



In vitro Antidiabetic Activity of a Polyherbal Tea and Its Individual Ingredients

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Authors' contributions

This work was carried out in collaboration between all authors. Author VP conducted experimental work and wrote the first draft of the manuscript. Author JJVT co-supervised the study and edited the manuscript. Author VS designed and supervised the study and reviewed and edited the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aim: To assess the antidiabetic potential of a polyherbal tea, Diabetea, and its individual ingredients; *Achillea millefolium* L., *Agathosma betulina* Bartl. & Weidl., *Salvia officinalis* L., *Taraxacum officinalis* L., *Thymus vulgaris* L., *Trigonella foenum-graecum* L. and *Urtica urens* L.
Study Design: An *in vitro* laboratory-based study with appropriate positive and negative controls.
Place and Duration of Study: Department of Pharmacology, February 2011 to August 2013.
Methodology: The α -amylase and α -glycosidase enzyme inhibitory activity of hot water- and dichloromethane extracts (HWE and DCME) of Diabetea and its constituents were assessed spectrophotometrically and data interpreted using the Michaelis-Menten model. Glucose uptake into C2C12 myotubes was determined using a fluorometric method.
Results: *A. betulina* (DCME) and *U. urens* (DCME) significantly ($p < 0.05$) inhibited the activity of α -glucosidase (non-competitively) and α -amylase (un-competitively). The inhibitory activity of these extracts significantly ($p < 0.05$) compared with the positive control, acarbose. The DCME of Diabetea, *T. officinalis*, *U. urens*, *A. millefolium* and the HWE of *A. betulina*, *T. officinalis*, *T. foenum-graecum*, *S. officinalis*, *U. urens* and *T. vulgaris* caused a significant ($p < 0.05$) uptake of glucose into C2C12 myotubes compared to the control. *S. officinalis* (HWE) and *T. vulgaris*

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(DCME) were found to be more active in reducing the blood sugar level than insulin ($p < 0.05$) at 3 and 20 $\mu\text{g/ml}$, respectively.

Conclusion: Diabetea, *T. officinalis*, *U. urens*, *A. millefolium*, *A. betulina*, *T. foenum-graecum*, *S. officinalis* and *T. vulgaris* contain bioactive compounds that act as insulin mimetics. It can be concluded that *U. urens* (DCME), *A. betulina* (HWE) and *T. vulgaris* (HWE and DCME) were the most promising *in vitro* antidiabetic preparations due to their potent hypoglycaemic activities.

Keywords: α -amylase; α -glycosidase; diabetea; diabetes mellitus; glucose uptake.

ABBREVIATIONS

2-NBDG	2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino]-2-deoxy-D-glucose
ANOVA	Analysis of variance
DCFH-DA	2',7'-dichlorodihydrofluorescein diacetate
DCM	Dichloromethane
DCME	Dichloromethane extract
dH ₂ O	Distilled water
DI H ₂ O	Deionised water
DM	Diabetes mellitus
DMEM	Dulbecco's Modified Eagle's Medium
DMEM+	DMEM with 1% penicillin G/ 1% streptomycin
DMSO	Dimethyl sulfoxide
DNSA	3,5-dinitrosalicylic acid
DPPH [•]	1,1-diphenyl-2-picryl-hydrazyl free radical
ES	Enzyme-Substrate
EDTA	Ethylenediaminetetraacetic acid
FCS	Foetal calf serum
FPG	Fasting plasma glucose
GLUT	Glucose transporter
HBSS	Hanks balanced salt solution
HWE	Hot water extract
KM	Michaelis Menten constant
LW-B	Lineweaver-Burk
MeOH	Methanol
PBS	Phosphate buffered saline
pNPG	p-nitrophenyl- α -Dglucopyranoside
RFI	Relative fluorescence intensity
[S]	Substrate concentration
SEM	Standard error of the mean
V _{max}	Maximum enzyme reaction velocity

1. INTRODUCTION

Chronic hyperglycaemia is the main characteristic of the metabolic disease, diabetes mellitus (DM). DM-related hyperglycaemia is diagnosed as an incessantly high fasting plasma glucose level of ≥ 7.0 mmol/l and a two hour, or at random, plasma glucose level of ≥ 11.1 mmol/l [1]. Untreated hyperglycaemia leads to incapacitating comorbidities such as gastroparesis, gangrene, myocardial infarction, kidney failure, blindness, stroke, atherosclerosis and paralysis [2-4]. DM is generally asymptomatic and is often diagnosed at perilous stages of the disease, when unremitting

symptoms such as polydipsia, polyuria, polyphagia, syncope, emesis and insomnia are present [1]. DM manifests as a result of insulin resistance, insufficiency and/or inactivity [5,6], which is associated with obesity, gestation, sedentary lifestyle and/or a hereditary predisposition [7]. Currently, DM has reached epidemic proportions [6], making research for new and improved treatments crucial.

