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AN EXPERIMENTAL EVALUATION OF ANTI-DIABETIC AND SUB CHRONIC TOXICITY STUDIES OF ORALLY ADMINISTERED PROTEASE INHIBITOR PURIFIED FROM THE SYZYGIUM CUMINI (L.) SKEELS SEEDS

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ABSTRACT

Introduction: *Syzygium cumini* (SC) L skeels seeds were traditionally used as anti-diabetic plant in India. Protease inhibitors are the small defensive proteins present in the seeds that interfere with the growth and development of phytophagous insects.
Objective: To partially purify the protease inhibitors from the seeds of SC and to evaluate the sub chronic toxicity and anti-diabetic activities of the SC derived protease inhibitors
Methods: Acute and sub chronic toxicity studies were evaluated for the protease inhibitor purified from the SC seeds. Various physiological, biochemical parameters were evaluated for the treated rats. Anti diabetic activity of the above purified protease inhibitor was evaluated by checking the food intake, water intake, body weight, SGPT, SGOT, ALP, creatinine, blood glucose, insulin, oral glucose tolerance studies and histopathology study of pancreatic slides.
Results: We observed no lethality upto dose of 2000 mg/kg body weight. After fifteen days of oral administration we observed no significant difference was observed in the rats treated with SCPI (200mg/kg) for 15 days on the body weight, food intake, and water consumption. Various biochemical, hematological and histopathological studies had shown no significant difference compared to the normal animals. Diabetes was induced by streptozotocin and various serum parameters were investigated. We observed a significant decrease in the physiological, biochemical and histopathological parameters in the protease inhibitors treated groups in dose dependent.
Conclusion: The above result confirms that protease inhibitor derived from the SC has no toxicity in acute and sub chronic toxicity, can be effectively used as anti-diabetic herbal drug

INTRODUCTION

Diabetes mellitus is a chronic, prevalent endocrinological disorder characterized by decreased insulin secretion or action or both. Globally it is estimated that 366 millions of people suffer and estimated that it may reach 552 million by 2030[1]. There has been increased use of complementary and alternative medicines for the use of various ailments. Herbs are being a source of such, for the treatment of diabetes mellitus. Even though there are many synthetic drugs, these drugs have got limitations in adverse effects. Among all the alternative systems of medicines herbs remained top for the treatment of diabetes [2][3].

Protease inhibitors are small proteins distributed in seeds, tubers and aerial parts. They are produced in response to the injury against the insects or pathogens[4]. These protein are known as anti metabolites, as they interfere with the digestive process of the insects. They act by forming a stable complex with the target enzyme, thereby preventing the blocking or altering the target enzyme. These protease inhibitors are involved in the various cellular responses like cell signaling, digestion, differentiation and apotopsis[5]. These plant derived protease inhibitors have already been proved to be affective in treating the pancreatitis, cancer, shock and allergy [4][5].

The *Syzygium cumini* belongs to Mytraceae family distributed in the forests of Asia and African continents. These fruits of the plants are edible and have been traditionally used. The plant parts like bark, leaves and seeds have used as anti-bacterial [6], anti-diabetic[7], anti-diarrohoeal[8] etc. The objective of the present study is to partially purify the protease inhibitor from the Syzygium cumini seeds and to evaluate the toxicity and anti-diabetic activity. Even thought anti diabetic activity has

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been proven, protease inhibitors purified from these plant has never been investigated for the anti-diabetic activity.

MATERIALS AND METHODS

Plant collection

The plant material was collected from the local forest areas of Belagavi, Karnataka. The Plant has been identified and authenticated by the Scientist C, ICMR (RMRC), Belagavi as Syzygium Cumini (L.)Skeels (Myrtaceae) voucher specimen (RMRC 1253) was preserved in the same department.

Partial purification of the protease inhibitor

The 500 grams of the seeds were made to coarse powder and homogenized in 1000 ml of the 0.1M Phosphate buffer (pH 7.0). The solution was then filtered through the four layered cheese cloth. The filtered solution was then centrifuged at 10,000rpm/15mins/4^0C (Kubato cooling centrifuge). The supernatant was collected and the precipitate was discarded. The supernatants were further fractionated by ammonium sulfate (30, 60, 90%). The 90% fraction was collected by adding minimum quantity of the phosphate buffer. The collected suspension was dialyzed for 24 hours at 4^0C in 0.1 M phosphate buffer (pH 7.0). The dialyzed suspension was then lyophilized and stored in -80 ^{0} C until further use. The proteins were assayed by Lowry et al method using bovine serum albumin as standard.

