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INSIGHT INTO ANTI-DIABETIC POTENTIAL OF *Clematis apiifolia* : GLUCOSE CONTROL IN DIABETES

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Antidiabetic activity of the methanolic extract of *Clematis apiifolia* was investigated in this research study. Methanolic, ethanolic and aqueous extracts were prepared and evaluated for three of the major (α-glucosidase, Antiglycation and Protein Tyrosine Phosphatase 1B) inhibitory assays. Cytotoxicity and Glucose consumption were studied in L6 cell line followed by PCR and Western blotting. Chemically induced diabetic mice model was used for *in vivo* antidiabetic activity. All the three extracts have shown significant enzymatic inhibitory activities while methanolic extract proved to be more compelling. Methanolic extract has shown no cytotoxicity in L6 cell line and promoted glucose consumption, with the highest glucose uptake at 1mg/mL, after treatment for 12 hours. These treatment conditions (1mg/mL, incubation time 12 hours) resulted in up regulation of mRNA level of GLUT4 while PTP1B get downregulated. Protein expression studied in western blot has given the results in line with results of PCR. *In vivo* study has shown the hypoglycemic effect of methanolic extract in diabetic mice comparable to standard drug glibenclamide.

Keywords: Diabetes, *Clematis apiifolia*, α-glucosidase, Antiglycation, Protein tyrosine phosphatase 1B(PTP1B), L6 cell line.

INTRODUCTION

Diabetes is characterized by hyperglycemia caused due to either less insulin production or cells become resistant and fail to sense insulin level (Alam *et al.*, 2014). Biochemical and physiological reactions get severely altered in diabetes. Insulin resistance is mainly caused by dysregulation in glucose metabolism, resulting in development of diabetes mellitus type 2 (Eriksson, 2007). Glucose transportation is mediated by either Glucose transporters (GLUTs) or Sodium dependent Glucose Transporters known as (SGLTs) (Harada and Inagaki, 2012). According to published data, any disturbance which can cause upregulation of any of these can lead to hyperglycemia (Hansen *et al.*, 2014; Jie *et al.*, 2015)

Skeletal muscles are the major target site for insulin mediated glucose homeostasis. Glucose transporter is a family of protein responsible for glucose translocation in muscles and fat tissues. Glucose transporter-4 (GLUT4) is one of the important member of this family and according to recent studies it is suggested that loss of its expression or translocation results in pathogenesis of Type 2 diabetes (Abel *et al.*, 2001; Lacombe, 2014). L6 cells are extensively used in antidiabetic research as they present a good model for glucose uptake (Ammerman *et al.*, 2008). Insulin is being negatively regulated by Protein tyrosine phosphatase 1B, which has proven to be a major target in treatment of diabetes type 2 (Wang *et al.*, 2012).

Many synthetic drugs are being used to lower hyperglycemia but still there is no such therapy which can lead to improvements in repercussion of insulin resistance. Majority of pharmacological approaches are focused on reducing hyperglycemia and intervening the complications through diet but there is no direct remedy to treat diabetes is available yet (Patil *et al.*, 2017).

One of the ways to control diabetes is use of traditional medicines. These herbs are cost effective with very fewer side effects when compared to drugs. More than 800 medicinal plants are being reported to have anti diabetic properties. These plants inhibiting the enzymes involved in glucose production or by enhancing insulin secretion (Arumugam *et al.*, 2013).

Clematis apiifolia is well known for its anti-obese properties but some of its biochemical aspects like antiglycation and PTP1B inhibition still needs to be explored. This paper describes inhibitory potential of various extracts of *Clematis apiifolia* against alpha glucosidase, AGES formation and PTP1B enzymatic activity along with cell line and *in vivo* study to have a deeper look into hypoglycemic potential of the plant.

MATERIALS AND METHODS

***Collection and preparation of plant extracts*:** *Clematis apiifolia* plants were collected from Elum Mountain, Swat Pakistan during spring season 2018. Plants were identified by Mehboob ur Rahman from Govt. Post Graduate Jehanzeb College Swat, Pakistan, and submitted to herbarium. Collected plants were thoroughly washed with water. Aerial parts of plants were shed dried with complete loss of moisture; stem and leaves were finally ground to fine powder.

