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Amelioration of Streptozotocin Induced Cytotoxicity by *Mimosa pudica* Root Extract - A Proteomic based in-vitro Study

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Abstract

The in-vitro cytotoxicity was studied in a pancreatic cell line (RIN5F) using a streptozotocin induced cellular damage. The Cytotoxic dose of aqueous and hydro-alcoholic extracts of *Mimosa pudica* root powder before and after streptozotocin treatment was studied and protein expression was explored. Both the extracts were subjected to metabolite analysis by LCMS. *Mimosa pudica* root powder extracts prevented Streptozotocin induced cellular damage. Proteomics studied indicated both aqueous and hydro-alcoholic extracts of *Mimosa pudica* regulates proteins related to insulin secretion, glucose uptake and insulin resistance with marked changes in proteins involved insulin secretion. The study first time reports safety of *Mimosa pudica* in RIN5F cells and explores protein expression after Mimosa pudica treatment. It was also observed that abundance of Mimosine was present in Hydro-alcoholic extracts as compared to aqueous extracts.

1. Introduction

Mimosa pudica family Fabaceae is a creeping annual or perennial herb. This herb is found in Central America, South America and Asia[1]. *Mimosa pudica* exhibits typical characteristics as its compound leaves fold and droop themselves upon touch which is a persistent focus of researchers' curiosity. It is commonly known as Lajjalu (लज्जालू) in Sanskrit, Chuimui in Hindi, lajalu in Marathi and Touch-me-not in English[2].

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A detailed review of literature indicated wound healing activity[3], regenerative action on sciatic nerve, anti-depressant, anti-convulsant, hyperglycemic, diuretic activities and effect on uterine bleeding[3], anti-fertility[4]; anti-oxidant[5], aphrodisiac activity[6], piles-fistula[7], anti-venom[8] and anti-microbial[9,10] activities. In Ayurveda, *Mimosa pudica* root is widely used in different preparations viz; *Pusyanuga Churna*, *Lavangadi Churna* and *Khadiradi Gutica* in various diseases of the female genital system, gastrointestinal diseases and diseases related to oral cavities respectively[11]. Whole plants are also used in Ayurvedic preparations like *Piyusavalli Rasa*, *Samangadi Churna*, *Kutjavleha* and *Brahat Gangadhar Churna*[12].

Chorei and *Hmar* tribes of Assam[13] use whole plant as a remedy for diabetes. Tincture of leaves in used as a remedy for diabetes by local tribes of Ghana[14]. Leaves of *Mimosa pudica* exhibited anti-diabetic activity in alloxan-induced diabetic rats[15] and in streptozotocin induced diabetes model[5]. Despite the traditional use of root of this plant in traditional medicine for diabetes the study delineating effect of root powder on pancreatic cells *in-vivo* or in-vitro are not traceable. Further, proteome after *Mimosa pudica* treatment is also not explored. Hence, present study was undertaken with an objective to study effect of *Mimosa pudica* root powder extract on protein expression involved in glucose regulation using pancreatic RIN5F cell line with and without streptozotocin treatment.

2. Materials and Methods

2.1 Source of Plant Material and its Authentication

The *Mimosa pudica* roots were collected from local garden. The plant was duly authenticated by the Botanist and sample was deposited to the Herbarium of the Institute.

2.2 Preparation of Extracts

Mimosa pudica (*lajjalu*) root powder was subjected for preparation of hydro-alcoholic and aqueous extracts by cold maceration method as described by Azwanida (2015)[16].

2.2.1 Aqueous Extract Preparation of Mimosa pudica Root Powder

50 gm powder was soaked in distilled water (1 part powder: 5 part distilled water) for 24 hours at room temperature. After soaking it was filtered through a muslin cloth and this filtrate was kept in incubator shaker for 24 hours. The filtrate obtained was lyophilized and the powder was stored in an airtight container at room temperature.

2.2.2 Hydro-alcoholic Extract of Mimosa pudica Root Powder

Water and alcohol were mixed in 1:1 proportion. 50 gm powder was soaked in water:alcohol (1 part powder: 5 part Hydro-alcohol) solution for 24 hours at room temperature. After soaking it was filtered through a muslin cloth and this filtrate was kept in incubator shaker for 24 hours. The homogenate was lyophilized and the powder was stored in an airtight container at room temperature.

