Protective effect of aqueous seed extract of *Vitis Vinifera* against oxidative stress, inflammation and apoptosis in the pancreas of adult male rats with diabetes mellitus

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**Abstract**

**Introduction:** Protective effects of *Vitis Vinifera* seed aqueous extract (VVSAE) against pancreatic dysfunctions and elevation of oxidative stress, inflammation and apoptosis in the pancreas in diabetes were investigated. Histopathological changes in the pancreas were examined under light microscope. Methods: Blood and pancreases were collected from adult male diabetic rats receiving 28 days treatment with VVSAE orally. Fasting blood glucose (FBG), glycated hemoglobin (HbA1c), insulin and lipid profile levels and activity levels of anti-oxidative enzymes (superoxide dismutase-SOD, catalase-CAT and glutathione peroxidase-GPx) in the pancreas were determined by biochemical assays. Histopathological changes in the pancreas were examined under light microscopy and levels of insulin, glucose transporter (GLUT)-2, tumor necrosis factor (TNF)-\(\alpha\), Ikk\(\beta\) and caspase-3 mRNA and protein were analyzed by real-time PCR (qPCR) and immunohistochemistry respectively. Radical scavenging activity of VVSAE was evaluated by in-vitro anti-oxidant assay while gas chromatography-mass spectrometry (GC-MS) was used to identify the major compounds in the extract.

**Results:** GC-MS analyses indicated the presence of compounds that might exert anti-oxidative, anti-inflammatory and anti-apoptosis effects. Near normal FBG, HbA1c, lipid profile and serum insulin levels with lesser signs of pancreatic destruction were observed following administration of VVSAE to diabetic rats. Higher insulin, GLUT-2, SOD, CAT and GPx levels but lower TNF-\(\alpha\), Ikk\(\beta\) and caspase-3 levels were also observed in the pancreases of VVSAE-treated diabetic rats (\(p < 0.05\) compared to non-treated diabetic rats). The extract possesses high in-vitro radical scavenging activities.

**Conclusion:** In conclusions, administration of VVSAE to diabetic rats could help to protect the pancreas against oxidative stress, inflammation and apoptosis-induced damage while preserving pancreatic function near normal in diabetes.

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**1. Introduction**

Defective insulin secretion by the pancreas was reported in type-1 and type-2 diabetes mellitus (DM) [1]. Under normal condition, pancreatic insulin secretion correlates with the blood glucose level [2]. Prior to insulin release, glucose moves into the \(\beta\)-cells via glucose transporter-2 (GLUT-2) which then activates the insulin upstream factor-1 transcription factor and transcription of insulin gene [3]. Glucose also stimulates intracellular pathways that lead to exocytosis of insulin-containing granules [4]. In diabetes, expression of GLUT-2 in \(\beta\)-cells was decreased [5]. There were evidences that exposure to high blood glucose level could lead to decreased binding of transcription activator to insulin gene, thus reduces insulin synthesis [6].

High glucose can also cause increase in oxidative stress, inflammation and apoptosis [7,8]. Free radicals generated from glucose auto-oxidation and protein glycosylation [9] can cause oxidative stress level in the pancreatic \(\beta\)-cells to increase [10]. This will result in pancreatic inflammation and apoptosis [11]. Conversely, inflammation can also cause elevated oxidative stress
2. Materials and methods

2.1. Chemicals and reagents

Streptozotocin (STZ), glibenclamide, epinephrine, thiobarbituric acid (TBA) and reduced glutathione (GSH) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were of analytical grade.

2.2. Preparation of V. vinifera aqueous seed extract (VVASAE)

Ripe fruits of V. vinifera (Muscat variety) were collected in Tirupati district, Andhra Pradesh, India in October 2014. Botanical identification and authentication were performed by botanist and voucher specimen was deposited at local herbarium. Seed extract was prepared as described earlier [15]. Briefly, seeds were separated from pulp, air dried and grounded into powder (1 kg). The powder was then soaked in 2.5 l distilled H2O for 3 days with intermittent shaking, then filtered through Whatman filter paper. The filtered extract was concentrated at 50 ± 5°C by using a rotary evaporator. The solid extract was stored at 4°C for further use.

2.3. Assessment of VVASAE anti-oxidant activity in-vitro

Antioxidant power of VVASAE was evaluated by using DPPH, superoxide, hydroxyl and H2O2 radical scavenging assays. DPPH radical-scavenging activity was determined according to the method by Katalinic, Milos [19]. Scavenging activity of superoxide radical was measured based on the method by Stewart and Bewley [20]. Hydroxyl radical scavenging activity was measured by using a modified method by Halliwell et al. [21]. Ability of VVASAE to scavenge H2O2 was determined according to the method by Ruch et al. [22]. Ascorbic acid (10–200 µg/ml) was

