Antioxidant activities and inhibitory effects of free and bound polyphenols from date (*Phoenix dactylifera* L.) seeds on starch digestive enzymes

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Abstract

Date (*Phoenix dactylifera* L.) seeds have gained interest as a valuable by-product of the date fruit industry and have been identified as a rich source of functional and bioactive ingredients. In this study, date seeds from five varieties (*Medjool, Deglet Nour, Barhee, Bou Sthammi* and *Dayrie*) cultivated in Australia were analysed for their total phenolic and flavonoid contents, ferric reducing antioxidant power (FRAP), Trolox equivalent antioxidant capacity (TEAC), and *in-vitro* inhibition against α-amylase and α-glucosidase. The date seed powders (DSP) were extracted with acetone-methanol-water (2:2:1, v:v:v) to obtain free polyphenols. The bound polyphenols were extracted from the residual solids with butanol-HCl (97.5:2.5; v/v) treatment at 100 °C. The greatest quantities of total phenols (4166±227 mg Gallic Acid Equivalents 100g^{-1} DSP), total flavonoids (52.1±9.6 mg Quercetin Equivalents 100g^{-1} DSP), FRAP (1589±47 μmol Iron (II) Equivalents g^{-1} DSP) and TEAC (45.2±1.4 μmol Trolox Equivalents g^{-1} DSP) were detected in seeds from *Deglet Nour* variety.

Both free and bound polyphenol fractions showed significant ($p<0.05$) inhibition against rat intestinal α-glucosidase with little or no inhibition against pancreatic α-amylase. Bound polyphenols showed stronger α-glucosidase inhibition compared with free polyphenols. The IC$_{50}$ for α-glucosidase were reported as mg GAE mL^{-1} ranged from 0.39±0.02 (*Deglet Nour*) to 0.68±0.02 (*Medjool*) for bound polyphenols, and from 0.907±0.08 (*Medjool*) to 1.75±0.15 (*Barhee-khalal*) for free polyphenols. Bound polyphenol fraction, with strong α-glucosidase inhibition and weak α-amylase inhibition, was suggested as a significant source of functional food ingredients with anti-hyperglycaemic properties.

Keywords: Date seed; Free polyphenols; Bound polyphenols; Type-2 diabetes

1 Introduction

The Date crop has been identified as a suitable crop for the arid regions of Australia, with a very good marketing potential for both date fruit industry and its by-products (Reilly, Reilly, & Lewis, 2010) The total world production of date fruits was 7.5 million tons in 2012 (FAO-STAT, 2015), and date seeds (approximately 10% of fruit weight) are an abundant by-product from this well-established industry (Besbes et al., 2004). Date fruit processing plants produce large quantities of date seeds as a result of value addition processing operations which involve pitting (deseeding) dates to manufacture dates filled with edible seeds, date paste and syrup. It has

Nomenclature

BPP	Bound polyphenols	PC	Proanthocyanidins
DSP	date seed powder	TEAC	Trolox equivalent antioxidant capacity
FPP	Free polyphenols	TFC	Total flavonoid content
FRAP	Ferric reducing antioxidant power	TPC	Total phenol content

been suggested that utilizing this by-product in profitable ways will benefit the date industry (Al-Farsi & Lee, 2008a).

It has been reported that date seeds contain large amounts of polyphenols (Al-Farsi & Lee, 2008b). Plant polyphenols are considered to be the key components responsible for many of the health benefits attributed to the consumption of fruits and seeds (Macedo et al., 2013; Mahbub et al., 2012; Gondoin, Grussu, Stewart, & McDougall, 2010). Health benefits of consuming date seed products have been known in middle-eastern folk medicine. Date seed infusions have been consumed in Turkey and Arab countries as herbal coffees with memory enhancing properties (Habib & Ibrahim, 2009). A recent *in-vitro* study has indicated that date seed extracts can inhibit enzymes related to neurodegeneration (Sekeroglu et al., 2012). *In-vivo* studies on rats have shown that date seed extract could reduce DNA binding and methylation with N-Nitroso-N-methyl urea, which is a well known carcinogen (Diab & Aboul-Ela, 2012).

