Sains Malaysiana 52(2)(2023): 501-512
http://doi.org/10.17576/jsm-2023-5202-14

An *in vitro* Adipogenic Potential and Glucose Uptake Stimulatory Effect of Betulinic Acid and Stigmasterol Isolated from *Tetracera indica* in 3T3-L1 Cell Line

(Potensi Adipogenik *in vitro* dan Kesan Rangsangan Pengambilan Glukosa Asid Betulinik dan Stigmasterol Dipencilkan daripada *Tetracera indica* dalam Titisan Sel 3T3-L1)

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Received: 17 February 2022/Accepted: 16 December 2022

ABSTRACT

Aerial parts of *Tetracera indica* Merr. (Dilleniaceae) are rich in betulinic acid and stigmasterol and traditionally used to treat diabetes. This study was aimed to evaluate an *in vitro* antidiabetic potential of betulinic acid and stigmasterol to ascertain whether they may contribute antidiabetic effect to *T. indica*. Initially, betulinic acid and stigmasterol were isolated from the most effective subfraction (ethyl acetate) and subjected to an *in vitro* antidiabetic investigation through adipogenesis and fluorescence glucose (2-NBDG) uptake assays using 3T3-L1 fibroblast. MTT viability assay was performed at 0.78 to 100 µg/mL for 48 h to determine the safe concentration. Both compounds were subjected to 2-NBDG uptake test on the differentiated adipocytes. The cells were treated in safe concentrations (25-100 µg/mL) as well as in different adipogenic cocktails, which were modified by the addition of compounds to be investigated and in the presence or absence of insulin (10 µM). Rosiglitazone (10 µM) was used as standard. Stems ethanol extract and its fractions (hexane and ethyl acetate), betulinic acid and stigmasterol were found safe at their highest concentration (100 µg/mL) by inhibiting cells well below their IC_{50} values viz. 18.60, 35.27, 21.40, 28.86 and 33.06%, respectively. Both betulinic acid and stigmasterol at the highest safe concentration (100 µg/mL) significantly ($p < 0.05$) induced adipogenesis like insulin, enhanced adipogenesis like rosiglitazone and exhibited glucose uptake activity. The present study demonstrates that both betulinic acid and stigmasterol possess an *in vitro* antidiabetic potential. However, *in vivo* antiglycemic study on these compounds and their chemical analogs are still warranted to ensure their therapeutic potential as safe antidiabetic agents.

Keywords: Adipogenesis; betulinic acid; insulin like activity; insulin sensitizing activity; stigmasterol; 2-NBDG uptake activity; 3T3-L1 preadipocyte cells

ABSTRAK

Bahagian udara *Tetracera indica* Merr. (Dilleniaceae) kaya dengan asid betulinik dan stigmasterol serta digunakan secara tradisi untuk merawat diabetes. Kajian ini dijalankan untuk menilai potensi antidiabetik asid betulinik dan stigmasterol secara *in vitro* untuk memastikan sama ada kedua-dua sebatian ini menyumbang kepada kesan antidiabetik

oleh *T. indica*. Asid betulinik dan stigmasterol diasingkan daripada subfraksi (etil asetat) yang paling berkesan. Kesan antidiabetik *in vitro* dikaji melalui asai adipogenesis dan asai pengambilan glukosa berpendaflour (2-NBDG) menggunakan sel fibroblas 3T3-L1. Asai kebolehhidupan MTT dijalankan pada kepekatan antara 0.78 hingga 100 µg/mL selama 48 jam bagi menentukan kepekatan yang selamat. Akhir sekali, kedua-dua sebatian diuji dengan asai 2-NBDG ke atas sel adiposit terbeza. Sel tersebut dirawat pada julat kepekatan selamat (25-100 µg/mL) dengan koktel adipogenik yang berbeza dengan pengubahsuaian adalah pada penambahan sebatian kajian dan dalam kehadiran (10 µM) atau tanpa insulin. Rosiglitazon (10 µM) digunakan sebagai sebatian piawai. Ekstrak etanol batang, fraksi (heksana dan etil asetat), asid betulinik dan stigmasterol dikenal pasti selamat pada kepekatan tertinggi (100 µg/mL), dengan merencat pertumbuhan sel di bawah nilai IC_{50} masing-masing iaitu 18.60, 35.27, 21.40, 28.86 dan 33.06%. Asid betulinik dan stigmasterol, kedua-duanya pada kepekatan selamat tertinggi (100 µg/mL), secara signifikan ($p < 0.05$) mengaruh adipogenesis seperti insulin, meningkatkan adipogenesis seperti rosiglitazon dan mempamerkan aktiviti pengambilan glukosa. Kajian ini menunjukkan kedua-dua asid betulinik dan stigmasterol berpotensi sebagai antidiabetik *in vitro*. Walau bagaimanapun, kajian antiglisemik *in vivo* terhadap kedua-dua sebatian dan terbitannya masih diperlukan untuk memastikan potensi terapeutik sebatian sebagai agen antidiabetik yang selamat.

Kata kunci: Adipogenesis; aktiviti pengambilan 2-NBDG; aktiviti pensensitifan insulin; aktiviti seperti insulin; asid betulinik; stigmasterol; sel preadiposit 3T3-L1

INTRODUCTION

Diabetes mellitus (DM) is a metabolic disorder which originates due to partial or complete loss of insulin as well as the resistance of the insulin receptor. The ancient Egyptians date back more than 300 years ago reported for the first time about this disease. Currently, the prevalence of this particular disease has increased so much that it is recognized as an alarming global health issue. About 20% of the world's population is anticipated to be diabetic within the next 10 years if no significant improvement takes place in the preventive measurements of diabetes. It is considered as one of the largest epidemics in human history (Zimmet 2017). According to the International Diabetic Federation (IDF), about 415 million people are affected by the syndrome worldwide and the number is set to further escalate even beyond 600 million by 2040 (Ogurtsova 2017). The leading pharmaceuticals around the world are yet to produce an inexpensive and side effects free drug which may possess insulin-like and insulin-sensitizing effects to fight this epidemic worldwide more effectively (Drzewoski & Hanefeld 2021).

