An in vitro study reveals nutraceutical properties of Ananas comosus (L.) Merr. var. Mauritius fruit residue beneficial to diabetes

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Abstract

BACKGROUND: Rapid urbanisation and nutritional transition is fuelling the increased global incidence of type 2 diabetes. Pineapple fruit residue was explored for its nutraceutical properties as an alternative or adjunct to currently available treatment regime. Ethyl acetate and methanolic extracts of pineapple fruit residue were evaluated for anti-diabetic activity in cell free and cell based systems. Specifically, we assessed: (1) antioxidant potential, (2) anti-glycation potential, (3) carbohydrate digestive enzyme inhibition, and (4) lipid accumulation and glycerol-3-phosphate dehydrogenase activity in differentiating 3T3-L1 cells.

RESULTS: The active components in the ethyl acetate and methanolic extracts were identified as sinapic acid, daucosterol, 2-methylpropanoate, 2,5-dimethyl-4-hydroxy-3(2H)-furanone, methyl 2-methylbutanoate and triterpenoid ergosterol using DART/HRMS and ESI/HRMS. Micronutrient analysis revealed the presence of magnesium, potassium and calcium. Adipogenic potential, anti-glycation property of the ethyl acetate extract, and DNA damage protection capacity of the methanolic extract are promising.

CONCLUSION: Results from this study clearly indicate that pineapple fruit residue could be utilised as a nutraceutical against diabetes and related complications.

Supporting information may be found in the online version of this article.

Keywords: pineapple fruit residue; Ananas comosus (L.) Merr. var. Mauritius; bromeliaceae; antioxidant; anti-glycation; α-glucosidase; adipogenesis

INTRODUCTION

Epidemiological studies indicate that a diet rich in fruits and vegetables impart significant health benefits.1 The peels of fruits such as apples, peaches2 and star fruits3 have been found to contain higher amounts of phenolics than the edible fleshy parts. Thus, scientific processing of under-utilised fruit waste is important in the development of functional food/nutraceutical.

The global epidemic of type 2 diabetes is increasing unabated in Asian populations.5 Genetic predispositions with rapid changes in dietary patterns significantly contribute to the early onset. It is estimated that 1 in 20 deaths worldwide is attributed to diabetes and related complications.5 Initial mild postprandial hyperglycaemia due to alterations in carbohydrate and lipid metabolism, impairs immediate beta cell response, resulting in sustained hyperglycaemia. A major consequence of hyperglycaemia is excessive non-enzymatic glycosylation of proteins, lipids or nucleic acids, referred to as advanced glycation endproducts accompanied by increased free radical activity. They form stable cross-links with other peptides, permanently altering their structure and function.6,7

Pineapple [Ananas comosus (L.) Merr. var. Mauritius], an edible fruit native to Central and South America is grown in several tropical and subtropical countries including India. The total pineapple production worldwide is 16 to 18 million tons.8 Ethanolic extract of pineapple leaves have been explored for their anti-diabetic and dyslipidaemia properties.9,10 Residual fibre and skin which...
constitutes approximately 30–35% of pineapple fruit are usually discarded as low-value by-products. The peel and residual pulp of pineapple fruit are collectively referred to as pineapple fruit residue. Valuable phytochemicals have been previously reported from pineapple fruit residue.11 However, there has been no systematic evaluation of its nutraceutical property relevant to diabetes. In the present study, the effects of pineapple ethyl acetate extract (PAE) and pineapple methanol extract (PAM) have been systematically evaluated for their anti-diabetic potential against key targets such as carbohydrate digestive enzyme inhibitor, DNA damage protection, anti-glycation activity and glitazone-like property.

MATERIALS AND METHODS

Materials

4-Nitro-phenyl-α-D-glucopyranoside, dinitro-saliclycic acid, ellagic acid (≥95%), α-glucosidase, α-amylase, acarbose (≥95%), quercetin (≥95%), Folin–Ciocalteu reagent, isobutyl-3-methylxanthine, dexamethasone, insulin, and rosiglitazone were from Sigma (St Louis, MO, USA). Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS) and penicillin–streptomycin were from Invitrogen (Carlsbad, CA, USA). One gram of pineapple residue was digested in 10 mL dilute hydrochloric acid and filtered. The concentration of various minerals was quantified by atomic absorption spectrophotometry (AAS; Perkin Elmer A Analyst 100; Perkin Elmer, MA, USA) using a hollow cathode lamp.

