ABSTRACT

Objective: The objective of the present study was to evaluate the inhibitory activity of alpha-amylase and alpha-glucosidase activity of various extracts of *Hybanthus enneaspermus*. Methods: The whole plant of *H. enneaspermus* was dried under shade and powdered. The powdered material was used for preparing the various solvent extracts, which were used for the *invitro* antidiabetic study. Results: Various extracts of *H. enneaspermus* showed alpha-amylase and alpha-glucosidase inhibitory effect in a concentration-dependent manner. Ethanolic extract possessed great inhibitory activity compared with other extracts. Conclusion: The present study confirms the evidence of hypoglycemic effect of *H. enneaspermus* through enzyme inhibitory activity and gives a clear evidence that *H. enneaspermus* has antidiabetic activity.


INTRODUCTION

Diabetes mellitus (DM) is a chronic disease characterized by a deficiency in insulin production and its action or both. That leads to prolonged hyperglycemia with disturbances in most metabolic processes inside the human body.[1] Untreated cases show severe tissue and vascular damage leading to serious complications such as retinopathy, neuropathy, nephropathy, cardiovascular complications and ulceration.[2-6] Moreover, diabetes has an indirect relation with a many other diseases being the most common endocrine disorder. It was estimated that about 200 million people worldwide suffered from DM in 2010, and it is expected to reach 300 million by 2025.[7] The common strategy for treatment focused mainly
on regulating and decreasing blood sugar to fall within the normal level. The main mechanisms in both traditional and western medicines involve decreasing blood sugar through stimulating pancreatic β-cells inhibiting other hormones elevating blood sugar; increasing the affinity and sensitivity of insulin receptor. On the other hand, lowering glycogen release; enhancing glucose utilization within many tissues and organs; clearing free radicals, resisting lipid peroxidation, correction of the lipid and protein metabolic disorders and improving human blood circulation are also involved. The alpha-glucosidase inhibitors “starch blockers” inhibit certain enzymes responsible for the breakdown of carbohydrates in the small intestine. They act mainly by decreasing the rate of carbohydrate absorption in the body. Moreover, acarbose, an important example in this class, reversibly inhibits both pancreatic alpha-amylase and alpha-glucosidase enzymes by binding to the carbohydrate-binding region and interfering with their hydrolysis into mono-saccharides. This results in a slower absorption together with a reduction in postprandial blood-sugar levels. Although, synthetic oral hypoglycemics together with insulin are the main route for controlling diabetes but they exhibited prominent side effects and failed to reverse the course of its complications. This constitutes the major force for finding alternatives, mainly from plant kingdom that are of less severe or even no side effects. Natural products are the major mine for discovering promising lead candidates, which play an important role in future drug development programs. Easy of availability, least side effects and low cost make the herbal preparations are the main key player of all available therapies, especially in rural areas. Since centuries, many plants are considered a fundamental source of potent anti-diabetic drugs. Hybanthus enneaspermus (Family; Violaceae) is well known for its different therapeutic uses. The whole plant of H. enneaspermus is very acid to relining strangury, painful dysentery, vomiting burning sensation, wandering of the mind. Leaves of the plant are used as an external application for wound. It has been as an antimalarial, antirheumatic, emmenagogue, sedative, antispasmodic and anti-asthmatic. H. enneaspermus is also attributed to its antimicrobial and anti-plasmodial action, and it has been reported, in ancient ayurvedic literature, to cure conditions of urinary calculi. The roots of this H. enneaspermus have been indigenously used in epilepsy and hysteria. Early documented the presence of flavonoids, xanthine, terpenoids and glycosides in the H. enneaspermus. The intestinal digestive enzymes alpha-glucosidase and alpha-amylase plays a vital role in the carbohydrate digestion. One antidiabetic therapeutic approach reduces the postprandial glucose level in blood by the inhibition of alpha-glucosidase and alpha-amylase enzymes. Inhibition of alpha-amylase and alpha-glucosidase enzymes can be an
important strategy in management of postprandial blood glucose level in type 2 diabetes patient.\textsuperscript{[20]} Thus, the objective of the present study is to investigate the phytochemical and enzyme (alpha-glucosidase and alpha-amylas) inhibitory activity of various extracts of \textit{H. enneaspermus} by invivo.

