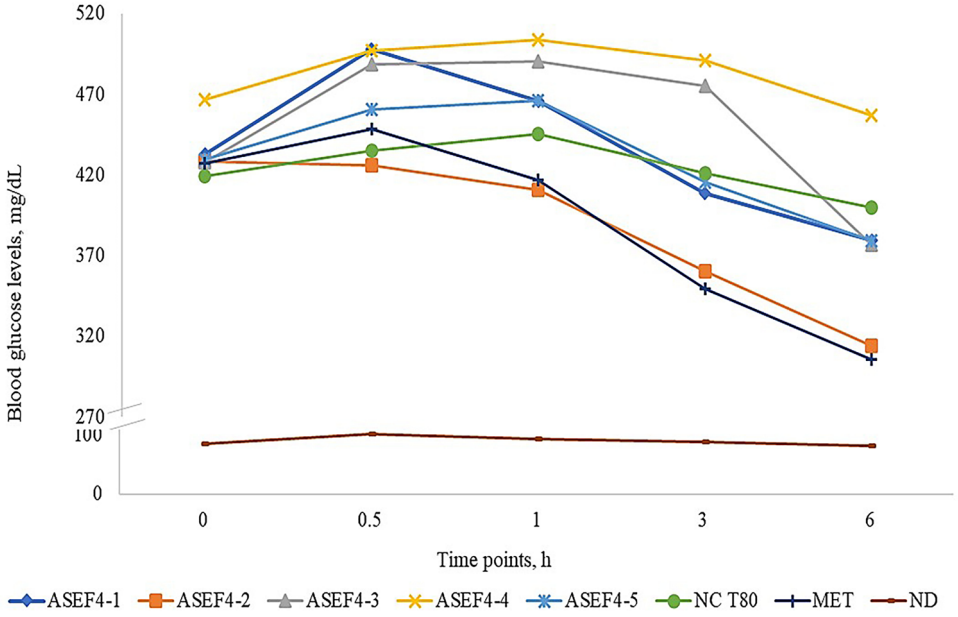

FIGURE 6 - Blood glucose levels of diabetic mice treated with 100 mg/kg *A. sessilis* ethyl acetate subfraction 4 (ASEF4) subfractions measured at different time points. ASEF4-1 to 4-5 – ASEF4 subfractions 1 to 5, MET – metformin (100 mg/kg), NC T80 – negative control 0.1% Tween 80 (10 mL/kg), ND – nondiabetic control (10 mL/kg saline).

The % blood glucose reduction of 100 mg/kg ASEF4 subfractions is presented in Figure 7. Prominent blood glucose lowering of more than 5% at 6-h post-treatment was observed in ASEF4-1, ASEF4-2, ASEF4-3, and ASEF4-5 compared to baseline. Conversely, ASEF4-4 did not show a reduction in blood glucose in comparison with its baseline. This activity is similar to NC T80 and ND.