2.2.3 LC-MS Analysis of Mimosa pudica Root Powder Extracts

Chromatographic analysis was performed using the Agilent 6500 system with a Poroshell C18 column (2.1 × 150 mm, 1.8 µm). The mobile phase was composed of acetonitrile (A) and water (B) in gradient manner. The gradient was as follows: 0–10 min, 20% A; 10–14 min, 20%–40% A; 14–25 min, 40% A. The flow rate was 0.40 mL·min^{-1} and the fluid temperature was kept at 40°C; the sample injection volume was 10 µL.

2.3 Cytotoxicity Studies

2.3.1 Procurement and Maintenance of the Cell Line

RIN5F cell line was procured from NCCS, Pune along with authentication certificate by PCR method and Certificate that the cell line is *Mycoplasma* free. Upon receipt the cell line was confirmed by observing under microscope and was preserved under the vapor phase of Liquid Nitrogen for further use. Cell number was decided by seeding cells at different concentrations and performing MTT assay. The cell number showing absorbance near to 1 was selected for further studies.

2.3.2 Cytotoxicity Studies on RIN5F Cells

Cytotoxicity of *Mimosa pudica* extracts was studied in the RIN5F cell line. For this, 3 x 10^3 cells/well were seeded in 96 well plates and allowed to adhere for 18 hours. Cells were treated with 0, 5, 10, 20, 40, 60, 80, 100, 150, 180, 200 µg/

ml *Mimosa pudica* aqueous or hydro-alcoholic extract for 24 hours prepared in fresh media. After 24 hours of drug treatment 50 µl of MTT solution was added and the plate was incubated for 4 hours at 37°C, 5% CO_2. After 4 hrs, the plate was wrapped with foil and centrifuged for 5 min @3500 rpm. Later supernatant solution was decanted and formazan crystals were solubilised using DMSO (100 µl/well). The absorbance (570 nm) was measured using plate reader (Mfg. Thermofisher Scientific). The absorbance was recorded and analysis of data was done.

2.3.3 Standardization of Streptozotocin Dose for RIN5F Cell Line

RIN5F cells (35000 cells/well) were treated with Streptozotocin (Mfg. Himedia) @ 0, 10 mM, 20 mM, 25 mM, 30 mM and 40 mM for one hour and cytotoxicity assay was performed as mentioned above. Dose for further studies was selected in such a way that the dose should initiate cellular damage but dose should not cause decrease in cell population. Cytotoxicity study of *Mimosa pudica* root extract before and after the Streptozotocin treatment was performed and doses were compared.

2.4 Chromatographic Analysis

Chromatographic analysis was performed using the Agilent 6500 system with a C18 column (2.1 × 150 mm, 1.8 µm). The mobile phase was composed of acetonitrile (A) and water (B) in a gradient manner. The flow rate was 0.40 $mL{\cdot}min^{-1}$ and the fluid temperature was kept at 40°C; the sample injection volume was 10 µL.

2.5 Study of Proteomics in RIN 5F cells treated with *Mimosa pudica* Root Powder Aqueous and Hydro-alcoholic Extract

Cells were seeded in 6-well plate @ 1.2 x 10^6 per well and after 18 hours cells were treated with 25 mM Streptozotocin, 60 µg/ml and 150 µg/ml of Aqueous extract of *Mimosa pudica* root powder or 150 µg/ml Hydro-alcoholic extract of *Mimosa pudica* root powder.

After 24 hours of treatment, cells were harvested. Further processing of samples for proteomics was done as per standard in-solution digestion protocol[17] and samples were analyzed on the LC-MS system.

2.6 Analysis of Proteins expressed after *Mimosa pudica* Treatment

Uniprot Entry keys[18] were used to compare and draw commonly expressed proteins by Venn diagram[19]. Proteins expressed during cell treatment were compared to the proteins expressed in diabetes; insulin regulation; insulin resistance and glucose uptake.

