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Research Article

Comprehensive Biological Activities Evaluation and Quantification of Marker Compounds of *Ficus deltoiea* Jack Varieties

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ABSTRACT

Ficus deltoidea Jack (FD) is a plant belonging to the Moraceae family and found in tropical and subtropical countries. Reports indicate that 13 varieties of this plant can be found in Malaysian forest. Scientific evidences revealed that FD possesses several biological activities such as antioxidant, anti-inflammatory and antidiabetic. The issue on varieties raised the question on whether all variety can be utilized medicinally. In this study, we investigate several biological activities on seven FD varieties namely var. *deltoidea*, var. *kunstleri*, var. *bilobata*, var. *angustifolia*, var. *motleyana*, var. *trengganuensis*, var. *intermedia* and var. *borneensis*. We found that var. *deltoidea* has the highest inhibition of nitric oxide and ferric reducing power with IC_{50} value of 42 µg/ mL and 3.67±0.07 nm, respectively. FD var. *motleyana* has lowest activity on both assays. In α-glucosidase inhibition, FD var. *deltoidea* also showed the strongest inhibition with IC_{50} value of 6 µg/mL. However, all varieties did not show any cytotoxic effect on HepG2, MCF7, RAW 264.7 macrophage and NIH-3T3 cell lines. Phytochemical screening revealed that var. *deltoidea* contains the highest amount of total phenolic but total flavonoid content occur the most in FD var. *angustifolia*. Quantification of bioactive markers by HPLC indicated that FD var. *deltoidea* has the highest content of vitexin while FD var. *borneensis* has the highest amount of isovitexin. Finding from this study demonstrates that it is very important to select suitable variety in order to maximized the benefit of this plant.

Keywords: *Ficus deltoidea*, antidiabetic, antioxidant, cytotoxicity, HPLC.

INTRODUCTION

Ficus deltoidea Jack (FD) from Moraceae family is considered both medicinal and ornamental plant. It can be found in tropical and subtropical countries. Infamously known as mas cotek or mistletoe fig, this plant was identified by the fine spots with gold coloured and forked midrib on the surface of each leaves as well as a yellow-olive leathery structure on the dorsal surface (Kochummen, 1978)[1]. Identification of FD is a bit confusing due to its variability in leaf and fig shapes and sizes. Based on plant morphology, Berg (2003)[2] reported that FD could be divided into two subspecies and thirteen varieties. Seven of those varieties can be found in Peninsular Malaysia and two in Malaysian Borneo[3,4]. *Ficus deltoidea* have been use traditionally to treat illnesses such as diabetes mellitus, heart problem, high blood pressure, gout, improve blood circulation, pneumonia, diarrhea and skin infections[5,6]. Pharmacological studies demonstrated that this plant possess antioxidant, antidiabetic, anti-inflammatory, wound healing, anti-melanogenic and anti-nociceptive[7-13]. All parts of *Ficus deltoidea* including the root, bark, leaf and fig have been reported to have medicinal properties[14]. Phytochemical profiling studies on plants of *F. deltoidea* discovered that they contain secondary metabolites such as saponins, flavonoids, tannins, polyphenols, triterpenoids, and proanthocyanins[6,15]. The leaf extracts were also reported to be rich of phenolic and flavonoid compounds[13]. The complexity of the varieties could affect the selection of suitable variety for certain illnesses. Previous reported that different accessions of *F. deltoidea* possibly contain different types of phenolic compounds, which have different antioxidant activities[16]. As reported by Mohd et al.[17], FD varieties showed significant differences in chemical profile and amount of bioactive markers, vitexin and isovitexin. Lack of variety specific biological study and quantification of marker for each varieties impetus us to conduct this study.

MATERIALS AND METHODS

Chemicals and Reagent

Methanol (HPLC grade), formic acid, dimethyl sulfoxide (DMSO), aluminium chloride, potassium acetate, gallic acid, L-ascorbic acid, Phosphate buffer (pH- 7.4), potassium ferricyanide, trichloroacetic acid, ferric chloride, sodium Nitroprusside, were purchased from Merck Sdn. Bhd. (Petaling Jaya, Selangor, Malaysia). Standard quercetin, *p*-Nitrophenyl α-D-glucopyranoside

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(*p*NPG), *α*-glucosidase (E.C. 3.2.1.20), Folin-Ciocalteu reagent, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), acarbose, Griess reagent (1% of sulphanilamide and 0.1% of naphthylethylenediamine in 2.5% HPO_3) were from Sigma-Aldrich (St. Louis, MO, USA); Milli Q water, the pure compounds vitexin and isovitexin were purchased from Fluka-Sigma.