Anti-hyperglycaemic agents such as acarbose, which act by inhibiting starch digestion enzymes, such as α-amylase and α-glucosidase, are widely used in combination with drug treatment for Type 2 *diabetes mellitus* (Tundis, Loizzo, & Menichini, 2010). Inhibition of α-amylase and/or intestinal α-glucosidase delays the rise in blood glucose levels, thus alleviating the complications arising from postprandial hyperglycaemia for diabetics. However, the generally prescribed anti-hyperglycaemic agents are strong α-amylase inhibitors and are known to exert unpleasant side effects (flatulence, bloating, abdominal distention) due to suppression of starch digestion in

the small intestine and bacterial fermentation in the colon (Yee & Fong, 1996). Consequently, searching for more specific polyphenolic compounds from plant sources that could inhibit α-amylase and α-glucosidase might provide a solution to these side effects, and be cheaper and more tolerable alternatives. Plant polyphenols (free and bound) is expected to delay postprandial hyperglycaemia by specifically inhibiting intestinal α-glucosidase (Phan, Wang, Tang, Lee, & Ng, 2013), and provide additional benefits against oxidative diseases such as diabetics due to their antioxidant properties (Maritim, Sanders, & Watkins, 2003).

Recent studies have demonstrated that the health benefits attributed to the plant polyphenols should include both free (also referred to as extractable) polyphenols (FPP) and bound (also referred to as non-extractable) polyphenols (BPP) (Saura-Calixto, 2012; Tow, Premier, Jing, & Ajlouni, 2011). Most of the reported polyphenol contents considered the fraction extracted into aqueous-organic solvent system, whereas the residual solids are not analysed. However these residual solids have been found to contain a large amount of polyphenols including polymeric proanthocyanidins (PC). Some of the PCs are strongly bound to cell wall components (Hamauzu & Mizuno, 2011), and Perez-Jimenez, Elena Diaz-Rubio, and Saura-Calixto (2013) indicated that BPPs contribute 60-90% to the total polyphenol content in most fruits. These studies have emphasised the importance of including the bound (non-extractable) polyphenols in plant polyphenol analysis.

The aim of this study was to determine the effect of date seeds' free and bound polyphenols

on the activity of starch the digesting enzymes, α-amylase and α-glucosidase. The phenolic contents and antioxidant capacities of seeds from those Australian grown date varieties were also reported.

2 Materials and Methods

2.1 Chemicals

Acetone, butanol, ethanol, methanol, acetic acid, aluminium trichloride, dimethyl sulfoxide (DMSO), ferric chloride hexahydrate, hydrochloric acid, potassium acetate and potassium persulphate used were of the highest analytical reagent or chemical grade. Acarbose (A8980), 2,2'-azinoBis[3-ethylbenzo]thiazoline-6-sulfonic acid (ABTS), 3,5-Dinitrosalicylic acid (DNS), gallic acid, Folin-Ciocalteu reagent (F-C reagent, 2N), 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox; 238813), p-nitrophenyl-α-D-glucopyranoside (PNPG; N1377), 4-nitrophenyl-α-D-maltopentaoside (PNPG5; N1519), porcine pancreas α-amylase (A3176), rat intestinal acetone powder (I1630), soluble potato starch (S9765), 2,4,6-tripyridyl-s-triazine (TPTZ T1253), and quercetin (Q4951) were purchased from Sigma-Aldrich Co, NSW, Australia.

2.2 Preparation of date seed powder (DSP)

Fruits of the *Deglet Nour* (also spelled *Deglet Noor*), *Medjool, Bou Sthammi , Dayri* in rutab-maturity stage, and *Barhee* varieties in both rutab and khalal stages, were kindly provided by Arid Zone Research Institute in Alice Springs. The botanical varieties were verified by molecular methods using established DNA markers (Dr Vivek Bhat, Senior Scientist, Arid Zone Research Institute, Alice Springs, Northern Territory 0870, Australia). Seeds were separated manually from the fruits (Figure 1) washed, then left to air dry for 5 days. Seeds were then ground using a cutting mill (Retsch SM100, Germany) to pass a 1mm sieve.
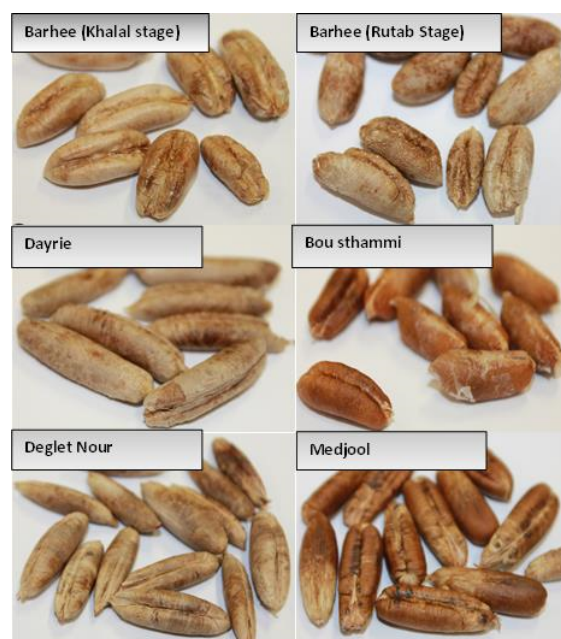

