**Original article**

**EVALUATION OF INSULIN LIKE PROTEIN FROM DOLICHOS LABLAB LINN**

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**ABSTRACT**

Leguminous plants are outstanding for their high levels of bioactive compounds, which can impact glucose metabolism by various mechanisms. Antidiabetic activity of leguminous plants is due to bioactive compounds such as genistein and daidzein, alpha-amylase inhibitors, alpha-glucosidase inhibitors. Present work focuses on assessment of edible bean, Dolichos lablab L. for the presence of insulin like proteins (ILP). Extraction of ILP was carried out by a modified, less laborious protocol based on fractional solubilization from germinating seeds of D. lablab L. Extracted ILP was purified by Reverse phase HPLC and collected fraction was assessed by SDS PAGE. The molecular weight was found to be 6KD. Further the in vitro assessment of the ILP was carried out using glucose uptake by yeast cells. Based on present work, it's evident that there are high chances of having insulin like proteins having antidiabetic potential in D. lablab L.

1. **Introduction**

Diabetes Mellitus (DM) is a metabolic disorder probably one of the oldest disorder to man characterized by the presence of chronic hyperglycemia with more or less damage to the metabolism of carbohydrates, lipids and proteins. The global pervasiveness of diabetes is increasing constantly. As per the International Diabetes Federation Report of 2011 around 366 million people had DM and by 2030 this number is estimated to almost around 552 million (Wild et al., 2000). The number of people with type 2 DM is increasing in every country with 80% of people with DM living in developing countries. Treatment of DM involves tablets lowering blood sugar, insulin injections or medication called incretin mimetics i.e. hormone-like substances that are designed to in crease the body’s in sulin production.

Legumes are a source of wholesome protein, alimentary fiber, and bioactive substances displaying antioxidant activity together with anti-inflammatory and antineoplastic properties. These plants should be employed in the promotion of a healthy lifestyle as a form of functional foods. Leguminous plants like dry beans, chickpeas, lentils, and soybeans play a crucial role in the diabetic diet. Legumes contain vegetable proteins ranging from 20% in beans and peas up to 38–40% in soya beans. These lysine rich proteins increase the nutritional value of beans. The legumes are known to have a low glycaemia index (<50) and they are alkalogenic products, which is especially vital in acid-alkaline balance maintenance organisms.

Leguminous plants are outstanding for their high levels of bioactive compounds, which can impact glucose metabolism by various mechanisms. Antidiabetic activity of leguminous plants is due to bioactive compounds such as genistein and daidzein, alpha-amylase inhibitors, alpha-glucosidase inhibitors.

Insulin-like proteinaceous active compounds were found in leaves of a legume plant Bauhinia variegata L. Ethanolic extracts of the same displayed the blood glucose reducing effect for white albino mice (Azevedo et al., 2006).

Khanna et al. (1974) reported the presence of insulin in the fruits of Momordica charantia (bitter gourd) and patented a process for its production from the same. Hirano H. (1996) isolated Leg-insulin from radishes of germinated soybean seeds by affinity chromatography. This Soybean seed glycoprotein, basic 7S globulin, which was capable of binding to bovine insulin and insulin-like growth factors was having similarity to the animal insulin receptor in protein structure, subcellular localization and protein kinase activity.

Oliveira et al. (1999) displayed that the seed coat of the legume Canavalia ensiformis contain a protein with a sequence equal to that of bovine insulin. A similar protein involved in carbohydrate transport to fruits was detected in empty pods and seed coats of cowpea (Vigna unguiculata) by Western blotting (VENÂNCIO et al., 2003).

Orally active fraction of Insulin like protein was purified from Costus igneus which found to have similar therapeutic effectiveness like animal insulin. The characterization of insulin like protein exposed that it is structurally different from insulin but functionally similar (Joshi et al., 2013). The prime focus of the current research work is to assess the papilionaceous member
Dolichos lablab Linn. (Valpapdi) for the presence of orally active plant Insulin like protein (ILP) having insulin mimicking property which will be a great support to the population suffering from diabetes.

