Inhibition of Key Digestive Enzymes Involved in Glucose Metabolism by Biosynthesized Zinc Oxide Nanoparticles from Syzygium cumini (L.): An in vitro and in silico Approach

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Submitted: 04-06-2019 Revised: 22-08-2019 Published: 28-11-2019

ABSTRACT

Background: In recent years, fusion of nanotechnology and medicine represents one of the major breakthroughs of modern science with the aim of developing nanomaterials for diagnosis, treatment, and overall improving health for the benefit of humankind. Objective: The aim of the study was to synthesize, characterize, and evaluate the antidiabetic effect of zinc oxide nanoparticles (ZnO NPs) from Syzygium cumini (SC). Materials and Methods: ZnO NPs were synthesized using SC seed extract and was characterized by ultraviolet-visible spectroscopy, Fourier transform-infrared (FT-IR) spectroscopy, X-ray diffraction (XRD), and transmission electron microscopy (TEM). Inhibitory potential of the ZnO NPs against α-amylase and α-glucosidase was analyzed in vitro, while AutoDock was used to analyze its potential in silico. Results: The FT-IR analysis suggested that the obtained ZnO NPs have been stabilized through the interactions of phenolic present in the seed extract. XRD pattern analysis confirmed the crystalline nature of the nanoparticles and TEM images revealed the polygonal structure of ZnO NPs of 16.7–22.9 nm in size. Molecular interaction studies of the phenolics from the SC seed extract with α-amylase and α-glucosidase have outlined its high affinity toward it relative to acarbose, while in vitro inhibitory potential studies of the ZnO NPs revealed that it could inhibit the enzymes similar to acarbose. Conclusion: Overall observations imply that phenolics of the SC seed extract helped in synthesis of the NPs and also its role in inhibitory potential which can be used as a potent antidiabetic drug.

Key words: Antidiabetic activity, Syzygium cumini, zinc oxide nanoparticles, α-amylase, α-glucosidase

SUMMARY

- Syzygium cumini (SC) seed extract reduced zinc nitrate to zinc oxide nanoparticles (NPs) which on characterization revealed its size of 16.7–22.9 nm and its crystalline nature
- Phenolic compounds present in SC seed extract were found to be responsible for the synthesis of the NPs and its nature
- In vitro and in silico studies on the synthesized NPs validated its inhibiting efficacy against digestive enzymes α-amylase and α-glucosidase, including the role of seed extract phenolics in it.

INTRODUCTION

Diabetes is one of the most common and complex endocrine metabolic disorders which causes both microvascular (retinopathy, neuropathy, and nephropathy) and macrovascular (stroke and heart attack) complications. As on 2014 World Health Organization report on diabetes states that 422 million individuals are affected by diabetes around the globe. Diabetes can be characterized into Type I and Type II, which is insulin dependent and insulin independent, respectively. Type II diabetes is the most common disorder that accounts for 90%–95% of the cases overall. In Type II diabetes, either the body cannot produce sufficient insulin or it cannot use the insulin properly. The most widely used therapies for diabetes are antidiabetic agents such as glinides, biguanides, sulfonlyureas, and insulin. Since these agents hold adverse side effects along with its benefits, the search for less adverse hypoglycemic agents is welcoming in the pharmaceutical market. Use of medicinal plants and herbs are being employed for 100s of years, which has insulin-mimetic property and also helps in glucose utilization by cells. The traditional medicinal plants provide the possibility for the discovery of potential antidiabetic compounds.

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Syzygium cumini (SC) belongs to the Myrtaceae family is widely used as medicinal plants from the ancient time for the treatment of various diseases including diabetes. It exhibits hypoglycemic property and protects the pancreas from the damage. The bioactive compounds such as anthocyanins, flavonoids, and phenolic acids in SC have potent antioxidant properties that help to prevent various metabolic disorders. Nanoparticles (NPs) are those which are <100 nm size in one dimension, which exhibits atom-like behavior. NPs can be synthesized either by chemical methods or by biological methods. Biosynthesis of NPs is mainly a bottom-up approach, where the NPs are synthesized from the scale of an atom to NPs. The main principle behind the biosynthesis of NPs is the reducing capacity of plants and micro-organisms. They are used in the reduction of atoms to NPs and also as a capping agent to stabilize the NPs formed.