Figure 1: Date seeds from the studied varieties, including seeds from two maturity stages of *Barhee (khalal*-early and *rutab*-later stages)

2.3 Preparation of free polyphenols (FPP) extract

DSP (2 g) was mixed with 50 mL of acetone: methanol: water (2:2:1, v:v:v), following method of (Al-Farsi & Lee, 2008b) with modifications. The mixture was placed in a shaking incubator (Labwit ZWYR-240, China) at 40 °C, at 150 rpm for 1 h and centrifuged at 10,000 rpm for 10 min. After 3 repeat extractions, the supernatants were combined and the solvent was rotary evaporated under vacuum at 40 °C (Buchi Vac V500 system, Switzerland). This concentrated FPP extract was re-dissolved in a minimum volume of water and freeze dried (Dyna Vac FD3, USA). The freeze-dried extract was stored at -20 °C until further analysis. The solids remaining after the extraction were used for the extraction of BPPs.

2.4 Preparation of bound polyphenols (BPP) extract

The generally used method for depolymerisation of the proanthcyanidins is HCl-butanol reaction with Fe^{3+} catalyst (Schofield, Mbugua, & Pell, 2001). Following this reaction, the presence of Fe^{2+}/Fe^{3+} ions in the final BPP extract was inevitable, thus rendering extract not suitable for subsequent analyses. Several trials showed that Fe^{2+}/Fe^{3+} ions interfered with total flavonoid assay and antioxidant activity measurements using FRAP and TEAC assays. Therefore the depolymerisation was performed without the ferric chloride catalyst. Optimization of the extraction was monitored *via* measurements of TEAC and TPC, and photometric scans showed absorption maxima at λ 550 nm characteristic to anthocyanidins which were significantly enhanced after 3 h of extraction (data not shown).

The residues from the FPP preparation were used for BPP extraction following the method of Perez-Jimenez and Saura-Calixto (2005), with modifications. From the FPP residue, 2 g was mixed with 25 ml butanol/HCl (97.5:2.5 v/v) and heated at 100 ˚C for 3 hours in a water bath. The mixture was centrifuged at 10,000 rpm for 10 minutes, supernatant was collected and residue was subjected to two washings with 10 mL butanol. Combined supernatants were rotary evaporated at 60 ˚C, and pH of the concentrated extract was adjusted to ᴗ4 using 1 M sodium hydroxide. pH above 5 caused precipitation of the extract, therefore pH 4 was selected to maintain both an intact extract and the compatibility with buffers. Extract was freeze-dried and stored at -20 ˚C until analysis.

2.5 Total polyphenol content (TPC)

Assay for total phenol content was based on reduction of the molybdenum-tungstate complex in F-C Reagent by phenols (Herald, Gadgil, & Tilley, 2012). The procedure was adapted to 96-well microplate platform (250 μL assay volume per well) with absorbance acquired at λ 765 nm using a microplate reader (Multiskan GO, Thermo Scientific, Australia) at 25 ˚C. Gallic acid (0-60 μg) was used to generate the standard plot, and TPC was expressed as mg Gallic Acid Equivalent (GAE) 100^{-1} g date seed powder (DSP).

2.6 Total flavonoid content (TFC)

Assay for total flavonoid content was based on chelation with Al^{3+} ion and absorbance measurement of the flavonoid-Al^{3+} complex as described by Xu, Wang, Hu, Lee, and Wang (2010), and was adopted to the microplate platform. Absorbance was acquired at λ 430 nm instead of 415 nm based on our optimisation study, which showed that quercetin-aluminium complex exhibit maximum absorbance at 430 nm (data not shown). Quercetin (0-30 μg) was used to generate the standard plot and TFC was expressed as mg Quercetin Equivalents (QE) 100^{-1} g DSP.

2.7 Trolox equivalent antioxidant activity (TEAC)

Free radical scavenging antioxidant activity was measured as TEAC using ABTS radical as the organic radical (Re et al., 1999). The assay was adapted to 96 well microplate platform and absorbance was measured at λ 734 nm after 10 min at room temperature (RT). ABTS radical scavenging standard curve was obtained using Trolox $(0 - 400\ \mu mol)$ and the TEAC expressed as μmole Trolox Equivalents g^{-1} DSP.

