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ISOLATION OF AN ALPHA AMYLASE INHIBITOR FROM *COSTUS SPECIOSUS* LEAVES

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ABSTRACT
Costus speciosus (*C. speciosus*), commonly known as keu, is a medicinal plant. Although the plant is being utilized in traditional medicine for treatments of diarrhea, dysentery, cold and cough, rheumatism, dyspepsia, skin diseases, fever, bronchial asthma, pneumonia, dropsy, urinary diseases, jaundice, eye and ear infections and snake bites etc. special interest was taken for its use in diabetes. Recently we have confirmed *in vitro* alpha amylase inhibitory activity of summer samples of *C. speciosus* leaves grown in this region. Since alpha amylase inhibitor can keep postprandial blood glucose level of Type-2 diabetic patients under control by inhibiting split of complex carbohydrate to free sugar, alpha amylase inhibitor is now being used in the treatment of diabetes. Acarbose, one alpha amylase inhibitor, has already been included in the list of drugs of Type - 2 diabetes mellitus. The present worked was, therefore, aimed to isolate alpha amylase inhibitor from the leaves of *C. speciosus.* Leaves of *C. speciosus* were collected from the local market during summer and identified by the taxonomist. Ethanol extract of the plant leaves was processed for isolation work by standard methods. Solvent extraction, 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 was used as control. Results showed that the isolated compound had maximum alpha amylase inhibitory activity which was comparable to that of acarbose. The isolated compound may, therefore, be used in the management of Type-2 diabetes mellitus.

KEYWORDS: *C. speciosus* Leaves, isolated compound, alpha amylase inhibitory activity, acarbose, diabetes.

INTRODUCTION
Diabetes mellitus is a chronic metabolic non-communicable disease. The disease is characterized by hyperglycemia with disturbances of carbohydrate, fat and protein metabolism. This is due to defects in insulin secretion, insulin action or both. Diabetes mellitus is of three types. Type – 1, Type – 2 and gestational diabetes. Polyuria, thirst, weight loss and blurred vision are usually symptoms of the disease. Long-term damage due to diabetes mellitus is dysfunction and failure of various organs.[1]

Diabetes mellitus is increasing rapidly. In 1980 there were approximately 108 million diabetic patients in the world 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.[2]

Diabetes mellitus specially Type – 2 diabetes mellitus is characterized by postprandial hyperglycemia. Therefore, to reduce postprandial hyperglycemia there are several therapeutic approaches. One such approach is to reduce postprandial hyperglycemia by inhibiting carbohydrate splitting enzymes such as alpha amylase.[3] Alpha amylase 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.[4] Acarbose, one alpha amylase inhibitor, has already been included in the list of drugs of Type - 2 diabetes mellitus.[5] Still search is going on for more alpha amylase inhibitors. Medicinal plants were also investigated and many plants are now-a-days known having alpha amylase inhibitory activity.[6]

Recently we observed that ethanol extract of summer sample of *C. speciosus* leaves has maximum *in vitro* alpha amylase inhibitory activity. Paper is under communication. Aim of the present study, therefore, was to isolate alpha amylase inhibitor from *C. speciosus* leaves.

2. METHODOLOGY

2.1 Collection of plant materials

Leaves of *C. speciosus* of summer sample 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 ^{0}C until further use.

***Costus speciosus* leaves**

2.2 Solvent extraction

Test drug (75g) was extracted separately with 500 ml of ethanol in soxhlet at 37^{0}C for 20 minutes. Ethanol was chosen as solvent as in our earlier study we found that ethanol extract of *C. speciosus* leaves had maximum *in vitro* alpha amylase inhibitory activity. Results were under communication. The extract was filtered and the filtrate was evaporated to dryness *in vacuo* with rotary evaporator at 40 – 50 ^{0}C. Brown mass obtained was used for *in vitro* alpha amylase inhibition assay.

2.3 Chemicals

Chemicals required for the study were purchased from Himedia Lab, Loba Chem. Lab, India and from Merck, Germany and Sigma Chemicals Co., USA.

2.4 Isolation of alpha amylase inhibitor from *C. speciosus* Leaves

Applying principles of standard isolation procedures of chemical compounds from plant sources[7,8], this was done by the following scheme.

Diagrammatic scheme for isolation of alpha amylase inhibitor from *C. speciosus* leaves

Powdered leaves of *C. speciosus* (75 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.

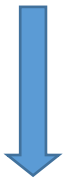
Active brown mass

↓ **ACID REFLUX**
Refluxed with 25 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 25 ml benzene on a rotary shaker for 10 min. It was then centrifuged. Supernatant was evaporated to dryness.

Active brown mass



TREATMENT WITH ETHYL ACETATE

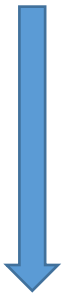
Treated with 25 ml ethyl acetate on a rotary shaker for 10 min. It was then centrifuged. Supernatant was evaporated to dryness.