***Extract preparation*:** Plant extracts were prepared in three solvents i.e., ethanol, methanol, and water. Dried powder was extracted in ethanol (1:10), kept in shaking incubator for 24 hours followed by centrifugation at 10,000 rpm for 15 min. The extract was then filtered into labeled container and residue was re-dissolved in methanol, followed by extraction in water. The evaporation of all extracts was carried out in water bath until complete drying. For enzyme assays the dried extracts were re-dissolved in DimethylSulfoxide (DMSO), while for cell based and *in vivo* study, dissolved in respective biological buffers.

***α-Glucosidase Inhibitory Assay*:** The α-glucosidase inhibitory activities of methanolic, ethanolic and aqueous extracts were carried out according to previous study (Shai *et al.*, 2011). Briefly, a 50µL of plant extracts at varying concentrations was mixed with 50µL of 50 mM phosphate buffer (pH 6.8) and 50µL of α-glucosidase (Cat. No. #G5003, Sigma-Aldrich) (0.5 unit/mL), followed by incubation at 37°C for 5 min. Substrate of the enzyme (1 mM p-nitrophenol- α-D glucopyranoside) (Cat. #N1377, Sigma-Aldrich) was then added to reaction mixture followed by incubation at 37°C for 20 min. By addition of 50µL Na2CO3 (100µM), reaction was being stopped. The absorbance was read at 405 nm. Control used has same ratio of all components except that sample was replaced by buffer. Following equation was used to calculate percentage of inhibition:

$$\% \text{ Inhibition} = (1 - \mathbf{A_T}/A_C) \times 100$$

Where A_T: absorbance of test, A_C: absorbance of control.

***Advanced Glycation end products formation Inhibitory Assay*:** Already well known method was adopted for advanced glycation end products inhibition assay with little modification (Choudhary *et al.*, 2011). Final reaction mixtures in all the experiments were 1200µL comprising of 400µl bovine serum albumin (BSA) (10 mg/mL), 400µL of glucose anhydrous (50 mg/mL) and 400µLof *Clematis apiifolia* extracts. Enzyme assay was done in 96 well plates. Glycated control had same components except sodium phosphate buffer in place of plant extract while blank control contained sodium phosphate buffer and BSA only. Plates were then incubated at 37 °C for 7 days. After incubation period, 120µL of trichloroacetic acid (TCA) was added followed by centrifugation at 15,000 rpm for 4 min at 4 °C. Pellets formed were then re-washed with 120µL of 10% TCA. Supernatant was discarded, and phosphate buffer solution (PBS) was used to dissolve the pellets. Fluorescence spectrum was assessed by using Spectro-fluorophotometer (RF-5301PC, Shemadzu, Japan). The results were calculated as:

% Inhibition = [1 - (Absorbance extract/Absorbance control)] x 100.

***Protein tyrosine phosphatase 1B Inhibitory Assay*:** Ethanolic, methanolic and aqueous extracts of *Clematis apiifolia* were being assessed for their PTP1B inhibitory potential. Enzyme inhibitory activities of the extracts were evaluated by using p-nitrophenyl phosphate (p-NPP) as a substrate for PTP1B(Nguyen *et al.*, 2015). PTP1B (0.05-0.1µg) was dissolved in buffer containing 50 mM citrate (pH 6.0), 0.1 M NaCl, 1 mM EDTA, and 1 mM dithiothreitol (DTT). Reaction mixture contained 2 mM pNPP and PTP1B with or without plant extract, followed by incubation at 37 °C for 30 min. Reaction was then terminated by addition of 10N NaOH. Absorbance was measured at 405nm to assess the amount of p-nitrophenol produced. Methanolic extract was further being run in dilutions to calculate IC_{50} value. Percentage of inhibition was calculated by formula.

$$\% \text{ Inhibition} = 1 - [(A_{\text{Sample}} - \mathbf{A}_{\text{Blank}}/A_{\text{Control-Blank}})] * 100$$

***Cell culture*:** Rat L6 myoblasts were cultured and maintained in Dulbecco's modified eagle's medium containing 10% fetal bovine serum and 1% Pen-Strep in incubator at 37°C with 5% CO_2. After 24h of incubation plates get confluent with cells, media were then replaced with Dulbecco's modified eagle's medium containing 2% fetal bovine serum and 1% Pen-Strep to induce differentiation of myotubes. After 48 h of differentiation, L6 myotubes were serum starved for 18 h in Dulbecco's modified eagle's medium having 0.2% bovine serum albumin and 1% Pen-Strep prior to experiments (Yap *et al.*, 2007).

