ORIGINAL ARTICLE

In Vitro Investigation of Antioxidant and Antidiabetic Properties of Phenolic-Rich Extract from Stingless Bee Honey (*Heterotrigona itama*)

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ABSTRACT

Introduction: The increase of Type 2 diabetes mellitus has prompted numerous research toward finding an alternative to manage the disease through the oxidant-antioxidant balance, mainly through bioactive compounds in natural products. This study explored the antioxidant and antidiabetic properties of phenolic-rich extract (PRE) from Stingless bee honey (SBH) (*Heterotrigona itama*) as therapeutic agent to restore the redox balance. **Methods:** The total phenolic content (TPC), total flavonoid content (TFC) and antioxidant assays of PRE and SBH, were determined to provide preliminary insight into the sample's antioxidant properties, followed by high-performance liquid chromatography analysis of PRE. The antidiabetic potential of PRE and SBH were determined based on their inhibition against α-amylase and α-glucosidase enzymes. The cytotoxicity analysis of PRE was conducted on 3T3-L1 adipocytes and L6 muscle cells before the glucose uptake and cellular antioxidant analyses were performed on both cell lines, respectively. **Results:** PRE yielded higher TPC, TFC and antioxidant activities than SBH. The phytochemical profile of PRE comprises gallic acid, myricetin, kaempferol, epicatechin, chlorogenic acid, quercetin, syringic acid, and cinnamic acid. The results from carbohydrate enzymatic inhibitory assays collectively suggested that PRE exhibited more robust antidiabetic activities than SBH. PRE showed good glucose uptake stimulating and reactive oxygen species scavenging effects in those cell lines. **Conclusion:** Overall, PRE from SBH showed higher carbohydrate enzymatic inhibition, glucose uptake, and protection against intracellular oxidative stress, primarily due to its high antioxidant content and may serve as an alternative therapeutic agent for managing T2DM.
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Keywords: Stingless bee honey, Phenolic-rich extract, Antioxidant, Antidiabetic, in vitro

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INTRODUCTION

The overproduction of free radicals during metabolism may attack cellular components, leading to many diseases, including Type 2 diabetes mellitus (T2DM) (1,2). T2DM is a complex metabolic disorder distinguished by compromised blood glucose homeostasis due to defective insulin secretion and reduced insulin sensitivity (3). Chronic hyperglycemia may cause hypertension and hyperlipidemia, contributing to other pathological problems, including cardiovascular and kidney diseases (4). Although several medications are available for managing diabetes mellitus, such as metformin, thiazolidinediones, and sodium-glucose co-transporter-2 inhibitors, many displayed various adverse effects that impeded their usefulness (5). In this regard, developing complementary alternative therapeutic agents with higher efficacy and lesser side effects is urgently needed.

Since ancient times, natural products have been used as therapeutic agents, and many effective modern drugs have been derived from there (6). Honey is a nutritious food produced from plant nectar in bees' hypopharyngeal glands (7,8). Stingless bee honey (SBH) exhibited high nutritional and therapeutic values as it contains a significant amount of health-promoting

components such as polyphenols, which can act as antioxidant, anticancer, anti-inflammatory, and wound healing agent (9,10). Accumulated research suggests that dietary antioxidants (e.g., phenolics) play a substantial role in preventing and managing T2DM through various mechanisms, including attenuating oxidative stress, inhibiting digestive enzymes, and so on (1,2). Previous research has reported that SBH has demonstrated an antidiabetic effect by improving lipid profiling and reducing cholesterol levels in high fat diet-STZ-induced diabetic rats (11). Despite the high antioxidant potential in SBH, there is also a high sugar content. With a high phenolic recovery rate, solid-phase extraction (SPE) was selected to extract further the bioactive compound responsible for the antioxidant and antidiabetic effect of SBH. Since SBH has high phenolic content (12,13), it was hypothesized that the phenolic-rich extract (PRE) concentrated from SBH could possibly possess relatively high antioxidant and antidiabetic activities.

To our best knowledge, in vitro, antioxidant and antidiabetic studies on PRE from SBH still need to be included. Thus, the present study aimed to provide fundamental findings on in vitro properties of PRE extracted from SBH using solid-phase extraction. Additionally, the study sought to identify the specific bioactive compound responsible for its antioxidant and antidiabetic effects.

MATERIALS AND METHODS

Sample

Stingless bee (*Heterotrigona itama*) honey (SBH) samples were harvested freshly from sealed stingless bee honey pots in Sibu, Malaysia (2°41′38.3″N 111°41′03.3″E) in July 2017. The collected honey samples were kept in sterilised bottles at 4 °C till further extraction process and analyses.

