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EVALUATION OF ANTIDIABETIC EFFECT OF FOUR EGYPTIAN PLANTS AND THEIR PHYTOCONSTITUENTS

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ABSTRACT

Postprandial hyperglycaemia is a major contributory factor in the development of diabetes which can be clinically targeted via a number of independent mechanisms such as the inhibition of postprandial glucose absorption and through potentiating glucose dependent insulin release. This study was carried out to evaluate antidiabetic effect of four Egyptian medicinal plants, *Sonchus oleraceus, Lagerstroemia tomentosa, Diospyros virginiana,* and *Toona ciliate*, and to investigate the phytoconstituents from the bio-active plant extracts. The results revealed that *L. tomentosa* leaves methanol 80% extract was the most effective inhibitor of carbohydrate digestion which showed strong inhibition against both alpha-amylase and alpha-glucosidase. The relevance to mammalian alpha-glucosidase was confirmed using a rat intestinal maltase assay however the inhibitory activity was considerably weaker relative to the yeast enzyme. None of the extracts displayed any significant inhibition against DPP-iv, an additional target to regulate postprandial hyperglycaemia. Cytotoxicity screening against Chang liver cells did not reveal any significant toxicity suggesting a favourable safety profile. Phytochemical studies of methanol 80% extract of *L. tomentosa* leaves produced lupeol (1), corosolic acid (2), gallic acid (3), ellagic acid (4), apigenin (5), vitexin (6), apigenin 7-O-β-glucoside (7), and kaempferol 3-*O*-β-galactoside (8). These results gave scientific evidence that *L. tomentosa* leaves may provide anti-diabetic effect through the inhibition of postprandial glucose absorption.

INTRODUCTION

Diabetes is a chronic metabolic disorder characterised by persistent hyperglycaemia that results from defects in both insulin action and insulin secretion. Due to changes in lifestyle and diet there has been a dramatic increase in the number of diabetic patients worldwide. Consumption of high-carbohydrate diets causes elevated postprandial hyperglycaemia, a recognised

underlying factor in the development of type-II diabetes. Accordingly the control of postprandial hyperglycaemia represents an important therapeutic target in the treatment and prevention of diabetes and the associated complications like hypertension, dyslipidemia, obesity, and cardiovascular diseases. Currently there are a number of recognized therapeutic approaches for decreasing postprandial hyperglycemia and the concomitant negative effects of elevated blood glucose. Retarding intestinal glucose digestion and uptake is clinically recognized as an effective strategy to limit postprandial glucose elevation. Anti-diabetic drugs such as acarbose and voglibose target intestinal carbohydrate digestion to inhibit the release and subsequent uptake of glucose following a carbohydrate rich meal. Alpha-amylase and alpha-glucosidase function to break down complex dietary carbohydrates into forms that are suitable for the intestinal absorption. Alpha-amylase catalyses the hydrolysis of α-1, 4-glucosidic linkages of starch, glycogen, and various oligosaccharides, while alpha-glucosidase further breaks down the disaccharides into simple sugars. Therefore, inhibition of its activity in the digestive tract is an effective mechanism to control postprandial hyperglycaemia and considered a feasible therapeutic target in the treatment of diabetes [1]. Rapid-onset insulin secretagogues, such as nateglinide, are also effective in reducing postprandial hyperglycaemia. Likewise, the incretin hormones glucagon like peptide-1 (GLP-1) and glucagon inhibitory peptide (GIP), which play a major role in regulating postprandial insulin secretion, function to counteract elevated glucose levels following a meal. Consequently incretin hormone action may provide an additional target for the therapeutic control of postprandial hyperglycaemia. GLP-1 is secreted from cells in the gastrointestinal tract into the circulation in response to nutrient absorption. Incretins do not lead to insulin release perse, but potentiate the physiological release of insulin from the pancreas in response to increases in plasma glucose. Circulatory levels of active GLP-1 and GIP are controlled by the enzyme dipeptidylpeptidase-iv (DPP-iv) which rapidly inactivates these hormones. Therefore, inhibiting DPP-IV prolongs the action of GLP-1 and GIP, which in turn, improves glucose homeostasis and postprandial hyperglycaemia [2]. Medicinal plants remain a valuable resource both in terms of potentially novel compounds for the development of effective anti-diabetic drugs and as an alternative or complementary therapy to treat this disease [3]. Unfortunately there is no single experimental model, whether *in vitro* or *in vivo*, which can be applied to accurately identify anti-diabetic medicinal plants suitable for therapeutic intervention. The complex heterogeneous nature of type-II diabetes possess a significant challenge in identifying the anti-diabetic activity of composite medicinal plant extracts as it is not possible to accurately select appropriate animal models and experimental designs without some knowledge of the mechanism through which these medicinal plants elicit a therapeutic effect. Target directed *in vitro* screening provides a simple strategy to prioritise such plant extracts for potential efficacy against a specific therapeutic mechanism and consequently allow the selection of appropriate animal models and experimental designs to confirm *in vivo* potential. *Lagerstroemia tomentosa* is a medicinal and ornamental, handsome decidous, small tree native of China. The bark of the plant is considered stimulant and febrifuge, leaves and flowers are used as purgative [4], the roots are astringent [5]. Only few reports about biological activities of the plant, aerial parts showed antioxidant and antimicrobial activities [6]. In the present study we have used target directed *in vitro* screening as a tool to characterize four anti-diabetic Egyptian medicinal plants with respect to their potential to inhibit postprandial hyperglycaemia and to investigate the bioactive constituents from the bioactive extract.