***Cytotoxicity in L6 cell line*:** L6 myoblasts were cultured and incubated for 24h in 96 well plate at a cell count of $1*10^5$ cells/ml prior to experiments. *Clematis apiifolia* methanolic extracts at the concentration of 250µg/mL,500µg/mL and 1000µg/mL were added, followed by incubation for 48h. MTT assay was used to evaluate cell viability (Yao *et al.*, 2017). Cells having no treatment were used as control.

***Glucose consumption Assay*:** In order to have optimized conditions, methanolic extract was studied for glucose consumption at three diffferent concentrations with varying incubation time. Differentiated L6 myotubes were treated with 250µg/mL,500µg/mL and 1000µg/mL of methanolic extracts and incubated for 1h,2h, and12h. Afterwards glucose consumed in media was determined by O-Toluidine method (Godkar, 1994).Glucose consumed was calculated by following equation:

$$\text{Glucose uptake} = [\text{Glucose}]_{\text{blank}} - [\text{Glucose}]_{\text{treated}}$$

***Quantitative Real Time PCR (RT-qPCR)*:** Differentiated myotubes were treated with *Clematis apiifolia* methanolic extract (1mg/mL) for 12 h followed by extraction of total RNA with TRIzol reagent. SuperScript II reverse transcriptase was used to synthesize cDNA. SYBER Green

master mix was used for PCR. To normalize cDNA levels, we used β - actin as internal control.

***Western blotting*:** Ice cold PBS was used to wash cell lysate prepared from treated and control groups followed by addition of lysis buffer (50 mM TRIS pH 8; 150 mM NaCl, 1% Nonidet-P40, 0.25% sodium desoxycholate and protease inhibitors). Proteins were separated on 10% SDS/PAGE and then transferred to polyvinylidene difluoride membrane. Membranes were then probed with primary antibodies for anti-GLUT4 and anti-PTP1B followed by washing with TBST, afterwards incubated with secondary antibodies. Chemiluminescence reagent was used to visualize the bands. Internal control used for normalization of data was GAPDH. Image J software was used for densitometric analysis (Schindelin *et al.*, 2012).

***In vivo study*:** Male albino mice (2 months old) weighing from 22g to 30g were purchased from Animal Facility at the Department of Pharmacy in University of Peshawar, Pakistan. Animal study was approved by Bioethical Committee of Hazara University, Mansehra, Pakistan.

Animals were acclimatized for one week before the start of *in vivo* study. Animals were allowed to fast for 12 hours, afterwards intraperitoneal injection of 230mg/kg (in 0.9% saline) of Nicotinamide, followed by injection of STZ (65 mg/kg in 0.9% saline) was used to induce diabetes. Blood glucose was checked on 7th day and mice with blood glucose higher than 250mg/dL were chosen for antidiabetic study (Lee *et al.*, 2010). Animals were divided into four groups, normal healthy mice, diabetic/untreated mice, diabetic/glibenclamide treated (5mg/kg, *i.p.*) while mice treated with extract were in group diabetic/extract treated (100mg/kg, *i.p.*). For next 24 days animals were given treatment with blood glucose monitored on every 4th day, by OneTouch®glucometer (LifeScan, Inc.). At the end of study animals were killed by cervical dislocation.

***Statistical analysis*:** The data were expressed as mean ± standard deviation (S.D.) and analyzed by Student's t-test using GraphPad Prism6 software. Groups were considered significantly different from the control group if $P< 0.05$.

