ABSTRACT

Patients of type-2 diabetes mellitus have high postprandial blood glucose level. One of the therapeutic approaches, therefore, is to reduce postprandial hyperglycemia. This can be achieved by inhibiting carbohydrate splitting enzymes. One such enzyme is alpha amylase which hydrolyses complex carbohydrates of food to free sugars. Inhibition in alpha amylase activity reduces hydrolysis of complex carbohydrate thereby postprandial hyperglycemia may be kept under control. Acarbose, one alpha amylase inhibitor, has already been included in the list of drugs of type - 2 diabetes mellitus. Still there is continuous search for alpha amylase inhibitors from different sources which even extended to the field of medicinal plants. Recently we have shown that ethanol extract of Murrya koenigii Linn. Spreng Wettst (M. koenigii L.) leaves has maximum in vitro alpha amylase inhibitory activity. Aim of the present work, therefore, was to isolate alpha amylase inhibitor from M. koenigii L. leaves. M. koenigii L. was collected from the local market and identified by the taxonomist. Ethanol extract of the plant leaves was processed for isolation work by standard methods. Solvent extraction and acid hydrolysis were done followed by solvent treatment and chromatographic experiments. Finally a compound was crystallized. In vitro alpha amylase inhibitory activity of the isolated compound was checked by standard method. Acarbose, an alpha amylase inhibitor, was used as control. Results showed that the isolated compound had strong alpha amylase inhibitory activity which was comparable to that of acarbose. The isolated compound may, therefore, be used in the management of diabetes.
KEYWORDS: *Murrya koenigii* linn. Leaves, isolated compound, alpha amylase inhibitory activity, Acarbose, diabetes.

1. INTRODUCTION

Diabetes mellitus, a chronic metabolic non-communicable disease, is increasing rapidly. There were approximately 108 million diabetic patients in the world in 1980 but in 2014 the number has been increased to 422 million. Presently highest population of diabetics are found in China, India, USA, Brazil, Mexico and Indonesia. In India diabetes is increasing so fast that the number of adults with diabetes is expected to reach 87 million by the year 2030. Due to this increasing trend diabetes took several lives till today. Only in 2015 diabetes mellitus was the cause of death for 5 million people worldwide.\[1\] Diabetes mellitus may be Type – 1 or Type-2. Type – 2 diabetes mellitus is characterized by postprandial hyperglycemia. One of the therapeutic approaches of Type – 2 diabetes mellitus, therefore, is to reduce postprandial hyperglycemia.\[2\] This can be done by inhibiting carbohydrate splitting enzymes. One such enzyme is alpha amylase which hydrolyses complex carbohydrates of food to free sugars. Inhibition of alpha amylase activity reduces hydrolysis of complex carbohydrate thereby postprandial hyperglycemia may be kept under control.\[3\] Acarbose, one alpha amylase inhibitor, has already been included in the list of drugs of Type - 2 diabetes mellitus.\[4\] Still search is going on for more alpha amylase inhibitors. In this context medicinal plants were also investigated and many plants are now-a-days known having alpha amylase inhibitory activity.\[5\]

Recently we observed that ethanol extract of *M. koenigii* L. leaves of rainy season has maximum *in vitro* alpha amylase inhibitory activity. Results are under communication. Aim of the present study, therefore, was to isolate alpha amylase inhibitor from *M. koenigii* L. leaves.

2. METHODOLOGY

2.1 Collection of plant materials

Leaves of *M. koenigii* L. of rainy season were purchased from the local market and authenticated by the taxonomist of the department of Botany of the University of North Bengal, Siliguri, Dist. Darjeeling, West Bengal, India. Voucher specimens of the leaves were deposited in the department for future references. Leaves of plants were washed thoroughly, shed dried and powered. The powder, used as test drug, was stored desiccated at 4 °C until further use.
2.2 Chemicals
Chemicals required for the study were purchased from Himedia Lab, Loba Chem. Lab, India and from Merck, Germany and Sigma Chemicals Co., USA.

2.3 Isolation of alpha amylase inhibitor from *M. koenigii* L. Leaves
Applying principles of standard isolation procedures of chemical compounds from plant sources\(^6,7\), this was done by the following scheme.

Diagrammatic scheme for isolation of alpha amylase inhibitor from *M. koenigii* L. leaves.

