

INVESTIGATING THE POTENTIAL ANTI-DIABETIC MECHANISMS OF WATER-SOLUBLE PALM FRUIT EXTRACT

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ABSTRACT

Water-Soluble Palm Fruit Extract (WSPFE) obtained from the aqueous vegetation liquor of oil palm (*Elaeis guineensis*) fruit milling demonstrated anti-diabetic effects in Nile rats, but the related mechanisms were unknown. Here, we investigated the potential effects of WSPFE samples on glucose uptake and enzymes involved in carbohydrate hydrolysis and incretin degradation. Glucose uptake assays were performed using spray dried (SD) WSPFE, freeze dried (FD) WSPFE, WSPFE ethyl acetate fraction (EAF) and seven individual WSPFE fractions obtained from WSPFE EAF on everted mouse intestinal sacs *ex vivo* and Caco-2 cell monolayers *in vitro*. α -glucosidase, α -amylase and dipeptidyl peptidase-4 (DPP-IV) enzymatic assays were performed *in vitro*. Glucose uptake assays revealed that all the WSPFE samples tested did not inhibit glucose absorption. However, WSPFE EAF consistently had stronger inhibitory effects on α -glucosidase, α -amylase and DPP-IV enzymes compared to SD WSPFE and FD WSPFE. SD WSPFE inhibited α -amylase better than FD WSPFE. In terms of individual fractions, F2 demonstrated the strongest inhibitory effects against α -glucosidase and DPP-IV. Hence, although WSPFE samples did not inhibit glucose uptake, they showed inhibitory effects on the three enzymes tested, especially WSPFE EAF and F2. Further studies to investigate their effects on carbohydrate digestion and postprandial hyperglycaemia are warranted.

Keywords: glucose, metabolic syndrome, oil palm phenolics.

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INTRODUCTION

Rising physical inactivity, obesity and consumption of energy-dense diets have resulted in an unprecedented increase in the incidence and prevalence of type 2 diabetes mellitus (T2DM), with a projected increase to 642 million cases by

2040 (Chatterjee *et al.*, 2017). T2DM is characterised by insulin resistance, declining insulin production and pancreatic β -cell failure (Lyssenko *et al.*, 2008). In overcoming T2DM, plant phenolic compounds may influence glucose metabolism via different mechanisms (Cuervo *et al.*, 2014; Hanhineva *et al.*, 2010; Moco *et al.*, 2012). These include inhibition of intestinal carbohydrate digestion, inhibition of intestinal glucose absorption, stimulation of pancreatic insulin secretion, modulation of hepatic glucose release, as well as activation of insulin receptors and glucose uptake in insulin-sensitive tissues. Modulation of gut flora activities that effect changes in energy metabolism, as well as modulation of gene expression and intracellular signalling pathways may also occur. An important property of phenolic compounds identified is their preventive effects against long-term T2DM

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complications, such as retinopathy, nephropathy and neuropathy, which may help improve quality of life in diabetic patients (Bahadoran *et al.*, 2013).

An extract obtained from the aqueous vegetation liquor produced from the milling of the oil palm (*Elaeis guineensis*) fruit, termed Water-Soluble Palm Fruit Extract (WSPFE), Palm Fruit Bioactives, palm fruit juice or oil palm phenolics (Sambanthamurthi *et al.*, 2011a), is rich in phenolic acids, including three caffeoylshikimic acid isomers, *p*-hydroxybenzoic acid and protocatechuic acid (Sambanthamurthi *et al.*, 2011a). It also contains an indoleacetic acid derivative (Sambanthamurthi *et al.*, 2014) and shikimic acid (Sambandan *et al.*, 2011). Pre-clinical *in vitro*, *ex vivo* and *in vivo* studies have shown that WSPFE has beneficial bioactive properties, while clinical studies in healthy volunteers have demonstrated that it is safe for human consumption and confers antioxidant as well as anti-inflammatory effects (Leow *et al.*, 2021a). We previously showed that WSPFE blocked T2DM progression in 12 week-old male Nile rats (*Arvicanthis niloticus*), with a significant decrease in blood glucose after 17 weeks of treatment (Sambanthamurthi *et al.*, 2011b). We also demonstrated that WSPFE delayed T2DM onset, completely prevented it and even reversed advancing T2DM in Nile rats (Bolsinger *et al.*, 2014). WSPFE thus, has excellent anti-diabetic effects. It would therefore be important to identify the potential mechanisms involved and the components which confer these effects, if possible, via bioactivity-guided fractionation. The underlying mechanism is not likely a rise in insulin secretion, as insulin levels were not significantly increased after WSPFE supplementation in Nile rats (Bolsinger *et al.*, 2014; Sambanthamurthi *et al.*, 2011b). This necessitates investigations into other possible mechanisms, which may include inhibition of glucose absorption, carbohydrate hydrolysis or incretin degradation.

In addition, different drying methods may have effects on the biological activities of polyphenols. For example, higher chokeberry polyphenol levels were present after drying at high temperatures as compared to after freeze drying (Horszwald *et al.*, 2013), while spray dried (SD) papaya products also retained higher levels of flavonoids and phenolic compounds as compared to freeze dried (FD) products (Gomes *et al.*, 2018). As such, investigating different drying methods would also be important for preparation of related products with optimal biological activities.

When compared to other routes of exposure, the gastrointestinal tract is highly relevant to the effects of phenolic compounds, as the areas of exposure of the gastrointestinal tract to these ingested compounds are extensive. *Ex vivo* everted

intestinal sacs have been used in various drug pharmacokinetic and pharmacodynamic studies (Alam *et al.*, 2012). Caco-2 cell monolayers have also been used as an *in vitro* model of the intestinal barrier for studies on metabolism and transport kinetics of dietary phenolic compounds (Sambuy *et al.*, 2005).

In humans, dietary glucose intake primarily comes from the hydrolysis of carbohydrates, such as starch. Salivary and pancreatic α -amylases, as well as intestinal α -glucosidases, are the carbohydrate-hydrolysing enzymes involved in the generation of dietary glucose. Inhibition of these carbohydrate-hydrolysing enzymes can be an important concept for T2DM management (Apostolidis and Lee, 2010; Striegel *et al.*, 2015). In addition, the dipeptidyl peptidase-4 (DPP-4) enzyme is involved in the degradation of incretins, which are gut peptides that are secreted after nutrient intake and stimulate insulin secretion. Glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) are the known incretin hormones from the cells of the upper and lower gut, respectively. These two hormones are responsible for the incretin effect, a phenomenon where there is a two- to three-fold higher insulin secretory response to oral as compared to intravenous glucose administration. In subjects with T2DM, this incretin effect is diminished or no longer present (Nauck and Meier, 2018). The inhibition of DPP-4 is thus, another possible anti-diabetic mechanism (Fan *et al.*, 2013).

In the present study, we were interested to find out the possible mechanisms by which WSPFE samples confer potential protection against T2DM. Thus, we sought to investigate whether they have inhibitory activities on glucose absorption, as well as their potential effects on enzymes involved in carbohydrate hydrolysis and incretin degradation. We compared the effects of WSPFE samples produced using two different drying methods and fractions prepared using preparative high-performance liquid chromatography (HPLC), so as to inform future studies on carbohydrate digestion and postprandial hyperglycaemia using WSPFE samples.

