Attenuation of palmitate induced insulin resistance in muscle cells by harmala, clove and river red gum

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Abstract: The present study aimed to decipher the mechanism of action of selected anti-diabetic plants extracts on palmitic acid mediated insulin resistance in muscle cells. Our results showed that extract from *Peganum harmala* seeds, *Eucalyptus camaldulensis* and *Syzygium aromaticum* leaves, showed significant antioxidant activity. We found that these extracts were able to affect stress signalling by reducing p-38 MAP kinase phosphorylation. They also reduced phosphorylation of substrate for insulin receptor (IRS) at serine residues and increased its phosphorylation at tyrosine residues and also enhanced PKB phosphorylation. Glucose uptake was also enhanced in muscle cells after treatment with these extracts. Extracts from Lantana camara, Psidium gujava fruit and different parts of *Cassia alata* did not affect FFA mediated down-regulation of insulin signalling. The study conclude that seeds of *Peganum harmala* and leaves of Eucalyptus camaldulensis and *Syzygium aromaticum* enhanced insulin signal transduction and glucose uptake in muscle cells via reducing oxidative stress. As a result, these herbal extracts may be considered useful to protect from insulin resistance.

Keywords: Insulin signalling; medicinal plants; antioxidant; insulin resistance; diabetes.

INTRODUCTION

Approximately, 382 million people worldwide have diabetes. In 2013, Pakistan had 7.1 million people suffering from diabetes. This number is likely to rise to 13.8 million by 2030 (Federation, 2013). Insulin is the major hormone that maintains metabolic homeostasis in the body (Taniguchi *et al*., 2006). Defects in insulin signal transduction are the key events in the progress of insulin resistance and diabetes mellitus (Bruning *et al*., 1997). Muscles are responsible for the highest levels of insulin-stimulated glucose clearance (Tsuchiya *et al*., 2010). Excess dietary lipid intake and exposure to free fatty acids (FFAs) are strongly associated with the pathogenesis of muscle insulin resistance. In humans, enhanced plasma FFAs cause insulin resistance (Reaven *et al*., 1988). FFA mediates muscle insulin resistance by several mechanisms including inflammation and oxidative stress by activating several kinases such as PKCs, IKK β and JNK and p38 MAP kinase (Bonnard *et al*., 2008). These kinases play major role in blocking insulin signaling by serine phosphorylation of insulin receptor substrate proteins (Martins *et al*., 2012). Consequently, downstream of insulin receptor signalling, uptake of glucose and other cellular functions are diminished (Boden & Shulman, 2002). Though, there are well known approaches to alleviate insulin resistance in type 2 diabetes patients, nevertheless, there is still need for alternative therapies. South Asian region has a large distribution (36%) of anti-diabetic herbal plants (Chan *et al*., 2012). Paradoxically, anti-diabetic plants are not being used in standard diabetes care because of lack of knowledge about their mechanism of action. Present situation necessitate studies on action mechanism of anti-diabetic folk medicinal plants. We hypothesized that folklore anti-diabetic plants could alleviate insulin resistance in muscle cells. We selected 6 plants used in the folk medicinal system for the treatment of diabetes but their mechanism of action is still unknown (Debjit bhowmik, 2012; Karou *et al*., 2011; Palanichamy *et al*., 1988; Patel *et al*., 2012; Saxena, 2012; Varghese *et al*., 2013).

MATERIAL AND METHODS

Preparation of plant extracts

The plant materials were taken from Karachi. Senior taxonomist from department of Botany, university of Karachi, identified the plants. The voucher specimens were also deposited in the herbarium. Each plant, *Lantana camara* L. (LC) (KUH No. 63482), aerial parts; Carissa carandas L. (KUH No. 86465) leaves; *Syzygium aromaticum* L. (KUH-GH No. 06235) leaves; *Eucalyptus camaldulensis* leaves (GH No. 85851); Psidium gujava (KUH-GH No. 53976) fruit; *Peganum harmala* (KUH-GH No. 53856) seeds; *Cassia alata* (KUH No. 86465) stem, *Cassia alata* beans, and *Cassia alata* leaves; were cut into small pieces and drenched in methanol (HPLC grade) at room temperature. The extract was decanted

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after 5-7 days. The process of soaking residue in methanol continued for 5-7 days and decanting was done five times and the extracts were combined. The solvent was removed under vacuum, using rotary evaporator to obtain residue which was used for testing antidiabetic activity. The concentrated extract was filtered, and dried under vacuum by using a rotary evaporator to obtain the experimental extract. The stock solution of the extract was prepared (100mg/ml) by dimethyl sulfoxide (DMSO).

