Seeds of *Syzygium cumini* (L.) Skeels: potential for islet regeneration in experimental diabetes

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**Objective:** The present study deals with the islet-regenerative potential of purified fraction of *Syzygium cumini* (L.) Skeels seeds (SC2) in streptozotocin (STZ)-induced diabetic mice.

**Methods:** Diabetes was induced in Swiss mice by single intraperitoneal injection of STZ (120 mg/kg). The treatment group mice were treated by administering oral dose of isolated SC2 fraction of *S. cumini* (2 g/L) for 21 d. Blood glucose level and body weight measurements were conducted regularly during the 21 d. On the 20th day of the experiment, oral glucose tolerance test was performed on overnight fasted mice. Experimental mice were sacrificed at the end of the treatment and tissues were separated. The liver glucose-6-phosphate-dehydrogenase (G6PD) activity and contents of hepatic and muscle glycogen were measured; levels of plasma insulin and C-peptide were also measured.

**Results:** SC2-treated mice showed sustained reversal in experimental diabetes as evidenced by restoration of normoglycemia, increases in G6PD and hepatic and muscle glycogen along with increases in plasma insulin and C-peptide levels. The occurrence of neo-islets in histological studies suggested regenerative property of SC2. These neo-islets were found to be producing insulin in *in vivo* STZ-induced diabetic mice.

**Conclusion:** These findings substantiate the action of SC2 fraction isolated from *S. cumini* seeds in islet regeneration and insulin secretion. Such regenerative approaches, in combination with other therapeutic strategies may provide a better means for the control and management of diabetes in the future.

**Keywords:** diabetes mellitus, experimental; *Syzygium*; plant extracts; hypoglycemic agents; islets of Langerhans; mice

*Syzygium cumini* (L.) Skeels commonly known as Jambu or Jambolan, synonyms *Syzygium jambolanum, Eugenia cumini* and *Eugenia jambolan*, belongs to the Myrtaceae family and is native to the subtropical Himalayas, India, Sri Lanka, Malaysia and Australia. *S. cumini* has been utilized in the traditional
system of medicine (Ayurveda) for the treatment of diabetes\textsuperscript{[3]}. \textit{S. cumini} seeds have been considered as an indigenous source of medicines possessing hypoglycaemic, antipyretic, anti-inflammatory, antioxidant, and antihyperglycemic properties\textsuperscript{[25,57]}. \textit{S. cumini} is most often recommended as an adjuvant therapy in type 2 diabetes. It is one of the extensively studied plants in the last 125 years\textsuperscript{[81]}. In spite of various bioactive phytochemical constituents and diverse medicinal properties attributed to \textit{S. cumini}, there are no detailed studies carried out to identify the mechanistic basis of its antidiabetogenic property. We have previously reported the glucosidase-inhibitory activity of \textit{S. cumini} seed extracts against murine pancreatic and intestinal glucosidas\textsuperscript{[6]}.

Diabetes mellitus (type 1 and type 2) is a metabolic disorder characterized by dysregulation in carbohydrate, protein and fat metabolisms caused by insufficient insulin secretion and/or insulin action\textsuperscript{[10,11]}. Various oral antidiabetic agents such as sulfonylureas, biguanides, α-glucosidase inhibitors, glinides are used to achieve glycemic control. All these medicines have limited efficacy and have been reported to be associated with undesirable side effects\textsuperscript{[15-16]}. As a result, attempts have been made to discover new antidiabetic regimens derived from plant sources\textsuperscript{[10,16]}. The mechanism of islet regeneration is poorly understood, but the identification of islet progenitor sources is critical for understanding β cell regeneration. Unraveling the mechanism of islet cell development and regeneration represents an important medical issue because it could lead to strategies aimed at restoring the functional β cell mass which is known to be deficient or even absent in diabetes\textsuperscript{[17]}. Therefore, expansion of the β cell mass from endogenous sources, either \textit{in vivo} or \textit{in vitro}, represents an area of increasing interest. Banerjee and Bhonde\textsuperscript{[18]} have demonstrated that streptozotocin (STZ)-induced diabetic mice retained their pool of intra-islet precursor cells and can give rise to mature functional islets if triggered externally. In such a scenario, any agent that can increase the level of regeneration, by triggering islet regeneration (nesidioblastosis) in the precursor cells, would be able to retard the progression of diabetes. Researchers are now shifting their area of interest towards to the herbal plants that may possess islet-neogenic activity.