2. Material & Methods:

Plant Material:

Authentic dry seeds of Dolichos lablab Linn. were procured from Agricultural Research Station, Navsari. The seeds were washed with plenty of sterile distilled water and dried again in the oven at 400C till complete dryness. Healthy, non-infected and viable seeds were used for the entire research work. Dried plant material was finely pulverized using a domestic grinder and then sieved through a No. 180 sieve in order to obtain fine powder of uniform particle size. The fine powder was stored in air tight polyethylene containers at -200 C till use. Similarly the powder was also prepared using germinating seeds.

All the chemicals used for the analysis were of Analytical grade and were procured from Merck India Pvt. Ltd. Lyophilized powder of Standard Bovine Serum Insulin was procured from Sigma Aldrich Pvt. Ltd. Analytical Standard was 99.99% pure and was obtained in amber bottle which was stored at -200 C.

Extraction of crude insulin like proteins:

10 g of dry, pulverized fine seed powder was defatted with 50 mL of mixture of n-Hexane:petroleum Ether (2:1 v/v) for 8-10 hour followed by air-drying to remove the residual solvents. 10 g defatted, air dried germinating seed powder was extracted in 100 mL of 60% ethanol containing 0.1% concentrated Sulfuric Acid. The mixture was next kept on magnetic stirrer for one hour in ice to ensure the complete extraction of insulin like proteins. After one hour, the mixture was filtered through the whatman filter paper and filtrate was collected and precipitated using equal volume of chilled acetone. Tubes were maintained at -200C for 10-12 hours (overnight) for complete precipitation of insulin like proteins (figure 1). Precipitate was collected by centrifugation and washed with deionized water followed by chilled alcohol. The precipitate was air dried (figure 1) for removal of traces of alcohol and dried precipitated proteins were reconstituted in 2mL of 0.05 M Sodium Phosphate buffer containing 0.1% Trifluoroacetic acid. Above mentioned protocol was also used to extract insulin like protein from soaked seeds. germinating seeds (24 hours, 36 hours, 48 hours and 72 hours) of Dolichos lablab Linn. This was performed to discover the stage at which maximum insulin like proteins are synthesized.

Analysis of Extracted ILP from Dolichos lablab L.

Analysis of ILP was carried out by thin layer chromatography, Sodium Dodecyl sulphate-Polyacrylamide Gel Electrophoresis, High performance Liquid Chromatography (HPLC) and UV-Visible Spectrophotometry.

Extracted protein was separated on 12% polyacrylamide gel, reducing conditions and stained using Coomassie brilliant blue.

Extracted protein was quantified by UV-visible spectrophotometry and HPLC. For Spectrophotometric Analysis, extracted protein was solubilized in 0.1% TCA and quantified by using Bovine serum insulin as Standard. Absorbance was recorded at 280 nm.

Reverse Phase High Performance Liquid Chromatography was carried out on isocratic mode for the separation of Insulin like proteins. Dionex C-18 column (5 mm, 120 A0 4.6 x 150 mm) was used for the RP-HPLC Analysis. Sample was prepared by dissolving 10 mg of precipitated protein in 2 mL of 0.1m L0.1% TFA in deionized water to obtain 5000 ppm of sample. The column was initially flushed using Flushing solvent Water: Acetonitrile (50:50 v/v) & then Mobile Phase Water: Acetonitrile (95.5 v/v) was injected for baseline correction. Flow rate of mobile phase was maintained at 1 mL per minute. 10 microliters of sample or standard was injected in HPLC system. The analyte was detected at 280 nm using Photodiode array detector.

In Vitro Assessment of Insulin Like protein using Glucose uptakeby Yeast cells

The In vitro assessment was carried out by the method reported by Cirillo et al., 1963 & Sinhu et al., 2013. 10 mg/mL Metronidazole Standard prepared in Phosphate buffered saline was used for the present study. Glucose was estimated from the media using DNSA method.

