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Diospyros lotus L. fruit: A potential antidiabetic functional food targeting intestinal starch hydrolysis

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Abstract

Post-meal hyperglycaemia is considered a prominent therapeutic target to attenuate the progression of diabetes and its associated complications. The present study identified fruit extract of *Diospyros lotus* Linnaeus, of the *Ebenaceae* family, as an inhibitor of starch digestion through the inhibition of both alpha amylase and alpha glucosidase. The extract inhibits porcine and human pancreatic amylase with IC_{50} values of 82.5 ± 2.0 and 130.4 ± 24 μg/ml respectively. The inhibition of intestinal sucrase and maltase activity was however considerably weaker. *In vitro* hydrolysis of solubilised potato starch into glucose yielded comparable inhibition kinetics for 100 μg/ml *D. lotus* L. extract and 3.5 μM acarbose. Screening the major phenolic constituents revealed that quercetin and myricetin were the strongest alpha amylase inhibitors. *D. lotus* L. extract showed strong antioxidant activity; however, this provided no meaningful protection against 2-deoxy-ribose induced oxidative stress in INS-1 cells. Taken together these findings identify *D. lotus* L. fruit as a multi-component functional food with potential to dampen the onset and development of diabetes through the inhibition of post meal hyperglycaemia.

Keywords: *Diospyros lotus*; diabetes; starch digestion; alpha-amylase; alpha-glucosidase; functional food

1. Introduction

The prevalence of Type 2 diabetes has steadily increased over the past few decades and predictions are that it will become a serious economic burden to many healthcare systems in the not too distant future [1]. Excess calorie intake, accompanied with a sedentary lifestyle, is a key factor leading to the development of diabetes and its associated complications [2].

Functional foods have been suggested as a potential intervention to limit the development of many lifestyle diseases, including diabetes [3]. Functional foods carry value not only in the management of established diseases, but also as a preventative approach to target high risk individuals prior to the development of overt symptoms and subsequent dependence on pharmacological mediation [4]. Clinical studies, although sometimes controversial, provide acceptance that functional foods have potential as a complementary approach to lessen the burden of metabolic diseases like diabetes [5]. Identification of their therapeutic mechanisms is thus an important initial step to justify integrating functional foods into healthcare programs.

Fasting hyperglycaemia is a conclusive feature of advanced diabetes and a recognised therapeutic target [6]. The sharp increase in blood glucose levels following a carbohydrate rich meal represents a major challenge for diabetic patients to manage hyperglycaemia. Furthermore, post-meal hyperglycaemic spiking is strongly implicated as an early causal factor in the initiation and progression of Type 2 diabetes [7, 8]. Controlling post-meal hyperglycaemia therefore represents a wide-ranging approach to target both established diabetic, as well as pre-diabetic, patients and subsequently it may be anticipated that functional foods which diminish the extremities of post-meal glucose fluctuations can contribute to management and progression of diabetes [9]. Effective antidiabetic drugs such as acarbose and

miglitol, which target post prandial hyperglycaemia, reinforce the appropriateness of this approach.

Diospyros lotus Linnaeus, also known as the date plum, is found naturally in the Middle East and south Asia and is widely cultivated for its small edible fruits. *D. lotus* L. is part of the *Ebenaceae* family and in traditional medicine the plant is used in the treatment of several diseases including diabetes, however the precise antidiabetic mechanism(s) have not been identified. Previous studies have recommended that nutrient rich *D. lotus* L. fruits be considered a functional food based on characteristics such as a high antioxidant content [10] and its capacity to reduce fasting hyperglycaemia in streptozotocin (STZ) induced diabetes [11]. Here we provide further evidence to support the potential of *D. lotus* L. fruits as a functional food through its capacity to inhibit enzymes involved in starch digestion, a critical factor in post-meal hyperglycaemia, and an established antidiabetic target.