These findings are useful to prioritise the selected anti-diabetic plants based on specific molecular mechanism(s) and allow the choice of an appropriate animal model and experimental design to accurately investigate the *in vivo* anti-diabetic efficacy.

Materials and Methods

UV/VIS: Shimadzu UV-visible recording spectrophotometer model-UV 240 (NRC, Egypt). ^{1}H-NMR and ^{13}C-NMR (Varian Unity Inova). MS (Finnigan MAT SSQ 7000, 70 ev). (Silica gel (0.063-0.200 mm for column chromatography) and Sephadex LH-20 (Pharmacia Fine Chemicals). Thin layer chromatography (TLC) F_{254} plates. Solvent mixtures, BAW (*n*-butanol:acetic acid:water 4:1:5 upper phase, 15% acetic acid: water: glacial acetic acid: 85:15). Paper Chromatography (PC) Whatman No.1 (Whatman Led.Maid Stone, Kent, England) sheets for qualitative detection of flavonoids and sugars.

Material of the plants

Leaves of *Lagerstroemia tomentosa* were collected from Al-Zohiriya garden, Giza, Egypt in May 2012, and leaves of *Sonchus oleraceus*, *Diospyros virginiana* and *Toona ciliate* were collected from Agricultural Research Centre, Giza, Egypt. All the plants were identified by Dr. Mohammed El-Gebaly, Department of Botany, National Research Centre (NRC) and by Mrs. Tereeza Labib consultant of plant taxonomy at the Ministry of Agriculture and director of Orman botanical garden, Giza, Egypt.

Preparation of the extracts

Air-dried powdered leaves of *Lagerstroemia tomentosa* (760 g), *Sonchus oleraceus* (540 g), *Diospyros virginiana* (490 g), and *Toona ciliate* (370 g) were extracted with methanol 80% at room temperature until exhaustion by maceration method. Each extract was concentrated under reduced pressure to give 32.5 g, 25.5, 26.5 and 19.5 of the crude extract, respectively. All the extracts were subjected to different phytochemical tests that described by Yadav and Agarwala [7].

Isolation of bio-active compounds from methanol 80% extract *Lagerstroemia tomentosa* leaves

Methanol extract of *L. tomentosa* (30 g) was fractionated on silica gel column chromatography eluting with n-hexane, dichloromethane, ethyl acetate and methanol gradually. The fractions that showed similar Paper Chromatography (PC) in butanol–acetic acid–water 4:1:5 (BAW) and 15% acetic acid were collected to give 4 main fractions (I, II, III, and IV).

Fraction I (3.85 g) was subjected to sub–column of silica gel eluted with dichloromethane and ethyl acetate. At elution with n-hexane: dichloromethane: (60:40) yielded compound 1 and further elution with dichloromethane: ethyl acetate (80:20) gave compound 2. Fraction II (6.35 g) was subjected to sub–column of silica gel eluted with dichloromethane and ethyl acetate. At elution of dichloromethane: ethyl acetate (85:15) yielded compound 3 and further elution with ethyl acetate (90:10) gave compound 4. Fraction III (5.75 g) was subjected to sub–column of silica gel eluted with dichloromethane and methanol. At elution of dichloromethane: methanol (98:2) yielded compound 5 and elution of dichloromethane: methanol (92:8) yielded compound 6. Fraction IV (6.25 g) was subjected to sub–column of silica gel eluted with dichloromethane and methanol. At elution of dichloromethane: methanol (85:15) yielded compound 7 and elution of dichloromethane: methanol (80:20) yielded compound 8. All the isolated compounds were purified on sephadex LH–20 column using different systems of methanol and mixture of methanol and distilled water.

General method for acid hydrolysis of flavonoid glycosides

Five milligrams of each flavonoid glycoside 6, 7, and 8 in 5 ml 10% HCl was heated for 5h. The aglycones were extracted with ethyl acetate and identified by co-TLC with authentic standards. The sugars in the aqueous layer were identified by co-paper chromatography (co-PC) with authentic markers on Whatman No. 1 sheets in solvent system (*n*-BuOH-AcOH-H_2O 4:1:5 upper layer).

Antidiabetic assay

Inhibition of pancreatic alpha-amylase

Alpha-amylase inhibitory activity was determined using the method described by Ali

et al., [8], with minor modification. Equal volumes of test extract and porcine pancreatin were pre-incubated for 5min at 37°C. The reaction was started by adding 100µl of potato starch (1%) dissolved in 20mM phosphate buffer (pH6.9) containing 6.7mM sodium chloride. The reaction mixture was incubated for a further 20 min at 37° and the reaction terminated by adding 100µl 3, 5-dintrosalicylic acid (96mM). The reducing groups liberated from starch hydrolysis by amylase reduce 3, 5-dinitrosalicylic acid resulting in formation of a coloured product which was measured spectrophotometrically at 540 nm. Acarbose, was used as a positive control.

