IN-VITRO ANTIDIABETIC EFFECT OF POLYHERBAL FORMULATION

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ABSTRACT
Diabetes mellitus is characterised by hyperglycaemia and unbalanced carbohydrate metabolism. Under hyperglycaemic condition, regulation of blood glucose level happens to be indispensable for preventing various diabetes related complications. Treatment with herbal drugs has always remained a choice of treatment for protecting or rejuvenating β-cells and smoothing out fluctuation in glucose levels. A polyherbal medicine (PHM) formulated and tested with different in-vitro anti-diabetic assays in present study indicate that PHM possesses good antidiabetic activity. In vitro human salivary amylase (HSA) inhibition assay exhibited 81.27 % amylase inhibition and yeast glucose uptake assay showed 25% increase in glucose uptake at the concentration of 250 mg ml⁻¹. Generally the haemoglobin glycosylation is determined mainly to identify the average glucose concentration over a prolonged period of time. Higher values of glycated haemoglobin indicate poor control of blood glucose level under diabetic condition. The in-vitro haemoglobin glycosylation inhibition assay showed considerable inhibition of glycosylation over a period of 72 hrs as compared to gallic acid, which clearly indicates the capacity of PHM to decrease the formation of glucose-haemoglobin complexes at physiological glucose concentration. Overall results of in-vitro antidiabetic assays clearly indicate that PHM has considerable antidiabetic activity and may be used in management of hyperglycaemia in diabetic patients.

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INTRODUCTION

Diabetes mellitus is a disease of the pancreas caused by the diminished effectiveness of endogenous insulin. It is characterised by hyperglycaemia and unbalanced carbohydrate metabolism. In diabetic patients pancreas produce insufficient insulin or insulin may not be used properly. Insulin manages glucose (sugar) entry from the bloodstream into the body cells to be used as energy. If the function of insulin is impaired, glucose cannot enter the body cells. Such conditions result in hyperglycaemia or diabetes that makes the cells inadequate in energy.

Based on the WHO recommendations hypoglycaemic agents of plant origin used in traditional medicine are important [1]. The anti-hyperglycaemic effects of plant based drugs are due to their ability to restore the function of pancreatic tissues or inhibit the intestinal absorption of glucose or to assist metabolites in insulin dependent processes. Hence, treatment with plant drugs has an effect on protecting β-cells and smoothing out fluctuation in glucose levels [2, 3].

In the traditional system of Ayurveda, polyherbal formulations are used as drug of choice rather than individual plant extract. Various herbal formulations such as DIAMED [4] COAGENT db [5] and HYPONID [6] are known for their antidiabetic effect [7]. However, these formulations cannot be said ideal in the treatment of diabetes.

Therefore, eight different herbs having β-cells rejuvenation properties and hypoglycaemic activities were blended to form a polyherbal formulation. In addition, polyherbal blend was subjected to bioconversion with fruit yeast to yield a liquid polyherbal formulation (PHM).

MATERIALS AND METHODS

The polyherbal medicine was formulated with 8 herbs viz. Tinospora cordifolia, Adhatoda vasica, Stevia rebaudiana, Pterocarpus marsupium, Withania somnifera, Tridax procumbens, Boerhaavia diffusa and Syzygium cumini. The formulation was subjected to bioconversion for 40 days with fruit yeast.

Glucose uptake by yeast cells

Yeast cells (1 g) were washed thoroughly for 4-5 times in distilled water and final cell suspension volume was made to 10 ml with distilled water. Polyherbal medicine at the concentration of 50 to 250 μg ml⁻¹ was added to 1ml of 10 mM glucose solution. These mixtures were incubated for 10 min at 37 °C and glucose uptake was started by adding and mixing 200 μl of yeast suspension and incubating at 37 °C for 60 min. After 60 min, the content was centrifuged (2,500 × g, 5 min) and amount of glucose was estimated with DNSA reagent. The percent increase in glucose uptake by yeast cells was calculated with following formula.

\[
\% \text{ Glucose uptake} = \frac{(A_{\text{control}} - A_{\text{Sample}})}{A_{\text{control}}} \times 100
\]

