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RESEARCH ARTICLE

Screening for Anti-Diabetic Peptides from *Moringa oleifera* leaves

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ABSTRACT:
Moringa oleifera commonly called as Drumstick tree is a multipurpose tree and it is widely present in the places like India, Asia, Africa, etc. Its leaves are emetic and their juices are used for medicinal purposes. Diabetes mellitus is a clinical syndrome with insufficient insulin secretion and abnormal glucose tolerance. It is a group of diseases which results in too much sugar in the blood. Leaves from Moringa oleifera were collected, analysed for their anti- diabetic activity. First the proteins are extracted using protein extraction methods, estimated using Lowry's method, and then anti- diabetic activity was checked using Glucose uptake assay by yeast cells, where the plant extract (small proteins) was able to uptake glucose. SDS-PAGE was carried out to check the protein's size and molecular weight. The characterization of Moringa oleifera conducted in this study shows that the small peptides from the leaves of this plant can contribute significantly in the daily recommended intake of it, since it serves as a rich source of all minerals, vitamins etc. This study has confirmed that the small peptides from the leaves of *M.oleifera* can fight against diabetics and the result indicated that Murungai (Moringa) leaves are suitable source of green leaf to reduce the diabetic complications in diabetic patients.

KEYWORDS: *Moringa oleifera*, Diabetes mellitus, peptides, Lowry's method, antidiabetic screening.

INTRODUCTION:
Diabetes mellitus (DM) is a severe chronic syndrome or disease which affects millions and millions of people worldwide. It is a group of disease characterized by hyperglycaemia, or high blood sugar, which often results from insulin secretion defects. The early symptoms are related to hyperglycaemia which includes polydipsia, polyuria, polyphagia, and blurred vision. Hyperglycaemia develops when insulin secretion no longer deals for the insulin resistance.[1] There are two types of diabetes, Type 1 diabetes, Type 2 diabetes. The type 1 diabetes, is loss of tissue response to insulin, which leads to type 2 diabetes. It is also called as insulin- dependent diabetes, and juvenile-onset diabetes since it begins in childhood. *Moringa oleifera* belongs to the species of the genus Moringa of the family Moringaceae. *Moringa oleifera* is described as the miracle tree, God's gift to man.[2 3] People use the leaves, flowers and fresh pods of Moringa as vegetables.[4] This tree has much potential in the improvement of nutrition, boost food security.[5 3]

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Diabetes was found to be at maximum in India for the past few years according to International Diabetes Foundation, it is said that it affects about 62 million Indians, which is around 7.2% of the adult population. It is a fast gaining status of potential epidemic in India. The leaves of moringa have been reported to be rich source of protein, b- carotene, calcium, vitamin C and potassium. Moringa oleifera has been reported to possess various pharmacological activities such as analgesic [6 7], antipyretic, antifertility [8], antioxidant[9 10], antiulcer[11 10], antimicrobial [12], antifungal [13 10], anticancer [14]. This study has confirmed that the small peptides from the leaves of *M.oleifera* can fight against diabetics and the result indicated that Murungai (Moringa) leaves are suitable source of green leaf to reduce the diabetic complications in diabetic patients.

MATERIALS AND METHODS:
Plant material and preparation of extract:
The *Moringa oleifera* leaves were collected naturally from the Murungai Tree, during July, in Chennai. The sample was collected manually using hands. The collected leaves were washed with water to get rid of some insects, pests. The leaves were made to store and shade dry at room temperature for a period of 7 days. After the process of shade drying, the dried leaves were

crushed and ground coarsely, and powdered into fine powder. The blended sample was stored in an air-tight polythene bags and was stored at room temperature in a dry dark place.