MATERIALS AND METHODS

Preparation of Water-Soluble Palm Fruit Extract (WSPFE) Samples

The solvents used were purchased from Merck KGaA (Darmstadt, Germany) unless otherwise stated. Liquid WSPFE was produced at the Malaysian Palm Oil Board (MPOB) Phenolic Antioxidant Pilot Plant as described by Sambanthamurthi *et al.* (2008) in Labu, Negeri Sembilan, Malaysia. Spray dried (SD) WSPFE

was obtained through the spray drying process carried out on liquid WSPFE at Biotropics Malaysia Berhad, Shah Alam, Selangor, Malaysia. This spray drying process was performed using the Agridon AG-10 spray dryer (Agridon Technologies, Sungai Buloh, Selangor, Malaysia), with the following conditions: inlet temperature of 185°C-200°C, outlet temperature of 110°C-130°C, aspirator value of 85%-100%, feed rate of 83-116 mL/min, air for drying and compressed air with a flow of 150-200 kPa for pneumatic liquid atomiser. Freeze dried (FD) WSPFE was obtained by freeze drying liquid WSPFE at MPOB using a FreeZone 2.5 L Benchtop Freeze Dry System (Labconco Corp., Kansas City, Missouri, United States of America) at -54°C. WSPFE ethyl acetate fraction (EAF) was obtained by extracting liquid WSPFE with ethyl acetate using a sample:solvent ratio of 1:3 and then collecting the upper solvent layer for rotary evaporation and freeze drying. Compared to using other solvents such as hexane, chloroform, methanol and water, extraction using ethyl acetate has been previously found to result in plant extracts containing high total phenolic content (Ooi *et al.*, 2016), as well as high carbohydrate-hydrolysing enzyme inhibitory activities (Ablat *et al.*, 2017; Nguyen *et al.*, 2020a) and anti-diabetic properties in animal models (Ablat *et al.*, 2017; Nguyen *et al.*, 2020b; Olatunji *et al.*, 2017; Ooi *et al.*, 2018).

Individual WSPFE fractions (F1-F7) were prepared by subjecting WSPFE EAF to preparative HPLC using a Waters Preparative AutoPurification HPLC System (Waters Corp., Milford, Massachusetts, United States of America) as described previously (Leow *et al.*, 2021b). Separation was achieved by using a reverse phase Waters Atlantis C18 5 µm column (Waters Corporation, Milford, Massachusetts, United States of America). A binary gradient system was used as the mobile phase, with phase A comprising 0.02% (v/v) trifluoroacetic acid in distilled water and phase B comprising 70%:30% (v/v) methanol-acetonitrile. A flow rate of 20 mL/min and a pressure limit of 2.76×10^4 kPa were used. The gradient elution with a total run time of 55 min was as follows: Started from 100% (v/v) phase A and 0% (v/v) phase B, increased to 32.5% (v/v) phase B over 40 min, then increased to 62.5% (v/v) phase B over 6 min and finally decreased to 0% (v/v) phase B over 9 min. Seven fractions (F1-F7) as characterised by ultraviolet/visible (UV/VIS) detection at 280 nm UV wavelength were collected. The identities of the major components in these fractions were confirmed by the retention times and UV/VIS spectra of the peaks which had already been identified in WSPFE previously. The related preparative HPLC chromatogram of these prepared fractions had been published previously (Leow *et al.*, 2021b).

Glucose Uptake of Everted Mouse Intestinal Sacs

Male, Institute of Cancer Research (ICR) mice aged between nine to 19 weeks (30 to 44 g) obtained from the Laboratory Animal Resources Unit, Universiti Kebangsaan Malaysia (UKM), Kuala Lumpur, Malaysia were maintained in a controlled environment of 25°C with a 12-hr/12-hr light/dark cycle. Twelve hours prior to the preparation of the everted intestinal sacs, the mice were only given access to water but not food. These animals were then euthanised using the cervical dislocation technique. Everted mouse intestinal sacs were prepared according to adapted protocols (Hamilton and Butt, 2013; Mary and Rao, 2002), with the relevant animal care and use protocols approved by the UKM Animal Ethics Committee with the approval number MPOB/2016/LEOW/23-MAR./735-APR.-2016-MAR.2019-AR-CAT2.

The chemicals used for glucose uptake experiments were purchased from Sigma-Aldrich (Saint Louis, Missouri, United States of America) unless otherwise stated. Ringer's solution was used as the buffer in the glucose uptake experiments on everted mouse intestinal sacs (Luz-Madrigal *et al.*, 2015). This buffer consisted of 123 mM sodium chloride (NaCl), 1.53 mM calcium chloride (CaCl₂), 5 mM potassium chloride (KCl), 0.8 mM disodium hydrogen phosphate (Na₂HPO₄) and 0.1 mM potassium dihydrogen phosphate (KH₂PO₄) [pH 7.4, adjusted with 0.1 M hydrochloric acid (HCl) and 0.1 M sodium hydroxide (NaOH)]. SD WSPFE samples for final concentrations between 0 to 500 µg/mL were prepared. Serial dilutions of phloretin (Cayman Chemical Company, Ann Arbor, Michigan, United States of America) and phloridzin (Cayman Chemical Company, Ann Arbor, Michigan, United States of America) were also prepared as positive controls. Glucose uptake assays were conducted by introducing Ringer's solution into everted mouse intestinal sacs (inner serosal sides) and immersing these sacs into Ringer's solution containing 10 mM of D-glucose as well as each prepared concentration of either one of the positive controls or SD WSPFE (outer mucosal sides) for 30 min. These assays were conducted in a 37°C oxygenated [95% v/v oxygen (O₂) and 5% v/v carbon dioxide (CO₂)] water bath (Memmert GmbH, Buchenbach, Germany). For the measurement of glucose, samples from the inner serosal sides were collected before and after the glucose uptake assays. Glucose measurement was performed using the Glucose Oxidase Assay Kit (Sigma-Aldrich, Saint Louis, Missouri, United States of America).

Glucose Uptake of Human Colonic Caco-2 Cell Monolayers

Human colonic Caco-2 cells (ATCC® HTB-37™) (passage numbers 20-60) were utilised for

the *in vitro* cell culture experiments. Appropriate aseptic mammalian cell culture techniques were applied throughout the cell culture experiments described in this study. The complete medium used for maintenance and subculturing of the cells was Dulbecco's Modified Eagles' Medium (DMEM) containing 10% v/v fetal bovine serum (FBS) and 1% v/v non-essential amino acids, obtained from Gibco (Thermo Fisher Scientific, Waltham, Massachusetts, United States of America). Dimethyl sulphoxide (DMSO) (Sigma-Aldrich, Saint Louis, Missouri, United States of America) was added at 5% v/v to the complete medium for cell cryopreservation.

For CellTiter-Glo (CTG) luminescent cell viability assays, Caco-2 cells grown to confluence were trypsinised with Gibco trypsin ethylenediaminetetraacetic acid (EDTA) (0.25% w/v) containing phenol red (Thermo Fisher Scientific, Waltham, Massachusetts, United States of America) and centrifuged at $130 \times g$ for 5 min. Following cell counting, 75 μL of complete Gibco DMEM medium containing 2.5×10^4 cells was pipetted into each well of a 96-well white opaque tissue-culture treated microplate (SPL Life Sciences Co. Ltd., Pocheon-si, South Korea). The microplate was then incubated overnight at 37°C in a 5% v/v CO₂ incubator (NuAire, Inc., Plymouth, Minnesota, United States of America). Appropriate media controls were also prepared in other wells within the same plate. WSPFE samples were prepared the following day using complete Gibco DMEM medium containing 0.4% v/v DMSO as the vehicle. A total of 25 μL of each WSPFE sample was added into the wells of the 96-well microplates prepared previously, before being subjected to incubation at 37°C in 5% v/v CO₂ for 24 hr. WSPFE samples were tested at varying final concentrations between 0 to 2000 $\mu\text{g}/\text{mL}$ to determine the half maximal inhibitory concentration (IC₅₀) values of the samples on Caco-2 cell viability. The CTG Luminescent Cell Viability Assay Kit (Promega Corp., Madison, Wisconsin, United States of America) was used for this purpose.

The protocols used to prepare Caco-2 monolayers were adapted from literature (Hubatsch *et al.*, 2007). Each sterile filter insert in 12-well plates (Corning, New York City, New York, United States of America) was coated with 250 μL of type I collagen solution from rat tail (Sigma-Aldrich, Saint Louis, Missouri, United States of America) prepared in 0.02 M acetic acid (Merck KGaA, Darmstadt, Germany) at a coating density of 10 $\mu\text{g}/\text{cm}^2$. This coating step was carried out at 37°C for 1 hr. Excess collagen solution was then pipetted out and 400 μL of complete Gibco DMEM medium containing 20% v/v FBS, 1% v/v non-essential amino acids and 1% v/v penicillin-streptomycin was then added to each well. The plates were then incubated at 37°C for 4 hr, after which the medium was removed prior to cell seeding.