Experimental animals

The experimental protocol was approved by the Institutional Animal Ethics Committee of KLEU`s College of Pharmacy, Belagavi which has registered with CPCSEA, Govt. of India (registration no 221/CPCSEA) with resolution no (KLECOP/IAEC/Res 18-19/05/2014). The wistar rats were procured from the Venketeshwara Enterprises, Bangalore and placed in individual cages with *ad libitum* water and rodent chow pellet diet. The animals were sacrificed at the end of the experiment by decapitation.

Proteolysis assay

The 100 µL of the suitable dilution of the protease inhibitor and trypsin are incubated at 37 ^{0}C for 15 mins. To the above mixture 200 µL of the 1 % casein was added and further incubated for 37 ^{0}C for 30 mins. The reaction was terminated by addition of 250 µL of 0.44 m trichloroacetic acid solution. The reaction mixture was centrifuged (Sigma, Germany) at 10,000 rpm for 15 mins to remove the precipitated protein. The 100 µL of the supernatant was further diluted with 500 µL of sodium carbonate (10%) and 100 µL Folin Ciocaltechu reagent (50%) was added and vortexed for 2 mins and incubated in dark for 30 mins. The absorbances were read at 750 nm in 96 well plate reader (Thermo Scientific) against the appropriate blanks. L-tyrosine was used as standard. Inhibitory activity was calculated.

Acute Toxicity studies

Acute toxicity studies were performed according to OECD guide lines 423. The five rats were fasted for six hours and administered with single oral dosage of 2000 mg/kg and observed for mortality for 24 hours. 200 mg/kg/BW of SCPI was administered for the rats (n=3) and control group received water was administered and food intake and water intake was observed for 1, 2 and 24 hours.

Sub chronic oral administration of SCPI

The six rats of (n=3) were fasted for 6 hours and administered with 200mg/kg of SCPI to treatment group and other group received the water ad libitum for 15 days. Food intake and water intake was measured on day 1, 7, 15th day prior to the treatment. Blood was collected by retro orbital plexus in EDTA coated tubes. Body organs like liver, kidney, heart, spleen and pancreas were separated and weighed. Liver function was analyzed by Asparate aminotransferase (AST), SGOT, SGPT, glucose, insulin, and creatinine was measured using the commercial kits.

Induction of Diabetes

Diabetes was induced by single injection of freshly prepared streptozotocin (STZ) injection in normal saline (0.9% w/v) in overnight fasted rats (50 mg/kg,ip). The blood glucose levels are measure after 72 hours, rats with serum glucose levels more than 200 mg/dL were considered as diabetic animals and grouped.

Experimental design

The animals were grouped of six animals in each, normal (treated with normal saline), diabetic control, diabetic treated with 100mg/kg and 200 mg/kg. The extracts were orally administered at same time for 14 days. Blood glucose levels and body weights were estimated on the 0, 7 and 15th day of treatment.

Assessment of the various blood parameters

Normal and diabetic control group were administered with saline water and the remaining groups were treated with the low and high doses of the SCPI. On the 15th day, animals were anaestheitized by anesthetic ether and blood was collected by retro orbital plexus. Glucose, triglycerides, cholesterol, SGOT, SGPT, ALP, creatinine and total protein was estimated by using the auto analyzer (Star 21Plus).

Effects of SCPI on food intake, body weight and water consumption

Food and water intake was noted on the 0, 7 and 14th day of the study period.

Histopathology study

After the study period of 15 days, animals were sacrificed and pancreatic samples are fixed in 10 % buffered formalin. The sections of the pancreas were stained by Hematoxylin-Eosin and analyzed under light microscopy (Olympus)

Oral Glucose Tolerance Test

Animals were fasted for 6 hours and randomly grouped into 6 animals each. Two groups were given the low and high doses of the SCPI and control group was treated with the vehicle. After treating the animals with respective doses, glucose was orally ingested (2g/kg p.o) after one hour. Blood was withdrawn by tail vein method at 0. 30, 60, 90 and 120 mins after the oral administration of glucose and estimated for the

serum blood glucose levels. Blood glucose analysis were performed using Free style optium blood glucose monitoring system (Abbott).

Statistical Analysis

All the parameters were expressed as Mean ±SD and ANOVA was carried out followed by post Dunnett`s multilple comparison t- test using Graph pad Prism 5 statistical software. Difference between the groups were considered significant at $p \leq 0.05$.