3. Results

3.1 Results of Cytotoxicity Study of Streptozotocin Treatment in RIN5F Cell Lines

Cytotoxicity study of Streptozotocin was performed to find the dose where cytotoxic changes initiate. The study

Table 1. Cytotoxicity dose calculation for Streptozotocin treatment in RIN5F cell lines

Parameter	Value
IC_{50}	**25.8236**
Equation	$Y = 72.9727 + \frac{101.0683 - 72.9727}{1+(\frac{X}{25.8236})19.2465}$
Equation Form	$Y = Min + \frac{Max - Min}{1+(\frac{X}{IC_{50}})\ Hill\ coefficient}$

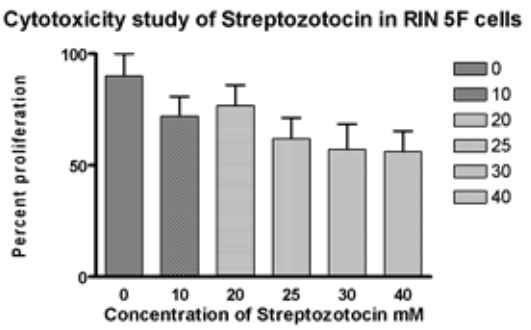


Figure 1. Cytotoxicity study of streptozotocin.

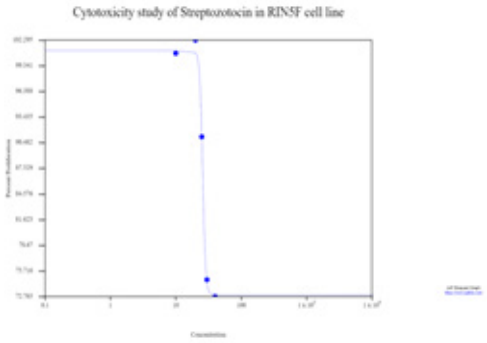


Figure 2. A four parameter logistic regression of Cytotoxicity of Streptozotocin in RIN 5F cells.

indicated 25.82 mM as IC_{50} of Streptozotocin in RIN5F cells (Table 1, Figure 1). Since, 20 mM was not showing much cellular damage and 30 mM was showing higher cellular damage, 25 mM was chosen as challenge dose to study cytoprotective effect of *Mimosa pudica root* powder extracts.

3.2 Results of Cytotoxicity Study of *Mimosa pudica* with and without 25 mM Streptozotocin Treatment in RIN5F Cell Lines

The studies in RIN5F cells indicated both the extractsare safe upto 150 µg/ml on the RIN5F cell line (since the maximum solubility of both the extracts was up to 2 mg/ml) hence for further study 60 µg/ml was chosen as middle dose and 150 µg/ml was chosen as maximum dose to be used.

3.3 Results of Metabolite Analysis

LC-MS based metabolite profile was studied to identify differences in both the extracts. Figure 3 and 4 indicate results of LC-MS analysis of Aqueous and Hydro-alcoholic Extracts of *Mimosa pudica* roots.

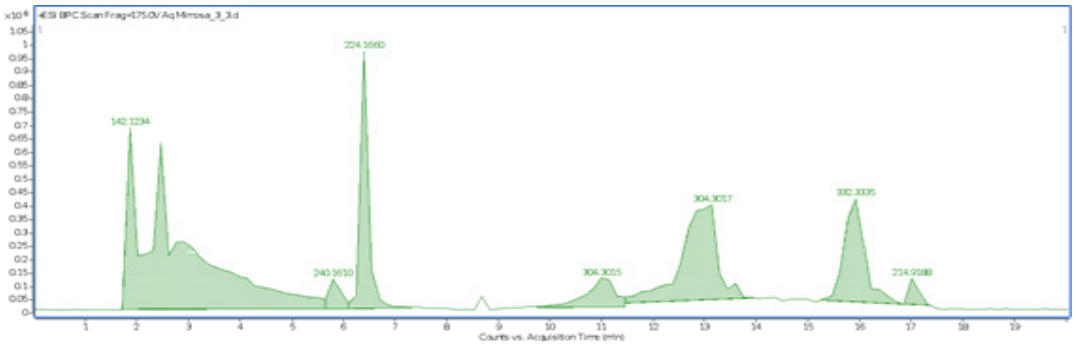


Figure 3. LC-MS based metabolite analysis of *Mimosa pudica* root aqueous extract.