Caco-2 cells grown to confluence were then trypsinised with Gibco trypsin-EDTA (0.25% w/v) containing phenol red and centrifuged at $130 \times g$ for 5 min. Following cell counting, 500 μL of complete Gibco DMEM medium containing 3×10^5 cells was pipetted into each collagen-coated filter insert (apical layer). The bottom well (basolateral layer) was filled with 1500 μL of complete Gibco DMEM medium. Incubation was then carried out at 37°C in 5% v/v CO₂ overnight before the apical medium was replaced with fresh complete Gibco DMEM medium. Both apical and basolateral media were changed twice a week to allow cell monolayers to form and differentiate for three weeks. The integrity of the cell monolayers was assessed by measuring the transepithelial electrical resistance (TEER) using the Millicell® ERS-2 Voltohmmeter (Merck KGaA, Darmstadt, Germany). Caco-2 monolayers with TEER values $>200 \Omega \text{ cm}^2$ were used in the glucose uptake experiments.

The chemicals used for glucose uptake experiments were purchased from Sigma-Aldrich (Saint Louis, Missouri, United States of America) unless otherwise stated. One day before the glucose uptake experiments, both apical and basolateral media of the cell monolayers were discarded. The monolayers were then washed with Gibco phosphate buffered saline (Thermo Fisher Scientific, Waltham, Massachusetts, United States of America). Serum-free Gibco DMEM medium containing 1% v/v non-essential amino acids and 1% v/v penicillin-streptomycin was then added to the apical and basolateral sides. The plates were then incubated at 37°C in 5% v/v CO₂ overnight to increase glucose uptake.

The following day, WSPFE samples were dissolved in DMSO and added to serum-free Gibco DMEM medium containing 1% v/v non-essential amino acids and 1% v/v penicillin-streptomycin at a final 0.4% v/v DMSO concentration. The dissolved test compounds were then serially diluted to the concentrations required using serum-free Gibco DMEM medium containing 1% v/v non-essential amino acids, 1% v/v penicillin-streptomycin and 0.4% v/v DMSO. Serial dilutions of phloretin (Cayman Chemical Company, Ann Arbor, Michigan, United States of America) and phloridzin (Cayman Chemical Company, Ann Arbor, Michigan, United States of America) were also prepared as positive controls. Following this, the apical and basolateral media of the cell monolayers were discarded. The prepared test compounds were then added to the apical sides. Serum-free Gibco DMEM medium containing 1% v/v non-essential amino acids, 1% v/v penicillin-streptomycin and 0.4% v/v DMSO was then added to the basolateral sides. The plates were then incubated at 37°C in 5% v/v CO₂ for 1 hr in acute studies, or 20 hr in chronic studies.

Following the required incubation time, the test compounds were discarded. The apical and basolateral sides were then washed with glucose-free sodium uptake buffer, which contained 140 mM NaCl, 5 mM KCl, 2.5 mM CaCl₂, 1 mM magnesium sulphate (MgSO₄), 1 mM sodium dihydrogen phosphate (NaH₂PO₄), 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES), 1% v/v L-glutamine, 1% v/v non-essential amino acids and 1% v/v penicillin-streptomycin (pH 7.4, adjusted with 0.1 M HCl and 0.1 M NaOH). Following the wash step, 250 µL of 10 mM D-glucose solution prepared in glucose-free sodium uptake buffer was added to the apical sides, while 750 µL of the glucose-free sodium uptake buffer was added to the basolateral sides. The plates were then incubated at 37°C in 5% v/v CO₂ for 1 hr. Following this, samples from the basolateral sides were collected for glucose measurements. These glucose uptake experiments using human colonic Caco-2 cell monolayers were repeated using sodium-free uptake buffer, whereby NaCl was replaced with KCl and NaH₂PO₄ was replaced with KH₂PO₄. The Glucose Hexokinase Assay Kit (Sigma-Aldrich, Saint Louis, Missouri, United States of America) was used for glucose measurement of samples collected from the basolateral sides of the Caco-2 monolayers.

Assays on Carbohydrate Hydrolysis and Incretin Degradation Enzymes

The Alpha-Glucosidase Activity Assay Kit (Colorimetric) (ab174093) (Abcam PLC, Cambridge, United Kingdom) was utilised to perform assays on α -glucosidase, a carbohydrate-hydrolysing enzyme. Assays on another carbohydrate-hydrolysing enzyme, α -amylase, were carried out using the Alpha-Amylase Activity Assay Kit (Colorimetric) (ab102523) (Abcam PLC, Cambridge, United Kingdom). For the incretin degradation enzyme DPPIV, assays were performed using the DPPIV-Glo Protease Assay Kit (G8351) (Promega Corp., Madison, Wisconsin, United States of America) and the recombinant human DPPIV high purity dimer enzyme (R&D Systems, Inc., Minneapolis, Minnesota, United States of America).

WSPFE samples were tested at varying final concentrations between 0 to 500 µg/mL to determine the IC₅₀ values of the samples on α -glucosidase, α -amylase and DPPIV. Negative control wells containing the substrate and enzyme without inhibitor samples, positive control wells containing the substrate, enzyme and the positive control inhibitor, as well as colour control samples which functioned as blanks for the corresponding samples were also prepared in these experiments. Acarbose (Sigma-Aldrich, Saint Louis, Missouri, United States of America) was used as the positive

control inhibitor in the α -glucosidase and α -amylase assays, while sitagliptin phosphate (Cayman Chemical Company, Ann Arbor, Michigan, United States of America) was used as the positive control inhibitor in the DPPIV assays. Acarbose which is a pseudo-tetrasaccharide obtained from the fermentation processes of the microorganism *Actinoplanes utahensis* is commonly used as an oral reversible competitive inhibitor of α -glucosidase and α -amylase enzymes in T2DM patients (Rosak and Mertes, 2012; Yee and Fong, 1996). On the other hand, sitagliptin which is a synthesised beta-amino amide incorporating fused heterocycles called triazolopiperazines is an oral reversible DPPIV inhibitor that is generally well tolerated when administered in T2DM patients (Bergman *et al.*, 2007; Kim *et al.*, 2005).

