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Chemical composition of the dietary supplement *Coccinia grandis* (L.) Voigt, and its effect on antioxidant status and inflammatory markers in patients with type 2 diabetes mellitus

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Abstract

Herbal dietary supplements are widely used throughout the world with reports of their use among patients with type 2 diabetes mellitus (T2DM). The present work aimed to provide a comprehensive depiction of the dietary supplement *Coccinia grandis* (L.) Voigt (in the form of aqueous extract of freeze-dried powder), and to determine its antioxidant and anti-inflammatory effects in patients with T2DM. The antioxidant and anti-inflammatory effects of the dietary supplement were evaluated through a three-month long, randomised, double-blind, placebo-controlled clinical trial involving 158 newly diagnosed patients with T2DM. The dietary supplement consisted of phytoconstituents including loliolide, neophytadiene, palmitic acid methyl ester. The absence of microorganisms was observed for a month at 40°C. *In vitro* antidiabetic assays revealed the inhibition of α-amylase, α-glucosidase, and dipeptidyl peptidase-IV enzymes, and also the enhancement of glucose uptake in cells. Administration of *C. grandis* dietary supplement (500 mg/day) for three months was able to change variables from the baseline to the end of the intervention, in the test and placebo groups, as -3.25 ± 3.93 and 1.42 ± 4.84 U/L for glutathione reductase ($p < 0.001$), 12.75 ± 33.35 and -1.45 ± 41.93 nmol/dL for malonaldehyde ($p = 0.025$), and 5.89 ± 11.49 and 0.46 ± 13.11 pg/mL for interleukin-6 ($p = 0.002$), respectively. The standardised dietary supplement showed antidiabetic activity *in vitro*. The clinical study revealed its promising commercial application as a dietary supplement, by improving antioxidant status and reducing inflammation in newly diagnosed patients with T2DM.

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Introduction

In recent decades, most of the world's population has used dietary supplements as alternatives or adjuncts to existing medication regimens to slow the risk of developing type 2 diabetes mellitus (T2DM), and delay the occurrence of complications associated with diabetes. Generally, dietary supplements contain vitamins, minerals, antioxidants, and amino acids (Rajakumari *et al*., 2018; Wierzejska, 2021). The use of herbal dietary supplements for the primary or secondary treatment of T2DM is of increasing interest. Antioxidants and anti-inflammatory agents are promising sources to get rid of underlining pathologies such as oxidative stress and inflammation in diabetes mellitus. Herbal dietary supplements consist of various phytoconstituents such as alkaloids, phenolics, tannins, and steroids that have been empirically reported as natural antioxidants and anti-inflammatory agents (Dahanayake *et al*., 2019). It is imperative to emphasise that the intake of herbal dietary supplements with potent antioxidant and anti-inflammatory activities has an additional meaning in the management of diabetes mellitus, and therefore controls cellular oxidative stress and inflammation.

Coccinia grandis (L.) Voigt (family Cucurbitaceae) is a popular leafy vegetable widely distributed in Sri Lanka, India, and Pakistan. Locally, it is known as kowakka or kem wel (English name: ivy gourd). Due to its therapeutic values, people in Asian countries prepare salads from the tender leaves

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of *C. grandis* mixed with grated coconut, and consume them with or without cooking. The results of a cross-sectional survey carried out using an interviewer-based questionnaire reported that *C. grandis* leaves are widely used as a dietary adjunct in the treatment of mild hyperglycaemia and/or impaired glucose tolerance (Medagama *et al*., 2014). Preclinical studies revealed that the leaves of *C. grandis* had a well-balanced efficacy and safety profile (Deshpande *et al*., 2011; Attanayake *et al*., 2013). The glucose- and lipid-lowering properties of *C. grandis* herbal supplement were proven in patients with newly diagnosed T2DM (Wasana *et al*., 2021).

In general, consumers, manufacturers, and regulatory officers consider the safety and quality of herbal supplements using standardised quality control measures and well-defined proximate composition before commercialisation. Additionally, it is important to describe the stability of herbal dietary supplements since herbal supplements are more prone to develop microbial contamination. Well-defined proximate nutrient composition is also important for a commercially viable dietary supplement. Although several herbal dietary supplements have been hosted for the diabetic population, limited evidence on clinical efficacy and safety, quality control measures, and stability restrict their wider use in clinical practice. Addressing these limitations in *C. grandis* supplementation is important before the commercialisation of *C. grandis* herbal supplement as a value-added product that targets the dietary management of diabetes. Therefore, the objectives of the present work were to standardise *C. grandis* herbal supplement, determine its shelf life and proximate and nutrient compositions, evaluate the antidiabetic and antioxidant potential *in vitro*, and assess the effect of the dietary supplement on oxidative stress and inflammation in newly diagnosed patients with T2DM.

Materials and methods

Plant material

Leaves of *C. grandis* were collected at the flowering stage, during October - November 2017 in Galle Southern Province, Sri Lanka. The botanical identity of *C. grandis* was confirmed by comparing it with authentic samples deposited in the National Herbarium of the Royal Botanical Gardens, Peradeniya, Sri Lanka. A voucher specimen of the plant was deposited in the Department of Biochemistry, Faculty of Medicine, University of Ruhuna, Sri Lanka (voucher no.: B/2017/Wasana 01).

Preparation of C. grandis dietary supplement

Leaves were washed with tap water, and oven-dried at 40°C for 3 d until a constant weight was reached. The dried plant material was ground, and the powdered material (60 g) was refluxed in distilled water (1.9 L) for 4 h. The hot aqueous extract prepared from the leaves of *C. grandis* was freeze-dried and filled into a gelatine capsule as *C. grandis* dietary supplement (Wasana *et al*., 2021).

