

Antidiabetic and antioxidants activities of *Clinacanthus nutans* (Burm F.) Lindau leaves extracts

¹Murugesu, S., ^{1*}Khatib, A., ¹Ibrahim, Z., ¹Ahmed, Q. U., ¹Uzir, B. F.,
¹Nik-Yusoff, N. I., ¹Perumal, V., ²Abas, F. and ²Saari, K.

¹Department of Pharmaceutical Chemistry, Kulliyah of Pharmacy, International Islamic University Malaysia, Jalan Sultan Ahmad Shah, Bandar Indera Mahkota, 25200 Kuantan, Pahang Darul Makmur, Malaysia.

²Laboratory of Natural Products, Institute of Bioscience, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor Darul Ehsan, Malaysia.

Article history

Received: 12 February, 2018

Received in revised form:

24 August, 2018

Accepted: 5 September, 2018

Abstract

Clinacanthus nutans (Acanthaceae) is a local plant consumed as tisane in Indonesia and 'ulam' in Malaysia. This plant has been claimed for its ability to prevent many diseases including diabetes. However, the scientific proof on this claim is still lacking. Therefore, the present work study was designed to evaluate the antidiabetic potential and antioxidant capacity of *C. nutans* leaves extracts using *in vitro* bioassay tests. The 80% methanolic crude extract of this plant was further partitioned using different polarity solvents namely hexane, hexane:ethyl acetate (1:1, v/v), ethyl acetate, ethyl acetate:methanol (1:1, v/v), and methanol. All the sub-fractions were analysed for antioxidant effect via 2, 2-diphenyl-2-picrylhydrazil (DPPH) scavenging activity, ferric reducing power (FRAP) and xanthine oxidase (XO) assays followed by antidiabetic evaluation via α -glucosidase and dipeptidyl peptidase-IV (DPP-IV) inhibitory assays and glucose uptake experiment. The ethyl acetate fraction showed a good antioxidant potential while the hexane fraction exhibited high α -glucosidase and DPP-IV enzyme inhibition. The hexane fraction also improved glucose uptake in a dose-dependent manner. The present work thus provides an informative data on the potential of *C. nutans* to be developed as a functional food in preventing diabetes.

Keywords

Antidiabetic

Antioxidant

Clinacanthus nutans

α -glucosidase inhibition

© All Rights Reserved

Introduction

Various plants that have been used or consumed for the prevention and treatment of various diseases since ancient times are distributed all over Malaysia (Bahmani *et al.*, 2014). One of the valuable herbs that has undergone multiple research studies in the past decades is *Clinacanthus nutans* (Burm F.) Lindau. This medicinal plant is native to several Southeast Asian countries including Malaysia (Arullappan *et al.*, 2014; Alam *et al.*, 2016). The Indonesians prepare decoction or tisane by boiling a handful of fresh *C. nutans* leaves and consume it to prevent several illnesses including diabetes (Globinmed, 2015). In fact, the plant has gained popularity in Malaysia and Singapore, where various varieties of commercialised products such as teas and drinks have been introduced because of its acclaimed anticancer properties (Zulkipli *et al.*, 2017). Some of the reported pharmacological properties of this

plant leaves include anticancer, anti-inflammatory, antiviral, antidiabetic and antigout (Arullappan *et al.*, 2014; Alam *et al.*, 2016). The leaves are typically used for the treatment of burns, allergic reactions, mucositis, skin rashes and lesions of varicella-zoster virus (VZV) and herpes simplex virus (HSV) (Alam *et al.*, 2016). Several bioactive compounds are reported to be present in *C. nutans* leaves such as caffeic acid, chlorogenic acid, quercetin, lupeol, betulin, stigmasterol and β -sitosterol (Alam *et al.*, 2016).

The present work was aimed to evaluate the antidiabetic and antioxidant potentials of the plant leaves extract via *in vitro* bioassays. *In vitro* bioassay is an important analytical method that has high capacity, low-cost and provides rapid results that could aid in pre-screening, screening, monitoring, and secondary testing of natural products (Panuganti, 2015). The bioassay tests conducted include 2, 2-diphenyl-2-picrylhydrazil (DPPH) scavenging activity, ferric

*Corresponding author.

Email: alfikhatib@iiu.edu.my

reducing power (FRAP) and xanthine oxidase (XO) assays followed by antidiabetic evaluation *via* α -glucosidase and dipeptidyl peptidase-4 (DPP-IV) inhibitory assays and glucose uptake experiment.

Materials and methods

Materials

All organic solvents of analytical grade (hexane, ethyl acetate, and methanol) and 1, 1-diphenyl-2-picrylhydrazil (DPPH) reagent were purchased from Merck (Darmstadt, Germany). The enzymes, α -glucosidase (yeast maltase, *Saccharomyces cerevisiae*) and xanthine oxidase (butter milk) were purchased from Megazyme (Ireland) and Millipore (USA), respectively. The p -nitrophenyl- ρ -D-glucopyranosidase (PNPG), 2, 4, 6-tripyrindyl-s-triazine (TPTZ), xanthine and quercetin were purchased from Sigma Aldrich (St. Louis, MO, USA). Allopurinol and glycine were obtained from Nacalai Tesque (Kyoto, Japan). The buffer solution was prepared using potassium dihydrogen phosphate, and dimethylsulfoxide (DMSO) was used as solvent, where both were purchased from HmbG Chemicals (Germany).