Table 1

<table>
<thead>
<tr>
<th>S. No</th>
<th>RT</th>
<th>Name of the compound</th>
<th>Molecular formula</th>
<th>MW</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>9.80</td>
<td>Formamide, N-formyl-N-methyl-</td>
<td>C9H10N2O</td>
<td>187.07</td>
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<tr>
<td>2</td>
<td>13.14</td>
<td>Guanidine, N,N-dimethyl-</td>
<td>C8H12N2</td>
<td>138.22</td>
</tr>
<tr>
<td>3</td>
<td>23.63</td>
<td>Oxime-, methoxy-phenyl-</td>
<td>C10H10N2O</td>
<td>184.16</td>
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<tr>
<td>4</td>
<td>30.25</td>
<td>Seychellene</td>
<td>C16H16</td>
<td>204.35</td>
</tr>
<tr>
<td>5</td>
<td>33.58</td>
<td>Benzene, (3-methylcyclopentyl)-</td>
<td>C12H10</td>
<td>160.25</td>
</tr>
<tr>
<td>6</td>
<td>36.07</td>
<td>9-Octadecanamide, (2)-</td>
<td>C28H52NO</td>
<td>430.87</td>
</tr>
<tr>
<td>7</td>
<td>38.32</td>
<td>Phenol, 2,5-bis(1,1-dimethyl-</td>
<td>C14H12O2</td>
<td>206.32</td>
</tr>
<tr>
<td>8</td>
<td>40.86</td>
<td>Octanal, 2-(phenylmethylene)-</td>
<td>C13H20O</td>
<td>216.31</td>
</tr>
</tbody>
</table>

Fig. 1. GC–MS tracing of aqueous seed extract of V. vinifera. Multiple peaks could be seen in the chromatogram indicating the presence of several active compounds.
used as standard. Ability of extract to scavenge or inhibit free radicals was expressed as% inhibition and was calculated by using this formula.

\[
\% \text{of inhibition} = \left( \frac{A_0 - A_t}{A_0} \right) \times 100
\]

\(A_0\) = absorbance of control group (without VVSAE) and \(A_t\) = absorbance of VVSAE group. Absorbance was measured by using a spectrophotometer (UV-1700, Shimadzu, Kyoto, Japan) at different wavelengths, which depend upon type of assay performed. All assays were carried out in triplicate.

2.4. GC–MS analyses of VVSAE

GC–MS analysis was carried out by using Trace GC ultra-gas chromatograph connected to a Quantum XLS mass spectrometer (Thermo Scientific, FL, USA). GC was equipped with TG-5MS capillary column (30 m × 0.25 mm i.d. × 0.25 μm film thickness) consisting of a stationary phase 5% phenyl and 95% methyl polysiloxane. Samples were dissolved in water and 1 μl of VVSAE was injected into CT splitless mode at an injector temperature of 280°C. The flow rate of the carrier gas (helium) was 1 ml/min. The oven temperature was programmed from 110°C (isothermal for 2 min), with an increase of 10°C/min to 200°C, then 5°C/min to 280°C, ending with a 9 min isothermal at 280°C. Mass spectra were taken at 70 eV; a scan interval of 0.5 s and fragments from 45 to 450 Da. The solvent delay was 0–2 min, and total GC/MS running time was 42 min. Interpretation on mass-spectrum GC–MS was conducted by using the database from National Institute Standard and Technology (NIST). The spectrum of the unknown components was compared with the spectrum of known components stored in NIST library. The name, molecular weight and structure of components of the test materials were identified.

2.5. Animal preparation

Adult male albino Wistar rats (9 weeks-old, 180–200 g) were purchased from local supplier and were maintained at room temperature of 23 ± 2°C and 12/12 h light/dark cycle. Animals were fed with standard pellet diet (Harlan, UK) and tap water ad libitum. Experimental procedures were in accordance with ARRIVE guidelines (Animals in Research: Reporting In-Vivo Experiments) and were approved by Institutional Ethics Committee, Andhra University, Visakhapatnam, India. Overnight (12 h) fasted animals were rendered diabetes by injecting a freshly prepared streptozotocin (STZ) at 55 mg/kg bw dissolved in 0.1 M citrate buffer (pH 4.5) with nicotinamide [23]. STZ-nicotinamide injected animals were given 5% glucose for 24 h to prevent mortality caused by drug-induced hypoglycemia. Diabetes was confirmed in animals exhibiting FBG levels greater than 300 mg/dl. Treatment was commenced four days after confirmation of diabetes and the first day of treatment was considered as day one (1). VVSAE was orally administered at 250 and 500 mg/kg/day in a form of suspension in 1% sodium carboxy methyl cellulose (Na-CMC) in distilled water. Glibenclamide (600 μg/kg) was used as reference (positive control) and was orally given [24].

2.6. Experimental design

The animals were randomly divided into five (5) groups with six animals in each group, receiving the following treatment for 28 consecutive days.

- Group I – Control, non-diabetic rats – treated with 1% Na-CMC (vehicle) only.
- Group II – Non-treated diabetic rats – receiving 1% Na-CMC (vehicle) only.
- Group III- Diabetic rats treated with VVSAE at 250 mg/kg b.w.
- Group IV- Diabetic rats treated with VVSAE at 500 mg/kg b.w.
- Group V- Diabetic rats treated with standard drug, glibenclamide at 600 μg/kg b.w.