RESULTS

Proteolysis assay

The total protein was estimated was 22.13±3.20, the percentage protease inhibitory activity was calculated was 30.88±2.21%

Acute oral toxicity studies

The protease inhibitor was safe up to a dose of 2000 mg/kg of body weight. There was no toxicity and lethal effect was observed in the treatment groups. There was no significant decrease in the food intake and water consumption.

Sub chronic toxicity studies

Even after oral administration of SCPI for 14 days we observed no mortality and lethality. We observed no significant difference in the food intake, body weight and water consumption illustrated in the table-3. At end of the study, we observed no significant difference in the hematological illustrated in table-4, biochemical parameters illustrated in table-1, and histopathological difference illustrated in figure-1 and organs weights illustrated in table-5 compared with the normal group.

Effects of SCPI on the biochemical parameters

There was significant reduction in the levels of cholesterol, triglycerides, serum insulin, creatinine when compared with the diabetic group. Liver enzymes (SGOT, SGPT, ALP) levels were also elevated when compared with the diabetic group. Total proteins levels were decreased in the diabetic group and the levels are reduced in the treatment groups. Results are illustrated in the table-1

Effects of SCPI on food intake, body weight and water consumption

Polyphagia and polydipsia are the symptoms of the diabetic, these complications were overcame by decrease in the food intake and water consumption. The diabetic animals consumed larger extent of food and water, these were decreased in the treatment groups. The diabetic animals were shown to decrease in body weight and the treated groups had shown non-significant increase in body weight. The data is explained in the Table 3.

Histopathology study of pancreas

The diabetic animals had shown a decrease in the β cells with degenerative necrosis and reduced dimensions of the islets, where there was partial restoration of cells in the treatment group. There was remarkable difference in the size, shape of the pancreatic β cells. The histopathology slides are represented in the Table 2.

Oral Glucose Tolerance test

The highest blood glucose levels were observed at 30 mins after the glucose ingestion. There was steep decrease in the blood glucose levels in both the treated animals, but the higher dosed animals almost reached the normal blood glucose levels than compared with the diabetic animals. The data is illustrated in the table-6.

Table 1 Effect of protease inhibitors of SCPI on various biochemical parameters in rats**.**

S No	Serum parameters	Control	SCPI (200mg/kg)	Diabetic	D+ SCPI (100mg/kg)	D+ SCPI (100mg/kg)
1	Insulin (µIU/ml)	3.38±0.35	3.43±0.33	1.10±0.16^{c}	1.72±0.24^{c}	2.36±0.25^{c}
2	Total protein (g/dL)	7.74±0.52	7.36±0.14	5.46±0.37^{c}	5.87±0.11^{c}	6.69±0.22^{c}
3	Cholesterol (mg/dL)	67.9±4.85	67.56±2.56	134.5±8.01^{c}	86.96±2.15^{c}	73.5±9.17
4	SGOT (U/L)	118±3.18	121.5±4.72	262±9.40^{c}	184±8.17^{c}	160±4.71^{c}
5	SGPT(U/L)	96.5±7.86	98.3±5.81	213±10.38^{c}	149.5±15.39^{c}	103.6±5.31
6	ALP (U/L)	180.3±3.77	187.6±5.50	300.3±7.68^{c}	221.5±6.53^{c}	205±5.92^{c}
7	Triglycerides (mg/dL)	60.73±3.51	62.53±2.96	121.7±3.30^{c}	95.3±2.36^{c}	82.5±4.11^{c}
8	Creatinine (mg/dL)	0.72±0.12	0.71±0.10	2.07±0.37^{c}	0.86±0.06	0.83±0.10

All the data are compared with the control group, ns-non significant, c-highly significant ($p<0.05$)

Anti hyperglycemic activity of the SCPI

Blood glucose levels were lowered in both the treatment groups. High dose (200mg/kg) have shown 53% reduction in the blood glucose levels and low dose (100 mg/kg) has shown 45 % reduction when compared with the blood glucose levels of diabetic group. Results are illustrated in the table-2

Table2 Effect of protease inhibitors of SCPI seeds on fasting blood glucose levels of rats on different days.