Sample collection and preparation

Matured leaves of *C. nutans* were harvested from EES Herbs Farm, Penang. The plant sample was submitted to the Herbarium, IIUM for species authentication. The specimen's voucher number is PIIUM 0238-1. The leaves were dried under shade at room temperature ($\pm 25^\circ\text{C}$) for 1 w. The dried leaves were then grounded to powder by using Universal cutting mill (Fritsch, Germany; Abdul *et al.*, 2011). Powdered leaves of *C. nutans* of about 6 kg were subjected to extraction using maceration technique with 80% methanol (Yi and Wetzstein, 2011). The powdered leaves were macerated into the solution at the ratio of 1:3 w/v where the solvent was changed each consecutive day. The extracts were filtered and recovered using a rotary evaporator at 40°C before freeze-drying to remove any remaining solvents. The concentrated extracts were then stored at -80°C for further analysis (Alothman *et al.*, 2009; Abdul *et al.*, 2011).

The dried crude extract obtained were subjected to further partitioning using 1:3 (w/v) of hexane (H), hexane:ethyl acetate (H:E), ethyl acetate (E), ethyl acetate:methanol (E:M) and methanol (M) (1:1 v/v). The fractions were filtered using Whatman filter paper No. 1 and were recovered using a rotary evaporator at 40°C before freeze-dried to remove any remaining solvents. The fractions were prepared in six replicates

and stored at -80°C for further analysis (Alothman *et al.*, 2009). The yield of extractions was determined in the percentage of 100 g ground sample on dry weight basis as described by Javadi *et al.* (2014):

$$\text{Percentage yield (\%)} = \frac{\text{Mass of extract (g)}}{\text{Mass of dry sample (g)}} \times 100$$

In-vitro bioactivity assays

2, 2-diphenyl-2-picrylhydrazil (DPPH) radical scavenging activity

The antioxidant activity using DPPH scavenging assay was carried out following the modified method described by Pieroni *et al.* (2002). Accurately, 1 mg extract was dissolved in 1 mL methanol. About 20 μL sample solution was treated with 80 μL 0.1 mM DPPH solution prepared in methanol as well. A sample solution of 100 μL was used as blank, and ascorbic acid as the positive control to validate the assay. The absorbance was measured at 517 nm after 10 min using spectrophotometer (Tecan Nanoquant Infinite M200, Switzerland). The half-maximal inhibition concentration (IC_{50}) value was determined.

$$\text{Inhibition (\%)} = \left[\frac{A_0 - A_1}{A_0} \right] \times 100$$

where A_0 and A_1 corresponds to the absorbance at 517 nm of the radical (DPP^\bullet) in the absence and presence of antioxidant respectively.

Ferric reducing power (FRAP)

A modified method described by Ghasemzadeh *et al.* (2015) was adopted for the FRAP assay. The FRAP reagent was prepared freshly by mixing 25 mL acetate buffer (0.1 M), 2.5 mL TPTZ (10 mM) and 2.5 mL FeCl_3 (20 mM) solutions, and allowed to stand for 10 min at 37°C . Plant fractions (20 μL) were reacted with FRAP solution (40 μL) for 20 min in the dark. The absorbance was measured at 593 nm. The antioxidant activity was expressed as ascorbic acid equivalent (mg AAE/g extract), which served as positive control, using the standard calibration curve developed.

Xanthine oxidase inhibition activity

Xanthine oxidase (XO) inhibitory activity was measured under an aerobic condition (Gliozzi *et al.*, 2016). Samples (2 mg) were dissolved in 1 mL 5% dimethyl sulfoxide (DMSO) in 50 mM buffer. Allopurinol (100 $\mu\text{g}/\text{mL}$), a known XO inhibitor, was used as positive control. The reaction mixture contained 50mM phosphate buffer (pH 7.5), 0.15 mM xanthine and 0.3 U/mL of xanthine oxidase from buttermilk. The absorption increment was measured at 295 nm. All determinations were done in triplicates, and percentage of inhibition was determined. XO

inhibition activity was expressed as the percentage inhibition of XO as follows:

$$\text{Inhibition (\%)} = \{(A-B) - (C-D)\} / (A-B) \times 100$$

where A was absorbance of the negative control (5% DMSO in 50 mM buffer), B was the blank of A which was without the enzyme, C was the sample with enzyme, and D was the blank of the extract without enzyme.

α-glucosidase inhibition (AGI) activity

The α-glucosidase inhibitory activity assay was performed based on the protocol mentioned by Javadi *et al.* (2014) which mimics the condition of intestinal fluid. All samples and quercetin (positive control) were prepared in methanol and p-nitrophenyl-p-D-glucopyranosidase (PNPG, substrate) was prepared in 50 mM phosphate buffer (pH 6.5). Accurately, 10 μL samples, quercetin, and negative control (solvent) each in 100 μL phosphate buffer (30 mM) were incubated for 5 min with 15 μL 0.02 U/μL α-glucosidase enzyme at room temperature. Meanwhile, the blank consisted of 115 μL buffer without the enzyme. All mixtures were then treated with 75 μL substrate. The reaction was stopped using glycine (pH 10) after another 15 min of incubation. The amount of p-nitrophenol released from PNPG was measured at 405 nm. The IC₅₀ value was determined using linear regression analysis. All determination was done in triplicates. The inhibitory activity (%) of each extract was calculated using the following formula:

$$\text{Inhibition (\%)} = [(A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}}] \times 100\%$$

where A_{control} was the absorbance of the negative control, and A_{sample} is the absorbance of sample or positive control. The half-maximal inhibition (IC₅₀) value was determined using linear regression analysis.

Dipeptidyl peptidase-IV (DPP-IV) Inhibitory Assay

Dipeptidyl peptidase-IV inhibitory activity was measured using a commercially available kit (Cayman Chemical Company, Ann Arbor, MI, USA) according to the supplier's recommendation. In brief, samples (30 μL diluted in assay buffer), 10 μL diluted DPP-IV, and 10 μL sample were added to each well, followed by incubation with 50 μL substrate for 30 min at 37°C. Then the fluorescence intensity was measured at 450-465 nm, with an excitation at 350-360 nm. DPP-IV inhibitory activity was calculated using the following equation:

$$\text{Inhibition (\%)} = [(\text{Initial Activity} - \text{Inhibitor}) / \text{Initial Activity}] \times 100 \%$$

where initial activity was the fluorescence of negative control and inhibitor was the fluorescence of the sample. The result was expressed as IC₅₀ value. All determination was done in triplicates.