Small protein extraction:

The dried leaf powders were measured using measuring or weighing balance. The total quantity of dried leaf powder was measured to be 75 grams. Then 1:3 ratio of diethyl ether was added, kept undisturbed for 16 hours at room temperature. The sample was diluted with diethyl ether in a 2000ml conical flask. The sample was diluted at 5.30 p.m. and after 16 hours, at 10.30 a.m. the sample was made prepare for further steps. The sample with diethyl ether was filtered using Whatman No.1 filter paper. This filter paper takes some time to filter out. The residue was extracted out very carefully from the filter paper. The filtrate was discarded. The extracted residue was treated with 33ml of cold distilled water. It was mixed continuously for few minutes. The extract with distilled water was centrifuged at 6000rpm for 15minutes.After the process of centrifugation, the supernatant was carefully transferred to separate 100ml flask by discarding the pellets completely. The supernatant was then treated with cold distilled water and again centrifuged for 15minutes at 6000 rpm. The process was repeated twice and making the volume up to 100ml.[15]

Acetone precipitation of proteins:

First the required volume of acetone was cooled at -20ºC. The protein samples were kept into acetone compatible tubes. About four times the sample volume of cold acetone was added to the tube. Then the tubes were vortexed so that the sample and the acetone are equally mixed together. It is then incubated for 60minutes at -20ºC. After the 1 hour of incubation, it was transferred to Eppendorf tubes and centrifuged for 10mins at 13,000 – 15,000 rpm. After centrifugation, the supernatant was properly discarded and being very careful by not dissolving the pellets. Then the acetone was allowed to evaporate from the uncapped tubes at room temperature for almost 30minutes. Do not over dry the pellet or it may not dissolve properly. Addition of appropriate buffer, the buffer used here is PBS (Phosphate Buffer Saline) pH 5.2 and vortexed thoroughly to dissolve protein pellet.

Estimation of proteins:

Lowry's method of protein estimation:

Different dilutions of BSA solution were prepared by mixing the stock BSA solution (1mg/ml) and water in test tube. Final volume of each of the test tube is 5ml. It ranges from 0.05 to 1mg/ml. To different dilutions, 0.2 ml of protein solution was pipetted out to different test tubes. 2ml of alkaline copper sulphate reagent was added. The solutions were mixed well and incubated at room temperature for 10 minutes. 0.2ml of Folin ciocalteau solution was added to each tube. It was then incubated for 30mins. The calorimeter was made zero with blank and OD was taken at 660nm. The absorbance was plotted against protein concentration to get a standard calibration curve. Absorbance of unknown sample was checked and the concentration of unknown sample was determined using the standard curve plotted. [16- 17]

Glucose uptake test:

Yeast cells were prepared and suspended in distilled water and then it was washed by repeated centrifugation (4200 rpm for 5mins) in water, until supernatant appeared clear, 10% (v/v) suspension was prepared in distilled water. 5 concentrations 25, 50, 75, 100,125µg/ml were prepared out of given compound. Standard glucose solution of concentration 100ml was prepared (1mg/ml). Acarbose was used as a standard solution. Various concentrations of the extract were added to 1ml of glucose solution. It was then incubated for 10mins at 37ºC. The reaction was started by adding 100µl of yeast suspension vortexed, incubated at 37º for 1 hour. After incubation, the tubes were centrifuged at 3,800rpm for 5mins. Glucose was estimated in the supernatant using anthrone method at 630nm.[18]

$$\text{Increase in glucose uptake\%} = \frac{\text{A630 of control} - \text{A630 of sample}}{\text{A630 of control}} \times 100$$

Anthrone method:

Preparation of anthrone reagent:

0.2 % of anthrone was dissolved in ice cold concentrated sulphuric acid. It was prepared fresh before use. Weighed 100mg of the sample into a boiling tube, hydrolysed by keeping it in a boiling water bath for three hours with 5.oml of 2.5N HCL and cooled to room temperature.

Procedure for anthrone method:

1ml of Sample was taken in 6 test tubes. 1ml of distilled water was taken in another tube as control. 2ml of anthrone reagent was added to all the tubes. All the tubes were mixed thoroughly. The colour change in dark bluish green was observed.[19]

Sodium Dodecyl Sulphate – Polyacrylamide Gel Electrophoresis (SDS-PAGE):

SDS-PAGE was performed by standard method. The marker used was M.GENEi Protein Molecular Weight Marker, which is a low range marker. The gel was placed in UV light and visualized.[20-21]

RESULTS AND DISCUSSION:

Estimation of protein by Lowry's method:

Basically, proteins are estimated i.e. the total concentration of proteins are estimated by Lowry's method. In this method, the protein sample was treated with the alkaline copper sulphate, BSA (Bovine Serum Albumin) solution and with the Folin Ciocalteau reagent. On treating the proteins which folin ciocalteau reagent, the copper is reduced to Cu++, produced by the oxidation of peptide bonds. Finally, the total concentration of proteins was analysed by the colour change and its absorbance was measured at 660nm. The following table.1 provides the total protein concentration. The graph is plotted according to the values, the concentration of the protein is calculated and represented in table.1 and figure 1.