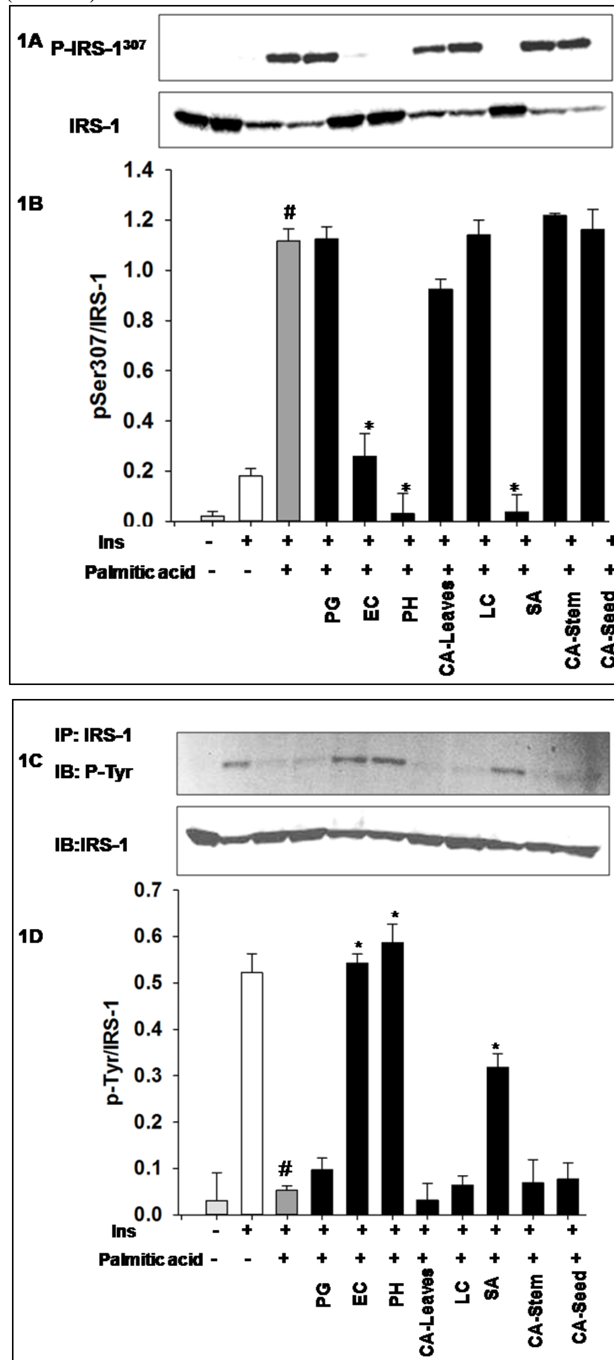

Fig. 1: Determination of effect of plant extracts on phosphorylation of insulin receptor substrate-1 (IRS-1). (A) C2C12 cells were treated with insulin (100nM, 30 min) alone as positive control or also with palmitic acid (200µM) and/or plant extracts (50µg/ml) for 24 hr. Immunoblotts with phospho-Ser307 antibody. (B) Phosphorylation intensity of serine phosphorylation of IRS-1 quantified based on densitometry of immunoblots normalized for IRS-1 protein. (Mean ± SE, n=3, # $p<0.05$ insulin vs. FFA alone, *$p<0.05$ extract vs. FFA alone). (C) Cells were lysed and IRS-1 was immunoprecipitated with a polyclonal IRS-1 antibody (IP) and immunoblotted (IB) with the phosphotyrosine antibody. (D) Tyrosine phosphorylation of immunoprecipitated IRS-1 was quantified based on densitometry of immunoblots normalized for IRS-1. (Mean ± SE, n=3, # $p<0.05$ insulin vs. FFA, *$p<0.05$ extract vs. FFA).