The cause of diabetes varies between individuals, but the major cause in the current world is environment and lifestyle. It has caused a severe burden on the physiological conditions of the body which lead to insufficient insulin production required for food intake and subsequent ineffectiveness leading to the hyperglycemic condition. This has caused the endoplasmic reticulum to produce more transcriptional proteins to meet the insulin demand which accumulates leading to endoplasmic reticulum stress that sets off the unfolded protein response leading to beta-cell destruction.

Zinc is a crucial metal that acts as an enzyme activator for more than 300 enzymes in the human body. It also has a role in glucose metabolism, which improves the utilization of glucose via hepatic glycogenesis that acts through the insulin pathway and insulin signaling via enhanced PI3K activity, glycogen synthesis kinase-3 inhibition, and increased phosphorylation of insulin receptor. Previous reports have revealed that the zinc concentration in the pancreas of healthy individuals was considerably high when compared to diabetic patients, and most of the zinc content is located in the β-cells of the pancreas. Pancreatic β-cells have diverse zinc transporters, such as zinc transporter-8 that helps in insulin secretion. Zinc deficiency leads to the reduction of insulin secretion in pancreatic β-cells. Thus, developing zinc-based therapy is attractive due to the connection between zinc and diabetes. Although there are several reports showing the beneficial role of zinc supplement in animal models, the toxicity and poor bioavailability are the major limitations that need to be rectified.

**MATERIALS AND METHODS**

**Syzygium cumini** seed extract preparation and characterization

Fruits of SC were collected from Vellore district (12°56’15.8”N 79°11’48.3”E), Tamil Nadu, India and authenticated in Plant Anatomy Research Centre, Chennai, India (voucher no: PARC/2018/3807). The pulp of fruits was removed and seeds were shade-dried and powdered. SC seed extract was prepared by maceration at a concentration of 10 g of seed powder in 100 ml of distilled water; the extract was then filtered in Whatman® filter paper 1 and used to synthesize NPs. The phytocompounds in the seed extract were separated by liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) on Waters® UPLC®-TQD equipped with SUNFIRE C-18 column (250 mm × 4.6 mm, 5 µm) using water and methanol as mobile phase at a flow rate of 1 ml/min. The spectral data obtained from the extracted ion chromatogram were processed and the spectra obtained were matched with the entries of the mass spectral repository of Massbank of North America (MoNA) to ascertain the compounds present in the SC seed extract.

**Synthesis and characterization zinc oxide nanoparticles from Syzygium cumini**

NPs were synthesized by dissolving 10 g of zinc nitrate (Zn(NO₃)₂) in 100 ml of SC seed extract and constantly stirred at 70°C–80°C using a...
heated stirrer to reduce the solution to pale yellow-colored paste. The mixture was then collected in a ceramic crucible and calcined in a muffle furnace at 400°C for 1 h. The white-colored powder obtained from the above process was further characterized to obtain its optical and structural properties.

Characterization of the synthesized zinc oxide (ZnO) NPs was carried out by various analyses to determine its nature, composition, diffraction, charge, size, and distribution. Ultraviolet-visible (UV-Vis) spectroscopy analysis was done to determine its absorption wavelength. Surface diffraction was analyzed by X-ray diffraction (XRD); the phase identification was done using Match! version 1.11-Crystal Impact, Germany. Fourier transform-infrared (FT-IR) analysis was carried out to find the functional groups present in the NPs. The total phenolic content of the synthesized ZnO NPs was determined spectrophotometrically at 760 nm using the Folin–Ciocalteau method described by Singleton et al.[17] using gallic acid as a standard reference and expressed in gallic acid equivalent (GAE)/g of sample. The stability of the synthesized NPs was estimated by measuring the attraction or repulsion forces between the NPs using Horiba SZ-100 NP analyzer which gives the zeta-potential. The morphological properties and size of the synthesized NPs were observed using transmission electron microscopy (TEM).