For determination of FBG, blood was collected by pricking the tail vein. FBG levels were measured at day 0, 7th, 14th, 21st and 28th of the experimental period by using a digital glucometer (Accu-Chek®, Roche, Mannheim, Germany). The initial and final body weight, daily food and water intakes were determined. At the end of 28-days treatment, animals were fasted overnight. Rats were then sacrificed and pancreas was excised and stored at –80°C for later analyses. Meanwhile, blood was collected via heart puncture and serum was separated by centrifugation at 4000g for 15 min at 4°C by using a centrifuge machine (Thermo Scientific, Model 75005286, USA).

### Table 2

<table>
<thead>
<tr>
<th>Extract/Standard</th>
<th>DPPH (IC50) [μg/ml]</th>
<th>Hydroxyl (IC50) [μg/ml]</th>
<th>Superoxide (IC50) [μg/ml]</th>
<th>Hydrogen peroxide (IC50) [μg/ml]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascorbic acid</td>
<td>38.23</td>
<td>40.28</td>
<td>32.15</td>
<td>44.64</td>
</tr>
<tr>
<td>V. vinifera</td>
<td>144.56</td>
<td>149.33</td>
<td>138.43</td>
<td>158.15</td>
</tr>
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</table>

### Table 3

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Normal</th>
<th>Diabetic 250 mg/kg vinifera</th>
<th>Diabetic 500 mg/kg vinifera</th>
<th>Diabetic 600 μg/kg glibenclamide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial body weight (g)</td>
<td>187.68 ± 7.94</td>
<td>189.84 ± 9.68</td>
<td>186.98 ± 7.45</td>
<td>185.18 ± 6.73</td>
</tr>
<tr>
<td>Final body weight (g)</td>
<td>229.76 ± 8.67</td>
<td>164.15 ± 8.92</td>
<td>196.18 ± 6.84</td>
<td>218.11 ± 6.76</td>
</tr>
<tr>
<td>Water intake (ml/rat per day)</td>
<td>32.43 ± 4.25</td>
<td>93.24 ± 5.46</td>
<td>67.23 ± 6.87</td>
<td>55.98 ± 6.35</td>
</tr>
<tr>
<td>Food intake (g/rat per day)</td>
<td>16.35 ± 2.46</td>
<td>26.47 ± 2.64</td>
<td>21.78 ± 2.78</td>
<td>18.34 ± 2.12</td>
</tr>
</tbody>
</table>

Value represents means ± S.E.M. for 6 rats per group. ns: non-significant.

* p < 0.05 compared to normal, non-diabetic rats.

** p < 0.01 compared to non-treated diabetic rats.
2.7. Determination of lipid profile levels

The serum lipid profile levels i.e. high density lipoprotein (HDL), total cholesterol (TC) and triglyceride (TG) were determined by using a diagnostic kit (BioSystems S.A. Costa Brava 30, Barcelona, Spain). Serum low density lipoprotein (LDL) — and very low density lipoprotein (VLDL) were estimated based on Friedewald formula:

\[
LDL = \text{Total cholesterol} - \text{HDL} - \left( \frac{\text{TG}}{5} \right)
\]

\[
VLDL = \frac{\text{TG}}{5}
\]

2.8. Determination of HbA1c and insulin levels

HbA1c in whole blood was estimated by using a commercially available kit (BioSystems S.A. Costa Brava 30, Barcelona, Spain) while serum insulin level was measured by using enzyme-linked immunosorbent assay (ELISA) kit (EIA-2048, 96 wells, DRG Instruments GmbH, Marburg, Germany), according to manufacturer’s guideline. Briefly, during incubation, insulin in the sample reacted with peroxidase-conjugated anti-insulin antibodies bound to micro-titer wells. Washing steps removed the unbound enzyme-labeled antibody. The bound conjugate was detected by the reaction with 3,3’5,5’-tetramethylbenzidine. The reaction was stopped by adding acid to give a colorimetric end-point and optical density was measured by using a micro plate auto reader (iMark™; Bio-Rad, Hercules, CA, USA) at a wavelength of 450 nm.

