Teucrium polium extracts stimulate GLUT4 translocation to the plasma membrane in L6 muscle cells

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ABSTRACT

Teucrium polium L. (TP) is recommended by herbal and integrative practitioners for the treatment of diabetes. However, its mechanism of action in stimulating GLUT4 activity and translocation to the plasma membrane is still unknown. This in vitro study examined the chemical composition, cytotoxicity, and antidiabetic activity of three TP distinct extracts: water/ethanol (WTP), methanol (MTP), and hexane (HTP). The compositions of the TP extracts were determined by GC/MS. MTT and LDH assays were used to assess the toxicity of the extracts. The efficacies of the TP extracts in enhancing glucose transporter-4 (GLUT4) translocation to plasma membrane (PM) were tested in L6 muscle cells, stably expressing myc-tagged GLUT4 using the cell-ELISA test. GC/MS phytochemical analysis of MTP and HTP extracts revealed 10 compounds in each extract, and only palmitic acid was communal in these two extracts. WTP, MTP, and HTP extracts were safe up to 63, 63, and 250 µg/ml, respectively. The HTP extract was the most efficient in GLUT4 translocation enhancement, while the least efficient was the WTP extract. In addition, 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. In contrast, WTP extract in the presence of insulin had no effect and in the absence of insulin had slightly enhanced GLUT4 translocation. These findings indicate that TP antidiabetic activity is mediated in part by enhancing GLUT4 translocation to the PM in skeletal muscle.

Keywords: GLUT4, GC/MS, phytochemicals, diabetes, Teucrium polium.

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INTRODUCTION

Herbal-based antidiabetic medicines have been a part of traditional medicine for centuries. The Chinese were the first to detect diabetes mellitus in the third century, and they noticed that the sweetness of urine attracts dogs. Later, Indian physicians in the sixth century related to diabetes as Honey urine and prescribed several herbs to treat it (Saad et al., 2017; Zaid and Saad, 2013). Diabetes was then recognized by medieval Greco-Arab physicians by its main symptoms: increased thirst, frequent urination, and tiredness. Greco-Arab physicians and practitioners then used a series of medicinal plants for treating these combined symptoms (Saad et al., 2017; Zaid and Saad, 2013).

Modern treatment of diabetes mellitus revolves around
controlling blood glucose levels either through glucose production or use, through increasing insulin secretion and effectiveness, reducing energy intake, or increasing energy expenditure (Kadan et al., 2013; Zaid et al., 2008). Unidirectional glucose uptake into skeletal muscle is mediated by the facilitative glucose transporter-4 (GLUT4), a membrane protein that continuously recycles between intracellular vessels and the plasma membrane (PM). Insulin receptor mediated signals significantly enhance the rate of GLUT4 traffic towards and fusion with the PM; this process is called GLUT4 translocation (Zaid et al., 2008).

Reports on natural herbs for diabetes treatment focus on lowering blood sugar and reducing the damaging effects of the disease. Interestingly, a single medicinal plant may include biochemically different antidiabetic mechanisms to stimulate insulin secretion; inhibit intestinal carbohydrate digestion and absorption; and enhance GLUT4 translocation to the plasma membrane, insulin sensitivity, and activation of MAPK and PPARγ. Some antidiabetic herbs even possess anti-inflammatory and immunomodulatory action (Ota and Ulrich, 2017; Rios et al., 2015; Saad et al., 2017; Zaid et al., 2016).

Insulin sensitizers include plants that increase glucose uptake and disposal by muscle, fat, and hepatic cells, as well as cells that regulate the hepatic glycolgen metabolism. We had recently tested the mechanism of action of several medicinal plant extracts in increasing the glucose uptake. *Trigonella foenum-graecum*, *Urtica dioica*, *Atriplex halimus*, *Cinnamomum officianalis* (Kadan et al., 2013), and *Ocimum basilicum* (Kadan et al., 2016) increased glucose disposal by enhancing the glucose transporter 4 (GLUT4) translocation to the plasma membrane.