Heavy metal and phytochemical analyses of C. grandis dietary supplement

The content of the dietary supplement was subjected to the following tests. The presence of heavy metals, including mercury (Hg), lead (Pb), cadmium (Cd), and arsenic (As), was tested following the protocol prescribed by the standards of the Association of Analytical Communities (AOAC, 2005). The qualitative preliminary phytochemical screening for the presence or absence of phenolics, flavonoids, tannins, alkaloids, saponins, steroids, terpenoids, and cardiac glycosides was carried out according to Edeoga *et al*., 2005 with slight modifications.

For TLC, the dietary supplement (10.0 g) was dissolved in methanol (50.0 mL), and the mixture was filtered. The filtrate was concentrated using a rotary evaporator at 40°C. The TLC fingerprint profile was developed using the mobile phase of methanol, dichloromethane, and cyclohexane in the ratio of 0.2:3.6:2 (v/v/v). Spots were observed under ultraviolet (UV) light (both at 254 and 366 nm). The retention factor (R_f) values of the spots were recorded.

For FT-IR, the dietary supplement (10.0 mg) was encapsulated in a KBr pellet (100 mg), and the spectrum was recorded in the mid-IR region (3800 - 800 cm^{-1}) at a resolution of 4 cm^{-1}. The spectrum was obtained after scanning the sample 16 times per second using an FT-IR spectrometer (Bruker, Germany).

For GC-MS, the dietary supplement was dissolved in methanol, and subjected to an Agilent 6,890 series gas chromatograph equipped with an HP-5 MS capillary column (5% phenylmethyl siloxane) (30 m × 0.25 mm id, film thickness; 0.25 µm) interfaced to an Agilent 5,973 N series mass selective detector. The oven was set up initially at 40°C, and it was maintained for 5 min before increased to 300°C

at 10°C/min. The inlet temperature was 280°C. Helium was used as the carrier gas at a flow rate of 0.9 mL/min. The MS source temperature and the MS quadrupole temperature were 230 and 150°C, respectively. The parameters were scanned at 15 and 550 amu. A library search for the identification of compounds was undertaken using the Wiley W9N08 and NIST databases.

Shelf life determination of C. grandis dietary supplement

The dietary supplement was kept in the incubator at 40°C for 30 d. At the baseline (day 0) and on the final day, microbial analysis was performed to determine the presence of aerobic plate count, *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), coliforms, yeasts and moulds, and *Salmonella* (ATCC 700623) following the Bureau of Ceylon Standards 1973 methods.

The vacuum oven method was performed to determine the moisture content of the dietary supplement on days 0 and 30. The dietary supplement (1.0 g) was kept in the vacuum oven at 105°C until a constant weight was reached. Moisture content was determined based on the weight loss of the sample.

Proximate and nutrient composition analyses of C. grandis dietary supplement

Proximate analysis in terms of total, acid-insoluble, and water-soluble ash contents was carried out following the AOAC standards (AOAC, 2005). The protein content was determined using the Kjeldahl method whereas the fat content was determined using the Soxhlet method. A digestion method was applied to determine the crude fibre content. Carbohydrate content was calculated by difference; carbohydrate (g) = 100 – [moisture (g) + lipid (g) + protein (g) + ash (g)]. Each analysis was carried out in three replicates to minimise experimental errors. Vitamins B_1, B_2, and C were determined using the HPLC method. The content of minerals; Ca, Mg, and Fe were determined using inductively coupled plasma-mass spectrometry (AOAC, 2005).

Total phenolic contents and total flavonoid contents of C. grandis dietary supplement

The total phenolic and flavonoid contents of the dietary supplement were determined using the Folin-Ciocalteu method as described by Siddhuraju and Becker (2003). In the determination of the total phenolic content, the results were obtained using the standard curve of gallic acid, and expressed as mg of gallic acid equivalent to the dry weight of the freeze-dried content of *C. grandis* (mg GAE/g). In the determination of the total flavonoid content, the results were obtained using the standard curve of quercetin, and expressed as mg of quercetin equivalent to the dry weight of the freeze-dried content of *C. grandis* (mg QE/g).

In vitro antidiabetic activity of C. grandis dietary supplement

The pre-incubation method was followed to determine the α-amylase (EC 3.2.1.1) inhibitory activity using porcine pancreatic α-amylase (Geethalakshmi *et al*., 2010). The reaction mixture consisted of porcine pancreatic α-amylase solution (0.050 mL), ice-cold phosphate buffer (20 mM, pH 6.9, 0.100 mL), 1% (w/v) potato starch in the prepared buffer as substrate solution (0.100 mL), the plant extract (0.050 mL), dinitrosalicylic acid colour reagent (0.100 mL), and distilled water (0.900 mL). Finally, the absorbance was measured at 540 nm. Acarbose was used as the standard inhibitor.

The method described by Elya *et al*. (2012) was used in determining the inhibitory activity of α-glucosidase (EC 3.2.1.20). The mixture of sodium phosphate buffer saline (30 mM, pH 6.8, 0.100 mL), the plant extract (0.010 mL), 0.5 units/mL α-glucosidase from *Saccharomyces cerevisiae* (0.010 mL), 1 mM *p*-nitrophenyl-D-glucopyranoside (0.020 mL), and 0.2 M sodium carbonate (0.040 mL) was used as reaction medium. The absorbance was measured at 405 nm. Acarbose was used as the standard inhibitor.

The dipeptidyl peptidase-IV (DPP-IV) (EC 3.4.14.5) inhibitory activity was determined according to Chakrabarti *et al*. (2011) with slight modifications. 1 unit/mL DPP-IV enzyme solution (0.015 mL) made by dissolving the DPP-IV enzyme in ice-cold Tris-HCl buffer (50 mM pH 7.5), 0.1 mM Gly-pro-*p*-nitroanilide solution as substrate solution (0.050 mL), plant extract (0.035 mL), and 25% glacial acetic acid (0.050 mL) were included in the reaction medium. The absorbance was measured at 380 nm. Diprotin A was used as the standard inhibitor. α-amylase, α-glucosidase, and DPP-IV inhibitory activities were represented in terms of IC_{50} values.