Different types of therapeutic approaches are taken into consideration in response to treat type-2 diabetes mellitus (T2DM). Most importantly, by stimulating pancreas to produce more insulin, enhancing the sensitivity of insulin to target receptor, or increasing glucose uptake in adipocyte cells. As insulin-like and insulin-sensitizing effects are considered major concerns in the antidiabetic research, the pre-adipocyte differentiation and glucose uptake on adipocyte have been reported widely as the effective *in vitro* bioassay models to evaluate the antidiabetic effect of any compound under investigation. Hence, adipocytes have been evolved as a key drug target for diabetes and obesity-mediated metabolic syndrome. Adipogenesis is a complex process in which pre-adipocytes become mature adipocytes with hundreds of gene alterations in insulin's presence. Several transcriptional factors have been extensively documented as being involved in the expression of adipogenesis, glucose uptake, and glycolysis pathway. These transcription factors chiefly comprise adipokines, for instance, leptin, glucose transporter-4 (GLUT4) and peroxisome proliferator-activated receptor-γ (PPARγ). A morphological change from fibroblastic to spherical takes place in the initial step of the adipogenesis when the change of the cell shape is accompanied by changes in the level of cytoskeletal and extra cellular matrix (ECM) components. Morphological alteration of cells, gene expression and lipid accumulation occur progressively by the proteolytic reaction of the stromal ECM. Lastly, an increased enzymatic activity in protein and mRNA level occur which comprises the augmented insulin sensitivity as well as the glucose transporters. Therefore, these transcription factors serve as model systems for assessing the differentiating program in normal conditions or in conditions of diabetes, insulin resistance and obesity (Li et al. 2020).

The exploration for a new class of safe antidiabetic agents is considered crucial to tackle diabetes. Hence, there have always been incessant explorations for the discovery of alternative drugs. Traditional medicinal plants have been acknowledged as the preeminent source to find a variety of drugs according to the

World Health Organization (2020). To overcome the hypoglycaemic effect along with other harmful properties of synthetic drugs, innumerable traditional medicinal plants are considered as the focal point to ethno-botanical community as they have been proved to show important medicinal characteristics, such as, anti-hyperglycaemic and hypoglycaemic properties (Kooti 2016). As a result, many traditional medicinal plants have been explored and have proved invaluable in discovering biologically active compounds with preferred pharmacological properties to treat the infirmities such as type-1 DM and type-2 DM (Ahmed et al. 2020).

Currently, scientists have become more engrossed towards traditional medicinal plants across the world. Several research studies are currently underway to find safe antidiabetic agents from the ethnomedicinal plants. In this respect, *Tetracera indica* (Houtt. Ex Christm. & Panz.) Merr. (Family: Dilleniaceae) is one of the Malaysian medicinal plants that can address this issue efficiently. It is commonly known as *Mempelas paya* or sandpaper in Malaysia. It is a large, woody, and rainforest climber. Its leaves have medium shaped; flowers are white, and fruits are berry-like in shape which taste sour (Alhassan et al. 2019). *T. indica* has traditionally been used for treating mouth ulcer, piles, diarrhea, insect's bites, flue, skin rashes, itching, sinus, fever, and diabetes. Leaves of *T. indica* are also used as one of the active components in a local herbal preparation viz., Plantisol® to treat diabetes in Malaysia (Hasan et al. 2017). *T. indica* leaves polar extracts have been reported to display significant *in vitro* 2-deoxy-D-[3H] glucose uptake effect which proves its potential of being an antidiabetic agent (Ahmed et al. 2012). *T. indica* leaves aqueous extract has been reported to decrease triglyceride accumulation on 3T3-L1 adipocyte cells in a dose-dependent manner, whereas cells treated with methanol extract significantly induced lipid accumulation. Three flavonoids, viz., wogonin, norwogonin and techtochrysin from the *T. indica* stems ethanol extract have been reported to induce significant ($p < 0.05$) adipogenesis like insulin and enhance adipogenesis like rosiglitazone. Wogonin and norwogonin also exhibited significant ($p < 0.05$) glucose uptake activity in 3T3-L1 adipocyte cells further confirming its antidiabetic potential (Hasan et al. 2017). However, all active principles responsible for the antidiabetic effect of *T. indica* are yet to be isolated and thoroughly investigated for their antidiabetic potential. Moreover, *T. indica* has been reported to contain triterpenes, especially pentacyclic ones, as the major phytoconstituents along with flavonoids.

Though, flavonoids from this plant have been reported for their antidiabetic effect, betulinic acid (pentacyclic triterpenoid) and stigmasterol (unsaturated phytosterol) isolated from this plant have never been investigated for their role as antidiabetic agents to support the traditional claims of *T. indica* towards the management of diabetes. The curative potential of betulinic acid and stigmasterol is very high yet still poorly recognized. Hence, in this research, our aim was to evaluate the *in vitro* antidiabetic potential of betulinic acid and stigmasterol isolated from the *T. indica* stems ethanol extract.

Materials and Methods

INSTRUMENTS

Melting point was measured using STUART SCIENTIFIC SMP10 instrument and uncorrected. The UV spectra were taken with a Double beam UV-Visible Spectrophotometer 1700 (SHIMADZU Japan). AVANCE III Bruker Spectrometer (600 and 150 MHz) was used for ^{1}H- and ^{13}C-NMR spectra. TMS was used as an internal standard. Bruker microTOF-Q spectrometer was used to obtain ESI-MS spectra. BUCHI rotary evaporator R-200 (Switzerland), water bath and freeze dryer (ALPHA 1-4 LD-2) were used to concentrate the plants' extract. Fritsch Universal Cutting Mill-Pulverisette 19-Germany was used to pulverize dried plant material to obtain the dry powdered form. Absorbance was measured on TECAN micro detection microplate reader (M 200). The fluorescent absorbance of 2-NBDG was detected using the Perkin Elmer Multi label HTS reader. Pictures of the cells were taken through Dino Eye and Evos Microscope.