Insulin defect(s) lead to an atypical metabolism of carbohydrates in adipocytes, myocytes and hepatocytes [8]. The greater part of carbohydrate digestion and absorption occurs within the small intestine, mediated through digestive enzymes

such as maltase, sucrase, lactase, α -glucosidase and pancreatic α -amylase [2]. Pancreatic α -amylase is produced by the β -cells of the pancreas and is released into the intestinal lumen in response to an increased carbohydrate concentration [2]. It mediates the hydrolysis of the internal 1,4- α -glycosidic linkages of saccharides into di- and tri-saccharides, which is further digested by α -glucosidase. α -Glucosidase is involved in the digestion of dietary carbohydrates and is found in the brushborder of the enterocytes of the small intestinal wall [2]. This enzyme mediates the hydrolysis of external 1,4- α -D-glucose linked residues from its non-reducing ends forming monosaccharides such as glucose [2]. Monosaccharides are absorbed into the circulatory system through the enteric wall by glucose transporters or via passive diffusion [2]. Consequently, the inhibition of α -amylase and α -glucosidase will aid in the deterrence of postprandial hyperglycaemia by preventing the hydrolysis and absorption of glucose into circulation.

Subsequent to the absorption of glucose into systematic circulation, glucose is transported into insulin-dependent cells such as myocytes and adipocytes, by means of glucose transporters (GLUT) e.g. GLUT-4 [2]. The binding of insulin to the insulin-receptor complex on the insulin-dependent cell activates the GLUT-4 transporters [2]. In the case of insulin inactivity, resistance or insufficiency, glucose will remain in the circulatory system, leading to chronic hyperglycaemia. This makes insulin the primary glucoregulatory hormone in carbohydrate metabolism [2].

The active site of an enzyme is highly selective to its substrate [9]. The inhibition of the action of an enzyme occurs when a compound, other than the substrate, weakens the interaction of the enzyme active site with the substrate. The type of inhibition is dependent on the type of inhibitor present. Michaelis-Menten kinetics dictates four types of inhibitors; competitive, non-competitive, un-competitive and mixed. The type of inhibitor is determined by evaluating the relationship between the maximum reaction velocity of the enzyme (V_{max}) and E-to-S affinity (Michaelis-Menten constant (KM)), using a Lineweaver-Burk (LW-B) plot. A LW-B plot is a double reciprocal plot of the Michaelis-Menten reaction graph (Velocity vs. substrate concentration [S]). Knowing the type of inhibitor provides insight into the physiological conditions that may, or may not be conducive to its action [9].

Diabetes has been treated with herbs long before the use of modern medicine [10]. Traditional medicine is often the only readily available and affordable source of treatment for the majority of people in developing countries [11]. The use of herbal remedies is said to be favoured over conventional treatment due to the perception that it is safer owing to its natural origin [12]. Various plant species have been reported to possess antidiabetic activities [13-16]. The conventional antidiabetic drug, metformin, is derived from *Galega officinalis* [3].

Diabetea is a commercial polyherbal tea mixture, used to treat diabetes, and consists of *Achillea millefolium*, *Agathosma betulina*, *Salvia officinalis*, *Taraxarum officinalis*, *Thymus vulgaris*, *Trigonella foenum-graecum* and *Urtica urens*. Some of the ingredients of Diabetea such as *S. officinalis*, *T. officinalis* and *T. foenum-graecum* have been shown to possess *in vitro* and or *in vivo* hypoglycaemic activities [17-19]. However, the herbs comprising Diabetea have never been tested in the form of a mixture before. Therefore, the aim of the present study was to assess the *in vitro* hypoglycaemic potential of Diabetea and its individual herbs.

2. MATERIALS AND METHODS

2.1 Plant Material and Extraction

Diabetea was obtained from Sing-Fefur organic herbs (Robertson, South Africa). The dried plant material of *A. millefolium* L. (Asteraceae, whole plant), *A. betulina* Bartl. Wendl. (Rutaceae, leaves), *S. officinalis* L. (Lamiaceae, leaves), *T. officinalis* L. (Asteraceae, aerial part), *T. vulgaris* L. (Lamiaceae, leaves and stems), *T. foenum-graecum* L. (Fabaceae, seeds) and *U. urens* L. (Urticaceae, whole plant), prepared by Pharma Germania, was purchased at a local health shop (Pretoria, South Africa). Each plant sample was ground into a fine homogenous powder (IKA-Werke Yellowline A10 analytical grinder) before extraction to maximise surface area.

Hot water (HW) and dichloromethane (DCM) were used as extraction solvents because of their differences in polarity. The HW extraction mimicked a decoction method, whereby 2 g homogenous plant powder was mixed with 20 ml of distilled water (dH₂O). The solution was left on an electronic shaker (Beckman Coulter, VRN-200) for 1 h and sonicated (Bransonic 52 Cleaning Equipment Co.) for an additional hour, after which the mixtures were macerated for 24 h

at 4°C. Thereafter the mixtures were left on a shaker for 30 min to reach room temperature before being boiled (Labotec, Büchi Heating Bath, B-490) for 15 min. Mixtures were centrifuged for 30 min at 1000 g (Allegra X-22, Beckman Coulter), filtered (0.22 µm) and kept at 4°C overnight. The following day the mixtures were dried by lyophilisation (Freezone 6, Labconco) and stored at -70°C. The DCM extraction was performed in a similar fashion to that of the HW extraction, up to the centrifugation step, whereafter the supernatant was concentrated by means of *in vacuo* rota-evaporation (Labotec, Büchi Rotavapor) at 60°C. The concentrate was reconstituted in dimethyl sulfoxide (DMSO) and stored at -70°C. Yields were determined gravimetrically.