2. Materials and Methods

2.1. Materials

Porcine pancreatin, human pancreatic amylase, yeast alpha-glucosidase, p-nitrophenyl α-D-glucopyranoside, rat intestinal acetone powder, myricetin, gallic acid, methyl gallate, ellagic acid, quercetin, 2-deoxy-ribose, N-Acetyl cysteine (NAC), 3-(4,5-dimethiltiazole-2-yl)-2,5-diphenyltetrazolium (MTT), 2,2-diphenyl-1-picrylhydrazyl (DPPH), glucose oxidase and horse radish peroxidase were obtained from Merck. INS-1 cells were kindly donated by Professor Guy Rutter from the University of Bristol, England. Tissue culture medium (RPMI) and fetal bovine serum (FBS) were purchased from Lonza.

2.2. Plant Material

The ripe fruits of *D. lotus* L. were collected from the Agricultural Research Centre, Giza, Egypt and identified by Dr. Mohammed El-Gebaly, Department of Botany, National Research Centre (NRC) and by Mrs. Tereez Labib Consultant of Plant Taxonomy at the Ministry of Agriculture and director of Orman botanical garden, Giza, Egypt. A voucher specimen No. 2457 is deposited in the herbarium of Agricultural Research Centre, Giza, Egypt.

2.3. Extract preparation and identification of phenolic constituents

The ripe fruits of *D. lotus* L. (500 g) were extracted with 70% methanol several times until exhaustion. The extract was concentrated under reduced pressure and the dried extract (115 g) stored at 4°C. For *In vitro* assays the extract was reconstituted in DMSO and diluted in buffer or culture medium to the concentrations indicated. Identification of the major phenolic constituents was conducted as follows: 100 g of dried methanol extract was dissolved in 500 ml of distilled water and fractionated with N-hexane, dichloromethane and butanol, respectively. Butanol fraction (12.5 g) was subjected to polyamide column chromatography (300 g) and the column was eluted with H_2O to methanol gradually. Eighty-four fractions were collected. The fractions that showed similar Paper Chromatography (PC) in Butanol-Acetic Acid-Water 4:1:5 (BAW) and 15% acetic acid were combined to give 4 fractions (I, II, III, and IV). Fraction I (800 mg) was subjected to sephadex LH-20 column which was eluted with methanol (50%) to give compound 1 (ellagic acid). Fraction II (720 mg) was eluted with water:methanol (70:30) to give compound 2 (methyl gallate) and compound 3 (gallic acid). Fraction III (650 mg) was subjected to Preparative Paper Chromatography (PPC), two dark purple bands appeared under UV light, changed with ammonia to yellow colour, each band eluted with MeOH (80%) and then purified on sephadex column eluted with methanol (50%) to give compound 4 (myricetin-3-O-β-glucuronide) and compound 5 (myricetin-3-O-α-rhamnoside), Fraction IV (350 mg) was subjected to sephadex LH-20 column which eluted with methanol 20% to give compound 6 (myricetin) and compound 7 (quercetin).

2.4. Inhibition of starch digesting enzymes and starch hydrolysis

Alpha-amylase activity was determined using the microplate method described by Xiao *et al.*, 2006 [12]. Yeast α-glucosidase was measured spectrophotometrically using the synthetic substrate p-nitrophenyl α-D-glucopyranoside in 0.01 M phosphate buffer pH 7.0. The inhibitory activity was determined after a 5 min pre-incubation at 37°C with the test sample (0-200 µg/ml) after which 10 mM substrate solution was added. The reaction was allowed to proceed for 30 min and the amount of p-nitrophenol released measured at 405 nm after the reaction was stopped with 100 mM sodium carbonate solution. Intestinal alpha-glucosidase solution was prepared using commercial rat intestinal acetone powder. Exactly 100 mg of acetone powder was suspended in

3 ml of 0.01 M phosphate buffer (pH 7.0) and sonicated twelve times for 30 s each in an ice bath followed by centrifugation at 3000 rpm, 4°C for 20 min. The supernatant containing the enzyme was kept on ice prior to assay. Both sucrase and maltase activity were determined in a mixture of 500 mM sucrose or maltose (10 μl), the test sample (5 μl), and 0.1 M maleate buffer (pH 6.0, 75 μl). The mixture was pre-incubated at 37°C for 5 min, and reaction was initiated by adding rat intestinal α-glucosidase (10 μl). The mixture was incubated at 37°C for 60 minutes. The glucose released in the solution was determined using a modified glucose oxidase/peroxidase method based on the assay by Trinder, 1969 [13, 14].