Chemicals and reagents

Formic acid, orthophosphoric acid, acetonitrile, methanol, aluminum chloride, DPPH (sodium carbonate, 2,2-dipheny1-l-hydrazy1-hydrate), ABTS (2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)), potassium ferricyanide $\{K_3(Fe(CN)_6)\}$, $FeCl_3$ (ferric chloride), phenolic standards (chlorogenic acid, epicatechin, gallic acid, quercetin, syringic acid, cinnamic acid, kaempferol and myricetin), trolox, porcine α-amylase, α-glucosidase, pNPG (p-nitrophenyl-α-D-glucopyranoside), fetal calf serum, D-glucose, metformin, insulin, AAPH (2-2′-azobis (2-amidinopropane) dihydrochloride), H_2O_2 (hydrogen peroxide) and 2′,7′-dichlorofluorescin were purchased from Sigma Chemical Co (St. Louis, Missouri, United States). DMEM (Dulbecco's modified eagle medium), MTT (methylthiazoliyldiphenyl tetrazolium bromide), and RPM1 (Roswell Park Memorial Institut 1640 medium), were obtained from Nacalai Tesque Inc. (Kyoto, Japan).

Solid-phase extraction (SPE) of phenolic-rich extract (PRE) from stingless bee honey (SBH)

SPE Agilent Bond Elut C18 column (3 mL x 1 g) (Agilent, Santa Clara, United States) was used for the preparation of PRE from SBH (14) with a slight adjustment. Briefly, 2 g of SBH was diluted with 100 mL of acidified water (0.5% orthophosphoric acid). The SPE C18 column was preconditioned with 5 mL of methanol, followed by 5 mL of acidified water. The column was then loaded with 5 mL of diluted SBH sample, eluted with 5 mL of 90 % methanol, and the PRE yielded was dried at 40 °C for 24 hours. The PRE were stored in 4 °C for further analyses. The phenolic recovery was calculated as TPC/(TPC+Eluent)*100.

Phytochemical analysis

Total phenolic content (TPC)

TPC of PRE and SBH was determined using Folin Ciocalteu assays (15). An aliquot of the extract/sample (100 µL) was dissolved with 500 µL of 10% Folin Ciocalteu reagent. A 400 µL of 7.5 % sodium bicarbonate solution was added to the mixture. The mixture was incubated for 30 min at 40 °C and absorbance was measured at 760 nm using Biotek Synergy H1 Multimode reader (Winooski, VT, USA). Gallic acid was used as a standard and TPC of the samples were expressed in microgram gallic acid equivalents per gram sample (µg GAE/g sample).

Total flavonoid content (TFC)

TFC in PRE and SBH samples was measured in 96-well plate using the aluminium chloride colorimetric method (15). Briefly, 150 µL of PRE and SBH diluted samples (1 g in deionized water) were homogenised with 50 µL aluminium chloride. The reaction was then incubated for 10 min at room temperature. The absorbance was measured at 435 nm using Biotek Synergy H1 Multimode reader (Winooski, VT, USA). Quercetin was used as standard and the flavonoid content of the samples was expressed in microgram quercetin equivalents per gram sample (µg QE/g sample).

High-performance liquid chromatography (HPLC) analysis for phenolic detection

HPLC analysis of phenolic composition in PRE was conducted using Agilent 1200 series HPLC (Agilent, Stevens Creek Blvd, Santa Clara, CA, USA) with a C18 column (4.6 x 150 mm), and ultraviolet (UV) visible detector (Agilent Technologies, Santa Clara, CA) (16), with minor modification. The samples were dissolved with water and passed through a 0.45 µm nylon syringe filter. Two mobile phase solvents were used for elution, Solvent A; 0.5 % formic acid in water and Solvent B; acetonitrile. The samples (10 µL) were injected with 0.50 mL/min flow rate following an elution program of 0 min (5 % B); 10 min (35% B); 15 min (65 % B), and 18 min (5 % B). The UV detector detected the separation at 280 nm. The samples retention times were recorded and compared with known standards for polyphenol

identification.

Antioxidant assays

DPPH free radical scavenging activity assay

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity of the samples was performed by following the method by Chan et al. (17) with minor adjustments. Briefly, 80 µL DPPH methanolic solution (0.1 mM) was added to 50 µL samples in a 96-well plate followed by one hour incubation in the dark. The plate was measured spectrophotometrically at 540 nm using Biotek Synergy H1 Multimode reader (Winooski, VT, USA). Trolox was used as a positive control. The sample's scavenging activity were expressed in microgram trolox equivalent per gram sample (µg TE/g sample).