**MATERIALS AND METHODS**

**Collection of plant material**

The plant \textit{H. enneaspermus} was collected from college campus, DGGA College(W), Mayiladuthurai, Tamilnadu, India, where it was found naturally. The whole plant was washed thoroughly in running tap water to remove soil particles and other adhered debris and then finally washed with sterile distilled water. The leaves were shade dried and ground well into fine powder. The powdered materials were stored in air tight container until the time of use.\textsuperscript{[21,22]}

**Phytochemical screening**

10 grams of \textit{H. enneaspermus} powder was soaked in 50 ml of various solvents (ethanol, acetone, petroleum ether and aqueous) separately and kept at room temperature for 12 hours.\textsuperscript{[21,22]} The samples were filtered and used for phytochemical screening. The various solvent extracts of this plant were screened for the presence of various phytochemical constituents such as alkaloids, flavonoids, phlobatannins, anthroquinones, steroids, tannins, phenols, terpenoids, saponins, resins, carbohydrate, protein and amino acids.\textsuperscript{[23,24,25]}

**Test for alkaloids**

Crude extract was mixed with 2 ml of Wagner’s reagent. Reddish brown colored precipitate indicates the presence of alkaloids.

**Test for flavonoids**

5 ml of dilute ammonia solution were added to a portion of the crude extract followed by addition of concentrated H\textsubscript{2}SO\textsubscript{4}. A yellow coloration observed in each extract indicated the presence of flavonoids. The yellow coloration disappeared on standing.

**Test for resins**

To 1ml of crude extract was treated with few drops of acetic anhydride solution followed by 1ml of concentrated H\textsubscript{2}SO\textsubscript{4}. Resins give coloration ranging from orange to yellow.
Test for saponins
Crude extract was mixed with 5 ml of distilled water in a test tube and it was shaken vigorously. Add some drops of olive oil. The formation of stable foam was taken as an indication for the presence of saponins.

Test for glycosides
5 ml of extract was treated with 2 ml of glacial acetic acid containing one drop of ferric chloride solution. This was underlaid with 1 ml of concentrated H$_2$SO$_4$. A brown ring of the interface indicates a deoxy sugar characteristic of cardenolides. A violet ring may appear below the brown ring, while in the acetic acid layer, a greenish ring may form just gradually throughout thin layer.

Test for steroids
2 ml of acetic anhydride was added to 0.5 ml crude extract of plant sample with 2 ml H$_2$SO$_4$. The colour changed from violet to blue or green in samples indicates the presence of steroids.

Test for tannins
1 ml of the sample was taken in a test tube and then 1 ml of 0.008 M potassium ferric cyanide was added. 1 ml of 0.02 M ferric chloride containing 0.1 N HCl was added and observed for blue-black coloration.

Test for phenols
To 1 ml of various solvent extract of sample, 2 ml of distilled water followed by a few drops of 10% aqueous ferric chloride solution was added. Formation of blue or green colour indicated the presence of phenols.

Test for terpenoids
5 ml of extract was mixed with 2 ml of chloroform and 3 ml of concentrated H$_2$SO$_4$ was carefully added to form a layer. A reddish brown coloration of the inter face was formed to show positive results for the presence of terpenoids.

Test for anthroquinones
Dilute NaOH was added to the 1 ml of crude extract. Blue green or red coloration indicates the presence of anthroquinones.
In-vitro methods employed in antidiabetic studies

Preparation of plant extracts
50 grams of *H. enneaspermus* powder were soaked in 250 ml of various solvents (aqueous, acetone and ethanol) separately and kept at room temperature for 12 hours and kept at shaker for 3 hours. The samples were filtered and through a single layer of muslin cloth, and then final filtrate was collected by passing it through a Whatman grade 1 filter paper in a Buchner funnel under vacuum. The filtrate was evaporated to dryness. The crude extract of *H. enneaspermus* was obtained. Different concentrations (40, 80, 120, 160 and 200µg) of each extract solution were prepared.[21,22]

Inhibition of alpha-amylase enzyme
A starch solution (0.1% w/v) was obtained by stirring 0.1g of potato starch in 100 ml of 16 mM of sodium acetate buffer. The enzyme solution was prepared by mixing 27.5 mg of alpha amylase in 100 ml of distilled water. The colorimetric reagent is prepared by mixing sodium potassium tartarate solution and 3, 5 di-nitro salicylic acid solution 96 mM. Both control and plant extracts were added with starch solution and left to react with alpha-amylase solution under alkaline conditions at 25°C. The reaction was measured over 3 minutes. The generation of maltose was quantified by the reduction of 3, 5 dinitro salicylic acid to 3-amino-5-nitro salicylic acid. This reaction is detectable at 540 nm.[26]