Inhibition of yeast alpha-glucosidase

Alpha-glucosidase inhibition was measured using ρ-nitrophenyl–α-D-glucoside as substrate in a 96-well plate format. The assay mixture contained 60mM potassium phosphate buffer (pH 6.8), 0.5mM glutathione, and 10µg/ml yeast enzyme. The reaction was initiated by adding 10mM ρ-nitrophenyl–α-D-glucoside and incubated at 37°C for 20min. followed by the addition of 100µl sodium carbonate solution (100mM). The absorbance was read at 400nm. Acarbose, was used as a positive control.

Inhibition of intestinal alpha-glucosidase

Alpha-glucosidase solution was prepared from rat intestinal acetone powder. Exactly 100 mg of acetone powder was suspended in 3ml of 0.01M phosphate buffer (pH 7.0) and sonicated twelve times for 30s each in an ice bath followed by centrifugation at 3000 rpm, 4°C for 20 min [9]. The supernatant containing the enzyme was kept on ice prior to assay. The enzyme inhibition activity of alpha-glucosidase was evaluated using sucrose and maltose as substrates as previously described by Takahashi, et al., [10]. Both sucrase and maltase activity were determined in a mixture of 500mM sucrose or maltose (10µl), the test sample (5µl), and 0.1M maleate buffer (pH 6.0, 75µl). The mixture was pre-incubated at 37°C for 5min, and reaction was initiated by adding rat intestinal α-glucosidase (10µl). The mixture was incubated at 37°C for 60 minutes. The glucose released in the solution was determined using a standard glucose oxidase/peroxidase method.

Inhibition of DPPiv activity

Inhibitory activity was determined using the chromogenic substrate Gly-Pro-pNA and recombinant human DPP-IV. The assay mixture contained 50mM Tris/HCl buffer (pH 8.0), 0.8mU/µl DPP-IV and plant extracts at a final concentration of 50µg/ml. The reaction was initiated by adding 10mM Gly-Pro-pNA substrate and the absorbance (405 nm) read after incubating at 37°C for 20min. PMSF (phenylmethylsulfonyl fluoride), a known serine protease inhibitor was used as a positive control.

Cytotoxicity in Chang liver cells

Dried extracts were reconstituted in DMSO to a concentration of 100µg/µl and then diluted with complete medium to the concentrations indicated. The final DMSO content in the assay is maintained below 1%, a concentration which has minimal effect on cell viability. Chang liver cells were seeded into 96-well culture plates at 10 000 cells/well in EMEM supplemented with10% fetal bovine serum (FBS) and left for 24 hours. Plant extracts were added and the cells incubated for a further 48 hrs after which the medium was replaced with 200µl MTT (Sigma) (0.5mg/ml in EMEM). After further 2hr incubation at 37°C, the MTT was removed and the purple formazan product dissolved in 200µl DMSO. The absorbance was measured at 560nm using a multiwell scanning spectrophotometer (Multiscan MS, Labsystems). All incubation steps were carried out in a 37°C humidified incubator with 5% CO_2. EC_{50} values were calculated from a minimum 5-point dose response curve using GraphPad Prism 4 software package.

Results and discussion

A marked hyperglycaemia is observed by following the ingestion of carbohydrate-rich foods, however under normal conditions subsequent hyperinsulinemia allows homeostatic glucose levels to return. Extended postprandial hyperglycaemia is one of the earliest metabolic defects to occur in type-II

diabetes and is considered to play a major role in the development of this disease and its associated complications. Therefore, mechanisms which control both intestinal glucose uptake as well as the natural hyperinsulinemic response, provide therapeutic opportunities to restore defective postprandial hyperglycaemia. In the present study four Egyptian medicinal plant extracts were tested for their potential to control postprandial hyperglycaemia through the inhibition of carbohydrate digestion and potentiate incretin hormone action and the bio-active extract chemical compounds were investigated.

Phytochemical composition of the different plants extracts.

Phytochemical analysis of *D. virginiana* leaves extract has shown that it contained phyto-constituents as triterpenes, carbohydrates, flavonoids, and tannins, *L. tomentosa* leaves extract has triterpenes, carbohydrates, flavonoids, alkaloids, tannins, and coumarins while *S. oleraceus* leaves extract contained triterpenes, carbohydrates, flavonoids, and saponins, and *T. ciliata* leaves extract had triterpenes, carbohydrates, and flavonoids (Table 1).

Table 1: Phytochemical analysis of the selected plants methanol 80% extracts

Phytoconstituents	*D. virginiana*	*L. tomentosa*	*S. oleraceus*	*T. ciliata*
Triterpenes and /or Sterols	+	+	+	+
Carbohydrates and/or glycosides	+	+	+	+
Flavonoids	+	+	+	+
Coumarins	-	+	-	-
Alkaloids and/or nitrogenous compounds	-	+	-	-
Tannins	+	+	-	-
Saponins	-	-	+	-
(+) presence of the constituents, (-) absence of the constituents				

Phytochemical characterization of *L. tomentosa* leaves methanol 80% extract

Chromatographic separation of *L. tomentosa* leaves extract resulted in the identification of lupeol (1), corosolic acid (2), gallic acid (3), ellagic acid (4), apigenin (5), vitexin (6), apigenin 7-*O*-*β*-glucoside (7) and kaempferol 3-*O*-*β*-galactoside (8) as shown in Fig.1. The structural identification of these components was elucidated by different spectroscopic analyses.