ABTS radical cation scavenging assay

The 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) scavenging assay was evaluated following the methods of Chan et al. (17). For this assay, 1 mL of potassium persulfate was added to 1 mL of ABTS stock solution followed by 12 hours incubation at room temperature in the dark to produce ABTS radical cation stock solution. A test solution with an absorbance of 0.70 ± 0.02 at 734 nm was formed by adding deionized water to the ABTS stock solution. Then, 20 µL of the diluted samples were added to 200 µL of the test solution to determine the scavenging activity, followed by 10 min of incubation at room temperature. The reaction mixture was measured using Biotek Synergy H1 Multimode reader (Winooski, VT, USA) at 734 nm. Trolox was used as a positive control. The radical scavenging activity of the samples was expressed in microgram Trolox equivalent per gram sample (µg TE/g sample).

Ferric reducing antioxidant power assay

The assay was conducted as performed by Cheng et al. (13). Approximately 1 mL of the diluted samples was homogenised with 1.5 mL hydrochloric acid and 5 mL deionized water. Next, 0.5 mL of 0.2% (w/v) ferric chloride, 1.5 mL of 1% (w/v) potassium ferricyanide, and 0.5 mL of 1% (w/v) sodium dodecyl sulfate were added to the mixture. The mixture was then incubated for 20 min at 50 °C before being measured spectrophotometrically at 750 nm by Biotek Synergy H1 Multimode reader (Winooski, VT, USA). Trolox was used as a positive control and the samples were expressed in microgram Trolox equivalents per gram sample (µg TE/g sample).

Determination of enzymatic inhibitory activity

α-amylase inhibition assay

The determination of the inhibitory effect of PRE and SBH against pancreatic α-amylase was performed following the method by Hemalatha et al. (18). In brief, 50 µL of the samples (diluted in DMSO) was mixed with 150 µL of the substrate (1 % starch solution in NaCl). The assay was initiated by adding 10 µL α-amylase enzyme (0.5 mg/mL) and followed by a 30-minute incubation period at room temperature. Consequently, 20 µL of NaOH was added to stop the reaction before adding 20 µL of dinitrosalicylic acid reagent (DNS). The mixture was heated for 30 min at 100 °C and further incubated at room temperature for cooling purposes. Deionized water was added to the mixture until the final volume of 10 mL was reached. Finally, the mixture was measured spectrophotometrically at 540 nm using Biotek Synergy H1 Multimode reader (Winooski, VT, USA). The percentage of enzyme inhibition was calculated using Equation (1) with acarbose used as the positive control.

Inhibition (%) = (1 – (sample absorbance/negative control absorbance)) x 100 (1)

α-glucosidase inhibition assay

The inhibiting effects on α-glucosidase by PRE and SBH were evaluated by the method described by Hemalatha et al. (18) with minor adjustment. The enzymatic reaction was initiated by adding 1 uL sample and 0.25 mL of pNPG (3 mM) to a pre-incubated mixture of 0.6 mL of phosphate buffer (0.05 M) and 0.25 µL of α-glucosidase solution (25 mg/mL) for 40 min at 37 °C. Then, 0.25 mL of sodium carbonate solution (0.6 M) was added to stop the enzymatic reaction. The assay absorbance was measured at 405 nm using Biotek Synergy H1 Multimode reader (Winooski, VT, USA). The percentage of enzyme inhibition was calculated using Equation (1) with acarbose used as the positive control.

Cell culture

The 3T3-L1 adipocytes and L6 muscle cells were acquired from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in DMEM supplemented with 4 mM glutamine, 10% fetal calf serum, 100 µg/mL penicillin/streptomycin and 4.5 g/L of D-glucose at 37 °C. The passage number of the cell lines was maintained at 3–12.

