**Abstract**

Diabetes type II, known as diabetes mellitus (T2DM), is a very common chronic metabolic disorder. DMT2 leads to high blood glucose levels due to in the cells’ inability to respond normally to insulin (insulin resistance), excessive hepatic glucose production and eventually decreased insulin secretion. Several types of anti-diabetic drugs are available, but unfortunately, most glucose-lowering drugs have side effects. Hence, it is crucial to develop new drugs with minimal side effects. In addition, these drugs become less effective over time when used chronically, and most patients need combination therapy with several anti-hyperglycemia drugs. These factors complicate the treatment of diabetes, and reduce the patient’s response to these drugs. Therefore, many research projects are focused on the development of new drugs for treating DMT2, in particular from natural resources as they potentially cause fewer side effects and may be less expensive. Discovery and development of a single new synthetic drug typically takes about 10-15 years with a cost of $300-500 million. In comparison, plant-based drug development involves much less time and cost than synthetic drugs.

The aim of this study was to investigate the anti-diabetic effect of crude extracts and fractions of several medicinal plants and to isolate the active compound(s) that enhance(s) glucose disposal into muscle cells. An L6 skeletal muscle cell line stably expressing myc epitope at the exofacial loop of the glucose transporter-4 (GLUT4), named L6-GLUT4myc, was used as a model to follow GLUT4 translocation to the plasma membrane (PM). We tested the cytotoxicity and anti-diabetic activity of 12 plants. *Ocimum basilicum* (OB),Gundelia tournefortii (GT) and Teucrium polium (TP) extracts were found to be the most effective. Chemical composition analysis was performed for the extracts of these three plants; 17 compounds in OB and 44 compounds in GT were detected for the first time. We decided to focus on GT extract in our search for active compounds. The concentrated bioactive GT MeOH extract was subjected to flash gradient silica gel column chromatography yielding 10 fractions. L6-GLUT4myc cells were treated with these fractions and cytotoxicity and GLUT4 translocation to the PM were assessed. The results indicated that fraction 6 was the most potent as it increased GLUT4 translocation about 3.5 and 5-fold when used at a concentration of 250 µg/ml in the absence and presence of insulin, respectively. 97 distinct phytochemicals were detected in GT fraction 10 and 25 of these are known to possess anti-diabetic activity. 20 compounds out of the 25 enhanced glucose disposal and GLUT4 translocation to the PM.

These findings indicate that GT fractions are potential candidates for isolating new anti-diabetic drugs. The activity of the most active fraction should be examined in diabetic animal models and human subjects before prescribing them as anti-diabetic therapy.

**Summary and Conclusions**

In T2DM, insulin resistance - particularly in skeletal muscle- is associated with insufficient recruitment of GLUT4 to the cell surface in the face of normal GLUT4 expression and elevated insulin. The treatment of T2DM relies largely on the use of drugs that stimulate insulin secretion (sulphonylureas), lower hepatic glucose output (metformin) or improve insulin action via the stimulation of the PPAR-gamma transcription factor (glitazones) or the inhibition of SGLT2 (dapagliflozin), or on injected insulin. Glucose-lowering drugs however, are either suboptimal or cause side effects, such as severe hypoglycemia, idiosyncratic liver cell injury, lactic acidosis, permanent neurological deficit, digestive discomfort, headache and dizziness. Hence, it is crucial to search for novel drugs that mimic insulin action and could potentially have no or less side effects.

Muscle, hepatic and adipose cells selectively increase their glucose uptake capacity in response to insulin. In these tissues, GLUT4 is the predominant glucose transporter, distinguished by its continuous cycling between the PM and intracellular stores. Modulations of the cycling rate result in different steady-state distributions that determine net glucose influx. Skeletal muscle is the major tissue absorbing circulating glucose during a meal. In T2DM, insulin resistance -particularly in skeletal muscle- is associated with insufficient recruitment of GLUT4 to the cell surface in the face of normal GLUT4 expression and elevated insulin.