Active brown mass

ALUMINA COLUMN CHROMATOGRAPHY

Extracted with 25 ml of ethanol for 10 min. It was then filtered. With filtrate alumina column chromatography was performed. Elution was done by benzene, chloroform mixture (50:50 v/v).

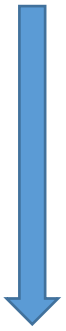
Third band was found active



POLYAMIDE COLUMN CHROMATOGRAPHY

Eluent of active third 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 benzene, chloroform mixture (50:50 v/v).

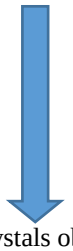
Fourth band was active



SILICA GEL G COLUMN CHROMATOGRAPHY

Eluent of active fourth 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 benzene, chloroform mixture (50:50 v/v).

Second band was found active



CRYSTALLIZATION

Eluent of the active second band obtained from the above step was evaporated to dryness. Repeated crystallization was done from Chloroform : isopropanol (60:40, v/v) mixture.

Crystals obtained (6.4 mg)

2.5 Alpha amylase inhibition assay

Alpha amylase inhibition assay of the isolated compound was carried out by the method described by Deguchi *et al.*[9] with slight modifications. 400 µl of 0.1 M sodium phosphate buffer (pH 7.0), 500 µl of 1% starch solution, isolated compound in varying concentrations (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 isolated compound and acted as a negative control. Acarbose was used as positive control. Inhibition capacity of test drug as well as acarbose was calculated as following:

Inhibition Percentage (%) = 1 − DO sample/DO control × 100.

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

2.6 Statistical calculation

This was 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.[10]

3. RESULTS

3.1 Isolation of compound

One compound was isolated from leaves of *C. speciosus*.

3.2 Alpha amylase inhibition activity of the isolated compound

Results are summarized in Table -1.

Table 1: Alpha amylase inhibitory activity of acarbose (standard alpha amylase inhibitor) and the isolated compound from *C. speciosus* leaves.

Drug/solvent extract	Concentration (µg/ml)	% of inhibition	IC_{50}Value (µg/ml)
Acarbose	**10**	**20.7±1.0**	**62.2±1.0**
	20	**28.9±1.1**	
	40	**38.5±1.2**	
	60	**48.2±1.3**	
	80	**56.8±1.3**	
	100	**63.5±1.4**	
Isolated compound from *C. speciosus* leaves	**10**	**26.2±0.7**	**60.2±1.1**
	20	**32.3±1.0**	
	40	**43.1±1.1**	
	60	**49.8±1.2**	
	80	**60.3±1.3**	
	100	**70.1±1.4**	

Values are mean ± SE *Significant

Isolated compound from *C. speciosus* leaves in the concentrations of 10, 20, 40, 60, 80 and 100 µg/ml showed 26.2±0.7, 32.3±1.0, 43.1±1.1, 49.8±1.2, 60.3±1.3 and 70.1±1.4 percent of inhibitions in alpha amylase activity respectively with IC_{50} value 60.2±1.1 µg/ml. Acarbose, standard alpha amylase inhibitor, in the concentrations of 10, 20, 40, 60, 80 and 100 µg/ml showed 20.7±1.0, 28.9±1.1, 38.5±1.2, 48.2±1.3, 56.8±1.3 and 63.5±1.4 percent of inhibitions in alpha amylase activity respectively with IC_{50} value 62.2±1.0 µg/ml.

4. DISCUSSION

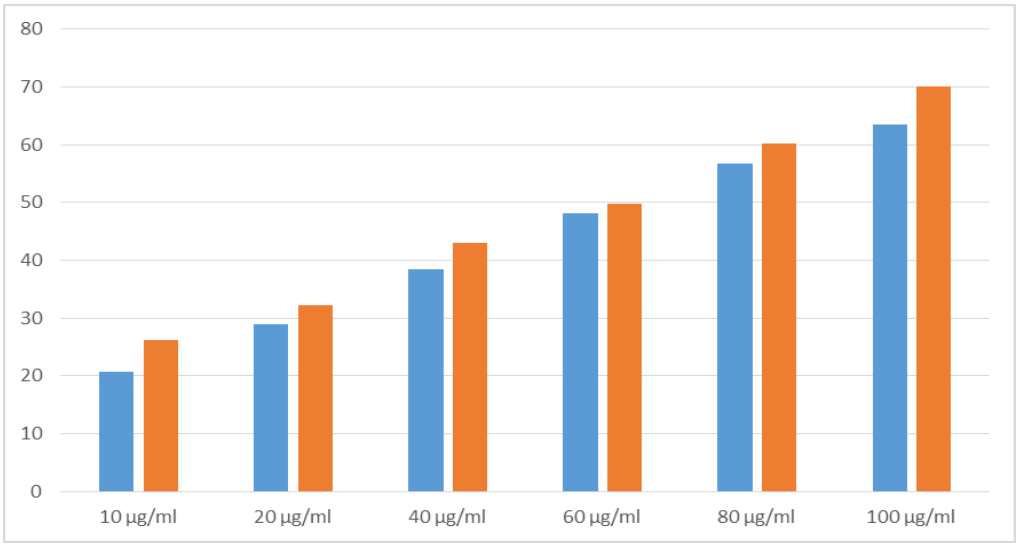
C. speciosus (family, Costaceae) is a medicinal plant. Native to the Malay Peninsula of Southeast Asia the plant is being used in treatments of various diseases since long. In India the plant is found in Sub-Himalayan tract, up to an altitude of 1200 m, in moist tropical evergreen forests, along roadsides, streams and in wastelands of many places of central India and in the Western Ghats of Karnataka, Maharashtra and Kerala. There are many species of the genus Costus but seven species are usually cultivated. The plant is commonly known as keu.[11,12]

