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Antioxidant, anti-alpha-glucosidase and pancreatic beta-cell protective effects of methanolic extract of *Ensete superbum* Cheesm seeds

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ABSTRACT

Objective: To investigate the antioxidant, anti-α-glucosidase and pancreatic β-cell protective potential of *Ensete superbum* (*E. superbum*) seeds.
Methods: A variety of *in vitro* assays including radical scavenging, reducing power potential, phenolic content determination, α-glucosidase assay and pancreatic β-cell (1.4E7 cells) viability were employed for assessing the effect of methanolic extract of *E. superbum* seeds.
Results: The radical scavenging and reducing power effects comparable with the standard rutin were obtained while the enzyme inhibitory activity of the extract was 68-fold better than the standard antidiabetic drug, acarbose. The seed extract of *E. superbum* was packed-full of polyphenols with mean percentage gallic acid equivalent value of (38.2 ± 1.8) (n = 3). The protection of pancreatic cells from massive onslaught of hydrogen peroxide was far superior to that obtained for rutin.
Conclusions: The reputed antidiabetic therapeutic uses of the seeds extract of *E. superbum* may be justified on the basis of inhibition of carbohydrate enzymes, antioxidant effects and pancreatic β-cell protection.

1. Introduction

Ensete superbum (*E. superbum*) is endemic to India where it is known to grow in most rocky mountainous edges and some barren lands of Western Ghats, north-eastern sub-Himalayan regions and some dry deciduous forests of Central and Western India. The plant is monocarpic, non-stoloniferous, unbranched herb that reproduces only through seeds with a life cycle of about four years. The comprehensive list of the ethnobotanical uses of the plant has been published by Vasundharan *et al.* [1] and includes the stem/pseudostem being widely used as food. The seeds are however by far the most exploited in the traditional medicine of India where they are popularly employed for treating diabetes, kidney stones, painful urination, urinary calci and leucorrhoea [2–4].

The antifertility activity of the alcoholic extract of *E. superbum* seeds has been established about half a century ago. At doses from 100 to 250 mg/kg body weight for the crude extract and doses as small as 2.5–5.0 mg/kg for the isolated active compound [4-(4-hydroxy-3-methyl-hex-5-enyl)-chroman-2,7-diol], anti-implantation activity has been effectively demonstrated [5,6]. With the suggested anti-ovulatory mechanism and very high lethal dose (3 235.9 mg/kg), the ethanol extract has been acclaimed as a potential natural antifertility drug [5,6]. There are also some reports showing the direct spermicidal effect of the seeds extract at a rather high doses (~100 mg/mL) [7] but perhaps the most interesting activity so far of therapeutic significance is inhibition of calcium hydrogen phosphate dihydrate crystals formation *in vitro* that might explain its uses for treating kidney stones, painful urination and urinary calci [8]. Although some preliminary studies on the

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antidiabetic properties of the plant have been reported [9,10], the possible mechanisms of action remains unknown. The present study was designed to investigate the antioxidant, anti-α-glucosidase and pancreatic β-cell protective potential of *E. superbum* seeds.

2. Materials and methods

2.1. Chemicals and reagents

Acarbose, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 5,5-dithiobis (2-nitrobenzoic acid), yeast α-glucosidase (from *Saccharomyces cerevisiae*, 0.5 IU/mL), Dulbecco's phosphate buffered saline, ferric chloride, Folin–Ciocalteu reagent, hydrogen peroxide (H_2O_2), *p*-nitrophenyl-α-D-glucopyranoside, potassium ferricyanide, rutin, sodium carbonate and all tissue culture media and supplements were products of Sigma (Sigma–Aldrich Chemical Company, Dorset, UK). Alamar Blue™ was a product of Serotec (Oxford, UK). High performance liquid chromatography (HPLC) solvents and other reagents were obtained from Fisher Scientific UK Ltd. (Loughborough, UK). All solvents and chemicals used in the experiments were of analytical reagent grades.