Structure elucidation of the isolated compounds

Lupeol (1): 18 mg, white powder, ^{1}H-NMR ($CDCl_3$, 400 MHz): δ ppm 0.75, 0.8, 0.85, 0.96, 0.98, 1.08, 1.75 (each 3H, s), 3.25 (1H, dd, J = 5.6, 10.8 Hz, H-3), 4.58 (1H, s, H-29a), 4.68 (1H, s, H-29b). ^{13}C-NMR ($CDCl_3$, 100 MHz): δ ppm 151.48 (C-20), 108.72 (C-29), 78.64 (C-3), 55.86 (C-5), 50.78 (C-9), 48.74 (C-18), 48.48 (C-19), 43.24 (C-17), 43.28 (C-14), 40.82 (C-8), 39.72 (C-22), 38.78 (C-4), 38.54 (C-1), 38.75 (C-13), 37.68 (C- 10), 35.74 (C-16), 34.54 (C-7), 29.46 (C-21), 28.46 (C-23), 27.64 (C-2), 27.62 (C-15), 25.48 (C-12), 21.46 (C-11), 19.48 (C-30), 18.75 (C-6), 18.46 (C-28), 16.54 (C-25), 16.26 (C-26), 15.75 (C-24), 15.25 (C-27).

Corosolic acid (2): 19 mg, White amorphous powder. ^{1}H-NMR (Pyridine-d_5, 400 MHz): δ ppm 4.07 (1H, ddd, J = 3.8, 9.4, 11.0 Hz, H-2), 3.41 (1H, d, J = 9.5 Hz, H-3), 5.42 (1H, s, H-12), 2.61 (1H, d, J = 10.9 Hz, H-18), 1.36 (3H, s, H-23), 1.12 (3H, s, H-24), 1.09 (3H, s, H-25), 1.04 (3H, s, H-26), 1.21 (3H, s, H-27), 1.03 (3H, d, J =6 Hz, H-29), 0.96 (3H, d, J = 6.0 Hz, H-30). ^{13}C-NMR (Pyridine-d_5, 100 MHz) δ ppm □48.58 (C-1), 69.14 (C-2), 84.35 (C-3), 40.39 (C-4), 56.46 (C-5), 19.35 (C-6), 34.94 (C-7), 41.28 (C-8), 49.52 (C-9), 39.26 (C-10), 23.75 (C-11), 126.28 (C-12), 139.85 (C-13), 43.82 (C-14), 29.54 (C-15), 25.96 (C-16), 48.65 (C-17), 54.28 (C-18), 40.22 (C-19), 40.45 (C-20), 31.58 (C-21), 38.44 (C-22), 29.78 (C-23), 19.15 (C-24), 18.68 (C-25), 17.95 (C-26), 25.24 (C-27), 181.26 (C-28), 18.24 (C-29), 21.95 (C-30). EI-MS: m/z 472.

Gallic acid (3): 22 mg, white amorphous powder. ^{1}H-NMR (DMSO-d_6, 400 MHz): δ ppm 7.12 (2H, s, H-2, 6). ^{13}C-NMR (DMSO-d_6, 100 MHz): δ ppm 166.95 (-COOH), 145.48 (C-3, 5), 137.83 (C-4), 121.42 (C-1), 109.62 (C-2, 6).

Ellagic acid (4): 15 mg, white amorphous powder. ^{1}H-NMR (DMSO-d_6, 400 MHz): δ ppm 7.35 (2H, s, H-4, 9). ^{13}C-NMR (DMSO-d_6, 100 MHz): δ ppm 158.26 (5, 10-CO), 147.84 (C-3, 8), 139.75 (C-2, 7), 136.72 (C-1a, 6a), 112.54 (C-4b, 9b), 110.72 (C-4, 9), 107.35 (4a, 9a).

Apigenin (5): 9 mg, yellow powder. ^{1}H-NMR (DMSO-d_6, 400 MHz) : δ ppm 12.82 (s, 1H, 5-OH), 7.64 (d, J = 8 Hz, 2H, H-2´, 6´), 6.86 (d, J = 8 Hz, 2H, H-3´, 5´), 6.15 (s, 1H, H-3), 5.83 (d, J = 2 Hz, 1H, H-8), 5.42 (d, J = 2 Hz, 1H, H-6). (-) ESI-MS: m/z 269 $[M-H]^-$.