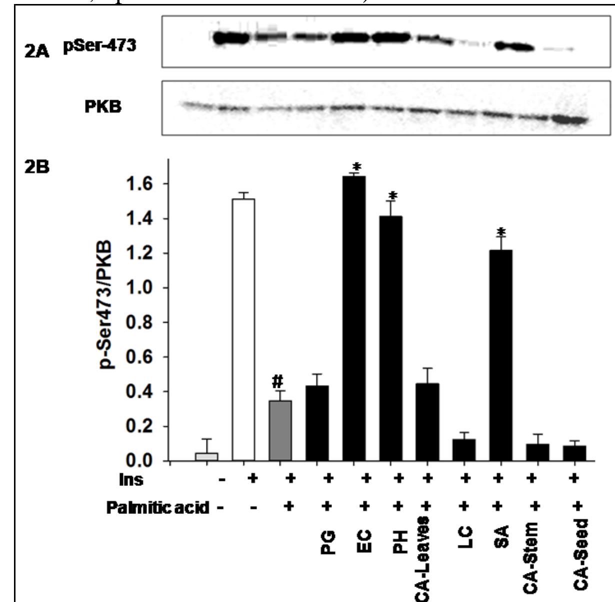

Fig. 2: Determination of effect of plant extracts on PKB phosphorylation. (A) C2C12 cells were stimulated with insulin (100nM, 30min) alone as positive control or also with Palmitic acid (200µM, 24 hr) or plant extracts (50 µg/ml, 24hr). The phosphorylation of Ser 473 of Akt was investigated. (B) Phosphorylation intensity of Ser 473 of PKB was quantified based on densitometry of immunoblots normalized for PKB protein (Mean ± SE, n = 3, # $p<0.05$ insulin vs. FFA, *$p<0.05$ extract vs. FFA).

Cell culture, cell lysis and immunoprecipitaion

Dulbecco's modified Eagle's medium (with 25mM glucose) was used as culture medium for the growth of cells. The medium was supplemented with, 100 units/ml penicillin, 2mM glutamine, 100µg/ml, streptomycin and 10% fetal calf serum. Muscle cells were stimulated (for 24 hours) with plant extracts and/or palmitic acid (200µM). Since insulin activate insulin signal transduction therefore cells treated with insulin alone were taken as positive control. Cells were either stimulated or without insulin (100nM). Protein was extracted from muscle cells with lysis buffer (containing protease and phosphatase inhibitors along with 1% Triton X-100, 150mM NaCl and 50mM Tris, pH 7.6). Volumes

constituting 50µg proteins were separated by sodium dodecyl sulfate, polyacrylamide gel electrophoresis. Western blot was performed as described previously (Waraich *et al*., 2008). For immunoprecipitation, 400µg protein was used followed by sodium dodecyl sulfate, polyacrylamide gel electrophoresis and Western blot analysis (Waraich *et al*., 2008).