Determination of total phenolic content

Total phenolic content of PAE and PAM was determined as described previously.14 Briefly, to 100 μL extracts, 500 μL of Folin–Ciocalteu and 1 mL of Na2CO3 (20%) were added and incubated at ambient temperature of 30°C for 90 min. The colour developed was measured at 760 nm using a UV–visible spectrophotometer (UV-2450PC; Shimadzu, Kyoto, Japan). Results were expressed as milligrams gallic acid equivalents per gram of extract (mg GAE g⁻¹).

Analysis of minerals by atomic absorption spectrophotometry

One gram of pineapple residue was digested in 10 mL dilute hydrochloric acid and filtered. The concentration of various minerals was determined by atomic absorption spectrophotometry.

Determination of antioxidant potential

The total antioxidant activity assay is based on a previous report.15 Three hundred microlitres of the extract at various concentrations in distilled water was mixed with 3 mL of reagent solution (0.6 mol L⁻¹ sulfuric acid, 28 mmol L⁻¹ sodium phosphate and 4 mmol L⁻¹ ammonium molybdate) and incubated at 95°C for 90 min. After the mixture had cooled to room temperature, the absorbance of the solution was measured at 695 nm against the blank. The antioxidant activity was expressed as the milligram equivalents of ascorbic acid per gram of extract. The antioxidant activity of extracts was assessed by 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging activity using the Zen-Bio ABTS assay kit (Zen Bio Inc, NC, USA). The IC₅₀ value was calculated and results were compared with standard Trolox.

DNA damage protection assay

The hydroxyl radical-induced DNA damage in plasmid pUC18 as described previously16 was performed with slight modifications. Ellagic acid (5 ng and 50 ng) was used as a positive control. The level of DNA damage was expressed as % DNA in OC form = [OC density/(OC density + SC density)] × 100%. The higher the OC percentage, the greater will be the DNA damage. (OC and SC denote open circular and supercoiled forms of plasmid pUC18.)

Determination of anti-glycation activity

Bovine serum albumin (BSA) derived advanced glycation endproducts were measured as per previous report with slight modifications.17 The fluorescence (λ_em = 370 nm; λ_ex = 440 nm) of the advanced glycation endproducts was measured using a Biotek microplate reader after 24 h. BSA (25 mg mL⁻¹) in the presence of ribose (500 mmol L⁻¹) in phosphate buffered saline (PBS) was used as control. The data was compared with the reference compound, quercetin (100 μmol L⁻¹). In addition,
Samples were also processed for scanning electron microscopy (SEM) following a slightly modified method reported previously. For this, the sample was sputter coated with gold on a Polaron SC 7620 sputtering machine fitted with gold–palladium target for a duration of 270 s at 10 mA current. It was then analysed on an EVO 18 special edition model of a Carl Zeiss SEM (Carl Zeiss, Munich, Germany) with an accelerating voltage ranging from 12 kV to 20 kV. For comparing the complexity of the microstructures, all samples were visualised at a magnification of 100 000.

α-Glucosidase and α-amylase inhibition assay
α-Glucosidase (EC 3.2.1.20) and α-amylase (EC 3.2.1.1) activity were determined spectrophotometrically at 405 nm and 540 nm, respectively. The percentage inhibition was plotted against the corresponding concentration of the extract to obtain the IC50 value. Results were compared with the standard drug acarbose.

Cell culture and treatment
Mouse 3T3-L1 fibroblast cells were purchased from the National Centre for Cell Sciences, Pune, India. 3T3-L1 cells were cultured in DMEM containing 25 mmol L−1 glucose supplemented with 10% FBS, 50 U mL−1 penicillin and 50 µg mL−1 streptomycin. Cultures were maintained at 37 °C in 5% CO2 incubator. At 70% confluence, cells were trypsinised and seeded in 24-well plates. 3T3-L1 pre-adipocytes were used within 10 passage numbers to limit batch-to-batch variations.

Studies on adipogenesis
Cells were seeded in 24-well plates at a density of 4 x 104 cells well−1 in 24-well plates and incubated for 24 h. Cells were then treated with various concentrations (10, 50, 100 and 200 µg mL−1) of PAM and PAE. Forty-eight hours after incubation, the cell viability was evaluated using an MTT assay kit (Cayman Chemical, MI, USA).