Nature is the best designer of medicines since natural products can interact optimally with biological systems through a long natural selection process. Indeed, more than 50% of modern small drug molecules were extracted and purified from plants or synthesized on the basis of products of plants. Accordingly, the rationale of the present study is to detect and isolate anti-diabetic phytochemicals from medicinal plants and test their mechanism of action *in vitro* in a well-established muscle cell model, the L6-GLUT4myc cell line, which stably expresses myc-tagged GLUT4.

The present research work comprised of screening extracts prepared from 12 selected plants for anti-diabetic activity. The plants were selected based on two criteria; reports from local alternative medicine practitioners of effective anti-diabetic medicinal plants and reports in the scientific literature describing plants with GLUT4-inducing activity. The plants used in this screen were: *Trigonella foenum-graecum* (fenugreek, seeds), *Atriplex halimus* (saltbush, aerial part), *Olea europaea* (olive, leaves), *Urtica dioica* (nettle, aerial part), *Allium sativum* (garlic, clove), *Allium cepa* (onion, bulb), *Nigella sativa* (blackcumin, seeds), and *Cinnamomon verum* (cinnamon, bark), *Portulaca oleracea* (PO; common purslane, aerial part), *Ocimum basilicum* (OB; basil, aerial part),Gundelia tournefortii (GT; tumble thistle, aerial part) and Teucrium polium (TP; felty germander, aerial part). Extracts were prepared from these plants by continuous hot extraction, Soxhlet extraction and sonicator apparatus methods with water/ethanol, dichloromethane, hexane and methanol. The resulted extracts were concentrated, dried and stored in a freezer at -20°C for further analysis.

To determine cell viability, MTT and the LDH leakage assays were performed. Muscle L6-GLUT4myc cells were treated with increasing concentrations of the plant extracts (up to 1 mg/ml) for 24 h. The results were used to determine the safe concentrations for further analysis. Extract concentration was considered safe if cell viability was at least 90% at that concentration. The plant extracts of *Trigonella foenum-graecum*, *Allium sativum* and *Allium cepa*, were found to be safe up to 1 mg/ml. *Atriplex halimus*, *Olea europaea*, *Urtica dioica*, *Nigella sativa* and *Portulaca oleracea*, were found to be safe up to 0.5 mg/ml. *Cinnamomon verum* and *teucrium polium* extracts were found to be safe up to 0.125 mg/ml. *Ocimum basilicum* and *Gundelia tournefortii* extracts were found to safe up to 0.25 mg/ml.

Theeffect of these plant extracts on GLUT4 translocation to the PM was tested onL6-GLUT4myc cells using a cell-ELISA assay (Fig. 1). Glucose uptake into skeletal muscle is mediated by the facilitative hexose transporter, GLUT4, a membrane protein that continuously cycles between intracellular stores and the PM. Insulin primarily promotes the rate of GLUT4 exocytosis and fusion with the PM, a process termed GLUT4 translocation that results in a gain in surface GLUT4. Our findings indicate that a cohort of the selected plant extracts led to a significant gain in GLUT4 translocation at non-cytotoxic concentrations as measured by MTT assay and the LDH leakage assay.

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| **Figure 1: Diagram showing the principle of the cell-ELISA assay to measure** **GLUT4 translocation in L6-GLUT4myc cells**. |

1 mg/mL *Trigonella foenum* 50% ethanol extract almost doubled GLUT4 translocation to the PM. Insulin stimulated GLUT4 translocation was slightly increased from 170% in the non-treated cells to about 200% and 230% in the cells treated with 0.5 and 1 mg/mL extract, respectively.

The 50% ethanol extracts of 0.25 mg/mL *Nigella sativa* increased the GLUT4 translocation in the absence and presence of insulin at 20% and 70%, respectively. At the 0.5 mg/mL concentration in the absence and presence of insulin, GLUT4 translocation was increased by 30% and 80%, respectively.

Exposing L6-GLUT4myc cells to 0.125 and 0.25 mg/mL of nettle 50% ethanol extract almost doubled GLUT4 translocation to the PM in the basal state and it was increased by about 1.6 fold in the insulin-stimulated state.