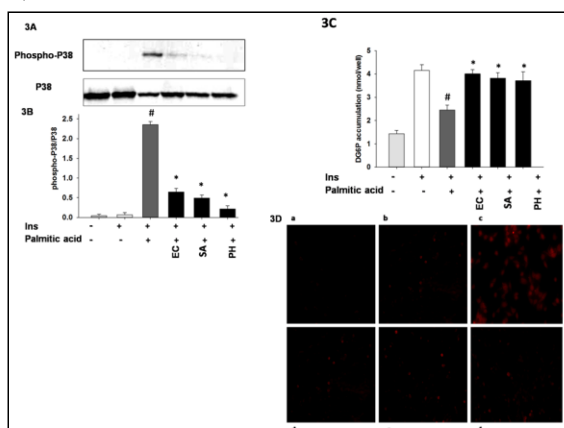

Fig. 3: Determination of effect of plant extracts on stress kinase. The phosphorylation of p38MAPK at Thr180/Tyr182 was investigated. (B) Phosphorylation intensity of phspho-p38MAPK was quantified based on densitometry of immunoblots normalized for p38MAPK protein (Mean ± SE, n = 3, # p<0.05 insulin vs. FFA, *p< 0.05 extract vs. FFA). (C) Effect of plant extracts on glucose uptake. C2C12 cells were treated with non-radiolabeled 2DG. Data are shown (Mean ± SE, n=3, # p <0.05 insulin vs. FFA, *p<0.05 extract vs. cells FFA). Abbreviations: DG6P, 2-deoxyglucose-6-phosphate; 2DG, 2-deoxyD-glucose. (D) Effect of plant extracts on ROS production. ROS generation was detected by DHE probe on fluorescence microscopy (×200): (a) Cells without FFA and insulin; (b) Cells with insulin alone (positive control) (c) Cells with insulin and FFA; (d) Cells with insulin, FFA and *Eucalyptus camaldulensis* extract; (e) Cells with insulin, FFA and *Peganum harmala* extract (f) Cells with insulin, FFA and *Syzygium aromaticum* leave extract.

Cell lines, reagents and antibodies

Cell culture media and supplements were from Gibco (Sigma, St.Louis, USA), fetal calf serum (Biowest, Brazil); and the antibodies against IRS-1 serine 307 from Millipore, IRS-1 protein, phospho Tyrosine (p)100, phospho Akt Serine 473, Akt protein, phospho-p38MAPK from Cell signaling (Beverly, US). Palmitic acid and insulin were from Sigma (St. louis, US). C2C12 cells were purchased from American Type Culture Collection (ATCC) (Virginia, USA). Glucose-6-phosphate dehydrogenase (G6PDH) from MP Biomedicals (France).

Cell viability assay

Viability of C2C12 cells post exposure to the plant extracts was determined by MTT assay. After seeding, cells were treated with different concentrations of the selected plant extracts. After 24 hrs, MTT was added for 3 h. DMSO was also added (to lyse the cells). The absorbance was measured using ELISA plate reader (Molecular devices Spectra Max 340, USA), at a wavelength of 540 nm (OD_{540}).

DPPH assay

DPPH assay was carried out to determine the ability of the plant extracts to scavenge free radicals as described by (Kerasioti *et al*., 2014). Scavenged percentage of the DPPH by the extract was calculated as difference of absorbance reading between plants extract and the control. (Ascorbic acid was used as the standard).

Glucose uptake assay

C2C12 cells were treated with palmitic acid and/or extracts for the indicated time. Then the cells were either stimulated or not stimulated with insulin. Muscle cells were then washed twice with HEPES buffer with 0.1% BSA. Myoblasts were incubated with KRPH buffer with 1mM 2DG and 0.1% BSA for 20 min in 5% CO_2 at 37°C. Muscle cells were washed twice with KRPH buffer then 25ul of 0.1N NaOH was added. The components in the wells were then neutralized by adding 25ul of 0.1N HCl. Uptake of 2DG6P into the mayoblasts was determined by enzymatic fluorescence assay using microplate reader (Molecular devices SpectraMax M5e. USA) as described by (Yamamoto *et al*., 2006).

Superoxide levels by dihydroethidium (DHE)

Superoxide was detected by DHE as described previously (Fiory *et al*., 2011). Mayoblasts were incubated with HBSS containing 5µM DHE. The fluorescence of muscle cells was recorded by fluorescent microscopy.

STATISTICAL ANALYSIS

Results presented as means ± SEM. Groups of data were compared using ANOVA. A p-value ≤0.05 was set as statistically significant.

RESULTS

Effect of anti-diabetic plant extracts on cell viability.

The ability of the cells to survive exposure to extracts of selected plants was evaluated by MTT cytotoxicity test. It was found that the cells exposed to *Syzygium aromaticum, Psidium guajava, Cassia alata* leaves, stem and seed extracts showed comparatively higher cytotoxicity against C2C12 cell line: whereas, *Eucalyptus camaldulensis, Peganum harmala* and *Lantana camara* were moderately cytotoxic to the cell line . CTC_{50} values (concentration of the sample required to kill 50% of the cells) were determined by MTT assay (table S1). The concentrations of the extracts not affecting cell viability were selected for the cell culture.