2-NBDG Uptake in 3T3-L1 Cells Assay

Another mechanism involved in type 2 diabetes mellitus includes glucose uptake by adipocytes *via* stimulation of the extract in the presence and absence of insulin in the system.

Cell culture

The 3T3-L1 cells were kindly provided by the Integrated Centre for Research Animal Care and Use (ICRACU) IIUM that was purchased from the American Type Culture Collection, ATCC (Virginia, USA). The cells were cultured in complete Dulbecco's Modified Eagle Media (DMEM) containing 10% foetal bovine serum (FBS) and 1% penicillin-streptomycin (Pen-Strep), maintained at 37°C in a humidified atmosphere with 5% CO₂, and sub-cultured every three to four days at approximately 70% confluence (Roffey *et al.*, 2007).

Cell viability assay

The cell viability assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was carried out with the method described by Roffey *et al.* (2007). The 3T3-L1 cells were seeded into 96-well plate at a density of 2.0 × 10⁵ cells/mL and grown until confluence. Treatment with *C. nutans* leaves hexane fraction dissolved in DMSO was proceeded for 48 h. At the end of the treatment period, cells were washed with phosphate buffer saline (PBS) and incubated with 20 μL MTT solution (5 mg/mL) for 4 h at 37°C. Next, 100 μL DMSO was added to each well to stop the MTT reaction and solubilise the purple formazan crystal. After 1 h, spectrophotometric absorbance (A) was measured at 570 nm with a reference wavelength of 630 nm. The absorbance reading correlated with viable cell number and metabolic activity of the cells (Roffey *et al.*, 2007). Cell viability (%) was calculated using the following equation:

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

where A sample was absorbance of the sample, A blank was absorbance of the blank, and A control was absorbance of the negative control.

3T3-L1 Preadipocyte Differentiation

Preadipocyte differentiation assay was assessed following the modified method of Manaharan *et al.* (2013). Firstly, 3T3-L1 pre-adipocytes were plated into 96-well plate at approximately 3×10^4 cells per well. The cells were incubated until 90% confluence and maintained in DMEM supplemented with 10% FBS, 1% penicillin and 1% hepes buffer at 37°C under a humidified 5% CO₂ atmosphere. The initiation of the cell differentiation from preadipocyte into adipocytes, occurred at two days after 90% confluence (defined as day 0), whereby the cells were incubated in differentiation initiation medium (DIM) containing 0.5 mM IBMX and 0.25 mM DEX in DMEM containing 10% FBS. After two days (defined as day 3), the culture media was changed to the differentiation progression medium (DPM) containing 100 nM insulin and 10% FBS in DMEM. At the next two days (defined as day 5), the medium was replaced again with fresh DMEM with/without insulin (100 nM), and the replacement of fresh medium every two days were continued until day 10. The effects of samples in 3T3-L1 pre-adipocyte differentiation were determined by treating the cells (from day 0) with hexane fraction of *C. nutans* leaves (0.04–5 µg/mL) for the entire ten days.

The control cultures were treated in basal medium (DMEM with 10% FBS and 0.1% DMSO) and insulin (100 nM). The cells were also treated with rosiglitazone, which served as positive control. The concentrations of samples used in this assay were determined to be non-cytotoxic to the 3T3-L1 cells, as established in the MTT viability assay. The cells were then maintained in the original propagation DMEM, changing medium every two to three days, until use. Plates where cells were >90% differentiated were used for experiments between day 9 to 12 post-induction. Percentage differentiation was observed by a visual method. On the day of the experiment, each well was examined under the microscope to identify the percentage of cells that had not yet become adipocytes, which are round and full of easily distinguishable fat globules. Wells that were used for experimentation contained little to none pre-adipocyte cells, and were easily distinguishable as >90% differentiated overall.

2-NBDG Uptake in 3T3-L1 Cells

The 2-NBDG uptake test in 3T3-L1 cells was performed by following the method described by Fang *et al.* (2008) and Saito *et al.* (2011) with slight modifications. Initially, 3T3-L1 cells were cultured and differentiated in fluorescent 96-well plate (SPL Life Sciences, Korea) and first starved in glucose-

free DMEM for 48 h. Then, glucose uptake was initialised by replacing the medium with serum and glucose-free DMEM containing 80 µM of 2-NBDG fluorescent glucose analogue added with varying extract (H_{fr}) concentrations with/without insulin (100 nM). Basal medium (DMEM with 10% FBS and 0.1% DMSO) and rosiglitazone-treated cells served as the control culture and positive control, respectively. After 48 h, the culture medium was discarded and washed with PBS to remove all traces of 2-NBDG. The fluorescence remaining in the cell monolayers were quantified with fluorescence microplate reader (Perkin Elmer, USA) set at a wavelength of 485 and 535 nm.

Results

Extraction yield and antioxidant activity (DPPH, FRAP and XO)

Table 1 displays the extraction yield, α-glucosidase inhibition activity (IC₅₀) and antioxidant activity assays (DPPH, FRAP, and XO) of *C. nutans* leaves methanolic extract's fractions. The result showed that the trend of extraction yield followed the polarities of the solvents used during extraction. The trend of this result was found as M_{fr} > E_{fr} > H_{fr} > HE_{fr} > H_{fr}. The most polar fraction, the methanolic fraction (M_{fr}), showed the highest extraction yield (64.3%), while the least polar fraction, the hexane fraction (H_{fr}) afforded the least yield (6.6%). Ethyl acetate fraction (E_{fr}) exhibited a good activity for all the assays with the lowest IC₅₀ value i.e. 37.40 ± 1.45 µg/mL for the scavenging action, while the rest of the fractions had IC₅₀ values of more than 100 µg/mL, which were comparably higher than that of the positive control. As for the FRAP value, the E_{fr} showed the highest FRAP activity (79.75 ± 0.17 mg AAE) as compared to other fractions with methanolic fraction (M_{fr}) showing the least FRAP activity (57.75 ± 0.86 mg AAE). However, all fractions of the methanol extract showed XO inhibitory activity below 50% at 720 µg/mL (assay concentration). Meanwhile, the IC₅₀ value of the allopurinol was determined to be 0.16 ± 0.00 µg/mL. Overall, E_{fr} exhibited promising antioxidant activity as compared to the other extracts.