**In vitro α-Amylase and α-glucosidase inhibitory and cytotoxicity assay**

The α-amylase inhibitory assay was accomplished by following the standard protocol of Kazeem et al.[18] The assay was carried out in a 96 well plate containing 50 µl of 0.5 mg/mL α-amylase solution in 0.02 M phosphate buffer (pH 6.9) with 50 µl of ZnO NPs and acarbose at different concentrations (20–100 µg/mL), and incubated at 37°C for 10 min then 50 µl of 3 mM p-nitrophenyl glucopyranoside was added to the reaction mixture. The reaction was stopped by adding 2 mL of 0.1 M Na2CO3 after 20 min of incubation at 37°C. Absorbance was measured at 405 nm which gives the α-glucosidase activity. A control was kept replacing the sample with distilled water in both the assays and the inhibition was calculated using the following equation:

\[
\%\ \text{Inhibition} = \left( \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \right) \times 100
\]

**Table 1: Mass spectral characteristics and composition of Syzygium cumini seed extract by liquid chromatography-electrospray ionization-tandem mass spectrometry**

<table>
<thead>
<tr>
<th>Ionization mode</th>
<th>Retention time</th>
<th>Compound name</th>
<th>m/z</th>
<th>Molecular formula</th>
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<tr>
<td>Negative</td>
<td>2.464</td>
<td>Isomaltulose</td>
<td>179</td>
<td>C9H14O11</td>
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<td></td>
<td>2.655</td>
<td>17-icosapentaenoic acid</td>
<td>195</td>
<td>C17H22O</td>
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<td></td>
<td>4.391</td>
<td>Citric acid</td>
<td>191</td>
<td>C6H8O7</td>
</tr>
<tr>
<td></td>
<td>3.401</td>
<td>Luteolin</td>
<td>133</td>
<td>C8H10O3</td>
</tr>
<tr>
<td></td>
<td>3.766</td>
<td>Tartarate</td>
<td>149</td>
<td>C4H6O5</td>
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<td></td>
<td>4.339</td>
<td>N, N-Dimethyl-N’-p-tolysulphamid</td>
<td>169</td>
<td>C10H17NO3</td>
</tr>
<tr>
<td></td>
<td>5.259</td>
<td>Gallic acid</td>
<td>169</td>
<td>C7H6O5</td>
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<tr>
<td></td>
<td>6.526</td>
<td>Propionic acid</td>
<td>198</td>
<td>C4H8O2</td>
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<td></td>
<td>21.73</td>
<td>Glycitin</td>
<td>469</td>
<td>C10H14O3</td>
</tr>
<tr>
<td></td>
<td>23.916</td>
<td>Kaempferol-3-Rhamnoside-7-Rhamnoside</td>
<td>433</td>
<td>C23H28O11</td>
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<td></td>
<td>24.159</td>
<td>naringenin-7-O-glucoside</td>
<td>433</td>
<td>C10H22O4</td>
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<tr>
<td></td>
<td>24.923</td>
<td>Hesperetin</td>
<td>301</td>
<td>C15H10O7</td>
</tr>
<tr>
<td></td>
<td>25.288</td>
<td>Quercetin</td>
<td>301</td>
<td>C15H10O7</td>
</tr>
<tr>
<td></td>
<td>39.173</td>
<td>N, N-Dimethyl-N’-p-tolysulphamid</td>
<td>213</td>
<td>C10H18O3</td>
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<tr>
<td>Positive</td>
<td>41.472</td>
<td>3,4’-Dimethoxy-7-hydroxyflavanone</td>
<td>311</td>
<td>C15H10O7</td>
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<tr>
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<td>51.634</td>
<td>2-Methoxyestradiol</td>
<td>151</td>
<td>C9H14O3</td>
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<tr>
<td></td>
<td>5.259</td>
<td>3′,4′-Dimethoxy-7-hydroxyflavanone</td>
<td>311</td>
<td>C15H10O7</td>
</tr>
<tr>
<td></td>
<td>33.28</td>
<td>Matricarin</td>
<td>230</td>
<td>C9H14O3</td>
</tr>
<tr>
<td></td>
<td>33.801</td>
<td>Quercetin</td>
<td>230</td>
<td>C9H14O3</td>
</tr>
<tr>
<td></td>
<td>46.193</td>
<td>all-trans-Retinoic Acid</td>
<td>301</td>
<td>C16H22O6</td>
</tr>
<tr>
<td></td>
<td>47.391</td>
<td>Thermoposide, crotonoyl</td>
<td>301</td>
<td>C16H22O6</td>
</tr>
<tr>
<td></td>
<td>49.317</td>
<td>(+)-Cinchonine</td>
<td>395</td>
<td>C16H22O6</td>
</tr>
<tr>
<td></td>
<td>50.011</td>
<td>Baquoloprim</td>
<td>309</td>
<td>C16H22O6</td>
</tr>
</tbody>
</table>
maintained at 37°C with 5% CO₂ condition. After the cells reached 90% confluence, it was used to analyze the cell cytotoxicity of the ZnO NPs by colorimetric assay using MTT. The cells were treated with ZnO NPs at different concentrations from 2.5 to 100 µg/ml. The morphological changes of the cells due to ZnO NPs were visualized under contrast microscope.