Vitexin (6): 20 mg, yellow amorphous powder. UV λmax (MeOH): 269, 331; (NaOMe): 279, 325 (sh), 391; ($AlCl_3$): 276, 303sh, 346, 382; ($AlCl_3$/HCl): 277; 303, 343, 380 (NaOAc): 278, 387 (NaOAc/H_3BO_3): 270, 319, 346. ^{1}H-NMR (DMSO-d_6, 400 MHz): δ ppm 8.04 (d, J = 8.5 Hz, 2H, H-2´, 6´), 6.88 (d, J = 8.5 Hz, 2H, H-3´, 5´), 6.42 (s, 1H, H-3), 6.74 (s, 1H, H-6), 4.65 (d, J = 9.6 Hz, 1H, H-1˝). (+) ESI-MS m/z: 433 $[M+H]^+$

Apigenin 7-*O*-*β*-glucoside (7): 16 mg, yellow powder. UV λmax (MeOH): 269, 335; (NaOMe): 276, 387; ($AlCl_3$): 282, 345, 360; ($AlCl_3$/HCl):282, 342, (NaOAc): 268, 392 (NaOAc/H_3BO_3): 266, 352 nm. ^{1}H-NMR (DMSO-d_6, 400 MHz): δ 7.94 (2H, d, J = 8.9 Hz, H-2ˊ, 6ˊ). 6.96 (2H, d, J = 8.9 Hz, H-3ˊ, 5ˊ), 6.84 (1H, s, H-3), 6.68 (1H, d, J = 2.2 Hz, H-8), 6.52 (1H, d, J = 2.2 Hz, H-6). Sugar moiety: 5.24 (1H, d, J = 7.2 Hz, H-1˝), 3.2- 4 (ramaining sugar protons).

Kaempferol 3-*O*-*β*-galactoside (8): 19 mg, pale yellow needles. UV λmax (MeOH): 266, 354; (NaOMe): 277, 329, 406; ($AlCl_3$): 276, 302 sh, 350, 398; ($AlCl_3$/HCl): 276, 303 sh, 348, 398 (NaOAc): 276, 304, 375, (NaOAc/H_3BO_3): 268, 354 nm. ^{1}H-NMR (DMSO-d_6, 400 MHz): δ ppm 12.58 (1H, s, 5-OH), 8.06 (2H, d, J =9 Hz, H-2´, 6´), 6.86 (2H, d, J =9 Hz, H-3´, 5´), 6.46 (1H, d, J =2.2 Hz, H-8), 6.24 (1H, d, J = 2.2 Hz, H-6), Sugar moiety: 5.36 (1H, d, J = 7.9 Hz, H-1´´) , 3.1- 4 (ramaining sugar protons). ^{13}C-NMR (DMSO-d_6, 100 MHz): δ ppm 177.75 (C-4), 164.58 (C-7), 161.24 (C-5), 160.28 (C-4´), 156.76 (C-2, C-9), 133.23 (C-3), 130.94 (C-2´, 6´), 121.12 (C-1´), 115.25 (C-3´,5´), 104.14 (C-10), 101.92 (C-1´´), 99.35 (C-6), 93.82 (C-8), 75.68 (C-5´´), 73.75 (C-3´´), 71.28 (C-2´´), 67.82 (C-4´´), 60.54 (C-6´´).

Identification of the isolated compounds of *L. tomentosa* leaves methanol extract

Compound 1 yielded a dark spot under short UV light and changed to pink to violet upon spraying with vanillin-sulphuric acid and heating in an oven at 110°C for 5 min. NMR spectral data showed signals very similar to lupeol [11]. Compound 2 was monitored by TLC, and the spot of the compound was detected by heating the plates at 110°C after spraying with p-anisaldehyde–sulfuric acid, the spectral data were in agreement with published data of corosolic acid [12]. Compound 3 was detected on paper chromatography as violet spot under short ultraviolet (UV) and its chemical structure was established by comparison of its spectral data with those reported in the literature for gallic acid [13]. Compound 4 was detected on paper chromatography (PC) as a shine spot under short ultraviolet (UV) and its spectral signals were very close to ellagic acid [14]. Compound 5 was detected as a deep purple spot under UV light and changed to yellow when subjected to ammonia and $AlCl_3$ and its spectral signals are very close to apigenin [15]. Compound 6 was detected as a deep purple spot under UV light and changed to yellow when subjected to ammonia and $AlCl_3$. With complete acid hydrolysis, there is no change for compound 8 and thus, it was subjected to ferric chloride degradation, the products being chromatographed with authentic flavonoid aglycone and sugar samples, where apigenin as an aglycone and glucose moiety were detected and all spectral data were very close to that of vitexin [16]. Compound 7 was detected as deep purple spot under UV light and changed to yellow when subjected to ammonia and $AlCl_3$. With acid hydrolysis, apigenin as an aglycone and glucose moiety was the result and comparison with literature, compound 7

was identified as apigenin 7-*O*-*β*-glucoside [15]. Compound 8 yielded a brown spot under UV light and changed to yellow when subjected to ammonia and $AlCl_3$. With acid hydrolysis, kaempferol aglycone and galactose sugar was the result and comparsion with literature, compound 8 was identified as kaempferol 3-*O*-*β*-galactoside [17].

