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INHIBITION OF CHOLINESTERASES BY WATER-SOLUBLE PALM FRUIT EXTRACT

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ABSTRACT

Cholinesterase (ChE) inhibitors are used for the symptomatic treatment of Alzheimer's disease and other neurological pathologies. There is interest in developing new ChE inhibitors from natural plant compounds. Water-Soluble Palm Fruit Extract (WSPFE) recovered from the aqueous oil palm vegetation liquor is rich in phenolic acids and has potential neuroprotective effects. Here, we investigated the effects of WSPFE samples on acetylcholinesterase (AChE) and butyrylcholinesterase (BChE). WSPFE ethyl acetate fraction (EAF) inhibited these enzymes the most (AChE IC_{50}: 0.218 ± 0.029 µg mL^{-1}; BChE IC_{50}: 222.860 ± 5.777 µg mL^{-1}) and had the highest AChE selectivity index (SI) value (1022.294) compared to whole samples and seven individual fractions but these effects were weaker than those of the AChE selective agent donepezil hydrochloride (DH) (AChE IC_{50}: 0.013 ± 0.001 µg mL^{-1}; BChE IC_{50}: 19.820 ± 1.415 µg mL^{-1}; AChE SI: 1524.615). Fractions containing p*-hydroxybenzoic acid and protocatechuic acid had the lowest AChE SI values (7.584 and 9.367 respectively) and may thus, function as dual ChE inhibitors. Binary mixtures of DH and WSPFE EAF might have more potent inhibitory effects against these enzymes, as well as higher BChE/AChE selectivity. Further studies to investigate the ChE inhibition potential of these WSPFE samples are warranted.*

Keywords: cholinesterases, neurodegenerative diseases, oil palm phenolics.

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INTRODUCTION

Neurodegeneration negatively affects mental and physical functioning in elderly people suffering from neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, multiple sclerosis and amyotrophic lateral sclerosis. The global population suffering from Alzheimer's disease, which is the main cause of dementia, was established to be around 45 million in 2015, and this number is expected to double by 2030 and triple by 2050 (Scheltens *et al.*, 2016). The cholinergic system appears to be the earliest and most affected molecular mechanism that describes Alzheimer's disease pathophysiology (Craig *et al.*, 2011). In the cholinergic hypothesis, damage to brain nerve cells by senile plaques leads to decreases in choline transferase activities and losses in cognitive functions (Davies and Maloney, 1976). At the heart of the cholinergic system are the cholinesterase (ChE) enzymes. ChEs are enzymes splitting esters of choline (Pohanka, 2011). There are two types of ChEs, namely acetylcholinesterase (AChE) and butyrylcholinesterase (BChE).

AChE (EC 3.1.1.7), also known as true ChE, catalyses the hydrolysis of acetylcholine (ACh) into choline and acetic acid, a reaction necessary to return a cholinergic neuron to its resting state after activation. AChE is localised in the synaptic gaps of the central and peripheral nervous systems. This membrane-bound enzyme is projected into the synapse and terminates nervous impulses by catalysing ACh hydrolysis. AChE is

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known to participate in vicious cycles resulting in the aggregation of beta-amyloid plaques and neurofibrillary tangles found in Alzheimer's disease (Garcia-Ayllon *et al.*, 2011). Strategies that increase ACh levels through the use of AChE inhibitors demonstrate symptomatic efficacy in Alzheimer's disease (Nordberg *et al.*, 2013). In addition to dementia, AChE inhibitors are also clinically used to counteract other neurological pathologies, such as myasthenia gravis (Colovic *et al.*, 2013). They are also used in pain management by decreasing the nociception pain response and generating analgesic actions (Eldufani and Blaise, 2019), as well as in postanaesthesia by recovering neuromuscular blockade induced by certain anaesthetics (Srivastava and Hunter, 2009).

On the other hand, BChE (EC 3.1.1.8), also known as pseudo ChE or plasma ChE, catalyses the hydrolysis of the neurotransmitter butyrylcholine (BCh) into choline and butyric acid. BChE can also hydrolyse ACh and compensate for AChE when its levels are depleted. BChE accounts for up to 90% total serum ChE, while its activity is 20-fold lower than AChE in hydrolysing ACh (Arbel *et al.*, 2014). Many studies have highlighted that BChE plays a more important role in Alzheimer's disease and selective inhibitors of BChE could be promising drug candidates (Greig *et al.*, 2005; Nordberg *et al.*, 2013).