α-glucosidase inhibitory activity

The α-glucosidase inhibitory activity of the *C. nutans* leaves methanol extract's fractions is displayed in Table 1 as the half-maximal concentration values (IC₅₀, µg/mL). A lower IC₅₀ value is desirable for higher α-glucosidase inhibitory activity (Nsimba *et al.*, 2008). The IC₅₀ values ranged from 3.05 to 133.57 µg/mL. The highest α-glucosidase inhibitory

Table 1 Extraction yield, α -glucosidase inhibition activity (IC₅₀) and antioxidant activity (DPPH, FRAP and XO) of *C. nutans* leaves methanolic extract's fractions

Samples	Percentage yield (%)	AGI IC ₅₀ (μ g/mL)	DPPH IC ₅₀ (μ g/mL)	FRAP mg AAE /g	XO Percentage inhibition (%)
Hexane (H _{fr})	6.508 \pm 0.324 ^c	3.05 \pm 0.05 ^d	> 400	72.64 \pm 0.28 ^c	12.93 \pm 0.33 ^c
Hexane: Ethyl acetate (HE _{fr})	10.074 \pm 0.333 ^d	5.54 \pm 0.09 ^d	140.98 \pm 2.04 ^b	66.48 \pm 0.82 ^d	20.61 \pm 0.36 ^b
Ethyl acetate (E _{fr})	12.558 \pm 0.364 ^c	8.42 \pm 0.17 ^c	37.40 \pm 1.32 ^c	79.75 \pm 0.12 ^a	41.25 \pm 0.54 ^a
Ethyl acetate: Methanol (EM _{fr})	23.398 \pm 0.707 ^b	37.45 \pm 0.90 ^b	328.89 \pm 6.33 ^a	77.50 \pm 0.67 ^b	9.25 \pm 0.21 ^c
Methanol (M _{fr})	64.282 \pm 0.493 ^a	133.57 \pm 0.30 ^a	325.74 \pm 1.21 ^a	57.75 \pm 0.60 ^c	10.19 \pm 0.46 ^d
Quercetin	nd	5.77 \pm 1.01	nd	nd	nd
Ascorbic acid	nd	nd	8.21 \pm 0.02 ^d	nd	nd
Allopurinol	nd	nd	nd	nd	91.40 \pm 0.00

Data expressed as mean \pm SD. SD = standard deviation.

Means in the same column that do not share the same letter are significantly different with p value < 0.05. nd = not determined.

activity was observed for the hexane fraction (3.07 μ g/mL), which was found better as compared to the IC₅₀ value obtained for quercetin (positive control). Meanwhile, the methanolic fraction exhibited the lowest activity with the highest IC₅₀ viz., 133.57 μ g/mL. The fractions exhibited the following trend: H_{fr} > HE_{fr} > EA_{fr} > EAM_{fr} > M_{fr} (Table 1). As the polarity increased, the α -glucosidase inhibitory activity of *C. nutans* leaves decreased significantly.

Table 2 The percentage inhibition for DPP(IV) inhibitory assay for *C. nutans* leaves methanol extract's hexane fraction

Samples	Percentage inhibition (%)	IC ₅₀ (μ g/mL)
Sitagliptin	93.92 \pm 0.08	0.007
Hexane (H _{fr})	91.40 \pm 0.18	0.034

Data expressed as mean \pm SD. SD = standard deviation.

Dipeptidyl peptidase- IV (DPP-IV) inhibitory activity

In order to assess the ability of the hexane extract (H_{fr}) in different mechanism involved in type 2 diabetes mellitus, the dipeptidyl peptidase-IV inhibition assay was also carried out and the results are presented in Table 2 with the percentage of inhibition with IC₅₀ value also calculated. The hexane fraction of *C. nutans* leaves methanolic extract (which showed the lowest IC₅₀ value for α -glucosidase) and sitagliptin (positive control) showed comparably good inhibition of the dipeptidyl peptidase-IV at 91.40% and 93.92%, respectively. The reported IC₅₀ value of sitagliptin is 0.007 μ g/mL (18 nM) according to the value given by Cayman, Anbor, USA. Meanwhile, the IC₅₀ value of the hexane fraction was calculated as 0.034 μ g/mL. The result showed that *C. nutans* leaves methanolic extract's hexane fraction had the capability to inhibit the DPP-IV enzyme as well.

2-NBDG Uptake in 3T3-L1 Cells Assay Cell Viability

Figure 1 shows the cell viability of the positive control, rosiglitazone and the *C. nutans* leaves methanolic extract's hexane fraction. Adipocytes are the major target cells of PPAR γ -agonists in vitro and in vivo, and PPAR γ is well demonstrated as a major regulator of adipogenesis. Ligand activation of PPAR γ can promote adipocyte differentiation. Thus, we examined the effects of the promising extract (leaves methanolic extract's hexane fraction) on adipocyte differentiation. The viability of 3T3-L1 cells was determined using the MTT method, and results showed no significant toxic effects at the concentrations range observed. There were no inhibitory effects manifested at different concentrations considered. There were no significant ($p > 0.05$) differences in viability of 3T3-L1 cells at the concentration of up to 100 μ g/mL as compared to the control basal (0 μ g/mL). Meanwhile, the control (rosiglitazone) showed no significant ($p > 0.05$) differences in the viability of 3T3-L1 adipocyte cells at a concentration up to 40 μ M as compared to the control basal (0 μ M).