Calculation

$Y = 0.0042x - 0.0572$

$1.0457 = 0.0042x - 0.0572$

$1.0457 + 0.0572 = 0.0042x$

$1.1029 = 0.0042x$

$x = \frac{1.1029}{0.0042}$

x = 262.60 µg/ 100 µl

Table. 1. Estimation of total protein concentration by Lowry's method

Concentration	Absorbance at 660nm
20	0.0413
40	0.0891
60	0.1925
80	0.2763
100	0.3641
125	1.0457

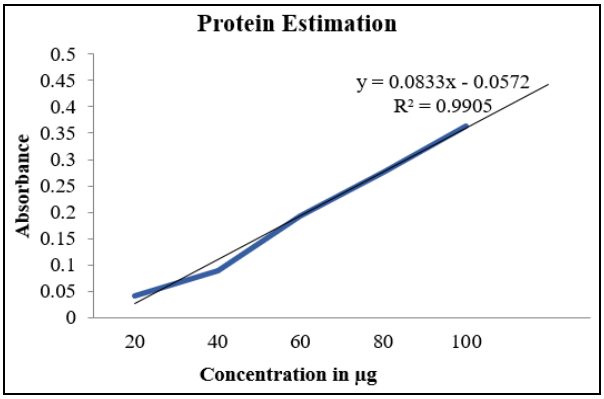

Fig.1 Chart showing the result of Lowry's assay

Glucose uptake assay:

The glucose uptake test is general highly sensitive and non-radioactive assay which detects glucose uptake in various cells. This method was done using baker's yeast in distilled water. To initiate this reaction, yeast suspension is poured in mixture of glucose and plant extract, it was incubated and centrifuged. The glucose was estimated finally by following the anthrone method, which resulted in colour change from yellow to dark bluish colour. Then the glucose was estimated using spectrophotometer at 630nm and finally the glucose percentage was calculated using a standard formula. The results of glucose uptake percentage are represented in table 2 and figure 2.

Table.2 Determination of glucose uptake percentage using the concentration and OD values

Con-centration (µg)	Sample	Acarbose	Control	%uptake of Acarbose	%uptake of Sample
25	1.06	1.6173	1.8991	44.1840	14.8360
50	0.956	1.5412	1.8991	49.6603	18.8457
75	0.901	1.5256	1.8991	52.5564	19.6672
100	0.865	1.5077	1.8991	54.4521	20.6097
Control	0.752	1.4491	1.8991	60.4022	23.6954