Starch hydrolysis was assessed using a combination of alpha-amylase and yeast glucosidase. To 20 μl of test sample, 20 μl enzyme solution (3.5mg/ml porcine pancreatin and 1.9 U/ml glucosidase), 20 μl assay buffer (PBS pH 7.4) and 20μl soluble starch (2 mg/ml) were added to the wells of a 96-well plate. After 30 min incubation at 37°C, 50 μl was transferred to a new 96-well plate and 200 μl glucose reagent added. Plates were then incubated for 20 min at 37°C and the absorbance read at 510 nm. Concentration glucose released from starch was calculated from a glucose standard curve and the kinetics of starch hydrolysis analysed according to the method described by Butterworth *et al.*, 2012 [15].

2.5. Antioxidant activity

DPPH titration curves were obtained using a modified method based on the assay by Brand-Williams *et al.*, 1995 [16, 17]. Briefly, 5 μl of a serial dilution of the extract was placed in the wells of a 96-well plate followed by 120 μl Tris-HCl (50 mM, pH 7.4) and 120 μl of freshly prepared DPPH solution (0.1 mM in absolute ethanol). The plate was incubated at room temperature for exactly 20 min and the absorbance measured at 593 nm. Percentage DPPH scavenging was determined as [(A-B/A) x 100], where A represents the absorbance in the absence of test sample and B represents the absorbance with test sample. Ascorbic acid was used as a positive control and IC_{50} values calculated using GraphPad prism software.

To assess the capacity of the extract to attenuate oxidative stress induced β-cell death, INS-1 cells were seeded into 96-well plates at a density of 10 000 cells per well and allowed to attach overnight. Cells were then treated with the indicated concentrations of the plant extract or NAC in RPMI medium containing 0.5% FBS for 30 min followed by the addition of 2-deoxyribose (40 mM final concentration) and a further incubation at 37°C for 8 hrs. Thereafter the spent culture medium was removed, and the cell viability

assessed using the MTT assay as described by Holst-Hansen and Brünnner, 1998 [18].

2.6. Statistical analysis

Three independent experiments were conducted for each assay. IC_{50} values were calculated using GraphPad Prism version 5.01. The two-tailed Student t-test was used to determine statistical significance. Error bars represent the standard deviation of the mean (SD).

3. Results

3.1. D. lotus L. extract inhibits α-amylase and α-glucosidase activity

The inhibitory effects of *D. lotus* L. extract on enzymes involved in intestinal starch digestion are presented in Table 1. Inhibition characteristics were similar for the human and porcine amylase enzymes with IC_{50} values of 82.5±2.0 and 130.4±24 μg/ml respectively. As expected, acarbose was a potent inhibitor of pancreatic amylase ($IC_{50} < 3$ μM). *D. lotus L.* extract was found to be a strong inhibitor of yeast glucosidase but inhibition of rat intestinal glucosidases (sucrase and maltase) was considerably weaker yielding less than 20% inhibition at 100 μg/ml, a concentration calculated to represent an achievable gut concentration at a practical dose of 0.25 g of ripe fruit.

Table 1: Enzyme inhibitory properties of *D. lotus* L. extract.

| Enzyme	% inhibition (100 μg/ml)	IC_{50} (μg/ml)
Amylase		
Porcine pancreatic amylase	54.4±3.4	82.5±2.0
Human pancreatic amylase	44.3±1.8	130.4±24
Glucosidase		
Yeast alpha glucosidase	> 90	5.5±2.0
Rat intestinal sucrase	12.9±0.7	ND
Rat intestinal maltase	19.5±1.9	ND

a) ND = not determined.