Results and Discussions:

Dried and germinating Seeds of Dolichos lablab Linn. (Valpapdi) were used for extraction of insulin like protein. In the current work, modified quicker, simple and less laborious protocol for extraction of insulin like protein was developed after trying several protocols reported earlier (Khanna et al., 1981, Collier et al., 1986, Oliveira et al., 1999, Venâncio et al., 2003). Dried and germinating Seeds of Dolichos lablab Linn. were assessed by analytical techniques such as UV-Visible Spectrophotometry (table 1, 2; Fig.1) and SDS-PAGE for the presence of insulin like proteins. From the results obtained high probabilities of expression of insulin like protein was throughout the germination period, but maximum expression of insulin like protein was found in the germination stage of 36 hours.

Further, Chromatographic analysis was employed for detection of insulin like protein. TLC as well as HPLC analysis substantiated the presence of insulin like protein in D. lablab L.

TLC is not frequently used for the analysis of proteins. The first work carried out on insulin like proteins (Khanna et al., 1981) from plants was carried out using TLC and recently published application notes proves that quantification of insulin can be done by HPTLC(CAMAG). In spite of this it was decided to use the same as in accordance, the crude insulin like proteins was subjected to thin layer chromatography. Standard bovine serum insulin and extracted insulin like protein showed a band at r f 0.23±0.1.
Results of HPLC analysis are provided in table 3 and figures 4, 5, & 6. It was evident that the extracted protein is relatively similar to Humulin & recombinant insulin. There was a slight variation in retention times of Humulin, recombinant insulin & plant insulin. This must be due to the difference in initial sources of the insulin & biochemical form in which it is present in it.

Chromatographic analysis of insulin like protein from D. lablab L. revealed that there are high chances of having protein which may be similar to insulin. Although preliminary detection of Insulin like protein from D. lablab L. was carried out by HPLC, further characterization of the insulin like protein was essential.

After assessing the peak characteristics various efforts were taken for HPLC method development for insulin like protein from D. lablab L. Therefore, it was decided to use HPLC as tool for purification for insulin like protein from D. lablab L.

Similar HPLC analysis of Insulin like protein from Vigna unguiculata (L.) was reported by Venâncio et al., in 2003 where, the retention time for analyte was around 30 minutes. In the current work a more efficient method for faster separation has been developed.

Detection of Insulin like protein was also carried out using HPLC from a legume plant Bauhinia variegata Linn. (Rashid et al., 2014). Ethanolic extracts of leaves of B. variegata L. were subjected to HPLC analysis using mobile phase Methanol & water in proportion 70:30 v/v having pH adjusted to 2.5 using formic acid. Standard Human Insulin Peak was detected at retention time of 15.698 minutes and insulin like protein from B. variegata L. was detected at 15.240 minutes.

Resemblance in analysis of ILP from plant samples was observed in the form of split peaks. This is possibly due to complex nature of the protein such as glycoprotein. The slight difference in retention time is probably due to the variation in the source of insulin. So, there can be a possibility of having structural differences in the insulin like protein and humulin or Recombinant Insulin. Based on the HPLC analysis an effort was made for quantitation of insulin like protein from the various accessions of D. lablab L.

Therefore, in comparison with previously reported work an effort was made to establish the faster, efficient and less laborious protocol for extraction, purification and analysis of plant based insulin (Venâncio et al., 2003). The work also focused on the HPLC method for analysis of plant insulin was developed during the present work. (Table 3, Figure 4, 5, 6)

Refinement of the insulin like proteins was carried out using fractional solubilization from Dolichos lablab Linn. Seeds and purity of each fraction was evaluated by SDS PAGE. Molecular weight of the insulin like protein (migration velocity 0.9) from D. lablab L. was found to be around 6kDa. (Fig.7)

From the results, it was concluded that D. lablab L. seeds contain proteins which might be similar to insulin. SDS PAGE Analysis confirmed that HPLC purified fraction contains single protein having molecular weight similar to Standard bovine serum Insulin. Therefore, HPLC and SDS PAGE Analysis of insulin like protein fraction elucidated that D. lablab L. seeds contain a protein which is similar to insulin.

Effect of Standard metronidazole and insulin like protein extracted from D. lablab L on in vitro glucose uptake by yeast cells was studied. There was significant increase in the glucose uptake with increasing concentration of metronidazole (Fig.8). Similar effect on glucose uptake was demonstrated by insulin like protein extracted from D. lablab L. (Fig.8). From the in vitro studies based on yeast cells, it can be manifested that insulin like protein isolated from D lablab L. may have antidiabetic potential which can be an alternative to modern drug.