Exposing L6-GLUT4myc cells to 0.25 and 0.5 mg/mL *Atriplex halimus* 50% ethanol extract almost doubled GLUT4 translocation in the basal state. When treated with insulin, GLUT4 translocation was increased in the presence of 0.25mg/mL to about 180%, and was increased from 160% to 230% in the presence of 0.5 mg/mL.

*Cinnamomun verum* results obtained here demonstrate that GLUT4 translocation to the cell surface was increased by 1.5- and 2-fold in the basal state in the presence of 0.063 and 0.125 mg/mL of cinnamon 50% ethanol extract, respectively. When the L6-GLUT4myc cells were treated with 0.063 mg/mL and 0.125 mg/mL in the presence of insulin, GLUT4 translocation was increased to about 140% and 170%, respectively.

Exposing L6-GLUT4myc cells to 0.5 and 1 mg/ml of *Portulaca oleracea* 50% ethanol extract in the absence of insulin enhanced GLUT4 translocation by 1.6 and 2.6-fold respectively, and 2.6 and 4-fold respectively in the presence of insulin. GLUT4 translocation to the PM increased by 1.4 and 2-fold when exposed to 0.25 and 0.5 mg/ml of *Portulaca oleracea* methanol extract in the absence of insulin, respectively. The same extracts concentrations enhanced GLUT4 translocation by 1.7 and 3-fold in the presence of insulin, respectively.

TP aerial parts were used for preparing three distinct extracts: water/ethanol (WTP), methanol (MTP), and hexane (HTP). The HTP extract increased the GLUT4 translocation at 32 µg/ml by 2- and 3-fold relative to the control in the absence and presence of insulin, respectively. A similar result was obtained with the MTP extract at 63 µg/ml. WTP extract in the absence of insulin and in the presence of insulin did not affect GLUT4 translocation.

L6-GLUT4myc cells were treated with three distinct OB extracts (MeOH, hexane and DCM). A dose-dependent increase in GLUT4 translocation was observed. Methanol, hexane and dichloromethane extracts (0.125 mg/ml) increased GLUT4 translocation to the PM 3.5, 2.5 and 1.8 times in the absence of insulin, respectively. Highest levels (about 7-fold compared to control cells) of translocation were observed with hexane (0.25 mg/ml) and methanol (0.125 mg/ml) extracts in the presence of insulin.

The anti-diabetic activity of GT aerial parts extracts (methanol and hexane) was also tested. The hexane extract was found to have the lowest effects on GLUT4 translocation, and only 16% increase of GLUT4 translocation was obtained at 32 𝜇g/ml and 63 𝜇g/ml GT hexane extracts in the absence of insulin. A similar effect was seen in the presence of insulin. Results indicate that methanol extract was the most efficient at GLUT4 translocation enhancement. It increased GLUT4 translocation at 63µg/ml by 1.5 and 2-fold relative to the control in the absence and presence of insulin, respectively.

Overall, these findings indicate that these plant extracts possess anti-diabetic activity as measured by induction of GLUT4 translocation to the PM. For the next phase of the research, we selected the plant extracts that augmented GLUT4 activity but had been the subject of fewer previous studies of their chemical properties (based on a literature search in PubMed). The plants that matched these criteria were OB, TP and GT. This phase of this project aimed to detect the chemical composition of three selected plants. Library searches were carried out using the NIST GC/MS Library and with mass spectra from literature for identifying the chemical compounds. The percentage composition of the samples was computed from the GC peak areas.

Phytochemical analysis of MeOH, hexane and DCM OB crude extracts was carried out with GC/MS analysis. Silylation derivatization technique was applied to detect molecules containing labile polar functional groups that are usually undetected by standard GC/MS techniques. Using this technique, we detected 53 chemical compounds, 17 of which were revealed for the first time in OB.