COLLECTION AND PREPARATION OF PLANT MATERIAL

Fresh stems of *T. indica* (10 kg) were collected from the Taman Pertanian, Indera Mahkota, Kuantan, Pahang, Malaysia. The plant material was authenticated by the taxonomists of Taman Pertanian and the botanist of Kulliyyah of Pharmacy (Dr. Norazian Mohd. Hassan), IIUM. Subsequently, the plant material was deposited in the herbarium of Kulliyyah (NMPC-QSTI-39).

Plant sample was subjected to dryness in a laboratory dryer (30 to 40 °C) and was pulverized using Fritsch Universal Cutting Mill PULVERISETTE 19 (Germany). The pulverized material (4.7 kg, 47%) was subjected to the process of ethanol extraction to isolate betulinic acid and stigmasterol (Alhassan et al. 2019).

PREPARATION OF ETHANOL EXTRACT, FRACTIONATION,

AND ISOLATION OF BETULINIC ACID AND STIGMASTEROL

The powdered material of stems (4.7 kg) was initially *defatted* using *petroleum ether* and then extracted by soaking in 95% ethanol in a round bottom flask for 24 h at room temperature, filtered through Buchner funnel and finally concentrated in a reduced pressure using the Buchi rotary evaporator. Recovered ethanol was again poured into the already extracted powdered material and then refluxed on the water bath for another 2-3 h. This process was repeated about 4 times until the plant material stopped giving coloration. The extracts were combined and finally concentrated using the rotary evaporator to obtain 295.5 g crude ethanol extract (6.28%).

The crude ethanol extract (295.5 g) was fractionated using a separatory funnel. Initially, it was dissolved in distilled water and treated with excess hexane until the hexane portion became colourless. The hexane portion was recovered through a rotary evaporator. The combined hexane fraction (11.2 g) was considered as non-polar extract of the stems of *T. indica*. Subsequently, the remaining hexane insoluble portion of crude ethanol extract was treated with ethyl acetate (EtOAc) in the same manner to obtain EtOAc fraction (62.28 g) of *T. indica* stems ethanol extract. Finally, betulinic acid and stigmasterol of *T. indica* stems were isolated in pure form through silica gel 60 (63-200 µm) column chromatography followed by small preparative column chromatographies containing silica gel 60 (63-200 µm) and *Sephadex* LH-20 (Alhassan et al. 2019).

Betulinic acid White crystal; mp: 295-300 °C; IR (KBr) ν_{max} cm^{-1} : 3455. 8, 2938.9, 2865.1, 1737.2, 1682.5, 1639.5, 1448.1, 1375.7, 1318.6, 1295.3, 1234.8, 1186.0, 1106.7, 1042.7, 1007.0, 986.0, 969.7, 946.5, 918.6, 885.8, 793.0, 751.1, 625.5, 572.1, 545.0, 453.4; ^{1}H-NMR [600 MHz, $CDCl_3$, δ (ppm)]: δ 4.74 (1H, br s, H_a-29) 4.62 (1H, br s, H_b-29), 3.20 (1H, dd, J=11.4, 4.2Hz, H-3), 3.02 (1H, m, H-19), 1.77 (3H, s, Me-30), 0.99 (3H, s, Me-26), 0.94 (3H, s, Me-27), 0.83 (3H, s, Me-25), 0.76 (3H, s, Me-24), ^{13}C-NMR ($CDCl_3$, 150 MHz): 39.07 (C-1), 27.59 (C-2), 79.22 (C-3), 38.91 (C-4), 55.54 (C-5), 18.49 (C-6), 34.52 (C-7), 40.89 (C-8), 50.71 (C-9), 37.41 (C-10), 21.05 (C-11), 25.70 (C-12), 38.57 (C-13), 42.64 (C-14), 30.74 (C-15), 32.36 (C-16), 56.46 (C-17), 47.09 (C-18), 49.46 (C-19), 152.56 (C-20), 29.91 (C-21), 37.23 (C-22), 28.19 (C-23), 14.90 (C-24), 16.23 (C-25), 16.34 (C-26), 14.60 (C-27), 179.96 (C-28), 109.91 (C-29), 19.58 (C-30); ESI-MS m/z 457 corresponding to $C_{30}H_{48}O_3$ $[M+H]^+$ (Ahmed et al. 2014; Alhassan et al. 2019).

Stigmasterol White crystal; mp: 160-165 °C; IR (KBr) ν_{max} cm^{-1} : 3390.9, 2932.8, 2867.4, 1462.6, 1366.5, 1163.7, 1104.7, 1074.4, 1019.9, 799.9, 620.8; ^{1}H-NMR [600 MHz, $CDCl_3$, δ (ppm)]: δ 3.55 (1H, m, H-3), 5.36 (1H, t, H-6), 5.17 (1H, dd, J = 15, 9 Hz, H-22), 5.04 (1H, dd, J = 15, 9 Hz, H-23), 1.00 (3H, s, Me-10), 0.90 (3H, d, J=6.0 Hz, Me-20), 0.85 (3H, d, J=2.4, Me-27), 0.80 (3H, d, J=2.4 Hz, Me-26), 0.65 (3H, s, Me-13), ^{13}C-NMR ($CDCl_3$, 150 MHz): δ 32.43 (C-1), 35.53 (C-2), 77.24 (C-3), 42.23 (C-4), 140.77 (C-5), 129.28 (C-6), 31.54 (C-7), 26.08 (C-8), 42.23 (C-9), 39.69 (C-10), 19.41 (C-11), 31.91 (C-12), 40.51 (C-13), 45.95 (C-14), 21.23 (C-15), 21.13 (C-16), 45.84 (C-17), 18.99 (C-18), 18.80 (C-19), 33.72 (C-20), 19.84 (C-21), 129.84 (C-22), 127.24 (C-23), 45.95 (C-24), 31.67 (C-25), 21.09 C-26), 23.08 (C-27), 25.42 (C-28), 19.05 (C-29),; ESI-MS m/z 413 $[M+H]^+$ (Ahmed et al. 2014; Alhassan et al. 2019).