2.9. Histology and immunohistochemistry

Following sacrifice, pancreas were harvested and fixed overnight in 10% formalin. This organ was then embedded and were cut into 5 μm thickness. The sections were deparaffinized by immersing in xylene for 20 min, dropped in ethanol at decreasing concentrations (100%, 95%, 90% and 80%), 5 min each. The sections were stained with hematoxylin and eosin (H&E). Prior to immunohistochemistry procedures, antigen retrieval was performed by incubating the sections in 0.01 M citrate buffer, pH 6.0 for 10 min at 100 °C. Subsequently 3% H2O2 in phosphate buffered saline (PBS) was used to neutralize the endogenous peroxidase. Sections were blocked in normal serum (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for non-specific binding, prior to incubation with insulin, TNF-α, Ikk-β, caspase-3 and GLUT-2 polyclonal primary antibodies (sc-9168; sc-1350; sc-34674, sc-1225 and sc-9117, Santa Cruz, CA, USA respectively) at a dilution of 1:500 in 5% normal serum at room temperature for 1 h. After four times rinsing with PBS, sections were incubated with biotinylated secondary antibody for 30 min at room temperature, then exposed to avidin and biotinylated HRP complex in PBS for another 30 min.
Table 4
Effects of *V. vinifera* aqueous seed extract (VSAE) on serum lipid profiles in streptozotocin-nicotinamide-induced diabetic rats.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Normal</th>
<th>Diabetic</th>
<th>Diabetic 250 mg/kg Vinifera</th>
<th>500 mg/kg Vinifera</th>
<th>600 μg/kg Glibenclamide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>56.64 ± 5.14</td>
<td>124.48 ± 8.15</td>
<td>89.15 ± 6.48</td>
<td>75.94 ± 5.71</td>
<td>68.25 ± 6.85</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>74.35 ± 2.14</td>
<td>116.15 ± 4.18</td>
<td>92.25 ± 3.65</td>
<td>96.45 ± 5.13</td>
<td>83.63 ± 3.43</td>
</tr>
<tr>
<td>HDL (mmol/L)</td>
<td>32.64 ± 2.56</td>
<td>12.18 ± 1.89</td>
<td>16.15 ± 2.48</td>
<td>19.87 ± 1.82</td>
<td>24.71 ± 1.97</td>
</tr>
<tr>
<td>LDL (mmol/L)</td>
<td>9.13 ± 1.74</td>
<td>89.07 ± 2.67</td>
<td>54.55 ± 1.65</td>
<td>36.77 ± 3.83</td>
<td>26.81 ± 1.29</td>
</tr>
<tr>
<td>VLDL (mmol/L)</td>
<td>14.87 ± 0.46</td>
<td>23.23 ± 0.64</td>
<td>18.45 ± 0.73</td>
<td>19.29 ± 0.47</td>
<td>16.72 ± 0.60</td>
</tr>
<tr>
<td>HDL (mmol/L)</td>
<td>1.73 ± 1.18</td>
<td>10.22 ± 0.35</td>
<td>5.52 ± 0.43</td>
<td>3.82 ± 0.53</td>
<td>2.76 ± 0.45</td>
</tr>
</tbody>
</table>

Value represents means ± S.E.M for 6 rats per group.

* p < 0.05 compared to normal, non-diabetic rats group.
** p < 0.01 compared to non-treated diabetic rats.

Fig. 3. (A): Histopathological changes in the pancreas of different experimental rats. (B) Changes in the number of islet of Langerhans/pancreas (C) diameter of islet of Langerhans (μm) (D) number of β-cells/islet of Langerhans and (E) diameter of β-cells (μm). Arrows show islet of Langerhans. Scale bar = 50 μM. N: Normal control; D: Diabetic control; D+250 Vv: 250 mg/kg of V. vinifera, D+500 Vv: 500 mg/kg of V. vinifera, D+G: 600 μg/kg/day of glibenclamide. (H & E, 200×). *p < 0.05 when compared to non-diabetic rats, †p < 0.05 when compared to diabetic rats.
The site of antibody binding was visualized by 3,3'-diaminobenzidine (DAB) which gave dark-brown precipitate. Sections were counterstained with hematoxylin for nuclear staining.

For immunofluorescence, sections were blocked with 10% normal goat serum (sc-2043) (Santa Cruz, CA, USA) prior to incubation with rabbit insulin polyclonal antibody (Santa Cruz, CA, USA; sc-9168) at a dilution of 1:100 in PBS with 1.5% normal blocking serum at room temperature for 1 h. After three times rinsing with PBS, sections were incubated with goat anti-rabbit IgG–fluorochrome-conjugated secondary antibody (sc-2012) (Santa Cruz, CA, USA) at a dilution of 1:250 in PBS with 1.5% normal blocking serum at room temperature for 45 min. The slides were rinsed three times with PBS and were mounted with

### Table 5

Effects of *V. vinifera* aqueous seed extract (VVSAE) on LPO, SOD, CAT and GPx levels in the pancreas of streptozotocin-nicotinamide-induced diabetic rats.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Normal</th>
<th>Diabetic</th>
<th>Diabetic 250 mg/kg vinifera</th>
<th>Diabetic 500 mg/kg vinifera</th>
<th>Diabetic 600 µg/kg glibenclamide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipid peroxidation (µ moles of malondialdehyde formed/gram wet weight of tissue)</td>
<td>5.32 ± 1.68</td>
<td>13.58 ± 2.16</td>
<td>8.97 ± 2.57</td>
<td>6.65 ± 1.78</td>
<td>6.78 ± 1.86</td>
</tr>
<tr>
<td>Superoxide dismutase (units/mg protein/min)</td>
<td>1.25 ± 0.06</td>
<td>0.76 ± 0.07</td>
<td>0.92 ± 0.07</td>
<td>1.05 ± 0.06</td>
<td>1.17 ± 0.05</td>
</tr>
<tr>
<td>Catalase (H2O2 metabolized/mg protein/min)</td>
<td>0.48 ± 0.06</td>
<td>0.21 ± 0.07</td>
<td>0.34 ± 0.08</td>
<td>0.41 ± 0.06</td>
<td>0.44 ± 0.07</td>
</tr>
<tr>
<td>Glutathione peroxidase (µmol of GSH consumed/mg protein/min)</td>
<td>1.26 ± 0.05</td>
<td>0.82 ± 0.08</td>
<td>1.12 ± 0.06</td>
<td>1.21 ± 0.07</td>
<td>1.23 ± 0.05</td>
</tr>
</tbody>
</table>

Value represents means ± S.E.M for 6 rats per group.