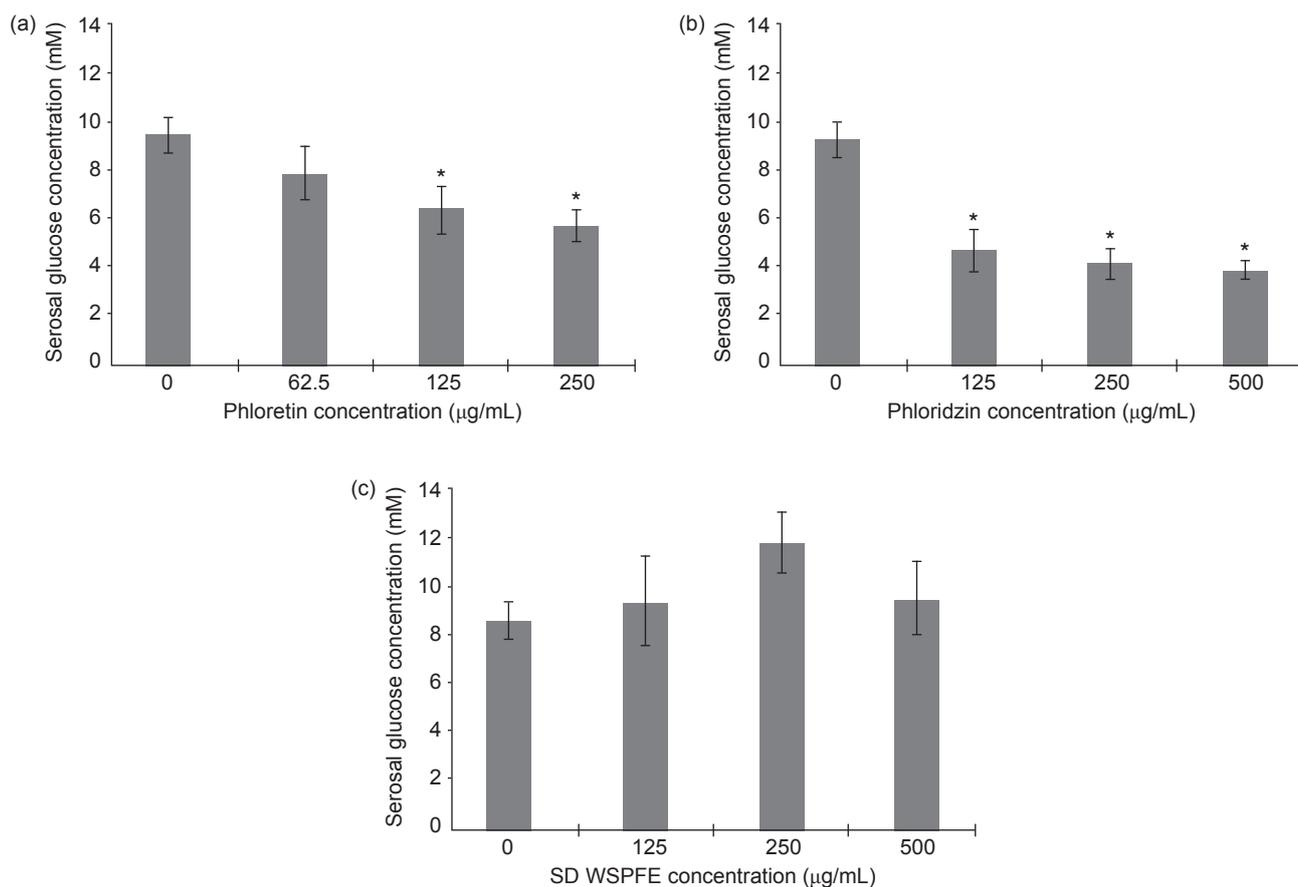
Statistical Analyses

Measurements of all enzymatic assays were carried out using the Tecan Infinite M200 microplate reader (Tecan Group Ltd., Zurich, Switzerland). Raw data was exported into the Microsoft Excel software (Microsoft Corp., Redmond, Washington, United States of America) for analysis. Data were expressed as means \pm standard error of the mean (SEM). Statistical analyses on the results obtained were performed using the SPSS Statistics software (IBM Corp., Armonk, New York, United States of America). One-way analysis of variance (ANOVA) with Tukey's honestly significant difference (HSD) *post-hoc* test or Dunnett's *post-hoc* test against Negative Control where appropriate was carried out. Differences were considered statistically significant at $p < 0.05$. IC₅₀ values were calculated using the Quest Graph™ IC₅₀ Calculator (AAT Bioquest, Inc., Sunnyvale, California, United States of America) (AAT Bioquest, 2019).

RESULTS AND DISCUSSION

Glucose Uptake of Everted Mouse Intestinal Sacs

Ex vivo everted mouse intestinal sac preparation protocols from previous studies (Hamilton and Butt, 2013; Mary and Rao, 2002) were adapted to elucidate the possible effects of WSPFE samples on glucose transport, since this model is considered to be one of the efficient tools used for studying intestinal absorption (Alam *et al.*, 2012). However, initial experiments conducted on SD WSPFE samples using *ex vivo* everted mouse intestinal sacs showed that while positive controls phloretin and phloridzin inhibited glucose transport from the mucosal to the serosal sides of the everted mouse intestinal sacs, SD WSPFE did not have any glucose absorption inhibition effects up to



Note: Error bars indicate standard error of the mean (SEM); $n=5$ biological replicates; * $p<0.05$ by one-way analysis of variance (ANOVA) with Dunnett's *post-hoc* test against Negative Control (0 µg/mL). SD - spray dried; WSPFE - Water-Soluble Palm Fruit Extract.

Figure 1. Glucose uptake assay results on everted mouse intestinal sacs using positive controls and SD WSPFE of different doses as measured using the absorbance-based Glucose Oxidase Assay Kit over the assay time period of 30 min after incubation with the respective test samples for 30 min. (a) phloretin, (b) phloridzin, and (c) SD WSPFE.

500 µg/mL (Figure 1). This suggested that the anti-diabetic effects of SD WSPFE were not due to inhibition of glucose absorption in the intestines. To further confirm this and to reduce the use of animals, another model of the intestinal barrier, the *in vitro* Caco-2 cell monolayer model, was utilised for glucose uptake evaluation. In addition, the glucose hexokinase assay was subsequently used for glucose measurements in the present study, since previous investigations had demonstrated the sensitivity and accuracy of this assay, which results from reduction of colour interference by plant phenolic compounds (Xu *et al.*, 2012).

Preparation of WSPFE Fractions

Further glucose transport investigations were conducted using *in vitro* Caco-2 cell monolayers. In addition to SD WSPFE, additional WSPFE samples were tested in this set of experiments, including FD WSPFE, WSPFE EAF, as well as seven individual WSPFE fractions (F1-F7) isolated using preparative HPLC. These seven individual fractions were prepared as previously described (Leow *et al.*,

2021b) and identification of the major components of the fractions was based on information obtained from our previous published work on WSPFE. F1 contained shikimic acid (Sambandan *et al.*, 2011), F3 contained protocatechuic acid (Sambanthamurthi *et al.*, 2011a), F4 contained *p*-hydroxybenzoic acid (Sambanthamurthi *et al.*, 2011a), F5 contained an indoleacetic acid derivative (Sambanthamurthi *et al.*, 2014), while F6 contained three caffeoylshikimic acid isomers (Sambanthamurthi *et al.*, 2011a). The components of F2 and F7 are presently unknown.

Caco-2 Cell Viability Assays

In cell viability and cytotoxicity studies, the IC_{50} of a compound defines its maximum non-toxic dose (Neubig *et al.*, 2003). IC_{50} thus, acts as a parameter to determine the maximum concentration of a tested compound which could be used to produce desirable effects without exceeding the range of acceptable toxicity. The IC_{50} value of a tested compound on cell lines is determined by the concentration needed to inhibit half of the maximum viability of the cell lines.

In the present study, before carrying out glucose uptake assays on Caco-2 cell monolayers, the IC₅₀ values of the WSPFE samples on the viability of the cell line were determined using the CTG Luminescent Cell Viability Assay Kit. The positive control phloridzin, SD WSPFE, FD WSPFE, WSPFE EAF and F1 were not cytotoxic to Caco-2 cells after incubation up to 2000 µg/mL for 24 hr (Table 1). On the other hand, the positive control phloretin and F2 to F7 showed cytotoxicity on the Caco-2 cells after incubation for 24 hr. F2 had an IC₅₀ value of 1265 ± 107 µg/mL, while F3 had an IC₅₀ value of 544 ± 48 µg/mL. The IC₅₀ value of F4 was 1007 ± 99 µg/mL, while F5 had an IC₅₀ value of 666 ± 107 µg/mL. F6 had an IC₅₀ value of 571 ± 178 µg/mL, while the last fraction, F7, had an IC₅₀ value of 737 ± 78 µg/mL. The positive control phloretin was most toxic to the Caco-2 cells, with an IC₅₀ value of 131 ± 31 µg/mL.

As such, the levels of cytotoxicity of WSPFE samples on Caco-2 cells followed the order of SD WSPFE = FD WSPFE = WSPFE EAF = F1 < F2 < F4 < F7 < F5 < F6 < F3. The results obtained suggested that the concentrations of WSPFE samples to be used in the glucose uptake experiments should

TABLE 1. IC₅₀ VALUES OF WSPFE SAMPLES AGAINST CACO-2 CELL VIABILITY

Sample	IC ₅₀ (µg/mL)
SD WSPFE	*
FD WSPFE	*
WSPFE EAF	*
F1	*
F2	1 265 ± 107 ^a
F3	544 ± 48 ^{bc}
F4	1 007 ± 99 ^{ab}
F5	666 ± 107 ^b
F6	571 ± 178 ^{bc}
F7	737 ± 78 ^b
Phloretin	131 ± 31 ^c
Phloridzin	*

Note: IC₅₀ indicates the dose that induced a 50% cell viability inhibition compared to Negative Control as measured using the luminescence-based CTG Luminescent Cell Viability Assay Kit over 10 min after incubation with the respective test samples up to 2000 µg/mL for 24 hr. These IC₅₀ values were expressed as means ± standard error of the mean (SEM) from three biological replicates. Means in a column with different letters are significantly different (*p*<0.05) by one-way analysis of variance (ANOVA) with Tukey's honestly significant difference (HSD) *post-hoc* test. *indicates IC₅₀ was not achieved at the highest concentration tested (2000 µg/mL). EAF - ethyl acetate fraction; F - fraction; FD - freeze dried; SD - spray dried; WSPFE - Water-Soluble Palm Fruit Extract.

be up to 500 µg/mL, as the IC₅₀ value of the most cytotoxic WSPFE sample, F3, was 544 ± 48 µg/mL. This would help to determine the effects of these samples in influencing glucose absorption, by discounting their effects on cell viability. Caco-2 cell monolayers have been widely used to determine the effects of dietary phenolic compounds on glucose transport (Alzaid *et al.*, 2013; Farrell *et al.*, 2013; Johnston *et al.*, 2005; Manzano and Williamson, 2010), as these cells abundantly express the active sodium-dependent glucose transporter 1 (SGLT1) and facilitated glucose transporter 2 (GLUT2) proteins (Sambuy *et al.*, 2005). Nevertheless, it should be noted that Caco-2 is a human cell line derived from human colon adenocarcinoma cancer tissues, and thus, these immortalised cell lines may have their own characteristics which may not be typical of primary cultures.