Supplementary material

Table S1: *In vitro* cytotoxicity screening

Psidium guajava, Fruit (PG)	500 250 125 62.5	325	Cassia alata, leaves (CA-Leaves)	500 250 125 62.5	345
Peganum harmala (PH)	500 250 125 62.5	430	*Syzygium aromaticum*, leaves (SA)	500 250 125 62.5	345
Eucalyptus camaldulensis (EC)	500 250 125 62.5	441	*Cassia alata* stem (CA-Stem)	500 250 125 62.5	300
Lantana camara (LC)	500 250 125 62.5	412	*Cassia alata,* seed (CA-Seed)	500 250 125 62.5	350

Determination of CTC_{50} of Plant extracts by MTT assay in C2C12 cell cultures. Viability of C2C12 cells was measured after exposure to different doses of plants extracts by MTT.

Fig. S1

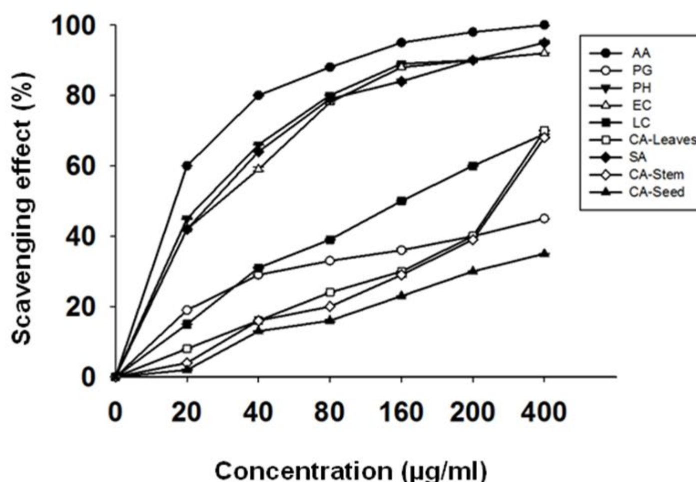

Fig. S1: Free radical scavenging activity of plant extracts. Antioxidant activity of plant extracts was measured using the DPPH assay. Results are Mean ± SE, n = 3: AA (Ascorbic acid).

Determination of antioxidant capacity of anti-diabetic plant extracts

The DPPH scavenging activity of the extracts enhanced in a concentration dependent manner. The extracts of *Peganum harmala, Eucalyptus camaldulensis* and *Syzygium aromaticum* demonstrated complete scavenging activity against the DPPH radical followed by rest of the extracts as shown in fig. S1.

Effect of plant extracts on phosphorylation at serine residues of insulin receptor substrate proteins

To determine the effect of potential local anti-diabetic medicinal plant extracts in reducing FFA induced serine phosphorylaion of IRS-1in muscle cells, C2C12 cells were stimulated with palmitic acid and treated with the plant extracts. It was found that extracts from *Peganum harmala, Eucalyptus camaldulensis* and *Syzygium aromaticum* showed significant diminished phosphorylation at serine 307 of insulin receptor substrate-1 in myoblasts as compared to cells stimulated with FFA alone (fig. 1A, 1B).

Effect of plant extracts on phosphorylation at tyrosine residues of insulin receptor substrate proteins

Muscle cells were treated with antidiabetic medicinal plants to evaluate their capability to restore FFA mediated

down regulation of tyrosine phosphorylation. Significantly enhanced phosphorylation was found for immunoprecipitated IRS-1 after FFA and *Peganum harmala, Eucalyptus camaldulensis* and *Syzygium aromaticum* treatment as compared to cells treated with FFA alone (fig. 1C, 1D).

Assessment of medicinal plants extracts on PKB phosphorylation

In order to decipher action mechanism of the selected antidiabetic plants i.e can they rescue FFA induced diminished PKB phsphorylation? When C2C12 cells were treated with *Peganum harmala, Eucalyptus camaldulensis* and *Syzygium aromaticum* plants extracts, and stimulated with insulin, PKB phosphorylation at serine 473 was restored, while rest of the extracts did not show their effects on PKB activation (fig. 2A, 2B).

