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FULL PAPER

The antioxidant properties and α-amylase inhibition activities of polyphyto mixture with honey formulations

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Abstract

Ficus carica, *Orthosiphon stamineus, Ficus deltoidea* and *Trigona* honey are known for their high antioxidant amount. In this research, these plants and honey were used to study their interactions in terms of the antioxidant properties and the inhibition of α-amylase enzyme. These plants samples were chosen as they are usually used in traditional medicine to treat diabetes. Therefore, the objectives of this study are to measure the antioxidant activity as well as the α-amylase enzyme inhibition properties of the polyphyto formulations and *Trigona* honey. Simplex Centroid Mixture Design (SCMD) was used to design the formulations. In this experiment, the highest (p<0.05) antioxidant content for 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay was a formulation of 100% *Trigona* honey (90.09%). Meanwhile, a mixture of 50% *F. carica* – 50% *O. stamineus* and 50% *O. stamineus* – 50% *Trigona* honey yield the highest (p<0.05) total phenolic content (TPC) value (133.77 and 133.17 µg/mL, respectively), which indicated a synergistic interaction effect. However, for the measurement of ferric reduction antioxidant potential (FRAP), the formulation of 100% *O. stamineus* and 100% *F. deltoidea* exhibits the highest (p<0.05) value (148.95 and 148.78 µg/mL, respectively). In terms of enzyme inhibition, *F. deltoidea* showed the highest inhibition activity for α-amylase (8.826%). In conclusion, different percentages of *F. carica*, *O. stamineus*, *F. deltoidea* and *Trigona* honey have different interaction effects on the antioxidant activity and α-amylase inhibition depending on the amount of each factor.

1. Introduction

People nowadays tend to find alternative medicine instead of only depending on medication prescribed by the doctors or pharmacists. Therefore, indicates a significant increase in sales of herbal supplements due to its numerous health benefits (Rashidi *et al.*, 2019). By years, a lot of researches has been done on finding the plants that may help to reduce or treat diabetes as diabetes is known as the most common global disease where it affects 200 million individual and around 300 million people are at risk by approximate (McCune and Johns, 2002). The alternatives of using plants as substitutes for chemical drugs is due to the research on the bioavailability presence in each plant. Upon generations, *F. carica* has been said to have abundant of health benefits to human as mentioned in the Holy Qur'an (Marwat *et al.*, 2011).

One of the causes of diabetes is the reduced plasma antioxidant level (Facchini *et al.*, 2000). Antioxidants are mostly obtained from plants and herbs as they may prevent the development of the disease (Thompson and Godin, 1995; McCune and Johns, 2002). According to Kelble (2005), a decreasing amount of plasma antioxidant level increase the induction of the vascular disease seen in type II diabetes. Inhibition activity of α-amylase enzyme can also stunt glucose uptake by the body as they function in promoting the uptake of glucose in the body.

F. carica, O. stamineus and *F. deltoidea* are the plants used in this experiment. The previous study reported that *F. deltoidea* may reduce the blood glucose level due to enhancement of glucose uptake in muscle and reduction of hepatic gluconeogenesis (Adam *et al.*, 2007). Plants and herbs formulation are regularly reckoned to have lesser toxicity and does not contribute to any synthetic medicine's side effects (Malviya *et al.,* 2010). *Trigona* honey was chosen to be added as it has strong antioxidant activities (Yazan *et al.,* 2016).

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Antioxidant compounds such as organic acids, carotenoids, phenolic and vitamin E may inhibit the oxidative mechanisms that will eventually cause to degenerative illnesses (du Toit *et al.,* 2001). The purpose of determining the total phenolic content of the formulation is to understand the relationship of the phenolic content to the antioxidant and anti-diabetic properties of the formulation. Therefore, the objectives of this study were to measure the antioxidant and α-amylase enzyme inhibition properties of polyherbal formulations and Trigona honey.

2. Materials and methods

2.1 Chemicals and instruments

2, 2-Diphenyl-1-picrylhydrazyl (DPPH), methanol, Folin-Ciocalteu reagent, sodium carbonate, acetate buffer, glacial acetic acid and TPTZ (2, 4, 6-tripyridyl-s-triazine) were purchased from Merck, Germany. 3, 5-Dinitrosalicylic acid were bought from QReC, Asia while α-amylase were bought from Sigma-Aldrich, USA. The instrument used was UV-Vis spectrophotometer (T60u, PG Instrument, USA) located in Food Analysis Laboratory, UTHM.