IN VITRO ANTIDIABETIC ACTIVITY

CELL CULTURE MATERIALS

Mouse 3T3-L1 fibroblast (CL-173) was obtained from the American Type Culture Collection (ATCC), Virginia, USA. Dulbecco's-modified Eagle medium (DMEM) (with glucose and without glucose), Tryple Express (trypsin) and human recombinant insulin (4 mg/mL) were procured from GIBCO, India. Dexamethasone was purchased from Calbiochem, EMD Chemicals, Inc. San Diego, CA. 2-[N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl) amino]-2-deoxy-D-glucose (2-NBDG) was purchased from Molecular Probes by Life Technologies, USA. 4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide (MTT) was obtained from Ameresco, Solon, Ohio 44139, USA. Rosiglitazone, Dimethyl Sulfoxide (DMSO) and Oil Red O staining (ORO) were procured from Sigma Aldrich, USA. 96 well flat bottom sterile microplates were obtained from Greiner Bio-one Cellstar and black microplates were obtained from SPL Life Science Limited, Korea. 3-isobutyl-1-methylxanthine (IBMX) was procured from EMD Millipore Corp., USA. Phosphate Buffered Saline (PBS) was bought from GIBCO by Life Technologies, Invitrogen, USA.

CELL CULTURE

3T3-L1 mouse pre-adipocyte cells were cultured according to the protocol provided by the ATCC, Rockville, MD, USA. The growth media was used as DMEM comprising 4.5 g/L D-glucose and L-glutamine with no sodium pyruvate and it was completed by adding 1% penicillin-streptomycin and 5% Fetal Bovine Serum (FBS). The cells were cultured in Greiner-bio CellStar flasks of 25 and 75 cm^2. The cells were incubated in a humidified atmosphere with 5% CO_2 supply at 37 °C. The

media was changed after every two days, and the cells were subcultured upon 80% confluence.

CELL VIABILITY ASSAY

The cell viability assay was carried out via MTT method on the 3T3-L1 mouse pre-adipocyte cells (2×10^5 cells) which were seeded in 96-well plates and allowed to get confluent for 48 h. Afterwards, they were treated with extract, fractions, betulinic acid and stigmasterol (dose: 0.78-100 µg/mL) for 48 h. Later, the cells were treated with MTT at 5 mg/mL. Every well was treated with 20 µL of MTT and incubated for 4 h. Finally, 100 µL of DMSO was transferred to every well to dissolve the water-insoluble purple formazan crystals (Choi et al. 2011). Plates were kept at normal temperature wrapped in the aluminum foil to avoid any chemical interaction with the light. TECAN micro detection microplate reader (M 200) was used to measure absorbance values at 570 nm. Cell percent viability percentage was calculated using the formula given as follows:

$$\text{Cell percent viability (\%)} = [(\text{mean } A_{sample} - \text{mean } A_{blank})/(\text{mean } A_{control} - \text{mean } A_{blank})] \times 100$$

ADIPOGENESIS

INDUCTION OF ADIPOGENESIS

The adipogenesis was induced according to the protocol reported by Hasan et al. (2017) and Park, Kim and Park (2012). Briefly, the pre-adipocytes were seeded in 96 well flat bottom sterile microplates. Afterward, the same cells were treated with the adipogenic cocktail after two days upon 90% confluence. The day was considered as day zero. At the end of the adipogenesis period, more than 90% of the cells contained lipid droplets that could be viewed under low power magnification. The adipogenic cocktail (differentiation media, DM) was modified by insulin, extract/betulinic acid and stigmasterol, and rosiglitazone. Oil Red O staining was used to confirm adipogenesis through transformation of fibroblasts into mature adipocytes.

Three different concentrations of stigmasterol and betulinic acid (25, 50 and 100 µg/mL) were evaluated based on the safety of the cells previously established via MTT assay. The 3T3-L1 pre-adipocyte cells were incubated for 2 days in the differentiation media. On 'day 2', the media was replaced by insulin media (IM) (10 µg/mL insulin in DMEM) for two days. Finally, on day four, the DMEM was applied and was changed every 2 days for 6-8 days.

OIL RED O STAINING

After the adipogenesis process, the cells were fixed with 10% formalin in phosphate-buffered saline (PBS) for 1 h at room temperature. Subsequently, the cells were washed thrice with PBS and stained with freshly prepared Oil Red O (3 parts of Oil Red O of 0.6% in 2 parts of deionized water) from the stock solution for 1 h. Cells were again washed with distilled water and approximately 1 mL isopropanol was further added. Oil Red O staining was extracted by isopropanol after 5 min. Finally, the absorbance was measured using a microplate reader at 520 nm (Hasan et al. 2017).

2-NBDG UPTAKE IN 3T3-L1 ADIPOCYTE CELLS

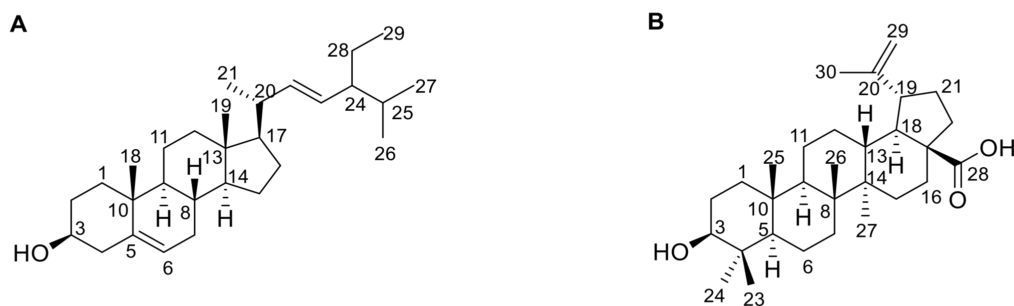
The 2-NBDG uptake test was done by following the protocol described by Hasan et al. (2017). Briefly, the pre-adipocytes were treated with the adipogenic cocktail to differentiate into mature adipocytes using the same protocol used for adipogenesis control groups. When the adipogenesis had been completed on day 8, the adipocytes were incubated in DMEM (serum and glucose free) for 2 days. Subsequently, the serum and glucose starving adipocytes were treated with 80 µM 2-NBDG and sampled (in different concentrations) with insulin (10 µg/mL) or with rosiglitazone (10 µM) for 48 h. At the same time, a sample was seeded with insulin to investigate the possible insulin-sensitizing effect. The cultures were washed with PBS to get rid of free 2-NBDG upon finishing the incubation. Finally, the fluorescence retained in the cell monolayer was measured by fluorescence microplate reader (Perkin Elmer Multi label HTS reader) at 485 nm and 535 nm, respectively. The 100% specific 2-NBDG uptake was determined by subtracting control having 2-NBDG and DMEM from control having 2-NBDG and insulin by following formula:

100% specific absorbance =

(Absorbance of insulin induced 2-NBDG uptake - Absorbance of non-insulin induced 2-NBDG uptake)

STATISTICAL ANALYSIS

Data were interpreted using IBM SPSS (version 20) and a minimum of three (n=3) replicates were performed for each data on different days. Graphical representations of all data were done by Microsoft Excel. The analysis was done by one-way ANOVA post hoc and followed by Dunnett multiple comparison test. The untreated control was taken as a dependent variable and the rest of the groups were compared with it. A $^{*}p < 0.05$ was considered statistically significant and $^{**}p < 0.005$ was considered highly significant.



No	Compound	IUPAC name	Molecular formula
A	Stigmasterol	(24*R*)-ethyl-cholesta-5,22-dien-3β-ol	$C_{29}H_{48}O$
B	Betulinic Acid	(3β)-Hydroxylup-(20)(29)-en-28-oic acid	$C_{30}H_{48}O_3$

FIGURE 1. Chemical structures of stigmasterol and betulinic acid

Results and Discussion

ISOLATION OF BETULINIC ACID AND STIGMASTEROL

Ethyl acetate fraction (active fraction) of *T. indica* stems ethanol extract using repeated silica gel and *Sephadex* LH-20 column chromatographies managed to provide betulinic acid (pentacyclic triterpenoid) and stigmasterol (unsaturated phytosterol) in pure form (Figure 1). Both compounds were characterized through spectroscopic analysis. Their spectral data were further compared with the data reported earlier for the similar compounds from other plants (Ahmed et al. 2014; Alhassan et al. 2019).

IN VITRO BIOACTIVITY DETERMINATION

MTT Assay

MTT viability assay helps to evaluate the safe concentration of the extracts/fractions/compounds investigated in the bioactivity experiments using pre-adipocytes or other types of cells (Choi et al. 2011). The inhibition of a drug is evaluated by comparing with untreated control when the safe concentration of the drug is considered below 50% inhibition which is called as IC_{50} (Roheem et al. 2020). Hence, in this study, a total of 8 different concentrations (0.78 to 100 µg/mL) were initially evaluated and finally 3 safe doses were selected to evaluate an *in vitro* antidiabetic effect against 3T3-L1 pre-adipocyte cells. Results showed that the *T. indica* stems ethanol extract inhibited 18.60% cells at its highest concentration (100 µg/mL) which was found highly significant (**p < 0.005) in comparison to the control group. Therefore, the ethanol extract was considered safe for the cells at its highest concentration (viz. 100 µg/mL). Similarly, both of its fractions (hexane and ethyl acetate) were also found safe at their highest concentrations viz. 35.27% and 21.40%, respectively, and both varied from the control significantly (**p < 0.005 inhibition). Betulinic acid and stigmasterol were found safe up to 100 µg/mL. The percent inhibitions at their highest concentrations of stigmasterol and betulinic acid were found 28.86% and 33.06%, respectively. It was concluded that these compounds up to 100 µg/mL can be safely used to investigate bioactivity owing to their safe nature at the tested concentrations via MTT assay.

Adipogenesis

3T3-L1 pre-adipocytes after undergoing the differentiation to adipocytes serve as excellent *in vitro* models and are considered valuable tools in understanding the glucose metabolism. In the adipogenesis process, the pre-adipocytes are undergone to growth arrest and subsequently differentiated to mature adipocytes. This experiment was done to ensure whether *T. indica* extract, its fractions, betulinic acid and stigmasterol can induce adipogenesis in the presence and absence of insulin.