2.2 Prevention of Hyperglycaemia

2.2.1 α-Amylase assay

The effect of each extract on the activity of α-amylase was determined using 3,5-dinitrosalicylic acid (DNSA), as described by Bernfeld et al. [20] with modifications, by adapting the method to a 96-well plate.

A 0.2 U/ml stock solution of porcine pancreatic α-amylase was prepared in a sodium phosphate buffer solution (9.66 mM sodium chloride, pH 6.9) and kept on ice for the duration of the experiment. Potato starch was used as the reaction substrate at a stock concentration of 0.5% w/v. The substrate was tested at a concentration range of 0.1 - 5 mg/ml to ensure that saturation of α-amylase activity was reached. The DNSA colour reagent was prepared at 80°C by mixing 5 M potassium tartrate (dissolved in 2 M sodium hydroxide) and 96 mM DNSA (dissolved in deionised (DI) water) at a ratio of 2:5. The colour reagent was diluted further with DI H₂O at a ratio of 1:0.7. This solution was stored in an amber container. Thirty microliters of extract (final concentration 20 µg/ml) and 60 µl of potato starch were added to experimental wells and the reaction was initiated by the addition of 30 µl of the α-amylase stock solution. The reaction took place for 5 min at 25°C and then 60 µl of DNSA colour reagent was added to each well. Termination of the reaction was induced by the semi-submersion of each plate into a 90°C water bath for 15 min. The plates were left to cool down and contents were transferred to a clear polystyrene 96-well plate. Acarbose (20 µg/ml) was used as the positive control and wells containing 120 µl of buffer with 60 µl of colour reagent served as blank.

Phytochemical interference was accounted for by the wells containing 90 µl buffer solution, 30 µl extract and 60 µl DNSA colour reagent. Wells containing 30 µl buffer, 30 µl α-amylase, 60 µl substrate and 60 µl colour reagent served as control. The plates were read at 540 nm (Biotech Instruments, ELX800UV). The results were expressed in terms of the type of inhibition exerted (using Lineweaver-Burk plots) and the percentage inhibition of α-amylase activity.

2.2.2 α-Glucosidase assay

The effect of each extract on the activity of α-glucosidase, was determined using a spectrophotometric, 96-well microplate method described by Collins et al. [21]. α-Glucosidase was sourced from *Bacillus stearothermophilus* (Sigma Aldrich, St. Louis, USA). A stock solution of α-glucosidase at 0.1 U/ml was prepared using a 0.01 M sodium phosphate buffer (pH 6.9) and kept on ice for the duration of each experiment. The substrate, *p*-nitrophenyl-α-*D*-glucopyranoside (pNPG), prepared in sodium phosphate buffer (0.01 M, pH 6.9) and glycine (pH 10) was used to terminate the reaction. Thirty microliters of plant extract at a reaction concentration of 20 µg/ml and 60 µl of p-NPG was pipetted into a well of a 96-well plate. The reaction was initiated by the addition of 30 µl of α-glucosidase and incubated for 5 min at 25°C. Acarbose was used as positive control at 20 µg/ml. Glycine (60 µl) was added and the plates were read at 405 nm (Biotech Instruments, ELX₈₀₀UV). Reactions containing 30 µl buffer, 30 µl α-glucosidase solution, 60 µl substrate and 60 µl glycine served as negative control. The reactions containing 30 µl plant extract, 90 µl buffer and glycine served as background control in order to eliminate any phytochemical interference. The mode of inhibition was determined using Lineweaver-Burk plots and the percentage inhibition against α-glucosidase activity was also determined.

2.3 Alleviation of Hyperglycaemia

2.3.1 C2C12 maintenance and differentiation

C2C12 mouse myoblasts were purchased from the American Tissue Culture Collection (ATCC CRL-1772). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM), which was supplemented with 1% penicillin/streptomycin and 10% foetal calf serum (FCS). Flasks were incubated in a 5% CO₂ incubator at 37°C.

C2C12 cells were differentiated into myotubes according to the method described by Burattini et al. [22]. The myoblasts were cultured until they

were 50% confluent, rinsed with 1 ml phosphate buffered saline (PBS) and medium replaced with 4 ml of DMEM+ containing 1% FCS and incubated for 7 days. The medium was changed daily until the cellular morphology resembled that of myotubes.

The differentiated C2C12 myotubes were harvested with 1 ml TrypLE™ Express in ethylenediaminetetraacetic acid (EDTA) for 3 min at 37°C, 5% CO₂. Ten milliliters of DMEM+ with 2% FCS was added to the dissociated cells and the suspension decanted into a 15 ml polypropylene tube. The homogenous cell suspension was centrifuged for 5 min at 200 g (Allegra X-22, Beckman Coulter centrifuge). Thereafter, the medium was replaced with 1 ml of DMEM+ and the cell pellet was carefully suspended into the medium. Cell density was determined using a haemocytometer.