Plant material

Plant materials were obtained from several locations around Terengganu, Kelantan, Pahang (Cameron Highland) and Sarawak of Malaysia. The eight varieties of *Ficus deltoidea* selected in this study were identified by Prof. Dr. Nashriyah Mat, Universiti Sultan Zainal Abidin (UniSZA), Terengganu, Malaysia. Those varieties were FD var. *angustifolia* (FDA), FD var. *bilobata* (FDB), FD var. *borneensis* (FDBN), FD var. *deltoidea* (FDD), FD var. *intermedia* (FDI), FD var. *kunstleri* (FDK), FD var. *motleyana* (FDM), and FD var. *trengganuensis* (FDTG).

Sample preparation

The leaves of *Ficus deltoidea* were washed and dried in fan assisted dryer at 45ºC for 2-3 days[17]. The dried leaves were then milled into powder and macerated with methanol at room temperature for 3 days. Methanol extract was then filtered and concentrated under pressure at 45°C. The crudes of methanol extract were stored into vials and kept in 4°C prior analysis.

Phytochemical Analysis

Total Phenolic Content

Total phenolic content (TPC) was determined by Folin-Ciocalteu method according to Sukjamnong and Santiyanont[18] with minor modifications. The assay was carried out in microplate. The volumes of 20 µL of extracts were mixed up with 100 µL of 1:10 Folin-Ciocalteu reagent. The volume 80µL of 7.5% of sodium bicarbonate solution (Na_2CO_3) were added into the mixture and incubated in room temperature for 30 minutes in the dark. The absorbance was recorded at 765 nm. Total phenolics were quantified by calibration curve of gallic acid equivalents. TPC was expressed as mg gallic acid equivalents per gram of dried extract (mg GAE g^{-1}). The samples were prepared in triplicate for each analysis.

Total Flavonoid Content

Total flavonoid content (TFC) of each sample extract was determined by aluminum chloride colorimetric method adapted from Mayur et al.[19] with slight modification. A volume of 20 µL of each extract was mixed with 20 µL of 10 % aluminium chloride, 20 µL of 1 M potassium acetate and 180 µL of distilled water. The mixture was incubated in room temperature for 30 minutes. The absorbance of the reaction was recorded at 415 nm. For total flavonoid determination, quercetin was used to make the calibration curve. TFC was expressed as mg quercetin equivalents per gram of dried extract (mg QE g^{-1}).

Quantification of marker compounds, vitexin and isovitexin by HPLC

Preparation of standard solution for calibration curve and extract samples

A mixture of vitexin and isovitexin with final concentration of 500µg/ mL were prepared and filtered through a 0.45 µm filter (Whatman). A series of working standard solutions were prepared by diluting the mixture of standards solution (500µg/ mL) mentioned above with methanol in concentration range of 15.6-500 µg/mL. Calibration curves for vitexin and isovitexin were prepared by injecting different concentrations of standard markers solutions. The peak areas were recorded and peaks areas against concentration of the standard were plotted. Methanol extract of eight FD varieties (10 mg) were dissolved in 1 mL of methanol and all prepared samples extracts were sonicated for 30 minutes. The samples were then centrifuged and transferred into new HPLC vials. The extract samples were injected; the peak areas of standard markers analytes in the samples were recorded.

Instrumentation and Chromatographic Condition

Agilent Technology Series 1100 HPLC equipped with an automatic injector, a column oven, vacuum degasser, quaternary pump and a UV detector was used in the analysis. The UV detector was operated via a sensitivity range of 0.005 AUFS, output of 15 mV. The HPLC qualitative and quantitative protocol in this study was adapted as described by Shafaei and Ismail[20]. The HPLC separation was performed by using a Eclipse C_{18} reversed phase column (250 mm x 4.6 mm), 5 µm diameter particle size (Agilent Tech, Palo Alto, CA), with flow rate of 1 ml/min and 10 µL of injection volume. The isocratic mobile phase constituted methanol: formic acid (1%) in Milli Q water, (33:67 v/v). The marker compounds of vitexin and isovitexin were detected at 330 nm at 30°C. The confirmation of the chromatographic peaks of the analytes was obtained by comparing their retention time with corresponding reference standards at 330 nm. All samples were injected for three replicates in HPLC system. The standard curves of vitexin and isovitexin were constructed and the regression equation obtained was used to calculate the amount of marker compounds in the samples extracts.