MTT assay

The cytotoxicity of PRE on 3T3-L1 adipocytes and L6 muscle cells were assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as outlined by Abel and Baird (19) with minor modifications. Cells were seeded at 1 x 10^5 cells per well into a 96-well plate overnight followed by 24 hours of sample treatment with varying concentrations of PRE (0-100 µg/mL) on the following day. At the end of the treatment, 20 µL of MTT (5 mg/mL) was added to the cells and incubated for 4 hours. Then, the media were removed before being rinsed with PBS. A 200 µL of DMSO was subsequently added to solubilize the formazan crystal. Metformin and DMEM were used as positive and vehicle control, respectively. The absorbance was measured at 570 nm (Biotek Synergy H1 Multi-Mode Reader) and the percentage of cell viability were calculated using equation (2):

Cell viability % = (Sample absorbance mean/ Vehicle absorbance control) x 100 (2)

Cellular glucose uptake assay

Cellular glucose uptake by 3T3-L1 adipocytes and L6 muscle cells was measured using a 2-NBDG uptake cell-based assay kit (Cayman Chemical, Ann Arbor, MI #600470). Briefly, the cell differentiation was induced by maintaining the cells in DMEM supplemented with 2% FBS for a period of 5-7 days. The differentiated cells were then incubated overnight in serum-free media before washing with HEPES buffered Kreb Ringer Phosphate solution (KRP buffer). The cells were further incubated with KRP buffer containing 0.1% BSA for 30 min at 37°C. Samples and metformin (positive control) treatment in glucose-free culture medium with and without 100 nM insulin were added and incubated for 4 hours. Then, 2-NBDG was added as a glucose analogue and the plate was incubated for 10 min. The plate was then centrifuged at 400 x g at room temperature for 5 min and the supernatant was aspirated. The plate was washed with 200 µL of wash buffer and the glucose uptake activity was determined by measuring the cellular uptake of 2-NBDG with fluorescent filters at 485/535 nm using Biotek Synergy H1 Multi-mode reader (Winooski, VT, USA).

Diacetyldichlorofluorescein (DCFH-DA) staining assay

The presence of intracellular reactive oxygen species (ROS) was measured according to Ooi et al. (20). Cells were first incubated with diluted samples for 24 hours in DMEM with 0.5% FBS. The cells were washed twice with PBS and 10 µM of DCFH-DA was added and further incubated for 30 min at 37 °C. The free remaining DCFH-DA was aspirated by washing the cells twice with PBS. Then, 1 mM AAPH and 4 mM H_2O_2 were added as a source of peroxyl and hydroxyl radicals, respectively. Fluorescent intensity measurements were performed using a Biotek Synergy H1 Multimode reader (Winooski, VT, USA) with a fluorescent filters. The excitation wavelength was set at 485 nm, and the emission wavelength was set at 530 nm. The measurements were taken instantaneously, and fluorescent intensity was recorded at 5-minute intervals for up to 2 hours. The integrated area under the curve (AUC) and cellular antioxidant activity (CAA) values were computed using equation (3) and (4):

$$AUC = 0.5 + (RFU1/RFU0) + (RFU2/RFU0) + (RFU3/RFU0) + \ldots + 0.5\ (RFUn/RFU0) \quad (3)$$

$$CAA\ units = 100 - (AUC_{sample}/AUC_{control}) \times 100 \quad (4)$$

where RFU0 represents the relative fluorescent value at time zero, RFUn represents the final relative fluorescent value, AUC_{sample} represents the integrated area under the curve for cells incubated with samples and $AUC_{control}$ represents the integrated area under the curve for cells not incubated with samples. Quercetin was used as a positive control. The results were expressed as µM quercetin equivalent (QE).

Statistical analysis

All data were reported as mean ± standard deviation. Analysis of variance-one way (ANOVA) and Student's T-test was carried out for statistical analysis with $p < 0.05$ and was regarded to be statistically significant.

RESULTS

Extraction yield of PRE

Since SBH comprises a mixture of sugars, minerals, vitamins, amino acids, organic compounds, polyphenols, and resin, the solid-phase extraction technique was chosen for the preparation of PRE. The utilization of a C18 (Bond Elut) SPE cartridge offers distinct advantages over conventional extraction methods. It effectively removes the aforementioned compounds while preserving a higher concentration of polyphenols and exhibit improved recovery rates (21). The PRE has a percentage of yield and phenolic recovery of 5% and 85.81%, respectively.

Phenolic composition profile of PRE

A total of eight phenolic compounds (14.63 mg/g) were detected in PRE (Table I), following the descending order of abundancy (Fig.1): gallic acid > myricetin > kaempferol > epicatechin > chlorogenic acid > quercetin > syringic acid > cinnamic acid. In addition to the phenolic compounds identified herein, Ranneh et al. (22) also reported that SBH contains caffeic acid, vanillic acid, and benzoic acid, with gallic acid and epicatechin served as the major phenolic compounds detected. For both TPC and TFC assays (Table II), PRE demonstrated substantially higher ($p < 0.05$) content of total phenolic