* p < 0.05 compared to non-diabetic rats.

** p < 0.01 compared to non-treated diabetic rats.

Fig. 4. (A) Immunoperoxidase images showing insulin expression in the islet of Langerhans in rats receiving different treatment. Dark-brown staining indicate expression of insulin. (B) Quantitative analyses of immunostaining intensity in the islet of Langerhans. N: Normal control; D: Diabetic control; D + 250 Vv: 250 mg/kg of *V. vinifera*, D + 500 Vv: 500 mg/kg of *V. vinifera*, D + G: 600 µg/kg/day of glibenclamide. Scale bar = 50 µM. Each value is expressed as mean ± S.E.M. (n = 6). *p < 0.05 when compared to non-diabetic rats, †p < 0.05 when compared to diabetic rats.
Ultracruz Mounting Medium (Santa Cruz, CA, USA) and counter-stained with DAPI to visualize the nuclei. Histological changes in the pancreas were viewed and micrograph by using a phase contrast microscope with attached photograph machine (Nikon H600L, Tokyo, Japan). Morphometric analyses were performed by using Image J software (Image J 1.39f, NIH-Bethesda, MD, USA). Histologically-stained sections were used for morphometric analysis (n = 30); 208 islet were examined at different magnifications in non-serial pancreatic sections to estimate (1) number of islets of Langerhans/pancreas, (2) diameter of islets, (3) number of β-cells/islets and (4) diameter of β-cells.

2.10. Real-time PCR (qPCR)

Pancreas was kept in RNA Later solution (Ambion, Austin, TX, USA) prior to RNA extraction. Total RNA was freshly isolated by using RNeasy Plus Mini Kit (Qiagen, Valencia, CA, USA). Purity and concentration of RNA was assessed by 260/280 UV absorption ratios (Gene Quant 1300, GE Healthcare UK Limited, Buckinghamshire, UK). Two steps Real Time PCR was used to evaluate gene expression. Reverse transcription into cDNA was performed by using high capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City, CA, USA). Controls included amplification on samples

![Image](https://example.com/image.png)
identically prepared with no reverse transcriptase (-RT) and amplifications performed with no added substrate. The assays used (Rn01477289_m1, Rn00560930_m1, Rn00577994_g1, Rn01525756_g1, Rn99999017_m1, Rn01756707_m1, Rn01488065_m1 and Rn00689321_m1) amplified 100 bp segment of Sod1, 107 bp segment of Cat, 77 bp segment of Gpx1, 137 bp segment of Rage (Ager), 108 bp segment of Tnf-α, 89 bp segment of Ikkb, 125 bp segment of caspase-3 and 111 bp segment of Glut2 (Slc2a2) respectively. Target assay was validated in-silico by using whole rat genome and in-vitro by using whole rat cDNA to ensure that target sequences were detected (Applied Biosystems, Foster City, CA, USA). Gapdh and Hprt were used as reference genes.

PCR program included 2 min at 50 °C for UNG activity, 20 ± 95 °C activation of ampliTaq gold DNA polymerase and 1 min

**Fig. 6.** (a) Immunofluorescence signals indicating RAGE expression in islet of Langerhans in rats receiving different treatment. Green signals indicate site of RAGE expression. D: Diabetic control; D + 250 Vv- 250 mg/kg of V. vinifera, D + 500 Vv- 500 mg/kg of V. vinifera, D + G - 600 μg/kg/day of glibenclamide. Scale bar = 50 μM. Each value is expressed as mean ± S.E.M. (n = 6). *p < 0.05 when compared to non-diabetic rats, †p < 0.05 when compared to diabetic rats.
denaturation at 95 °C, 20 s and annealing/extension at 60 °C for 1 min. Denaturing and annealing was performed for 40 cycles. All measurements were normalized using GenEx software (MultiD, Goteburg, Sweden) followed by Data Assist v3 software (Applied Biosystems, USA) which was used to calculate the RNA fold changes. All experiments were carried out in triplicates. Data were analyzed according to comparative Ct (2^−ΔΔCt) method.