2-NBDG uptake in 3T3-L1 cells

The ability of *C. nutans* leaves methanolic extract's hexane fraction to stimulate glucose uptake by adipocytes was evaluated on the 2-NBDG uptake in terminally differentiated 3T3-L1 adipocytes. The hexane fraction stimulated 2-NBDG uptake in these cells in a concentration-dependent manner. It confirmed insulin-sensitising property of the *C. nutans* leaves methanolic extract's hexane fraction by showing the stimulation of 2-NBDG uptake into adipocytes in the presence of insulin. The glucose uptake was also stimulated even without the presence of insulin, which increased with the concentration of

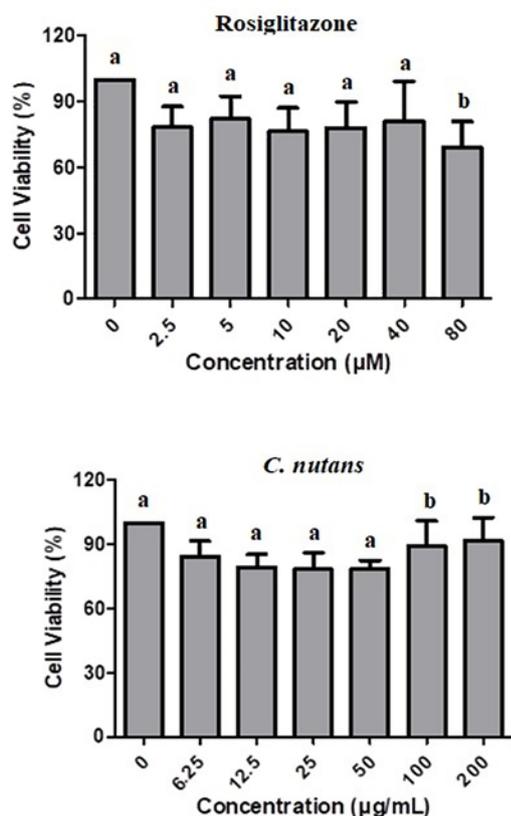


Figure 1 The cell viability of rosiglitazone-treated cells (A) and *C. nutans* leaves methanol extract's hexane fraction-treated cells (B). Data presented in mean \pm SD. Means that do not share a letter are significantly different with p value < 0.05 . SD = standard deviation.

the hexane fraction. This indicated that the leaves methanolic extract's hexane fraction has insulin-like activity from the concentration 25 $\mu\text{g/mL}$ to 200 $\mu\text{g/mL}$. The result (Figure 2) showed that the plant demonstrated its antidiabetic effect (stimulation of glucose uptake) far better in the presence of insulin than the absence of insulin like a PPAR γ agonist, the positive control rosiglitazone.

Discussion

Various methods have been developed and applied in natural product extraction to prepare plant extracts. The most conventional method used since decades ago includes boiling or decoction with water, which is simple and economically viable. In recent times, medicinal plant-based researches have been done continuously due to the variety of metabolites found in plants with health benefits. In more advanced researches nowadays, organic solvents are utilised to obtain plant extracts of various metabolites based on the polarity and the nature of the compound(s) of interest (Zulbadli *et al.*, 2011). Multiple factors that might also influence plant extraction procedure include the types of solvent used, nature of the plant

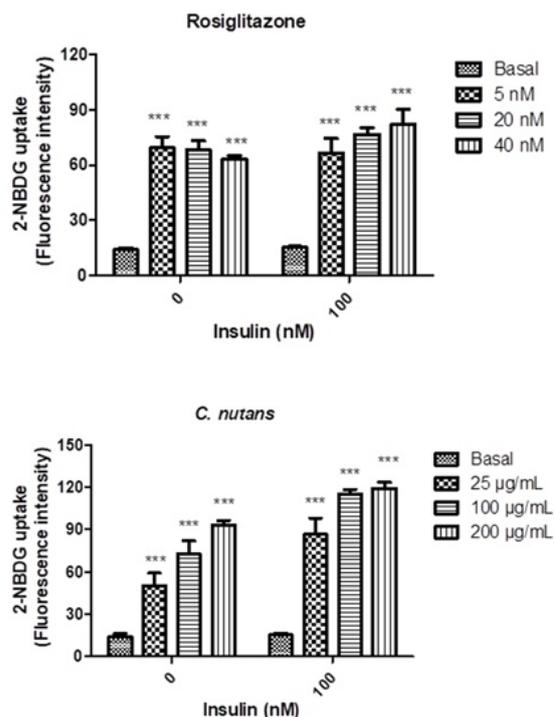


Figure 2 The 2-NBDG glucose uptake in differentiated 3T3-L1 cells in the absence (0 nM) and presence (100 nM) of insulin in positive control rosiglitazone (5–40 μM) (A) and at different concentrations of *C. nutans* leaves methanol extract's hexane fraction (25–200 $\mu\text{g/mL}$) (B). Data presented in mean \pm SD. Means that do not share a letter are significantly different with p value < 0.001 . SD = standard deviation.

material, temperature and target metabolites (John *et al.*, 2006). The variation in the solvent polarity results in remarkably different bioactivities and phytochemical constituents. Therefore, initially in the present work, 80% methanol was used to obtain *C. nutans* leaves methanolic extract's and subsequently, solvents of different polarities were used to obtain its different polar and non-polar fractions to evaluate the different metabolites that might have different polarity thus exhibiting different potentials in the bioactivity assay analysis.