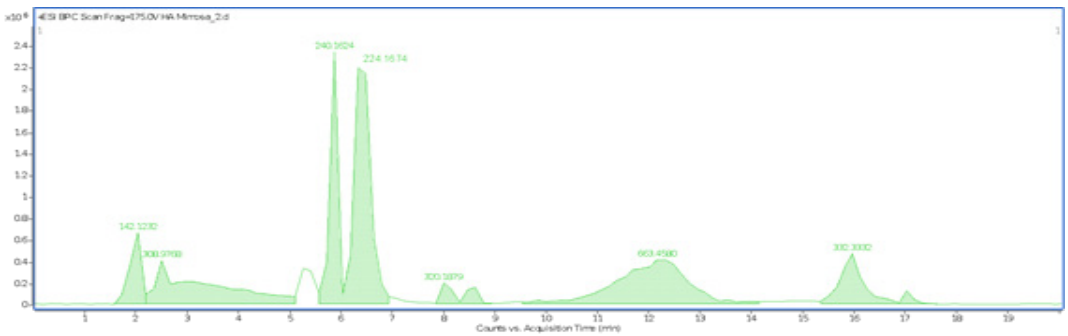


Figure 4. LC-MS based metabolite analysis of *Mimosa pudica* root hydro-alcoholic extract

3.4 Results of Proteomic Analysis

Proteins expressed are presented in the Venn diagram as follows: Number of proteins commonly expressed in each treatment is depicted in the form of Venn diagram (Figures 5 and 6).

The Venn diagram indicates 3 proteins were commonly expressed after treatment with aqueous extract. It also indicated 7 proteins were commonly expressed after hydro-alcoholic extracts in cells treated with streptozotocin.



Figure 5. Venn diagram of proteins expressed in cells treated with aqueous extracts of *Mimosa pudica* root powder before and after streptozotocin treatment.

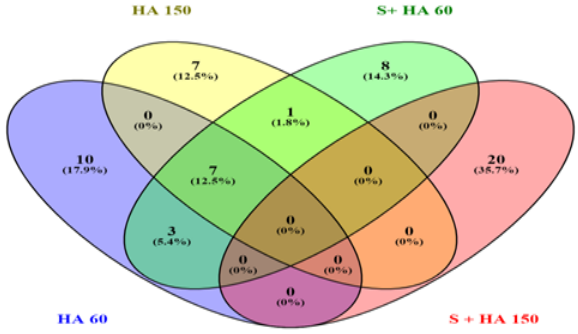


Figure 6. Venn diagram of proteins expressed in cells treated with hydro-alcoholic extract of *Mimosa pudica* root powder.