2.8 Ferric reducing antioxidant power (FRAP)

Reducing antioxidant activity was measured as FRAP based on the reduction of Fe^{3+}-TPTZ complex (Benzie & Strain, 1996). The absorbance was measured at λ 593 nm at a fixed incubation time of 10 min at RT. Reducing standard curve was obtained using Fe^{2+}-TPTZ (0-500 μmol Fe^{2+}), and the FRAP was expressed as μmol Fe^{2+} equivalents g^{-1} DSP.

2.9 α-Glucosidase assay

α-Glucosidase extract from rat intestine was prepared using rat intestinal acetone powder and activity assayed following the method of Kee, Koh, Oong, and Ng (2013). The protein content of the enzyme preparation was determined to be 12.65 ± 0.81 mg mL^{-1} using the Lowry protein determination method. Date seed freeze dried extract was reconstituted in DMSO and 10-75 μL was mixed with 700 μL of buffer (0.1 M potassium phosphate, pH 6.8 + 1 % (w:w) NaCl) and 100 μL of α-Glucosidase (in the same buffer), and pre-incubated for 5 minutes at RT. A control (100% enzyme activity) was prepared the same way with DMSO replacing the sample extract. Enzyme activity was compatible with 10% (v:v) DMSO with no inhibition of activity observed (data not shown). A colour correction solution was prepared for each extract with buffer replacing the enzyme to correct for background colour of extract. 100 μmol L^{-1} of p-nitrophenyl β-D-glucopyranoside (PNPG) substrate was added to initiate the reaction, and the mixture was incubated at 37 ˚C for 1 h. Reaction was terminated by chilling microplate on ice and the absorbance promptly measured at λ 410 nm with spectrophotometer (Novaspec II, USA). The % inhibition of α-glucosidase activity was calculated using following equation:

$$I = 1 - \frac{Abs_{ctrl} - Abs_{extract}}{Abs_{ctrl}} \times 100 \qquad (1)$$

Where I is the % Inhibition, Abs_{ctrl} is the Absorbance of control incubation corrected for enzyme color and $Abs_{extract}$ is the Absorbance of extract incubation corrected for extract and enzyme color

2.10 α-Amylase assay I (starch-DNS method)

α-Amylase activity was assayed by the starch-DNS method according to Wan, Yuan, Cirello, and Seeram (2012) with some modifications. Porcine pancreas α-amylase was diluted to 1 unit mL^{-1} in 20 mM sodium phosphate buffer containing 7 mmol L^{-1} NaCl and 1 mmol L^{-1} CaCl$_2$. Soluble potato starch (0.5%; w:w) was

prepared in the same buffer. 50 μL Sample (in DMSO), 100 μL α-amylase and 400 μL buffer were mixed in 10 mL screw capped plastic tube and incubated for 5 minutes at RT. Enzyme activity was compatible with 10% (v:v) DMSO and no inhibition of activity was observed (data not shown). 500 μL 0.5 % starch solution (w:w) was added and mixture was incubated at 37 ˚C for 20 min. then 500 μL dinitrosalicylic acid (DNS) was added. Tube was placed in boiling water bath for 15 minutes. It was then cooled and absorbance was measured at λ 540 nm. A colour correction solution was prepared for each extract. Percentage inhibition of the α-amylase activity was calculated using equation 1.

2.11 α-Amylase assay II (PNPG5 method)

α-Amylase assay II was performed using the chromogenic substrate p-nitrophenyl-α-D-maltopentaoside (PNPG5) method as described by Funke and Melzig (2006) with modifications. Porcine pancreas α-amylase was diluted to 50 units mL^{-1} in 20 mM sodium phosphate buffer (pH 6.8), containing 7 mM NaCl and 1 mM CaCl$_2$. PNPG5 was made up to 25 mM in the same buffer. The assay was performed in 96 well microplates. 50 μL of PNPG5, 10 μL of extract in DMSO, and 190 μL buffer were added to each well, and 50 μL of enzyme was added to initiate the reaction. Absorbance was measured at λ 405 nm using microplate reader at 3 minute intervals for 90 minutes. Absorbance of control (without inhibitor) and for extract background (without added enzyme) was also acquired. The percentage inhibition of amylase activity was calculated by the ratio of the linear gradients of control and extract incubation, using the linear range of the kinetic curve.

2.12 IC$_{50}$ determination

Active extracts against rat intestinal α-glucosidase were assessed for their potency by their IC$_{50}$ values. The data points (mean $\pm$ SD, n = 3) were fitted into a nonlinear sigmoid plot to take into account non-linear concentration dependent of enzyme inhibitor interaction at

low and high concentrations (Burlingham & Widlanski, 2003).