The method described by Riaz *et al*. (2020) was used in the assessment of antidiabetic activity *via*

glucose uptake by yeast cells. The reaction mixture consisted of commercially available 1% baker's yeast suspension (100 µL), 1 - 5 mg (w/v) freeze-dried powder of hot aqueous extract of *C. grandis* leaves in distilled water, and a glucose solution of 5 or 10 Mm. The glucose concentration was determined spectrophotometrically at a wavelength of 520 nm following the glucose oxidase method. Metronidazole was used as the standard. The percentage of glucose uptake was calculated at each of the plant material concentrations.

In vitro antioxidant activity of C. grandis dietary supplement

The total antioxidant activity of the dietary supplement was determined following the oxygen radical absorbance capacity (ORAC) assay (Ou *et al*., 2001). The reaction mixture consisted of an aqueous extract of plant material (12 µL) and 10 µM fluorescein (138 µL). The reaction was initiated with the addition of 768 µM 2,2′-azobis(2-amidinopropane) dihydrochloride (50 µL). The ORAC value of the dietary supplement was determined by referring to the area under the Trolox curve as the reference compound. The results were expressed as Trolox equivalent antioxidant capacity (TE).

The method described by Blois (1958) was followed to determine the DPPH radical scavenging activity of the dietary supplement dissolved in methanol. The freshly prepared 40 µg/mL DPPH solution (200 µL) was incubated with 60 - 180 µg/mL of each prepared plant extract (200 µL) for 30 min in a dark room at room temperature (25 ± 2°C). Absorbance was recorded against a blank at 517 nm. The capacity to eliminate the DPPH radical by 50% (IC_{50}) was calculated from the dose-effect curves.

Antioxidant and anti-inflammatory activities of C. grandis dietary supplement in patients with newly diagnosed type 2 diabetes mellitus

Study design and patients

A three-month long, placebo-controlled, double-blind, randomised clinical trial was carried out involving newly diagnosed patients with T2DM. A detailed study design with patients and the baseline characteristics of the entire group of patients have previously been published (Wasana *et al*., 2021). In summary, a total of 158 newly diagnosed T2DM patients referred to the University Medical Clinic, Karapitiya Teaching Hospital, Galle, Southern, Sri Lanka, were enrolled for the clinical trial based on their fasting plasma glucose concentration and/or percentage of glycated haemoglobin. The enrolled group of patients was equally randomised into two groups to receive the dietary supplement or placebo capsule. Subjects in the dietary supplement-treated group received capsules containing 500 mg of the freeze-dried powder of hot aqueous extract of *C. grandis* leaves once a day after lunch, and those in the placebo group received capsules containing 500 mg of corn starch the same way for three months. Ethical clearance was granted from the Ethical Review Committee, Faculty of Medicine, University of Ruhuna, Sri Lanka (approval no.: 14.06.2017:3.9). The clinical trial protocol was prospectively registered on 17th April 2018 at the Clinical Trial Registry, Sri Lanka (SLCTR/2018/012), https://slctr.lk/trials/slctr-2018-012.

Outcomes

The outcome was the determination of the effect of the dietary supplement on oxidative stress and inflammation in newly diagnosed patients having T2DM using oxidative stress markers; serum activity of catalase EC 1.11.1.6 (CAT), glutathione reductase EC 1.6.4.2 (GR), serum concentration of malonaldehyde (MDA) and anti-inflammatory markers; serum concentrations of tumour necrosis factor-alpha (TNF-α), and interleukin-6 (IL-6).

Biochemical measurements

At baseline (month 0) and at the end of the intervention (three months), a fasting venous blood sample (8 - 10 mL) was taken from each study participant. The blood samples were centrifuged, separated to obtain serum/plasma samples, and the samples were immediately stored (-80°C). Biochemical evaluations were performed on the collected serum samples. The serum activity of CAT was determined by the enzymatic method (Elabscience, USA), and the serum activity of GR was determined spectrophotometrically. The serum concentration of MDA was determined using thiobarbituric acid reactive substances. The serum concentrations of TNF-α and IL-6 were determined using the ELISA method (Elabscience, USA). All laboratory tests were quality-controlled, and biochemical estimations were performed in duplicate using the same kits and reagents.

Statistical analysis

The SPSS software version 25.0 was used. Repeated measure analysis was performed. *In vitro* and assays on antidiabetic activity and antioxidant activity were carried out in triplicates. The results were expressed as mean ± standard deviation (SD) of three separate determinations. The normality of the data sets was checked using the D'Agostino-Pearson omnibus K^2 test. The unpaired sample *t*-test and the Mann-Whitney U test were used to compare the variables with normal and non-normal distributions between the groups, respectively. Paired sample *t*-test or Wilcoxon signed-rank test, were used to compare the variables with normal and skewed distributions within the groups, respectively. A per-protocol analysis was carried out in the clinical trial. The statistical significance was set at $p < 0.05$ for each of the parameters.

Results

The selected heavy metals including Hg (< 0.05 ppm), Pb (0.10 ppm), Cd (< 0.05 ppm), and, As (< 0.05 ppm) were present in the dietary supplement. The preliminary screening for phytoconstituents revealed the presence of alkaloids, phenolics, saponins, flavonoids, tannins, steroids, and triterpenoids; and the absence of cardiac glycosides in the dietary supplement. As shown in Figure 1, the TLC fingerprint profile of the dietary supplement showed four prominent spots bearing R_f values of 0.64, 0.57, 0.16, and 0.10; and six prominent spots bearing R_f values of 0.86, 0.67, 0.60, 0.30, 0.21, and 0.17; at 254 and 366 nm, respectively.