Determination of mechanism of restoration of insulin signal transduction

P38MAPK activity was evaluated in response to combination of FFA and the selected plant extracts. It was found that after free fatty acid stimulation, insulin stimulated C2C12 cells showed increased p38MAPK phosphorylation, while this phosphorylation was significantly diminished after *Peganum harmala, Eucalyptus camaldulensis* and *Syzygium aromaticum* extract treatment (fig. 3A, 3B).

Determination of the effect of medicinal plants on glucose uptake in muscle cells

Activation of PKB indicated potential effect of these medicinal plants on glucose uptake. Therefore, we measured glucose uptake in C2C12 by enzymatic fluorescence assay (Yamamoto *et al*., 2006). It was found that treatment with extracts from *Syzygium aromaticum, Peganum harmala* and *Eucalyptus camaldulensis* was able to restore insulin effect on glucose uptake in C2C12 cells (fig. 3C).

Role of medicinal plants on reactive oxygen species (ROS) production in muscle cells

In order to further validate the antioxidant capacity of *Peganum harmala, Eucalyptus camaldulensis* and *Syzygium aromaticum*. The antioxidant property of plant extracts was evaluated with ROS-activated fluorescent dye DHE (fig. 3D). Superoxide level was increased in C2C12 cells stimulated with FFA as compared to the cells stimulated with insulin alone. Further, treatment of the cells with the three plant extracts showed significant reduction in FFA induced ROS production in muscle cells.

DISCUSSION

In this study the deciphering mechanism of action of the selected antidiabetic plants in muscles was studied. We found that extracts showed restoration of proximal insulin signalling (fig. 1A, C). Studies shows that, FFA and oxidative stress in muscle cells, activate stress signalling pathways such as JNK and p38 MAP kinase and thus mediate insulin resistance (Blair *et al*., 1999; Hemi *et al*., 2011; Ragheb *et al*., 2009). Since extracts showed comparable antioxidant activity as ascorbic acid (fig. S1) and reduced ROS production in muscle cells (fig. 3D), therefore, their ability to diminish stress signalling pathway was assessed. Accordingly, these extracts were able to reduce p38 MAP kinase phosphorylation (fig. 3A). Aditionally, extracts from seeds of Harmala and leaves of River Red Gum and Clove reduced serine phosphorylation of IRS-1, accompanied by increased tyrosine phosphorylation and enhanced Akt phosphorylation, thereby indicating the possible mechanism of reducing insulin resistance by these extracts. It was also, established that upregulation of insulin siganling by the extracts was also reflected in glucose uptake measurements (fig. 3C). It is for the first time been shown the effect of *Peganum harmala* and Eucalyptus camaldulensis leaves extract on decreasing FFA mediated insulin resistance in muscle cells. Though, *Syzygium aromaticum* have been reported to increase the glucose uptake in liver cells (Prasad *et al*., 2005) but role of its leave extract in muscle cells was poorly known. This study shows its positive role in glucose uptake and insulin signalling in muscle cells. Guava fruit, *Cassia alata* and *Lantana camara* did not showed rescue of insulin signalling after FFA exposure. Though, extracts of guava leaves enhanced glucose metabolism in rats by alleviating insulin resistance in muscle cells. Similarly *Cassia alata* reduces hyperglycemia in rats probably by inhibiting carbohydrate digestion (Palanichamy *et al*., 1988; Varghese *et al*., 2013). However, the presented data show that guava fruit has no effect on FFA mediated insulin resistance. In conclusion, it is reported that seeds of *Peganum harmala* and leaves of *Eucalyptus camaldulensis* and *Syzygium aromaticum* extracts have shown the potential to alleviate free fatty acid mediated down regulation of insulin signal transduction and hence can reduce insulin resistance. Further these data validate the use of these extracts as traditional medicine and support the value of these extracts as therapeutic targets for treatment of diabetes. Considering the modulating effect of the extracts on insulin resistance, further *in vivo* and clinical studies on these extracts are well merited.

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