Table I: Composition of polyphenol detected in PRE

Compounds	Retention time (min)	Content (mg/g sample)	Regression equation
Gallic acid	7.153	5.98	y = 203.88x + 3258.9, R = 0.9970
Epicatechin	9.062	0.97	y = 1158.6x +196.06, R = 0.9979
Chlorogenic acid	9.975	0.48	y = 10952x – 4731.5, R = 0.9975
Myricetin	10.333	3.60	y = 2239.6x – 4961.1, R = 0.9967
Syringic acid	12.596	0.31	y = 25.077x + 457.41, R = 0.9988
Quercetin	14.013	0.37	y = 24.95x + 501.84, R = 0.9965
Kaempferol	14.980	2.87	y = 160.26x + 1972.4, R = 0.9996
Cinnamic acid	15.378	0.05	y = 25.6x + 192.87, R = 0.9985

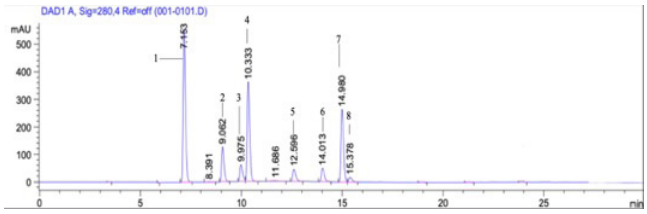

Figure 1: HPLC profiles of phenolic compounds detected at 280 nm. Chromatograms: gallic acid (1), epicatechin (2), chlorogenic acid (3), myricetin (4), syringic acid (5), quercetin (6), kaempferol (7), cinnamic acid (8).

Table II: TPC, TFC and antioxidant activity of PRE and SBH

Sample	Phenolic content		Antioxidant assays and scavenging activity		
	TPC (µg GAE/g)	TFC (µg QE/g)	DPPH scavenging activity (µg TE/g)	ABTS assay (µg TE/g)	FRAP assays (µg TE/g)
PRE	2027.14± 51.32[a]	609.62± 7.32[a]	1042.85± 22.98[a]	1206.91± 38.62[a]	1509.30± 33.16[a]
SBH	491.77± 5.11[b]	172.53± 11.18[b]	242.57± 11.83[b]	315.82± 9.23[b]	474.90± 29.32[b]

Different lowercase letters within the same column indicate significant differences ($p < 0.05$). Data are expressed as mean ± standard deviation. PRE, phenolic-rich extract; SBH, stingless bee honey

and flavonoid than SBH. The TPC of SBH and PRE were 491.77 ± 5.11 and 2027.14 ± 51.32 µg GAE/g, respectively. The TFC of SBH and PRE were 172.53 ± 11.18 and 609.62 ± 7.32 µg QE/g, respectively. These results indicate a better reconstitution of polyphenols on PRE upon extraction via SPE, which is approximately 4-folds (TPC) and 3.5-folds (TFC) compared to SBH.

Antioxidant activity of SBH and PRE

DPPH scavenging activity, ABTS scavenging activity, and FRAP assays were conducted to provide preliminary insight into the antioxidant activities of SBH and PRE, based on the ability to scavenge radicals in the respective assays. It was demonstrated in Table II that PRE has higher DPPH, ABTS and FRAP activities with 1042.85±22.98 µg TE/g, 1206.91±38.62 µg TE/g, 1509.30±33.16 µg TE/g, repectively ($p < 0.05$) compare to SBH with 242.57±11.83 µg TE/g (DPPH), 315.82±9.23 µg TE/g (ABTS), 474.90±29.32 µg TE/g (FRAP), indicative of a higher antioxidant capacity of PRE by the antioxidant assays mentioned above.

α-amylase and α-glucosidase inhibition activities of SBH and PRE

Table III demonstrated that both samples exhibited concentration-dependent inhibition against α-amylase and α-glucosidase. PRE inhibited both α-amylase and α-glucosidase more effectively than SBH, as evidenced by their lower IC_{50} values with 12.4 µg/mL and 15.4 µg/mL, respectively ($p < 0.05$). Although acarbose has shown the greatest inhibition against both enzymes, PRE is comparable to acarbose in regards to α-amylase inhibition.