2.11. Pancreas homogenates and estimation of LPO, SOD, CAT and GPx

Pancreas was washed in ice-cold saline to remove the blood and then sliced separately into pieces and homogenized in buffer containing 0.25 M sucrose and 0.1 M Tris–HCl (pH 7.4). Homogenate was centrifuged at 600g for 10 min at 4 °C in cold centrifuge. The mitochondrial pellet was obtained by centrifugation of post-nuclear supernatant at 10,000g for 15 min to obtain nuclear pellet. The microsomal fractions were prepared by using calcium chloride (CaCl₂) sedimentation method of Walawalkar, Serai [25] and were used for biochemical analyses. LPO was estimated by thiobarbituric acid (TBA) reaction with malondialdehyde (MDA), a product formed from peroxidation of membrane lipids [26]. In brief, to 1.0 ml supernatant, 0.25 ml of TBA reagent was added and the content was incubated at 95 °C for 1 h then cooled under tap water prior to adding 1 ml of n-butanol. After thorough mixing, the content was again centrifuged for 15 min at 4000g in a refrigerated centrifuge. The organic layer was transferred into a clear tube and absorbance was measured at 532 nm by using a spectrophotometer (UV-1700, Shimadzu, Kyoto, Japan). The rate of lipid peroxidation was expressed as μ moles of MDA formed/gram wet weight of tissue.

SOD activity level was assayed according to the method of Misra and Fridovich [27]. The assay procedures involve inhibition of epinephrine auto-oxidation in alkaline medium (pH 10.2) to adrenochrome, which was markedly inhibited in the presence of SOD. To 0.5 ml of supernatant, 1.5 ml of carbonate buffer (0.05 M, pH 10.2) and 0.5 ml of ethylene-diamine-tetra acetic acid (EDTA) solution (0.49 M) were added. The reaction was initiated by adding 0.4 ml of epinephrine (3 mM). Changes in absorbance were recorded at 480 nm for 1 min at 15 s interval, 3 min each by using a spectrophotometer (UV-1700, Shimadzu, Kyoto, Japan). A single unit of enzyme was expressed as 50% inhibition of epinephrine reduction/min/mg protein.

CAT activity level was determined on the basis of hydrogen peroxide decomposition according to the method by Bonaventura et al. [28]. The reaction solution contained 2.5 ml of 50 mmol phosphate buffer (pH 5.0), 0.4 ml of 5.9 mmol H₂O₂ and 0.1 ml
enzyme extract. The reaction was initiated by adding the enzyme extract. Changes in absorbance were measured at 240 nm every 30 s and was read by a spectrophotometer (UV-1700, Shimadzu, Kyoto, Japan). The activity level of this enzyme was expressed in μmol of hydrogen peroxide (H₂O₂) metabolized/mg protein/min. GPx activity level was measured according to the method by Rotruck et al. [29]. The reaction mixture consist of 0.2 ml of 0.8 mM EDTA, 0.1 ml of 10 mM sodium azide, 0.1 ml of 2.5 mM H₂O₂, 0.2 ml of glutathione (GSH), 0.4 ml of 0.4 mM phosphate buffer (pH 7.0) and 0.2 ml of homogenates incubated at 37 °C for 10 min. The reaction was arrested upon addition of 0.5 ml of 10% TCA and the tubes were centrifuged at 2000 rpm. To the supernatant, 3.0 ml of 0.3 M disodium hydrogen phosphate and 1.0 ml of DTNB were added and color changes were immediately read at 420 nm by using a spectrophotometer (UV-1700, Shimadzu, Kyoto, Japan). Activity level of GPx was expressed as μmol of GSH consumed/mg protein/min.

2.12. Statistical analyses

Statistical differences between group were evaluated by one-way analysis of variance (ANOVA) and Student’s t-test using a GraphPad Prism Software (GraphPad Software, Inc., CA, USA). A probability level of less than 0.05 (p < 0.05) was considered as significant. Post-hoc statistical power analysis was performed also using GraphPad Prism software and values obtained were >0.8 which indicate adequate sample size.

3. Results

3.1. GC–MS analyses

In Fig. 1, several peaks were observed in GC–MS chromatogram of VVSAE. In Table 1, the molecules at retention time (RT) 9.80 was identified as formamide, N-formyl-N-methyl, at RT 13.14 was guanidine, N,N-dimethyl, at RT 23.63 was oxime-, methoxy-phenyl, at RT 30.25 was seychellene, at RT 33.58 was benzene, (3-methylcyclopentyl), at RT 36.07 was 9-octadecenamide, (Z), at RT 38.32 was phenol, 2,5-bis(11-dimethylethyl) and at RT 40.86 was octanal, 2-(phenylmethylene).

3.2. In-vitro antioxidant assay

In Table 2, IC₅₀s of VVSAE for DPPH, hydroxyl, superoxide and H₂O₂ radical scavenging activities were 144.56, 149.33, 138.43 and
at when rats to VVSAE (p glibenclamide when treatment the 3.3. parameters VVSAE diabetic vinifera caspase-3 158.15 μg/ml respectively while IC_{50,5} of ascorbic acid for the above parameters were 38.23, 40.28, 32.15 and 44.64 μg/ml respectively. VVSAE IC_{50,5} was approximately 3–4 times lower than IC_{50,5} for ascorbic acid.