2.3.2 2-NBDG assay

Glucose uptake into myotubes was determined using a fluorescent *D*-glucose analogue, 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-D-glucose (2-NBDG), following a method described by Zou et al. [23]. C2C12 myotubes (80 µl) were seeded into white plates at a density of 1.5×10^4 cells/well and incubated for 48 h at 37°C with 5% CO₂. The medium was changed after 24 h. Fifty microliters of glucose free DMEM+, containing 400 µM of 2-NBDG and 80 µl of plant extract (1 - 20 µg/ml) was added and incubated at 37°C in 5% CO₂ for 1 h. The medium was removed and 50 µl Hanks balanced salt solution (HBSS) was added to each well. Metformin and insulin were used separately as positive controls at 20 µg/ml in reaction. Wells with 50 µl HBSS and no cells served as blank, while pre-seeded wells treated with 2-NBDG and glucose free DMEM served as control. The plates were read fluorometrically at $\lambda_{ex} = 460$ nm and $\lambda_{em} = 544$ nm. The results were expressed in terms of relative fluorescence intensity (RFI).

2.4 Statistical Analysis and Data Representation

All experiments were conducted in technical and biological triplicate. Values are expressed as mean ± standard error of the mean (SEM). Statistical analysis between extracts was performed using a one-way ANOVA test with Dunnet's multiple comparison. Student's *t*-tests were used to determine the significance of

extracts compared to the negative and positive controls. Lineweaver-Burk plots were constructed using Michaelis-Menten non-linear regression analysis. The statistical packages used included GraphPad Prism 6.0 and Microsoft Excel 2010. Statistical significance was regarded at $p < 0.05$.

3. RESULTS AND DISCUSSION

Diabetes mellitus is characterized by chronic hyperglycaemia primarily as a result of defective insulin function [1-3]. The prevention and alleviation of hyperglycaemia by inhibiting the activity of important carbohydrases and stimulating glucose absorption into peripheral tissue are two main mechanisms by which conventional treatments produce therapeutic effects [3]. There are several conventional drugs that act as hypoglycaemic agents by stimulating insulin secretion, increasing insulin sensitivity of cells or acting as insulin mimetics [3]. However, all of these drugs are associated with unwanted side-effects and regimen requirements [3]. Several plant extracts have been shown previously to possess antidiabetic activities similar to these conventional drugs [14].

3.1 Prevention of Hyperglycaemia

Both the HW and DCM extracts of *Diabetea* were ineffective at inhibiting α -amylase and α -glucosidase (Tables 1 and 2). The DCME of *U. urens* was the only preparation to significantly ($p < 0.05$) inhibit the activity of α -amylase, by exerting an inhibitory activity of $52.8 \pm 12.1\%$ in an uncompetitive manner (Fig. 1). This result was similar to the inhibitory activity exerted by acarbose, $57.0 \pm 7.4\%$ (Table 1). Acarbose is a conventional drug that acts as a carbohydrase inhibitor. However, it is associated with unwanted side-effects such as severe abdominal cramping, flatulence and requires multiple daily dosing [3].

The type of inhibition exerted by *U. urens* indicates that it contains compounds that interact with the enzyme-substrate (ES) complex once formed, thereby decreasing V_{max} and increasing E-to-S affinity. This implies that this inhibitor may be more effective when administered after a meal. *U. urens* contains hydroxylated flavonoids, such as patuletin, that act as potent α -amylase inhibitors [24]. A 50% methanol-water extract of *U. urens* was shown not to inhibit α -amylase activity, but rather to be stimulatory [25], which was also observed in the present study with the

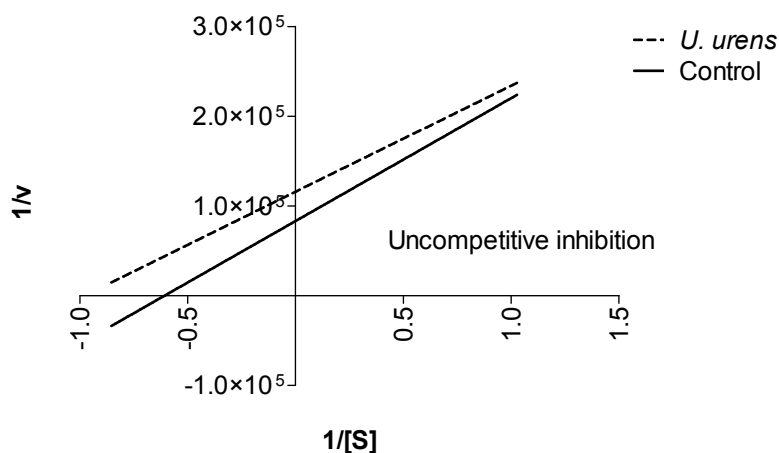


Fig. 1. Lineweaver-Burk plot of the reaction of the dichloromethane extract of *U. urens* against α -amylase activity

HWE of *U. urens* (Table 1). *U. urens* stimulated the activity of α -amylase by -31.9%, which is almost four times higher than what was observed by Hamdan et al. [25]. This discrepancy may be due to the difference in extract solvents used, where the solvent in the present study was inorganic and more polar than that used by Hamdan et al. [25]. This observation implies that a correlation exists between the hydrophilic nature of an extract and its stimulatory activity on α -amylase.