Groups/day	Day 1	Day 7	Day 14
Normal	79.2±4.51	78.3±4.51	82.7±5.62
SCPI (200mg/kg)	79.5±6.37^{ns}	81.1±10.1^{ns}	80.5±8.31^{ns}
Diabetes	366.5±4.66^{C}	391.5±6.9^{C}	402.3±6.68^{C}
SCPI (100 mg/kg)	348.3±5.95^{C}	313.6±4.7^{C}	265.3±3.82^{C}
SCPI (200mg/kg)	387±4.93^{C}	222.1±4.70^{C}	189.1±4.02^{C}

All the data are compared with the control group, ns-non significant, c-highly significant($p<0.05$)

DISCUSSION

Plant protease inhibitors are widely distributed in the in the families of Solanaceae, Graminiaceae, and Cucurbitaceae[10]. Among all the types of inhibitors most of them belong to the serine protease inhibitors [11]. Protease inhibitors represent 10% of the total protein in the seeds. Serine protease inhibitors mostly decrease the food intake when orally administered [12]. However, in all the previous studies protease inhibitors are completely purified and it was assessed for the appetite suppression and other physiological process[13].We therefore, hypothesized that by partially purifying the anti-diabetic plant

Syzygium cumini seeds and to evaluate the oral toxicity and anti-diabetic activity.

food intake upto a week days, it had overcome on the 14th day. These results confirms that food intake reduce the body

Table 3 Effect of SCPI on body weight, feed and water intake of the rats.

S No	Parameter	Control	SCPI (200mg/kg)	Diabetic	D+ SCPI (100mg/kg)	D+ SCPI (200mg/kg)
1	Food intake(g)	21.33±5.46	22.52±3.38^{ns}	28.9±4.47^{b}	26.11±5.90^{ns}	26.42±4.93^{ns}
2	Water intake (ml/day)	27.22±4.57	31.16±6.24^{ns}	72.4±6.01^{C}	54.2±5.70^{C}	50.4±7.19^{C}
	Mean Body weight (g)	183.8±20.6	174±16.1^{ns}	169.6±21.2^{ns}	188.1±7.54^{ns}	189±15.01^{ns}
3	Wt variation(g) 7th day (Avg)	+18.46	-17.9	-19	+9	+25
	Wt variation(g) 14th day (Avg)	+41.2	+14.4	-42	+15	+41

All the data are compared with the control group, ns-non significant, c-highly significant($p<0.05$)

Table4 CBC after 14 days of the treatment of SCPI.

S No	Haemotological markers	Control	SCPI (200mg/kg,po)
1	Hb (g/dL)	12.33±0.22	12.70±0.225^{ns}
2	Platelets (10^3/µL)	760.77±29.8	801.3±43.86^{ns}
3	RBC (10^6/µL)	6.72±0.17	6.38±0.06^{ns}
4	Granulocytes (10^3/µL)	0.46 ±0.08	0.47±0.08^{ns}
5	Lymphocytes (10^3/µL)	4.74±0.24	4.55±0.31^{ns}
6	WBC (10^3/µL)	5.44 ±0.201	5.51±0.40^{ns}

All the data are compared with the control group, ns-non significant, c-highly significant($p<0.05$)

Table5 Organ weights (g) of after SCPI administration for 14 days.

S No	Organ	Control	SCPI (200mg/kg,po)
1	Kidneys(g)	2.81±0.41	2.65±0.73^{ns}
2	Liver(g)	11.88±0.17	11.96±0.29^{ns}
3	Spleen(g)	0.85±0.04	0.86±0.14^{ns}
4	Pancreas(g)	1.58±0.14	1.59±1.19^{ns}
5	Heart(g)	1.69±0.07	1.65±0.10^{ns}

All the data are compared with the control group, ns-non significant, c-highly significant($p<0.05$)

There is no proper literature regarding the present plant further investigations has to been carried out to characterize these protease inhibitor. The reduced food intake may be associated with the proteolytic activity in the gastro intestinal tract. The adaptive response may be due to the increase in the nutrient density or release of the hormonal mediators [16]. To study the safety profiles of the SCPI, we used healthy rats. The 14 day treatment with SCPI (200mg/kg b.w) showed no adverse effects except in decreasing the body weights in the mid experiments.

Syzygium Cumini was traditionally proven to be anti-diabetic drug [17]. SCPI 200 mg/kg b.w administered orally for 15 days showed significant reduction in the blood glucose levels. The protease inhibitors have shown a dose dependent anti-hyperglycemic effect. The activity may be due to the secondary metabolites in the partially purified protease inhibitors from the seeds, and thus making as an effective anti-diabetic drug.

STZ causes the abnormal elevated levels of the liver enzymes (SGPT, SGOT, ALP) due to the hepatic dysfunction. Liver is the metabolic organ responsible for the glucose metabolism its function gets altered in the diabetic rats.