2.2 Preparation of leaves extracts

F. carica dried leaves were bought from Formeniaga Company in Selangor while *O. stamineus* and *F. deltoidea* dried leaves were bought from Ethno Resources, Sg. Buloh. All of these plants were ground before being extracted to reduce the size. Each plant has a different extraction method based on the optimum yield of extraction for each plant. *F. carica* was extracted with maceration of 70% ethanol as applied from Vongsak *et al.* (2013). A total of 165 g of *F. carica* dried ground leaves were weighed using the weighing machine model TX3202L, Shimadzu, Japan and macerated with 70% ethanol (1:40, w/v) for 72 hrs at room temperature. Next, it was agitated on a shaker with a speed of 70 rpm and the extract was filtered using Whatman No. 1 filter paper and the marc was re-macerated in the same solvent until the extraction was exhausted. The liquid extract was left in the fume hood at room temperature until the ethanol was removed completely. It was left refrigerated at 4°C until further use.

O. stamineus was extracted using soxhlet method as it gives comparatively high yield percentage of extract compared to other methods. A total of 5 g of the dried leaves powder was extracted in 60% aqueous ethanol (w/v), heated according to the solvent's boiling point (>78°C) for 2 hrs. All extracts were filtered and concentrated under a fume hood at room temperature. The extracts were kept refrigerated at 4°C until further use (Mansor *et al.*, 2016). *F. deltoidea* was extracted with the method outlined by Hasham *et al.* (2013) with a little bit of modification. A total of 30 g *F. deltoidea* leaves were extracted with 0.48 L of hot boiling water for 20 mins. Whatman No. 1 filter paper was used to filter the solid parts and the liquid was kept refrigerated until further use.

2.3 Development of plants formulation

The formulations are made up of different percentages of mixture generated by a statistical software program, Design Expert® 6.0.4. (Stat-Ease, United States) and it was standardized at 20 g/100 mL (100% w/v) of the formulation. Four components in the mixtures, including the extracts of *F. carica, O. stamineus, F. deltoidea* and *Trigona* honey were selected as causal factors. The interaction effects of phytochemicals in antioxidant and α-amylase inhibitory activity were determined by comparing the experimental result with the predicted value according to the method of Palafox-Carlos *et al.* (2012), with slight modification. The predicted value is the addition of the individual values of the samples according to each proportion in the formulation.

2.4 DPPH radical scavenging assay

Radical scavenging activity of this formulation was determined spectrophotometrically by using the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay. The DPPH is basically reduced due to the donation of hydrogen from the antioxidant compound when the DPPH reacts with it. The colour changes were measured on a UV-Vis spectrophotometer at 515 nm. In this method, the DPPH was diluted in methanol to a concentration of 6 x 10^{-5} M (2.37 mg per 100 mL) (Miliauskas *et al.*, 2004). Three (3) mL of DPPH solution was incorporated with 77 µl of extract in cuvettes and kept in dark condition for 15 mins at room temperature. The blank sample was prepared to contain the exact volume of methanol and DPPH, then its absorbance value was measured. The experiment was done in triplicate. The calculation of the percentage inhibition can be expressed using the equation shown:

$$\% \text{ inhibition} = \left[\frac{(x-y)}{x}\right] \times 100 \quad (1)$$

Where x = the blank absorption at t = 0 min; and y = the extract absorption at the 15th mins.

2.5 FRAP assay

Ferric reduction antioxidant potential (FRAP) assay was done conferring to Benzie and Strain (1996) with minor modification. Reagents used were 300 mmol/L acetate buffer, pH 3.6 (3.1 g $C_3H_3NaO{\cdot}3H_2O$), and 16 mL $C_2H_4O_2$ (glacial acetic acid) in 1 L of buffer solution; 10 mmol/L 2, 4, 6-tripyridyl-s-triazine (TPTZ) in 40 mmol/L HCl; 20 mmol/L $FeCl_3{\cdot}6H_2O$. Working FRAP

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reagent was prepared as required by mixing 25 mL acetate buffer, 2.5 mL TPTZ solution and 2.5 mL $FeCl_3 \cdot 6H_2O$ solution. Warmed (37°C) FRAP reagent (3 mL) and its absorbance was read at 593 nm. About 100 µL of the sample was then incorporated with 300 µL H_2O. Absorbance reading was measured after 4 mins. The experiment was done in triplicate. The absorbance change between the final value and the blank absorption value was quantified for every single formulation and compared with a standard curve. The standard curve was made by using different concentration of the reagents as shown in Table 1. The absorbance value was read at 593 nm after exposed in the dark for 30 mins.