Salivary alpha amylase inhibitory assay

For assaying amylase inhibitory action, 10 Units of human salivary amylase (HSA) was pretreated with PHM. The pretreatment assay mixture composed of 2.5 ml of 0.1 M sodium phosphate buffer (pH 6.9) containing 6 mM sodium chloride, 10 units of HSA and 50 to 250 μg ml⁻¹ PHM. These mixtures were incubated at 37°C for 30 min. After pre-incubation, 0.5 ml of 1% (v/v) starch solution was added to each tube and incubated at 37°C for 30 min. The reactions from each tube were terminated with addition of 1.0 ml 3, 5-Dinitro salicylic acid (DNSA) reagent and incubating reaction mixture in water bath at 100 °C for 10 min. The volume of reaction mixture in each tube was made to 10 ml with distilled water and absorbance was measured at 540 nm on UV-vis spectrophotometer (Shimadzu). The reaction mixture in control tube was prepared without medicine. For eliminating the absorbance produced by PHM, corresponding control without enzyme were also treated with DNSA reagent. The control HSA assay at 10 U without PHM represented 100% enzyme activity. The percent α-amylase inhibition was calculated with following formula.

\[
\% \alpha\text{-Amylase inhibition} = \frac{(A_{\text{control}} - A_{\text{Sample}})}{A_{\text{control}}} \times 100
\]

Haemoglobin glycosylation

Preparation of haemoglobin.

The blood of healthy volunteers was collected in EDTA vials from a pathology laboratory. Haemolysate was prepared based on the principle of hypotonic lysis [8]. The blood collected was washed thrice with 140 mM sodium chloride solution and one volume of red blood cells suspension was lysed with two volumes of 10 mM phosphate buffer, pH 7.4 and 0.5 volume of chloroform. The haemolysate was then centrifuged at 3000 x g for 15 min to achieve clear haemoglobin solution. The haemoglobin rich fraction i.e. the upper layer was separated and dispensed into vials for storage and refrigerated until required for further use [8].

Effect of PHM on Haemoglobin Glycosylation:

To study the effect of PHM on glycosylation, 1 ml of haemoglobin solution, 5μl of gentamycin and 50, 100,150,200, and 250 μg ml⁻¹ of PHM were added in test tubes. The reaction was started by the addition of 1 ml of 20 mM glucose in 10M phosphate buffer (pH 7.4) and incubated in dark at 27 ± 2 °C temperature. The amounts of glycated haemoglobin were estimated at 443nm on spectrophotometer at the incubation interval of 24, 48 and 72 hrs and percent haemoglobin glycosylation was calculated. [8]
Haemoglobin glycosylation at physiological glucose concentration
To 1 ml of haemoglobin solution, 1 ml of glucose solution (2, 10 and 20 mg ml\(^{-1}\)) and 5μl of gentamycin in 20 ml of 10 mM phosphate buffer (pH 7.4) were mixed and incubated in dark at room temperature in the presence of 50, 100,150,200, and 250 μg ml\(^{-1}\) of Gallic acid and PHM respectively. Amount of haemoglobin was measured at 443 nm on spectrophotometer over an incubation period of 72 hrs as an indicator of haemoglobin glycosylation. The assay was carried out in triplicates and percent haemoglobin glycosylation was calculated at physiological glucose concentration. [8]

RESULTS AND DISCUSSION
Data pertaining to the phytoconstituents estimation of PHM are given in Table-1. Data indicate that PHM contains 10.80 mg ml\(^{-1}\) of total reducing sugars, 8.84 mg ml\(^{-1}\) of total phenols, 2.36 mg ml\(^{-1}\) flavonoids including 1.02 mg ml\(^{-1}\) gallic acid and 4.73 mg ml\(^{-1}\) tannic acid.

Table-1: Amount of phyto-constituents in poly-herbal medicine.

<table>
<thead>
<tr>
<th>Sr No</th>
<th>Compounds/molecules</th>
<th>Amount (mg ml(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Reducing sugar</td>
<td>10.80</td>
</tr>
<tr>
<td>2</td>
<td>Total Phenols</td>
<td>8.84</td>
</tr>
<tr>
<td>3</td>
<td>Total flavonoids</td>
<td>2.36</td>
</tr>
<tr>
<td>4</td>
<td>Gallic acid</td>
<td>1.02</td>
</tr>
<tr>
<td>5</td>
<td>Tannic acid</td>
<td>4.73</td>
</tr>
</tbody>
</table>

Table-2: Alpha amylase inhibitory potential of poly herbal medicine.