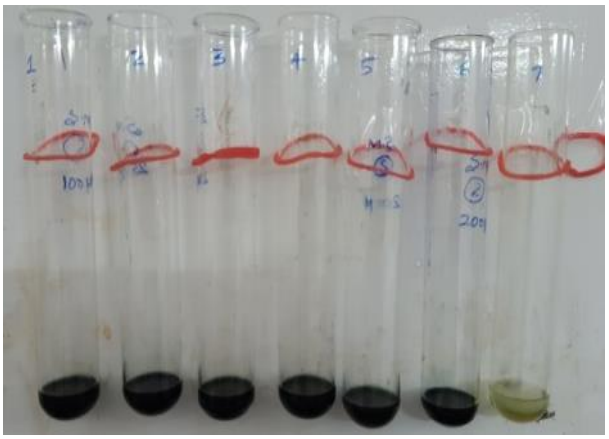

Fig.2 Result of Glucose uptake showing the colour change into Dark bluish green

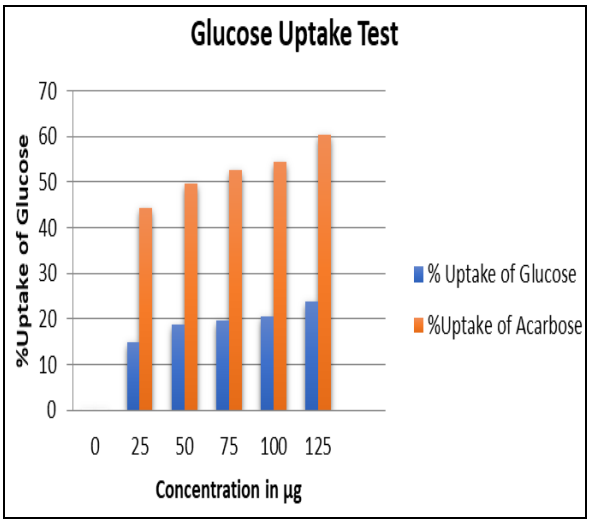

Fig. 3 Bar chart between the concentration of the sample and the percentage of uptake (absorbance)

Sodium Dodecyl Sulphate – Polyacrylamide Gel Electrophoresis:

SDS-PAGE method was performed to find out the particular size and molecular weight of the isolated protein by separating the charged molecules in mixtures by their molecular masses in an electric field. The sample and ladder were loaded into gel and made to run vertically, connected to power supply. The sample and ladder were added with dye. The Molecular marker used here is M-Gennie Protein molecular weight marker-lower range. The gel was stained and destained with reagents. After staining, different biomolecules appear as distinct bands within the gel. The resultant gel was viewed under UV light and the gel was illustrated in Fig. 4

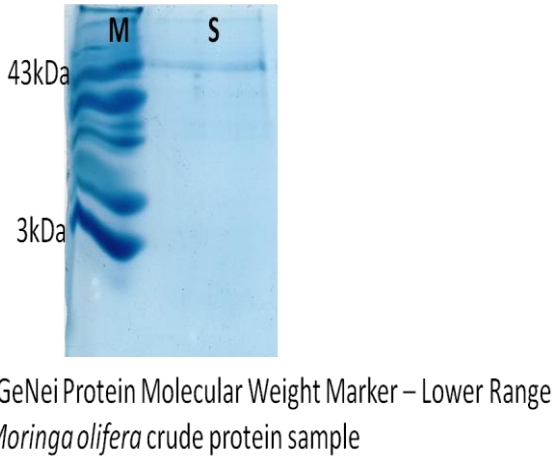

Fig.4 Result of SDS – PAGE

The size of the protein in the gel can be determined by calibrating the gel with molecular weight size marker or ladder, which is a set of standards that are used to identify the approximate size of a molecule. The resultant protein was found to be an average small peptide with a molecular size of 43Kda.

CONCLUSION:

Small proteins or peptides from *Moringa oleifera* leaves were isolated by using diethyl ether and acetone precipitation method. It was checked for the estimation by Lowry's method and the total concentration of small protein was calculated to be 262.59 µg /100µl. Then the small proteins were checked for anti- diabetic activity by Glucose uptake Assay using yeast cells, where the small proteins were found to be able to uptake the glucose. The uptake rate and percentage were calculated according to the standard Glucose uptake percentage formula. The glucose uptake percentage was calculated. Finally, SDS-PAGE was performed to find out the size of the isolated small protein with low molecular marker. The size of isolated small protein was 43kDa which is regarded as an average small protein and it is known to have anti-diabetic property.

People who are familiar with Moringa mainly have some sense with medicinal uses of it and it is easy for the introduction of Moringa leaves for daily consumption. It is very important to stress that a healthy diet is a key. Moringa leaves should be taken as a part of wholesome diet. It has all medicinal properties and it was found to have all anti- microbial, anti- bacterial activities. From this study, it is very clear that, the experiment revealed anti-diabetic activity of the peptides from *Moringa oleifera* leaves.

Collectively, this is a preliminary experiment, and the study has shown that *Moringa oleifera* may help in lowering the blood sugar levels. It is possibly safe. This resulted that the small peptides isolated from *Moringa oleifera* leaves has tendency to reduce blood pressure and high blood sugar levels and this result needs further investigation. *M. oleifera* was found to be more effective. Since it is a widely present perennial tree, and if further more research is done, this can be produced as a better drug for persons with diabetes.

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CONFLICT OF INTERESTS:

There is no conflict of interests.

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