*Teucrium polium* L. (TP) is one of the most used antidiabetic herbs, and it is a wild-growing flowering plant in the temperate parts of Europe, Africa, and Asia (mainly the Middle East) (Bahramikia and Yazdanparast, 2012). In Greco-Arab medicine, TP has been used for different pathological conditions including inflammation, gastrointestinal disorders, rheumatism, and diabetes mellitus (Saad et al., 2017). TP antidiabetic activity was evaluated in animal models, and some studies in diabetic animal models have shown that intravenous, intraperitoneal, or oral administration of a TP crude extract to STZ-induced diabetic rats significantly decreased serum glucose levels (Esmaeili and Yazdanparast, 2004; Gharaibeh et al., 1988; Shahraki et al., 2007). *T. polium* ethanol/water extract fed-diabetic rats experienced significant reductions in serum glucose levels and blood insulin level was enhanced by almost 160% (Shahraki et al., 2007). Gavage and oral administration of a hydroalcoholic and water extract of TP increased insulin secretion from rat-isolated islets (Mohseni Salehi Monfared and Pournourmohammadi, 2010) and insulin levels in rats (Esmaeili and Yazdanparast, 2004; Mohseni Salehi Monfared and Pournourmohammadi, 2010; Tabatabaie and Yazdanparast, 2017). *T. polium* crude extract enhanced insulin secretion by almost 135% after a single dose of plant aqueous extract (Esmaeili and Yazdanparast, 2004). In addition, TP ethyl acetate extract decreased serum, liver, and muscle triglyceride content of sucrose-induced insulin resistance in rats (Mousavi et al., 2012).

In the present study, the role of GLUT4 translocation in the traditionally known antidiabetic effects of TP was evaluated.

**MATERIALS AND METHODS**

**Plant extract preparation**

*Teucrium polium* (aerial parts) were purchased from Al Alim- Medicinal Herb Center, Zippori, Israel. TP air-dried aerial parts (40g) were powdered, packed in an Erlenmeyer flask, and extracted in 500 ml with 50% ethanol in water (WTP), methanol (MTP), or hexane (HTP) at room temperature for 72 h to give a dark green extract. The methanol extract was filtered and concentrated by a rotary vacuum evaporator under pressure at 50°C. The hexane extract was filtered and evaporated to dryness under pressure at 50°C and dissolved in DMSO for further studies. The water/ethanol was not concentrated. The yield of the extracts was 6.3, 11.1 and 4.6% for WTP, MTP and HTP extracts, respectively. The stock extracts were then preserved in airtight glass containers and kept at −20°C.

**Gas chromatography-mass spectrometry analysis**

GC/MS analysis was performed with HP5890 Series II GC equipped with a Hewlett-Packard MS Engine (HP5989A) single quadrupole MS, HP7673 auto sampler, HP MS-DOS Chemstation, and HP-5MS capillary column (0.25 μm × 15 m × 0.25 mm). The temperature program was as follows: injector temperature, 180°C; initial temperature, 40°C for 6 min; gradient of 20°C/min until 140°C; gradient of 10°C/min until 200°C; and hold time, 3 min. The MS parameters were set as follows: source temperature, 180°C; transfer line, 280°C; positive ion monitoring; and EI-MS (70 eV).

**Identification of components**

The percentage composition of the samples was computed from the GC peak areas, and library searches were performed using the NIST GC/MS Library, and the mass spectra from the literature were compared with the compositions (spectra) of the samples to define them. Component relative percentages were calculated based on GC peak areas without using correction factors.

**Cell culture**

Cells from the rat L6 muscle cell line, stably expressing myc-tagged GLUT4 (L6-GLUT4myc; a kind gift from Prof. Amira Klip) (Zaid et al., 2009), were maintained in myoblast monolayer culture. All cells were grown under an atmosphere of 95% air and 5% CO2 in α-MEM supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 0.1 mg/ml streptomycin.

**MTT and lactate dehydrogenase (LDH) assays**

Cells were subcultured into 96-well plates with 200 μl of medium (2 × 10^3/well) for 24 h and then exposed to (0 to 1 mg/ml) of TP extracts. Cell viability was assessed by following the amount of
formazan dye formed in alive cells (MTT) or by calculating the relevant activity of LDH released in dead cells, as described previously (Kadan et al., 2013). MTT and LDH kits were purchased from Promega (WI, USA).