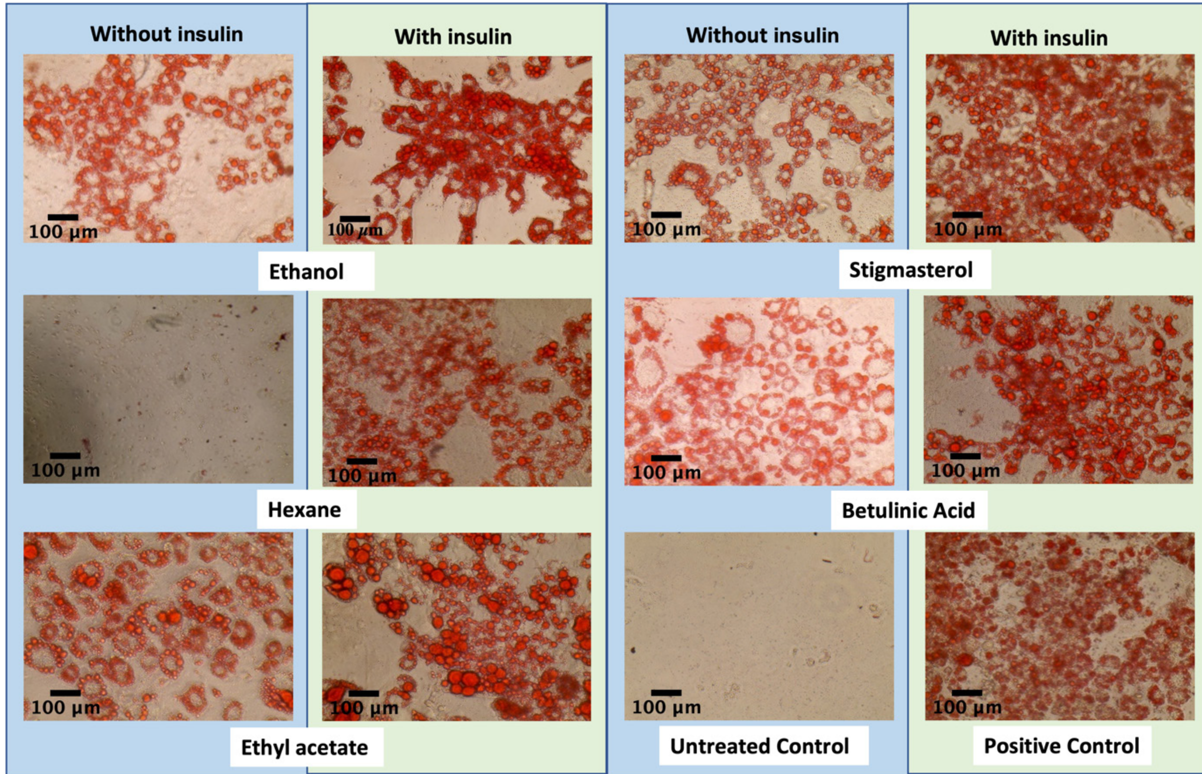


FIGURE 2. Oil-red-O staining of 3T3-L1 adipocytes on day-10. Results showed to induce differentiation (adipogenesis) at indicated concentrations in the presence or absence of insulin (10 µg/mL). Cultures in basal medium and insulin served as positive control. Cells treated with rosiglitazone served as drug control

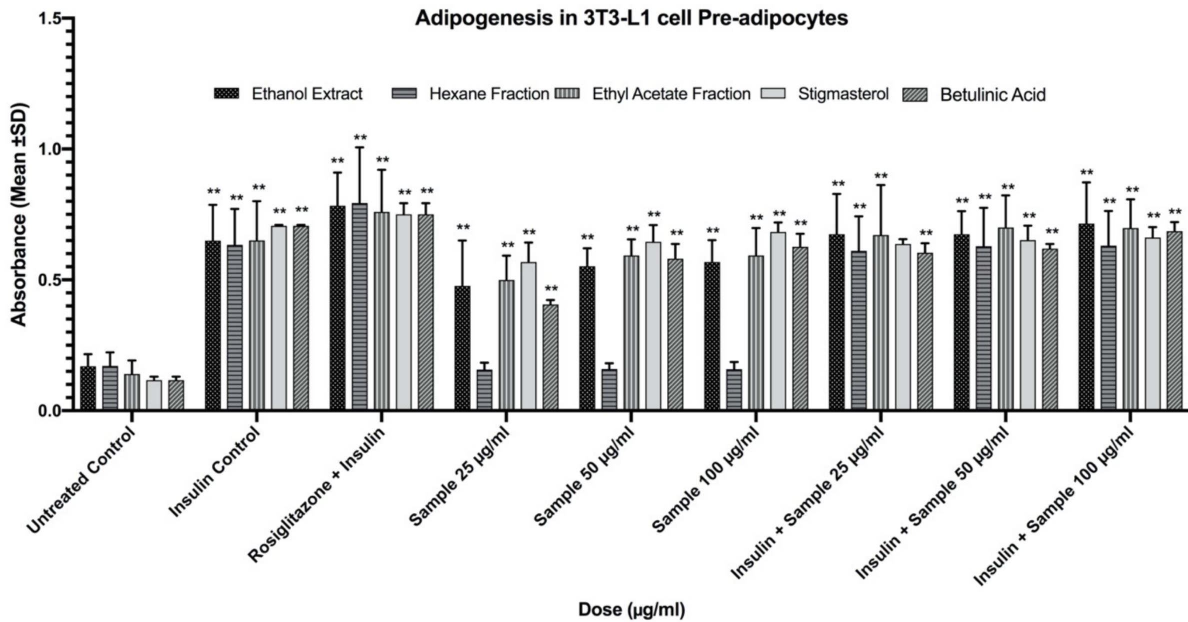


FIGURE 3. *T. indica* stems ethanol extract, it's two fractions (hexane and ethyl acetate) and isolated compounds (stigmasterol and betulinic acid) were investigated for their ability to enhance adipogenesis in the absence and presence of insulin (10 µg/mL) and rosigiltazone (10 µM). The results showed in absorbance (Mean± SD), n =3 per group and triplicate of each group

The results displayed that all substances exerted a dose-dependent effect similar to insulin-like and insulin sensitizing effects (Figures 2 and 3). In this experiment, rosiglitazone (positive control) was taken as a marker for insulin-sensitizing activity. The results further shown that the combination groups of betulinic acid and stigmasterol along with insulin did not show significant difference as of the only sample groups.

Pre-adipocytes are differentiated into adipocytes through an inducer known as adipogenic cocktail which includes insulin, phosphodiesterase inhibitor methylisobutylxanthine (IBMX), fetal bovine serum (FBS) and the synthetic glucocorticoid dexamethasone (Ruiz-Ojeda et al. 2016). Our study was designed to evaluate the activity by altering the adipogenic cocktail. The insulin-like activity of both betulinic acid and stigmasterol was determined by replacing the insulin from the adipogenic cocktail and the insulin-sensitizing activity was determined in the presence of insulin to evaluate whether it can sensitize or increase the effect of insulin. Rosiglitazone belongs to thiazolidinediones, also known as 'glitazones', was taken as positive control which is an insulin-sensitizing agent that acts by improving the sensitivity of peripheral tissues to insulin through activating PPARγ receptor (Hasan et al. 2017). The results showed that the *T. indica* stems crude ethanol extract, ethyl acetate fraction, betulinic acid and stigmasterol showed effects in a dose dependent manner similar to insulin-like- and insulin sensitizing activities. However, non-polar fraction (hexane fraction) showed no effect on stimulating adipogenesis in the absence of insulin. Moreover, the hexane fraction also did not show any insulin sensitizing effect in comparison to rosiglitazone. Stems' crude ethanol extract, its ethyl acetate fraction, betulinic acid and stigmasterol at their highest concentration showed absorbance almost similar to control (insulin) and also exhibited similar activity like rosiglitazone.