2.2. Extraction of the plant material

Dried seeds of *E. superbum* were purchased from Kerala Town Centre (India) herbal markets in September 2013 and authenticated in the Department of Botany, CMS College, Kottayam. The seeds were milled using domestic blender and the fine powders (500 g) were soaked in methanol (2.5 L) for 2 weeks. Removal of the solvent under reduced pressure using a rotary evaporator afforded 10.2 g of the extract residue.

2.3. HPLC analysis

An Agilent 1200 series gradient HPLC system composed of degasser (G1322A), quaternary pump (G1322A), auto sampler (G1329A), thermostat column compartment (G1316A) maintained at 25 °C and a diode array detector (G1315D) was used. Samples of plant extracts were injected (20 μL) into a reverse phase column (Agilent–Eclipse XDB-C18, 5 μm, 4.6 mm × 150 mm). The mobile phase was a mixture of water (A) and methanol (B). The composition of the mobile phase at a flow rate of 1 mL/min was rising from 10% to 90% methanol over a period of 50 min.

2.4. DPPH radical scavenging

The antioxidant activity of test samples was measured by using our established microtitre-based DPPH assay [11]. Briefly, the DPPH solution in methanol (0.1 mmol/L) was incubated with varying concentrations of test compounds for 20 min at room temperature. The bleaching effect was then assessed by measuring absorbance at 540 nm against a blank using Multiscan EX Reader (Thermo Labsystems, Altrincham, UK).

2.5. Total phenolic content determination

Our previously described method of total phenolic content determination was employed [12]. To the various concentrations of test samples in Dulbecco's phosphate buffered saline (1 mL), 1 mL of Folin–Ciocalteu reagent (previously diluted 10-fold with deionised water) was added. The reaction mixture was allowed to stand at room temperature for 5 min following which 1 mL of 7.5% (w/v, in deionised water) sodium carbonate was added. After further incubation at room temperature for 1 h, absorbance at 765 nm was read using a UV–vis spectrophotometer. The standard calibration curve was plotted using gallic acid (0.2–125.0 μg/mL) from which total phenolic content was expressed as percentage (w/w) gallic acid equivalent of extract.

2.6. Measurement of reducing power

The reducing power of test agents was quantified by using our previously published method [13]. Briefly, 3 mL of the reaction mixture, containing different concentrations of samples in Dulbecco's phosphate buffer saline (pH 7.0) was incubated with potassium ferricyanide (1%, w/v) at 50 °C for 20 min. Following termination of the reaction by trichloroacetic acid solution (10%, w/v), ferric chloride (0.1%, w/v) was added. The absorbance of the diluted (in deionised water) samples was then read at 700 nm. An increase in absorbance of the reaction mixture suggested a greater reducing power.

2.7. α-Glucosidase inhibition assay

Our published microtiter-based assay was employed [14]. The reaction mixture in a total volume of 100 μL in 250 mmol/L phosphate buffer (pH 6.8) contained 2.5 mmol/L *p*-nitrophenyl-α-D-glucopyranoside, experimental drugs and 1.2 IU/mL α-glucosidase. After incubation at 37 °C for 10 min, 25 μL of 0.2 mol/L sodium carbonate solution was added to each well to stop the reaction. The 4-nitrophenol absorption was measured at 405 nm using Multiskan plate reader (Thermo Labsystems, UK).

2.8. Cytoprotective assay

The human pancreatic β-cell line, 1.4E7, of European Collection of Cell Cultures origin was obtained from the Public Health England (Porton Down, Salisbury, UK). Cells were routinely maintained in RPMI medium supplemented with 10% (v/v) foetal bovine serum and 100 IU/mL penicillin, 100 μg/mL streptomycin in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. For the cytoprotection assay, cells were seeded in 96-well plates at a density of 10^5 cells per well and allowed to grow until confluence by incubating plates at 37 °C, 5% CO_2. Cultures were treated with various concentrations of the test agents in the presence or absence of H_2O_2. Drugs were normally added 30 min prior to H_2O_2 treatment and left incubated with the cells for either 24 or 48 h. After the indicated period of incubation, Alamar Blue™ was added and cell viability was assessed by measuring fluorescence [13].

2.9. Statistical analysis

All data are presented as mean ± SEM values from a minimum of four replicates. Where appropriate, the significance of difference between two means was analysed by using unpaired *t*-test. All experiments were repeated at least three times.