It is also interesting to note that the toxicity effects of F2 to F7 at various concentrations suggest the potential use of these fractions against colon cancer. Many studies have shown that phenolic acids were able to inhibit cancer (Abotaleb *et al.*, 2020), including colon cancer (Rosa *et al.*, 2016). The cell viability results obtained in the present study showed that F3 containing protocatechuic acid was more cytotoxic to the Caco-2 cells as compared to fractions containing the caffeoylshikimic acid isomers, the indoleacetic acid derivative and *p*-hydroxybenzoic acid. Protocatechuic acid was shown to be a potential cancer chemopreventive agent (Tanaka *et al.*, 2011), as it exerted pro-apoptotic and anti-proliferative effects (Kakkar and Bais, 2014). 3-O-caffeoylshikimic acid and 5-O-caffeoylshikimic acid from the fruits of the Chinese fan or fountain palm (*Livistona chinensis*) were shown to have potent anti-proliferative activities against several cancer cell lines (Zeng *et al.*, 2012). Indoleacetic acid was also found to induce cancer cell death in combination with horseradish peroxidase (Wardman, 2002) or ultraviolet B irradiation (Kim *et al.*, 2010). Derivatives of *p*-hydroxybenzoic acid also exhibited anti-cancer activities (Seidel *et al.*, 2014).

Key structural motifs in phenolic acids responsible for anti-cancer activities include the aromatic ring, number and position of free hydroxyl groups and unsaturated fatty acid chains (Anantharaju *et al.*, 2016). The simpler structure of protocatechuic acid containing an aromatic ring attached with one free hydroxyl group might have contributed to the cytotoxicity effects of F3, but future *in silico* molecular docking experiments would be helpful to better explain the structure-activity relationships between the individual compounds present in WSPFE and potential anti-cancer molecular targets (Chen *et al.*, 2012). However, it would be important to note that F1-F7 used in the present study were not

pure compounds, but only fractions enriched in the compounds mentioned. Therefore, their effects might not only be attributable to the compounds, but also to unidentified non-phenolic compounds and/or the complex nature of these fractions and interactions between phytochemicals present in them.

The cytotoxic properties of the WSPFE samples demonstrated in the present study are also consistent with the anti-cancer effects of WSPFE previously identified, in which FD WSPFE exhibited inhibitory effects on human A549 lung carcinoma, human MCF7 breast adenocarcinoma and mouse J558 immunoglobulin A-secreting myeloma cell lines (Sekaran *et al.*, 2010) when tested up to 2000 µg/mL. Nevertheless, FD WSPFE did not have an effect on the cell viability of the Caco-2 cells used in the present study when tested up to 2000 µg/mL. This suggests that FD WSPFE may not be as effective against human colon adenocarcinoma cancer as compared to other types of cancer. As such, additional experiments in the future on other types of colon cancer cell lines and on normal colon cell lines would be required to further supplement the present findings.

Glucose Uptake of Human Colonic Caco-2 Cell Monolayers

The uptake of glucose in the gastrointestinal tract occurs via the enterocyte membrane transporters SGLT1 and GLUT2. SGLT1 is involved in active glucose transport from the apical side of the intestinal lumen into enterocytes, while GLUT2 is involved in facilitated glucose diffusion from the basolateral side of the intestinal lumen into the hepatic portal vein (Scheepers *et al.*, 2004).

In the present study, positive controls phloretin and phloridzin dose-dependently inhibited glucose transport from the apical sides to the basolateral sides of the Caco-2 monolayers when tested in both sodium uptake buffer (Figure 2) and sodium-free uptake buffer (Figure 3). When tested using sodium uptake buffer, the inhibitory effects of phloretin on glucose uptake were more pronounced during acute treatment (Figure 2a), while those of phloridzin were more pronounced during chronic treatment (Figure 2b). On the other hand, SD WSPFE and FD WSPFE did not show any glucose absorption inhibition effects up to 500 µg/mL during acute treatment (Figure 2c) or chronic treatment (Figure 2d). In addition, glucose uptake assays carried out using WSPFE EAF and F1-F7 up to 500 µg/mL also did not show any effects of the WSPFE fractions on glucose uptake during acute treatment (Figure 2e) or chronic treatment (Figure 2f). Similar results were obtained when glucose uptake assays were performed on the Caco-2 cell monolayers using sodium-free uptake buffer (Figures 3a-f). These

results implied that the anti-diabetic effects of WSPFE were not due to glucose uptake inhibition, and hence other mechanisms might be involved instead.

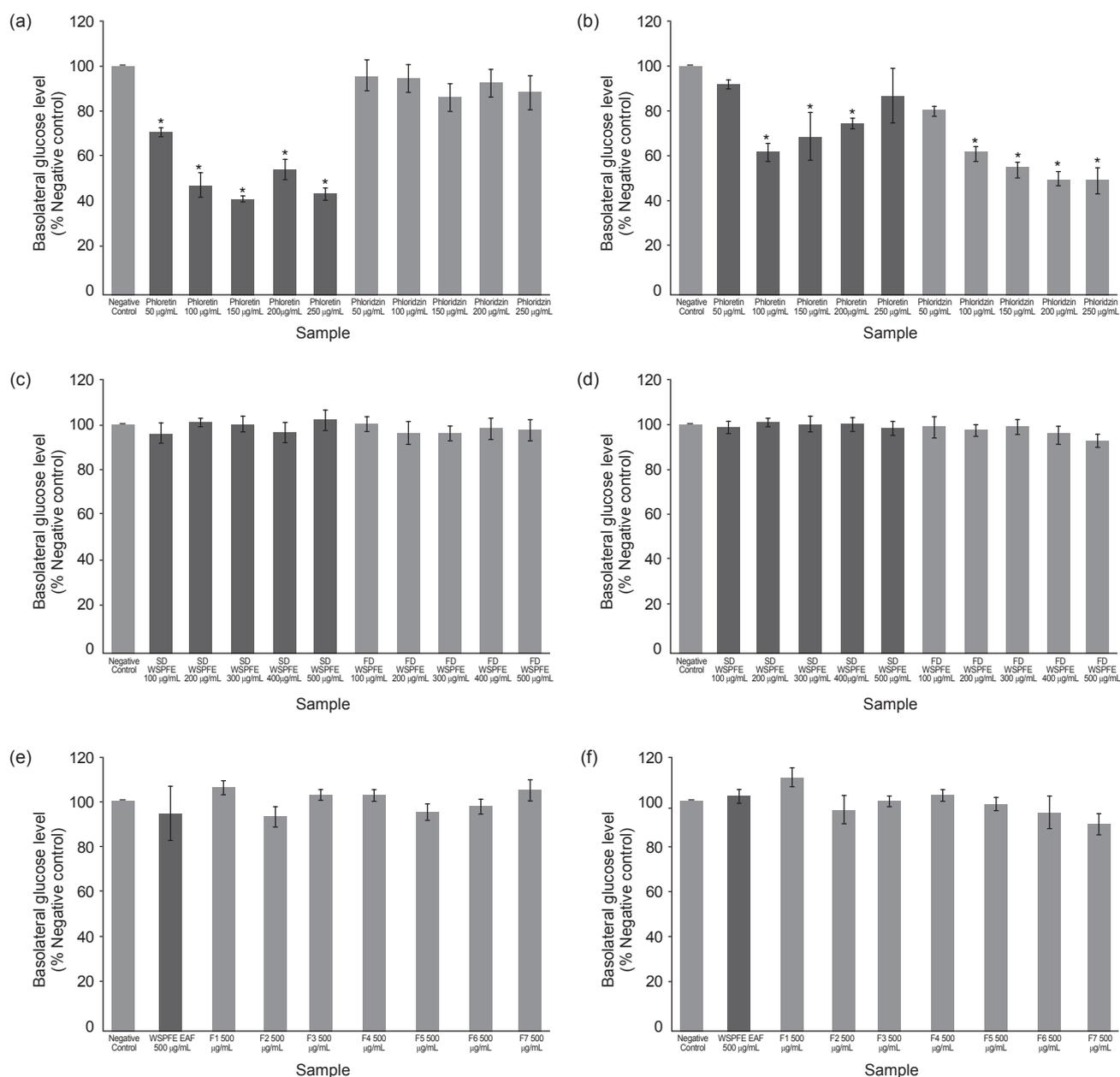
Although phloretin is known to inhibit GLUT2 and phloridzin is known to inhibit SGLT1 (and also renal SGLT2) (Ehrenkranz *et al.*, 2005), we did not find any differences in terms of the inhibitory patterns of these positive controls when using sodium uptake buffer or sodium-free uptake buffer in the present study (Figures 2a-b and 3a-b). The strong acute inhibitory effects of phloretin on glucose uptake compared to the strong chronic inhibitory effects of phloridzin observed in the present study would most likely be due to the degradation of phloridzin to its phloretin and glucose moieties (Ehrenkranz *et al.*, 2005), which increased the availability of phloretin in inhibiting glucose uptake of the Caco-2 cell monolayers.