Powdered leaves of *M. koenigii* L. (50 g)

**SOLVENT EXTRACTION**
Extracted with 500 ml of ethanol for 15 min at 37\(^0\)C in a Soxhlet apparatus. It was then centrifuged. Supernatant collected and evaporated to dryness.

Active brown mass

**ACID REFLUX**
Refluxed with 50 ml of 1(N) HCL for 10 min on a water bath at 100 \(^0\)C. It was then cooled and centrifuged. Supernatant was evaporated to dryness.

Active brown mass

**TREATMENT WITH BENZENE**
Treated with 50 ml benzene on a rotary shaker for 15 min. It was then centrifuged. Supernatant was evaporated to dryness.
Active brown mass

**ALUMINA COLUMN CHROMATOGRAPHY**
Extracted with 25 ml of methanol for 10 min. It was then filtered. With filtrate alumina column chromatography was performed. Elution was done by isopropanol, hexane mixture (60:40 v/v).

Fifth band was found active

**POLYAMIDE COLUMN CHROMATOGRAPHY**
Eluent of active fifth band was evaporated to dryness. The dry mass was extracted with 25 ml ethanol for 10 min. It was then filtered. With filtrate polyamide column chromatography was performed. Elution was done by isopropanol, hexane mixture (60:40 v/v).

Second band was active

**SILICA GEL G COLUMN CHROMATOGRAPHY**
Eluent of active second band was evaporated to dryness. The dry mass was extracted with 25 ml ethanol for 10 min. It was then filtered and the filtrate was subjected to silica gel column chromatography using silica gel G as adsorbent. Elution was done by isopropanol, hexane mixture (60:40 v/v).

Third band was found active

**CRYSTALLIZATION**
Eluent of the active third band obtained from the above step was evaporated to dryness. Repeated crystallization was done from Chloroform: ethyl acetate (40:60, v/v) mixture.

Crystals obtained (5.8 mg)

**2.4 Alpha amylase inhibition assay**
Alpha amylase inhibition assay of the test drug was carried out by the method described by Deguchi et al.\(^8\) with slight modifications. 400 μl of 0.1 M sodium phosphate buffer (pH 7.0), 500 μl of 1% starch solution, isolated compound (10 μg/ml, 20 μg/ml, 40 μg/ml, 60 μg/ml, 80 μg/ml 100 μg/ml) separately dissolved in DMSO and 50 μl of pancreatic α-amylase (Sigma, St. Louis, USA) solution (2 U/ml) were mixed and incubated at 37 °C for 10 min. 3 ml of 3,5-dinitrosalicylic acid (DNS) color reagent was then added. The mixture was kept in a
boiling water bath for 5 min and then diluted with 20 ml of distilled water. The absorbance was recorded at 540 nm. Control sample was prepared accordingly without test drug and acted as a negative control. Acarbose was used as positive control. Inhibition capacity of test drug and acarbose were calculated as following:

\[
\text{Inhibition Percentage (\%)} = 1 - \frac{\text{DO sample}}{\text{DO control}} \times 100.
\]

All tests were done for five sample replications. IC\text{50} value which is the concentration required to inhibit 50\% of alpha amylase activity was calculated in each case.

2.5 Statistical calculation
This was done by SPSS 20. The statistical significance of enzyme inhibitions between test drugs and acarbose, the known inhibitor, was evaluated with Duncan’s multiple range test (DMRT). 5\% was considered to be statistically significant.\(^{[9]}\)

3. RESULTS

3.1 Isolation of compound
One compound was isolated from leaves of \textit{M. koenigii} L.

3.2 Alpha amylase inhibition activity of the isolated compound
Results are summarized in Table -1.

<table>
<thead>
<tr>
<th>Drug/solvent extract</th>
<th>Concentration (µg/ml)</th>
<th>% of inhibition</th>
<th>IC\text{50} Value (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acarbose</td>
<td>10</td>
<td>22.2±1.1</td>
<td>71.1±1.1</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>28.1±1.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>36.4±1.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>47.5±1.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>56.2±1.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>62.4±1.7</td>
<td></td>
</tr>
<tr>
<td>Isolated compound from \textit{M. koenigii} L.</td>
<td>10</td>
<td>28.2±0.9</td>
<td>64.6±1.0*</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>34.7±1.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>42.5±1.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>49.6±1.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>61.1±1.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>70.5±1.8</td>
<td></td>
</tr>
</tbody>
</table>