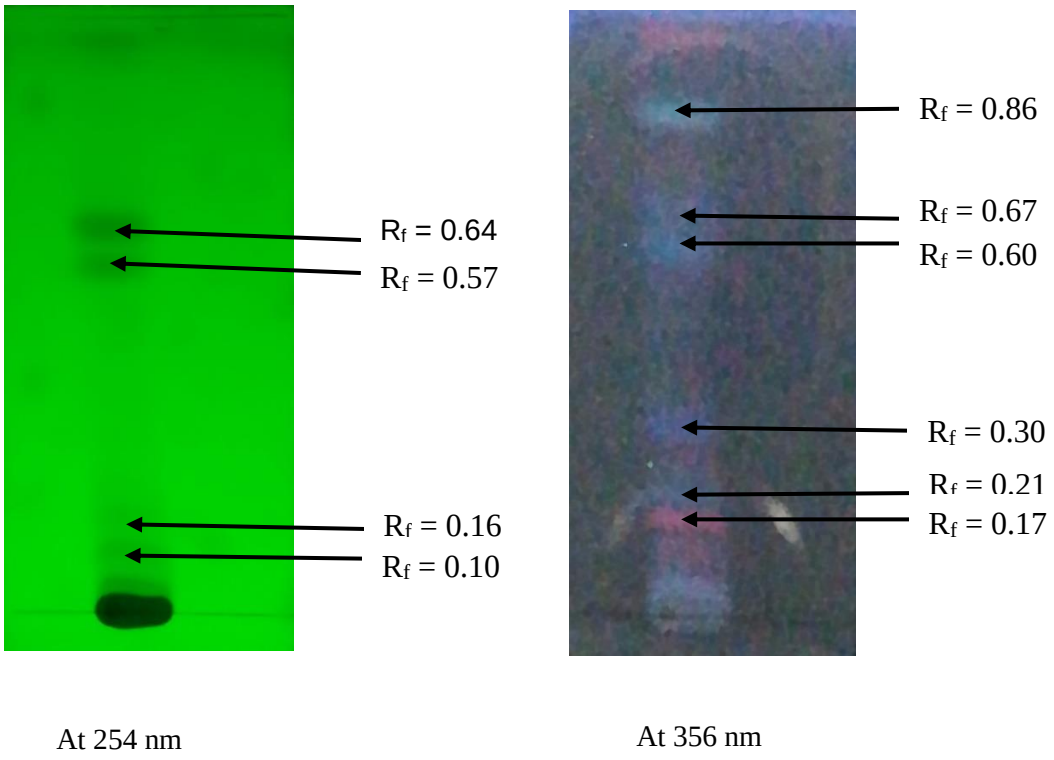

Figure 1. TLC fingerprint profile.

The FT-IR fingerprint (Figure 2) shows a sharp peak at 1021 cm^{-1}, which is characteristic of the freeze-dried powder of *C. grandis.* The GC-MS analysis revealed the presence of 12 compounds including γ-tocopherol, α-tocopherol, phytol, and others in the methanolic fraction of the dietary supplement (Table 1 and Figure 3).

In the determination of the dietary supplement's shelf-life, the microbial analyses yielded negative results for aerobic plate count, *Staphylococcus aureus*, *Escherichia coli*, coliforms, yeast and mould, and *Salmonella* on days 0 and 30. The moisture content of the dietary supplement was 0.20 ± 0.0 and 0.50 ± 0.0% (w/w) on days 0 and 30, respectively. The proximate and nutrient composition analyses in the dietary supplement revealed the contents of total ash, acid-insoluble ash, water-soluble ash, carbohydrate, protein, fat, and fibre as 42.9 ± 0.1, 0.2 ± 0.02, 22.4 ± 0.8, 26.7 ± 0.3, 29.8 ± 0.4, 0.3 ± 0.0, and 0.3 ± 0.1%, respectively.

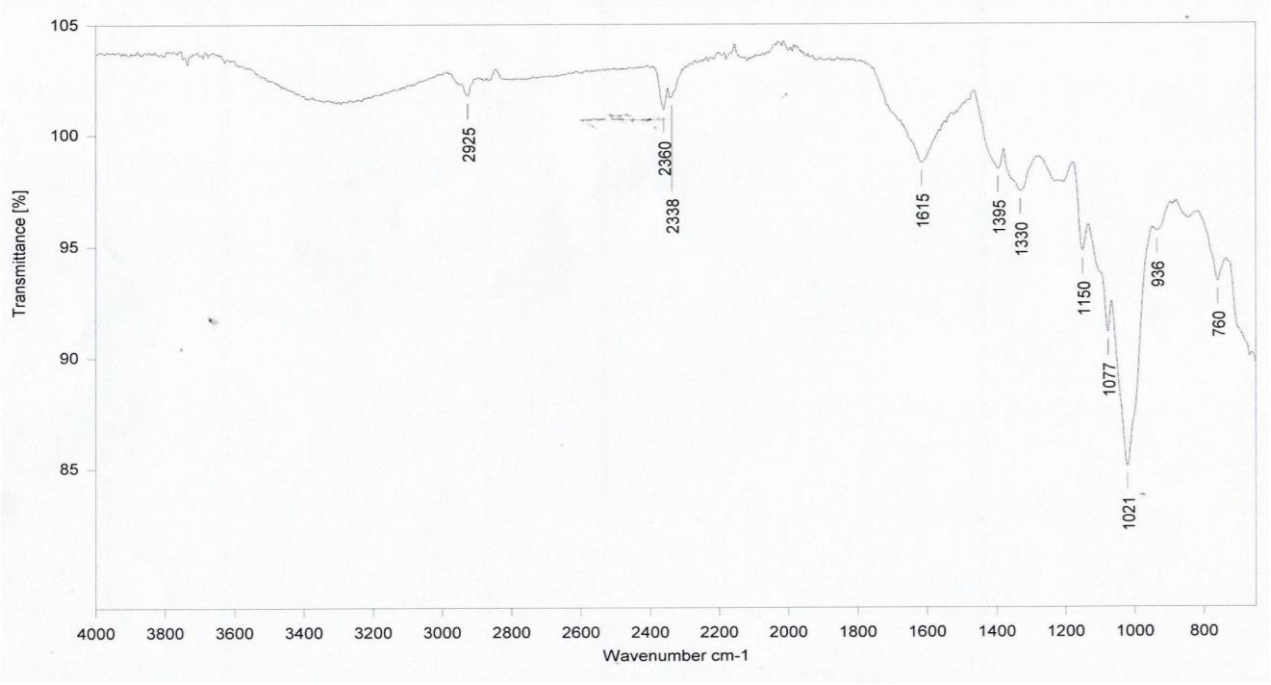

Figure 2. FT-IR fingerprint profile.