Cell viability assessment

The viability of 3T3-L1 adipocytes and L6 muscle cells

Table III: IC_{50} values of PRE, SBH and acarbose against α-amylase and α glucosidase

Sample	IC_{50} (µg/mL)	
	α -amylase	α -glucosidase
PRE	12.40 ± 1.7[a]	15.40 ± 2.21[b]
SBH	40.50 ± 3.7[b]	49.16 ± 0.83[a]
Acarbose	8.31 ± 0.43[a]	8.35 ± 0.30[c]

Different lowercase letters within the same column indicate significant differences ($p < 0.05$). Data are expressed as mean ± standard deviation. Acarbose was used as the positive control. PRE, phenolic-rich extract; SBH, stingless bee honey.

upon introducing test samples was measured using MTT assay (Fig. 2). There were no significant ($p > 0.05$) differences in cell viability of 3T3-L1 adipocytes and L6 muscle cells at a concentration up to 50 µg/mL of PRE compared to the control. However, PRE exhibited appreciable cytotoxicity only at ≥ 75 µg/mL. At 100 µg/mL, the sample inhibited the growth of 3T3-L1 and L6 by 35.22 and 30.4%, respectively, indicating that the cytotoxicity of PRE was in a concentration-dependent manner. No significant ($p > 0.05$) differences in both cell viability at a concentration up to 10 µM for the positive control (metformin) compared to the control. Thus, the subsequent cellular assays employed the concentration at a range of 6.25–50.00 µg/mL and 1.2–10 µM for PRE and metformin, respectively.

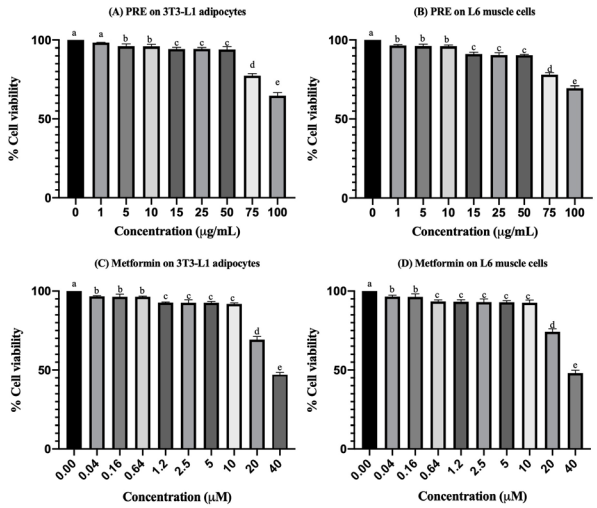

Figure 2: MTT cell viability of PRE on both (A) 3T3-L1 adipocyte cells and (B) L6 muscle cells and drug metformin on (C) 3T3-L1 adipocyte cells and (D) L6 muscle cells. Cultures in basal medium served as control. Values with different letters indicates significant differences ($p < 0.05$).

Glucose uptake stimulating effect of PRE

The study employed PRE to determine glucose (2-NBDG) uptake in both 3T3-L1 adipocytes and L6 muscle cells. As shown in Fig. 3, in the presence of insulin (100 nM), PRE significantly ($p < 0.05$) increased the uptake of 2-NBDG by both cells in a dose-dependent manner even at the lowest concentration tested (6.25 µg/mL), suggestive of its remarkable potential as an antidiabetic agent. Compared to the untreated control, PRE (50 µg/mL) significantly ($p < 0.05$) enhanced the glucose uptake of 3T3-L1 and L6 cell lines by 28.4- and 32.6-

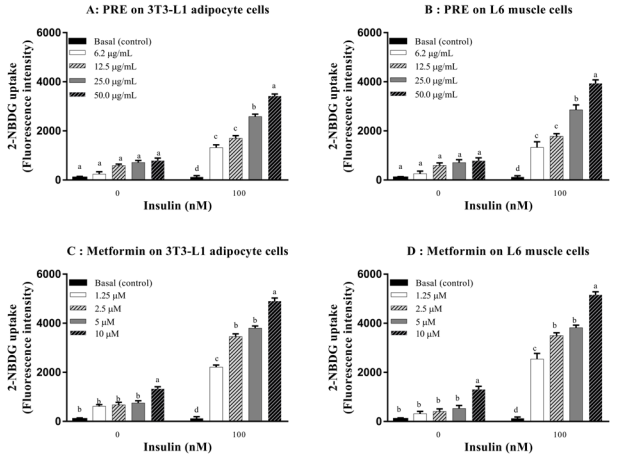

Figure 3: PRE and metformin (positive control) ability to stimulate 2-NBDG uptake in 3T3-L1 adipocytes and L6 muscle cells in the absence (0 nM) and presence (100 nM) of insulin at the indicated concentration. Cultures in basal medium and insulin served as control. Values with different letters indicates significant differences ($p < 0.05$).

fold, respectively. The effective insulin-sensitizing agent metformin expectedly showed substantial 2-NBDG uptake by both cell lines regardless of the presence of insulin.