3.3. Effects on body weight, food and water intakes

In Table 3, there was a decrease in final body weight of diabetic rats when compared to non-diabetic rats. Administration of VVSAE at 250 and 500 mg/kg/day resulted in higher final body weight when compared to non-treated diabetic rats. Glibenclamide treatment resulted in higher final body weight when compared to non-treated diabetic rats. The average daily water intakes were higher in diabetic rats when compared to normal, non-diabetic rats (p < 0.05). Diabetic rats treated with 250 and 500 mg/kg/day VVSAE or glibenclamide had lower daily water intakes when compared to non-treated diabetic rats (p < 0.05). In diabetic rats, the mean daily food intakes were higher when compared to non-diabetic rats. Treatment with 250 and 500 mg/kg/day VVSAE or glibenclamide to diabetic rats resulted in lower daily food intake when compared to non-treated diabetic rats.

3.4. Effects on FBG, HbA1c, insulin and lipid profile levels

In Fig. 2A, in diabetic rats, FBG levels at weekly interval were higher when compared to non-diabetic rats (p < 0.05). In diabetic rats receiving VVSAE or glibenclamide, FBG levels began to decrease from day 7th, further decline at day 14th and reached the lowest at day 28th. Meanwhile, in Fig. 2B, HbA1c levels were highest in non-treated diabetic rats. Treatment with VVSAE or glibenclamide resulted in lower HbA1c levels when compared to non-treated diabetic rats (p < 0.05). In Fig. 2C, serum insulin levels were lowest in non-treated diabetic rats (p < 0.05). Treatment with 250 and 500 mg/kg/day VVSAE or glibenclamide resulted in higher serum insulin levels when compared to non-treated diabetic rats (p < 0.05).

In Table 4, in non-treated diabetic rats, TC, TG, LDL, VLDL levels and LDL/HDL and TC/HDL ratios were higher than non-diabetic rats (p < 0.05), however HDL levels were lesser than in non-diabetic rats. Treatment with VVSAE at 250 or 500 mg/kg/day resulted in lower TC, TG, LDL, VLDL, LDL/HDL and TC/HDL respectively when compared to non-treated diabetic rats (p < 0.05). However, HDL levels were higher when compared to non-treated diabetic rats.
3.5. Histopathological changes in pancreas

In Fig. 3A, normal appearance of islets was seen in non-diabetic rats. However, in diabetic rats, pathological changes were observed in islet histology which include massive destruction of the islets, blood-filled interlobular ducts and disorganized acinar cells. Lesser signs of destruction could be seen in the islets following VVSAE or glibenclamide treatment with higher number and diameter of islets and β-cells (Fig. 3B–E).

3.6. Effects on pancreatic LPO, SOD, CAT and GPx activity levels

In Table 5, in non-treated diabetic rats, pancreatic LPO levels were higher than non-diabetic rats (p < 0.05). Treatment with 250 mg/kg VVSAE resulted in lower pancreatic LPO levels when compared to non-treated diabetic rats (p < 0.05). Treatment with 500 mg/kg VVSAE resulted in pancreatic LPO levels to be approximately 50% lower when compared to non-treated diabetic rats. In diabetic rats, pancreatic SOD activity levels were lower than non-diabetic rats (p < 0.05). Treatment with 250 or 500 mg/kg/day VVSAE resulted in higher SOD activity level when compared to non-treated diabetic rats (p < 0.05). In diabetic rats, CAT activity levels were lower when compared to non-diabetic rats (p < 0.05).

Treatment of diabetic rats with VVSAE at 250 mg/kg/day or 500 mg/kg/day resulted in higher CAT activity level when compared to non-treated diabetic rats (p < 0.05). In non-treated diabetic rats, GPx activity levels were lower when compared to non-diabetic rats. Treatment with 250 mg/kg or 500 mg/kg VVSAE resulted in higher GPx activity level when compared to non-treated diabetic rats (p < 0.05).

3.7. RAGE, insulin, TNF-α, ikkβ, caspase-3 and GLUT-2 expression levels in pancreas

In Figs. 4 and 5, in non-treated diabetic rats, low insulin level was observed in the islets (p < 0.05 when compared to non-diabetic rats). Treatment with VVSAE or glibenclamide resulted in higher insulin level when compared to non-treated diabetic rats (p < 0.05). Treatment with 500 mg/kg/day VVSAE resulted in higher insulin level when compared to 250 mg/kg/day VVSAE treatment.

In Fig. 6, in non-treated diabetic rats, high level of RAGE was observed in the islets (p < 0.05 compared to non-diabetic rats). However, treatment with 250 mg/day VVSAE resulted in lower RAGE expression level when compared to non-treated diabetic rats (p < 0.05). In diabetic rats treated with 500 mg/kg VVSAE or
glibenclamide, RAGE expression level in the islets was lower when compared to diabetic rats treated with 250 mg/kg/day VVSAE.

In Figs. 7 and 8, levels of inflammatory markers i.e. TNF-α and Ikkβ in the islets of non-treated diabetic rats were higher when compared to non-diabetic rats. Administration of VVSAE or glibenclamide resulted in lower inflammatory marker level when compared to non-treated diabetic rats.

In Fig. 9, high caspase-3 level was observed in the islets of non-treated diabetic rats. Treatment with 250 mg/day VVSAE resulted in lower caspase-3 level when compared to non-treated diabetic rats (p < 0.05). Caspase-3 level was even lower in diabetic rat islets treated with 500 mg/kg VVSAE or glibenclamide when compared to diabetic rats receiving 250 mg/day VVSAE.