Table 1. Compounds identified in methanolic extract of freeze-dried powder of hot aqueous extract of *Coccinia grandis* leaves through GC-MS analysis.

Retention time (min)	Compound name	Molecular formula	Molecular weight (g/mol)	Peak area (%)
13.272	2,4-Bis(1,1-dimethylethyl)-phenol	$C_{14}H_{22}O$	206.32	0.02
16.362	Loliolide	$C_{11}H_{16}O_3$	196.24	0.03
16.787	Neophytadiene	$C_{20}H_{38}$	278.50	0.01
17.672	Palmitic acid methyl ester	$C_{17}H_{34}O_2$	270.45	0.05
19.302	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	$C_{19}H_{34}O_2$	294.47	0.03
19.375	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)	$C_{19}H_{32}O$	292.50	0.08
19.467	Phytol	$C_{20}H_{40}O$	296.50	0.09
25.077	Squalene	$C_{30}H_{50}$	410.73	0.05
25.984	2H-1-Benzopyran-6-ol, 3,4-dihydro-2,5,7,8-tetramethyl-2-(4,8,12-trimethyl-3,7,11-tridecatrienyl)-	$C_{29}H_{44}O_2$	424.70	0.11
26.729	Gamma-tocopherol	$C_{28}H_{48}O_2$	416.68	0.12
27.366	Alpha-tocopherol	$C_{29}H_{50}O_2$	430.71	0.20
29.519	5Beta-pregn-7-en-3,20-dione	$C_{21}H_{30}O_2$	314.50	0.06

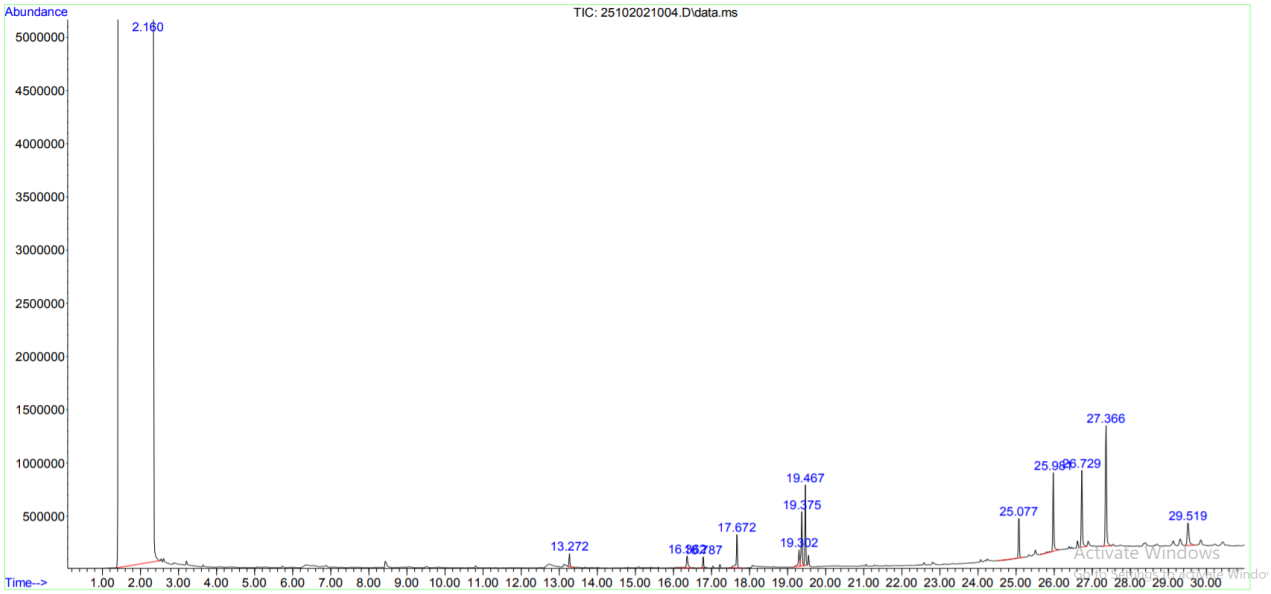

Figure 3. GC-MS fingerprint profile.

Furthermore, the dietary supplement consisted of vitamins B_1 and B_2 at 0.52 ± 0.01 and 0.38 ± 0.02 mg/100 g, respectively, and minerals of Ca as 3.7 mg/kg and Mg as 0.2 mg/kg. However, vitamin C and mineral Fe were not significantly detected.

The total phenolic content of the dietary supplement was 8.68 ± 0.83 mg GAE/g of dry weight, and the total flavonoid content was 18.0 ± 1.2 mg QE/g of dry weight.

The dietary supplement exerted significantly lower ($p < 0.05$) α-amylase (IC_{50} 1.70 ± 0.06 mg/mL), α-glucosidase (IC_{50} 0.70 ± 0.06 mg/mL), and DPP-IV (IC_{50} 2.32 ± 0.12 mg/mL) inhibitory activities than the respective standard compounds. The dietary supplement promoted glucose uptake across the plasma membrane of yeast cells (Figures 4a and 4b). When the concentration of the solution consisting of the dietary supplement was increased, the ability of the yeast cells to absorb glucose from the environment was dramatically increased. The effect of different concentrations of the dietary supplement (10, 15, and 20 mg/mL) on glucose uptake by yeast cells at a glucose concentration of 5 and 10 Mm was significantly higher than that of metronidazole.