The most commonly prescribed ChE inhibitors are donepezil, rivastigmine and galantamine. Among these inhibitors however, donepezil is the only ChE inhibitor approved for the treatment of all stages of Alzheimer's disease (Allgaier and Allgaier, 2014; Schneider *et al.*, 2014). Natural plant resources possessing ChE inhibitory activities may potentially improve dementia and other neurodegenerative symptoms (Tundis *et al.*, 2016). Galantamine and rivastigmine are plant-derived alkaloids (Balkrishna *et al.*, 2019). There is also interest in developing new ChE inhibitors from among plant non-alkaloid compounds, such as polyphenols (Jabir *et al.*, 2018; Khan *et al.*, 2018). As such, various plant extracts have been shown to have AChE and/or BChE inhibitory activities, including those from Africa (Adewusi and Steenkamp, 2011), China (Kaufmann *et al.*, 2016), Europe (Ferreira *et al.*, 2006; Wszelaki *et al.*, 2010), India (Kadiyala *et al.*, 2014; Mathew and Subramanian, 2014; Sheeja Malar *et al.*, 2017), Middle East (Orhan *et al.*, 2004; 2008), South America (Nino *et al.*, 2006) and Southeast Asia (Kumaran *et al.*, 2019; Nuria *et al.*, 2020; Tappayuthpijarn *et al.*, 2012).

The oil palm (*Elaeis guineensis*) is a high oil-producing tropical plant. There continues to be increasing evidence showing the potential health benefits of nutraceuticals and phytonutrients derived from the oil palm (Kushairi *et al.*, 2019). The water-soluble part of the oil palm fruit is rich in phenolic acids, including three caffeoylshikimic acid isomers, *p*-hydroxybenzoic acid, protocatechuic acid (Sambanthamurthi *et al.*, 2011) and an indoleacetic acid derivative (Sambanthamurthi *et al.*, 2014), as well as shikimic acid (Sambandan *et al.*, 2011). Termed Water-Soluble Palm Fruit Extract (WSPFE), palm fruit bioactives (PFB), palm fruit juice (PFJ) or oil palm phenolics (OPP), these compounds could be recovered from the aqueous vegetation liquor during the palm oil milling process (Sambanthamurthi *et al.*, 2011). WSPFE has been shown to have potential neuroprotective effects, such as reducing neuroinflammatory factors *in vitro* (Weinberg *et al.*, 2018a), inhibiting beta-amyloid peptide aggregation *in vitro* (Weinberg *et al.*, 2018b), up-regulating genes involved in brain development and activity *in vivo* (Leow *et al.*, 2013) and increasing brain tyrosine hydroxylase levels *in vivo* (Weinberg *et al.*, 2019). As such, we hypothesised that WSPFE might also have possible ChE inhibition effects in the present study.

MATERIALS AND METHODS

Preparation of WSPFE Samples

Liquid WSPFE was obtained from the Malaysian Palm Oil Board (MPOB) Phenolic Antioxidant Pilot Plant in Labu, Negeri Sembilan, Malaysia (Sambanthamurthi *et al.*, 2008). Spray dried (SD) WSPFE was obtained through the spray drying process carried out on liquid WSPFE at Biotropics Malaysia Berhad, Shah Alam, Selangor, Malaysia. Freeze dried (FD) WSPFE was obtained by freeze drying liquid WSPFE at MPOB. WSPFE ethyl acetate fraction (EAF) was obtained by fractionating liquid WSPFE with ethyl acetate, followed by rotary evaporation and freeze drying. The remaining water partition was also collected, followed by rotary evaporation and freeze drying to obtain WSPFE water fraction (WF).

The different WSPFE fractions (F1–F7) were prepared by subjecting WSPFE EAF to preparative HPLC using a Waters Preparative AutoPurification High Performance Liquid Chromatography (HPLC) System (Waters Corporation, Milford, MA, USA). Separation was achieved by using a reverse phase Waters Atlantis C18 5 μm column (Waters Corporation, Milford, MA, USA). A binary gradient system was used as the mobile phase, with phase A comprising distilled water containing 0.02% (v/v) trifluoroacetic acid and phase B comprising 70%:30% (v/v) methanol-acetonitrile. A flow rate of 20 mL min^{-1} 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 based on their retention time.

The total phenolic content of these samples at 5000 μg mL^{-1} was determined in terms of μg mg^{-1} gallic acid equivalent (GAE) by using the Folin-Ciocalteu reagent (Merck, Germany) and an absorbance reading at 765 nm using the U-2800 spectrophotometer (Hitachi, Japan) (Gao *et al.*, 2000). These prepared samples were stored at -20°C until use.

AChE Assays

AChE assays were carried out using the commercial AChE Assay Kit (Fluorometric-Red) (ab138873) (Abcam PLC, Cambridge, United Kingdom), according to manufacturer's instructions. The kit uses AbRed Indicator to quantify the choline produced from the hydrolysis of ACh by AChE through choline oxidase-mediated enzyme coupling reactions. The fluorescence intensity of AbRed Indicator is used to measure the amount of choline formed, which is proportional to the AChE activity and was measured at Ex/Em = 540/590 nm in a kinetic mode of 1-min intervals for 30 min using the Infinite M200 Microplate Reader (Tecan, Switzerland). WSPFE samples were tested at varying concentrations between 0 to 500 μg mL^{-1} to determine the half maximal inhibitory concentration (IC_{50}) values of the samples on AChE. Negative control wells containing the substrate and enzyme without inhibitor samples, positive control wells containing the substrate, enzyme and the positive control inhibitor donepezil hydrochloride (DH) (ab120763) (Abcam PLC, Cambridge, United Kingdom) (Augustin *et al.*, 2020; Sheeja Malar *et al.*, 2017; Suganthy and Devi, 2016), as well as colour control samples which functioned as blanks for the corresponding samples were also prepared in these experiments.