RESULTS

***α-Glucosidase Inhibitory Assay*:** To appraise anti diabetic potential of *Clematis apiifolia*; plant extracts were evaluated for their α-glucosidase inhibitory potential. All the extracts have shown enzyme inhibition in the concentration dependent manner. Methanolic extract has shown higher potency as compared to aqueous and ethanolic with IC_{50} values of 92.98µg/mL,183µg/mL and 212.9µg/mL respectively. Standard drug Acarbose was used as a reference which has shown IC_{50} value of 77.85 µg/mL (Table 1). These results have shown methanolic extract to be more effective among the three extracts.

Table 1. IC_{50} (µg/mL) of *Clematis apiifolia* extracts against α-Glucosidase and AGES activity.

	α-Glucosidase	AGES
Methanolic extract	90.69	36.63
Ethanolic extract	212.90	93.30
Aqueous extract	183.00	67.81
Acarbose	77.85	-
Rutin	-	35.62

***Advanced Glycation end products Inhibitory Assay*:** All extracts have shown good antiglycation activity. It is clear from data that methanolic extract had better inhibitory potential than aqueous and ethanolic extracts having IC_{50} values of 36.63µg/mL,67.81µg/mL and 93.3µg/mL, respectively. Rutin was used as standard and has shown IC_{50} value of 35.62 µg/mL. So methanolic extract was more active among these three extracts, having IC_{50} equivalent to Rutin (Table 1).

The inhibitory effect shown by extracts were in line with α-glucosidase inhibitory action. All three extracts have shown AGEs inhibition in a concentration dependent with best inhibition shown at 1mg/mL. So, this concentration was chosen to evaluate their PTP1B inhibitory potential.

***Protein tyrosine phosphatase 1 B Inhibitory Assay*:** *Clematis apiifolia* extracts were being evaluated for their PTP1B inhibitory potential. Results have shown that methanolic extract has highest potential with 79.3 ± 0.5 % inhibition of PTP1B while aqueous and ethanolic extract has shown 52.14 ± 1.1% and 31.28 ± 0.7% inhibition respectively at the same concentration(1mg/mL). Methanolic extract were then run-in dilutions and has shown IC_{50} value of 63.54 µg/mL (Table 1).

***Effect of methanolic extract on cell viability*:** In this study, the toxicity of *Clematis apiifolia*methanolic extract at various concentration was determined by MTT assay.The results (Fig. 1A) of this assay hasconfirmed that the methanolic extract was fairly safe, even at highest concentration of 1mg/mL cell viability was 100%.

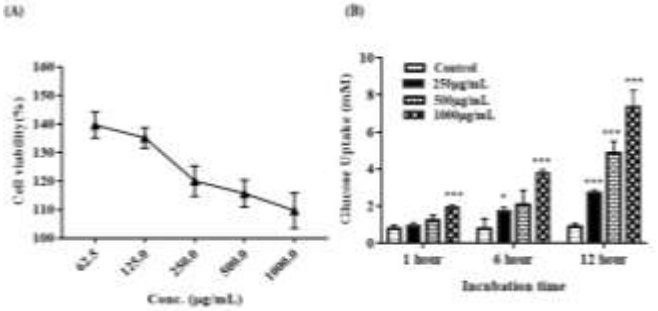

Figure 1. *In vitro* (cell based) assay of methanolic extracts of *Clematis apiifolia.* (A) Graph showing L6 cells viability in presence of methanolic extract, incubation time was 48h. (B)Effect of methanolic extract on glucose uptake. Data were expressed as mean ± SD. * P <0.05, **** P <0.0001. (n=3).

***Effect of methanolic extract on glucose consumption*:** Result of this experiment has revelead that glucose consumption in L6 myotubes was dose and time dependent i.e. glucose uptake increases with increase in incubation time(Fig. 1B).The results has also shown that glucose metabolism *in vitro*, was improved by methanolic extracts and was highest (7.3mM) 1000µg/mL at 12 h incubation time (P <0.0001), so these optimized conditions were furtherly used in real time PCR and western blot analysis.