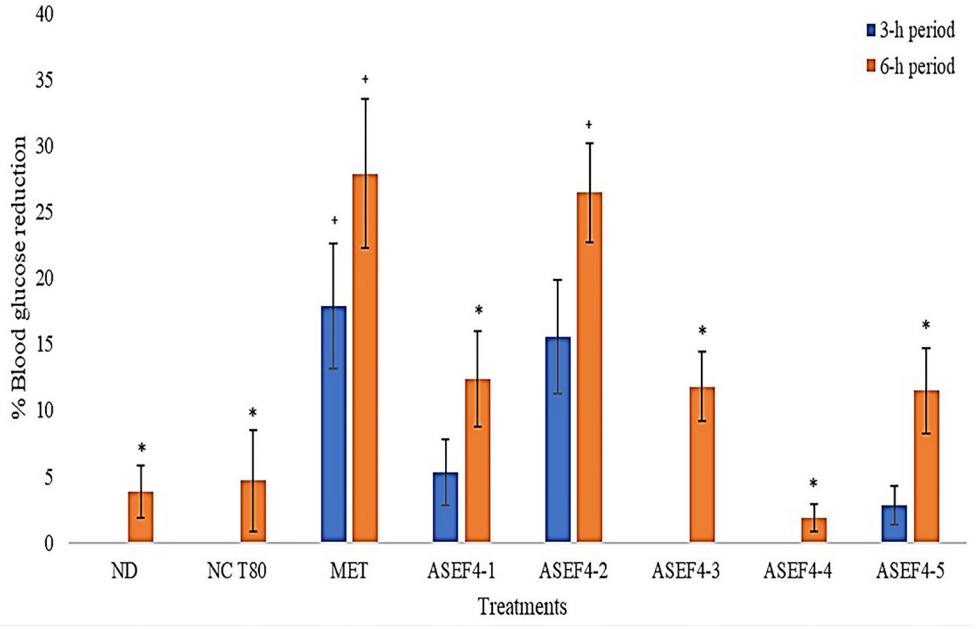

FIGURE 7- Blood glucose reduction values of diabetic mice treated with 100 mg/kg *A. sessilis* ethyl acetate subfraction 4 (ASEF4) subfractions at 3-h and 6-h period (less than 0 values not shown). ASEF4-1 to 4-5 – ASEF4 subfractions 1 to 5, MET – metformin (100 mg/kg), NC T80 – negative control 0.1% Tween 80 (10 mL/kg), ND – nondiabetic control (10 mL/kg saline). *,+ - Significant different compared to positive (MET) and negative controls, respectively.

The trend of anti-hyperglycemic activity based on the 6-h % blood glucose reduction values of the subfractions is as follows: ASEF4-2 (26.45±3.75%) > ASEF4-1 (12.36±3.63%) > ASEF4-3 (11.80±2.61%) > ASEF4-5 (11.47±3.25%) > ASEF4-4 (1.86±4.60%). ASEF4-2 ($p<0.0001$) and MET ($p<0.0001$) exhibited remarkable difference in % blood glucose reduction values relative to negative control at 6-h post-treatment.

MET exhibited the highest 6-h % blood glucose reduction values (27.87±5.65%) among the treatments. Comparison of the subfractions with MET revealed that only ASEF4-1 ($p=0.553$), ASEF4-2 ($p=1.000$), and ASEF4-5 ($p=0.332$) displayed almost similar % blood glucose reduction values at 3-h post-treatment. However, only ASEF4-2 manifested no significant reduction in blood glucose levels compared with MET after 6-h treatment ($p=0.999$). This signifies that ASEF4-2 has a potent anti-hyperglycemic activity and has comparable activity to that of metformin at 100 mg/kg. Thus, ASEF4-2 was subjected to UPLC/Q-TOF MS for metabolite identification.

Metabolite profiling by UPLC/Q-TOF MS

ASEF4-2 was subjected to UPLC/Q-TOF MS analysis to determine the metabolites present in the fraction. Figure 8 depicts the representative chromatogram of ASEF4-2 with its respective blank. Only eight peaks were discriminated from ASEF4-2 based on the criteria for the candidate peak. Table I shows the annotated peaks and the putative compounds identified from the Traditional Chinese Medicine library and the custom library of known compounds from *A. sessilis*.