Determination of surface GLUT4myc

Surface myc-tagged GLUT4 was measured in intact, non-permeabilized cells. Cells grown in 24-well plates for one day followed by the addition of the plant extracts for 20 h and serum-starved for 3 h were treated with or without 1 µM insulin for 20 min. GLUT4myc on the plasma membrane was then detected as previously described (Zaid et al., 2009). Briefly, the cells were reacted with polyclonal anti-myc antibody (1:200) (from Sigma-Aldrich) for 1 h at 4°C, washed with PBS, reacted with horseradish peroxidase-bound goat anti-rabbit secondary antibody (1:1000) (from Promega) for 1 h at 4°C, and washed with PBS. Cells then were incubated with 0.5 ml of o-phenylenediamine dihydrochloride reagent and allowed to develop for 20 to 30 min in the linear range in the dark at room temperature. The reaction was stopped with 0.5 ml/well of 3 N HCl. Supernatants were collected, and absorbance was measured at 492 nm. Background absorbance obtained in the absence of anti-myc antibody was subtracted from all values.

Statistical analysis

Error bars were plotted and represent simple standard deviations of the mean. When comparing different samples, results were considered to be statistically different when P < 0.05. T-test statistical calculations were conducted using SPSS version 21.0.

RESULTS

The current study evaluated the chemical composition, cytotoxicity, and antidiabetic activity of three distinct T. polium L. (TP) extracts: water/ethanol (WTP), methanol (MTP), and hexane (HTP).

Teucrium polium L. chemical composition

The chemical compounds in the HTP and MTP extracts were identified by GC/MS (Table 1), and 19 chemical compounds were detected by GC/MS analysis in TP extracts. They contained a complex mixture of chemical compounds, 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. Interestingly, only one mutual compound was found in the two extracts, namely, palmitic acid (Table 1). In addition, palmitic acid was recently reported by our group to be found in three different Ocimum basilicum L. extracts (methanol, hexane, and dichloromethane). O. basilicum-derived palmitic acid was suggested to play an essential anti-diabetic role that seems to be mediated through GLUT4 translocation (Kadan et al., 2016).

As seen in Table 1, (5E,8E,11E)-methyl heptadeca-5,8,11-trienoate (5.6%); (9Z,12Z)-octadeca-9,12-dienoic acid (4.5%); 3,7,11-trimethyltrideca-1,6,10-trien-3-ol (4.4%); and Palmitic acid (4.2%) are the major compounds in the MTP (Figure 1C). In the HTP, cis-linoleic acid (20%), butyl (2-ethylhexyl) phthalate (12.2%), and Palmitic acid (7.2%) are the main compounds (Figure 2C). Some of the detected compounds were reported to possess antidiabetic activity, namely, thymol (Saravanan and Pari, 2015), carvacrol (Ezhumalai et al., 2014), eugenol (Jeong et al., 2014), and cis-vaccenic acid (Alstrup et al., 2004).

Toxicity of Teucrium polium L. extracts

MTT and LDH leakage assays were used to evaluate the nontoxic concentrations of the three TP extracts, and the toxicities of the plant extracts were tested in vitro in L6-GLUT4myc cells. Cells were seeded in 96 well plates and were subjected to increasing concentrations of the extracts (0 to 1 mg/ml) for 24 h. Extract concentrations that led to less than 10% cell death were considered as safe, and WTP (Figure 3A), MTP (Figure 1A), and HTP (Figure 2A) extracts were found to be safe up to 63, 63 and 250 µg/ml, respectively. The efficacy studies were performed at concentrations equal or less than the safe concentration of each extract.

Effects of Teucrium polium extracts on GLUT4 translocation

Skeletal muscle and liver are the primary tissues responsible for dietary glucose uptake and disposal. In muscle and hepatic and adipose tissues, insulin promotes the exocytic traffic of intracellular GLUT4 vessels towards the plasma membrane to elicit a rapid increase in glucose uptake (Osorio-Fuentealba and Klip, 2015; Zaid et al., 2008; Zierath et al., 1996). In insulin resistance and type 2 diabetes, insulin fails to promote GLUT4 translocation to the PM, and some of the antidiabetic synthetic drugs and medicinal plants-based products bypass the insulin resistance by increasing GLUT4 translocation in insulin dependent and independent pathways (Zaid et al., 2012).