BChE Assays

BChE assays were carried out using the commercial BChE Assay Kit (Colourimetric) (ab241010) (Abcam PLC, Cambridge, United Kingdom), according to manufacturer's instructions. The kit is based on the ability of BChE to hydrolyse a substrate and produce thiocholine. Thiocholine reacts with 5, 5′-dithiobis (2-nitrobenzoic acid) and generates a yellow chromophore that can be quantified at 412 nm. This was measured in a kinetic mode of 1-min intervals for 30 min using the Infinite M200 Microplate Reader (Tecan, Switzerland). WSPFE samples were tested at varying concentrations between 0 to 500 μg mL^{-1} to determine the IC_{50} values of the samples on BChE. Negative control wells containing the substrate and enzyme without inhibitor samples, positive control wells containing the substrate, enzyme and the positive control inhibitor DH (ab120763) (Abcam PLC, Cambridge, United Kingdom), as well as colour control samples which functioned as blanks for the corresponding samples were also prepared in these experiments.

Synergistic Assays Using Binary Mixtures of DH and WSPFE EAF

To determine the potential synergistic effects of DH and WSPFE EAF on the inhibition of AChE and BChE, binary-mixture experiments were performed using the assays described in the previous section. Two sets of experiments were performed, *i.e.*, set A to identify the effects of adding DH on the IC_{50} value of WSPFE EAF, and *vice versa* in set B. For set A, fixed concentrations of DH used were 0.01 μg mL^{-1} and 20 μg mL^{-1} for AChE and BChE enzymatic assays respectively, chosen on the basis of the respective IC_{50} values. WSPFE EAF in varying concentrations between 0 to 500 μg mL^{-1} were used. For set B, fixed concentrations of WSPFE EAF used were 0.2 μg mL^{-1} and 200 μg mL^{-1} for AChE and BChE enzymatic assays respectively, also chosen on the basis of the respective IC_{50} values. DH in varying concentrations between 0 to 500 μg mL^{-1} were used.

Statistical Analyses

Statistical analyses were performed using SPSS Statistics (IBM Corporation, Armonk, New York, USA). Analysis of variance (ANOVA), repeated measures or one-way where appropriate, with Tukey's HSD (honestly significant difference) post-hoc test were performed and differences with *p* values of less than 0.05 were considered statistically significant. Pearson's correlation analysis was performed to correlate the total phenolic content and ChE inhibition in the WSPFE samples. IC_{50} values were calculated using the Quest Graph™ IC_{50} Calculator (AAT Bioquest, Inc., Sunnyvale, CA, USA) (AAT Bioquest, 2019). AChE Selectivity Index (SI) values were calculated in Microsoft Excel (Microsoft Corporation, Redmond, WA, USA) using the formula: AChE SI = IC_{50} of BChE/IC_{50} of AChE (Zhao *et al.*, 2013).

RESULTS AND DISCUSSION

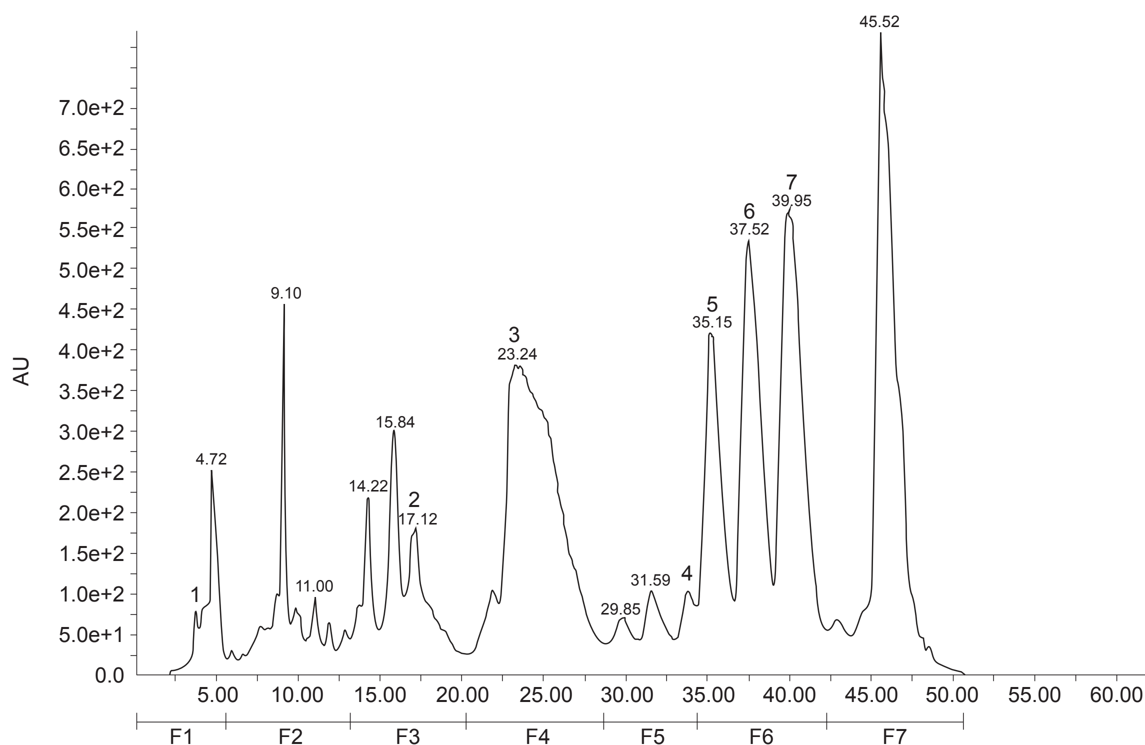
Cholinergic nerves are a major portion of the central, as well as peripheral parasympathetic and sympathetic nervous systems (Craig *et al.*, 2011). The main pathogenic feature connected with the progression of Alzheimer's disease is the weakening