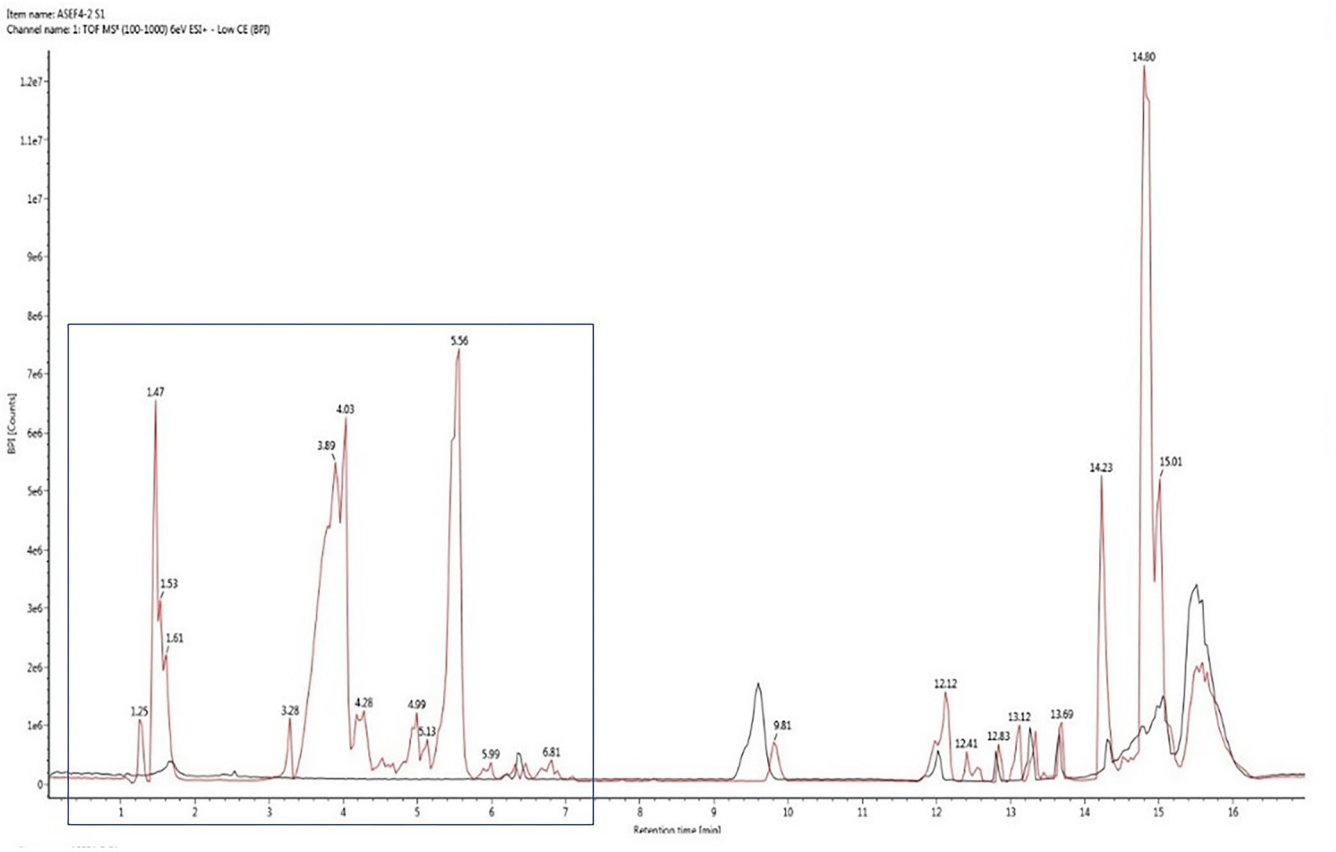

FIGURE 8 - Representative chromatogram of ASEF4-2 (red) and acetonitrile (black). Distinct peaks from ASEF4-2 were inside the blue rectangle.

TABLE I - Putative compounds identified in *A. sessilis* ethyl acetate subfraction 4-2 (ASEF4-2) using UPLC/Q-TOF MS

Peak	Retention time (min)	Compound ID	Molecular formula	Observed neutral mass (Da)	Observed m/z	Mass error (ppm)	Detector counts	Adducts	Common fragments found
1	1.25	Unknown	-	118.085	-	-	-	-	-
2	1.31-1.61	Unknown	-	136.061	-	-	-	-	-
3	3.28-4.03	Sophorabioside	C27H30O14	578.171	579.171	0.1	2512525	+H, +Na	417.116, 413.088, 399.106, 381.095, 329.065, 265.085
4	4.28	Unknown	-	685.226	-	-	-	-	-
5	4.96-5.13	Unknown	-	355.113	-	-	-	-	-
6	5.42-5.56	Luteolin	C15H10O6	286.048	287.054	-3.4	3634090	+H	153.018
7	5.8-5.99	Ononin	C22H22O9	430.125	431.132	-4.2	109254	+H	323.054, 283.059
8	6.73-6.81	Apigenin	C15H10O5	270.059	271.059	-5.4	442292	+H	153.017

The putative compounds identified belong to the class of flavonoids, which absorb at the 250 and 300 nm region due to their aromatic and carbonyl chromophores, respectively (Sisa *et al.*, 2010). These wavelengths were used in the HPLC profiling of ASEF4, confirming that flavonoids should be the major group of compounds present in ASEF4-2 subfraction.

