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Portulaca oleracea L. extracts enhance GLUT4 translocation to the surface of muscle cells in insulin dependent and independent fashion

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ABSTRACT

Portulaca oleracea L. is a grassy plant with a fleshy stem, succulent leaves, yellow or white small flowers and small black seeds. *P. oleracea* used as a medicinal plant in many countries, as a diuretic, febrifuge, vermifuge, antiseptic, anti-spasmodic, anti-bacterial, anti-inflammatory and anti-oxidant. It is in use also as anti-diabetic medicinal herb. The aim of this study was to evaluate the effect of the aerial parts of *P. oleracea* 50% ethanol/50% water and methanol based extracts on the glucose transporter-4 (GLUT4) translocation to the plasma membrane of muscle cells. Anti-diabetic activity of the extracts was examined by measuring the relative amount of GLUT4 translocation to the plasma membrane in the presence and absence of insulin. L6 muscle cells, stably expressing myc-tagged GLUT4, repeatedly shown to display insulin regulated GLUT4 traffic, were used as a model in this project. L6 myoblasts were pre-treated with increasing concentrations of *P. oleracea* extracts for 20 h, followed by 3 h serum-deprivation and insulin stimulation for 20 min and then GLUT4myc levels at the plasma membrane was determined. Toxicity of the extracts was determined by MTT assay and LDH leakage assay. Results obtained show no cytotoxic effects in cells treated for 24 h with 50% ethanol and methanol *P. oleracea* extract up to 1 mg/ml and 0.5 mg/ml extract respectively as measured with MTT and LDH-leakage assays. GLUT4 translocation to the plasma membrane was elevated by 2.6 and 4 folds (-/+ insulin) after treatment for 20 h with 1 mg/ml *P. oleracea* 50% ethanol extract and 1.7 and 3 folds (-/+ insulin) when treated with 0.5 mg/ml methanol extract. These results indicate that *P. oleracea* anti-diabetic proporties are mediated in part by increasing GLUT4 distribution in the muscle plasma membrane.

Keywords: *Portulaca oleracea* L., GLUT4 translocation, medicinal plants, diabetes.

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INTRODUCTION

P. oleracea is a warm-climate annual plant. It is widely used as an edible medicinal plant and as coocked food and salad. It is in use in many areas of the world including but not limited to the Middle East, America, Europe, China and other Asian regions. *P. oleracea* is used as anti-septic, anti-bacterial, anti-inflammatory, anti-oxidant and anti-diabetic herbal plant. The Chinese refer to *P. oleracea* as “vegetable for long life” (Rahimi et al., 2019).

P. oleracea phytochemicals content was analyzed and several chemicals were reported such as polysaccharides (Zhao et al., 2015), fatty acids including essential fatty acids: α-linolenic acid (omega-3) and linoleic acid (omega-6) (Uddin et al., 2014). Other compounds were isolated including terpenoids, alkaloids, flavonoids (Rahimi et al., 2019), coumarin (Zhou et al.,

2015) and vitamins (Rahimi et al., 2019). Among the interesting compounds detected in *P*. *oleracea* were noradrenaline and dopamine; two critical neurotransmitters that regulate human behavior and mood (Chen, 2007). *P*. *oleracea* extracts significantly lowered blood glucose levels in treptozotocin diabetic rat (Zhou et al., 2015). The crude polysaccharide extracts of *P*. *oleracea* decresed blood glucose and modulates the metabolism of blood lipids and glucose in diabetic mice model (Gong et al., 2009). *P*. *oleracea* seeds intake lowered the body mass index (BMI), total cholesterol, triglycerides, low density lipoprotein cholesterol fasting and postprandial blood glucose and body mass in human diabetic subjects (El-Sayed, 2011). Oral administration of crude *P*. *oleracea* polysaccharides reduced the fasting blood glucose level, and elevates the fasting insulin level and the insulin sensitivity value in diabetic rats (Bai et al., 2016). A clinical report published in 2016 showed positive effect for *P*. *oleracea*. HbA1c declined significantly more in the *P*. *oleracea* tablets treated group than the placebo group (Wainstein et al., 2016). *P*. *oleracea* has been recently reported to enhance insulin secretion from pancreatic betta cells (Hu et al., 2019). Moreover, Park and colleagues reported recently that *P*. *oleracea* extract enhance glucose uptake in 3T3L1 adipose cells by stimulating GLUT4 translocation to the plasma membrane. They found that (E)-5-hydroxy-7-methoxy-3-(2'-hydroxybenzyl)-4-chromanone is the active phytochemical leading to GLUT4 enhanced translocation to the plasma membrane by activating insulin signaling pathway (Park et al., 2019).