2.13 Statistical analysis

One-way analysis of variance (ANOVA) was used to analyse the data followed by Least Significant Difference (LSD) test ($p<0.05$) for significant differences using SAS software (Version 9.2). Uncertainty of replicate determinations was reported as Standard Deviation in 2 significant figures according to EURACHEM guidelines (Ellison & Williams, 2012).

3 Results and Discussion

As previously mentioned in the introduction (section 1), it is evident that analysis of antioxidant capacity and other health benefits derived from fruit polyphenolics must include both FPPs and BPPs. In this study we have taken into account FPPs and BPPs when discussing the polyphenol contents, antioxidant activity, and starch-digestive enzyme inhibition. BPP fraction has been subjected to acid-catalysed depolymerisation to study the above properties of the resulting degraded end-products.

3.1 Total phenol and flavonoid content

We found significant differences ($p<0.05$) between the total phenol contents (TPC) in FPP fractions of the date seed varieties (Table 1). *Deglet Nour* and *Dayrie* seeds showed significantly ($p<0.05$) high phenolic contents (4166±227 and 3777±309 mg 100g^{-1}, respectively) compared with other varieties, whereas *Medjool* showed the lowest (2088±124 mg 100g^{-1}). Considerable levels of TPC were found in the BPP fractions in all varieties (Table 1). The TPC quantities in BPP fraction (reported as mg 100g^{-1} DSP) were the greatest in *Medjool* seeds (514±13), followed by *Barhee* (khalal) (405.9±9.2), *Barhee* (rutab) (378.9±8.9), *Deglet Nour* (373±4.6) and *Dayrie* (345.2±5.0).

The reported TPCs of date seeds in the literature varied due to different methodologies used in extraction and quantification, as well as varietal

and geographic factors (Sirisena, Ng, & Ajlouni, 2015). That was also evident in the current study where varieties differed significantly ($p<0.05$) in terms of phenolic contents. Most authors have reported very high TPC for the FPP of date seeds, ranging from 2000-4500 mg 100g^{-1} DSP (Al-Farsi et al., 2007; Ardekani, Khanavi, Hajimahmoodi, Jahangiri, & Hadjiakhoondi, 2010). The TPCs in the FPP of the Australian date varieties in this study sit within that range. The detected amounts of TPC in the FPP from Australian date seeds were greater than those reported in cocoa, chestnut, hazelnut, pecan nut and walnut seeds (1104, 2757, 687, 1816 and 1576 mg GAE 100g^{-1} dry weight), respectively (Perez-Jimenez, Neveu, Vos, & Scalbert, 2010). Literature data were rather limited for comparing BPPs from seeds.

Since not all phenolic compounds are flavonoids, the flavonoids content are expected to be smaller than the phenolic contents. Results confirmed that analyses showed significantly ($p<0.05$) smaller quantities of TFCs in comparison with TPCs in both FPPs and BPPs (Table 1). The TFCs in *Medjool* seeds were the lowest (9.4±0.58 mg QE 100g^{-1} DSP) in the FPPs, and the highest in the BPPs (30.4±1.7 mg QE 100g^{-1} DSP), while other varieties showed the opposite trend. Measuring flavonoids contents can provide an indirect assessment of its proportional contribution to the total phenols and also its contribution to antioxidant activity.

3.2 Antioxidant properties of FPP and BPPs

Antioxidant activity of food components can provide functionality contributing to disease prevention by reducing oxidative stress in the gastrointestinal tract and, if absorbed, in body tissues. The antioxidant capacity in the FPPs and BPPs of date seeds were evaluated using TEAC and FRAP assays. These analyses were based on the ability of antioxidants to scavenge (by electron or hydrogen atom donation) an organic free radical or to reduce iron, respectively.

Significant differences in antioxidant activity were observed between the seed varieties as measured by TEAC and FRAP (Table 1). For the

FPPs, *Deglet Nour* and *Dayrie* seeds have the highest TEAC and FRAP, while *Medjool* seeds the lowest. FRAP values (μmol Fe^{2+} Equivalents g^{-1} DSP) in *Deglet Nour* (1589±47) and *Dayrie* (1445.2±5.4) of seeds FPPs are in a similar range with those of Avacado and Longan seeds (1484 and 1388 μmol Fe^{2+} Equivalents g, respectively) (Soong & Barlow, 2004). Additionally, TEAC value of *Deglet Nour* seed FPP (45.2±1.4) was rather similar to Avacado seed (42 μmole Trolox Equivalents g^{-1}) and smaller than Longan seed (75 μmol Trolox Equivalents g^{-1}) (Deng et al., 2012). However Al-Farsi et al. (2007) reported much larger TEAC values for Omani date varieties (580-929 μmol Trolox Equivalents g^{-1} fresh weight). With the exception of *Medjool* DSP, the TEAC values in BPPs were 2-4 folds smaller than in FPPs. Similarly, the FRAP values in BPPs of all DSP were 2-10 folds smaller than those in FPPs.