Table 1. Concentration reagents in making a standard curve for FRAP

Reagent	1	2	3	4	5	6	7	8
H_2O (µL)	1000	985	970	940	880	820	760	700
1mM Std (µL)	0	15	30	60	120	180	240	300
FRAP (mL)	2	2	2	2	2	2	2	2
Std Conc (µM)	0	5	10	20	40	60	80	100

2.6 Total phenolic content (TPC)

The total phenolic content (TPC) assay was experimented according to Miliaukas *et al.* (2004). The reference sample was made by taking 1 mL gallic acid solutions aliquots with the concentration of 10, 20, 40 and 80 µg/mL and incorporated with 5 mL of Folin-Ciocalteu reagent (diluted ten-fold) added with 4 mL (75 g/L) sodium carbonate. Absorbance reading was taken at 20°C at 750 nm after 30 mins to determine its calibration curve. About 100 µL of the sample was added to 2 mL sodium carbonate (2 g in 100 mL distilled water) for 2 mins at room temperature before mixing with Folin-Ciocalteu reagent. It was left for 30 mins before taking the reading at 750 nm. The experiment was done in triplicate and the absorbance value was compared to the standard curve.

2.7 α-Amylase inhibitory activity assay

Firstly, 500 µL of each sample and 500 µL of 0.02 M phosphate buffer (pH 6.9) containing porcine pancreatic α-amylase (EC 3.2.1.1) (0.5 mg/mL) were incubated for 10 mins at room temperature. Next, 500 µL of 1% starch solution in 0.02 M phosphate buffer (pH 6.9) was incorporated in the mixture solution. Then, the solution was incubated for 10 mins at room temperature before added with 1.0 mL of dinitrosalicylic acid (DNSA). Thereafter, the reaction in the solution was ceased by incubating it for 5 mins in a 100°C water bath before it was let to cool to room temperature. About 10 mL of distilled water was added to dilute the reaction mixture before measuring the absorbance at 540 nm (Worthington, 1993). All other reagents and the enzyme without the test sample was referred as blank sample. The α-amylase inhibitory activity was referred as percentage inhibition.

$$\% \text{ inhibition} = \left[\frac{(x-y)}{x}\right] \times 100 \qquad (2)$$

Where x = the reference sample absorption value; and y = the test sample absorption value.

2.8 Statistical analysis

The statistical analyses were done by a one-way ANOVA using IBM SPSS Statistics 20. The results were expressed as mean±SD, to show variations in the various experimental conditions. Differences are considered significant when $p<0.05$.

3. Results and discussion

3.1 DPPH radical scavenging assay

Table 2 shows the results for the DPPH assay. Formulation with 100% *Trigona* honey exhibits the highest scavenging activity with percentage of 90.09% followed by *F. carica* (88.09%) and *F. deltoidea* (81.93%). The antioxidant activity of the honey is attributed from its phenolic and flavonoids structure (Khalil *et al.*, 2011; Muhammad, 2012). This is because *Trigona* honey has bioactive compounds such as polyphenols, organic acids, vitamins, glutathione peroxidase and catalase that aids in improving the antioxidative property (Aljadi and Kamaruddin, 2004). However, formulations that were combined with *Trigona* honey has an antagonistic interaction. These results correspond to a previous study which shows that interaction between flavonoid compounds can work either synergistically or antagonistically (Hidalgo *et al.*, 2010). *O. stamineus* showed the least DPPH activity (24.29%) as compared to other studied samples (*F. carica*, 86.98%; *F. deltoidea* 82.93%; *Trigona* honey, 90.09%). It might be due to the extraction process which was done under high temperature with prolonged time. The previous study showed extraction at higher temperatures and longer extraction time decreased the free radical-scavenging activity of *O. stamineus* extracts (Chew *et al.*, 2011).