In the present *in vitro* study, the *P*. *oleracea* extracts toxicity and anti-diabetic activity on muscle cell line was evaluated. Results obtained indicate that water/ethanol and methanol extract of the plant, led to a significant gain in GLUT4 translocation at none cytotoxic concentrations as measured with MTT assay and the LDH leakage assay.

MATERIALS AND METHODS

Materials

Fetal bovine serum, α-MEM and all other tissue culture reagents used were from biological industries (Beit Haemek, Israel). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibodies were obtained from Promega (Madison, WI, USA). Polyclonal anti-myc antibody (A-14) and other standard chemicals were purchased from Sigma-Aldrich.

Plant extract preparation

P. *oleracea* (aerial parts) were purchased from (Al Alim- Medicinal Herb Center, Zippori, Israel). The air-dried aerial parts of the plant were powdered, 40 g of the ground plant was then packed in an Erlenmeyer and were extracted with 500 ml of 50% ethanol in water (or either 100% methanol) and heated at 70°C for 30 min and then stirred at room temperature for 24 h to give a dark green extract. The stock extracts were preserved in airtight glass container and kept at -20°C.

Cell culture

Cells from the rat L6 muscle cell line, stably expressing myc-tagged GLUT4 (L6-GLUT4myc), were maintained in myoblast monolayer culture. Cells were grown in incubator under an atmosphere of 95% air and 5% CO_2 in *α*-MEM supplemented with 10% fetal bovine serum (FBS), 0.1 mg/ml streptomycin and 100 U/ml penicillin.

MTT assay

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay was the first homogeneous cell viability assay developed for a 96-well form (Mosmann, 1983). The assay was optimized for the cell lines used in the experiments and was applied to assess cell viability as described by Kadan et al. (2013, 2016). Cells were plated in 96 well plate at a density of 2×10^4/200 µl of medium /well and were allowed to attach to the plate for 24 h. Cells were cultivated with 50% ethanol and methanol *P*. *Oleracea* extracts for 24 h at increasing concentrations up to 1 mg/ml for 24 h. The cells medium was then replaced with 200 µl fresh medium/well containing 0.5 mg/ml MTT and incubated for another 4 h darkened in the cells incubator. The supernatant was removed and 100 µl isopropanol/HCl (1 mM HCl in isopropanol) were added per well. The color density was measured at 620 nm with microplate reader (Anthos). Two wells per plate without cells served as blank. All experiments were repeated three times in triplicates. The effect of the plants extracts on cell viability was expressed using the following formula:

Percent viability = (A^{620nm} of plant extract treated sample/A^{620nm} of none treated sample) × 100

Lactate dehydrogenase assay (LDH)