Strong positive correlations were observed for the TPC content of FPPs with the corresponding FRAP (R^2=0.97), and TEAC (R^2=0.95) values. TFC content also positively correlated with FRAP (R^2=0.98) and TEAC (R^2=0.97). Similar correlations were observed for BPPs (FRAP, R^2=0.89; TEAC R^2=0.98), indicating that the radical scavenging and ferric reducing properties of both FPPs and BPPs fractions are associated with phenolic and flavonoid compounds.

3.3 Inhibition of carbohydrate hydrolysing enzymes

Inhibition of intestinal α-amylase and α-glucosidase (membrane bound sucrase-isomaltase and maltase-glucoamylase complexes) can prevent starch digestion and delay or reduce postprandial hyperglycaemia due to inhibition of starch digestion (Funke & Melzig, 2006). Since control of hyperglycaemia is an essential treatment strategy for diabetes, α-amylase and α-glucosidase inhibitors play a role in the management of post-prandial hyperglycaemia (Tundis et al., 2010). Ideally, the drugs should have mild α-amylase inhibition and moderate α-glucosidase inhibition, which would minimize the side effects such as flatulence and abdominal pain caused by colonic fermentation of intact

starch (Yee & Fong, 1996).

Inhibition of α-glucosidase

To our knowledge, no previous studies have reported the inhibitory effect of date seed extract against α-glucosidase. However, El-Fouhil, Ahmed, and Darwish (2010) reported that diabetic rats fed on boiled aqueous date seed extract resulted in significant hypoglycaemia. In addition, Takaeidi et al. (2014) reported that methanolic date seed extract was able to promote the activities of serum paraoxonase (PON1) enzyme, which is known to be protective against oxidative stress related to diabetic complications. These studies pointed to the possible role of date seeds polyphenol in diabetic control.

We showed that BPPs from DSP inhibited rat intestinal α-glucosidase (Table 2), ranging from 58-96%, with BPPs from Deglet Nour seeds showing the strongest percentage inhibition (96.1±1.9) at 1 mg GAE mL^{-1}. Inhibition from FPPs was moderate to low, ranging from 39-65 % at 1 mg GAE mL^{-1}, with FPPs from Medjool seeds showing the strongest percentage inhibition (65.3±1.5). Acarbose, which was used as a positive inhibitor of α-glucosidase, inhibited activity completely at a 1 mg mL^{-1}.

The IC_{50} values gave a clearer indication of the inhibitory potential of DSP against the α-glucosidase (Table 3). The IC_{50} of FPPs from the seeds ranged from 0.907-1.75 mg GAE mL^{-1}, and again showing Medjool seeds with the strongest effect (0.91±0.07 mg GAE mL^{-1}). BPPs from DSP showed a much stronger inhibition (smaller IC_{50}) than FPP, with values ranging from 0.39-0.68 mg GAE mL^{-1}. Deglet Nour BPP showed the strongest inhibition with IC_{50} value of 0.399±0.018 mg GAE mL^{-1}.

The difference in α-glucosidase inhibitory effect between FPPs and BPPs from the seeds reflects on the different phenolic composition between the two phenolic fractions. Detailed characterisation of the FPP and BPP fractions of date seeds has been performed using UHPLC-MS in a separate study, which showed that FPPs constitute mainly simple and glycosylated phenolic acids, flavonols and flavanols, while the BPPs contained partially depolymerised proanthocyanidins and

Table 1: Total phenol content (TPC), total flavonoid content (TFC), and antioxidant activities TEAC[3] and FRAP[4] of free polyphenols (FPP) and bound polyphenols (BPP) from date seeds