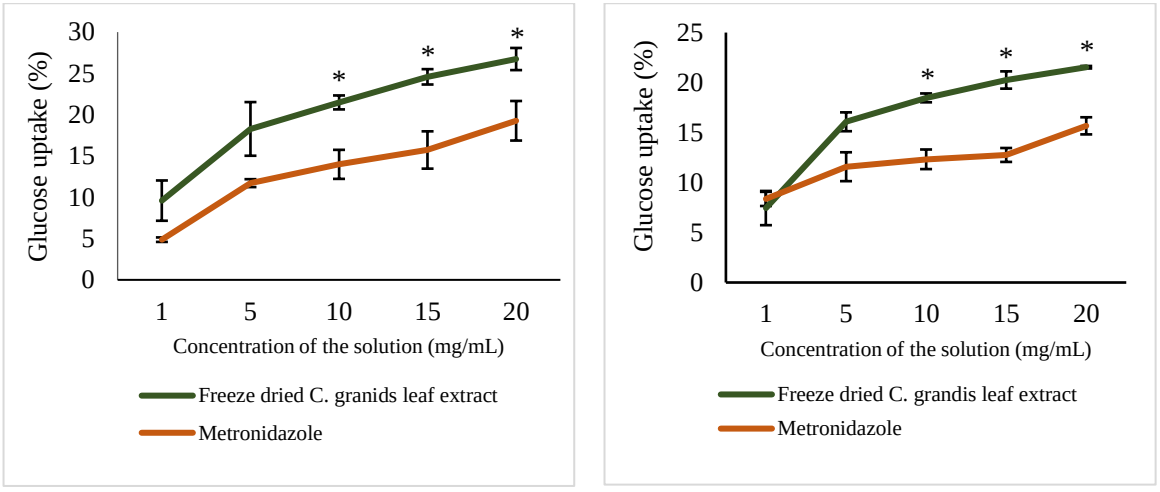

Figure 4. Glucose uptake by yeast cells in the presence of freeze-dried *C. grandis* leaf extract at **(a)** 5 mM and **(b)** 10 mM glucose concentrations. *Statistically significant different at $p < 0.05$. Each data point represents mean ± SD.

The ORAC of the dietary supplement was 3.37 ± 0.42 µmol TE/g of dry weight, while the IC_{50} value was 141.9 ± 1.6 µg/mL in the DPPH assay.

The changes in CAT and GR activities and MDA concentration at baseline and the end of the intervention upon administration of the dietary supplement for three months in patients with newly diagnosed T2DM are presented in Table 2. Significant mean ± SD changes in serum GR activity ($p < 0.001$) and serum MDA concentration ($p = 0.025$) were observed from baseline to the end of the intervention between the test and placebo groups. No significant changes in CAT activity were observed with the treatment of the dietary supplement in patients with newly diagnosed T2DM ($p > 0.05$). As shown in Table 2, there was a significant mean change ± SD ($p = 0.002$) in serum IL-6 concentration from baseline to the end of the intervention between the two groups. However, the decrement of serum concentration of TNF-α in the test group was not significant as compared to the placebo group ($p > 0.05$).

Table 2. Changes of oxidative stress and anti-inflammatory markers in study subjects from beginning to end of intervention (three months).

Variable	Group	Baseline mean ± SD	*p* value[#]	After intervention mean ± SD	*p* value[##]	Mean ± SD changes	*p* value[###]	*p* value[####]
CAT	Test	11.03 ± 10.72	0.016	11.66 ± 10.91	0.065	0.24 ± 7.30	0.404	0.378
(U/mL)	Placebo	16.76 ± 11.66		16.26 ± 11.47		0.50 ± 7.61		0.728
GR	Test	30.97 ± 7.82	0.095	34.22 ± 7.98	<	-3.25 ± 3.93	< 0.001	< 0.001
(U/L)	Placebo	29.14 ± 6.99		27.71 ± 7.50	0.001	1.42 ± 4.84		0.001
MDA	Test	195.31 ± 47.02	0.344	182.56 ± 43.46	0.419	12.75 ± 33.35	0.025	< 0.001
(nmol/dL)	Placebo	187.24 ± 39.98		188.68 ± 47.50		-1.45 ± 41.93		0.08
TNF-α	Test	6.92 ± 1.10	0.791	6.79 ± 1.25	0.102	0.13 ± 1.79	0.225	0.263
(pg/mL)	Placebo	6.82 ± 0.99		6.89 ± 1.00		-0.07 ± 1.22		0.870
IL-6	Test	49.81 ± 16.38	0.639	43.92 ± 13.06	0.217	5.89 ± 11.49	0.002	0.001
(pg/mL)	Placebo	48.14 ± 16.96		47.68 ± 15.60		0.46 ± 13.11		0.831

Data are means ± SD. CAT: catalase; GR: glutathione reductase; IL-6: interleukin-6; MDA: malonaldehyde; TNF-α: tumour necrosis factor alpha; *p* value[#] : values for comparing variables between test and placebo groups at baseline; *p* value[##]: values for comparing variables between test and placebo groups at the end of the intervention; *p* value[###]: values for comparing mean ± SD changes of the variables from the baseline to the end of intervention between test and placebo groups; and *p* value[####]: values for comparing variables within the group.