Effect of methanolic extract on mRNAs and Protein expression of GLUT4 and PTP1B in differentiated L6 myotubes: We have examined the effect of methanolic extract on the mRNA and protein levels expression of PTP1B and GLUT4 in differentiated L6 cells. We have found that methanolic extract of *Clematis apiifolia* treatment at optimized conditions, downregulated the mRNA expression of PTP1B (P <0.0001) and upregulated the expression of GLUT4 (P <0.0001) as shown in Fig. 2A and B, respectively. Our findings demonstrated that methanolic extract exposure significantly upregulated the GLUT4 protein level (P<0.05) while it has significantly reduced (P<0.01) the PTP1B protein level compared with control as shown in Fig.3A and B, respectively. Densitometric values for GLUT4 and PTP1B proteins level (fold change over control) are shown in Fig.3. Differentiated L6 cells without any treatment were used as control. The data were expressed as the mean percent of the control level ± SE for 3 independent experiments.

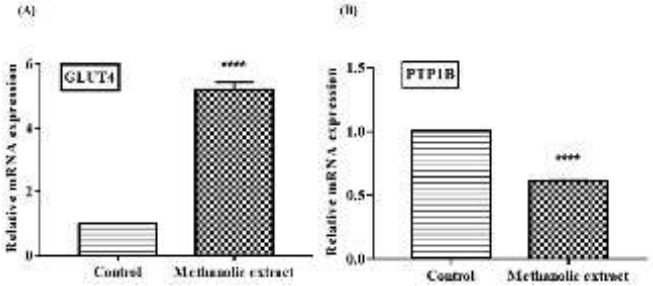

Figure 2. Effect of methanolic extract on mRNA expression level of GLUT4 and PTP1B in L6 cells. L6 cells were treated with methanolic extract treatment for 12h. Data were presented as mean ± S.D. (n=3). (** P <0.0001).**

***Induction of diabetes and Antidiabetic activity*:** Animals became hyperglycemic on 7th day, after induction with STZ/Nicotinamide. Animals were given treatment for 24 days and it was observed that diabetic/non-treated mice have shown stable hyperglycemia while diabetic/glibenclamide (commercial drug) treated mice, demonstrated significantly (P<0.0001) lowering of blood glucose level when compared with diabetic/untreated mice. Methanolic extract treated group has also shown significant lowering of blood glucose level when compared to diabetic/untreated group (Fig. 4). The hypoglycemic effects of methanolic extracts in *in vivo* study were comparable to glibenclamide.

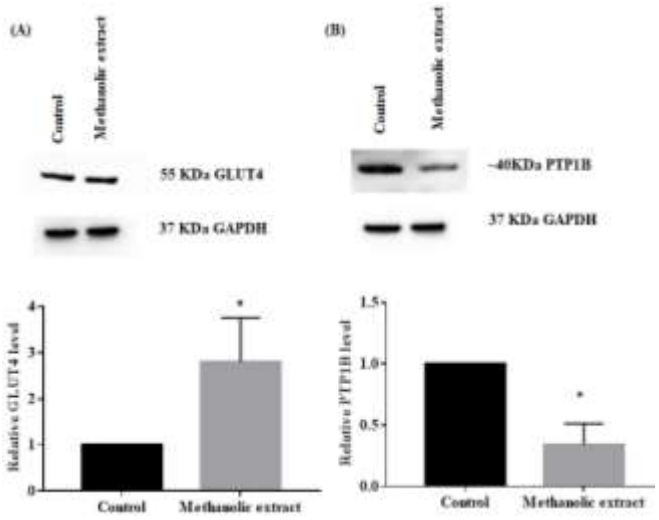

Figure 3. Effect of *Clematis apiifolia* methanolic extract on protein expression level of GLUT4 and PTP1B in L6 cells. L6 cells were treated with methanolic extract treatment for 12h. Densitometric analysis was carried out by ImageJ software. Data were presented as mean ± S.D. (n=3). (*P <0.05).