Glycerol-3-phosphate dehydrogenase activity
3T3-L1 adipocytes that were treated as described above, were washed twice with PBS and then harvested into 25 mmol L−1 Tris buffer (pH 7.5) containing 1 mmol L−1 EDTA and 1 mmol L−1 dithiothreitol (DTT). The cells were disrupted by sonication, centrifuged at 12 000 × g for 20 min at 4 °C. The supernatants were assayed for GPDH (EC 1.1.1.8) using a Takara GPDH Assay Kit (Takara Bio Inc, Otsu, Japan) and protein content was measured using a bicinchoninic acid kit (Pierce, Rockford, IL USA).

Flow cytometry analyses
The differentiation process was studied by a simple and sensitive flow cytometry method based on the method of Lee et al., which was slightly modified for the purpose of our work. Briefly, cells were trypsinised, incubated for 5 min at 37 °C, resuspended in PBS and kept on ice until analysis by fluorescence activated cell sorting (FACS) in a BD FACS Aria™ II (Becton Dickinson, CA, USA). The dot plot obtained was gated into four regions (I–IV) based on forward scatter and side scatter, which reflects the cell size and granularity, respectively. This directly correlates with the amount of lipid accumulation within the cells. Differentiated cells may be classified into three groups: (1) cells with lesser intracellular granularity containing fewer lipid droplets, (2) cells with intermediate granularity with medium sized lipid droplets, and (3) cells with greatest intracellular granularity containing largest lipid droplets. Region I (P1) contains cells in with a granularity similar to that of less differentiated cells whereas region IV (P4) contains cells with the greatest granularity because of the biggest droplets and highest lipid content. Regions II (P2) and III (P3) contain cells that exhibit intermediate granularity.

The results are expressed as the percentage of the cell population in each gated region and displayed in the form of a stacked column.

Statistical analysis
Data are presented as mean ± SD, n = 6, from three independent experiments with replicates. The results were analysed using a statistical program SPSS/PC+, version 11.0 (SPSS Inc., Chicago, IL, USA). One-way ANOVA was employed for comparisons between the groups. Pair-fed comparisons between the groups were made by Duncan’s multiple range tests. P < 0.05 was considered to be significant.

RESULTS
DART MS of PAE (Supplementary Fig. 1) showed a major peak at m/z = 89, illustrating the [M + H]+ peak for 2-methylpropanoate. The fraction also gave peaks at m/z = 127, corresponding to the [M + H]+ peak of 2,5-dimethyl-4-hydroxy-3(2H)-furaneone, and at m/z = 116, corresponding to the M− ion peak of methyl 2-methylbutanoate. All of them are reported as aroma-causing constituents of pineapple. The m/z at 397 may be due to the [M + H]+ peak corresponding to the triterpenoid ergosterol.

DART MS of PAM (Supplementary Fig. 2) also showed almost the same pattern of the spectra. The m/z at 180 may be due to the [M + H]+ peak of sugars such as fructose and galactose. ESI mass spectra of PAE, recorded on an LCQ Advantage MAX (ESI) spectrometer, showed the presence of sinapic acid at m/z = 224.9 (M+ peak). The identification of the compound from the spectrum was based on previously published literature. The fraction also gave major peaks having m/z values at 757 (may be the [M + H]+ peak for daucosterol) (Supplementary Fig. 3).
that differ significantly from the positive control group, i.e. L2 (Fig. 1), comparable with the standard Trolox (5 ± 0.98 μg mL⁻¹). We have also evaluated the DNA damage protection efficacy of the extracts using the pUC18 plasmid. PAM at 200 and 500 μg mL⁻¹ exhibited significant protection of super-coiled pUC18 from the harmful effects of DNA damage induced by Fenton’s reagent, lanes 3 and 4: positive control (plasmid + Fenton’s reagent), lanes 5 and 6: PAM (plasmid + Fenton’s reagent + PAM, 200 μg and 500 μg, respectively). The images are representative of six observations of replicate experiments. Magnification is × 16 000.