The percentage of cell viability was calculated using the following formula:

\[
\text{% Cell viability} = \frac{\text{absorbance of treated cells}}{\text{absorbance of control cells}} \times 100
\]

### Table 2: Binding energy and interactions of ligands with α-amylase and α-glucosidase from AutoDock analysis

<table>
<thead>
<tr>
<th>Compound name</th>
<th>Interaction with alpha-amylase</th>
<th>Interaction with alpha-glucosidase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Binding Energy (kcal/mol)</td>
<td>Inhibition constant (Ki)</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>−3.37</td>
<td>3.38 mM</td>
</tr>
<tr>
<td>Glycinit</td>
<td>−4.55</td>
<td>459.68 µM</td>
</tr>
<tr>
<td>Hesperetin</td>
<td>−5.14</td>
<td>169.51 µM</td>
</tr>
<tr>
<td>Hydroxydimethoxy flavones</td>
<td>−5.23</td>
<td>145.83 µM</td>
</tr>
<tr>
<td>Kaempferol</td>
<td>−5.18</td>
<td>158.84 µM</td>
</tr>
<tr>
<td>Luteolin</td>
<td>−5.88</td>
<td>49.01 µM</td>
</tr>
<tr>
<td>Naringenin</td>
<td>−5.91</td>
<td>46.75 µM</td>
</tr>
<tr>
<td>Propionic acid</td>
<td>−3.28</td>
<td>3.92 mM</td>
</tr>
<tr>
<td>Quercetin</td>
<td>−5.13</td>
<td>173.74 µM</td>
</tr>
<tr>
<td>Thermopsoside</td>
<td>−3.37</td>
<td>3.41 mM</td>
</tr>
<tr>
<td>Acarbose</td>
<td>−0.19</td>
<td>721.22 mM</td>
</tr>
</tbody>
</table>

### Interaction analysis using AutoDock

Molecular interaction study was carried out on the selected phytocompounds from the LC-ESI-MS/MS analysis using AutoDock version 4.2. The target chosen for the interaction study was α-amylase and α-glucosidase enzymes, the three-dimensional structure of the proteins was retrieved from Protein Data Bank (PDB) with ID 1B2Y and 5NN8, respectively. Autodock and AutoGrid algorithms were used for docking the protein target against...
various ligands to observe its inhibitory activity. Acarbose was used as a standard against α-amylase and α-glucosidase which has been commercially used for inhibitory properties. The algorithm used predicts the interaction of ligands with macromolecule (protein) targets. Kollman and Gasteiger charges were added. The selected docking parameters for the Lamarckian genetic algorithm are as follows: Number of docking runs were set to 10 with a population size of 150 with a maximum number of generations of 27,000. The least binding energy shows the highest binding affinity. Hydrogen bonds and hydrophobic interactions of all the docked complexes were viewed using LigPlot+ version 1.4.5.[22]

**RESULTS**

**Phytoconstituents of Syzygium cumini seed extract**

Phytocompounds present in the extract was determined by LC-ESI-MS/MS, the base peak intensity chromatogram was obtained for both negative and positive ionization mode [Figure 1]. The mass spectral data were obtained for the different fraction at various retention times; the spectrum obtained was used for similarity search in the spectral repository of MoNA [Table 1]. The major phytocompounds present in the seed extract was found to be phenols and flavonoids.