The chemical compounds of hexane and methanol TP (HTP and MTP) extracts were identified by GC/MS, and 19 chemical compounds were detected. They contained a complex mixture of chemical compounds, including aromatic, saturated and unsaturated fatty acids, and phenolic compounds. There were 10 compounds detected in the MTP extract, and nine compounds were found in the HTP. Only palmitic acid was found in both extracts.

Phytochemical screening using GC/MS analysis for silylated GT methanol and hexane extracts revealed 26 and 20 compounds respectively. The use of the silylation derivatization technique was found to be helpful due to presence of polar phytochemicals. Diverse components were detected, including sterols, esters, phenols, saturated and unsaturated fatty acids, and aromatic compounds. Only stigmasterol was found in both extracts. Thirty-nine out of the 45 detected compounds are reported here for the first time in GT. Only six components, namely, stigmasterol, 𝛽-sitosterol, palmitic acid, linoleic acid, 𝛼-linolenic acid, and stearic acid were reported elsewhere.

GT was the most recommended anti-diabetic herb by alternative medicine practitioners and the least researched chemically and biologically. GT methanol extract was more effective than the hexane extract at stimulating GLUT4 translocation to the PM. With these facts in mind, GT methanol extract was selected for further examination, in attempt to isolate active compound(s). To accomplish this, chemical fractionation was carried out. The crude dry extract was dissolved in EtOH then about 4 g of silica was added. The solvent was evaporated and 1.43 g dry extract was loaded onto a 40 g silica gel flash chromatography column at 40 ml/min flow rate. Ten fractions were separated. The MTT assay was used to assess the fractions toxicity in the L6-GLUT4myc cells. No toxic effect was observed with the fractions 1-4 and 8-10 up to 500 µg/ml, fraction 5 up to 125 µg/ml and fractions 6 and 7 up to 250 µg/ml. To assess the fractions efficacy at augmenting GLUT4 translocation to the muscle cell membrane, the L6-GLUT4myc cells were incubated in the presence and absence of insulin for 20 h with 125 and 250 µg/ml of each fraction, apart from fractions 9 and 10, for which 63 and 125 µg/ml were used due to limited quantity of the fractions. The results indicated that fraction 6 was the most efficient as it enhanced GLUT4 translocation about 3.5 and 5-fold at a concentration of 250 µg/ml in the absence and presence of insulin respectively.

97 distinct phytochemicals were detected in the 10 GT fractions and 25 of these have anti-diabetic activity (Fig. 2), while 20 have been reported to enhance glucose disposal and GLUT4 translocation to the PM. The number of the compounds in each fraction ranged from 3 to 30. One fraction had only two phytochemicals, GLUT4 translocation was enhanced by about 120% and 140% when cells treated with 125 µg/ml of this fraction in the absence of insulin. It contained three chemicals only, namely palmitic acid, β-amyrin and lupeol, all of which are known to enhance glucose disposal in muscle cells.

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| **Figure 2: The chemical structure of all anti-diabetic and GLUT4 translocation enhancer phytochemicals existing in the 10 GT fractions.** |

**Conclusions**

The selected plants in this study, namely *Trigonella foenum-graecum*, *Urtica dioica*, *Atriplex halimus*, *Cinnamomun verum , Portulaca oleracea*, *Ocimum basilicum*,Gundelia tournefortii and Teucrium polium, are known traditionally as anti-diabetic herbs. However, to the best of our knowledge, this is the first study examining the effect of these plant extracts on GLUT4 translocation as an anti-diabetic mechanism. Gundelia tournefortii has not been previously reported as anti-diabetic plant and it is the first study to isolate several active compounds in Gundelia tournefortii. Most telling fraction 2 that has only three compounds: palmitic acid, lupeol and β-amyrin. While palmitic acid and lupeol are known to stimulate GLUT4 activity and translocation to PM, β-amyrin has not been reported before as a GLUT4 activator. These findings indicate that GT fractions are potential candidates for isolating new anti-diabetic drugs. The activity of the most active fraction must be examined in diabetic animal models and human subjects before prescribing as an anti-diabetic therapy.