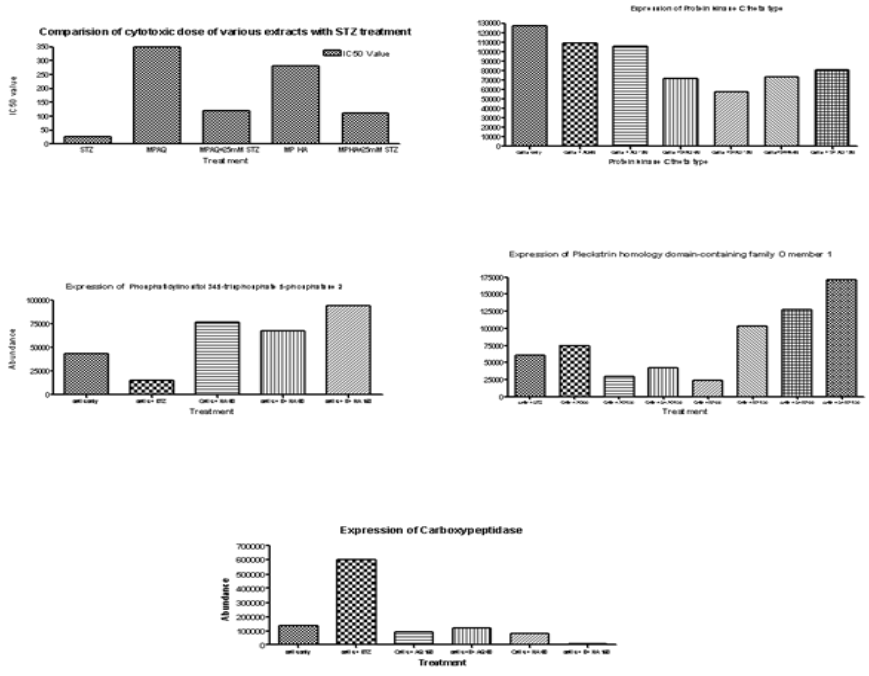


Figure 7. Graphical representation of protein expression in RIN5F cells treated with *Mimosa pudica* root extracts.

Two important proteins expression Protein kinase C and Phosphatidylinositol 3,4,5-trisphosphate 5-phosphatase 2 abundance in RIN5F cells treated with *Mimosa pudica* root powder extracts is depicted by graphical representation in Figures 3 and 4.

4. Discussion

The in-vitro anti-diabetic activity was studied in a pancreatic cell line (RIN5F) using a streptozotocin-induced cellular damage model. The study first time reports safety doses of *Mimosa pudica* aqueous and hydro-alcoholic extracts using RIF5F (Pancreatic) Cell line. Further both extracts were tested against streptozotocin challenge to explore whether *Mimosa pudica* root can inhibit oxidative stress induced by streptozotocin. The IC_{50} value of aqueous extract without streptozotocin treatment was from 349.77 µg/ml, which decreased to 118.53 µg/ml after streptozotocin treatment. Simillarly, IC_{50} of hydro-alcoholic extract without streptozotocin treatment 280.46 µg/ml which decreased to 110.92 µg/ml when cells were simultaneously exposed to streptozotocin at 25 mM concentration (Figure 2). However, no literature is available to compare the IC_{50} value of *Mimosa pudica* root extract with other or same cell lines. Further, Aqueous and hydro-alcoholic extracts were subjected to metabolite analysis by LC-MS which first time reports Mimosine content is 3-4 fold more in hydro-alcoholic extract as compared to aqueous extract. Since, there was not so significant difference in the IC_{50} dose of Aqueous and hydro-alcoholic extract indicates Mimosine content may not play major role in cytotoxicity.

Proteomic study indicated both aqueous and hydro-alcoholic extracts of *Mimosa pudica* regulated proteins related to insulin secretion, glucose uptake and insulin resistance. Mainly more proteins were involved in insulin secretion by the cells.

Streptozotocin up-regulated Serine/threonine-protein kinase D1 protein which is phosphorylated at Ser-403 and Ser-407 by MAPK13 during regulation of insulin secretion in pancreatic beta cells[18]. Down-regulation of 3-phosphoinositide-dependent protein kinase 1 due to STZ treatment was observed, which plays a central role in the transduction of signals from insulin by providing the activating phosphorylation to PKB/AKT1, thus propagating the signal to downstream targets controlling cell proliferation and survival, as well as glucose and amino acid uptake and storage. Down-regulation of Phosphatidylinositol 3,4,5-trisphosphate 5-phosphatase 2 which plays a central role in the regulation of PI3K-dependent insulin signaling was observed.

Streptozotocin (STZ) is an anticancer drug mainly used in experimental pharmacology to induce diabetes as it destroys the insulin-secreting β cells present in the pancreas. It contains a glucose molecule (in a deoxy form) that is linked to a highly reactive methyl nitrosourea moiety which exerts STZ's cytotoxic effects, while the glucose moiety directs the chemical to the pancreatic β cells. STZ recognizes the GLUT2 receptor that is abundant in β cell's plasma membranes. Therefore, pancreatic β cell is a specific target of STZ. Also, it is well established that glucose-stimulated insulin secretion is tightly linked to glucose-driven ATP production by β cell mitochondria[20].