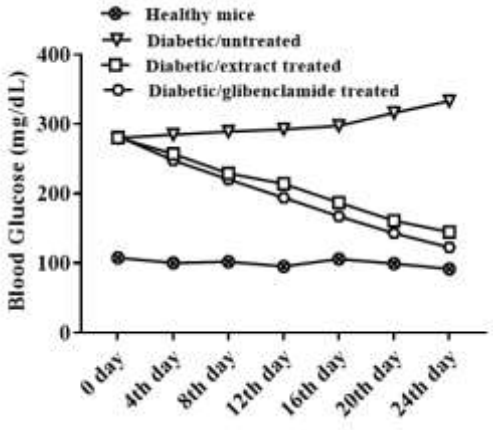

Figure 4. Anti-diabetic activity of the *Clematis apiifolia* methanolic extract in the diabetic mice. Postprandial glucose was measured by OneTouch®glucometer. Glibenclamide was used as positive control. Data were presented as Mean ± S.D.(n=5). (** P <0.0001).**

DISCUSSION

Genus *Clematis* comprises of 300 species all around the world, it belongs to Ranunculaceae. *Clematis apiifolia*, one of its species is being used to treat beriberi, travail, asthma, and urinary infections in Korean traditional medicines. But still there is no data explaining anti-diabetic aspects of this plant. In this study we have tried to explore and screen the anti-

diabetic potential of this plant by studying some of the *in vitro* enzymatic inhibitory activities along with possible mechanistic study in L6 cell line.

One of the targets for controlling postprandial glucose is alpha glucosidase enzyme. This enzyme is secreted from small intestine and digest carbohydrates (Kim *et al.*, 2005). So, inhibitors blocking the activity of this enzyme can suppress the hyperglycemia followed by eating a carbohydrate rich food so are helpful in management of type 2 diabetes mellitus (Watanabe *et al.*, 1997). There is always a search for natural based inhibitors as they have fewer side effects (Gholamhoseinian and Fallah, 2009).

Advanced glycation end products (Ages) are formed because of diabetic complications. Drugs or plants extracts having potential to stop or degrade AGES are used to control AGES relevant complications. In our study all three extracts (ethanolic, methanolic and aqueous) have shown AGES inhibitory activity with methanolic extract being most effective. So, it was being chosen for PTP1B inhibition study. Protein tyrosine phosphatase 1B is member of PTP family, expressed in insulin targeted tissues. Overexpression of this protein is linked to inhibition of insulin signaling pathways (Zhang and Lee, 2003). Over expression of PTP1B is linked to development of pathological complications like development of insulin resistant in obese patients. So, inhibiting the activity of PTP1B is the main strategy to control progression of diabetes mellitus (Koren and Fantus, 2007).

In our study we have evaluated that *Clematis apiifolia* methanolic extract possesses the highest inhibitory potential for all activities performed in cell free assay. So methanolic extract proved to be a potent candidate, was furtherly evaluated for cytotoxicity and glucose uptake study. For this purpose, L6 cell line was chosen as it presents best model for study of GLUT4 translocation (Yap *et al.*, 2007). Methanolic extract has proven to be safe up-to 1000µg/mL with highest glucose consumption studied at this concentration through glucose uptake assay. Previous research has shown that glucose consumption can be enhanced by plant extracts through upregulation of GLUT4 expression levels (Ueda *et al.*, 2013).

In order to find possible mechanism involved we furtherly studied effect of treatrment on GLUT4 levels. The treatment of methanolic extract to L6 myotubes has resulted in upregulation of mRNA levels of GLUT-4 and downregulation of PTP1B mRNA level. The results were furtherly verified through western blottingg and were in line with results of PCR.

When evaluated in chemically induced diabetic model, methanolic extract has shown its hypoglycemic effect by lowering the blood glucose level significantly. This could possibly be attributed to phytochemicals present in the extract.A previous studied conducted on Clematis montana has shown similar kind of hypoglycemic effect in diabetic mice (Singh *et al.*, 2015).

***Conclusion*:**Our study for the first time has shown the possibility of *Clematis apiifolia*methanolic extract as a novel hypoglycemic agent for the treatment of diabetes.Results of *in vitro* study, cell free and cell based enzymatic assays have supported the *in vivo* hypoglycemic action in diabetic mice. In order to explore the antidiabetic potential of *Clematis apiifolia*, further studies focusing identification and isolation of bioactive phytoconstituents will be required.

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