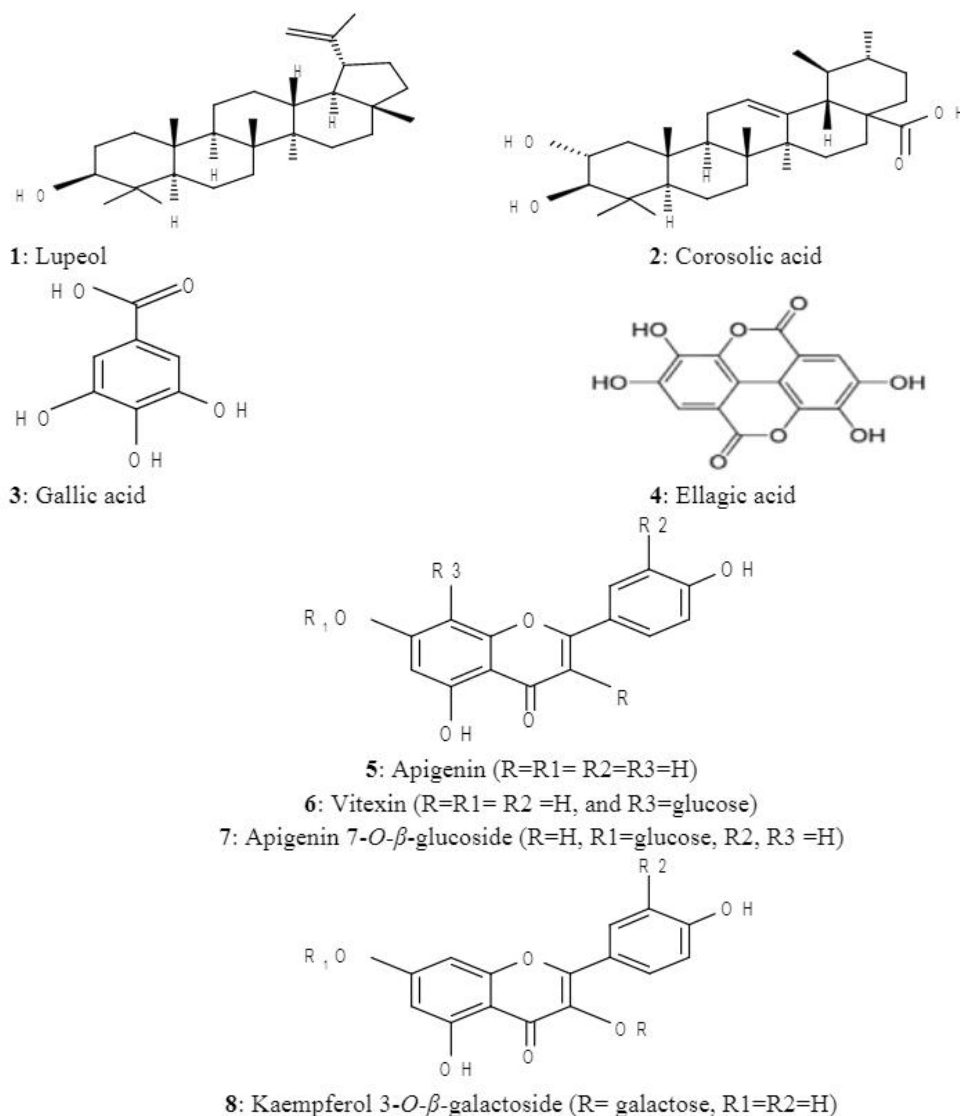

Fig. 1: Chemical structures of the compounds isolated from *L. tomentosa l*

Antidiabetic activity of the methanol extracts of the plants

Carbohydrates represent primary components of the human diet and consist mostly of complex polysaccharides such as starch and glycogen and to lesser extent disaccharides like sucrose. These carbohydrates need to be hydrolyzed by specific gastrointestinal enzymes before they can be absorbed through the intestinal mucosa. Alpha-amylase is a key enzyme responsible for hydrolyzing starch to maltose, which further breaks to glucose via the action of alpha-glucosidase. The inhibitory effects of the selected plant extracts on pancreatic alpha-amylase are presented in Table 2. Meaningful inhibition was observed