Table6 Oral glucose (mg/dL) Tolerance test for the different treatments

Groups/time(min)	0	30	60	90	120
Normal	83±3.40^{ns}	150.1±3.06	136.8±5.34	124.5±3.50	121.5±4.76
SCPI (100 mg/kg,po)	81.8±3.86^{ns}	135.8±6.21^{C}	123.1±5.19^{C}	113.1±3.43^{b}	101.1±4.87^{C}
SCPI (200mg/kg po)	82.1±4.21^{ns}	122.8±5.84^{C}	114. ±4.27^{C}	99.6±7.42^{C}	93.1±4.99^{C}

All the data are compared with the control group, ns-non significant, c-highly significant($p<0.05$)

Plant Protease Inhibitors (PPI) are small proteins mainly considered as anti-nutritional because it decrease the food intake when administered orally [14]. There are many claims stating that protease inhibitors decrease the food intake by having the proteolytic activity against the GIT enzymes like trypsin, chymptrypsin[15]. Even though, we administered partially purified SCPI, we observed there was no significant difference in the body weight, food intake, and water consumption. Most of the researchers used purified fractions and assessed the toxicity of protease inhibitors contrary to that we partially purified protease inhibitor, and assessed the toxicity of the SCPI and observed no toxic effect.

There was no significant decrease in the body weight, food intake and water consumption by oral intake of the SCPI. We observed body weight adaption to the protease inhibitors up to the 14th day. Indeed there was decrease in the body weight on the7th day, but on the final day body weight is similar to the control animals. These results were confirmed by the reduced

Orally administered SCPI significantly reduced the liver marker enzymes in a dose dependent manner may be due to the restoration of the β cells of the pancreas and thereby showing protective effect on the hepatocytes[18].

Diabetes is associated with the increased levels of the serum creatinine, due to the increased glucose levels [19]. However, there was decrease in the creatinine levels in the treatment groups in a dose dependent. Cholesterol levels are indication for hyperlipidemic activity, these values are altered in the streptozotocin induced diabetes in rats, treatment group had decreased levels of the serum cholesterol.

Serum protein levels are the indication for the protein degradation in the liver and other organs. These levels are lower in the diabetic animals and the levels are restored in the treatment animals. Polyphagia and polydipsia are characteristic features of the diabetes [20]. We observed decrease food intake and water consumption in the treated animals than compared

with the diabetic animals. This decrease in the water and food intake may be related to the decrease in the protein degradation by improving the glycemic levels. Due the glycemic controls there was indeed increased the body weight of the SCPI treated animals than compared with diabetic animals may be by lowering the utilization of glucose in the muscle and body fat[21].

Pancreas secretes the insulin from the β-cells of langerhans. Streptozotocin intraperioteneal administration generally causes the destruction of the β-cells [22]. Due to the absence of the anti-oxidant and the free radicals these β-cells are damaged by the nitric oxide [23]. Histopathology studies indicates the destruction of the islets cells in the diabetic animals and in the treatment group regeneration of the β-cells was observed possibly due to the anti-oxidant activity of the plant. Liver and pancreas has the special characterization of regeneration of the lost cells by the remaining cells [24]. These neogensis of the pancreatic cells were observed in the SCPI treated groups. These proliferations may be mainly due to the phenols. Many previous reports states that, medicinal plants with phenolic compounds possess anti-oxidant activity. These anti- oxidant effect might be reason for the restoration of the β-cells.

It was already reported that SC contains several secondary metabolites like phenols, alkaloids, flavonoids and terpens in varied concentrations. These secondary metabolites were also observed in the partially purified fractions. Plant derived protease inhibitors were already proven to be good anti-oxidant activity [25]. These anti-diabetic activities may be due to the phenolic compounds present in the 90% fraction.

The reduction in the blood glucose levels were observed in the oral glucose tolerance test. These effect may be due to the increased efficiency of uptake of glucose from the peripheral tissues for into the blood for maintaining the glucose homeostasis levels. Thus the protease inhibitors purified from the SC can be effectively used as the anti-diabetic herbal drug and future studies has to be carried out for validation of the above results.

CONCLUSION

In the present study, protease inhibitors were partially purified from the *Syzygium cumini* seeds and observed for the acute and chronic toxicity studies and observed that these protease inhibitors are safe. The protease inhibitor was also evaluated for the anti diabetic activity in streptozotocin induced diabetes in rats and found to be safe and effective.

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