3. Results

3.1. Antioxidant effect of E. superbum seeds

As shown in Figure 1, the crude extract showed a concentration-dependent potent DPPH radical scavenging effect with an IC_{50} value of (6.2 ± 0.3) μg/mL (n = 4). Both Figure 1 and the IC_{50} value data [(5.5 ± 0.6) μg/mL, n = 4] showed that the radical scavenging activity of the standard control, rutin, was comparable with *E. superbum* seeds extract (no significant difference, $P > 0.05$, between the two groups).

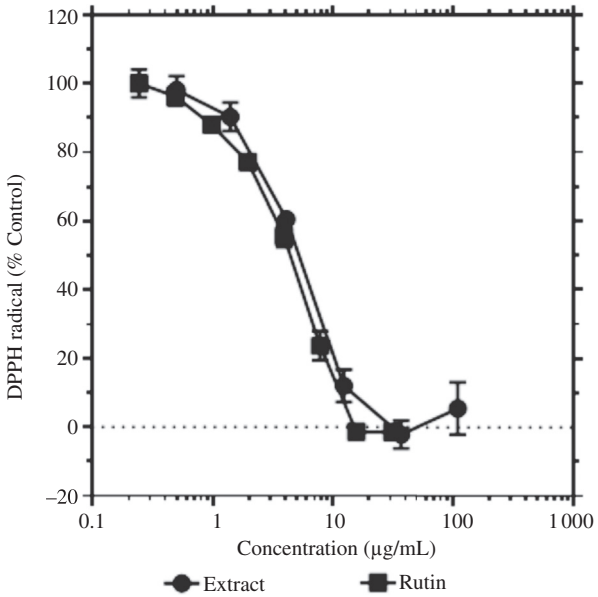

Figure 1. Antioxidant activity of the methanolic extracts of *E. superbum* seeds and rutin.
Data are mean ± SEM values (n = 4).

3.2. HPLC profiling of E. superbum seeds extract

The HPLC chromatogram of the crude extract (Figure 2) appears to show numerous constituents within the full range of the analysis time suggesting it being a mixture of several dozens of metabolites.

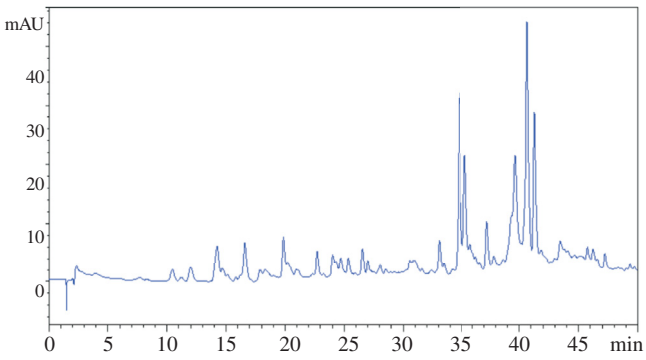

Figure 2. HPLC profile of the crude extract of *E. superbum* seeds.

3.3. Total phenolic content of E. superbum seeds extract

The Folin–Ciocalteu reagent assay-based standard curve obtained for gallic acid gave a straight line equation with r^2 value of 0.999 8. On this basis, the extract was found to contain a mean gallic acid equivalent of (38.2 ± 1.8)% (n = 3).

3.4. Reducing power of E. superbum seeds extract

Both the extract and rutin appeared to exhibit reducing power potential at relatively higher concentrations and their effect by and large appeared to be comparable (Figure 3).