One of the beneficial effects shown by antidiabetic plants in the diabetic environment is improving insulin action and/or enhancing insulin secretion\textsuperscript{[19]}. The plant extracts of \textit{Enicostemma littorale} is shown to be exerting beneficial effects in the diabetic environment by improving and/or mimicking insulin action via pancreatic islet regeneration in \textit{in vitro} studies\textsuperscript{[20]}. Various biomolecules like gastrointestinal neuropeptides and colecytokinin, have shown to stimulate pancreatic regeneration and restoration of islet cell mass \textit{in vivo}\textsuperscript{[17,21]}. \textit{Terminalia catappa} Linn fruits, \textit{Vinca rosea} extracts and alkaloid extract of \textit{Éphedra herba}, \textit{Evertiamia microphylla}, \textit{Enicostemma littorale}, \textit{Gymnema sylvestre}, \textit{Momordica charantia} and \textit{Beta vulgaris} have so far shown significant islet regeneration effect in diabetic animals\textsuperscript{[29-30]}. In the present study, we purified the active fraction from \textit{S. cumini} seeds and further investigated its role in islet regeneration and insulin-secretogogue activity in STZ-induced diabetic mice.

1 Materials and methods

1.1 Plant materials and extraction \textit{S. cumini} (voucher number MBBP 6) fruits were collected from the Western Ghats of Pune, India and were authenticated by the Botanical Survey of India (BSI), Pune, India. The seeds were collected after depulping the fruits. The chloroform, methanol and aqueous extracts were prepared sequentially in a Soxhlet extractor using 30 g of the dried tissue mixed with 150 mL of the respective solvent (100%, volume ratio) for 24 h\textsuperscript{[19,10]}. Chloroform, methanol and aqueous extracts were collected and dried under reduced pressure using a Buchi rotavapor (Buchi, Germany) and a lyophilizer respectively (Thermo Electron Corporation, Germany).


total of 25 mg dry weight of each crude extract was further reconstituted in 2.5 mL of distilled water. All the fractions were initially tested in the in vivo STZ-induced diabetic mice. The chloroform extract having significant antihyperglycemic activity was further purified to isolate the active fraction.

1.2 Purification of active component from the chloroform extract Chloroform extract (25 mg/mL) was subjected to purification using silica gel (60 to 120 mesh) column chromatography with 100% chloroform solvent system. Three fractions were eluted and out of them only one fraction (fraction 2) showed antihyperglycemic activity. The thin layer chromatography (TLC) of active fraction 2 (5.8 mg) showed mixture of three components under ultraviolet light. Therefore, fraction 2 was further purified by semipreparative high-performance liquid chromatography (HPLC. Waters, USA) system using C18 column (25 cm × 10 mm; 5 μm particle size) chromatography. The injection volume was 50 μL and elution was carried out with a flow rate of 0.6 mL/min using the acetonitrile-water (80:20 volume ratio) solvent system. The detection was set at 254 nm and the fractions were collected for further analysis. The active fraction (SC2, 1.2 mg) was obtained and rechromatographed on the HPLC system using the C18 column to check the purity. The mass of the fraction was determined by liquid chromatography-mass spectrometry (LC-MS. Waters, USA).

1.3 Oral administration of active fraction to the STZ-induced diabetic mice Male Swiss mice (6 to 8 weeks) were derived from a colony maintained at the Department of Zoology, University of Pune, India. The mice were in an air-conditioned room at 22 °C with a light/dark cycle of 12 h and were fed on a standard pelleted diet ad libitum. Mice were divided into three groups (six mice per group), namely, normal control, diabetic control and treatment groups, respectively. The diabetic control and treatment group mice were rendered diabetic using a single intraperitoneal injection of STZ (Sigma Chemical Co., USA) (120 mg/kg) in citrate buffer (pH 4.5). S. cumini active fraction SC2 (2 g/L, 8 mg/kg) was suspended in distilled water with 0.5% dimethyl sulfoxide and administered orally in group of six mice for 21 d. The remaining experimental procedure for in vivo studies was similar to what was previously described[30].