Table 1. The inhibitory effect of hot water (HW) and dichloromethane (DCM) extracts (n=9) on α -amylase activity

Herb	HW extract	DCM extract
<i>A. betulina</i>	-50.7±9.4 [#]	-1.1±10.3
<i>T. officinalis</i>	-29.7±10.7 [#]	10.6±5.8
<i>T. foenum-graecum</i>	-11.1±11.7	-5.2±7.9
<i>S. officinalis</i>	-27.6±8.0 [#]	10.3±7.3
<i>U. urens</i>	-31.9±7.0 [#]	52.8±12.1 [*]
<i>T. vulgaris</i>	-53.1±10.6 [#]	10.6±4.8
<i>A. millefolium</i>	-51.5±8.5 [#]	12.5±7.6
Diabetea	-31.8±9.5 [#]	0.8±10.7
Acarbose	57.0±7.4 [*]	57.0±7.4 [*]

Student's t-test, with $p < 0.05$; * = Significant inhibition; # = Significant stimulation

The HWE and DCME of *A. betulina* exerted the most efficacious inhibitory activity on α -glucosidase of all extracts tested (Table 2). The HWE of *A. betulina* inhibited α -glucosidase (42.6±5.5%) in a mixed manner (Fig. 2A), whereas the DCME showed a non-competitive inhibition (93.8±0.8%) (Fig. 3A). The inhibition exerted by the DCME of *A. betulina* was

comparable to the activity of acarbose, which inhibited the activity of α -glucosidase completely (100.0±0.6%) (Table 2). No literature regarding the activity of extracts of *A. betulina* against α -glucosidase could be obtained, making this the first to our knowledge to report these findings.

Table 2. The inhibitory effect of hot water (HW) and dichloromethane (DCM) extracts (n=9) on α -glucosidase activity

Herb	HW extract	DCM extract
<i>A. betulina</i>	42.6±5.5 [*]	93.8±0.8 [*]
<i>T. officinalis</i>	-11.9±6.6	10.9±2.3
<i>T. foenum-graecum</i>	-2.7±4.9	3.9±4.8
<i>S. officinalis</i>	0.3±6.2	52.7±4.7 [*]
<i>U. urens</i>	-12.5±6.0	10.4±3.5
<i>T. vulgaris</i>	24.5±4.1 [*]	46.1±5.1 [*]
<i>A. millefolium</i>	-2.0±6.1	-3.3±1.7
Diabetea	2.02±5.9	8.2±7.6
Acarbose	100.0±0.6 [*]	100.0±0.6 [*]

Student's t-test, with $p < 0.05$; * = Significant inhibition

The HWE and DCME of *T. vulgaris* significantly ($p < 0.05$) inhibited α -glucosidase activity (Table 2). The mode of inhibition exerted by both extracts were mixed (Figs. 2B and 3B), resulting in a decrease in reaction rate and E-to-S affinity. An essential oil extract of *T. vulgaris* was found to exert an inhibitory activity against both α -amylase and α -glucosidase [26]. A flavonoid isolated from *T. vulgaris* known as luteolin has been reported to inhibit α -glucosidase by 36% at 0.5 mg/ml [27], which is within the range of the inhibitory activity of *T. vulgaris* (HWE and DCME) observed in the present study (between 24 –

47% inhibition), although at a much lower concentration (20 µg/ml).

The DCME of *S. officinalis* significantly ($p < 0.05$) inhibited α -glucosidase in a mixed manner (Fig. 3C), which was similar to what was observed for the DCME of *U. urens*. In previous studies the HW and MeOH extracts of *S. officinalis* were reported to inhibit rat intestinal α -glucosidase activity by ~30 and 18%, respectively [28]. This is similar to the inhibitory activity of the HWE of *S. officinalis* observed in the present study using α -glucosidase from *Bacillus stearothermophilus* (Table 2). The different degrees by which α -glucosidase was inhibited could possibly be accounted for by the different sources of α -glucosidase used. Cazzola et al. [28] reported that a HWE and MeOH extract of *S. officinalis* inhibited 25 and 15% of the activity of porcine α -amylase activity, respectively [28]. This result by Cazzola et al. using the HWE is contrary to that observed in the present study (HWE -27%), where a significant stimulatory activity on α -amylase was seen (Table 1). The reason for this discrepancy could be due to the difference in duration of extraction time.

All hydrophilic plant extracts had a stimulatory effect on the activity of α -amylase (Table 1). This observation suggests that porcine α -amylase has a structural affinity to hydrophilic ligands/substrates. It is known that porcine α -amylase has one active site with five subunits for the binding of ligands, and its active site contains amino acids in close proximity with a network of H₂O molecules [29]. These amino acid residues in the active site have polar side chains that interact via hydrogen-bonding. The interaction of a ligand with the active site of α -amylase

involves a general acid-base reaction with acid hydrolyzing/nucleophilic substitution activity [29], indicating that the catalytic reaction mediated by α -amylase occurs with the addition of water molecules to the substrate. This may be the reason why the HWE (being more hydrophilic) caused a significant increase in the rate of substrate hydrolysis. It has also been shown that lignin activates the activity of α -amylase, and has an even greater activating activity on α -amylase than chloride [30]. Lignin is a type of dietary fiber abundant in plant material that is immune to enzyme digestion [30]. This may also be a reason for the enhanced activity of α -amylase observed.