From the results obtained, it can be deduced that the plant extract contained polar substances with the highest yield furnished by methanolic fraction which might include polyhydroxyl compounds as well as glycones of different classes of organic compounds while the hexane fraction possessed the least yield that could possibly contain non-polar compounds comprised of fatty acids, terpenoids and pigments that have been reported in previous studies (Teshima *et al.*, 1997; Sakdarat *et al.*, 2009; Chelyn *et al.*, 2014; Tu *et al.*, 2014; Kifayatullah *et al.*, 2015).

The *in vitro* antioxidant and antihyperglycaemia bioassays were used to screen the bioactivity of *C. nutans* leaves methanolic extract's different fractions.

Later, the capability of the most active fraction inhibiting α -glucosidase activity was further analysed for its antidiabetic potential through the inhibition of dipeptidyl peptidase-IV (DPP-IV) protein which is another therapeutic pathway to treat diabetes. Besides, cell viability and 2-NBDG uptake in 3T3-L1 adipocytes cells were also preliminarily measured to assess the insulin sensitising and mimicking effects of the resultant hexane fraction (Roffey *et al.*, 2007; Choi *et al.*, 2009).

This finding highlights the radical scavenging capacity of the leaves extracts and is consistent with the findings previously reported by Sulaiman *et al.* (2015) in which the DPPH activity of *C. nutans* leaves methanolic extract's ethyl acetate fraction showed the highest scavenging activity as compared to its hexane fraction which. Besides, Sarega *et al.* (2016) have also reported the poor scavenging activity manifested by methanolic and hexane fractions with an average scavenging percentage of 22% and 18%, respectively. The results of the present work however, were found comparable to what was reported by Sarega *et al.* (2016) with 100 mg GAE/g for ethyl acetate fraction. Other than that, the DPPH results were also found comparable to what had earlier been reported by Arullappan *et al.* (2014) using ethyl acetate and methanol fractions of the aerial part of the plant at 2 mg/mL with 50% and 37%, respectively. As for the reducing power assay, the results were considerably moderate. However, Sarega *et al.* (2016) reported a lower FRAP value for hexane fraction (55 mg GAE/g) and a slightly higher value for ethyl acetate and methanolic fractions with the FRAP value of 100 mg GAE/g and 95 mg GAE/g, respectively. The FRAP value for the methanolic fraction (57.75 ± 0.86 mg AAE/g) was found noticeably higher than that reported by Kong *et al.* (2016) i.e. 10.07 ± 0.01 mg TE/g. Meanwhile, the XO inhibitory activity of the plant exhibited a fairly low activity with only ethyl acetate fraction showing approximately 41% of inhibition. In general, the results were found to be contraindicated to what was recently reported by Duong *et al.* (2017) whereby an IC_{50} value of 30.4 μ g/mL was observed for the same species from Thailand.

As a matter of fact, variations in results could also be manifested due to geographical distribution of the plant itself that might influence the presence and the quantity of the bioactive compounds in the plant material, which might vary depending on the different quality of soil, fertiliser and other environmental factors (Figueiredo *et al.*, 2007). Apart from that, solvent(s) used for the extraction of the plant material also plays an important role

in extracting the bioactive compounds. Differences can be observed in terms of yield, composition and bioactivity of the extract. Variation in polarities of the solvents affects the compounds selectivity, which would be more selectively soluble in different solvents thus highlighting the importance of investigating and identifying the optimal extraction solvent for each sample type to evaluate its proper efficacy (Ngo *et al.*, 2017).

Furthermore, the α -glucosidase inhibitory activity of the fractions decreased with the increase in polarity. This indicates the presence of bioactive metabolites belonging to the non-polar to semi-polar groups such as the terpenoids, sterols and fatty acids group members possibly responsible for the above results. The result was found agreeable with that reported by Lee *et al.* (2014) which showed weak inhibitory activity (13.57%; IC_{50} value $> 1,000$ μ g/mL) for the methanolic fraction obtained from the *C. nutans* leaves. The hexane fraction might contain terpenoids such as betulin that has previously been reported for its α -glucosidase inhibiting potential (Raza *et al.*, 2015). Therefore, hexane fraction was selected for further investigation.

DPP-IV inhibitors are the most recent incretin therapy rapidly developed for the therapeutic use for the management of type 2 diabetes mellitus (Elya *et al.*, 2015). The result deduced that the hexane fraction had a remarkable potential to inhibit DPP-IV enzyme as well. This indicates that the phytoconstituents in the *C. nutans* leaves are highly applicable in the management of type 2 diabetes mellitus. To our knowledge, there is no literature reporting on the plants' antidiabetic activity through this assay.

Apart from this, assessment of the glucose uptake by adipocytes via stimulation of the extract (H_{fr}) in the presence and absence of insulin in the system along with its cell viability showed the potential of the fraction as effective in combating type 2 diabetes mellitus. The results showed the ability for the hexane fraction to react in the same way as the PPAR γ agonist by stimulating the glucose uptake even in the absence of insulin. However, better results were observed with the presence of insulin by exerting insulin-like activity in a dose dependent manner thus also indicating that the constituents present in the hexane fraction display synergistic action in this mechanism.

Phytochemical screening from previous study has shown the presence of phenolics, tannins, flavonoids, diterpenes and phytosterols (Yang *et al.*, 2013). Some of the vital compounds found abundantly in *C. nutans* leaves include stigmaterol, lupeol, β - sitosterol, betulin along with some C-glycosyl flavones which includes vitexin, isovitexin, schaftoside,

isomollupentin 7-O-b-glucopyranoside, orientin and isoorientin (Khoo *et al.*, 2015; Alam *et al.*, 2016). Some of these compounds are known as effective antidiabetic and antioxidant agents (Tanruean *et al.*, 2017). Conclusively, the plant leaves have potential to be developed as an ingredient in functional food for the management of diabetes and other disorders caused by oxidative stress.