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3.2. Inhibition of glucose release from starch

In view of the interdependence of starch digestion enzymes in the liberation of glucose from complex carbohydrates, intestinal starch digestion was modelled using a combination of both alpha amylase and alpha glucosidase and the rate of glucose released from solubilised starch measured as an indication of the rate of digestion. Alpha amylase cleaves linear portions of amylose and amylopectin within the starch molecule resulting primarily in maltose and maltotriose as end products which are substrates for glucosidase to produce glucose [19].

Irrespective of the inhibitor used, maximum inhibition of starch hydrolysis did not exceed much beyond 50%. *D. lotus* L. extract attenuated the release of glucose in a concentration dependent manner with a maximum percentage inhibition of 53.16 ± 6.2,

which is similar to that observed for acarbose at 56.57 ± 2.1 (Fig.1 A, B). Above a concentration of 100 µg/ml the inhibitory efficacy of the extract begins to plateau similar to that observed for acarbose. This non-linear kinetic response is not unusual for starch digestion and most likely relates to the structural changes occurring in the substrate molecule during enzymatic hydrolysis [20]. Furthermore, oligosaccharides released during hydrolysis themselves become new substrates which will have different kinetic properties.

At the respective IC_{50} values for amylase inhibition, *D. lotus* L. extract and acarbose were equally effective as inhibitors of starch digestion, at least within the context of the current model. Similarly, inhibition rate constants for acarbose and *D. lotus* L. extract were comparable (Fig. 1C), suggesting that the extract can be as effective as a pharmacological dose of acarbose when used at a concentration of 100 µg/ml.
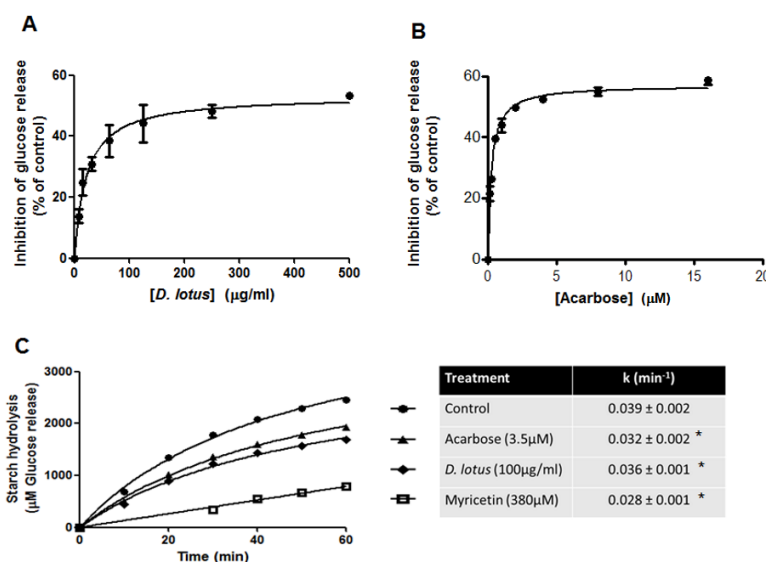
Figure 1: *In vitro* inhibition of starch digestion. Hydrolysis of solubilised potato starch was determined using a combination of porcine pancreatic alpha-amylase and yeast alpha-glucosidase and glucose released quantified enzymatically with glucose oxidase. A and B: dose dependent inhibition of glucose release for *D. lotus* and acarbose respectively. C: Rate of glucose release in the presence of acarbose (3.5 µM), *D. lotus* L. (100 µg/ml) and Myricetin (380 µM). Rate constants were calculated using Guggenheim plots as described by Butterworth *et al.,* 2012. Data represents the mean ± standard deviation from three independent experiments. *Indicates statistical significance compared to control $p < 0.05$ using two-tailed Student's t-test.