LDH, a cytoplasmic enzyme, release is the consequence of cell membrane breach. Activity of LDH released to the cell culture medium was monitored following the formation of formazan by coupled enzymatic reaction at 492 nm according to the manufacture kit (Promega). Cell membrane breach was defined as the ratio of LDH activity in the supernatant of cells treated with the plant extract compared to the LDH activity released in the vehicle. L6-GLUT4myc cells were seeded as in the MTT assay. After cell attachment, (24 h) cells were treated with increasing concentrations of the plant extracts (0 to 1 mg/ml). The extracellular LDH activity was measured in the medium after 24 h. Therefore, 50 µl from each well was transferred to a new 96 well plate; the enzyme reaction was carried out according to the manufacture kit (CytoTox 96, Promega) (Kadan et al., 2018). All experiments were repeated three times in triplicates. The effect of the plants extracts on cell viability was expressed using the following formula:

Percent viability = (A492 nm of plant extract treated sample/A^{492nm} of control) × 100

Determination of surface GLUT4myc

Surface myc tagged GLUT4 was measured in intact, non-permeabilized cells as previously described (Zaid et al., 2009) using anti-myc antibody followed by secondary antibody conjugated to horseradish peroxidase. Cells grown in 24-well plates for one day followed by addition of the plant extracts for 20 h and serum-starved for 3 h were treated without or with 100 nM insulin for 20

min. The cells washed twice with ice-cold PBS, fixed for 15 min with 3% paraformaldehyde, blocked 10 min with 5% (v/v) goat serum, and reacted with polyclonal anti-*myc* antibody (1:200) for 1 h at 4°C, washed 10 times with PBS and reacted with horseradish peroxidase-bound goat anti-rabbit secondary antibody (1:1000) for 1 h at 4°C, and washed 10 times with PBS. Cells then incubated with 1 ml *o*-phenylenediamine dihydrochloride reagent and allowed to develop for 20–30 min in the linear range in the dark at room temperature. The reaction was stopped with 0.125 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 limits cited and error bars plotted represent simple standard deviations of the mean.

RESULTS

The extracts effect on the muscle cells toxicity by examined MTT (Figure 1A and Figure 2A) and LDH leakage assay (Figure 1B and Figure 2B) as described in the methods section. The methanol extract was nontoxic on the L6-GLUT4myc cells up to 0.5 mg/ml as it led to about 8% death only. However, 1 mg/ml led to about 55% death (Figure 1A). The water/ethanol extracts was safer as it was not toxic at all the concentrations tested up to 1 mg/ml (Figure 2B).