Polyphenols	Medjool	Deglet Nour	Barhee (Rutab)	Barhee (Khalal)	Bou Sthammi	Dayrie
FPP						
TPC[1]	2088 ± 124^a	4166 ± 227^b	2482 ± 31^c	3105 ± 137^d	2580 ± 139^c	3777 ± 309^b
TFC[2]	9.38 ± 0.58^a	52.7 ± 9.6^b	23.6 ± 4.6^c	32.1 ± 2.0^c	24.7 ± 1.1^c	47.9 ± 6.0^b
TEAC[3]	12.23 ± 0.42^a	45.2 ± 1.4^b	24.64 ± 0.80^c	25.28 ± 0.23^c	25.15 ± 0.26^c	41.38 ± 0.65^b
FRAP[4]	635 ± 34^a	1589 ± 47^b	1157 ± 40^c	871 ± 35^d	894 ± 17^d	1445.2 ± 5.4^b
BPP						
TPC[1]	514 ± 13^a	373.6 ± 4.6^d	378.9 ± 8.9^c	405.9 ± 9.2^b	369.0 ± 8.2^d	345.2 ± 5.0^d
TFC[2]	30.4 ± 1.7^a	13.8 ± 2.5^b	11.7 ± 1.0^b	14.6 ± 2.1^b	5.94 ± 0.63^c	5.97 ± 0.40^c
TEAC[3]	28.77 ± 0.31^a	12.86 ± 0.40^b	16.26 ± 0.86^c	13.1 ± 1.1^b	14.68 ± 0.53^d	12.7 ± 1.1^b
FRAP[4]	197 ± 12^a	171.6 ± 5.9^a	155.7 ± 4.2^a	404.6 ± 4.1^b	238.1 ± 2.0^c	174 ± 10^a

Values are reported as mean±SD (n≥3) from replicates determination. Different letters[a,b,c...] within each row indicates significantly different means (p<0.05).
1- Total phenolic content was expressed as mg Gallic Acid Equivalents $100g^{-1}$ dry date seed powder (DSP)
2- Total Flavonoid Content (TFC) was expressed as mg Quercetin Equivalents $100g^{-1}$ DSP
3- Trolox equivalent antioxidant capacity (TEAC) values were expressed as μmol Trolox Equivalents g^{-1} DSP
4-Ferric reducing antioxidant power (FRAP) was expressed as μmol Fe^{2+} equivalents g^{-1} DSP

simple phenolic acids including several dihydroxybenzoic acid isomers (unpublished data).

Inhibition of α-amylase

We initially measured the inhibition of α-amylase using the starch-DNS method according to literature (α-amylase assay I), but observed significant absorbance interference due reduction of the DNS reagent by polyphenols (data not shown). The reducing effect of polyphenols with DNS have been documented before (Xu, Leng, Wang, & Zhang, 2012), thus this method is not suitable for the assay of α-amylase activity with polyphenols. Therefore an alternative method was adapted using a chromogenic maltopentaoside substrate (α-amylase assay II), which is similar to the α-glucosidase assay in that liberated p-nitrophenol is measured by absorbance to indicate enzyme activity.

Figure 2 shows the linear inhibitory kinetics of porcine pancreatic α-amylase exhibited by FPPs and BPPs from *Dayrie* seeds as measured by α-amylase assay II. Acarbose, used as a positive inhibitor of α-amylase, almost completely inhibited activity at 1 mg mL^{-1}. The percentage inhibition can be obtained by the ratio of the linear gradient of extract incubation over that of control incubation, which were 18.8% and 40.8% for FPP and BPPs, respectively at 1 mg GAE mL^{-1} of *Dayrie* seed extract. Table 2 summarised the

% α-amylase inhibition by FPPs and BPPs for the other seed varieties. It showed that inhibition by BPPs (ranged from 9-57%) were generally stronger than inhibition by FPPs (ranged from 7-18%) for the same seed variety. Furthermore, the inhibition of α-glucosidase was generally stronger than the inhibition of α-amylase for the same seed variety. IC50 was not determined for α-amylase due to the low inhibitory activity shown by both fractions.

The data did not show a clear correlation between α-glucosidase and α-amylase inhibition with phenolic content and antioxidant capacity of the FPP and BPP fractions. This could be due to different polyphenolic 'acids profile' of the 2 fractions. How polyphenols mediate enzyme inhibition still require more thorough investigation. At the molecular level, the number and position of free hydroxyl groups in the polyphenol structure play an important role in both α-amylase and α-glucosidase inhibition (Funke & Melzig, 2006). Simple phenolic acids such as hydroxybenzoic acids and hydroxycinnamic acids were not inhibitory (Phan et al., 2013). Tannins showed mild inhibitory activity towards α-amylase, but, ellagitannins with β-galloyl groups at the glucose C-1 position showed a higher inhibition than those with α-galloyl and desgalloyl compounds (Li, Tanaka, Zhang, Yang, & Kouno, 2007). In a plant extract however, the

Table 2: Inhibition of rat intestinal α-glucosidase and porcine pancreatic α-amylase by date seed free polyphenol (FPP) and bound polyphenol (BPP) extracts