PPARγ ligands can affect the adipocyte differentiation and are reported to have an effect on glucose uptake in 3T3-L1 adipocytes (Moseti, Regassa & Kim 2016). Hence, the next objective of this research was an attempt to assess the effect of betulinic acid and stigmasterol on the glucose uptake and insulin sensitizing effects. In adipocytes, basal (cells treated with normal glucose without the presence of insulin and 2-NBDG) and insulin-stimulated glucose uptake activities require a glucose transporter. Insulin can accelerate glucose entry by affecting the translocation of GLUT4 from intracellular stores to the plasma membrane. In general, it is known that GLUT4 provides insulin-stimulated glucose transport in adipocytes. Furthermore, glucose uptake in adipocytes is the consequence of stimulation of insulin receptors by insulin. This process consists of translocation of GLUT4. During adipogenesis, the expression of GLUT4 is increased and in the presence of insulin, the GLUT4 is translocated to the plasma membrane. Subsequently, the glucose uptake by the cells is increased (Fazakerley, Koumanov & Holman 2022). Moreover, GLUT4 is expressed in the adipocytes and its expression is regulated by PPARγ. Our experiments were designed to evaluate insulin-like and insulin sensitizing activities for both betulinic acid and stigmasterol. Hence, we evaluated the effect of stigmasterol and betulinic acid on the matured adipocytes to stimulate 2-NBDG in the presence as well as absence of insulin and rosiglitazone (insulin-sensitizer).

All groups were compared with the untreated control group to check the significant differences. It was observed that the insulin control and rosiglitazone control groups were significantly different from the untreated control group. Keeping in mind the mechanism of action for insulin sensitizer is to increase the insulin activity, we treated rosiglitazone along with insulin to observe the insulin-sensitizing activity. Moreover, the treatment groups without insulin were compared with the insulin groups to understand the insulin-like activity of the groups whereas the groups containing the insulin and sample (betulinic acid and stigmasterol/extracts) were compared to the rosiglitazone group to check the insulin-sensitizing activity. It was observed that the effect of adipogenesis was manifested in a dose dependent manner. Stems' crude ethanol extract, its ethyl acetate fraction, stigmasterol and betulinic acid showed insulin-like activity. Moreover, at their highest dose, all were also found to exhibit activity similar to insulin. The groups also showed the same trend when they were compared with the rosiglitazone-insulin combination.

In vitro fluorescence glucose uptake (2-NBDG Uptake)

This experiment was done to ensure whether stigmasterol and betulinic acid may demonstrate the insulin-like and insulin sensitizing activities. Therefore, both compounds were investigated on the matured adipocytes to stimulate 2-NBDG in the absence and presence of insulin and rosiglitazone (insulin-sensitizer). Glucose uptake in adipocyte cells occurs with the introduction of insulin through the translocation of GLUT4 to the cell surface (Fazakerley Koumanov & Holman 2022). The results displayed an increased stimulation of the 2-NBDG uptake in the absence of insulin. A dose dependent increase for both betulinic acid and stigmasterol were clearly observed as the activity was found to enhance

with the increased concentration. It was also noticed that 2-NBDG uptake by stigmasterol was higher than the insulin (control) at its highest concentration (100 µg/mL). Same trend was observed with betulinic acid as well, however, insulin-like activity was way greater than the insulin even at its lowest concentration (25 µg/mL). The fluorescence absorbance at the higher concentration (100 µg/mL) (Figure 4) was found much higher than the insulin which clearly indicated that both betulinic acid and stigmasterol could well be suggested to be used as the replacement of insulin in future research studies. Moreover, in the same experiment, rosiglitazone was taken as positive control to determine insulin-sensitizing effect on 3T3-L1 adipocytes. The results suggested insulin sensitizing effect on a dose dependent manner for both stigmasterol and betulinic acid. The effect was found to be similar to the insulin-rosiglitazone combination.

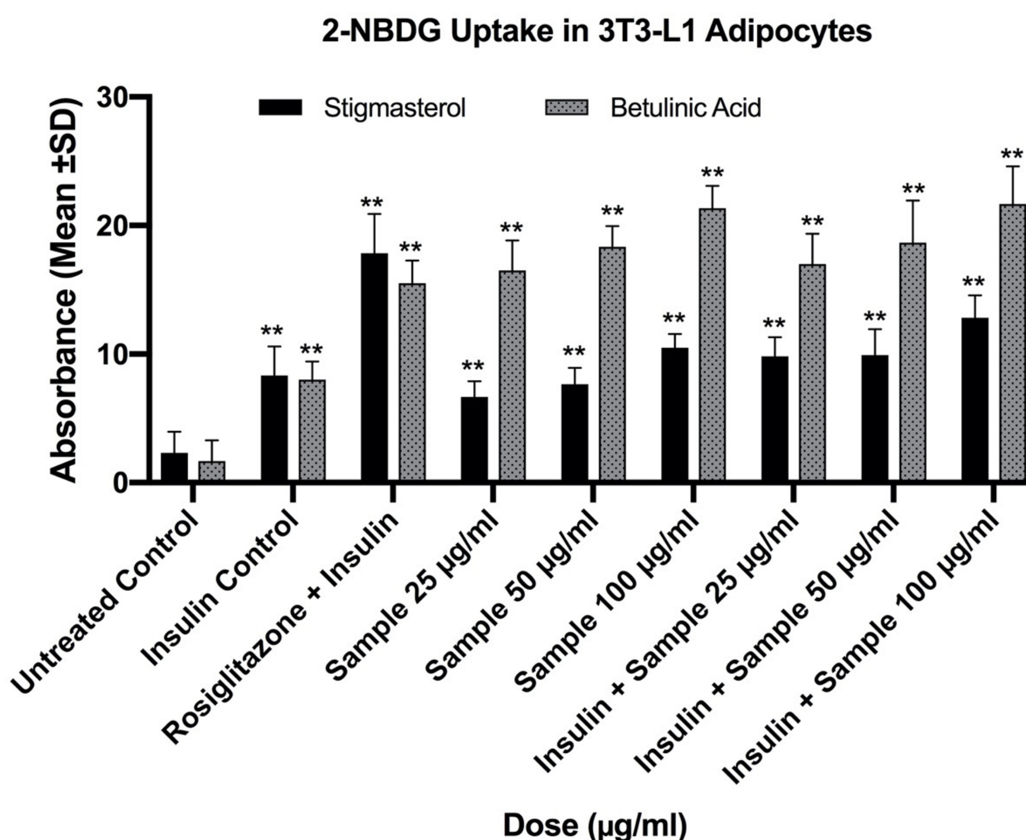


FIGURE 4. Stigmasterol and betulinic acid were evaluated to stimulate 2-NBDG uptake in 3T3-L1 adipocytes in the absence and presence of insulin at indicated concentration 25-100 µg/mL, respectively. Cultures in basal medium and insulin served as control. Cells treated with rosiglitazone were considered as positive control which represents insulin-sensitizing activity. Data expressed in mean ± SD, n = 9 (Three biological triplicate each containing minimum n=3). Student paired t-test showed significant value, **$p < 0.005$