Furthermore, the DCM herb extracts generally exerted a greater inhibitory activity against α -glucosidase than that of the HWE. Li et al. [31] suggested that the interaction between the active site on α -glucosidase and inhibitors are mainly hydrophobic. This could be the reason why DCM extracts were more active against α -glucosidase than α -amylase in the present study.

The DCME of Diabetea caused a significant ($p < 0.05$) dose-dependent increase in the rate of 2-NBDG absorption into C2C12 myotubes (Fig. 4). Myotubes are insulin-dependent cells, which absorb glucose by means of GLUT that are activated by insulin [2]. The absorption of glucose via insulin and GLUT takes place by the binding of insulin to an insulin receptor on the membrane of an insulin-dependent cell, causing the activation of protein cascades, which initiates the translocation of GLUT-4 transporter to the plasma membrane, with the subsequent influx of glucose [2]. In the case of insulin inactivity,

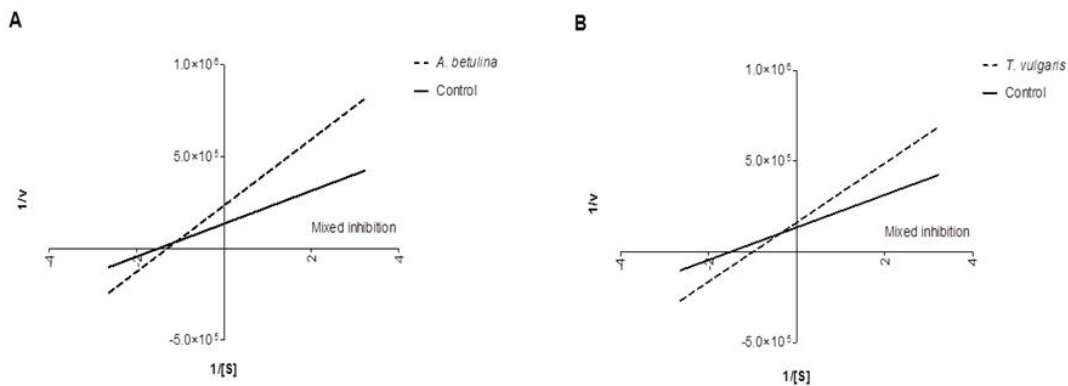


Fig. 2. Lineweaver-Burk plots of the hot water extracts of (A) *A. betulina* and (B) *T. vulgaris* showing a mixed inhibitory effect against the activity of α -glucosidase

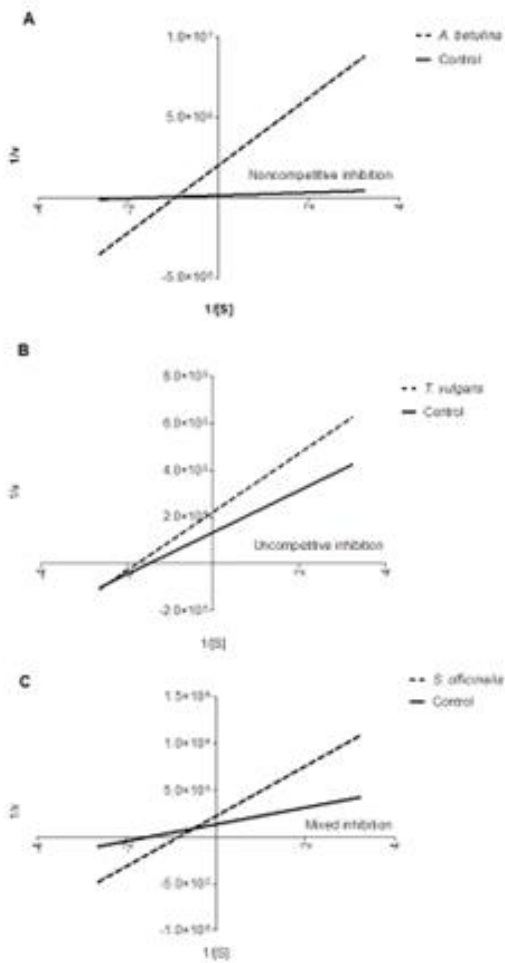


Fig. 3. Lineweaver-Burk plots of the dichloromethane extracts of (A) *A. betulina*, (B) *T. vulgaris* and (C) *S. officinalis* showing a non-competitive, un-competitive and mixed inhibitory effect against the activity of α-glucosidase, respectively

3.2 Potential to Alleviate Hyperglycaemia

resistance or insufficiency, glucose will remain in the circulatory system, leading to chronic hyperglycaemia. This makes insulin the primary glucoregulatory hormone in carbohydrate metabolism [2]. The significant ($p < 0.05$) dose-dependent increased uptake of 2-NBDG into C2C12 myotubes (Fig. 4), as exerted by the DCME of *Diabetea*, suggests that it possesses phytochemical constituents that activate the GLUT4 cascade by mimicking the action of insulin. However, the possibility of alternative molecular mechanisms by which glucose is

transported into C2C12 myotubes, other than as mediated by insulin, are not disregarded here. The HWE of *Diabetea* did not possess any potent *in vitro* antidiabetic activity (Tables 1 and 2, Fig. 5), which is contrary to what was expected since it is traditionally used as an antidiabetic treatment in the form of a hot water infusion.