Blood glucose level and body weight measurements were conducted regularly during the 21 d. All the procedure was carried out under the guidelines of the Animal Ethical Committee of the University of Pune, India. The toxicity analysis of SC2 fraction on brine shrimp (Artemia salina) developed by Michael et al[31] was performed as described earlier.

1.4 Oral glucose tolerance test On the 20th day of the experiment, oral glucose tolerance test was performed on overnight fasted mice. Glucose (2 g/kg body weight) was administered and the blood was collected using tail prick method. The reduction in glucose levels was monitored at 0, 30, 60, 90 and 120 min after glucose administration using an AccuCheck Active Glucometer[32].

1.5 Biochemical analysis Experimental mice were sacrificed by cervical dislocation at the end of the treatment and tissues (pancreas, livers and skeletal muscles) were utilized to check the overall glucose homeostasis. Liver glucose-6-phosphate dehydrogenase (G6PD) activity was estimated by Langdon’s method[33]. Contents of hepatic and muscle glycogens were estimated according to the method developed by Sadasivam and Manickam[34]. The levels of plasma insulin (Merodia, Sweden) and plasma C-peptide (Yanaihara Institute, Japan) were measured using enzyme-linked immunosorbent assay (ELISA) kits.

1.6 Histological and immunohistochemical stainings The animals were sacrificed on the 21st day of treatment and the pancreas was excised and fixed in 4% formaldehyde. The multiple parallel sections (6 μm) of the pancreas from experimental mice were taken on rotary microtome (Thermo Electron Corporation, Germany). The sections were stained with hematoxylin and eosin (HE) for histological analysis. Immunohistochemical analysis was performed using fluorescein isothiocyanate (FITC)-labeled murine anti-insulin antibody followed by image analysis using a fluorescent microscope (Nikon, Japan).

1.7 In vitro islet differentiation Human pancreatic carcinoma epithelial-like cell line (PANC-1) (American type culture collection No. CRL 469) procured from the National Centre for Cell Sciences, Pune, India was used to check the formation of neo-islet and insulin-secretory activity for the in vitro experiment. PANC-1 cells were cultured in 24-well plates at the density of 10^6 cells/well with Dulbecco’s minimal essential medium (DMEM) containing 10% fetal bovine serum and 0.1% antibiotic solution at 37 °C and 5% CO2. After adherence, cells were cultured in serum-free culture medium containing Krebs-Ringer bicarbonate buffer (KRB) (containing D-glucose 1.8 g/L, magnesium chloride 0.046 g/L, potassium chloride 0.34 g/L, sodium chloride 7.0 g/L, sodium phosphate dibasic 0.1 g/L and sodium phosphate 0.18 g/L). The experimental wells were supplemented with KRB along with different concentrations (1, 2 and 4 μg) of SC2 active fraction. The control wells were supplemented with KRB. The cells were observed regularly and the medium was changed every alternate day till the 8th day. On the 8th day the cells were washed with KRB. The cell differentiation was observed on the 8th day for islet-like cellular aggregates (ICAs) under an inverted microscope. To evaluate the total number and assess the specificity of the newly generated islets, these ICAs
were washed with Hank’s balanced salt solution (HBSS) and stained with insulin-specific dye dithizone (DTZ) (Sigma, St. Louis, USA), and visualized under an inverted microscope (Olympus, Tokyo, Japan).

1.8 Immunohistochemistry of functional cellular aggregates In order to check the functionality of the islets to produce insulin with SC2, the ICAs were fixed with 4% paraformaldehyde solution. The fixed cells were incubated in blocking solution (1% bovine serum albumin and 1% phosphate buffer saline) for 1 h. After incubation, ICAs were stained with murine anti-insulin antibody (dilution 1:1000) overnight at 4 ºC. The ICAs were then washed with washing buffer and further incubated with FITC-labeled secondary antibody (dilution 1:500) for 1 h at room temperature. The ICAs were observed for insulin-positive, functional islets under a fluorescent microscope (Nikon, Japan).