Intracellular antioxidant activity of PRE

The DCFH-DA assay was used to determine the intercellular antioxidant activities in 3T3-L1 adipocytes and L6 muscle cells upon introducing free radicals, AAPH and H_2O_2. As shown in Fig. 4, pre-treatment with PRE exerted a protective effect on 3T3-L1 adipocytes against both hydroxyl and peroxyl radicals at their highest concentration of 50 µg/mL with 71.36±4.12 uM QE for AAPH and 50.71±3.21 uM QE for H_2O_2. In contrast, PRE effectively shielded L6 skeletal muscle cells from both radicals at ≥ 25 µg/mL in a dose-dependent manner.

DISCUSSION

This present study aimed to evaluate the antioxidant and antidiabetic properties of phenolic-rich extract (PRE) from stingless bee honey (SBH) (*Heterotrigona itama*). Superior results are seen in PRE compared to SBH in total phenolic and flavonoid contents, antioxidants, and enzymatic activities. The comparatively high phenolic and flavonoid contents in PRE compared to SBH were due to the SPE process, which purified and concentrated the polyphenols in the sample by using a column packed with alkylated silica gel (C18), where the C18 column has strong hydrophobicity which effectively extracts non-polar compounds from aqueous samples (23). This finding shows that antioxidant in SBH is mainly contributed by its phenolic compound. There is a strong correlation between the antioxidant activity of the samples and their TPC and TFC. Phenolics and flavonoids are recognized for their antioxidant potential (24), and their presence in the samples contributes to their observed antioxidant activity. Notably, the study's SBH sample demonstrated comparatively elevated levels of TPC and TFC compared to previous studies (22, 25, 26) investigating stingless bee honey collected from Malaysia. This observation indicates that the study's SBH sample contains a substantial amount of phenolic compounds and flavonoids, contributing to its exceptional antioxidant properties.

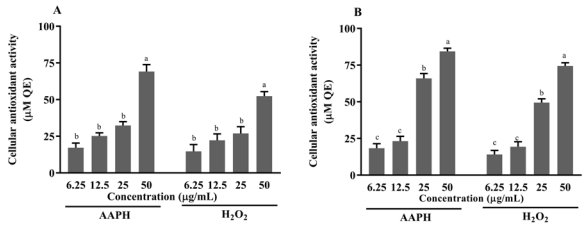

Figure 4: Cellular antioxidant activities of PRE in 3T3-L1 adipocytes (A) and L6 muscle cells (B) under influence of AAPH and H_2O_2. Data are expressed as mean ± standard deviation following triplicate experimentation. Different letters indicate significant differences ($p < 0.05$) within treatment concentrations. QE, quercetin equivalent; PRE, phenolic-rich extract.

Alpha-amylase regulates glucose homeostasis by acting as a catalyst in the 1,4-glycosidic linkage hydrolysis of starch and glycogen. The enzyme is responsible for further starch digestion, which involves breaking down the polymer into simple sugar for intestinal digestion (27). Meanwhile, α-glucosidase is a vital regulator for blood glucose level control, effectively suppressing postprandial hyperglycemia, ultimately leading to T2DM prevention (28). Therefore, inhibiting these enzymes will delay carbohydrate hydrolysis and reduce the postprandial blood glucose excursion in diabetic patients.

Since PRE has a higher content of antioxidant activity and enzyme inhibitory activity, it was therefore chosen for the subsequent cell-based assays. In line with the results of phenolic content and antioxidant activities assays, PRE showed significantly higher ($p < 0.05$) enzyme inhibition activity than SBH, which could be attributed by a higher relative abundance of polyphenols in PRE specifically gallic acid, epicatechin, chlorogenic acid, myricetin, syringic acid, quercetin, kaempferol and cinnamic acid. For instance, Huang et al. (29) have previously reported that myricetin is a potent inhibitor against porcine pancreatic amylase. As the main phenolic found in PRE, gallic acid appeared to have superior synergistic inhibition against both α-amylase and α-glucosidase enzymes when combined with commercial acarbose (30). Kaempferol was found to reduce blood glucose levels in obese diabetic mice by Alkhalidy et al. (31). Liao and Gong et al. (32) discovered that the same compound inhibited both α-amylase and α-glucosidase. Hence, the phenolic is the phytochemical that contributes to the enzymatic inhibition activities.