Form the present work we can conclude that there are high chances of Insulin like protein in germinating seeds of Dolichos lablab L. However, the further confirmation for the presence of ILP is essential by Mass spectroscopic methods and in vivo assessment using a suitable animal model.

Figure 1. Crude precipitate of insulin like protein extracted from D. lablab L.

Fig. 1 Calibration graph of Standard Insulin at 280 nm

Table 1 Insulin like protein in germinating seeds of the edible legumes assessed

<table>
<thead>
<tr>
<th>Name of the plant</th>
<th>Absorbance at 280 nm</th>
<th>Amount of insulin like protein per 10 g of seeds</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dolichos lablab Linn. (Valpapdi)</td>
<td>0.128</td>
<td>44.50 mg</td>
</tr>
</tbody>
</table>

Table 2. Insulin like protein in germinating seeds of Dolichos lablab Linn.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Absorbance at 280 nm</th>
<th>Amount of insulin like protein in mg /10 g of seeds</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seeds soaked for 12 hours</td>
<td>0.110</td>
<td>43.64</td>
</tr>
<tr>
<td>Germinating Seeds (24 hours)</td>
<td>0.128</td>
<td>44.50</td>
</tr>
<tr>
<td>Germinating Seeds (36 hours)</td>
<td>0.142</td>
<td>43.36</td>
</tr>
</tbody>
</table>
Fig. 3. Expression of insulin like proteins in germinating seeds of *D. lablab* L.

![Expression of Insulin Like Protein In Germinating Seeds of Dolichos lablab Linn.](image)

Germinating Seeds (48 hours) 0.131 44.88
Germinating Seeds (72 hours) 0.110 43.64

Table 3: HPLC analysis of Insulin like protein from *Dolichos lablab* Linn.

<table>
<thead>
<tr>
<th>Name of the Peak</th>
<th>Retention time (minutes)</th>
<th>AUC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recombinant Insulin Standard</td>
<td>1.861</td>
<td>161396</td>
</tr>
<tr>
<td>Humulin</td>
<td>1.830</td>
<td>2954982</td>
</tr>
<tr>
<td>Leginsulin/ Insulin like protein from <em>Dolichos lablab</em> Linn.</td>
<td>1.524</td>
<td>3892745</td>
</tr>
</tbody>
</table>

Fig. 4 HPLC chromatogram of Humulin Standard

![HPLC chromatogram of Humulin Standard](image)

Fig. 6 HPLC chromatogram of protein extracted from *D. lablab* L.

![HPLC Chromatogram of Protein Extracted from D. lablab L.](image)

Table 4:

<table>
<thead>
<tr>
<th>Track</th>
<th>Details</th>
<th>No of proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>Protein Marker having range of molecular Weight from 9 KDa to 20 KDa</td>
<td>07</td>
</tr>
<tr>
<td>T</td>
<td>Protein Fraction extracted in 60% acetic acid before precipitation</td>
<td>13</td>
</tr>
<tr>
<td>P1</td>
<td>Protein Fraction Reconstituted in 0.15 M Sodium Phosphate buffer (pH 7.6) after precipitation after washing with sterile deionized water</td>
<td>01</td>
</tr>
<tr>
<td>P2</td>
<td>Standard Bovine serum Insulin</td>
<td>01</td>
</tr>
</tbody>
</table>

Fig. 7. Electrophoresis gel showing separation of insulin like protein from plant

![Electrophoresis Gel Showing Separation of Insulin Like Protein from Plant](image)

Fig. 8 Glucose Uptake after the treatment of Standard Metronidazole and Insulin like protein from *D. lablab* L.

Glucose Uptake by yeast cells mg/mL

![Glucose Uptake by Yeast Cells mg/mL](image)

Fig. 8 Glucose Uptake after the treatment of Standard Metronidazole and Insulin like protein from *D. lablab* L.

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4. P.S. Ramanathan Advance Instrumentation Centre, Ruia College
References