Values are mean ± SE *Significant
Acarbose, standard alpha amylase inhibitor, in the concentrations of 10, 20, 40, 60, 80 and 100 μg/ml showed 22.2±1.1, 28.1±1.0, 36.4±1.3, 47.5±1.5, 56.2±1.3 and 62.4±1.7 percent of inhibitions in alpha amylase activity respectively with IC₅₀ value 71.1±1.1 μg/ml. Isolated compound from M. koenigii L. leaves in the concentrations of 10, 20, 40, 60, 80 and 100 μg/ml, however, showed 28.2±0.9, 34.7±1.0, 42.5±1.2, 49.6±1.7, 61.1±1.3 and 70.5±1.8 percent of inhibitions in alpha amylase activity respectively with IC₅₀ value 64.6±1.0 μg/ml.

4. DISCUSSION

Since long M. koenigii L. (family, Rutaceae), a semi deciduous aromatic herb or small tree with dark green bark., is being used as medicinal plant. The plant is found in India, Bangladesh, Nepal, Bhutan, Thailand, Pakistan, Vietnam and Sri Lanka in plenty amount and commonly known as curry leaf as leaves of the plant are often used in curries for flavouring due to their typical flavour. The plant is also known as ‘meehi saag’ in Nepali, ‘bursunga’ in Hindi etc. M. koenigii L. is widely distributed at foothills of Himalayas from Kumaon to Sikkim, Bengal, Assam, middle and lower hill forests up to the height of 5000 ft. February to May is the flowering time of the plant.[10] In traditional medicine M. koenigii L. has many uses. Roots and leaves are used to treat kidney pain, piles, leukoderma and blood disorders. Burk is used to cure eruptions and poisonous animal bites. The herb is also used for its stomachic and tonic properties.[11]

Several phytochemicals like 6, 7-dimethoxy-3-methyl carbazole-1, 4- quinone, murrayazolidine, girinimibine, murrayacinine, 9- formyl –3- methyl carbazole, mukonidine, mahanimbinol, mahanimbilol, murrayazolinol, girinimbinol, 7- methoxy- 3 methyl carbazole-1,4- quinone, 1- hydroxy –3- methyl carbazole, Me- 2- methoxy carbazole –3- carboxylate, 9- carbothoxy-3-methyl carbazole etc. were isolated and characterized from M. koenigii L.[12]

Different parts of M. koenigii L. possess pharmacological activities like anti ulcer, cytotoxic, antitumor, antidiabetic, antimicrobial, anthelmintic, photoprotective, hepatoprotective, antioxidant, analgesic, anti diarrheal, and anti inflammatory properties.[13]

Recently we have shown that ethanol extract of M. koenigii L. leaves has maximum in vitro alpha amylase inhibitory activity. Results are under communication. We, therefore, intended to isolate alpha amylase inhibitor from M. koenigii L. leaves. Adopting standard techniques of isolation we isolated one compound from the plant leaves. The compound showed alpha
amylase inhibitory activity which was comparable to that of acarbose, the standard alpha amylase inhibitor, both in terms of concentrations (Figure – 1) and IC₅₀ Value (Figure – 2).

Figure – 1: Alpha amylase inhibitory activity in different doses of acarbose and in the same doses of the compound isolated from *M. koenigii* L. leaves.

Alpha amylase inhibitors are being isolated from medicinal plants.¹⁴ The present work also described isolation of one alpha amylase inhibitor from *M. koenigii* L. leaves. The compound now needs characterization. Work in this direction is presently in progress in our laboratory.

Figure – 2: IC₅₀ values (μg/ml) in alpha amylase inhibitory activity of acarbose and the compound isolated from *M. koenigii* L. leaves.
5. CONCLUSION

One of the therapeutic strategies of Type -2 diabetes mellitus is to keep postprandial blood glucose of the patients under control. These can be achieved by alpha amylase inhibitors. By inhibiting splitting of complex carbohydrate into free sugars alpha amylase inhibitors keep postprandial blood glucose within normal range. In the present work the compound which was isolated from *M. koenigii* L. leaves showed strong alpha amylase inhibitory activity. The compound, therefore, may be used in future as a drug for Type – 2 diabetes mellitus.

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