Conclusion

Conclusively, the present work has shown that *C. nutans* leaves extract possesses promising effects to be utilised as a potential antidiabetic and antioxidant agent *via* different mechanisms that can lead to type 2 diabetes mellitus. *C. nutans* leaves extract might contain phytoconstituents that can be further explored to be used as essential ingredient in functional food in the management of metabolic disorders. The plant leaves also have the potential to be utilised in pharmaceutical, cosmeceutical and other industries.

Acknowledgement

The authors wish to thank the Malaysian Ministry of Agriculture and Agro-based Industry for the NRGs fund (NRGS SP15-060-0182) and International Islamic University Malaysia for the Publication Research Initiative Grant Fund (PRIGS18-027-0027).

Conflict of Interest

No conflict of interest.

References

- Abdul, A. S. M., Low, C. N., Chai, L. C., Abd-Razak, S. S. N., Selamat, J., Son, R., Sarker, M. Z. I. and Khatib, A. 2011. Screening of selected Malaysian plants against several food-borne pathogen bacteria. *International Food Research Journal* 18(3): 1195-1201.
- Alam, A., Ferdosh, S., Ghafoor, K., Hakim, A., Juraimi, A. S., Khatib, A. and Sarker, M. Z. I. 2016. *Clinacanthus nutans*: A review of the medicinal uses, pharmacology and phytochemistry. *Asian Pacific Journal of Tropical Medicine* 9(4): 402-409.
- Alothman, M., Bhat, R. and Karim, A. A. 2009. Antioxidant capacity and phenolic content of selected tropical fruits from Malaysia, extracted with different solvents. *Food Chemistry* 115(3): 785-788.
- Arullappan, S., Rajamanickam, P., Thevar, N. and Kodimani, C. C. 2014. In vitro screening of cytotoxic, antimicrobial and antioxidant activities of *Clinacanthus nutans* (Acanthaceae) leaf extracts. *Tropical Journal of Pharmaceutical Research* 13(9): 1455-1461.
- Bahmani, M., Zargaran, A., Rafieian-Kopaei, M. and Saki, K. 2014. Ethnobotanical study of medicinal plants used in the management of diabetes mellitus in the Urmia, Northwest Iran. *Asian Pacific Journal of Tropical Medicine* 7(1): 348-354.
- Chelyn, J. L., Omar, M. H., Mohd Yousof, N. S. A., Ranggasamy, R., Wasiman, M. I. and Ismail, Z. 2014. Analysis of flavone C-glycosides in the leaves of *Clinacanthus nutans* (Burm. f.) Lindau by HPTLC and HPLC-UV/DAD. *Scientific World Journal*: 1-6.
- Choi, J. S., Nurul Islam, M., Yousof Ali, M., Kim, E. J., Kim, Y. M. and Jung, H. A. 2014. Effects of C-glycosylation on anti-diabetic, anti-Alzheimer's disease and anti-inflammatory potential of apigenin. *Food and Chemical Toxicology* 64: 27-33.
- Duong, N. T., Vinh, P. D., Thuong, P. T., Hoai, N. T., Thanh, L. N., Bach, T. T., Nguyen, H. N. and Anh, N. H. 2017. Xanthine oxidase inhibitors from *Archidendron chypearia* (Jack.) I.C. Nielsen: Results from systematic screening of Vietnamese medicinal plants. *Asian Pacific Journal of Tropical Medicine* 10(6): 549-556.
- Elya, B., Handayani, R., Sauriasari, R., Azizahwati, Hasyiyati, U. S., Permana, I. T. and Permatasari, Y. I. 2015. Antidiabetic activity and phytochemical screening of extracts from Indonesian plants by inhibition of alpha amylase, alpha glucosidase and dipeptidyl peptidase IV. *Pakistan Journal of Biological Sciences* 18: 279-284.
- Fang, X. K., Gao, J. and Zhu, D. N. 2008. Kaempferol and quercetin isolated from *Euonymus alatus* improve glucose uptake of 3T3-L1 cells without adipogenesis activity. *Life Sciences* 82(11-12): 615-622.
- Figueiredo, C., Barroso, J., Pedro, L. and Scheeffer, J. 2007. Factors affecting secondary metabolite production in plants: volatile components and essential oils. *Flavour and Fragrance Journal* 24(4): 206-213.
- Ghasemzadeh A., Jaafar H. Z. E. and Rahmat A. 2015. Phytochemical constituents and biological activities of different extracts of *Strobilanthes crispus* (L.) Bremek leaves grown in different locations of Malaysia. *BMC Complementary and Alternative Medicine* 15(1): 422-432.
- Glozzi M., Malara, N., Muscoli, S. and Mollace, V. 2016. The treatment of hyperuricemia. *International Journal of Cardiology* 213: 23-27.
- Globinmed. 2015. *Clinacanthus nutans* (Burm.f.) Lindau. Retrieved from website: http://www.globinmed.com/index.php?option=com_content&view=article&id=79320:clinacanthus-nutans-burmf-lindau.
- Javadi, N., Abas, F., Hamid, A. A., Simoh, S., Shaari, K., Ismail, I. S., Mediani, A. and Khatib, A. 2014. GC-MS-based metabolite profiling of *Cosmos caudatus* leaves possessing alpha-glucosidase inhibitory activity. *Journal of Food Science* 79(6): 1130-1136.
- John, K. M. M., Vijayan, D., Kumar, R. R. and Premkumar, R. 2006. Factors influencing the efficiency of extraction of polyphenols from young tea leaves. *Asian Journal of Plant Sciences* 5(1): 123-126.