Assays on Carbohydrate Hydrolysis and Incretin Degradation Enzymes

The IC₅₀ results obtained from the α-glucosidase assays showed that F2, WSPFE EAF, F1, F5, F3 and F4 inhibited this enzyme more significantly than the positive control acarbose, while SD WSPFE, FD WSPFE, F6 and F7 did not (Table 2). F2 and WSPFE EAF inhibited α-glucosidase the most, with F2 having stronger inhibitory effects compared to WSPFE EAF, as indicated by their IC₅₀ values of 162 ± 23 µg/mL and 179 ± 11 µg/mL, respectively.

The IC₅₀ results obtained from the α-amylase assays showed that WSPFE EAF, SD WSPFE, F7, FD WSPFE and F5 inhibited this enzyme, but their inhibitory activities were significantly weaker than that of the positive control acarbose by several orders of magnitude. On the other hand, F1, F2, F3, F4 and F6 did not inhibit α-amylase (Table 2). Among the WSPFE samples which inhibited α-amylase, WSPFE EAF inhibited the enzyme the most, as indicated by its lowest IC₅₀ value of 108 ± 13 µg/mL. This was followed by SD WSPFE and F7, as indicated by their IC₅₀ values of 158 ± 1 µg/mL and 160 ± 2 µg/mL, respectively. As such, WSPFE EAF which contained all the seven individual WSPFE fractions had the strongest inhibitory effects on this enzyme, as compared to whole SD WSPFE and FD WSPFE samples. Nevertheless, SD WSPFE had stronger inhibitory effects on α-amylase as compared to FD WSPFE. In terms of individual fractions, F7 followed by F5 inhibited α-amylase the most, but their inhibitory effects were generally weaker than that of WSPFE EAF.

Acarbose is a known anti-hyperglycaemic agent which acts by inhibiting starch digestion enzymes and is widely used in combination with other anti-diabetic drugs (Derosa and Maffioli, 2012). Acarbose is known to inhibit α-amylase

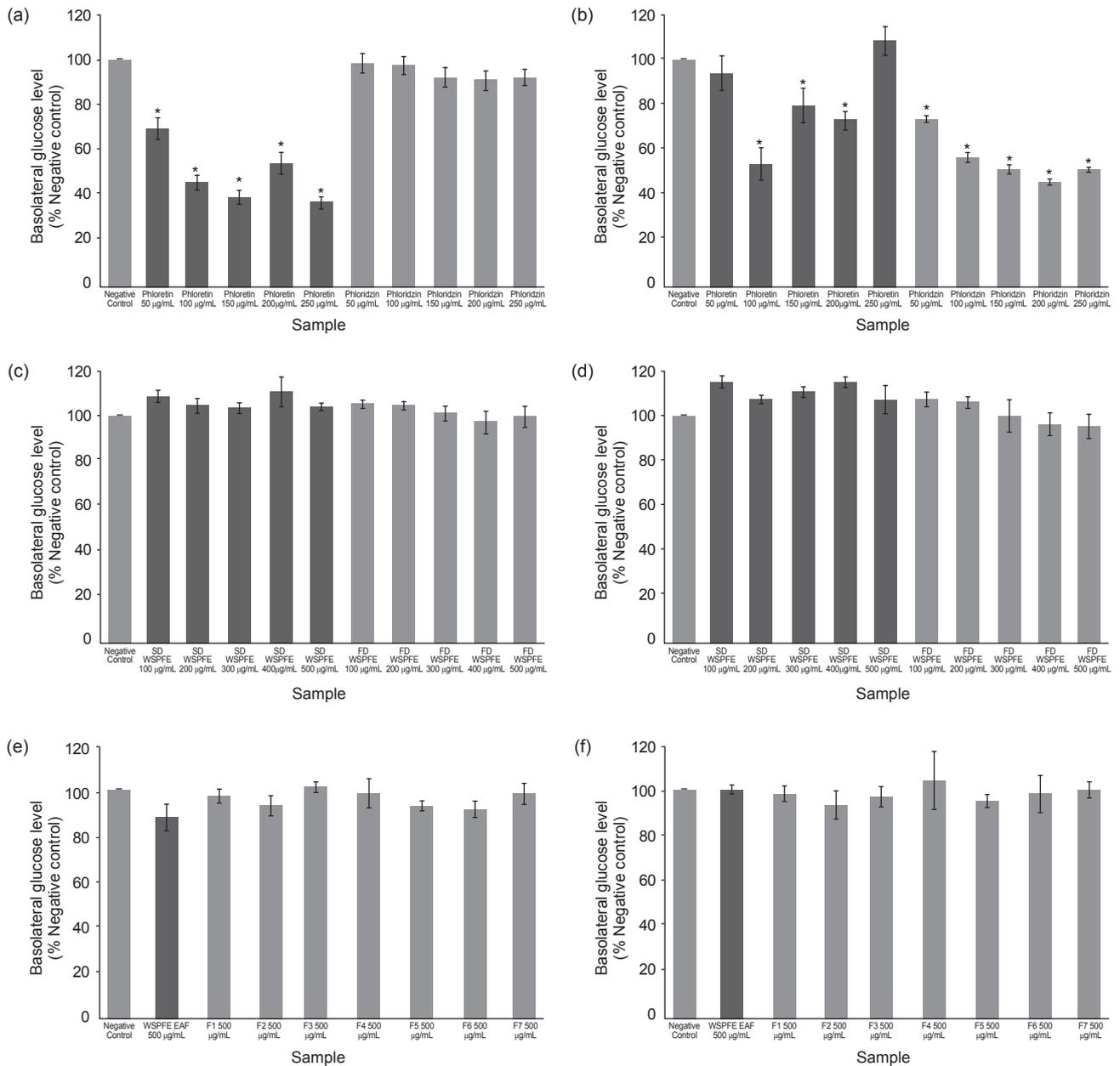


Note: Error bars indicate standard error of the mean (SEM); $n=3$ biological replicates; * $p<0.05$ by one-way analysis of variance (ANOVA) with Dunnett's *post-hoc* test against Negative Control. EAF - ethyl acetate fraction; F - fraction; FD - freeze dried; SD - spray dried; WSPFE - Water-Soluble Palm Fruit Extract.