of the brain cholinergic system. ChE inhibitors are recognised as one of the choices in treating Alzheimer's disease, approved as a therapeutic strategy to reduce symptoms and prevent its progression (Hussein *et al.*, 2018).

The neuroprotective effects attributed to plant phenolic compounds could be mediated by their AChE inhibitory activities, in addition to other mechanisms of action, such as antioxidant, anti-inflammatory and anti-amyloid production activities, as well as interactions with brain cell signalling (Nwidu *et al.*, 2017; Szwajgier *et al.*, 2017; 2018). Among plant phenolic compounds, the pharmacokinetic properties of phenolic acids make them suitable drugs for Alzheimer's disease, owing to their simplicity and structural similarity to popular ChE inhibitors (Szwajgier *et al.*, 2018). They are not degraded in the gastrointestinal tract prior to absorption (Rechner *et al.*, 2002), easily released from foods in the gastrointestinal tract by bacterial esterases and directly absorbed (Rondini *et al.*, 2002), as well as being transformed only to a limited extent (Couteau *et al.*, 2001).

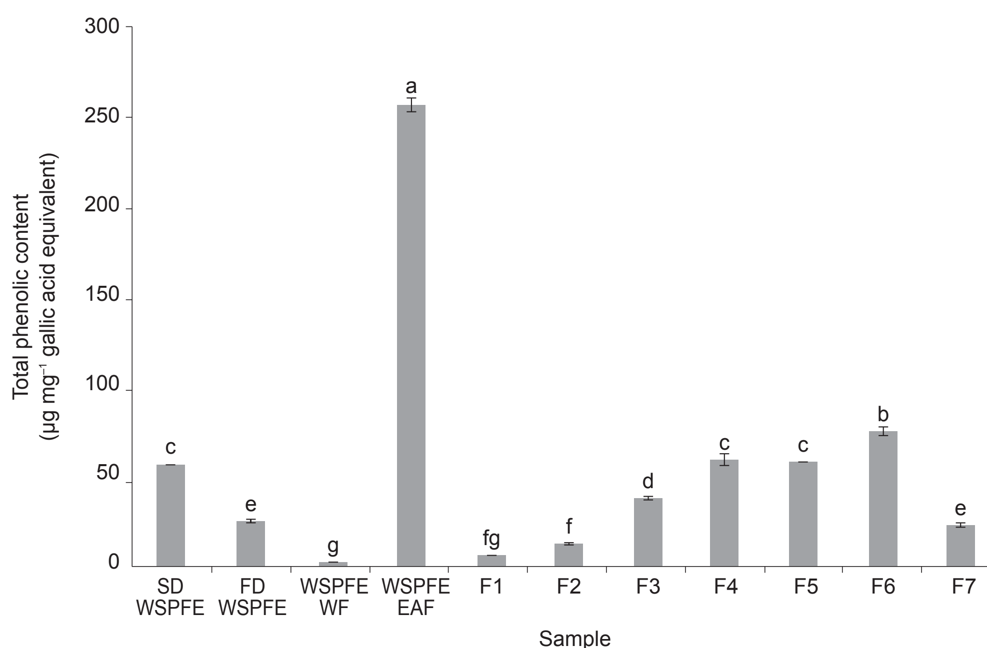
The majority of phenolic compounds present in WSPFE are phenolic acids. In the present study, seven preparative liquid chromatographic fractions (F1-F7) of WSPFE were prepared based on information obtained from previous literature on WSPFE (*Figure 1*). F1 contained shikimic acid (Sambandan *et al.*, 2011), F3 contained protocatechuic acid (Sambanthamurthi *et al.*, 2011), F4 contained *p*-hydroxybenzoic acid (Sambanthamurthi *et al.*, 2011), F5 contained an indoleacetic acid derivative (Sambanthamurthi *et al.*, 2014), while F6 contained three caffeoylshikimic acid isomers (Sambanthamurthi *et al.*, 2011). The components of F2 and F7 are still unknown.