1.9 Statistical analysis Results were expressed as mean ± standard deviation. Statistical analysis was carried out using one-way analysis of variance followed by Dunnett t test. P value less than 0.05 was considered to be statistically significant.

2 Results

2.1 HPLC analysis of active fraction SC2 S. cumini chloroform extract showing significant antihyperglycemic activity was subjected to purification. Crude extract (25 mg/mL) was first fractionated with silica gel (60 to 120 mesh) column chromatography using 100% chloroform. Forty fractions were collected and analyzed on TLC. The fractions showing similar profile on TLC were pooled yielding three major fractions. The fraction 2 (5.8 mg) exhibiting significant antidiabetic activity

in vivo was further purified by semipreparative HPLC system using C18 column and denoted as SC2 (1.2 mg). The purified fraction was rechromatographed on HPLC to check the purity of the fraction. Figure 1 shows the single peak obtained after purification indicating the purity of the fraction. The mass of active fraction SC2 was estimated to be 336.5 m/z.

2.2 Toxicity analysis The lethal concentration (LC50) value obtained after brine shrimp toxicity analysis of SC2 fraction was 12.33 mg/mL. The total dosage of 2.1 mg of SC2 fraction was administered to mice within 21 d which is very minimal to show any toxicity.

2.3 Oral glucose tolerance and antihyperglycemic activity of SC2 Supplementation of SC2 improved the oral glucose tolerance in the STZ-induced diabetic mice after 20 d of treatment. The fasting blood glucose levels of diabetic control mice were significantly higher than those of the normal control mice (P<0.01). SC2-treated mice showed reduced fasting glucose level, acquiring good glycemic control which reveals its antihyperglycemic activity (Table 1).

2.4 Biochemical analysis Glycolytic enzyme G6PD was increased after treatment with SC2 fraction. SC2 treatment also improved the levels of hepatic and muscle glycogens, respectively. Glycogen contents of the diabetic mice were significantly lower than those of the normal control mice (P<0.01). This indicated the role of SC2 in normalization of glycogenesis and carbohydrate metabolism in STZ-induced diabetic mice. Plasma insulin and C-peptide levels of diabetic mice treated with SC2 were increased when compared with those of the untreated diabetic control mice (Table 2).

![Figure 1 HPLC chromatogram of SC2 fraction](image-url)

HPLC chromatogram of SC2 fraction using semipreparative HPLC system using C18 column (25 cm × 10 mm; 5 µm particle size) in acetonitrile-water (80:20 volume ratio) solvent system. HPLC: high-performance liquid chromatography; SC2: purified fraction of Syzygium cumini.
Table 1  Oral glucose tolerance test results of each group

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Fasting blood glucose level (mg/L)</th>
<th>(mean±standard deviation)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0 min</td>
<td>30 min</td>
</tr>
<tr>
<td>Normal control</td>
<td>5</td>
<td>820.0±91.2</td>
<td>1 490.0±87.5</td>
</tr>
<tr>
<td>Diabetic control</td>
<td>5</td>
<td>4 550.0±102.2**</td>
<td>4 750.0±112.8**</td>
</tr>
<tr>
<td>SC2 treatment</td>
<td>5</td>
<td>840.0±32.5</td>
<td>890.0±81.2</td>
</tr>
</tbody>
</table>

** P<0.01, vs normal control group. SC2: purified fraction of *Syzygium cumini*.

Table 2  Biochemical and enzymatic analysis results of each group

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>G6PD (U/mg)</th>
<th>Glycogen (μg/L)</th>
<th>Plasma insulin (ng/L)</th>
<th>Plasma C-peptide (μg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Hepatic</td>
<td>Muscle</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal control</td>
<td>5</td>
<td>0.013 0±0.000 1</td>
<td>30±2</td>
<td>41±4</td>
<td>130±10</td>
</tr>
<tr>
<td>Diabetic control</td>
<td>5</td>
<td>0.003 4±0.000 2**</td>
<td>30±5**</td>
<td>42±3**</td>
<td>34±10**</td>
</tr>
<tr>
<td>SC2 treatment</td>
<td>5</td>
<td>0.003 9±0.000 4</td>
<td>51±3</td>
<td>63±4</td>
<td>110±20</td>
</tr>
</tbody>
</table>

** P<0.01, vs normal control group. SC2: purified fraction of *Syzygium cumini*; G6PD: glucose-6-phosphate dehydrogenase.