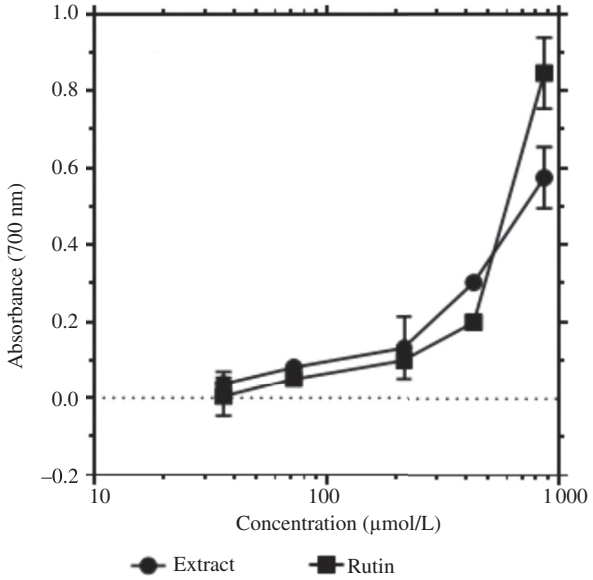

Figure 3. Reducing power of ethanolic extract of *E. superbum* seeds and rutin based on measurement of Fe^{3+}–Fe^{2+} transformation.
Data are expressed as mean ± SEM (n = 4).

3.5. Pancreatic β-cell protective effect of E. superbum seeds extract

While the antioxidant rutin failed to protect pancreatic β-cells from a massive onslaught of H_2O_2 toxicity induced by 5 mmol/L concentrations (data not shown), it displayed some protective effects against 1 mmol/L H_2O_2 (Table 1). This protective effect was also evident even when cells were exposed to H_2O_2 for 48 h though the effect evidently reduced during long exposure to H_2O_2 (Table 1). In comparison to rutin, the cytoprotective potential of *E. superbum* extract was evident even when cells were exposed to 5 mmol/L H_2O_2. As shown in Table 1, a concentration-dependent protective effect was demonstrated at both (24 and 48 h) time points.

Table 1
Protection of cultured human pancreatic β-cells (1.4E7) from hydrogen peroxide toxicity.

Treatment (μg/mL)	Rutin + 1 mmol/L H_2O_2[a]		Extract + 5 mmol/L H_2O_2	
	24 h	48 h	24 h	48 h
0.0	24.5 ± 0.8	11.8 ± 3.1	0.6 ± 3.4	1.3 ± 6.9
7.4	22.7 ± 4.5	10.4 ± 2.5	0.0 ± 0.6	–0.4 ± 1.5
22.2	26.7 ± 5.2	11.0 ± 1.8	9.0 ± 2.3	10.3 ± 3.4
66.7	40.2 ± 4.0	4.0 ± 0.9	27.4 ± 2.5	56.4 ± 2.8
200.0	53.0 ± 5.5	23.9 ± 2.1	25.2 ± 0.8	58.3 ± 4.6

[a]: Rutin failed to show protection when 5 mmol/L H_2O_2 was used. Drugs (rutin or *E. superbum* seeds extract) were added 30 min prior to H_2O_2 treatment and left incubated with the cells for either 24 or 48 h.

3.6. Effects of *E. superbum* seeds extract on α-glucosidase activity

The comparative enzyme inhibitory activity of the crude extract in comparison with the known antidiabetic drug, acarbose, is shown in Figure 4. The IC_{50} values obtained for acarbose and the extract in this assay were (188.3 ± 24.7) μmol/L, or (121.5 ± 15.9) μg/mL and (1.8 ± 0.1) μg/mL (n = 3) respectively.

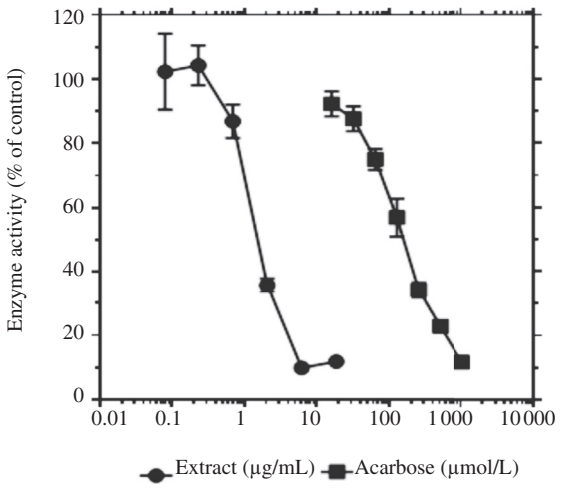

Figure 4. Anti-α-glucosidase activity of the crude methanolic extracts of *E. superbum* seeds and acarbose.
Data are expressed as mean ± SEM (n = 4).