Total phenolic content analysis of these samples at 5000 μg mL^{-1} (*Figure 2*) showed that SD WSPFE had a higher total phenolic content compared to FD WSPFE ($p<0.05$). This was similar to a previous study in which higher chokeberry polyphenol levels were present after drying at high temperatures, compared to after freeze drying (Horszwald *et al.*, 2013). SD papaya products also retained higher levels of flavonoids and phenolic compounds compared to



Note: The different Water-Soluble Palm Fruit Extract (WSPFE) fractions (F1–F7) were prepared using a Waters Preparative AutoPurification High Performance Liquid Chromatography (HPLC) System (Waters Corporation, Milford, MA, USA). Stationary phase: Reverse phase Waters Atlantis C18 5 μm column (Waters Corporation, Milford, MA, USA). Mobile phase: Binary gradient system, with phase A comprising distilled water containing 0.02% (v/v) trifluoroacetic acid and phase B comprising 70%:30% (v/v) methanol-acetonitrile. Flow rate: 20 mL min^{-1}. Pressure limit: 2.76 × 10^{4} kPa. Total run time: 55 min. Gradient elution: 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. Detection: Ultraviolet/visible (UV/VIS) at 280 nm UV wavelength. Peaks: 1: Shikimic acid; 2: Protocatechuic acid; 3: *p*-hydroxybenzoic acid; 4: Indoleacetic acid derivative; 5: 5-O-caffeoylshikimic acid; 6: 3-O-caffeoylshikimic acid; 7: 4-O-caffeoylshikimic acid.

Figure 1. Preparative liquid chromatogram of WSPFE fractions viewed at 280 nm ultraviolet wavelength.



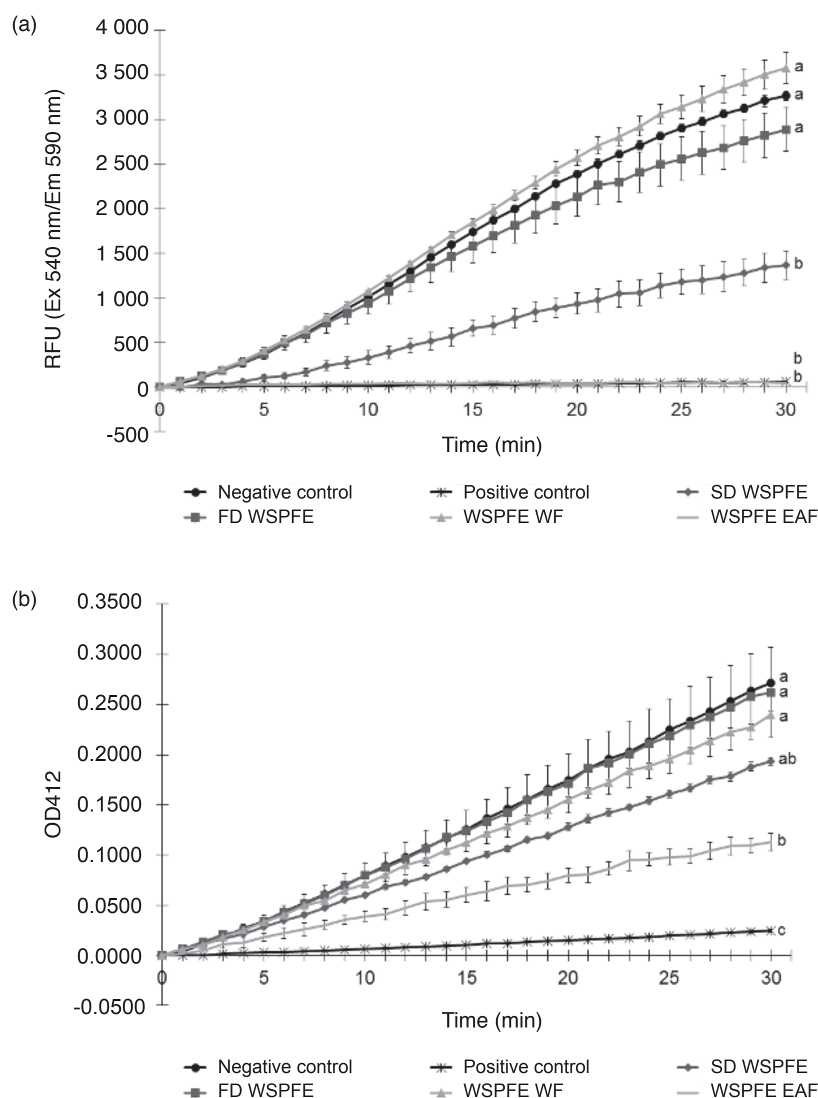
Note: Values are means ± standard error of the mean (SEM) from triplicate determinations. Means with different letters are significantly different (p<0.05) by one-way analysis of variance (ANOVA) with Tukey's honestly significant difference (HSD) *post-hoc* test. WSPFE - Water-Soluble Palm Fruit Extract; EAF - ethyl acetate fraction; WF - water fraction; SD - spray dried; FD - freeze dried.

Figure 2. Total phenolic content of WSPFE samples at 5000 µg mL^{-1}.