for *L. tomentosa,* with IC_{50} value of 136±14µg/ml. *S. oleraceus*, *D. virginiana*, and *T. ciliate* on the other hand did not reveal any significant inhibition against alpha-amylase. A similar trend was also observed for the inhibition of yeast alpha-glucosidase with *L. tomentosa,* and *D. virginiana* extracts providing near complete inhibition at a concentration of 100µg/ml (Table 3). Several studies have raised concern that non-mammalian alpha-glucosidases may not be accurate surrogates for intestinal digestion due to considerable differences in the structure and catalytic activity of these enzymes [18]. Furthermore, a variety of alpha-glucosidase enzymes exist which possess different substrate specificities (maltose, sucrose and isomaltose) and susceptibility to inhibitors. When using rat intestinal alpha-glucosidase and maltose as substrate, acarbose provided similar inhibition relative to the yeast enzyme (Table 3 and 4), however only *L. tomentosa* is considered significantly inhibitory to the mammalian enzyme preparation albeit with much reduced potency relative to the yeast enzyme system. Bearing in mind the interdependence between alpha-glucosidase and alpha-amylase in the digestion of starch, it is reasonable to assume that concurrent inhibition of both enzymes will lead to a cumulative inhibition of starch digestion and thus be more effective in the reduction of postprandial hyperglycaemia. Therefore *L. tomentosa* would appear as the more promising extract for further *in vivo* analysis (Fig. 2). While current literature evidence supports the clinical efficacy of known α-glucosidase inhibitors such as acarbose and voglibose in reducing postprandial hyperglycaemia in diabetic patients [19], it is also reported that α-glucosidase and pancreatic α-amylase inhibitors produce undesirable gastrointestinal side effects such as abdominal pain, flatulence, and diarrhoea in treated patients [20]. With regard to the gastrointestinal side effects associated with the use of acarbose, it has been suggested that excessive inhibition of pancreatic α-amylase results in abnormal bacterial fermentation of undigested carbohydrates in the large intestine which consequently gives rise to many of the observed gastrointestinal side effects [21, 22]. Therefore, although the combined effect of alpha-amylase and alpha-glucosidase inhibitors are envisaged to provide a more potent inhibition of carbohydrate hydrolysis, strong alpha-amylase inhibitors are at higher risk of producing more severe side effects. Therefore the strong inhibition of alpha-amylase by *L. tomentosa* extract put this plant extract at risk of producing undesirable side effects as in the case of acarbose. Given that crude plant extract is complex mixture of natural compounds, the potential for an extract to possess more than one pharmacological activity is not unrealistic [23]. The overall health benefit of a plant extract may be a result of a range of biological effects which function together. With this in mind we sought to further prioritise the selected medicinal plant extracts through other mechanisms which may target postprandial hyperglycaemia in addition to the inhibition of carbohydrate hydrolysis and thus potentially provide a therapeutic effect which is greater than the later activity on its own. Since incretin hormones potentiate glucose induced insulin release from the pancreas, regulation of GLP-1 and GIP levels represent an addition therapeutic option to control postprandial hyperglycaemia. Inhibition of DPP-iv, the enzyme responsible for removing active circulatory incretin hormones, is now recognised as an effective therapeutic target to lower postprandial hyperglycaemia [2]. Testing for potential DPP-iv inhibition revealed that none of the selected plant extracts significantly inhibited this enzyme (data not shown), ruling out this as an additional mechanism.

Toxicity related to traditional medicines is becoming more widely recognized with hepatotoxicity a major concern. Cell based cytotoxicity analysis of botanical or natural extracts are difficult to interpret in terms of directly predicting toxicity *in vivo*, because these extracts contain mixture of compounds and their concentrations will differ depending on their oral bioavailability. However, cell culture provides an acceptable model to explore many of the known drug-induced cytotoxic mechanisms including inhibition of mitochondrial function, disruption of

intracellular calcium homeostasis, activation of apoptosis, oxidative stress, inhibition of specific enzymes and transporters and formation of reactive metabolites that cause direct toxicity are common to most cells in culture. In this context, screening against chang liver cells is useful to identify mechanism based potential toxicity and consequently to flag high risk extracts prior to *in vivo* anti-diabetic screening. The extracts screened in this study do not raise any direct significant concern as they are considered essentially non-toxic to chang liver cells (Fig. 3). The mild toxicity observed for *L. tomentosa and D. virginiana* are unlikely to be physiologically relevant as the concentrations required to produce a significant toxic effect is greater than can be extrapolated to occur in the *in vivo* situation considering a practical dose and is in agreement with their use in traditional medicine.

Table 2: Inhibition of pancreatic alpha-amylase

Plant species	% inhibition (100μg/ml)	IC_{50} (μg/ml)
Sonchus oleraceus	2.35±4.2	NI
Lagerstroemia tomentosa	42.94±4.1	136±14
Diospyros virginiana	36.33±0.4	>100
Toona ciliata	3.31±3.3	NI
Acarbose	54.14±3.5	ND

Table 3: Inhibition of yeast alpha-glucosidase

Plant species	% inhibition (100μg/ml)	IC_{50} (μg/ml)
Sonchus oleraceus	22.28±1.5	>100
Lagerstroemia tomentosa	94.45±0.4	24±5
Diospyros virginiana	93.11±0.7	12.5±4
Toona ciliata	3.42±3.1	NI
Acarbose	32.93±3.5	>100

Table 4: inhibition of intestinal alpha-glucosidase

Plant species	% inhibition (100μg/ml)	
	Sucrose	Maltose
Sonchus oleraceus	2.06±1.4	8.22±0.6
Lagerstroemia tomentosa	13.40±1.0	27.44±1.4
Diospyros virginiana	9.27±1.4	4.03±1.7
Toona ciliata	9.28±2.2	4.73±0.8
Acarbose	32.64±3.6	40.67±2.2

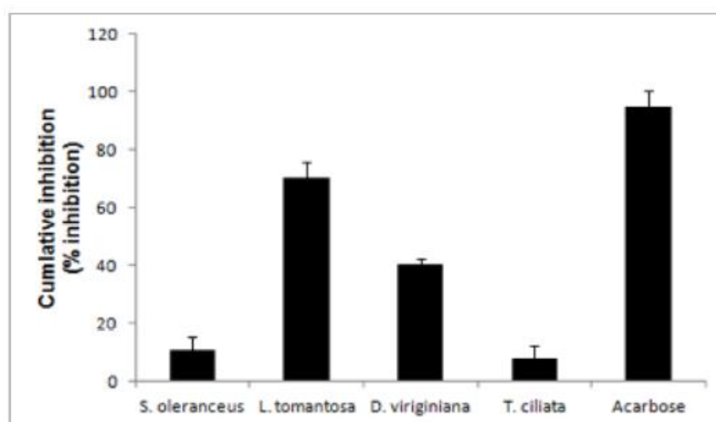

Fig. 2: Predicted cumulative inhibition of starch digestion. Data extrapolated from Table 2 and 4 and indicates the sum of both alpha-amylase and maltose inhibition at a fixed concentration of 100μg/ml.