Inhibition of alpha-glucosidase enzyme
The inhibitory activity was determined by incubating a solution of starch substrate (2 % w/v maltose or sucrose) 1 ml with 0.2 M Tris buffer pH 8.0 and various concentration of plant extract for 5 min at 37°C. The reaction was initiated by adding 1 ml of alpha-glucosidase enzyme (1U/ml) to it followed by incubation for 40 min at 35°C. Then the reaction was terminated by the addition of 2 ml of 6N HCl. Then the intensity of the colour was measured at 540 nm.[27] The results were expressed as % inhibition calculated using the formula:

\[
\text{% Inhibition activity} = \frac{\text{Abs (Control)} - \text{Abs (Extract)}}{\text{Abs (Control)}} \times 100
\]

where Abs is the absorbance
RESULTS

The phytochemical active compounds of *H. enneaspermus* were analyzed by qualitatively and the results are presented in Table 1. In these screening process alkaloids, flavonoids, phlobatannins, anthroquinones, steroids, tannins, phenols, terpenoids, saponins, resins, carbohydrate, protein and amino acids shows different types of results in different extracts of *H. enneaspermus*. Alpha-amylase and alpha-glucosidase inhibitory activity of various solvent extracts of *H. enneaspermus* showed in table 1 and 2 respectively. All the extracts showed inhibitory activity against both enzymes significantly in dose dependent response. The ethanol extract of *H. enneaspermus* has maximum inhibitory effect for alpha-amylase and alpha-glucosidase than other solvent extracts is shown in figure 1 and 2 respectively. The inhibitory effect was represented as follows ethanol> petroleum ether>aqueous> acetone.

**Table-1 Phytochemical analysis of various extracts of *H. enneaspermus***

<table>
<thead>
<tr>
<th>S. No</th>
<th>Phytochemical constituents</th>
<th>Ethanol</th>
<th>Acetone</th>
<th>Petroleum ether</th>
<th>Aqueous</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Alkaloids</td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>Flavonoids</td>
<td>+</td>
<td>_</td>
<td>+</td>
<td>+</td>
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<tr>
<td>3</td>
<td>Glycosides</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>Steroids</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>Tannins</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>Phenols</td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>Terpenoids</td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>Saponins</td>
<td>_</td>
<td>_</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>9</td>
<td>Resins</td>
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<td>+</td>
<td>_</td>
<td>+</td>
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<td>10</td>
<td>Carbohydrate</td>
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<td>+</td>
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<td>+</td>
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<td>11</td>
<td>Phlobatannins</td>
<td>+</td>
<td>+</td>
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<td>_</td>
</tr>
<tr>
<td>12</td>
<td>Anthroquinones</td>
<td>_</td>
<td>_</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>13</td>
<td>Protein and aminoacid</td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
</tr>
</tbody>
</table>

(+ = presence, - = absence)

**Table-2 Alpha-amylase inhibitory activity of various extracts of *H. enneaspermus***

<table>
<thead>
<tr>
<th>Concentration of plant extract (μg)</th>
<th>Ethanol</th>
<th>Acetone</th>
<th>Petroleum ether</th>
<th>Aqueous</th>
</tr>
</thead>
<tbody>
<tr>
<td>40</td>
<td>38.46</td>
<td>11.53</td>
<td>19.23</td>
<td>15.38</td>
</tr>
<tr>
<td>80</td>
<td>57.69</td>
<td>23.07</td>
<td>34.61</td>
<td>26.92</td>
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<td>120</td>
<td>65.38</td>
<td>30.76</td>
<td>46.15</td>
<td>42.30</td>
</tr>
<tr>
<td>160</td>
<td>76.92</td>
<td>61.53</td>
<td>73.07</td>
<td>69.23</td>
</tr>
<tr>
<td>200</td>
<td>84.61</td>
<td>73.07</td>
<td>80.76</td>
<td>79.43</td>
</tr>
</tbody>
</table>
Table-3 Alpha-glucosidase inhibitory activity of various extracts of *H. enneaspermus*

<table>
<thead>
<tr>
<th>Concentration of plant extract (μg)</th>
<th>Ethanol</th>
<th>Acetone</th>
<th>Petroleum ether</th>
<th>Aqueous</th>
</tr>
</thead>
<tbody>
<tr>
<td>40</td>
<td>15.15</td>
<td>7.62</td>
<td>11.35</td>
<td>10.13</td>
</tr>
<tr>
<td>80</td>
<td>29.19</td>
<td>17.26</td>
<td>24.55</td>
<td>21.29</td>
</tr>
<tr>
<td>120</td>
<td>35.48</td>
<td>20.04</td>
<td>32.58</td>
<td>31.83</td>
</tr>
<tr>
<td>160</td>
<td>68.20</td>
<td>53.19</td>
<td>61.06</td>
<td>60.78</td>
</tr>
<tr>
<td>200</td>
<td>83.02</td>
<td>71.61</td>
<td>79.64</td>
<td>79.24</td>
</tr>
</tbody>
</table>

Figure 1. Alpha-amylase inhibitory activity of various extracts of *H. enneaspermus*