2.5 Histological and immunohistochemical analysis

The histological and immunohistochemical stainings were performed on pancreas of experimental mice to check the role of SC2 in retaining islet morphology. SC2-treated pancreas revealed distinct (neodioblastosis) islet regeneration with a connection between the newly formed islets and the ductal precursor cells (Figure 2A). The functionality of newly formed islets was confirmed with positive reaction with FITC-labeled anti-insulin antibody (Figure 2B), whereas diabetic pancreas had shrunken, deformed islets, negative for insulin staining (Figures 2E and 2F).

2.6 Immunohistochemical analysis of *in vitro* islet regeneration

PANC-1 of pancreatic nature having stem cell-like property was chosen to verify the islet-neogenic activity of SC2 fraction. Different concentrations of SC2 were added to PANC-1 cell line and it was observed that maximum concentration 4 μg showed cell differentiation after 8 d in the serum-free culture medium. The presence of islet-like clusters (ICAs) (54.00±10.26 islets per a 24-well plate (where P = 0.05) was observed in turn, developed into well-defined islets. The generated ICAs were stained positive for DTZ and appeared crimson red in color, confirming the differentiation of the cells into newly formed islets after culturing with SC2 (Figure 3A). Immunopositivity of ICAs with FITC-labeled anti-insulin antibody proved that the newly formed islets in *in vitro* culture are functionally differentiated islets (Figure 3B).

![Histological and Immunohistochemical analysis of pancreatic sections from SC2-treated mice (A, B), normal control mice (C, D) and diabetic control mice (E, F)](image)

The development of newly formed neo-islets from the pancreatic duct can be seen with SC2 treatment with 1: newly formed islet; 2: connection between the duct and the islet; 3: duct from which the islet emerged. A, C and E are the HE staining results under a light microscope with magnification of 400; B, D and F are the FITC staining results under a fluorescent microscope with magnification of 480. SC2: purified fraction of *Syzygium cumini*; HE: hematoxylin and eosin; FITC: fluorescein isothiocyanate.
3 Discussion

*S. cumini* is an important medicinal plant known to possess numerous therapeutic properties such as antihyperglycemic and antioxidant activities\(^\text{34,35}\). In our earlier study, we had demonstrated the *in vitro* glucosidase-inhibitory activity of chloroform extract of *S. cumini* seeds in porcine pancreatic and murine intestinal and pancreatic glucosidases\(^\text{36}\). The *in vivo* study using *S. cumini* chloroform extract revealed the significant islet regeneration and insulin secretagogue activity in STZ-induced diabetic mice along with moderate glucosidase-inhibitory activity. Based on these findings, in the present study we purified the active fraction from *S. cumini* chloroform extract with islet-regenerative activity. The active fraction was denoted as SC2.

In diabetes there is a decrease in functional mass of β cells, so replacing missing β cells or triggering their regeneration may allow for improvement in the control of diabetes. Since the replicative potential of β cells is limited, therefore search for factors that trigger islet neogenesis is important\(^\text{36}\). In this study we showed the ability of *S. cumini* active fraction SC2 to regenerate pancreatic islets in STZ-induced diabetic mice. The regeneration of islets was observed histologically with a presence of neoductoblastosis, that is, connection between the islets and the ductal precursor cells (Figure 2A). These newly formed islets were confirmed to be functional with evidence of insulin-producing β cells as seen in immunohistochemical analysis using FITC-labeled murine anti-insulin antibody (Figure 2B). The islet regeneration and stimulation of insulin-secretory effect observed from these newly formed islets after SC2 treatment could be responsible for the homeostasis of the glucose metabolism, suggesting SC2's role in normalization of glucose metabolism (Table 2).