The study indicates Streptozotocin treatment at a sub-lethal dose (25 mM) to pancreatic cell lines (RIN5F) mimics diabetes-like protein changes. Protein kinase C theta type which may mediate inhibitory effects of free fatty acids on insulin signaling by phosphorylating IRS1, which in turn blocks IRS1 tyrosine phosphorylation and downstream activation of the PI3K/AKT pathway was down-regulated by aqueous extract treatment after streptozotocin treatment. Protein Kinase C and Diacetyl Glycerol up-regulation lead to advanced glycation end product overproduction leading to diabetic vascular complications[21]. Hence, it may be predicted that *Mimosa pudica* root powder may have anti-diabetic activity but further studies on insulin-resistant diabetes model in-vitro and its further evaluation in *in-vivo* diabetes models are suggested.

Inositol polyphosphate phosphatase-like protein 1Uniprot Entry key Q9WVR3) was down-regulated in mere streptozotocin treatment but was up-regulated by hydro-alcoholic extract treatment alone after streptozotocin treatment to RIN 5F cells. This protein plays a central role in the regulation of PI3K-dependent insulin signaling[18], although the precise molecular mechanisms and signaling pathways remain unclear. While over expression reduces both insulin-stimulated MAP kinase and Akt activation, its absence does not affect insulin signaling or GLUT4 trafficking.

Cdc42-interacting protein 4 (Uniprot Entry key P97531) which was solely expressed in RIN5F cells and may be down-regulated by streptozotocin and *Mimosa pudica* treatment is required for translocation of GLUT4 to the plasma membrane in response to insulin signaling[18].

3-phosphoinositide-dependent protein kinase 1 (Uniprot Entry key O55173) which plays a central role in the transduction of signals from insulin by providing

the activating phosphorylation to PKB/AKT1, thus propagating the signal to downstream targets controlling cell proliferation and survival, as well as glucose and amino acid uptake and storage[18] was down-regulated by mere streptozotocin treatment and remained unexpressed in treatment by aqueous and hydro-alcoholic extracts of *Mimosa pudica* treatment.

Insulin receptor substrate 1 (IRS-1) (Uniprot entry key P35570) was expressed in hydro-alcoholic extract-treated cells with high intensity as compared to cells treated with streptozotocin alone. This protein may mediate the control of various cellular processes by insulin[21]. These types of proteins have no intrinsic enzymatic activity. However, they function as adaptors and contribute to signaling and to organize signaling complexes[22]. Upon phosphorylation by the insulin receptor it binds specifically to various cellular proteins containing SH2 domains such as phosphatidylinositol 3-kinase. In this study, phosphatidylinositol 3-kinase showed highest abundance in Cells treated with streptozotocin with *Mimosa pudica* root powder hydro-alcoholic extract. Its binding to SH2 domain regulates binding of growth factors[23].

Pleckstrin homology domain-containing family O member 1 (Uniprot entry key Q5BJM5) is predominantly localized to the plasma membrane through the binding to phosphatidylinositol 3-phosphate[18]. It rapidly translocates to the plasma membrane from cytoplasm after insulin stimulation[24]. This protein was up-regulated in cells treated with Hydro-alcoholic extract of *Mimosa pudica* at 150 µg/ml after streptozotocin treatment which indicates hydro-alcoholic extract may enhance insulin stimulation.

Konstantinova *et al.* (2007)[25] reveal that signaling via an ephrin ligand and its Eph receptor mediate communication between β cells. Both the extracts showed positive effect on diabetes regulating proteins but the same has to be validated *in-vivo*. The model used in this study was streptozotocin induced stress model. Further studies are required to be done on insulin resistance cellular models and on primary pancreatic cells so that more relevant results could be obtained. Although many proteins related to diabetes were expressed; further studies are required to refine proteomics procedures and obtain more protein expression for more comparison.

5. Conclusion

The study gives bird's eye view of the protein-expression due to streptozotocin challenge and its amelioration with the aqueous and hydro-alcoholic extracts of *Mimosa pudica* root. Hydro-alcoholic extract of *Mimosa pudica* L. modifies proteins like phosphatidylinositol 3-kinase, Insulin receptor substrate 1 and Pleckstrin homology domain-containing family O member 1 which play an important role in insulin regulation. Further studies in this regard are warranted.

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