The antioxidant potential of PAE and PAM were assessed in terms of relative fluorescence units (RFU). The antioxidant potential of PAE and PAM were assessed in terms of total antioxidant capacity and ABTS cation decolorisation activity (IC₅₀ = 26.36 ± 0.62 μg mL⁻¹). The IC₅₀ value of Trolox was 5 ± 0.98 μg mL⁻¹. Values are means ± SD; n = 6. *Represents groups differ significantly from the control group (P < 0.05).

Anti-glycation. (A) Anti-glycation property of various concentrations of pineapple ethyl acetate extract (PAE; 100, 250, 500 and 1000 μg mL⁻¹). Quercetin (100 μmol L⁻¹) was used as the reference compound. Relative fluorescence units (RFU) are normalised to 100. Values are means ± SD; n = 6. *Represents groups differ significantly from the control group (P < 0.05). (B) SEM microstructures of glycated products formed under various conditions. Control (panel a) and 50 treated groups (panels b–d; quercetin 100 μmol L⁻¹, PAE 100 μg mL⁻¹, and PAE 1000 μg mL⁻¹ respectively). The images are representative of six observations of replicate experiments. Magnification is × 16 000.
DISCUSSION

Natural products represent an alternative source of anti-diabetic medication and complement the limited number of anti-diabetic drugs currently on the market. Currently available drugs are essentially limited to the sulfonylureas, the biguanides and the thiazolidinediones. Long-term treatment with some of these drugs is fraught with adverse effects such as weight gain, feeling of fullness and cardiovascular disorders. Plant-derived agents have been used since time immemorial by indigenous physicians to treat a variety of diseases including diabetes. In the present study, we have evaluated the anti-diabetic potential of pineapple fruit residue using an in vitro model.

The chemical composition of the fractions showed the presence of sinapic acid, daucosterol and ergosterol, which are reported to have various types of therapeutic properties, such as immunomodulatory, anti-hyperglycaemic, antifungal etc. Minerals such as sodium, potassium, calcium, magnesium, chromium and vanadium are reported to have a protective property against diabetes. AAS analysis of the fruit residue showed the presence of moderate amounts of magnesium, calcium and potassium, and traces of manganese, sodium and zinc.

Studies have shown that depletion of antioxidant status in patients with type 2 diabetes mellitus is responsible for the early onset of secondary complications. Supplementation with antioxidants offers protection against oxidative stress and free radical damage. PAE and PAM exhibited promising total antioxidant potential. PAE showed significant ABTS radical decolourisation potential (Fig. 1). Significant DNA damage protection efficacy was observed for PAM (Fig. 2A and B).

Preventing the formation of advanced glycation endproducts is another attractive strategy to prevent the early onset of diabetic complications. We have obtained a significant difference in anti-glycation potential only for two doses, i.e. for 100 and 1000 µg mL⁻¹. This may be due to the heterogeneous composition of PAE. SEM has been variously employed to examine the microstructure of food. Here, we have employed SEM microstructure analysis (Fig. 3B, panels a–d) to complement the fluorescence data (Fig. 3A).

Inhibition of digestive enzymes is a therapeutic approach to decrease the postprandial rise in blood glucose and delays the early onset of secondary complications. We found that PAE is a promising inhibitor of α-glucosidase (Fig. 4), comparable with acarbose. The excessive inhibition of α-amylose could result in the abnormal fermentation of undigested starch in the intestine and, therefore, low α-amylose inhibitory activity is useful. In our study both fractions did not show any α-amylose inhibitory potential.

During the process of differentiation, increase in lipid accumulation and GPDH activity, a key enzyme in lipogenesis is observed. In this study, we used three criteria for adipocyte conversion: (1) morphological changes i.e. formation of visible lipid droplets; (2) increase in GPDH activity; and (3) TG accumulation. Phase contrast microscopy images clearly display lipid accumulation in differentiating 3T3-L1 adipocytes treated with various concentrations of PAE (Fig. 5, panels d–f) and 100 nmol L⁻¹ rosiglitazone (Fig. 5, panel c). A small percentage cells in vehicle control also showed lipid accumulation (Fig. 5, panel b). An increase in GPDH is observed for rosiglitazone and all three doses of PAE treatment (Fig. 6A). However, the increase in TG is significant for rosiglitazone and PAE at 100 µg mL⁻¹ (Fig. 6B). This implies that a maximal increase in GPDH activity is required for TG accumulation.