Seed Variety	EPP (% Inhibition at 1mg GAE mL^{-1} reaction)		BPP (% Inhibition at 1 mg GAE mL^{-1} reaction)	
	α-Glucosidase	α-amylase	α-Glucosidase	α-amylase
Deglet Nour	47.4 ± 1.4^a	11.27 ± 0.93^a	96.1 ± 1.9^a	23.98 ± 0.52^a
Dayrie	42.5 ± 1.0^a	18.82 ± 0.41^b	71.4 ± 1.1^a	40.76 ± 0.20^b
Barhee (Khalal)	39.65 ± 0.66^b	7.20 ± 0.86^b	68.26 ± 0.49^b	17.26 ± 0.91^c
Barhee (Rutab)	43.24 ± 0.73^c	8.841 ± 0.060^c	58.79 ± 0.62^a	21.01 ± 0.41^c
Bou Sthammi	52.7 ± 1.1^b	9.63 ± 0.74^b	67.1 ± 1.0^b	9.55 ± 0.69^b
Medjool	65.3 ± 1.5^d	15.56 ± 0.85^a	68.58 ± 0.60^b	57.84 ± 0.42^d

Percentage inhibition is relative to control without added inhibitor, and values listed are mean % inhibition $\pm$ SD (n$\geq$3)
a,b,c,d: Values sharing different superscript letters in a column are significantly different (p<0.05)

Table 3: IC$_{50}$ values for the inhibition of rat intestinal α-glucosidase by FPP and BPP of date seed extract

Date Seed Variety	FPP (mg GAE mL^{-1} reaction)	BPP (mg GAE/mL^{-1} reaction)
Deglet Nour	1.28 ± 0.16^a	0.399 ± 0.018^a
Dayrie	1.483 ± 0.089^b	0.612 ± 0.011^b
Barhee (Khalal)	1.752 ± 0.093^c	0.585 ± 0.091^d
Barhee (Rutab)	1.23 ± 0.75^a	0.582 ± 0.029^d
Bou Sthammi	1.219 ± 0.083^a	0.648 ± 0.038^c
Medjool	0.907 ± 0.072^d	0.681 ± 0.013^c

Values of IC$_{50}$ reported are mean$\pm$SD (n=3)
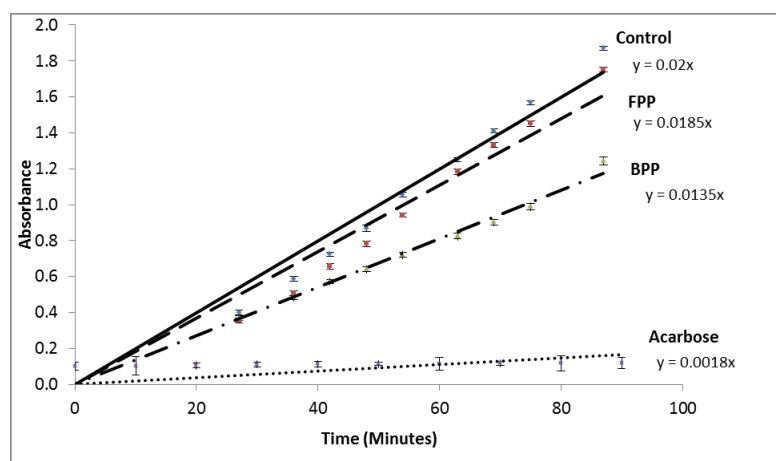a,b,c,d: Values sharing different superscript letters in a column are significantly different (p<0.05)

Figure 2: Inhibition of porcine pancreatic α-amylase using PNPG5 as substrate (α-amylase assay II). The absorbance at different time points was plotted as mean$\pm$SD (n$\geq$3). FPP (long dash) and BPP (long dash and dot) of *Dayrie* variety at 1 mg GAE mL^{-1} reaction was compared to control (solid line) without added inhibitor. Acarbose (dots) at 1 mg mL^{-1} reaction was used as a positive inhibition control

mechanisms of inhibition might be enhanced by purported existence of synergistic action between polyphenols or other components which requires more thorough investigation. Stronger inhibitory activity of BPPs against α-glucosidase may be attributed to the different phenolic profile obtained as a result of depolymerisation. Data on the chemical compositions of bound polyphenols in date seeds are very rare, which calls for further investigation of this area. Characterisation of phenolic profiles of FPPs and BPPs of the date seed is required to correlate inhibitory properties with specific compounds.

4 Conclusions

Our study showed that Australian date seeds have high polyphenol contents and in-vitro antioxidant activities. Bound polyphenol fraction from date seeds showed larger inhibitory capacity towards both starch digestive enzymes; α-amylase and α-glucosidase, compared to the free polyphenol fraction. Also, the bound polyphenol fraction showed strong inhibition towards α-glucosidase but weak inhibition towards α-amylase, indicating they are good source of functional ingredients with potent antihyperglycaemic properties.

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