Discussion

The present work focused on the determination of the chemical composition of the dietary supplement of *C. grandis*. Heavy metal analysis is important for assuring the safety of the supplement. Excessive content of heavy metals in dietary supplements is associated with toxic/adverse effects with diseases related to cardiovascular, renal, hepatic, nervous, and skeletal systems (Alwakeel, 2008). Interestingly, the present work found that heavy metals were not significantly detected in the dietary supplement. Preliminary phytochemical screening carried out on supplements includes all possible information generated concerning the presence of secondary metabolites. In the present work, the presence of secondary metabolites such as phenolics, flavonoids, tannins, alkaloids, saponins, steroids, and triterpenoids of the dietary supplement was observed. In line with the present work, previous studies reported that the identified plant secondary metabolites were present in the ethanolic, methanolic, and aqueous extracts of *C. grandis* leaves (Attanayake *et al*., 2016; Pavithra *et al*., 2017; Al-Madhagy *et al*., 2019). Polyphenolics and flavonoids are reputed for having multiple hydroxyl groups on their aromatic rings. Due to this structural feature, polyphenolic and flavonoid compounds are good electron and proton donors, thus able to scavenge free radicals, and reduce oxidative stress by transferring

H-atom from their hydroxyl group(s) to free radicals (Heim *et al*., 2002; Papuc *et al*., 2017). In the present work, the hot aqueous extract of *C. grandis* leaves contained total phenolic content of 8.68 ± 0.83 mg GAE/g of dry weight, and total flavonoid content of 18.0 ± 1.2 mg QE/g of dry weight. These could contribute to its antioxidant potential. Several previous studies also reported remarkable total phenolic and flavonoid contents of the different extracts of *C. grandis* leaves (Namchaiw *et al*., 2021; Putra *et al*., 2021). In this context, TLC, FT-IR, and GC-MS fingerprints were obtained as quality control tools to maintain batch-to-batch consistency in the mass production of the dietary supplement for successful commercialisation. Previous findings have also reported the importance of fingerprint profiles to maintain batch-to-batch consistency in commercially viable dietary supplements (Xie *et al*., 2006; Gupta *et al*., 2019). Among the identified secondary metabolites of the dietary supplement *via* GC-MS analysis, loliolide, has been well-established as an antioxidant and anti-inflammatory compound (Yang *et al*., 2011; Silva *et al*., 2021). Phytol was reported to exert an insulin sensitiser/antidiabetic effect in streptozotocin-induced diabetic rats (Elmazar *et al*., 2013). Gamma-tocopherol and alpha-tocopherol are compounds that exert antioxidant, anti-inflammatory, and anti-hyperglycaemic activities, and therefore supplementation with *C. grandis* could be beneficial for patients with T2DM (Jamalan *et al*., 2015; Lee and Lim, 2019).

Contamination with microorganisms irrespective of whether it is harmful or not can change the physicochemical characteristics of plant-based dietary supplements. Several scientific investigations reported that herbal products are associated with a broad variety of microbial contaminants (Kneifel *et al*., 2002; Alwakeel, 2008; Vuuren *et al*., 2014). Microbial contamination of dietary supplements may occur during the period of handling of the products by people who are affected with pathogenic bacteria during harvesting or collection, post-harvest processing, and manufacturing process. Collectively, all the results obtained in the present work indicate that the herbal capsules of *C. grandis* were safe for human consumption.

Moreover, the present work also revealed a lower amount of moisture content of the freeze-dried powder of hot aqueous extract of *C. grandis* leaves as compared to the powered material of its dry leaves (Attanayake *et al*., 2016). Indeed, the presence of a low amount of water in a dietary supplement causes less chance for the growth of bacteria and fungi as well as for the hydrolysis of phytoconstituents. Interestingly, freeze-drying of the aqueous extract removes up to 90% of the water from the target material, and the water is removed in a frozen state as compared to oven drying of the fresh plant material. Hence, lower moisture content of the dietary supplement *C. grandis* associates with a lesser chance of microbial degradation during storage, thus promoting long-term stability.

For proximate and nutrient composition analyses, the determination of the contents of ash, carbohydrate, lipid, protein, fibre, vitamins, and minerals are important as these provide the nourishment which is essential to prevent or slow down the risk of developing diabetes mellitus. Indeed, some of the parameters are quality measures of herbal capsules. High ash values indicate the presence of contamination, substitution, or adulteration in herbal preparations (Chandel *et al*., 2011). The acid-insoluble ash is a part of the total ash which is insoluble in hydrochloric acid, and determines the level of silica, especially sand and siliceous earth present in the herbal preparations (Rao and Xiang, 2009). The water-soluble ash is a part of the total ash which is soluble in water, and an indicator for water-soluble matter present in herbal preparations. Based on the findings of the present work, the acid-insoluble ash value was low, and total ash and water-soluble ash values were higher in the freeze-dried powder of the hot aqueous extract as compared to the published reports on the dry leaves of *C. grandis* (Attanayake *et al*., 2016). A low value of acid-insoluble ash would be beneficial in the manufacturing processes of herbal capsules as it would indicate low contamination with silica.

A sudden increase in blood glucose concentration after a meal occurs mainly due to starch hydrolysis by pancreatic α-amylase and glucose uptake by intestinal α-glucosidase. The products of starch hydrolysis are not absorbed into the duodenum and upper jejunum, and therefore are further hydrolysed into glucose by α-glucosidase. Therefore, the inhibition of the α-amylase and α-glucosidase enzymes suppresses carbohydrate digestion, delays glucose uptake, thus reducing the blood glucose level. Even though the freeze-dried *C. grandis* leaf extract showed considerable α-amylase and α-glucosidase inhibitory activities, those activities were lower than that of acarbose. The DPP-IV enzyme is reported to

inactivate the incretin hormones, and further hampers insulinotropic activity. DPP-IV inhibitors block DPP-IV enzyme activity, extending the half-life of incretins, and this phenomenon has become one of the modest pharmaceutical targets in the treatment of T2DM (Wasana *et al*., 2020). This is the first attempt in assessing the inhibitory activity of the DPP-IV enzyme in the leaves of *C. grandis.* The inhibitory activity of DPP-IV of the freeze-dried powder of the hot aqueous leaf extract of *C. grandis* was significantly lower as compared to the standard drug diprotein A. Glucose transportation *via* yeast cell membrane may involve facilitated diffusion. Glucose concentration within the cell is decreased when glucose is used or converted into other metabolites. This phenomenon inspires the high uptake of glucose within cells. In the present scenario, the percentage of glucose uptake was increased when the freeze-dried *C. grandis* leaf extract concentration was increased.