Both the DCME and HWE of *U. urens* significantly ($p < 0.05$) increased the amount of glucose absorbed into the myotubes (Figs. 4 and 5). However, this was not applicable to all concentrations tested as there was a concentration-dependent decrease in its glucose uptake activity, which may indicate an inhibitory effect, whereby compounds either form complexes with 2-NBDG, interfere with glucose receptors/insulin receptors or cause a rapid degradation of 2-NBDG. No other literature could be found for the glucose uptake activity mediated by *U. urens* making this the first report thereof. This finding implies that in order to maintain the desired antidiabetic effect of *U. urens*, caution should be exercised with its dosing.

The HWE of *A. millefolium* significantly ($p < 0.05$) inhibited the normal cellular glucose uptake (Fig. 5), which may also be explained by any one of the mechanisms proposed above. Irrespective of this result, the DCME of *A. millefolium* significantly ($p < 0.05$) increased glucose uptake into myotubes (Fig. 4). *A. millefolium* has been reported to exert a hypoglycaemic effect, both *in vitro* and *in vivo* [32,33]. A commercial drug combination known as Liv.52, includes *A. millefolium* as one of its main ingredients, which acts as an insulin mimetic for glucose uptake into steatotic HepG2 cells [32]. Both aqueous and methanol extracts of *A. millefolium* have been shown to contain hypoglycaemic activity in rats, ascribed to possible insulin secretory effects or a direct insulin-type of action [33]. Current results indicate that *A. millefolium* has an insulin mimicking effect, rather than an insulin secretory or synergistic effect. This observation strengthens the antidiabetic evidence that already exists for *A. millefolium*.

The HW extract of *A. betulina* significantly ($p < 0.05$) increased the amount of glucose absorbed into the myotubes at 20 µg/ml (Fig. 5) with no concentration-dependent relationship or stimulation observed. Bioactive flavonoids, such as diosmin and hesperidin, isolated from the essential oil of *A. betulina*, have been shown to exert antidiabetic properties [34]. A micronised purified flavonoid fraction called Daflon 500 (90%

diosmin and 10% hesperidin), has been tested in a long term study for its antidiabetic activities and was found to possess hypoglycaemic, antiglycating and antioxidant activities in type 1 diabetic patients [35-37]. A study performed on streptozotocin nicotinamide-induced diabetic rats using diosmin showed that it caused an increase in insulin secretion from pancreatic β -cells [35]. The results of the present study support an

insulin mimetic activity exerted by the extract, rather than that of the stimulation of insulin secretion.

The HW extract of *T. foenum-graecum* exhibited potent ($p < 0.05$) *in vitro* antidiabetic activity at 10 and 20 $\mu\text{g/ml}$ (Fig. 5). Compounds such as nicotinic acid, nicotinamide and coumarin

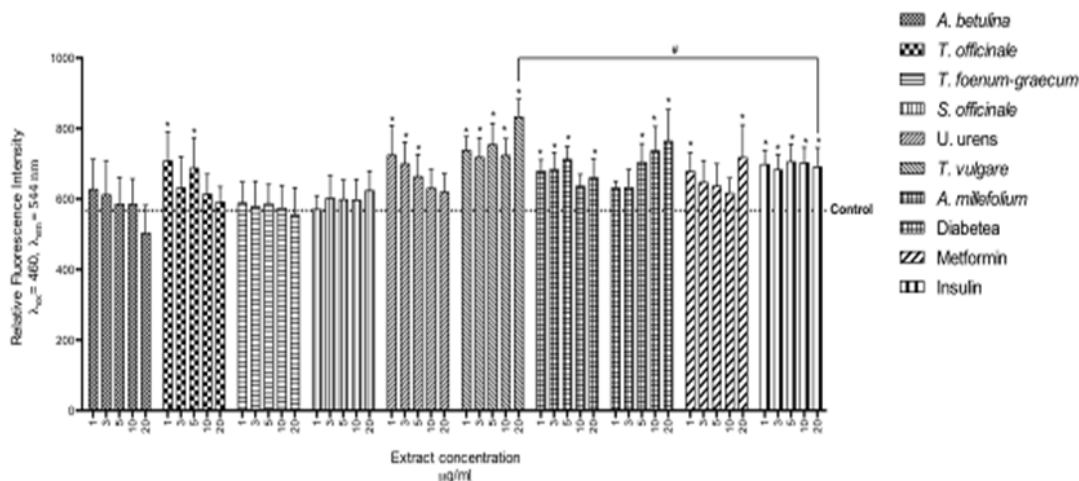


Fig. 4. The ability of dichloromethane herb extracts to mediate glucose uptake into C2C12 myotubes. Significance ($p < 0.05$) was determined against control (*) and positive controls (#) using the student's *t*-test. Metformin and insulin served as positive controls