4. Discussion

Reactive oxygen species (ROS) generated under normal physiological conditions are known to be involved in several cell signalling process that are considered vital for maintaining cellular homeostasis. When ROS are generated at excessively high amount (*e.g.* by toxicants, drugs and infections) or the body's antioxidant system is inadequate, a state of oxidative stress is initiated. On the basis that oxidative stress is now recognised to play central role in the pathogenesis of various disease conditions, including neurodegenerative diseases and diabetes, the role of antioxidant therapy has been scrutinised in recent years [12,15,16]. Not surprisingly, the antioxidant rutin has been demonstrated to display remarkable biological effect both *in vitro* and *in vivo* through antioxidant mechanisms [15,16]. In the present study, the antioxidant activity of the crude methanolic extract of *E. superbum* seeds was first assessed using the standard DPPH assay with the well-known natural antioxidant, rutin [13,17,18], as a positive control. Although the HPLC profiling showed that the seeds extract is a crude mixture of dozens of constituents, its radical scavenging activity was comparable with the standard control, rutin. The presence of a highly active therapeutically useful antioxidant compound(s) that explain some of the observed therapeutic claims could thus be present in the seeds. Given the potent antioxidant activity of the crude extract, it was worthwhile to analyse the total phenolic content that could contribute to the observed antioxidant activity. The Folin–Ciocalteu reagent assay indicated an incredibly high polyphenolic value in the extract. As evidenced from our previous studies [13,19], the antioxidant effect of many compounds could be in part attributed to their reducing power. Hence, the reductive capacity of the crude extract was also assessed by measuring Fe^{3+}–Fe^{2+} transformation in the presence or absence of test agents. The demonstrated concentration-dependent reducing power effect, which by and large was comparable with the standard rutin, was a further demonstration of the potent antioxidant potential of the *E. superbum* seeds extract. The fact that the crude extract happens to display antioxidant activity comparable with rutin in a variety of assays confirms its therapeutic potential for a range of disease conditions.

Hydrogen peroxide is often employed to assess the direct cytotoxicity of ROS in mammalian cells and identification of potential antioxidant compounds. The effect of the antioxidant rutin on the protection of pancreatic β-cells from a massive onslaught of H_2O_2 toxicity was in agreement with previous reports from our laboratories [20]. Incredibly, the protective effect of *E. superbum* seeds extract was far superior to the positive control, rutin.

In order to see whether the claimed antidiabetic effect of the seeds extract of *E. superbum* was mediated via inhibition of key carbohydrate digestive enzymes, an α-glucoside inhibition assay was also employed in our study. The observed activity for the standard positive control was consistent with our previous reports [14,18,21,22]. The seeds extract of *E. superbum* showed in this assay a remarkable level of enzyme inhibition potency with about 68-fold better activity than the standard antidiabetic drug, acarbose. Our data therefore provided the scientific basis for the claimed use of *E. superbum* seeds for treating diabetes. Moreover, the potential antidiabetic effect of the seeds extract could be in part due to direct inhibition of carbohydrate digesting enzymes in the gut.

The loss of pancreatic β-cells in diabetes is linked to ROS-mediated toxicity and prevalent both in type-1 and type-2 diabetes. Protection of β-cells death by compounds like rutin is now thus considered to be one of the many therapeutic approaches employed in reversing and/or reducing the deterioration of diabetes pathology [12,23]. A protective effect by *E. superbum* extract far better than rutin must therefore be considered as a significant milestone to justify the use of the seeds in diabetes treatment. Overall, these data together with the potent α-glucosidase inhibitory activity of the extract implies the possible therapeutically relevant compounds in *E. superbum* seeds that target diabetes and other oxidative stress-associated disease through multiple mechanisms.

In conclusion, the crude methanolic extract of seeds of *E. superbum* showed potent antioxidant, anti-α-glucosidase and β-cell protective activity *in vitro*. Given the high phenol content of the extract and numerous constituents detected by HPLC, further studies on the identification of the active principles are well warranted.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

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