FD products (Gomes *et al.*, 2018). Drying is a major food processing operation to increase shelf life. The choice of drying method influences product quality, as it is related to the retention of bioactive compounds and antioxidant activities (Abascal *et al.*, 2005). Freeze drying is a technique based on water removal by sublimation under low pressure and is used to obtain various industrial products (Santo *et al.*, 2013). It makes a product lightweight, prevents yeast and bacteria survival, 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. Conversely, spray drying removes moisture from products by rapid evaporation on spray droplet under high temperature exposure. Spray drying produces a dry powder from a liquid or slurry by rapidly drying with a hot gas in a single processing step. Spray drying is suitable for heat-sensitive materials, despite the high temperatures of the drying gas, owing to the cooling effect of the evaporating solvent which keeps the droplet temperature relatively low (Haggag and Faheem, 2015). The heat and mass transfer occurs in the air with vapour films surrounding the product droplets, which form protective envelopes to keep product particles from approaching the dryer outlet temperature. Spray drying also has high performance due to low residence time of a few seconds (Verma and Singh, 2015).

In the present study, fractionation of liquid WSPFE with ethyl acetate resulted in WSPFE EAF which had the highest total phenolic content among all of the WSPFE samples, with the remaining components in WSPFE WF having the least. WSPFE EAF had a total phenolic content of around 25%. The total phenolic content of the WSPFE fractions increased from F1 to F6, but dipped down in F7, *i.e.,* the total phenolic content of the WSPFE fractions followed the ascending order of F1 < F2 < F7 < F3 < F5 < F4 < F6. Initial comparison of whole WSPFE, *i.e.,* SD WSPFE and FD WSPFE, showed that SD WSPFE had higher AChE and BChE inhibition activities compared to FD WSPFE, while WSPFE EAF had higher AChE and BChE inhibition activities compared to WSPFE WF (*Figure 3*). Although these results appeared to reflect the total phenolic content of the samples, in which samples with higher total phenolic content had higher AChE and BChE inhibition activities, there was weak positive correlation between the total phenolic content of all the WSPFE samples with AChE (R^2=0.527, p>0.05) and BChE (R^2=0.411, p>0.05) inhibition potential, which was in line with previous studies (Elufioye *et al.*, 2019; Zengin *et al.*, 2020). As the fractions used in the present study were not pure compounds, unidentified non-phenolic inhibitors might justify the activities observed. In addition, these findings might be due to the complex nature of these fractions and interactions between phytochemicals present in them.

Further AChE and BChE assays were then carried out using WSPFE samples of varying concentrations between 0 to 500 µg mL^{-1}, alongside



Note: Values are means ± standard error of the mean (SEM) from triplicate determinations. Means with different letters are significantly different (p<0.05) by repeated measures analysis of variance (ANOVA) with Tukey's honestly significant difference (HSD) *post-hoc* test. AChE - acetylcholinesterase; BChE - butyrylcholinesterase; WSPFE - Water-Soluble Palm Fruit Extract; EAF - ethyl acetate fraction; WF - water fraction; SD - spray dried; FD - freeze dried.

Figure 3. Effects of WSPFE samples on (a) AChE at 100 μg mL^{-1}, and (b) BChE at 400 μg mL^{-1}.

the positive control DH, in order to obtain the IC_{50} values on the respective enzymes (*Table 1*). Based on the IC_{50} values determined, the inhibitory effects of WSPFE samples on AChE followed the ascending order of SD WSPFE < F4 < F2 < F3 < F5 < F1 < F6 < F7 < WSPFE EAF. The IC_{50} values of FD WSPFE and WSPFE WF were not achieved as they were higher than the highest concentration tested (500 μg mL^{-1}). For BChE, the inhibitory effects of WSPFE samples on this enzyme were not strong, since the IC_{50} values of several samples, *i.e.*, SD WSPFE, FD WSPFE, WSPFE WF, F2, F6 and F7 were not achieved as they were higher than the highest concentration tested (500 μg mL^{-1}). On the other hand, among the WSPFE samples of which their IC_{50} values were determined, their inhibitory effects on BChE followed the ascending order of F4 < F1 < F5 < F3 < WSPFE EAF. Hence, in both assays, WSPFE EAF showed the highest inhibitory activities (AChE IC_{50}: 0.218 ± 0.029 μg mL^{-1}; BChE IC_{50}: 222.860 ± 5.777 μg mL^{-1}). However, the positive control DH still had lower IC_{50} values (AChE IC_{50}: 0.013 ± 0.001 μg mL^{-1}, BChE IC_{50}: 19.820 ± 1.415 μg mL^{-1}) and hence higher inhibitory potency when compared to WSPFE EAF.