The involvement of glucose transporter (GLUT4) in the observed antidiabetic effects of T. polium extracts was evaluated by applying the GLUT4 translocation assay. In addition, insulin increases the GLUT4 translocation to the myoblasts surface, thus enhancing glucose uptake (Osorio-Fuentealba and Klip, 2015; Zaid et al., 2008). L6 skeletal muscle cell lines expressing myc epitope at the exofacial loop of the GLUT4, named L6-GLUT4myc, were used as a model to demonstrate GLUT4 translocation to the plasma membrane (Zaid et al., 2008). The extracts were added to the L6-GLUT4myc cells in the absence or presence of insulin, and the translocation of GLUT4myc to the plasma membrane was assessed as described in
Afifi et al. (2017) used methanol and hexane in preparing TP extract and colleagues (2010). In this study, only compounds that showed at least 90% similarity to the GC/MS library were detected and reported. This explains the short list of compounds listed here. Afifi and colleagues (2009) on the other hand have used methanol and hexane in preparing TP extract and yet they detected deferent compounds compared to our study. Several factors especially the method of extraction and the accuracy of compounds detection by GC/MS. In this study, only compounds that showed at least 90% similarity to the GC/MS library reference were listed. This explains the short list of compounds listed here. Afifi and colleagues (2009) on the other hand have used methanol and hexane in preparing TP extract and yet they detected deferent compounds compared to our list. In addition to the previous possible reasons, we have used deferent extraction method to prepare the extracts and the plants were grown freely in the nature where in the presence of insulin (Figure 3B).

MTP extract (63 µg/ml) increased GLUT4 translocation to the PM by two and three times in the absence and presence of insulin, respectively (Figure 1B). HTP extract (at 32 µg/ml) led to similar results (Figure 2B) obtained with MTP extract (at 63 µg/ml). These findings indicate that the HTP extract was the most efficient extract in enhancing GLUT4 translocation.

DISCUSSION

TP is one of the traditional medicinal plants well known for its antidiabetic property in the Middle East (Saad et al., 2017). TP extracts anti diabetic activity was evaluated in vitro, in vivo and in situ. TP aqueous extract supplemented rats had significant decrease in cornstarch induced acute hyperglycemia 45 min post starch intragastric administration (Kasabri et al., 2011). TP antidiabetic activity was further evaluated in animal models, and through gavage and oral administration of a hydroalcoholic and water extract of TP, insulin secretion levels increased in the circulating blood in rats (Esmaeili and Yazdanparast, 2004; Mohseni Salehi Monfared and Pournourmohammadi, 2010; Tabatabaie and Yazdanparast, 2017). Insulinotropic properties of TP extracts was attributed to the presence of apigenin (5-hydroxy-4', 7-dimethoxyflavone) existing only in methanolic, ethanolic, but not in aqueous/ethanolic and fractions (Mirghazanfari et al., 2010). In this study however, apigenin was the sole compound detected by GC/MS.

The chemical compounds in the HTP and MTP extracts in this study were identified by GC/MS. Ten compounds detected in the MTP extract, and nine compounds were found in the HTP. The extracts contained aromatic, saturated and unsaturated fatty acids, and phenolic compounds. Previous studies have studied the chemical content of TP extracts (Mahmoudi and Nosratpour, 2013; Djabou et al., 2012; Guetata and Al-Ghamdia, 2014). They reported several distinct compound compered to the list of compounds reported here. This could be due to several factors especially the method of extraction and the accuracy of compounds detection by GC/MS. In this study, only compounds that showed at least 90% similarity to the GC/MS library reference were listed. This explains the short list of compounds listed here. Afifi and colleagues (2009) on the other hand have used methanol and hexane in preparing TP extract and yet they detected deferent compounds compared to our list. In addition to the previous possible reasons, we have used deferent extraction method to prepare the extracts and the plants were grown freely in the nature where in