3.3. Phenolic constituents of the D. lotus L. extract and their inhibitory properties

Phenolic compounds from plants are often reported to inhibit amylase and glucosidase activity. Identification of the major phenolic constituents in the *D. lotus* L. extract yielded seven known compounds namely: gallic acid, methyl gallate, ellagic acid, quercetin, myricetin, myricetin-3-O-β-glucuronide and myricetin-3-O-α-rhamnoside [21]. Screening these compounds for potential enzyme inhibition indicated that quercetin and myricetin were the strongest alpha-amylase inhibitors (Table 2).

Table 2: Inhibitory capacity of the major phenolic constituents of *D. lotus* L. fruit extract.

| Type	Compound	Amylase IC_{50} (μM)
Phenolic acid	Gallic acid	NI
	Methyl gallate	NI
	Ellagic acid	680.7 ± 42
Flavanoid	Quercetin	231.9 ± 18
	Myricetin	384.8 ± 23
Flavanoid glycoside	myricetin-3-O-β-glucuronide	ND
	myricetin-3-O-α-rhamnoside	ND

a) NI = no inhibition; ND = not determined.

3.4. Antioxidant capacity of D. lotus L. extract

The radical scavenging activity of *D. lotus* L. fruit extract was evaluated using the well-known DPPH assay which revealed strong activity similar in magnitude to that of the pure compound ascorbic acid. DPPH titration curves for the extract and ascorbic acid are presented in figure 2. IC_{50} values were calculated as 4.6 and 24.07 μg/ml for *D. lotus* L. extract and ascorbic acid respectively. This strong radical scavenging activity is perhaps not surprising considering the abundance of phenolic compounds in the extract.

Treatment of INS-1 cells with 2-deoxy-ribose resulted in an approximate 40% reduction in cell viability which was strongly attenuated by the antioxidant NAC (Figure 2). In contrast, *D. lotus* L. extract did not provide any degree of protection. Thus, despite the seemingly robust antioxidant potential of the extract as determined using the DPPH assay, it afforded no measurable protection against oxidative stress induced cell death, at least within the context of the 2-deoxy-ribose model.

Figure 2: Antioxidant potential of *D. lotus* L. extract. A: DPPH titration curves for *D. lotus* L. extract and ascorbic
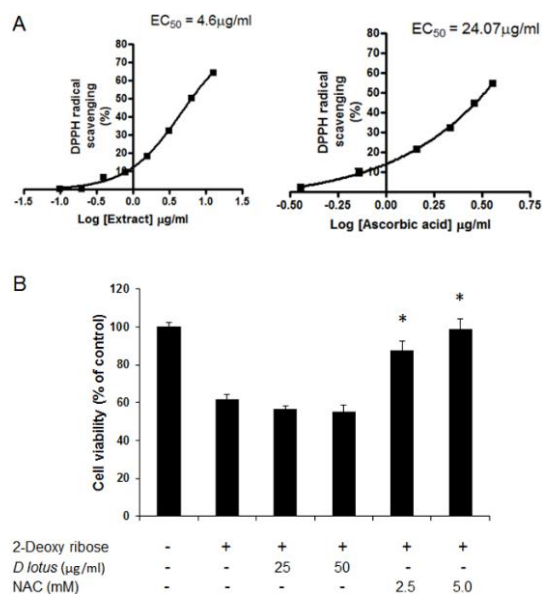
acid. B: 2-deoxy-ribose was used to induce oxidative stress and cell death determined using the MTT assay. Data is expressed as a percentage of the untreated control and represents the mean ± standard deviation for three independent experiments. NAC refers to N-Acetyl cysteine. * Indicates statistical significance compared to 2-Deoxy ribose control $p < 0.05$ using two-tailed Student's t-test.

4. Discussion

Type 2 diabetes is recognised to become a global pandemic as it affects both developed and developing countries. While the prospect to develop new and affordable drugs in time to circumvent further crippling of poorer healthcare systems appears weak, functional foods have emerged as a possible strategy to attenuate the impact [4]. Considering the key contribution of dietary carbohydrates in the development and progression of diabetes, interventions aimed at reducing glycaemic load and post-meal blood glucose spiking, represent a sensible approach [22].