- Khoo, L. W., Mediani, A., Zolkeflee, N. K. Z., Leong, S. W., Ismail, I. S., Khatib, A., Shaari, K. and Abas, F. 2015. Phytochemical diversity of *Clinacanthus nutans* extracts and their bioactivity correlations elucidated by NMR based metabolomics. *Phytochemistry Letters* 14: 123-133.
- Kifayatullah, M., Mustafa, M. S., Senguptha, P., Sarker, M. R., Das, A. and Das, S. K. 2015. Evaluation of the acute and sub-acute toxicity of the ethanolic extract of *Pericampylus glaucus* (Lam.) Merr. in BALB/c mice. *Journal of Acute Disease* 4(4): 309-315.
- Kong, H.S., Musa, K.H. and Abdullah Sani, N. 2016. *Clinacanthus nutans* (Belalai Gajah / Sabah Snake Grass): antioxidant optimization on leaves and stems. *AIP Conference Proceedings* 1784(1): 1-5.
- Lee, S. Y., Mediani, A., Nur Ashikin, A. H., Azliana, A. B. S. and Abas, F. 2014. Antioxidant and α -glucosidase inhibitory activities of the leaf and stem of selected traditional medicinal plants. *International Food Research Journal* 21(1): 165-172.
- Manaharan, T., Hwee, C. and Palanisamy, U. D. 2013. *Syzygium aqueum* leaf extract and its bioactive compounds enhances pre-adipocyte differentiation and 2-NBDG uptake in 3T3-L1 cells. *Food Chemistry* 136(2): 354-363.
- Ngo, T. V., Scarlett, C. J., Bowyer, M. C., Ngo, P. D. and Vuong, Q. V. 2017. impact of different extraction solvents on bioactive compounds and antioxidant capacity from the root of *Salacia chinensis* L. *Journal of Food Quality* 2017: 1-8.
- Panuganti, S. J. 2015. Principles involved in bioassay by different methods: a mini-review. *Research and Reviews: Research Journal of Biology* 3(2): 1-18.
- Pieroni, A., Janaik, V., Durr, C. M., Ludeke, S., Trachsel, E. and Heinrich, M. 2002. *In vitro* antioxidant activity of non-cultivated vegetables of ethnic Albanians in Southern Italy. *Phytotherapy Research* 16(5): 467-473.
- Raza, R., Ilyas, Z., Ali, S., Nisar, M., Khokhar, M. Y. and Iqbal, J. 2015. Identification of highly potent and selective α -glucosidase inhibitors with antiglycation potential, isolated from *Rhododendron arboreum*. *Records of Natural Products* 9(2): 262-266.
- Roffey, B. W. C., Atwal, A. S., Johns, T. and Kubow, S. 2007. Water extracts from *Momordica charantia* increase glucose uptake and adiponectin secretion in 3T3-L1 adipose cells. *Journal of Ethnopharmacology* 112(1): 77-84.
- Saito, K., Lee, S., Shiuchi, T., Toda, C., Kamijo, M., Inagaki- Ohara, K., Okamoto, S. and Minokoshi, Y. 2011. An enzymatic photometric assay for 2-deoxyglucose uptake in insulin-responsive tissues and 3T3- L1 adipocytes. *Analytical Biochemistry* 412(1): 9-17.
- Sakdarat, S. and Shuyprom, A. 2009. Bioactive constituents from the leaves of *Clinacanthus nutans* Lindau. *Bioorganic and Medicinal Chemistry* 17(5): 1857-1860.
- Sarega, N., Imam, M. U., Ooi, D. J., Chan, K. W., Esa, N. M., Zawawi, N. and Ismail, M. 2016. Phenolic rich extract from *Clinacanthus nutans* Attenuates Hyperlipidemia-Associated Oxidative Stress in rats. *Oxidative Medicine and Cellular Longevity*: 1-16.
- Sulaiman, I. S. C., Basri, M., Chan, K. W., Ashari, S. E., Masoumi, H. R. F. and Ismail, M. 2015. In vitro antioxidant, cytotoxic and phytochemical studies of *Clinacanthus nutans* Lindau leaf extracts. *African Journal of Pharmacy and Pharmacology* 9(34): 861-874.
- Tanruean, K., Suwannarach, N., Choonpicharn, S. and Lumyong, S. 2017. Evaluation of phytochemical constituents and biological activities of leaves and stems of *Marsdenia glabra* Cost. *International Food Research Journal* 24: 2572-2579.
- Teshima, K., Kaneko, T., Ohtani, K., Kasai, R., Lhieochaiphant, S., Picheansoonthon, C. and Yamasaki, K. 1997. C-Glycosyl Flavones from *Clinacanthus*. *Natural Medicines* 51(6): 557.
- Tu, S. F., Liu, R. H., Cheng, Y. Bin, Hsu, Y. M., Du, Y. C., El-Shazly, M. and Chang, F. R. 2014. Chemical constituents and bioactivities of *Clinacanthus nutans* aerial parts. *Molecules* 19(12): 20382-90.
- Nsimba, Y. R., Kikuzaki, H. and Konishi, Y. 2008. Antioxidant activity of various extracts and fractions of *Chenopodium quinoa* and *Amaranthus* spp. seeds. *Food Chemistry* 106(2): 760-766.
- Yang, H. S., Peng, T. W., Madhavan, P., Shukkoor, M. S. A. and Akowuah, G. A. 2013. Phytochemical analysis and antibacterial activity of methanolic extract of *Clinacanthus nutans* leaf. *International Journal of Drug Development and Research* 5: 349-355.
- Yi, W. and Wetzstein, H. Y. 2011. Effects of drying and extraction conditions on the biochemical activity of selected herbs. *HortScience* 46(1): 70-73.
- Zulbadli, N., Alwi, H. and Hamid K. H. K. 2011. Study on Important Factors Affecting the extraction of *Momordica charantia* using pressurized boiling system. Langkawi, Malaysia: IEEE Symposium on Business, Engineering and Industrial Applications (ISBEIA).
- Zulkipli, I. N., Rajabalaya, R., Idris, A., Sulaiman, N. A. and David, S. R. 2017. *Clinacanthus nutans*: a review on ethnomedicinal uses, chemical constituents and pharmacological properties. *Pharmaceutical Biology* 55(1): 1093-1113.