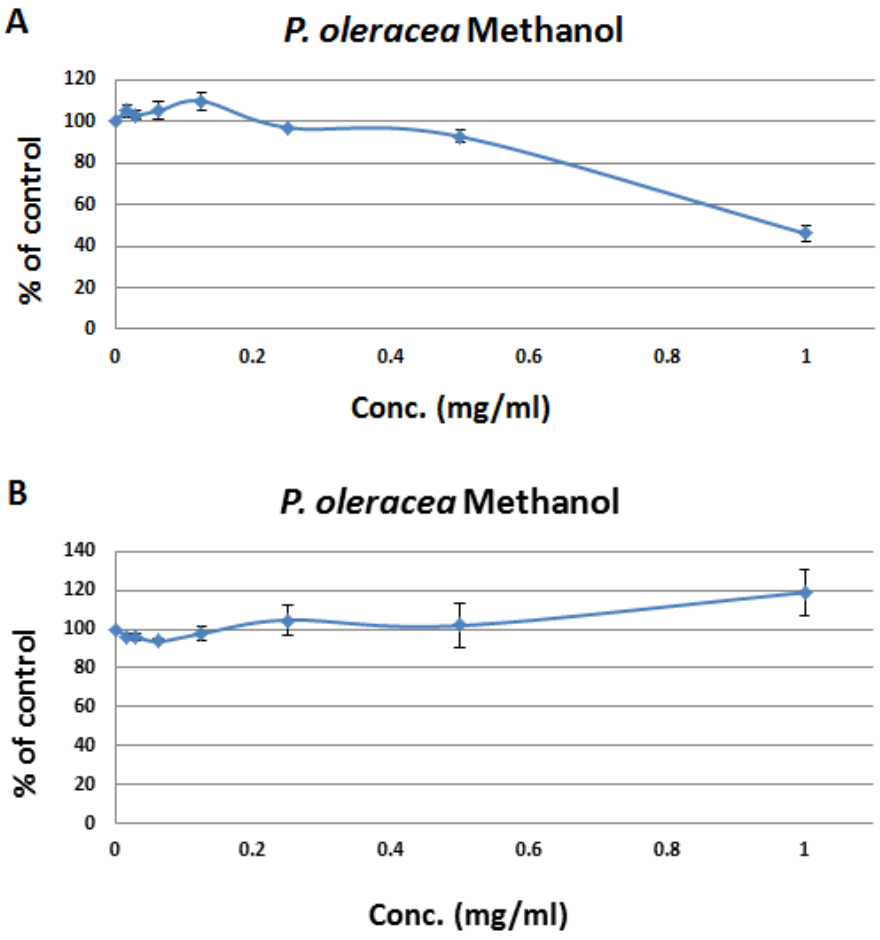

Figure 1. Effect of *P. oleracea* methanol extract on cell viability by MTT assay (A) and LDH leakage assay (B). L6-GLUT4myc cells (20,000 cell/well) were exposed to methanol extract for 24 h. Values given represent means ± SEM (% of untreated control cells) of three independent experiments carried out in triplicates.

The efficacy of the *P. oleracea* Methanol and 50% ethanol extracts in treating insulin resistance were examined by the GLUT4 translocation assay. Insulin causes GLUT4 translocation to the surface of myoblasts, where it mediates the increase in glucose uptake. To examine the contribution of the plant extract to GLUT4

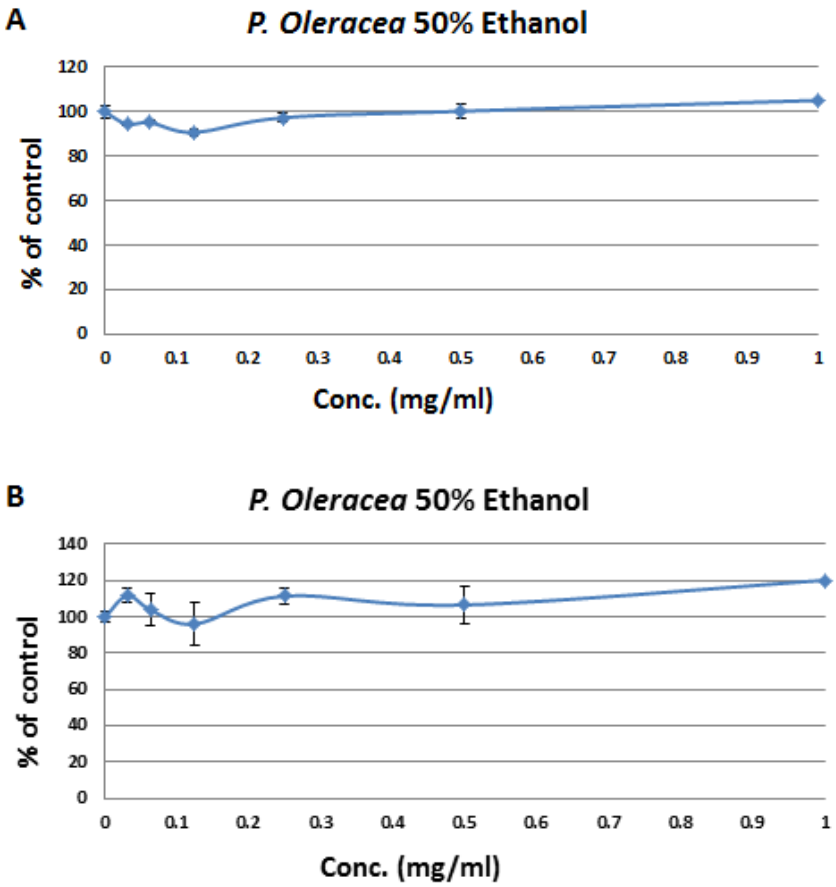

Figure 2. Effect of *P. oleracea* 50% ethanol extract on cell viability by MTT assay (A) and LDH leakage assay (B). L6-GLUT4myc cells (20,000 cell/well) were exposed to 50% ethanol extract for 24 h. Values given represent means ± SEM (% of untreated control cells) of three independent experiments carried out in triplicates.