Figure 4. α-Glucosidase inhibitory potential of various concentrations (50–250 µg mL⁻¹) of pineapple ethyl acetate extract (PAE; IC₅₀ = 85.26 ± 1.35 µg mL⁻¹). The IC₅₀ value of the standard drug acarbose is 45 ± 1.21 µg mL⁻¹. The values are means ± SD; n = 6.
Figure 5. Cellular morphology. (Panels a–f) Micrographs (×10) showing (a) pre-adipocytes, (b) vehicle control, (c) lipid accumulation in differentiating 3T3-L1 adipocytes treated for 8 days with rosiglitazone (100 nmol L\(^{-1}\)), and (d–f) various concentrations of PAE (10, 50 and 100 µg mL\(^{-1}\), respectively). DMSO (0.1%, vehicle) in differentiation media served as the vehicle control.

Figure 6. Glycerol-3-phosphate dehydrogenase (GPDH) activity and triglyceride content. (A) Glycerol-3-phosphate dehydrogenase activity in various groups (the vehicle control was rosiglitazone at 100 nmol L\(^{-1}\)) and various concentrations of pineapple ethyl acetate extract (PAE) treatment (10, 50, 100 µg mL\(^{-1}\)). Data are expressed as the means ± SD; \(n=6\). *Represents groups that differ significantly from the vehicle control group (\(P \leq 0.05\)). (B) The triglyceride content in various groups (the vehicle control was rosiglitazone at 100 nmol L\(^{-1}\)) and various concentrations of PAE treatment (10, 50, 100 µg mL\(^{-1}\)). Data are expressed as the means ± SD; \(n=6\). *Represents groups that differ significantly from the vehicle control group (\(P \leq 0.05\)).

In this study we have further analysed the adipogenic potential of PAE using flow cytometry. The flow cytometry analysis reveals four populations of cells based on size and granularity, i.e. P1, P2, P3 and P4. Rosiglitazone (100 nmol L\(^{-1}\)) and extract-treated groups (50 µg mL\(^{-1}\) and 100 µg mL\(^{-1}\)) exhibit more small cells with less granularity (P1 population; Fig. 7A and 7B panels b, d and e) as observed in the case of thiazolidinedione group of drugs which are reported to induce differentiation of more small adipocytes.\(^{36,39}\) It is observed that there are fewer large adipocytes in the P4 population of 50 and 100 µg mL\(^{-1}\) (Fig. 7A and 7B panels b and d).

The anti-diabetic property evident from in vitro assays could be an individual or synergistic effect of these compounds. The results of the present study reveal nutraceutical properties of pineapple fruit residue. However, these promising in vitro results must be ascertained using in vivo models.

ACKNOWLEDGEMENTS
M.P. Riya and K.A. Antu acknowledge the Council of Scientific and Industrial Research and Indian Council of Medical Research, respectively, for the financial assistance as research fellowships. The partial financial assistance from CSIR 12th five year plan project “UNDO” is also acknowledged. We thank the Director, CSIR-NIIST, Thiruvananthapuram and Dr A. Sundaresan, Head, Agroprocessing and Natural Products Division, for providing the necessary laboratory facilities. We thank Dr N.S. Pradeep, Senior Scientist, Jawaharlal Nehru Tropical Botanical Garden Research Institute (JNTBGRI), Palode, Thiruvananthapuram, for the valuable advice regarding the collection of plant material. We also thank Dr G. Valsala, Curator, Department of Botany, University of Kerala, for identification of the plant material. Special thanks to the members of the Biotechnology Division for assisting in some of the studies.
**Figure 7.** Effect of pineapple ethyl acetate extract (PAE) on adipocyte differentiation as assessed by flow cytometry. (A) Statistical stacked column graph of the flow cytometric data. Data are means ± SD; n = 6. (B) The dot plot of cytometric forward scatter (FSC) and side scatter (SSC) was gated into four regions (P1, P2, P3, P4) based on their size and granularity distribution. The various groups are: (panel a) vehicle control, (panel b) rosiglitazone (100 nmol L\(^{-1}\)), and (panels c, d and e) various concentrations of PAE treatment (10, 50, 100 µg mL\(^{-1}\)).

**SUPPORTING INFORMATION**

Supporting information may be found in the online version of this article.

**REFERENCES**