Figure 2. Glucose uptake assay results on human colonic Caco-2 cell monolayers in sodium uptake buffer as measured using the absorbance-based Glucose Hexokinase Assay Kit over the assay time period of 30 min after incubation with the respective test samples. (a) Different doses of positive controls phloretin and phloridzin in 1-hr acute treatment, (b) different doses of positive controls phloretin and phloridzin in 20-hr chronic treatment, (c) different doses of SD WSPFE and FD WSPFE in 1-hr acute treatment, (d) different doses of SD WSPFE and FD WSPFE in 20-hr chronic treatment, (e) WSPFE EAF and F1-F7 (500 µg/mL) in 1-hr acute treatment, and (f) WSPFE EAF and F1-F7 (500 µg/mL) in 20-hr chronic treatment.

significantly more than it inhibits α -glucosidase (Oboh *et al.*, 2016). However, strong α -amylase inhibitors such as acarbose cause unpleasant side effects including flatulence, bloating and abdominal distention, due to suppression of starch digestion and bacterial fermentation in the gut (Yee and Fong, 1996). As such, anti-hyperglycaemic agents, drugs or phytochemicals should ideally have mild α -amylase inhibition but moderate α -glucosidase

inhibition, in order to minimise their side effects. In line with this, bound polyphenol fractions from date (*Phoenix dactylifera* L.) seeds for example, were found to have strong α -glucosidase inhibition but weak α -amylase inhibition (Sirisena *et al.*, 2016). Similar results were found for ethyl acetate extract from the roots of *Smilax glabra* Roxb, an Asian medicinal plant used for the treatment of various chronic diseases (Nguyen *et al.*, 2020b).



Note: Error bars indicate standard error of the mean (SEM); $n=3$ biological replicates; * $p<0.05$ by one-way analysis of variance (ANOVA) with Dunnett's *post-hoc* test against Negative Control. EAF - ethyl acetate fraction; F - fraction; FD - freeze dried; SD - spray dried; WSPFE - Water-Soluble Palm Fruit Extract.

Figure 3. Glucose uptake assay results on human colonic Caco-2 cell monolayers in sodium-free uptake buffer as measured using the absorbance-based Glucose Hexokinase Assay Kit over the assay time period of 30 min after incubation with the respective test samples. (a) Different doses of positive controls phloretin and phloridzin in 1-hr acute treatment, (b) different doses of positive controls phloretin and phloridzin in 20-hr chronic treatment, (c) different doses of SD WSPFE and FD WSPFE in 1-hr acute treatment, (d) different doses of SD WSPFE and FD WSPFE in 20-hr chronic treatment, (e) WSPFE EAF and F1-F7 (500 µg/mL) in 1-hr acute treatment, and (f) WSPFE EAF and F1-F7 (500 µg/mL) in 20-hr chronic treatment.

In the present study, SD WSPFE and FD WSPFE inhibited α -amylase but not α -glucosidase. These results indicate that the anti-diabetic effects of WSPFE may be due to α -amylase inhibition and not α -glucosidase inhibition, especially more so when SD WSPFE is supplemented. WSPFE EAF and most of the individual WSPFE fractions, as compared to the positive control acarbose, had stronger inhibitory effects on α -glucosidase but weaker inhibitory effects on α -amylase. Several

of the individual WSPFE fractions, *i.e.*, F1-F5, also preferably inhibited α -glucosidase than α -amylase, hence these fractions might be better anti-diabetic oral supplement candidates worthy of further investigations.

The IC_{50} results obtained from the DPPIV assays showed that F2, F5, F4, WSPFE EAF and F3 inhibited this enzyme, while SD WSPFE, FD WSPFE, F1, F6 and F7 did not (Table 2). F2 inhibited DPPIV the most, as indicated by its lowest IC_{50}

TABLE 2. IC₅₀ VALUES OF WSPFE SAMPLES AGAINST α -GLUCOSIDASE, α -AMYLASE AND DPPIV

Sample	IC ₅₀ (μ g/mL)		
	α -glucosidase	α -amylase	DPPIV
SD WSPFE	*	158 \pm 1 ^b	*
FD WSPFE	*	232 \pm 13 ^a	*
WSPFE EAF	179 \pm 11 ^b	108 \pm 13 ^b	224 \pm 6 ^b
F1	210 \pm 10 ^b	*	*
F2	162 \pm 23 ^b	*	162 \pm 7 ^c
F3	213 \pm 18 ^b	*	291 \pm 16 ^a
F4	214 \pm 18 ^b	*	196 \pm 2 ^{bc}
F5	210 \pm 13 ^b	239 \pm 29 ^a	185 \pm 4 ^c
F6	*	*	*
F7	*	160 \pm 2 ^b	*
Positive Control	440 \pm 64 ^a	0.324 \pm 0.016 ^c	0.006333 \pm 0.000384 ^d

Note: IC₅₀ indicates the dose that induced a 50% enzymatic inhibition compared to Negative Control as measured using the respective enzymatic assays kits over 60 min. These IC₅₀ values were expressed as means \pm standard error of the mean (SEM) from three technical replicates. Means in a column with different letters are significantly different ($p < 0.05$) by one-way analysis of variance (ANOVA) with Tukey's honestly significant difference (HSD) *post-hoc* test. * indicates IC₅₀ was not achieved at the highest concentration tested (500 μ g/mL). Acarbose was used as the positive control for α -glucosidase and α -amylase assays, while sitagliptin was used as the positive control for DPPIV assays. DPPIV - dipeptidyl peptidase-4; EAF - ethyl acetate fraction; F - fraction; FD - freeze dried; SD - spray dried; WSPFE - Water-Soluble Palm Fruit Extract.

value of 162 \pm 7 μ g/mL. This was followed by F5 and F4, as indicated by their IC₅₀ values of 185 \pm 4 μ g/mL and 196 \pm 2 μ g/mL, respectively. WSPFE EAF which contained all the seven individual WSPFE fractions was also found to have better inhibitory effects on DPPIV, as compared to SD WSPFE and FD WSPFE. In terms of individual fractions, F2 followed by F5, F4 and F3 inhibited DPPIV the most with their inhibitory effects being generally stronger than those of WSPFE EAF, except for F3. However, the inhibitory effects of all the WSPFE samples towards DPPIV were several orders of magnitude less effective than that of the positive control sitagliptin, which had an IC₅₀ value of 0.006333 \pm 0.000384 μ g/mL.

The incretin effect, which refers to the amplification of insulin secretion after oral *vs.* intravenous glucose, is responsible for the disposal of most ingested glucose and is hence, essential for glucose tolerance. It is mainly due to the actions of the gut hormones GIP and GLP-1. GIP is essential for insulin secretion, while GLP-1 is important for inhibition of glucagon secretion (Holst, 2019). DPPIV inhibitors help to protect both GIP and GLP-1 from degradation, hence suppress postprandial glucagon release, delay gastric emptying and regulate satiety (Singh *et al.*, 2017). Natural phenolic compounds such as curcumin have been shown to reduce blood glucose via DPPIV inhibition as one of their mechanisms (Huang *et al.*, 2019).

In the present study, WSPFE EAF and most of the individual WSPFE fractions inhibited DPPIV, although their inhibitory effects were several orders of magnitude less effective than that of the positive control sitagliptin. Clinical studies to date indicate that DPPIV inhibitors increase native GLP-1 to effectively improve glucose control. These agents have low adverse effects and are well tolerated (Ling *et al.*, 2019), hence they could be better than α -glucosidase inhibitors for improved glycaemic control and lower gastrointestinal side effects in T2DM patients (Li *et al.*, 2019).

Among the individual WSPFE fractions, F2 demonstrated the strongest inhibitory effects against both DPPIV and α -glucosidase, but not α -amylase. However, the components in F2 are presently unknown. On the other hand, F7 only inhibited α -amylase, similar to SD WSPFE and FD WSPFE, but the components of F7 are also unknown. Further studies on the components in these two fractions are warranted.

F1 which contained shikimic acid was found to only inhibit α -glucosidase in the present study, although shikimic acid itself was found inactive as an α -glucosidase inhibitor in a previous study (Pham *et al.*, 2014). However, this discrepancy might be because a fraction, and not pure shikimic acid was used in the present study. In line with this, *Juniperus oxycedrus* subsp. *oxycedrus* berry extracts containing shikimic acid were previously shown to be hypoglycaemic (Orhan *et al.*, 2012).