<table>
<thead>
<tr>
<th>Compound</th>
<th>MTP (%)</th>
<th>HTP (%)</th>
<th>*rt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thymol</td>
<td>1.5</td>
<td>11.49</td>
<td></td>
</tr>
<tr>
<td>5-isopropyl-2-methylphenol</td>
<td>1.6</td>
<td>11.58</td>
<td></td>
</tr>
<tr>
<td>Eugenol</td>
<td>1</td>
<td>12.05</td>
<td></td>
</tr>
<tr>
<td>(E)-7,11-dimethyl-3-methylenedeca-1,6,10-triene</td>
<td>0.96</td>
<td>13.43</td>
<td></td>
</tr>
<tr>
<td>2,6-di-t-tert-butylphenol</td>
<td></td>
<td>13.58</td>
<td></td>
</tr>
<tr>
<td>(2E,6E)-3,7,11-trimethylbenzade-2,6,10-trien-1-yl acetate</td>
<td>1.6</td>
<td>13.67</td>
<td></td>
</tr>
<tr>
<td>3,7,11-trimethylbenzade-1,6,10-trien-3-ol</td>
<td>4.4</td>
<td>13.94</td>
<td></td>
</tr>
<tr>
<td>(S)-2-methyl-6-((p-toly)hept-2-en-4-one</td>
<td>2.2</td>
<td>15.04</td>
<td></td>
</tr>
<tr>
<td>1,3,5-tr-tert-butylbenzene</td>
<td>3.4</td>
<td>15.22</td>
<td></td>
</tr>
<tr>
<td>2-(t-tert-butyl)-4-(2,4,4-trimethylpentan-2-yl)phenol</td>
<td>5.2</td>
<td>16.1</td>
<td></td>
</tr>
<tr>
<td>Palmitic acid</td>
<td>4.2</td>
<td>17.3</td>
<td></td>
</tr>
<tr>
<td>butyl (2-ethylhexyl) phthalate</td>
<td>12.2</td>
<td>17.33</td>
<td></td>
</tr>
<tr>
<td>(9E,12E,15E)-methyl octadeca-9,12,15-trienoate</td>
<td>3</td>
<td>18.08</td>
<td></td>
</tr>
<tr>
<td>(9Z,12Z)-octadeca-9,12-dienoic acid</td>
<td>4.5</td>
<td>18.37</td>
<td></td>
</tr>
<tr>
<td>cis-vaccenic acid</td>
<td>20</td>
<td>18.4</td>
<td></td>
</tr>
<tr>
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<td>5.6</td>
<td>18.42</td>
<td></td>
</tr>
<tr>
<td>Oleamide</td>
<td>2.6</td>
<td>19.52</td>
<td></td>
</tr>
<tr>
<td>2-[(2-ethylhexyl)oxy]carbonyl]benzoic acid</td>
<td>3.1</td>
<td>20.31</td>
<td></td>
</tr>
<tr>
<td>Henicosane</td>
<td>2.2</td>
<td>20.76</td>
<td></td>
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</table>

*rt, retention time (min).

Table 1. Chemical composition of Teucrium polium methanol (MTP) and hexane (HTP) extracts.
Figure 1. Effect of MTP extract on cell viability (A), GLUT4 translocation (B) and major active compound (C). For measuring cell viability (MTT assay and LDH leakage assay) L6/GLUT4myc cells (20,000 cell/well) and exposed to methanol extract for 20 h. For the evaluation of the GLUT4, L6/GLUT4myc cells (150,000 cell/well) were exposed to MTP for 20 h. Serum depleted cells were treated without (-) or with (+)1 µM insulin for 20 min at 37°C and surface myc-tagged GLUT4 density was quantified using the antibody coupled colorimetric assay. Chemical composition analysis was carried out using GC/MS analysis. Values given in A and B represent means ± SEM (% of untreated control cells) of three independent experiments carried out in triplicates. T-test of statistical calculations were conducted using SPSS version 21.0. ** p < 0.01, significant as compared with controls.

Afifi’s report, some of the plants were grown in greenhouse and they might be collected in different season.

Some of the detected compounds in this study were previously reported to possess antidiabetic activity, namely, thymol (Saravanan and Pari, 2015), carvacrol (Ezhumalai et al., 2014), eugenol (Jeong et al., 2014), and cis-vaccenic acid (Alstrup et al., 2004). Moreover, Palmitic acid detected in HTP and MTP was previously reported in active anti diabetic extracts of Ocimum basilicum (Kadan et al., 2016). However, palmitic acid cannot be the sole active compound in HTP and MTP since water extract (where palmitic acid is not expected to be present) also increased GLUT4 activation although it was less effective than HTP and MTP.