3.2 FRAP assay

Based on Table 3, the highest FRAP value was found from 100% *O. stamineus* and 100% *F. deltoidea* (148.95 and 148.78 µg/mL, respectively) with no significant difference ($p<0.05$). Although formulation 6, 7 and 9 showed synergistic interaction effect, the FRAP values are still lower significantly ($p<0.05$) than the individual *O. stamineus* and *F. deltoidea* extract. In contrast to formulation 5, indifferent interaction can be

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Table 2. DPPH scavenging activity in each formulation

No	Description	Predicted value	DPPH scavenging activity (%)	Type of interaction
1	100% D	-	90.09±0.51[a]	-
2	100% B	-	24.29±1.03[i]	-
3	100% A	-	86.98±0.51[b]	-
4	100% C	-	82.93±1.02[c]	-
5	50% A + 50% B	56.19±0.44	60.90±0.05[e]	Synergistic
6	50% B + 50% D	57.19±0.46	46.85±0.10[g]	Antagonistic
7	50% C + 50% D	86.01±0.53	79.28±0.09[d]	Antagonistic
8	33.33% C + 33.33% B + 33.33% D	65.44±0.48	60.00±1.32[e]	Antagonistic
9	33.33% A + 33.33% B + 33.33% D	67.49±0.47	57.09±0.55[f]	Antagonistic
10	12.5% A + 12.5% C + 12.5% D + 62.5% B	47.69±0.43	40.01±1.33[h]	Antagonistic

(A) *F. carica*, (B) *O. stamineus*, (C) *F. deltoidea*, (D) *Trigona* honey. Different superscript within the column indicates significant differences (p<0.05)

Table 3. FRAP content in each formulation

No	Description	Predicted value	FRAP value (µg/mL)	Type of interaction
1	100% D	-	119.55±1.342[f]	-
2	100% B	-	148.95±0.801[a]	-
3	100% A	-	143.73±2.165[c]	-
4	100% C	-	148.78±0.394[a]	-
5	50% A + 50% B	143.99±1.40	144.11±0.08[bc]	Indifferent
6	50% B + 50% D	132.52±1.26	136.46±0.68[e]	Synergistic
7	50% C + 50% D	131.51±1.09	139.46±1.75[de]	Synergistic
8	33.33% C + 33.33% B + 33.33% D	137.32±0.99	145.18±1.52[abc]	Synergistic
9	33.33% A + 33.33% B + 33.33% D	134.69±1.51	141.23±0.98[cd]	Synergistic
10	12.5% A + 12.5% C + 12.5% D + 62.5% B	143.35±1.02	148.13±0.51[ab]	Synergistic

(A) *F. carica*, (B) *O. stamineus*, (C) *F. deltoidea*, (D) *Trigona* honey. Different superscript within the column indicates significant differences (p<0.05)

seen between the combination of the equal concentration of *F. carica* and *O. stamineus*. These phenomena are in accordance with reports by Hajimehdipoor *et al.* (2014) which indicates that when an individual extract is mixed, there is a risk of interfering and even neutralizing the antioxidant effects. In the case of the lowest FRAP value, 100% *Trigona* honey showed FRAP values of 119.55 µg/mL. However, it is considered as relatively high FRAP content if compared to other types of honey due to the contributions of flavonoids and phenolic compounds to the antioxidative capacity of the honey (Alzahrani *et al.*, 2012). *Trigona* honey yield lower FRAP value compared to other formulations because FRAP assay does not react immediately with some antioxidants compound such as glutathione found in honey (Guo *et al.*, 2003).

3.3 Total phenolic content (TPC)

The highest TPC content would be a mixture of 50% *F. carica* – 50% *O. stamineus* and 50% *O. stamineus* – 50% *Trigona* honey (Table 4). Notably, all formulation showed synergistic interaction effect for TPC content. This effect might be contributed from the positive interaction between phenolic compounds in each mixture of plant extract that attributed to their antioxidant activity (Muhammad *et al.,* 2012; Widyawati *et al.*, 2017; Rahim *et al., 2019*). Such phenolic compounds which are eupatorin, sinensetin, 3'-hydroxy-5,6,7,4'-tetramethoxyflavone and rosmarinic acid, were reported contains in *O. stamineus* (Abdullah, 2012). While *F. carica* leaves contain phenolic acids such as oxalic, citric, malic, quinic, shikimic and fumaric acids (Oliveira *et al.,* 2008). Other than that, *Trigona honey* main compounds are salicylic, p-coumaric acid, aromadendrin and taxifolin (Biluca *et al.*, 2020). These findings also supported by Liu (2003) which reported that the synergistic effects of plant combinations may be due to the increased interaction possibilities which allowing more cooperative effects between compounds in the mixtures (Liu, 2003).