AChE selectivity index (SI) is defined as IC_{50} BChE/IC_{50} AChE ratio, with a higher IC_{50} BChE/ IC_{50} AChE ratio indicating a higher selectivity towards AChE rather than BChE. All of the WSPFE samples tested in the present study were found to be more AChE selective. AChE SI values calculated based on IC_{50} values which could be determined indicated that the AChE selectivity of the WSPFE samples followed the ascending order of F4 < F3 < F5 < F1 < WSPFE EAF. The AChE SI value of

WSPFE EAF was 1022.294. This was lower than that of the AChE selective positive control DH, which had the highest AChE SI value of 1524.615. Two of the fractions, F4 and F3, which contained *p*-hydroxybenzoic acid and protocatechuic acid, respectively, had the lowest AChE SI values (7.584 and 9.367, respectively), indicating that these fractions were less AChE selective compared to the other WSPFE samples tested and may thus, function better as dual ChE inhibitors. The samples F5 and F1 which contained an indoleacetic acid derivative and shikimic acid, respectively, had AChE SI values of 27.879 and 38.897, respectively. F6 which contained three caffeoylshikimic acid isomers showed mainly AChE inhibitory properties.

Protocatechuic acid and *p*-hydroxybenzoic acid in particular have been shown to have potential neuroprotective properties (Winter *et al.*, 2017). The amount of *p*-hydroxybenzoic acid present in plant extracts has been shown to be significantly correlated only with BChE inhibition (Kobus-Cisowska *et al.*, 2019a; 2019b). On the other hand, the amount of protocatechuic acid present in plant extracts was significantly correlated with both AChE and BChE inhibition (Kobus-Cisowska *et al.*, 2019b). In the present study however, we found that both fractions containing protocatechuic acid and *p*-hydroxybenzoic acid respectively demonstrated dual ChE inhibitory properties. This discrepancy might be because fractions and not pure compounds were used in the present study. Indoleacetic acid derivatives have been shown to have inhibitory activities against both AChE (Dileep *et al.*, 2013) and BChE (Bodur and Cokugras, 2005) as well. Shikimic acid and caffeoylshikimic acids have not been shown to have ChE inhibitory activities as pure compounds, but plant extracts containing caffeoylshikimic acids and other shikimic acid derivatives possessing these properties have been documented in the literature (Kim *et al.*, 2018; Song *et al.*, 2020).

The possibility to isolate pure lead compounds from crude plant extracts or to administer these as nutraceuticals or cheap alternatives to drugs makes plants a versatile source of natural ChE inhibitors. However, plants produce a variety of secondary metabolites representing a complex mixture of compounds from several chemical classes. The action modes of most plant metabolites cannot be attributed to one single lead chemical compound, but to their pleiotropic effects (Wink, 2015). Hence, synergies within and between chemical groups of different compounds in plant extracts may take place and should thus, be considered (Kaufmann *et al.*, 2016). In the present study, WSPFE EAF which contained all the seven WSPFE fractions had the strongest inhibitory effects on AChE and BChE. This suggests that the seven WSPFE fractions when given together have synergistic inhibitory effects against these enzymes and would work better in attenuating these enzymes compared to giving individual WSPFE fractions.

In order to identify whether WSPFE EAF has potential synergistic effects in inhibiting AChE or BChE when used in combination with DH, we tested binary mixtures of these two compounds in the respective assays (*Table 1*). We found that adding DH to WSPFE EAF (AChE IC_{50}: 0.218 ± 0.029 μg mL^{-1}; BChE IC_{50}: 222.860±5.777 μg mL^{-1}) resulted in lower IC_{50} values of WSPFE EAF for both enzymes (AChE IC_{50}: 0.041 ± 0.013 μg mL^{-1}; BChE IC_{50}: 40.127 ± 8.063 μg mL^{-1}), but only the differences for BChE were statistically significant ($p<0.05$). However, the AChE SI values were almost similar (WSPFE EAF: 1022.294; DH + WSPFE EAF doses: 978.707), indicating that BChE/AChE selectivity was maintained. Adding WSPFE EAF to DH (AChE IC_{50}: 0.013 ± 0.001 μg mL^{-1}; BChE IC_{50}: 19.820 ± 1.415 μg mL^{-1}) also resulted in lower IC_{50} values of DH for both enzymes (AChE IC_{50}: 0.008103 ± 0.000174 μg mL^{-1}; BChE IC_{50}: 1.643 ± 0.403 μg mL^{-1}), but these differences were not statistically significant ($p>0.05$). Nevertheless, the AChE SI value was 7.5-fold lower (DH: 1524.615; WSPFE EAF + DH doses: 202.764), indicating that BChE/AChE selectivity was higher.

Hence, although WSPFE EAF by itself had high AChE selectivity, it reduced rather than increased the AChE selectivity of DH. A previous study showed that phenformin with an AChE SI value of 0.052 indicating that it was BChE selective did not alter the AChE SI value of donepezil, whereas a metformin sulphonamide derivative with an AChE SI value of 3.23 increased the AChE SI value of donepezil around 200-fold higher. On the other hand, metformin with an AChE SI value of > 425.53 did not alter the AChE SI value of donepezil either (Markowicz-Piasecka *et al.*, 2018). Many factors may thus, be at work for this apparent discrepancy. The exact mechanism by which synergistic effects could be achieved could only be explained by conducting combination index-isobologram analysis and enzyme kinetic studies (Balkrishna *et al.*, 2019; Huang *et al.*, 2019; Kaufmann *et al.*, 2016). *In silico* molecular docking experiments would also be helpful to identify the molecular mechanistics of AChE and BChE inhibition by the compounds present in WSPFE, as well as the structure-activity relationships of individual compounds with these ChEs (Jang *et al.*, 2018).