**Syzygium cumini seed extract synthesized stable crystalline zinc oxide nanoparticles**

The synthesized ZnO NPs were optically confirmed by UV-Vis spectrum by a wave phenomenon. The λ<sub>max</sub> of ZnO NPs usually occurs in the range of 360–380 nm and the absorption maximum of the synthesized ZnO NPs was observed at 369 nm that confirms the nanoscale level of the NPs. XRD data obtained were analyzed using MATCH! version 1.11b software which confirmed the presence of zinc and oxide in the sample [Figure 2b]. The XRD pattern revealed the Bragg’s reflection at 2Θ 31.72, 34.37, 36.19, 47.48, 56.53, 62.80, 67.89 which corresponds to lattice planes of (1 0 0), (0 0 2), (1 0 1), (1 0 2), (1 1 0), (1 0 3) of the ZnO NPs.[23]

The average size of ZnO NPs was determined from the XRD pattern obtained using Debye-Scherrer equation D = 0.9 λ/β cos Θ,[24] which was found to be 18.92 nm and was in accordance with the TEM images obtained (16.7–22.9 nm). The diffraction peaks revealed the crystalline and polygonal nature of the synthesized NPs. The surface analysis showed the d-spacing of 0.27 nm and its corresponding diffraction pattern through selected area electron diffraction. TEM analysis revealed that the synthesized NPs were polygonal in structure [Figure 3]. The zeta-potential of the synthesized ZnO NPs was estimated to be −36.8 mV which shows the stable nature of the NPs.

The phytochemical compounds capped onto synthesized ZnO NPs were confirmed by FT-IR matching the acquired spectrum with a reference spectrum [Figure 2a]. The peaks at 1116/cm and 1215/cm corresponds to C-N stretching of aliphatic amines (alcohols and phenols) and the peak at 1739/cm represents the presence of the aromatic ring, thus confirms the presence of polyphenols from SC seed extract.[25] The total phenolics on the synthesized ZnO NPs were estimated to be 4.542 mg/g GAE.

**Biosynthesized zinc oxide nanoparticles posses α-amylase and α-glucosidase inhibitory activity**

The inhibitory potential of ZnO NPs against α-amylase and α-glucosidase were analyzed and found that it shows inhibition similar to that of the commercial drug acarbose [Figure 4]. The 50% of inhibitory concentration (IC<sub>50</sub>) (concentration at which the activity of the digestive enzymes is reduced by 50%) value of the ZnO NPs and acarbose was found out graphically. The IC<sub>50</sub> value of ZnO NPs and acarbose on the activity of α-amylase was estimated to 74.75 and 87.31 mg/ml, respectively, and in case of α-glucosidase activity the IC<sub>50</sub> value was
calculated to be 63.07 and 51.86 mg/ml, respectively. Although acarbose inhibited α-amylase at higher concentration, ZnO NPs reduced its activity by half at a lower concentration and inferred that the synthesized ZnO NPs could be used as a α-amylase inhibitor. When the inhibitory potential of acarbose and ZnO NPs against α-glucosidase is compared, the NPs had better inhibition and a lower IC₅₀ value.

Alpha-amylase and alpha-glucosidase are major digestive enzymes that help in converting starch to glucose, which in turn increases the postprandial blood glucose level. Phenolic compounds present in traditional medicines were proven to be the main component in having inhibitory effects on these enzymes.⁶⁴,⁶⁵ Most of the reported phenolics in this study have previously been reported to show antidiabetic activity. These phenolics may either work alone or synergistically to produce the hypoglycemic effect.

The inhibitory activity of the synthesized ZnO NPs on RIN-5F cell line was expressed at a various concentration from 2.5 to 100 µg/ml [Figure 5]. The IC₅₀ value of ZnO NPs on RIN-5F cells was estimated to be 62.06 µg/ml from the MTT assay. Morphological features of the β-cells were visualized and found that ZnO NPs did not cause major changes at the 60 µg/mL concentration, while in case of the cells treated with ZnO NPs, 100 µg/mL concentration had undifferentiated cells and were seen aggregated [Figure 5 a-c].