3.4 α-Amylase inhibitory activity assay

Based on Table 5, highest α-amylase percentage inhibition is exhibited by *F. deltoidea* (8.826%). This is might be due to the presence of a more potent compound called vitexin, sitosterol and isovitexin which reported could inhibit α-amylase enzyme (Joseph and Raj, 2011; Bakar *et al.*, 2018). The ability of *F. deltoidea* in

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Table 4. TPC content in each formulation

No	Description	Predicted value	TPC value (µg/mL)	Type of interaction
1	100% D	-	24.84±2.16[g]	-
2	100% B	-	132.71±0.56[ab]	-
3	100% A	-	54.76±1.32[f]	-
4	100% C	-	82.63±2.26[d]	-
5	50% A + 50% B	91.87±0.65	133.77±0.508[a]	Synergistic
6	50% B + 50% D	83.11±0.87	133.17±0.521[a]	Synergistic
7	50% C + 50% D	56.21±1.34	60.24±0.289[e]	Synergistic
8	33.33% C + 33.33% B + 33.33% D	81.71±1.08	124.86±0.86[c]	Synergistic
9	33.33% A + 33.33% B + 33.33% D	72.41±0.83	127.65±0.55[c]	Synergistic
10	12.5% A + 12.5% C + 12.5% D + 62.5% B	103.37±0.78	128.29±2.81[bc]	Synergistic

(A) *F. carica*, (B) *O. stamineus*, (C) *F. deltoidea*, (D) *Trigona* honey. Different superscript within the column indicates significant differences ($p<0.05$)

Table 5. α-Amylase percentage inhibition in each formulation

No	Description	Predicted value	α-Amylase inhibition activity (%)	Type of interaction
1	100% D	-	6.824±0.593[c]	-
2	100% B	-	4.828±1.133[e]	-
3	100% A	-	6.208±1.111[bc]	-
4	100% C	-	8.826±1.63[a]	-
5	50% A + 50% B	5.479±0.93	7.47±0.41[b]	Synergistic
6	50% B + 50% D	5.826±0.86	5.22±1.56[d]	Indifferent
7	50% C + 50% D	7.825±1.11	7.22±1.05[cd]	Indifferent
8	33.33% C + 33.33% B + 33.33% D	6.826±1.12	6.49±1.06[bc]	Indifferent

(A) *F. carica*, (B) *O. stamineus*, (C) *F. deltoidea*, (D) *Trigona* honey. Different superscript within the column indicates significant differences ($p<0.05$)

inhibiting α-amylase enzyme also reported in another study conducted by Adam and his colleagues (2011). It was also found in this study formulation 5 which contains 50% *F. carica* - 50% *O. Stamineus* gives synergistic interaction effect. However, its value of α-amylase inhibitory activity is still lower than individual inhibition of *F. deltoidea*. Farsi *et al.*, (2011) reported there was a direct correlation between antioxidant activity and α-amylase inhibitory activity. Hence, it is concluded that the highest α-amylase inhibitory activity of *F. deltoidea* may be contributed by its high antioxidant compounds.

4. Conclusion

In conclusion, formulation with 100% *Trigona* honey gives the highest DPPH free radical scavenging activity. While the formulation of 50% *F. carica* – 50% *O. stamineus* and 50% *O. stamineus* – 50% *Trigona* honey exhibits the highest TPC content. Other than that, the best formulation for the FRAP assay is 100% *O. stamineus* and 100% *F. deltoidea*. This indicates that these individual extracts and mixtures can be used to regulate free radicals. The highest percentage inhibition of the α-amylase enzyme is achieved by individual formulation of *F. deltoidea* which exhibit high antioxidant activity hence it can be concluded that α-amylase inhibitory activity of *F. deltoidea* may be contributed by the antioxidant compounds. More in-depth studies are required to isolate, classify and analyze the active ingredients of this plant for the management of diabetes.

Conflict of interest

Authors declare no conflict of interest.

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