A limitation of the present study is that the bioavailability and biotransformation of most of the phenolic compounds in WSPFE and their metabolites are unknown at the moment. This information must be considered and applied, as metabolism is important in defining actual activity. Drugs that cross the blood brain barrier do not have dissociable groups (Tayeb *et al.*, 2012). Increasing

TABLE 1. THE IC_{50} AND SELECTIVITY INDEX (SI) VALUES OF WSPFE SAMPLES AS WELL AS BINARY MIXTURES OF WSPFE EAF AND DH AGAINST AChE AND BChE

Sample	IC_{50} (µg mL^{-1})		AChE SI
	AChE	BChE	
SD WSPFE	110.177 ± 7.141^{a}	>500	*
FD WSPFE	>500	>500	*
WSPFE WF	>500	>500	*
WSPFE EAF	0.218 ± 0.029^{d}	222.860 ± 5.777^{d}	1 022.294
F1	9.903 ± 1.751^{cd}	385.193 ± 11.966^{ab}	38.897
F2	37.093 ± 7.439^{b}	>500	*
F3	33.530 ± 3.131^{bc}	314.087 ± 10.251^{c}	9.367
F4	53.393 ± 11.972^{b}	404.913 ± 12.793^{a}	7.584
F5	12.197 ± 2.038^{cd}	340.043 ± 24.633^{bc}	27.879
F6	3.327 ± 0.052^{d}	>500	*
F7	0.645 ± 0.283^{d}	>500	*
DH	0.013 ± 0.001^{d}	19.820 ± 1.415^{e}	1 524.615
DH + WSPFE EAF doses (set A)	0.041 ± 0.013^{d}	40.127 ± 8.063^{e}	978.707
WSPFE EAF + DH doses (set B)	0.008103 ± 0.000174^{d}	1.643 ± 0.403^{e}	202.764

Note: The IC_{50} indicates the dose that induced a 50% enzymatic inhibition as compared to negative control (enzyme only) over 30 min. These IC_{50} values were expressed as means ± standard error of the mean (SEM) from triplicate determinations. 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. >500 indicates IC_{50} was not achieved as it was higher than the highest concentration tested (500 µg mL^{-1}). AChE SI is the AChE selectivity index defined as IC_{50} BChE/ IC_{50} AChE ratio. * indicates AChE SI was not calculated. AChE - acetylcholinesterase; BChE - butyrylcholinesterase; WSPFE - Water-Soluble Palm Fruit Extract; EAF - ethyl acetate fraction; DH - donepezil hydrochloride; WF - water fraction; SD - spray dried; FD - freeze dried.

the availability of ACh at receptors in the brain would result in better neuron to neuron transport. However, the poor ability to cross the blood brain barrier can be an advantage when a compound to regulate the peripheral nervous system is needed (Pohanka, 2014), such as in the treatment of myasthenia gravis (Benatar and Kaminski, 2012) and in post-anaesthesia (Chambers *et al.*, 2010). While direct measurements have not been done to confirm the availability of WSPFE in the brain, a previous study confirmed the increased expression of tyrosine hydroxylase in the brains of Nile rats fed WSPFE (Weinberg *et al.*, 2019). In addition, the bioavailability of two of the components present in WSPFE, *i.e.*, protocatechuic acid and *p*-hydroxybenzoic acid, has been indicated before in the literature. Protocatechuic acid has been found to be the major human plasma metabolite of cyanidin-glucosides following oral consumption of blood orange juice (Vitaglione *et al.*, 2007), while *p*-hydroxybenzoic acid is the major human plasma metabolite of pelargonidin-glucosides following oral consumption of strawberries (Azzini *et al.*, 2010). However, while protocatechuic acid has been found to be present in the brain following oral supplementation in animals (Lin *et al.*, 2011), *p*-hydroxybenzoic acid was not (Margalef *et al.*, 2015). Hence, understanding the bioavailability of WSPFE components in either the central or peripheral nervous system would further help to determine the applications of the ChE inhibition properties of WSPFE samples found in the present study.

CONCLUSION

SD WSPFE had higher AChE and BChE inhibition activities compared to FD WSPFE. WSPFE EAF was found to possess the highest inhibitory activities against these enzymes and the highest AChE selectivity among all the WSPFE samples compared, but these effects were weaker than those of the positive control DH. Fractions containing *p*-hydroxybenzoic acid and protocatechuic acid had the lowest AChE selectivity indices and may thus, function as dual ChE inhibitors. Binary mixtures of DH and WSPFE EAF might have more potent inhibitory effects against these enzymes, as well as higher BChE/AChE selectivity. Further studies, especially *in vivo* ones, to further confirm the *in vitro* results obtained in the present study are warranted.

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