In Fig. 10, GLUT-2 expression level in the islets of non-treated diabetic rats was lower when compared to non-diabetic rats (p < 0.05). In diabetic rats, higher GLUT-2 expression level was observed following treatment with 250 mg/day or 500 mg/kg VVSAE or glibenclamide (p < 0.05 when compared to non-diabetic rats).

3.8. Sod-1, cat, gpx-1, rage, tnf-α, ikk-β, caspase-3 and Glut-2 mRNA levels in pancreas

In Fig. 11A & B, levels of Sod-1, Cat and Gpx-1 mRNA in diabetic rats were lower while levels of Rage mRNA were higher when compared to non-diabetic rats (p < 0.05). In diabetic rats, treatment with VVSAE resulted in higher Sod-1, Cat and Gpx-1 mRNA levels when compared to non-treated diabetic rats (p < 0.05). However, in diabetic rats, lower Rage mRNA levels were observed when treated with VVSAE when compared to non-treated diabetic rats (p < 0.05). In diabetic rats receiving VVSAE or glibenclamide, Tnf-α, Ikk-β and caspase-3 mRNA levels were lower when compared to non-treated diabetic rats (p < 0.05). Glut-2 mRNA levels were lower in diabetic rats when compared to non-diabetic rats (p < 0.05). However in diabetic rats, higher Glut-2 mRNA levels were observed following treatment with VVSAE or glibenclamide.

4. Discussion

In this study, we have shown that aqueous seed extract of V. vinifera (VVSAE) was able to preserve near normal pancreatic function and ameliorated pancreatic oxidative stress, inflammation and apoptosis in diabetic rat model. Administration of VVSAE to diabetic rats could help to preserve near normal plasma insulin level which could account for near normal serum FBG, HbA1c and lipid profile levels in diabetes. We have shown that administration of VVSAE was able to ameliorate pancreatic destruction as evidence from near-normal histological appearance of the pancreas which include near normal pancreatic islets and β-cell numbers and appearances. We have shown that treatment with VVSAE preserved near normal expression level of Glut-2 that is involved in glucose-stimulated insulin release [30]. The presence of adequate Glut-2 could help to enhance glucose influx into the β-cells that is required to induce insulin secretion by the pancreas.

These observed effects of VVSAE could be due to maintenance of low level of oxidative stress, inflammation and apoptosis in the pancreas (almost similar to the level observed in non-diabetic rats). Ability of the extract to prevent elevation of oxidative stress in diabetes was supported by its high in-vitro anti-oxidant capability. The extract was found able to scavenge free radicals such as DPPH, superoxide, peroxide and hydroxyls. The levels of free radicals in cells were reported to increase in diabetes [31]. Further, our findings have shown that the extract was able to preserve near normal activity and expression levels of antioxidative enzymes namely SOD, CAT and GPs in the pancreas which could help to scavenge the free radicals. The levels of these enzymes were found to markedly decrease in diabetes [32]. Administration of VVSAE was also able to maintain RAGE expression level close to the level seen in non-diabetic rats. RAGE levels were found to increase in diabetes and this compound was responsible for many diabetic complications [33].

In addition to a decrease in the level of oxidative stress, VVSAE also help to decrease the level of inflammation and apoptosis in the pancreas in diabetes. High inflammation and apoptosis levels in diabetic pancreas could be due to increase in oxidative stress. These might be responsible for the massive destruction of the islets as seen in diabetes. Amelioration of inflammation and apoptosis in diabetic rat pancreas as observed following treatment with VVSAE could explain near normal histological appearance of this organ in diabetic rats treated with this extract.

The effect of VVSAE could be contributed by the major compounds in the extract which include formamide N-formyl-N-methyl, guanidine N,N-dimethyl, oxime methoxy-phenyl, seychellene, benzene 3-methylcyclopentyl, 9-octadecenamide, phenol, 2,5-bis(1,1-dimethylethyl) and octanal, 2-(phenylmethylene),
N-formyl-N-methyl was proposed able to counter oxidative stress [34]. However, biological effects of guanidine N,N-dimethyl has never been reported. Meanwhile, oxime methoxy-phenyl and seychellenone have been suggested to play a role as anti-oxidant [35,36]. Benzene 3-methylcyclopentyl was proposed to display anti-inflammatory properties [37] and 9-octadecenamide, phenol was reported to display anti-oxidant properties [37]. Meanwhile, 2,5-bis(1,1-dimethylthyl) was reported to display both anti-oxidant and anti-inflammatory activities [37] and octanal, 2-(phenylmethylene) was reported to possess anti-apoptotic activity in cancer cells [38].

In conclusions, this study has confirmed the protective role of VVSae against pancreatic dysfunction and elevation of oxidative stress, inflammation and apoptosis in the pancreas in diabetes. In view of these, aqueous seed extract of V. vinifera may potentially be used as a supplement or a drug to maintain near normal pancreatic function in a patient with diabetes mellitus.

**Conflict of interest**

The authors reported no conflict of interest in this study.

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