<table>
<thead>
<tr>
<th>Concentration of PHM (µg ml(^{-1}))</th>
<th>Standard (Acarbose)</th>
<th>IC(_{50}) value (µg)</th>
<th>Polyherbal Medicine (PHM)</th>
<th>IC(_{50}) value (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>18.14</td>
<td>154.58</td>
<td>15.62</td>
<td>146.71</td>
</tr>
<tr>
<td>100</td>
<td>39.78</td>
<td>28.92</td>
<td>25.78</td>
<td>81.27</td>
</tr>
<tr>
<td>150</td>
<td>48.52</td>
<td>51.12</td>
<td>40.52</td>
<td>70.18</td>
</tr>
<tr>
<td>200</td>
<td>60.88</td>
<td></td>
<td>45.28</td>
<td></td>
</tr>
<tr>
<td>250</td>
<td>70.18</td>
<td></td>
<td>50.78</td>
<td></td>
</tr>
</tbody>
</table>

Data with respect to in-vitro human salivary α- amylase (HSA) activity indicate that percent HSA inhibition is dose dependent and showed increase in percent amylase inhibition with increase in concentration of PHM (50 to 250 µg ml\(^{-1}\)) from 15.62 to 81.27 % respectively with IC\(_{50}\) value of 146.71 µg which is comparatively less than the standard drug acarbose i.e. 154.58 µg (Table -2). This clearly indicates that PHM has more amylase inhibitory potential than acarbose.

Table-3: Effect of PHM on haemoglobin glycosylation.

<table>
<thead>
<tr>
<th>Concentration of PHM (µg ml(^{-1}))</th>
<th>Percent increase in glucose uptake by yeast cells</th>
<th>% Haemoglobin glycosylation inhibition 24 hrs</th>
<th>48 hrs</th>
<th>72 hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>11.20</td>
<td>12.14</td>
<td>14.48</td>
<td>28.42</td>
</tr>
<tr>
<td>100</td>
<td>15.57</td>
<td>18.16</td>
<td>21.48</td>
<td>48.56</td>
</tr>
<tr>
<td>150</td>
<td>19.55</td>
<td>20.24</td>
<td>23.18</td>
<td>62.68</td>
</tr>
<tr>
<td>200</td>
<td>22.58</td>
<td>23.18</td>
<td>30.19</td>
<td>76.82</td>
</tr>
<tr>
<td>250</td>
<td>25.60</td>
<td>26.88</td>
<td>42.17</td>
<td>88.84</td>
</tr>
</tbody>
</table>

Data given in Table- 3 indicate that % inhibition of haemoglobin glycosylation, which is dose dependent, and clearly point out that as the concentration of PHM increases formation of glucose- haemoglobin complex decrease thereby unshackling the haemoglobin from sugar. Similar trend was seen for the hemoglobin glycosylation at physiological glucose concentration, however higher glycosylation was reported at higher levels of glucose (Table-4).
Table 4: Effect of PHM on percent inhibition of haemoglobin glycosylation at physiological glucose concentrations in comparison with standard (gallic acid).

<table>
<thead>
<tr>
<th>Concentration of Gallic acid / PHM (µg ml⁻¹)</th>
<th>Glucose (2 mg ml⁻¹) Standard</th>
<th>Glucose (10 mg ml⁻¹) Standard</th>
<th>Glucose (20 mg ml⁻¹) Standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>32.45</td>
<td>25.52</td>
<td>24.96</td>
</tr>
<tr>
<td>100</td>
<td>43.99</td>
<td>43.79</td>
<td>33.84</td>
</tr>
<tr>
<td>150</td>
<td>52.57</td>
<td>57.38</td>
<td>40.44</td>
</tr>
<tr>
<td>200</td>
<td>58.81</td>
<td>68.94</td>
<td>45.24</td>
</tr>
<tr>
<td>250</td>
<td>59.90</td>
<td>75.46</td>
<td>46.08</td>
</tr>
</tbody>
</table>