F5 which contained an indoleacetic acid derivative was shown to inhibit all three α -glucosidase, α -amylase and DPPIV enzymes, in the present study. However, its quantity in WSPFE is the most limited. Indole compounds, which are aromatic nitrogen-containing heterocyclic organic compounds each consisting of a six-membered benzene ring fused to a five-membered pyrrole ring, have been found to have potential anti-diabetic activities (Zhu *et al.*, 2021), including α -glucosidase and α -amylase inhibitory properties (Sravanthi *et al.*, 2017).

F4 containing *p*-hydroxybenzoic acid and F3 containing protocatechuic acid had lower α -glucosidase and DPPIV inhibitory activities compared to F5. These phenolic acids have two and one hydroxyl groups attached to benzoic acid, respectively. *p*-hydroxybenzoic acid was shown to possess α -glucosidase inhibitory activities in a previous study (Ablat *et al.*, 2017). On the other hand, protocatechuic acid was previously shown to inhibit α -amylase more than α -glucosidase (Adefegha *et al.*, 2015). But this might be because a fraction, and not pure protocatechuic acid was used in the present study.

For α -glucosidase inhibition, hydroxyl groups in phenolic compounds such as flavonoids play an important role in binding with α -glucosidase (Sun and Miao, 2020). On the other hand, methyl and acetate groups decrease this binding (Sarian *et al.*, 2017). For DPPIV inhibition, hydrogen bonding was found to be the main binding mode of all berry and citrus phenolic compounds tested in a previous study (Fan *et al.*, 2013). It would again be helpful to perform *in silico* molecular docking experiments in the future to identify the molecular mechanistics of enzymatic inhibition by the major compounds in WSPFE, as well as the structure-activity relationships of individual compounds with these enzymes (Kim *et al.*, 2018; Rasouli *et al.*, 2017).

The α -amylase inhibitory activities of WSPFE samples found in the present study might be caused by non-phenolic rather than the phenolic compounds present in WSPFE samples, in line with a previous study on extracts obtained from bioprocessed pineapple and guava wastes, whereby α -amylase inhibition was caused by the presence of specific compounds, phenolic or not, rather than by the actual concentration of overall fruit phenolics (Sousa and Correia, 2012). Nevertheless, there might still be direct interactions between dietary plant phenolic compounds on starch properties and starch digestion (Sun and Miao, 2020). These might alter starch physical properties and micromolecular arrangements, such as its morphology, hydrogen bond intensity, crystalline structure, thermal stability and spatial configuration (Chen *et al.*, 2020).

F6 which contained three caffeoylshikimic acid isomers did not show inhibitory activities against α -glucosidase, α -amylase or DPPIV. This was in contrast with a previous study which showed that kitul palm (*Caryota urens* L.) inflorescence extract containing caffeoylshikimic acid isomers had significant α -glucosidase and α -amylase inhibitory properties (Ferrerres *et al.*, 2021). However, these kitul extracts not only contained caffeoylshikimic acid isomers, but caffeoylquinic acid isomers as well, the latter of which have been shown to have inhibitory effects towards carbohydrate-hydrolysing enzymes (Nyambe-Silavwe and Williamson, 2018).

Bioactivity Comparison Summary of Various WSPFE Samples

Different forms of plant phenolic compounds may confer different anti-diabetic effects. These mechanisms may also vary from one extract or component to another and they may thus, be harnessed to maximise the anti-diabetic effects of these compounds. In the present study, all of the WSPFE samples tested did not inhibit glucose transport, but several had inhibitory effects on α -glucosidase, α -amylase and DPPIV. It is interesting to note that SD WSPFE had stronger inhibitory effects on α -amylase as compared to FD WSPFE. Drying is a major food processing operation to increase shelf life. The choice of drying method however influences product quality, as it is related to the retention of bioactive compounds and biological activities (Abascal *et al.*, 2005). In spray drying, moisture is removed by rapid evaporation on spray droplet under high temperature exposure using hot gas. It is the preferred drying method for thermally sensitive materials such as foods and pharmaceuticals (Verma and Singh, 2015). Conversely, freeze drying involves water removal by sublimation under low pressure (Santo *et al.*, 2013), where much water is removed. This makes a product lightweight, prevents the survival of yeast and bacteria in it, as well as retains its taste, shape and appearance when water is reintroduced. However, freeze drying equipment is expensive, while the process is very time-consuming and labour-intensive. The results obtained in the present study suggest that the spray drying technique is preferable compared to the freeze drying technique in order to retain the potential anti-diabetic bioactivities of WSPFE.

On the other hand, WSPFE EAF had stronger inhibitory effects in all three enzymatic assays carried out, as compared to both SD WSPFE and FD WSPFE. WSPFE contains various components including phenolic acids, indoleacetic acid derivative, shikimic acid, fibre, *etc.*, while its EAF had a higher phenolic content (Leow *et al.*, 2021b).

When compared to most individual WSPFE fractions, WSPFE EAF also consistently inhibited all the three enzymes tested, the other being F5. This suggests that the seven individual WSPFE fractions when given together as WSPFE EAF had additive or synergistic inhibitory effects against these enzymes and may work better in attenuating diabetes as compared to giving individual WSPFE fractions, or even whole WSPFE samples. It was also interesting to note that while WSPFE EAF consistently inhibited all three enzymes tested in the present study, it was not cytotoxic to the Caco-2 colon cancer cells, although additional experiments on normal colon cell lines would be useful to indicate the implied safety of WSPFE EAF.

In terms of individual WSPFE fractions, F2 demonstrated the strongest inhibitory effects towards α -glucosidase and DPPIV, but not α -amylase. This individual fraction should be explored further in future anti-diabetic studies, but its possible cytotoxic effects must be taken into account when designing future experiments. It is again important to emphasise here that F1-F7 were enriched in certain phenolic compounds as mentioned previously and that their effects might also be due to other compounds besides those described.

The anti-diabetic mechanisms investigated in the present study are only a fraction of what is possible. Hence, it would be important that other possible anti-diabetic mechanisms besides those explored in the present study, such as hormonal response to food and modulation of gut flora activities, be investigated as well (Cuervo *et al.*, 2014; Hanhineva *et al.*, 2010; Moco *et al.*, 2012). As such, further pre-clinical studies which investigate the effects of WSPFE using T2DM animal models such as the Nile rat would be useful to explore other *in vivo* anti-diabetic mechanisms. Human intervention clinical trials administering different forms of carbohydrates in healthy volunteers, such as starch, disaccharides and glucose, together with WSPFE, such as that done by Kerimi *et al.* (2019) using oleuropein from olives, would also be useful to confirm the inhibitory effects of WSPFE on carbohydrate-hydrolysing enzymes *in vivo*. It would also be important to carry out studies on postprandial absorption and bioavailability of WSPFE after human consumption *in vivo* to determine its potential anti-diabetic effects on other types of functional insulin-sensitive tissues post-absorption, such as liver, muscle and adipose tissues (Sun and Miao, 2020). Nevertheless, although direct measurements have not been done to confirm the bioavailability of WSPFE, various *in vivo* physiological effects previously observed in animal models and human volunteers as reviewed by Leow *et al.* (2021a) indicate that WSPFE is bioavailable to exert these health functions.

CONCLUSION

Our previous studies in Nile rats demonstrated the potential anti-diabetic properties of WSPFE, but the related mechanisms were unknown. The present study indicated that WSPFE samples did not inhibit glucose uptake, as shown through experiments using *ex vivo* everted mouse intestinal sacs and *in vitro* Caco-2 cell monolayers. The concentrations of WSPFE samples used in the glucose uptake experiments were only up to 500 $\mu\text{g}/\text{mL}$, as the IC_{50} value of the most cytotoxic WSPFE sample against Caco-2 cells, F3, was $544 \pm 48 \mu\text{g}/\text{mL}$. Further experiments on α -glucosidase, α -amylase and DPPIV enzymes showed that, WSPFE EAF which contained all seven individual WSPFE fractions, consistently had stronger inhibitory effects as compared to SD WSPFE and FD WSPFE. SD WSPFE had better α -amylase inhibitory effects than FD WSPFE. In terms of individual fractions, F2 demonstrated the strongest inhibitory effects against α -glucosidase and DPPIV. Future studies to investigate the effects of these WSPFE samples on carbohydrate digestion and postprandial hyperglycaemia are thus warranted.

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