Different parts of *C. speciosus* are used in traditional medicine as expectorant, anthelmintic, purgative and stimulant. They are also used in treatments of bronchial asthma, dropsy, urinary diseases, jaundice, eye and ear infections, rheumatism, diabetes, cough and cold, pneumonia, diarrhea, dysentery, fever, dyspepsia, skin diseases and snake bites.[13]

Several phytochemicals like dioscin, gracillin, methyl protodioscin, methylprotogracillin, protogracillin, α-tocopherolquinone, 24-hydroxytriacontan-26-one, 8-hydroxy triacontane-25-one, methyl triacontanoate are present in *C. speciosus.*[14] Due to presence of these phytochemicals the plant exerts anti diabetic, diuretic, antipyretic, anti fungal, anti oxidant, anti cancer, anti bacterial, antifertility, anticholinestrase, antihelminthic, hepatopretective, hypolipidemic like pharmacological properties.[15,16] *C. speciosus* leaves also exert alpha amylase inhibitory activity.[17,18] Recently we have also shown that ethyl extract of summer sample of *C. speciosus* leaves of this region had maximum exert alpha amylase inhibitory activity. Results are under communication.

Patients of Type-2 diabetes mellitus have characteristic post prandial hyperglycaemia which can be kept under control by using alpha amylase inhibitor. Alpha amylase inhibitors, therefore, may be used in the treatment of Type-2 diabetes mellitus. In fact acarbose, one alpha amylase inhibitor, has already been included in the list of drugs of Type - 2 diabetes mellitus.[5]

In the present study the isolated compound from *C. speciosus* leaves showed strong *in vitro* alpha amylase inhibitory activity. The activity was comparable to that of acarbose, the standard alpha amylase inhibitor, both in terms of concentrations (Figure – 1) and IC_{50}Value (Figure – 2). The compound, therefore, may be used as drug for Type - 2 diabetic patients in future. Isolated compound now needs characterization. Work is going on presently in our laboratory in this direction.



■ Acarbose ■ Compound isolated from *C. speciosus* leaves

Figure 1: Alpha amylase inhibitory activity in different doses of acarbose and in the same doses of the compound isolated from *C. speciosus* leaves.

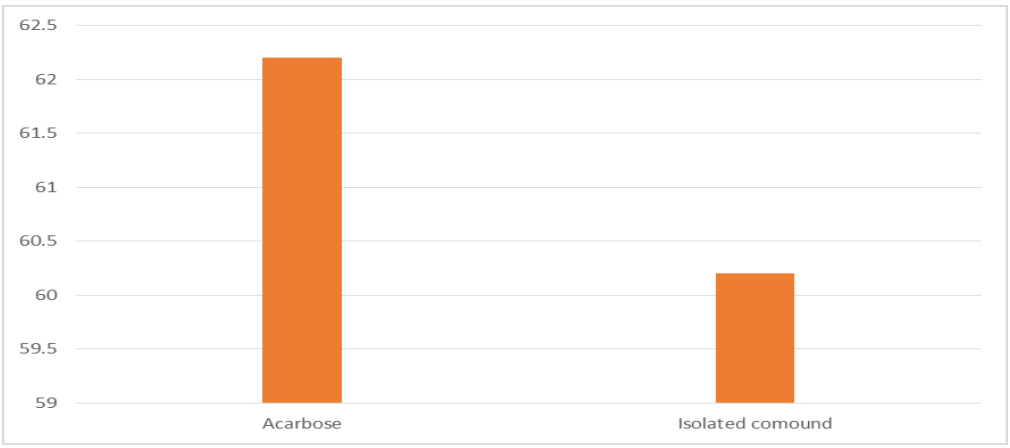


Figure 2: IC_{50} values (µg/ml) in alpha amylase inhibitory activity of acarbose and the compound isolated from *C. speciosus* leaves.

5. CONCLUSION

One of the therapeutic strategies of type -2 diabetes mellitus is to keep postprandial blood glucose level 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 level within normal range. In the present work the compound which was isolated from *C. speciosus* 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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