This was further supported by the increase in plasma insulin and C-peptide levels as valuable indices for endogenous insulin secretion after SC2 treatment (Table 2). Histological and immunohistological studies support the regeneration of islets by SC2 fraction, as a possible mechanism of their antidiabetogenic activity in diabetic mice (Figure 2). Many plants and their components such as pancreas tonic and ephedrine and *Gymnema* sylvestre leaf extracts has been reported to show pancreatic islet regeneration and stimulation of insulin release in diabetic animals\(^\text{37,38}\).

It was reported that intrinsic factors present within the pancreas could somehow "sense" the islet mass and manage homeostasis of the β cell mass in normal healthy condition\(^\text{39-43}\). However, although these factors are intrinsic, they can be modulated by extrinsic factors such as diet, pancreatectomy, etc\(^\text{44}\). This indicates that SC2 fraction can be acting on intrinsic factors which stimulates islet regeneration and plays a role in regeneration and insulin-secretory activity in *in vivo* diabetic mice. Therefore, we further tested SC2 in *in vitro* cell line PANC-1 of pancreatic nature having stem cell-like property for *in vitro* islet differentiation. PANC-1 cell line was chosen as a suitable model system because it was reported to show islet differentiation with active herbal components without any intrinsic factors\(^\text{45}\). The PANC-1 cells in presence of SC2 led to the formation of ICAs (Figure 3A) while there was no cell aggregation observed in the control wells, which confirms that the aggregation and maturation of the islets was brought about by SC2. The identity of these ICAs was established by the use of the insulin-specific D1TZ staining. The capacity of these aggregates to produce insulin was further confirmed by positive reaction to FITC-labeled anti-insulin antibodies which indicates the cellular aggregates to be newly formed islets in *vitro* (Figure 3B). This supports that SC2 has a potential to regenerate islets without any help from endogenous factors.

4 Conclusion

The present study demonstrates for the first time *S. cumini* SC2 fraction carries the potential to induce islet neogenesis and is capable of bringing
about nesidioblastosis in vivo as well as in vitro. On the basis of our findings, we hypothesize that newly formed pancreatic islets may be the source of β cells for subsequent β cell expansion and proliferation. Such regenerative approaches, in combination with other therapeutic strategies may provide a better means for the control and management of diabetes in the future.

5 Acknowledgements

The authors would like to acknowledge Department of Zoology, University of Pune, India for providing animal house facilities and Council of Scientific and Industrial Research (CSIR, Government of India) for financial support. Menakshi Bhat Dusane also would like to acknowledge CSIR, India for Senior Research Fellowship.

6 Competing interests

The authors declare that they have no competing interests.

REFERENCES

海南蒲桃种子促进实验性糖尿病胰岛细胞再生的研究

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目的：研究海南蒲桃（Syzygium cumini）种子提取物 SC2 对链脲霉素诱导的实验性糖尿病小鼠胰岛细胞再生的作用。

方法：经 SC2 诱导的胰岛细胞再生实验。SC2 经口服给予胰岛细胞再生小鼠，共 21 d，期间有规律地测量体重及血糖。第 20 d 进行血糖检测。实验结束后处死小鼠并分离组织。测量大鼠组织中葡萄糖-6-磷酸脱氢酶活力、肝糖元及肌糖元含量，血浆胰岛素及血浆 C 肽水平。

结果：经 SC2 治疗的实验性糖尿病小鼠血糖水平恢复正常，肝组织内葡萄糖-6-磷酸脱氢酶活力升高，肝糖元和肌糖元含量升高，血浆胰岛素和 C 肽水平升高。组织学结果显示，SC2 治疗组出现新生胰岛细胞，提示 SC2 具有促进胰岛细胞再生的作用。这些新生胰岛细胞可以在实验性糖尿病小鼠体内产生胰岛素。

结论：本研究结果证明了海南蒲桃种子提取物 SC2 在胰岛再生和胰岛素分泌中的作用。这种作用结合其他治疗策略可能在将来为临床控制糖尿病提供一个更理想的途径。

关键词：糖尿病，实验性；蒲桃属；植物提取物；降血糖药；胰岛；小鼠