**In silico analysis**

Molecular interaction of seed polyphenols of SC with α-amylase and α-glucosidase was studied using AutoDock version 4.2 tools, the binding energy (kcal/mol) and inhibition constant (Ki) are provided in Table 2. Graphical representation of the interactions involved with α-amylase and α-glucosidase are given in Figures 6 and 7,
respectively. When compared with the binding energy of reference drug acarbose (−0.19 kcal/mol); naringenin (−5.91 kcal/mol) and luteolin (−5.88 kcal/mol) had the highest binding energy through exhibiting good interaction with α-amylase. In case of interaction studies with α-glucosidase, hesperetin (−5.83 kcal/mol) and naringenin (−5.7 kcal/mol) had highest binding energy when compared with acarbose (−0.69 kcal/mol). At the same instance, the inhibition constant with α-amylase was considerably low of 46.75 and 49.01 µM for naringenin and luteolin, respectively, and with α-glucosidase, the inhibition constant was 53.52 and 65.97 µM for hesperetin and naringenin, respectively. The interaction studies have outlined the ability of the compounds present in the NPs as potent inhibitor against the enzymes.

**DISCUSSION**

Diabetes is a serious and complex disorder around the globe. The effective and highly available therapy is needed to counteract the increased incidence of the disorder. Although insulin and enzyme inhibitors have become a widely used therapeutic agent, there is always an effort to find new insulin substitute from other sources. In the present study, ZnO NPs synthesized from SC seed extract against streptozotocin (STZ)-induced rats have shown reduced blood glucose level. In the previous findings, SC has been reported to have antihyperglycemic and antihyperlipidemic effects on STZ-seed extract against streptozotocin (STZ)-induced rats. The phytochemical analysis of the biosynthesized ZnO NPs has confirmed the presence of phenolic content of SC seed extract coated on the NPs. The phenolic content of the plants was mainly responsible for the reduction of the chemical substrate to NPs, and the –OH group of the phenolics were involved in the reduction of zinc nitrate.[30]

Previous studies on α-amylase have revealed that the active site is situated in Domain A composed of 1–99 and 169–496 residues and the inhibitory potential of acarbose was due to interaction with residues 197, 233, and 300.[31] The interaction studies visualized from LigPlot has exposed the residues involved and all the compounds had interaction with Domain A. Naringenin and luteolin had the best binding energy and least inhibition constant and interacted with residues 197 and 300 which is responsible for inhibiting the enzyme. As of α-glucosidase, hesperitin and naringenin had the best binding energy and low inhibition constant. Most of the phytocompounds present in SC seed extract and the synthesized ZnO NPs have been previously reported to have inhibitory activity against α-amylase and α-glucosidase.[32] Previous studies show that 100 µg/ml of chemically synthesized ZnO NPs inhibits 75% on RIN-5F cells.[33] Cytotoxicity analysis of the biosynthesized ZnO NPs on RIN-5F cell lines revealed IC₅₀ value of 62.06 µg/ml. Thus, ZnO synthesized in our study showed less cytotoxic to pancreatic β-cells due to the presence of phytocompounds. The findings from the in vitro studies on the inhibitory potential of the ZnO NPs correlate with the computational studies which explain that the phenols present can be responsible for the inhibitory activity against the digestive enzymes such as α-amylase and α-glucosidase.

**CONCLUSION**

In summary, SC seed extract successfully reduced Zn (NO3)2 to ZnO NPs, which was confirmed by UV-Vis and XRD, further...
characterization by TEM and nanoanalyzer revealed its stable nanosized particles of 16–18 nm, while FT-IR and total phenolic assay confirmed the role of phenolics in synthesizing ZnO NPs and the presence of its molecular docking studies examined the interactions of phenolics from the SC seed extract with α-amylase and α-glucosidase enzymes which demonstrated the high binding affinity of naringenin, luteolin, and hesperetin with both the enzymes which is further supported by the in vitro inhibitory potential of α-amylase and α-glucosidase enzymes which reveals the role phenolics. Overall observations suggest that the ZnO NPs synthesized were stable and can be used for its antidiabetic property.

Acknowledgements
The authors acknowledge CSIR-Central Drug Research Institute, Lucknow, for carrying out LC-ESI-MS/MS analysis in their sophisticated analytical instrument facility.

Financial support and sponsorship
The authors would like to thank the Vellore Institute of Technology for the financial support and facilities to carry out this research work.

Conflicts of interest
There are no conflicts of interest.

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