Furthermore, targeting starch digestion in the gastrointestinal tract implies that the bioavailability and efficacy requirements may be less stringent and thus more readily achievable relative to most other pharmacological strategies [23].

The present study identifies *D. lotus* L. fruit as an inhibitor of starch hydrolysis and therefore can be considered a functional food with relevance to the development of diabetes. This inhibition can be attributed to its effects on amylase and to a lesser extent alpha glucosidase. Quercetin and myricetin were identified as the strongest inhibitors among major phenolic constituents. Amylase occurs at the top of the starch digestion cascade and thus its inhibition will also limit the availability of substrates

for other digestion enzymes which are ultimately responsible for liberating glucose. This prominent role for pancreatic amylase is emphasised by the finding that amylase activity directly correlates with post-prandial blood glucose levels [24, 25]. Although the inhibition of intestinal alpha glucosidases was relatively weak, the activity may nonetheless be physiologically relevant as such gut concentrations are practical.

Apart from a direct reduction in post meal hyperglycaemia, inhibition of starch hydrolysis can have further therapeutic benefits through targeting other mechanisms relevant to diabetes [26]. Inhibition of starch digestion elevates serum levels of glucagon-like peptide-1 (GLP-1, a gut derived incretin hormone), presumably due to increased carbohydrate delivery to the L cells in the distal intestinal tract [27]. Also, undigested starch is fermented by gut microbiota producing short chain fatty acids such as butyrate which stimulate the release of GLP-1 [28]. GLP-1 is associated with numerous benefits relevant to diabetes.

Oxidative stress is widely implicated as a negative component in human health leading to the belief that functional foods rich in antioxidants must possess health benefits. Previous studies regarding the antidiabetic activity of *D. lotus* L. fruits in STZ induced hyperglycaemia led the authors to hypothesise that the therapeutic effect may be attributed to antioxidants preventing oxidative stress to pancreatic β-cells [11]. It is well known that β-cells are particularly prone to oxidative stress due to the extremely low expression of antioxidant enzymes, especially catalase and glutathione (GSH) peroxidase [29] and several studies suggest that antioxidants can exert beneficial effects in diabetes by providing protection against β-cell toxicity [30-32].

To model potential cellular antioxidant capacity under hyperglycaemic conditions, oxidative stress was induced in INS-1 cells (pancreatic β-cell line) using 2-deoxy-ribose. 2-Deoxy-ribose is reported to provoke apoptosis in β-cells through protein glycation and oxidative stress and is suggested to represent a suitable model to identify agents that can protect against hyperglycaemia induced β-cell dysfunction [33, 34]. Despite the strong antioxidant capacity, as determined using the DPPH assay, *D. lotus* L. fruit extract offered no protective effects in the 2-deoxy-ribose model. Although not established in β-cells, previous studies have indicated that 2-deoxy-ribose interferes with glutathione homeostasis leading to the depletion of GSH with subsequent oxidative stress and apoptotic cell death [35, 36]. NAC, a precursor for GSH synthesis, prevents GSH depletion and rescues 2-deoxy-ribose induced cell death. In contrast to NAC, non-thiol antioxidants (ascorbic acid and pyruvate) are reported to provide no protection [35], suggesting that

cell death correlates with GSH depletion and not oxidative stress per se. Therefore, we cannot rule out a potential protective antioxidant role for *D. lotus* L. extract against hyperglycaemia induced oxidative stress.

5. Conclusion

The findings of this study identify *D. lotus* L. as a multi-component functional food which can target the progression of diabetes through inhibition of starch digestion and as such may be worthy of further investigation as a food supplement to diminish the anticipated health and economic burden that is predicted to emerge as a result of diabetes.

6. Conflicts of interest

There are no conflicts to declare.

7. Acknowledgments

8. References

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