Flavonoids have been reported to exemplify antidiabetic activities through numerous enzymatic inhibitions and biochemical modulations of cytokines linked to diabetes. Sophorabioside, an isoflavone glycoside, was identified in the seeds of *Sophora japonica* (Abdallah *et al.*, 2014) and the stems of *Astragalus membranaceus* (Jia *et al.*, 2016). This compound has been reported to affect glucose metabolism and transport by significantly increasing glucose consumption in an *in vitro* model to study GLUT4-dependent glucose uptake using L6 myotubes (Yap *et al.*, 2007). However, it showed inferior activity compared to that of insulin (Jia *et al.*, 2016).

Ononin, another isoflavone glycoside, was identified as one of the components present in the hydroethanolic extract of *Radix astragali* dried roots. This extract showed decreased levels of fed and fasting blood glucose, and serum triglyceride in db/db diabetic mice. It also reduced the secretion of inflammatory cytokines linked to diabetes (TNF-α, interleukin-6, and MCP-1) and alleviated insulin resistance (Hoo *et al.*, 2010). Flavonoid-rich extract of *Sophora tonkinensis*, which also contains ononin, demonstrated hypoglycemic activity by increasing insulin sensitivity and stimulating GLUT4 translocation in L6 cells (Huang *et al.*, 2016).

Among the putative compounds, luteolin, and apigenin, both under the flavone class of flavonoids, were previously identified as compounds present in *A. sessilis* varieties of other countries. Several studies have already presented the antidiabetic activity of luteolin through inhibition of α-amylase and α-glucosidase activities (Zhang *et al.*, 2017; Kim, Kwon, Son, 2000), including improvement of insulin sensitivity in adipocytes by increasing glucose uptake and reducing mRNA levels of cytokines linked to insulin resistance, such as TNF-α, interleukin-6, and MCP-1 (Zang, Igarashi, Li, 2016; Ding, Jin, Chen, 2010). Continuous oral feeding of 200 mg/kg dose of luteolin in streptozotocin-induced diabetic rats for 8 weeks demonstrated a significant fall in blood glucose levels compared to baseline values. Moreover, it displayed protective effects on diabetic nephropathy by modulating inflammatory cytokines, leading to decreased malondialdehyde content and increased superoxide dismutase activity in kidney homogenates of diabetic rats (Guo Guang *et al.*, 2011).

Previous literature reported that insulin resistance is alleviated by apigenin through upregulation of gene expression involved in fatty acid oxidation, tricarboxylic acid cycle, oxidative phosphorylation, and electron transport chain (Jung, Cho, Choi, 2016). It also enhanced GLUT4 translocation and downregulated CD38 protein expression resulting in reduced blood glucose levels in streptozotocin-induced diabetic rats (Hossain *et al.*, 2014).

From these results, it can be inferred that ASEF4-2 may be a potential source of anti-hyperglycemic agents.

CONCLUSIONS

Compounds attributed to the anti-hyperglycemic activity of the local *A. sessilis* have been poorly investigated. This study provided a bioassay-guided approach to identify the putative compounds present in the anti-hyperglycemic fraction of *A. sessilis* leaves, most of which belong to the class of flavonoids. Further studies are recommended to determine the anti-hyperglycemic activity of ASEF4-2 at a higher dose, including the main metabolite responsible for its bioactivity through further purification. Moreover, the mechanisms involved in the blood glucose-lowering activity of the putative metabolites from *A. sessilis* may be further explored.

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