The antidiabetic potential of PRE was evaluated from the ability of 3T3-L1 and L6 cells to take up 2-NBDG after treatment. Adipocytes and muscle cells were used in the glucose uptake assay as they are the primary insulin-responsive cells in maintaining glucose homeostasis, both involved in glucose uptake by GLUT4 (glucose transporter type 4) translocation (33). The fluorescent 2-NBDG (glucose analog) acts as a good marker and tracer for glucose metabolism and utilization, whereby an increase of 2-NBDG fluorescence intensity in the cells after treatment indicates that the samples may exhibit insulin-mimetic properties (34).

The results demonstrated that PRE might act as an insulin sensitizer by activating glucose uptake in 3T3-L1 adipocytes and L6 muscle cells when insulin is dose-dependent. As the primary polyphenol in PRE, gallic acid significantly affects PRE's antidiabetic properties. According to Variya et al. (35), the antidiabetic properties of gallic acid have improved glucose translocation and insulin sensitivity via the AKT signaling pathway. Gallic acid treatment of 3T3-L1 adipocytes resulted in a dose-dependent increase in the expression of GLUT4 and Peroxisome proliferator-activated receptor-gamma (PPARγ) proteins. For in vivo study, Garud and Kulkarni et al. (36) showed that gallic acid administration significantly decreased blood urea nitrogen, plasma creatinine, protein, and albumin levels and increased creatinine clearance streptozotocin-induced diabetic nephropathy rats. This is due to gallic acid's antioxidant and advanced glycation inhibitory characteristics, which ultimately suppressed TGF-1 expression by decreasing oxidative stress and advanced glycation (36).

On the other hand, epicatechin, quercetin and cinnamic acid constituents in the PRE may also contribute to the antidiabetic potential of the sample. Knezevic et al. (37) and Thielecke & Boschmann (38) revealed that catechin improves insulin sensitivity by activating the AMP-activated protein kinase (AMPK) pathway for glucose uptake and helps inhibit α-amylase, α-glucosidase and ROS formation. Quercetin helps ameliorate ROS by inhibiting the advanced glycation end product (39) and is associated with GLUT4 translocation and AKT signalling (40). Cinnamic acid is proven to inhibit adipogenesis and gluconeogenesis (41). Therefore, phenolic in PRE may contribute to the antidiabetic effect, particularly on glucose uptake.

Intracellular ROS generation is intrinsically linked to the cellular response to oxidative stress. Oxidative stress ensues when the antioxidant defense mechanism is overwhelmed by ROS, resulting in cellular damage. The hydroxyl and peroxyl radicals are the most abundant ROS produced during biological processes (20). Regarding the potent radical scavenging ability of PRE, the intracellular antioxidant activities of PRE on 3T3-L1 adipocytes and L6 skeletal muscle cells were evaluated using a DCFH-DA staining assay. The post-treatment with H_2O_2 and AAPH was to introduce hydroxyl and peroxyl radicals to the cells, thereby inducing oxidative stress. ROS is one of the culprits that caused T2DM insulin resistance by impairing the insulin signaling pathway, resulting in decreased expression of GLUT4 transporter in the cellular membrane. Thus, restoring antioxidant balance has been one of the therapeutic options in treating T2DM (42). PRE obtained from SBH exhibited high cellular antioxidant potential, which might ameliorate oxidative stress and improve insulin sensitivity in adipocytes and muscle cells. This was evidenced by the high number of antioxidant activity in assays conducted on the PRE, highlighting its robust antioxidant properties. For the relationship between antidiabetics and antioxidants, gallic acid (which abundantly presents in PRE) is well known for inhibiting advanced glycation and oxidative stress (35). Moreover, epicatechin and syringic acid present in PRE have appeared to moderate insulin sensitivity and glucose tolerance by lowering the high glucose plasma level in diabetes-induced hepatic dysfunction (43).

CONCLUSION

The present findings showed that PRE had been successfully extracted from SBH via SPE with an enhancement in TPC, TFC, antioxidant, and inhibition of digestive enzyme activities. HPLC analysis of PRE revealed eight phenolic compounds with gallic acid, myricetin and kaempferol as the prominent polyphenol in PRE. PRE obtained from SBH has also been identified as able to increase glucose uptake in 3T3-L1 adipocytes and L6 muscle cells in the presence of insulin and demonstrated a considerable protective effect against ROS in those cell lines. Therefore, phenolics are the contributing bioactive to the observed bioactivities. The findings support our hypothesis that PRE could be a promising antidiabetic agent through scavenging free radicals, implying that it can be considered as an alternative therapeutic component for T2DM management. Accordingly, in vivo studies need to be conducted to comprehend the antidiabetic mechanism of PRE and SBH.

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