However, both betulinic acid and stigmasterol showed no significant difference with the sample group when it was combined with insulin which indicated that both betulinic acid and stigmasterol are more likely to not possessing insulin sensitizing effects. A further thorough study could well be suggested based on this observation. An in-depth mechanism of action could lead us to uncover the possible reason why combinatorial effects were found insignificant. There could be the possibility of an interaction of betulinic acid and stigmasterol with insulin. Since the addition of insulin does not increase the glucose uptake significantly, there could be an event of structural alteration leading to hindering the GLUT4 translocation. As a hindrance occurred that could possibly be blocking the signaling pathway of glucose transport,

we suggest further studies on insulin receptor binding, GLUT4 translocation assay, and intracellular protein phosphorylation study for a better understanding of these observations.

These findings were found similar with the study carried out by Ma et al. (2014) in which a structural analogue of stigmasterol, namely, 6α-hydroxy-lup-20(29)-en-3-on-28-oic acid obtained from the *Viburnum odoratissimum* Ker Gawl. Adoxaceae aerial parts significantly stimulated glucose absorption in insulin resistant HepG2 cells without showing any deleterious effect on the cell viability for the same insulin resistant HepG2 cells. Moreover, the same unsaturated phytosterol also restored the glucose absorption in DXMS-induced insulin resistant 3T3-L1 cells. In another study, Panda et al. (2009) isolated stigmasterol from the bark of *Butea monosperma* (Lam.) Taub. (Fabaceae) and evaluated its glucose regulatory efficacy in mice. Its administration at 2.6 mg/kg/d for 20 days reduced glucose concentrations indicating its hypoglycemic property. Moreover, another similar study done by Wang et al. (2017) showed the antidiabetic activity and potential mechanism of stigmasterol. In the same study, stigmasterol exhibited a mild *in vitro* GLUT4 translocation property in L6 cells and also displayed significant effects on the enhancing glucose uptake at different concentrations in L6 cells. In the same research study, stigmasterol was given orally to the KK-Ay mice and significantly improved their insulin resistance and oral glucose tolerance. Moreover, it also managed to reduce fasting blood-glucose levels and blood lipid indexes viz., triglyceride and cholesterol. Furthermore, the GLUT4 expression in L6 cells, skeletal muscle and white adipose tissue were also boosted. These results further confirm potential beneficial effects of stigmasterol on the treatment of type 2 diabetes mellitus with the probable mechanism of targeting GLUT4 glucose transporter thereby increasing GLUT4 translocation and expression (Nazaruk & Borzym-Kluczyk 2015; Wang et al. 2020).

In addition to that, there is some experimental evidence that clearly shows the antidiabetic potential of betulinic acid. Singab et al. (2005) reported that 70% ethanol extract of the Egyptian *Morus alba* L. (Moraceae) root bark showed significant hypoglycaemic effect in streptozotocin induced diabetic rat when the extract was administered at 600 mg kg^{-1} day^{-1} for 10 days. It was confirmed through phytochemical analysis that the *M. alba* root bark ethanol extract contained betulinic acid in good amount. Likewise, the hypoglycaemic effect of betulinic acid was further evaluated by de Melo et al. (2009). In their study, the mice on a high-fat diet were observed to show glucose lowering effect by betulinic acid at 50 mg/L. Moreover, betulinic acid has also been reported to suppress protein tyrosine phosphatase 1B (PTP1B; plays a role in the regulation of insulin signalling) activity which was found comparable to the positive controls viz., ursolic acid and RK-682 (Choi et al. 2009). Besides, another study proved insulin sensitizing activity of betulinic acid as the PTP1B inhibitor which further confirms betulinic acid as a potential insulin sensitizer (Jin, Yu & Huang 2016).

Conclusion

The current study has shown that betulinic acid and stigmasterol isolated from *T. indica* stems crude ethanol extract do possess antidiabetic effect on 3T3-L1 adipocytes. Both betulinic acid and stigmasterol were found to show significant insulin-like and insulin-sensitizing activities at their safe doses during adipogenesis. Furthermore, both betulinic acid and stigmasterol displayed significant increase in stimulating 2-NBDG in absence of insulin. Betulinic acid showed higher 2-NBDG uptake activity than the rosiglitazone. This clearly proves insulin-like activity of betulinic acid and stigmasterol. Moreover, a further study on the mechanism of action, receptor binding, intracellular phosphorylation could pave a way to understand the mechanism of insignificant difference in combinatorial treatment. Our study suggests antidiabetic potential of betulinic acid and stigmasterol in terms of insulin-like effects. However, we still suggest further in-depth research study on the isolated betulinic acid and stigmasterol and their semisynthetic derivatives that might lead to the discovery of efficacious antidiabetic drugs.

Acknowledgements

Authors are grateful to the Universiti Malaysia Pahang (UMP), Malaysia for financial assistance to accomplish this work. Authors are also grateful to the Molecular Structure Determination Laboratory (MSDL), Centre for Research and Instrumentation Management, Universiti Kebangsaan Malaysia (CRIM, UKM) for conducting NMR/MS analysis. This research work was supported under the Joint Research Projects (UKM: GUP-2021-047; ID: SRCG20-002-0002; UMP-IIUM-UiTM Sustainable Research Collaboration Grant 2020). The authors declare that they have no conflict of interest.

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