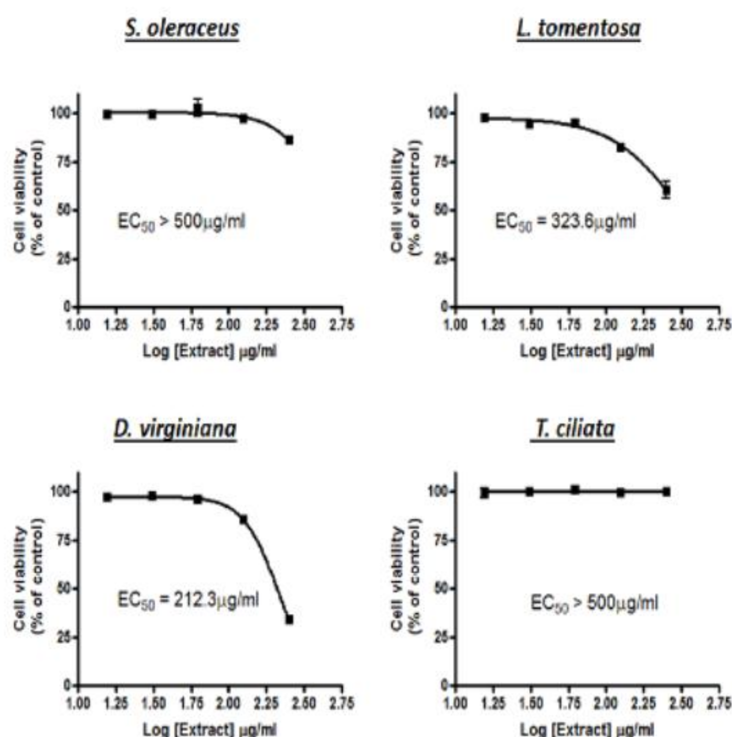

Fig. 3: Cytotoxicity in Chang liver cells. Dose dependent inhibition of Chang liver cell viability by selected plant extracts. Each data point represents the mean±ST Dev for quadruplicate assays. Dose response curves and EC_{50} values were calculated using GraphPad Prism 4 software.

The observed antidiabetic effect of *L. tomentosa* leaves methanol extract can be explained by the presence of isolated bio-active compounds. Triterpene compound (lupeol), isolated from *Solanum xanthocarpum*, caused decreases in glycated haemoglobin, serum glucose and nitric oxide, and treatment with lupeol also increased antioxidant levels, with a decrease in the level of thiobarbituric acid-reactive oxygen species [24]. Corosolic acid showed a significant antidiabetic activity. It reduced the blood glucose levels of KK-Ay mice after 4 hours after a single oral dose of (2 mg/kg body weight), and also it reduced the blood glucose levels in KK-Ay mice after two weeks after a single oral dose of (2 mg/kg body weight). It significantly lowered plasma insulin levels were in KK-Ay mice under similar conditions [25]. Aqueous extract of *Terminalia paniculata* bark showed a significant antidiabetic activity. At doses of 100 and 200 mg/kg, it significantly decreased blood glucose and glycosylated haemoglobin levels in diabetic rats than diabetic control rats. HPLC analysis of the extract proved the presence of gallic acid, ellagic acid, catechin, and epicatechin. Gallic acid (major compound) isolated from our plant extract showed significant enhancement of glucose uptake action in presence of insulin in muscle cells than vehicle control [26]. *Otostegia persica* (Burm.) Boiss. Plant from (Lamiaceae family) proved hypoglycemic and antioxidant effects. The ethyl acetate methanol fractions showed antidiabetic activity at a dose of 300 mg/kg and the isolation and the purification of compounds produced Chrysoeriol from ethyl acetate and three apigenin derivatives (6-methylapigenin, apigenin-7-O-glucoside, and echinaticin) from the methanol fraction and these bio-active compound can explain antidaibetic effect of the plant [27].

Conclusion

Target directed *in vitro* screening represents a feasible strategy to elucidate the potential molecular mechanism(s) through which a plant extract may produce an anti-diabetic effect and provides the rationale for selecting appropriate animal models and a context driven experimental design. In the present study, methanol extract of *L. tomentosa* leaves was shown to possess significant activity against starch hydrolysis and consequently represent a feasible therapeutic option to regulate postprandial hyperglycaemia and this is due to the presence of different bio-active compounds isolated from the extract. Therefore this medicinal plant may be considered suitable for further *in vivo* investigation in a carbohydrate challenged animal model, also it can serve as therapeutic agent and can be used as potential source of novel bioactive compounds for treating diabetes mellitus type 2.

Conflict of interest

There is no conflict of interest associated with the authors of this paper.

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