Different types of oral hypoglycaemic agents are available along with insulin for the treatment of diabetes mellitus; however, patients insist for natural antidiabetic products. Insulin cannot be used orally and continuous use of synthetic drugs has risk of side effects and toxicity [9, 10]. Insulin is a main factor which controls glucose homeostasis in normal human. However, under diabetic condition lack of insulin affects the metabolism of carbohydrates, proteins, fat and causes significant disturbance of water and glucose homeostasis [11]. In diabetic patients higher level of postprandial blood glucose is mainly responsible for micro and macro-vascular complications than the fasting blood glucose. It is well known that suppression of starch digestive enzymes reduce elevation of postprandial blood glucose level [12]. Hence, one of the remedial approaches for minimizing postprandial (PP) blood glucose level in diabetic patients is to reduce glucose production by suppressing amylases and glucosidases after food intake. The amylase inhibitors of plant origin can regulate glucose level in the blood of the diabetic patients and thereby prevent the various complications associated with this disease. The in vitro amylase inhibition assays carried out in present study indicate that 50 % inhibition of human salivary amylase was achieved with 146.71 µg ml⁻¹ of PHM, which about 5% more than standard drug i.e. acarbose. This indicates that PHM possesses good antidiabetic activity. A comparative in-vitro alpha amylase inhibitory activity study in Stevia rebaudiana, Tinospora cordifolia, Boerhaavia diffusa and Withania somnifera reported that these plants have considerable inhibitory activity [13].

The mechanism of glucose uptake through yeast cell membrane has been most recognized as in vitro screening protocol to study anti-hyperglycaemic effects of different phytoconstituents or compounds of plant origin. The study carried out in the recent past on the transport of non metabolizable sugars and certain metabolizable glycosides in yeast cells (Saccharomyces cerevisiae) suggest that sugar transport across the cell membrane is extremely complex and mediated by stereospecific membrane carriers. Such facilitated carriers are specific carriers that transport solutes down the concentration gradient. This means that effective transport is only attained if there is removal of intracellular glucose [14, 15, 16]. In present study when yeast cells were treated with the PHM, the glucose uptake was found to increase in a dose dependent manner. Result indicates that PHM has greater efficiency in increasing the glucose uptake by yeast cells.

A non-enzymatic reaction between free amino groups of proteins and reducing sugars is known as glycation [17]. The increased glycation is nothing but additional binding of glucose to hemoglobin which may leads to formation of reactive oxygen species (ROS). Such accelerated haemoglobin glycation can also cause pathogenic conditions like angiopathy, nephropathy and neuropathy in diabetic patients [18]. When oxidation occurs in glycation process, it leads to glycoxidation and formation of advanced glycation end products (AGEs) [19, 20]. It is well known that phytoconstituents play an important role in the inhibition of the AGEs. In present study increased glycosylation was noted when hemoglobin was incubated with glucose over a period of 72 hrs. However, the PHM significantly inhibited the haemoglobin glycosylation which was indicated by presence of increasing concentration of haemoglobin. The PHM also displayed the inhibition of haemoglobin glycosylation at different physiological concentrations of the glucose over the period of 72 hrs, which indicate that the PHM inhibits formation of the glucose-haemoglobin complex and thus allows haemoglobin to work freely. Results clearly indicate that PHM has an appreciable antidiabetic activity. This observed effect might be attributed to the presence of bioactive compounds like flavonoids and phenols in the PHM. Research studies carried out in recent past indicate that epicatechin and epigallocatechin gallate showed insulin like properties and hypoglycaemic activity respectively [21]. Such active phenolic and flavonoid compounds along with gallic acid have been reported in some herbs like Boerhaavia diffusa, Withania somnifera and Tinospora cordifolia used in PHM [22, 23, 24]. This antidiabetic activity may be attributed to a bioconversion process, which extracts phytoconstituents from the plant materials during fermentation and in addition modify and detoxify certain compounds available in the herbs. Yeast also produces its own vitamins and organic acids, which results in a value added form of medicine. However this needs further investigation of specific bioactive compound responsible for such activities.
CONCLUSION

Findings of in-vitro antidiabetic assay clearly indicate that PHM possesses considerable inhibitory activity against human salivary amylase, it also facilitates glucose uptake by yeast cells and inhibit the glycation of haemoglobin. This observed antidiabetic activity might be attributed to bioactive compounds like phenols, flavonoids, alkaloids extracted from herbs used in the formulation as well as organic acids and vitamins produced by the fruit yeast during bioconversion process. Results aforesaid of in-vitro antidiabetic assay clearly indicate that PHM could be used to reduce post-prandial blood glucose levels in diabetic patients. This needs further in-vivo investigations in animal models and identification of specific bio active compounds responsible for such activities.

Conflict of interest

Authors do not have any conflict of interest.

Abbreviations

PHM: Polyherbal medicine, HSA: Human salivary α- amylase; IC_{50} Inhibitory concentration where 50 % inhibition of enzyme is achieved; μg ml⁻¹: Microgram per millilitre; α: alpha; UV-Vis : Ultraviolet – Visible, ROS: Reactive oxygen species, AGEs: Advanced glycation end products

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