Figure 2. Alpha-glucosidase inhibitory activity of various extracts of *H. enneaspermus*

**DISCUSSION**

In present study photochemical screening of *H. enneaspermus* contained alkaloids, flavonoids, phlobatannins, anthroquinones, steroids, tannins, phenols, terpenoids, saponins, resins, carbohydrate and protein and amino acids. A wide and diverse range of plants have
been reported in the literature to prevent and treat diabetes. Several phytochemicals, including alkaloids, flavonoids, glycosides, glycolipid, galactomannan, polysaccharides, peptidoglycan, hypoglycans, guanidine, steroids, carbohydrates, glycopeptides, terpenoids, amino acids, saponins, dietary fibres and inorganic ions affect various metabolic cascades, which directly or indirectly affect the level of glucose in the human body. These have produced potent hypoglycemic, anti-hyperglycemic and glucose suppressive activities. The above effects achieved by either increase in serum insulin level or increase in the production of insulin from pancreatic β-cells, inhibit glucose absorption in the gut, stimulate glycogenesis in liver or increase glucose utilization by the body. These compounds also exhibit their antioxidant, hypolipidemic, anticataract activities, restored enzymatic functions, repair and regeneration of pancreatic islets and alleviation of liver and renal damage. Hence it is demonstrated that medicinal plant have potential effectiveness against diabetes and the phytochemicals play a major role in the management of diabetes.

Drugs that inhibit carbohydrate hydrolyzing enzymes have been demonstrated to decrease postprandial hyperglycemia and improve impaired glucose metabolism without promoting insulin secretion of NIDDM patients. The results of present invitro study showed that various extracts of *H. enneaspermus* inhibits alpha-amylase and alpha-glucosidase activity. Natural health products of vegetable origin were clearly indicated as a promising avenue for the prevention of chronic diseases. Ethanolic extracts of *H. enneaspermus* showed maximum inhibition of alpha-amylase, followed by petroleum ether, aqueous and acetone extracts (Table 2 and Figure 1). Alpha-amylase is an enzyme that hydrolyzes alpha bonds of large, alpha-linked polysaccharides, such as starch and glycogen. It is the major form of amylase found in humans and other mammals. Since alpha-amylase plays an important role in digestion of starch and glycogen, it is considered a strategy for the treatment of disorders in carbohydrate uptake, such as diabetes and obesity to reduce postprandial glucose level. Hence alpha-amylase inhibitors may be of value as novel therapeutic agents. However, inhibition of alpha-amylase by the phytochemicals of plants could be conclusively attributed to the presence of flavonoids, phenols.

The percentage inhibition of alpha-glucosidase activity at 200, 160, 120, 80 and 40 μg/ml concentration of crude plant extracts shown concentration dependent reduction in percentage inhibition. Table 3 and figure 2 showed significant inhibition of alpha-glucosidase activity. Here also ethanolic extract has greater inhibitory activity compared with petroleum ether,
aqueous and acetone extracts. alpha-glucosidase is an enzyme located on the brush border of enterocytes of jejunum.\textsuperscript{10} It binds to disaccharides and oligosaccharides, and cleaves terminal, non-reducing 1,4-alpha bonds and breaks down to single alpha-glucose molecule depending upon the substrate. It is proposed that alpha-glucosidase in the glucosidic path plays an important part in complementing phosphorolytic pathway in the liver’s metabolic response to energy demands.\textsuperscript{43} Alpha-glucosidase inhibitors block the action of enzyme in the small intestine, which is rate-limiting in the conversion of oligosaccharides to monosaccharides necessary for gastrointestinal absorption. The main benefits attributed to alpha-glucosidase inhibitors are, reduction in both postprandial glycemic levels and the total range of postprandial glucose levels.\textsuperscript{44} Derivatives of terpenoids are already proven as alpha-glucosidase inhibitors.\textsuperscript{45,46} Therefore, the antidiabetic effect of \textit{H. enneaspermus} might attribute to its inhibitory effect against alpha-amylase and alpha-glucosidase that retarding the digestion of carbohydrate leads to delay the postprandial rise in blood glucose. Regulation of glucose level in the blood of the diabetic patient can prevent the various complications associated with the disease.\textsuperscript{47}

CONCLUSION
In conclusion, our findings showed \textit{H. enneaspermus} have the potential to be explored further to identify the anti diabetic compounds in this plant. The ethanolic extract shows more anti-diabetic activity. This activity may be due to the presence of flavonoids, phenols, terpenoids, steroids, alkaloids and tannins and its quantification of individual phytoconstituents as well as pharmacological profile based on \textit{in vitro} and \textit{in vivo} studies and on clinical trial should be further investigated.

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