As one of the main objectives of the present work, we evaluated the importance of the dietary supplement *C. grandis* and its antioxidant and anti-inflammatory effects in patients with newly diagnosed T2DM. Intake of 500 mg of herbal supplement *C. grandis* daily for three months increased serum GR activity and decreased serum concentrations of MDA and IL-6 as compared to the placebo-treated group. However, the increase in CAT activity and the decrease in TNF-α concentration after administration of the herbal capsule of the dietary supplement *C. grandis* were not statistically significant as compared to the placebo drug administration. The hyperglycaemic status present in patients with T2DM enhances glucose flow through metabolic pathways, and causes an overdrive of the mitochondrial electron transport chain, thus resulting in an increased level of ROS formation (Fakhruddin *et al*., 2017). This leads to the development of oxidative stress, and has been often associated with cell dysfunction, altered cell cycle and cell signalling, inflammation, insulin resistance, and progression of diabetes and its associated complications (Giacco and Brownlee, 2010). Therefore, a high level of antioxidant enzymes is beneficial for patients with diabetes, as they can potentially reduce the level of ROS with the consequent slowing of complications associated with diabetes. Interestingly, the result of the present clinical trial as the mean change in GR of -4.67 U/L (test: -3.25 U/L; placebo: 1.42 U/L) from baseline to the end of the intervention between the test and placebo groups demonstrated the potent antioxidant activity of the dietary supplement *C. grandis*. GR facilitates the formation of reduced glutathione from glutathione disulphide in a reaction requiring NADPH as a cofactor. Reduced glutathione is highly effective in the detoxification of hydrogen peroxide, and therefore decreases oxidative stress in patients with T2DM (Matough *et al*., 2012). Furthermore, several biomarkers of oxidative stress are available, including ROS themselves. High reactivity and the short half-life of ROS restrict its wider practice. Therefore, it is more convenient to determine oxidative stress by measuring oxidation target products. MDA is one of the oxidation target products, and frequently used to determine the oxidative stress level. It is important to determine the serum MDA concentration in newly diagnosed patients with T2DM as a key pathogenic factor responsible for the progression of insulin resistance and β-cell dysfunction leading to the development of diabetic complications (Siddiqui *et al*., 2019). Importantly, the results of the present clinical trial revealed that there was a difference in the mean change in MDA of 14.2 nmol/dL (test: 12.75 nmol/dL; placebo: -1.45 nmol/dL) from baseline to the end of the intervention between the test and placebo groups. Therefore, the decrease in the extent of lipid peroxidation coupled with an increase in GR activity after-treatment of the *C. grandis* herbal capsule supported the idea that this dietary supplement could act as a potent antioxidant agent that could be beneficial in the treatment of diabetes, and delay the development of diabetic complications.

Apart from oxidative stress, increased concentrations of IL-6 and TNF-α are implicated in low-grade inflammation, thus activating the innate immune system, and facilitating the pathogenesis of some complications of diabetes (Mohammadi *et al*., 2017). Elevated serum IL-6 concentration in diabetic patients is likely to elicit its effects on adipocytes, vascular smooth muscle cells, hepatocytes, and endothelial cells, thus promoting the development of insulin resistance that leads to the development of T2DM, and ultimately to the progression of diabetes complications (Akbari and Hassan-Zadeh, 2018). The increased level of IL-6 promotes the expression of the suppressor of cytokine signalling 3 (SOCS3) in adipocytes. Furthermore, IL-6 decreases the expression of insulin signalling components; IR-β, IRS-1, glucose transporter-4, and insulin-sensitising factors (Akbari and Hassan-Zadeh, 2018). The effect of IL-6 on vascular smooth muscle cells and

endothelial cells by trans-signalling leads to atherosclerosis and macrovascular complications in patients with T2DM (Akbari and Hassan-Zadeh, 2018). Increased levels of TNF-α induce insulin resistance in adipocytes and peripheral tissues by altering insulin signalling through serine phosphorylation, thus leading to the development and progression of diabetes mellitus (Zhao *et al*., 2023). Therefore, it is important to have a low level of cytokines in patients with T2DM, which delays the development of complications associated with diabetes. Based on the findings of the present work, there was a significant difference in the mean change of serum concentration of IL-6 as 5.43 pg/mL (test: 5.89 pg/mL; placebo: 0.46 pg/mL) from baseline to the end of intervention between the test and placebo groups. Therefore, the dietary supplement *C. grandis* may inhibit the formation of cytokines, and act as an anti-inflammatory agent through the enhancement of the expression of SOCS3 in adipocytes and hepatocytes, and stimulation of the secretion of GLP-1 from pancreatic β-cells, in patients with newly diagnosed T2DM.

Conclusion

The data obtained from heavy metal and shelf life analyses of *C. grandis* dietary supplement supported its safety and stability for human consumption. Proximate analysis indicated significant contents for a commercially viable dietary supplement. Contents of carbohydrates, proteins, fibres, fats, vitamins, and minerals added values to *C. grandis* dietary supplement. Possible mechanisms that regulate blood glucose concentration include the inhibition of α-amylase, α-glucosidase, and DPP-IV enzymes; increased glucose uptake; and exhibition of antioxidant activity. Administration of *C. grandis* dietary supplement (500 mg/day for three months) had favourable effects on oxidative stress and inflammation in patients with newly diagnosed T2DM. Collectively, all findings showed the quality, stability, safety, efficacy, and commercial value of *C. grandis* dietary supplement that could be particularly recommended for patients with diabetes in the management of T2DM and its complications.

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