localization to the plasma membrane, *P. oleracea* extract was added to the L6-GLUT4myc cells in the presence or absence of insulin and GLUT4myc translocation to the plasma membrane was monitored by ELISA.

Exposing L6-GLUT4myc cells to 0.5 and 1 mg/ml of *P. oleracea* 50% ethanol extract enhanced GLUT4 translocation by 1.6 and 2.6 folds respectively in the absence of insulin and 2.6 and 4 folds respectively in the presence of insulin (Fig. 3 A). GLUT4 translocation to the plasma membrane increased by 1.4 and 2 folds when exposed to 0.25 and 0.5 mg/ml of *P. oleracea* methanol extract in the absence of insulin, respectively. The same extracts concentrations enhanced GLUT4 translocation by 1.7 and 3 folds in the presence of insulin, respectively (Figure 3B).

Taken together, the results clearly indicate that *P. oleracea* is an effective medicinal plant that enhances GLUT4 translocation to the plasma membrane in muscle cells in the presence and absence of insulin. These extracts might be effective in curing insulin resistance and type II diabetes.

DISCUSSION

Type II diabetes is a multi-factorial disease defined by hyperglycemia due to insufficient insulin secretion or insulin resistance in the liver, adipose tissue and muscle (Zaid et al., 2018). Insulin sensitizers and secretagogues are commonly used to lower blood glucose levels (Zaid et al., 2015). Nevertheless, these agents might lead to hypoglycemic shock. Accordingly many research groups seeking safer drugs especially from natural and herbal origin (Zaid et al., 2016).

P. oleracea is an annual plant used as a medicinal herb for type II diabetes patients(El-Sayed, 2011). In addition, *P. oleracea* is reported to be beneficial in diabetes related physiological disorders such as hyperlipidemia and obesity (Bai et al., 2016). *P. oleracea* comprises several compounds, mainly flavonoids, coumarins, alkaloids and