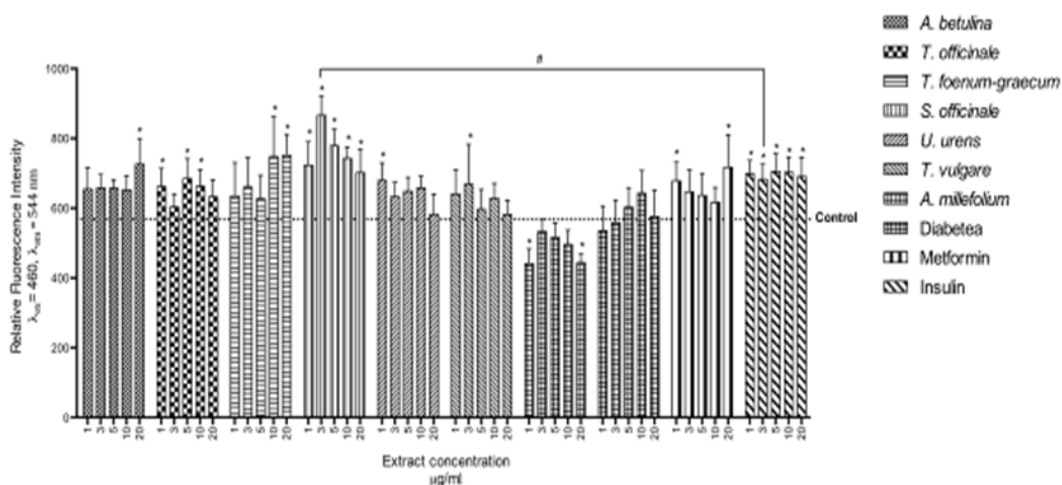


Fig. 5. The ability of hot water herb extracts ($n=9$) to mediate glucose uptake into C2C12 myotubes. Significance ($p < 0.05$) was determined against control (*) and positive controls (#) using the student's *t*-test. Metformin and insulin served as positive controls

have been isolated from *T. foenum-graecum* seeds and have indicated hypoglycaemic activity in alloxan-diabetic rats [38]. The aqueous extract of *T. foenum-graecum* leaves has also been reported to decrease blood glucose in alloxan-diabetic rats at a dose of 0.2 g/kg after 1 h [39]. The exact mechanism of action has not yet been established but may be explained by GLUT4 transporter cascade activation.

The glucose uptake activity induced by the DCME and HWE of *T. vulgaris* was significant ($p < 0.05$) (Figs. 4 and 5). These results are supported by a study in which DCM extracts of aerial parts of *T. vulgaris* were found to stimulate glucose uptake into 3T3-L1 adipocytes [40]. In addition, the DCM extract of *T. vulgaris* was considerably ($p < 0.05$) more active than insulin at 20 $\mu\text{g/ml}$ (Fig. 4) in the present study.

Treatment of C2C12 myotubes with HWE of *S. officinalis* resulted in glucose uptake at all concentrations tested, which was more efficacious than the activity of insulin at 2.5 $\mu\text{g/ml}$ (Fig. 5). Previously, a 15% ethanol-water extract was shown to have a hypoglycaemic effect in normal and mild alloxan-diabetic mice in the presence of insulin [41]. The present results on the HWE of *S. officinalis* showed that it acted as a hypoglycaemic preparation in the absence of insulin, indicating a mediated glucose transport across the cell membrane either by means of GLUT4 cascade activation or through other unknown mechanism(s). However, Cristovao et al. [42] found no hypoglycaemic activity in diabetic rats after the administration of *S. officinalis* HWE *ad libitum* for 14 days. This indicates an incongruity between the *in vitro* and *in vivo* hypoglycaemic activities of particular extracts of *S. officinalis*. This incongruity was also observed with *T. officinalis*, whereby its HWE and DCME were found to significantly ($p < 0.05$) increase the amount of glucose absorbed into the myotubes (Figs. 4 and 5). The HWE and alcoholic extract of *T. officinalis* was reported to have no hypoglycaemic activity in male Swiss mice [43], which was also observed by Swanston-Flatt et al. [44]. Due to the lack of data, the only means by which this discrepancy could be verified is by further investigation.

4. CONCLUSION

The results of this study indicate that the DCM extract of Diabetea, a commercial antidiabetic tea mixture, possesses significant *in vitro* hypoglycaemic activity by acting as possible

GLUT4 activator, mimicking the action of insulin. Contrary to that, the HWE of the Diabetea did not show any antidiabetic activity, which may dispute its traditional use as a hot water tea infusion. Most of the monoherb extracts were more active than the polyherbal Diabetea, which implicates that the mixture is not more active than its individual ingredients, contesting the presence of any positive synergistic effect. The most significant result for the prevention of hyperglycaemia was observed for the DCME of *A. betulina*, which exerted a potent non-competitive mode of inhibition of α -glucosidase. This result was similar to that of a known and effective α -glucosidase inhibitor, acarbose. The conventional use of *A. betulina*, *T. officinalis*, *T. foenum-graecum*, *S. officinalis*, *U. urens* and *T. vulgaris* as hypoglycaemic hot water infusions are supported in the present study in terms of their significant *in vitro* insulin mimetic potential. The organic preparations of *T. officinalis*, *U. urens* and *A. millefolium* resulted in an increase in glucose uptake into C2C12 myotubes, with the extract of *T. vulgaris* being significantly ($p < 0.05$) more active than insulin. The extracts that exerted a significant antidiabetic activity by both preventing and alleviating hyperglycaemia were the DCME of *U. urens*, HWE of *A. betulina*, and both the HWE and DCME of *T. vulgaris*. It can be concluded that these extracts are potent *in vitro* antidiabetic preparations exerting hypoglycaemic activity.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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