TP was reported as the insulin secretion enhancer by regulation transcription factors of the JNK pathway in the pancreatic β-cells (Tabatabaie and Yazdanparast, 2017). However, TP effect on glucose disposal in muscle cell line was not reported. To the best of the authors’ knowledge, this is the first report on the efficacy of the TP extract on GLUT4 activity and translocation to the PM.

Glucose transporter-4 (GLUT4) continuously recycles between the PM and the intra cellular vesicles, and insulin shifts GLUT4 translocation towards the PM. Glucagon, in contrast, shifts GLUT4 translocation towards the intracellular stores (Osorio-Fuentealba and Klip, 2015; Zaid et al., 2008; Zierath et al., 1996). We and others tested the hypoglycemic activity of traditionally used antidiabetic medicinal plants through increasing glucose transporter (GLUT) translocation to the plasma membrane in muscle and hepatic tissue (Kadan et al., 2013, 2016; Ota and Ulrih, 2017; Zaid et al., 2015, 2016). Although TP is recommended by herbal and integrative practitioners for the treatment of diabetes (Mousavi et al., 2015; Saad et al., 2017), the action mechanism whereby TP enhances glucose uptake is not fully understood. Therefore, the present study was conducted to evaluate the role of GLUT4 translocation in the observed antidiabetic TP effects. Three TP extracts (water/ethanol,
Figure 2. Effect of HTP extract on cell viability (A), GLUT4 translocation (B) and major active compound (C). For measuring cell viability (MTT assay and LDH leakage assay) L6-GLUT4myc cells (20,000 cell/well) and exposed to HTP extract for 20 h. For the evaluation of the GLUT4, L6-GLUT4myc cells (150,000 cell/well) were exposed to HTP extract for 20 h and tested as described in Fig. 1 legend. Chemical composition analysis was carried out using GC/MS analysis. Values given in A and B represent means ± SEM (% of untreated control cells) of three independent experiments carried out in triplicates. T-test of statistical calculations were conducted using SPSS version 21.0. *p < 0.05, **p < 0.01, significant as compared with controls.

Figure 3. Effect of WTP extract on cell viability (A), GLUT4 translocation (B). For measuring cell viability (MTT assay and LDH leakage assay) L6-GLUT4myc cells (20,000 cell/well) and exposed to methanol extract for 20 h. For the evaluation of the GLUT4, L6-GLUT4myc cells (150,000 cell/well) were exposed to methanol extract for 20 h as described in Figure 1. T-test of statistical calculations were conducted using SPSS version 21.0. *p < 0.05, significant as compared with controls.
methanol, and hexane) were prepared, and their effects on GLUT4 translocation were measured in L6 skeletal muscle cell line in the present and absence of insulin.

The extent of increase in the insulin-stimulated GLUT4 translocation was additive to that of basal GLUT4 translocation in TP-exposed cells, suggesting the possible synergistic effects between the TP active ingredients and the insulin. Alternately, TP active ingredients might activate GLUT4 translocation in non-insulin dependent pathways (e.g., AMP-Kinase). It is then possible that TP active ingredients might possess insulin-like or insulin-sensitizing activity/compounds. It is essential to dissect TP active compounds to identify its cellular molecular target and to show its specific antidiabetic mechanism(s) and cellular pathway(s).

Insulin enhances the mobilization of GLUT4-containing vesicles from intracellular stores to the muscle cell surface and thus promotes glucose uptake. Notably, the gain in GLUT4 at the muscle membrane is reduced in primary cells from diabetic animals and human diabetic subjects (Shamni et al., 2017; Zaid et al., 2008; Zierath et al., 1996). Hence, understanding the action mechanisms of antidiabetic medicinal plants and determining their potential active ingredients are of paramount importance in developing antidiabetic new drugs.

Conclusion

Phytochemical analysis with GC-MS technique revealed various phytochemical identities in both MTP and HTP extracts. Some antidiabetic compounds were identified in TP extracts, and these compounds maybe responsible on GLUT4 translocation. Further studies regarding the chemical profile of the most active extract (HTP) will be performed to identify the bioactive compound(s).

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