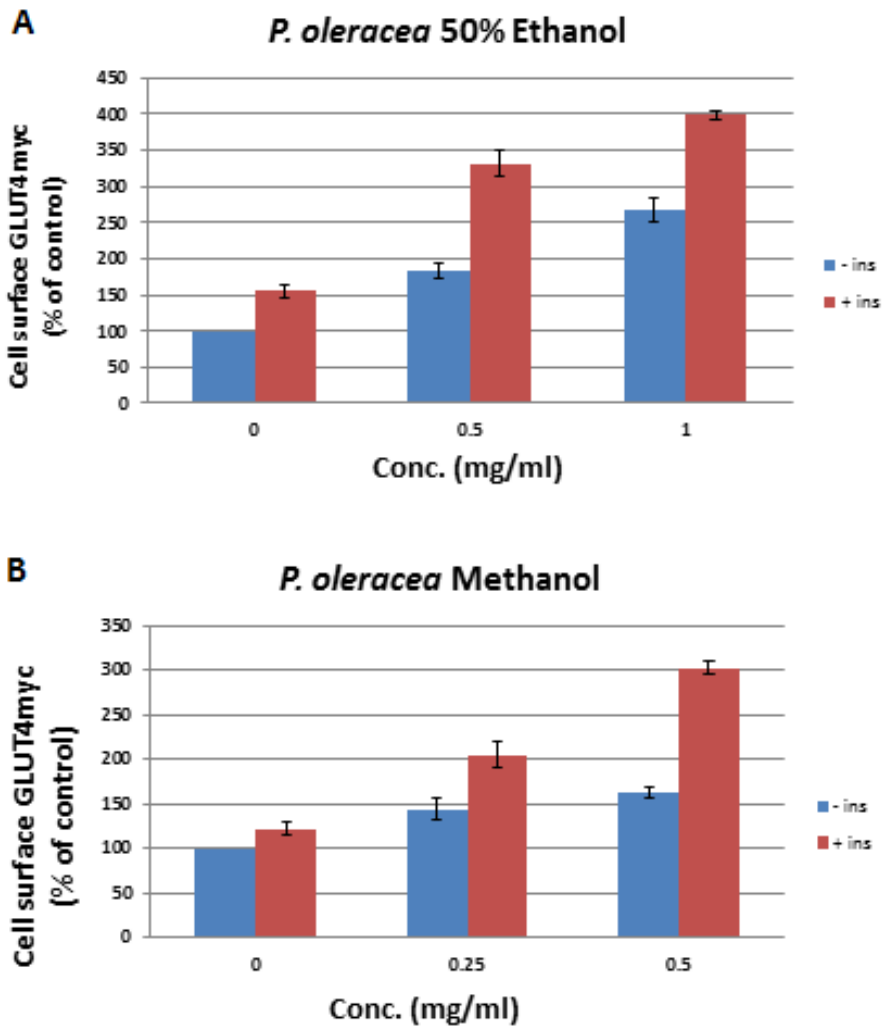

Figure 3: Effect of *P. oleracea* extracts on GLUT4 translocation. L6-GLUT4myc cells were seeded in 24 well plate (150,000 cell/well) and exposed to *P. Oleracea 50%* Ethanol extract (A) and Methanol extracy (B) for 24 h. Serum depleted cells were treated without (-) or with (+) 100 nM insulin for 20 min and at 37°C and surface *myc*-tagged GLUT4 density was quantified using the antibody coupled colorimetric assay. Shown are the means +/- SEM (standard error mean) relative to basal non treated cells from three independent.

terpenoids (Liu et al., 2015; Zhou et al., 2015). More telling, a report by Park and Han show that 80% ethanol *P. oleracea* extract stimulated insulin secretion via a K+/ATP channel dependent pathway in INS-1 β-cells (Park and Han, 2018). Interestingly, flavonoids isolated from *P. oleracea* increased glucose uptake through activating the AMPK and PI3K/AKT pathways in 3T3-L1 adipocytes (Park and Han, 2018). Concomitant with our results, these *P. oleracea* based chemicals induced GLUT4 translocation to the plasma membrane in 3T3L1 adipose cells (Park et al., 2019). Our study showed that treating L6 muscle cells with either methanol or 50% ethanol of *P. oleracea* extracts augmented GLUT4 translocation to the plasma membrane in insulin and non-insulin dependent manner. Both extracts displayed similar effect at the same concentration. Indeed, the increase in GLUT4 translocation to the plasma membrane plays a pivotal role in glucose clearance from the circulation.

Some studies proposed that GLUT4 intrinsic activity is regulated (Shamni et al., 2017) and that insulin might activate GLUT4 within the membrane (Ishiki et al., 2005). On the other hand, other studies proposed that GLUT4 activation in plasma membrane and translocation to the plasma membrane are distinct events (Teixeira et al., 2012). The finding that *P. oleracea* extracts augmented the insulin-stimulated GLUT4 translocation to higher extend comparer to non-insulin stimulated cells suggests that the membrane localized transporters were similarly amenable to *P. oleracea* extracts-induced modulation.

Taken together, the results presented here clearly indicate that *P*. *oleracea* is an effective herbal source for GLUT4 translocation enhancement to the plasma membrane in muscle cells. *P*. *oleracea* extracts seem to have no effect on GLUT4 intrinsic activity. *In vivo* studies are essential before prescribing these products for diabetics.

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