Biological Activities

In vitro α-glucosidase inhibitory assay

The effect of the plant extracts on α-glucosidase activity was performed on 96-well microplate according to the method reported by Mohd et al.[21]. Each sample extracts were dissolved in 10% DMSO. The mixtures of 10 µL of test sample at various concentrations, 50 µL of 0.1 M phosphate buffer (pH 7.0) and 25 µL of *α*-glucosidase solution (0.2 Unit/mL) of each well were pre-incubated at 37°C for 10 minutes. Then, 25 µL of 0.5 mM 4-nitrophenyl *a*-D-glucopyranoside (*p*NPG) was added to start the reaction. The reaction mixture was then incubated at 37°C for 30 minutes and 100µL of 0.2 M sodium carbonate (Na_2CO_3) were added into each well in order to terminate the reaction. The absorbance (A) of test samples was measured at 410 nm. Plant extract with 10% DMSO used as control and acarbose was used as positive control. In sample blank and control blank, *α*-glucosidase and *p*NPG was replaced with buffer in the mixtures, respectively. All experiments were carried out in triplicates. Inhibition percentage was calculated according to the formula:

$$\text{Percentage of Inhibitions (\%)} = 1 - \frac{(A_S - A_{SB})}{(A_C - A_{CB})} \times 100$$

Where, A_S, A_{SB}, A_C, and A_{CB} are the absorbance of sample, sample blank, control, and control blank, respectively. Concentrations of extracts resulting in 50% inhibition of enzyme activity (IC_{50}) were determined graphically.

Antioxidant Activity

Nitric Oxide scavenging assay

The nitric oxide (NO) scavenging activity of the extract was measured according to the method of Mayur et al.[13] and Jain and Agrawal[22] with modification. The volume of 25 µL of sodium nitroprusside (10 mM) in phosphate-buffered saline (PBS) was mixed with 25 µL of different concentrations of sample extracts (dissolved in the suitable solvent system) and was incubated in the dark at room temperature for 15 minutes. After incubation, 50 µL of Griess reagent were added. The absorbance of the chromophore formed was immediately read at 546 nm. A blank for each concentration was prepared with test samples, phosphate buffered saline (PBS), and Griess reagent. Ascorbic acid was used as positive control and results were expressed as percentage inhibition of nitric oxide.

$$\text{Percentages of Inhibitions (\%)} = 1 - \frac{(A_S - A_{SB})}{(A_C - A_{CB})} \times 100$$

Where, A_S, A_{SB}, A_C, and A_{CB} are the absorbance of sample, sample blank, control, and control blank, respectively. IC_{50}, which is an inhibitory concentration of each extract required to reduce 50% of the nitric oxide formation was determined.

Ferric (Fe^{3+}) reducing antioxidant power (FRAP) assay

The reducing antioxidant power method was performed according to Mayur et al.[19]. Ten microliters of different concentrations of extracts, 15 µL of phosphate buffer (0.1 M, pH 6.6) and 15 µL of 1% w/v potassium ferricyanide [$K_3Fe(CN)_6$] were added into 96-well plates and were mixed together. The mixture was incubated at 50ºC for 20 minutes. After incubation, 15 µL of 10% trichloroacetic acid solution was added to each well. The volume of 55 µL of distilled water and 110 µL of 0.1% (w/v) fresh ferric chloride were added and after 10 min reaction, the absorbance was measured at 700 nm. L-ascorbic acid was taken as a references standard. Increased absorbance of the reaction mixture indicates the increasing of reducing power.

Cell Culture

HepG2 cell line (Human liver hepatocellular carcinoma), MCF7 cell line (human breast adenocarcinoma), RAW264.7 macrophages cell line and NIH-3T3 cell line (mouse embryonic fibroblasts) were obtained American Type Culture Colection (ATCC). Cells were cultured in DMEM media supplemented with 10% heat-inactivated fetal bovine serum (FBS), and 1% antibiotics (100 U/ml penicillin and 0.1 mg/mL streptomycin) (GIBCO, USA) at 37ºC in 5% CO_2 incubator.

Cytotoxicity assay

Cell viability was assessed according to Soundararajan and Sreenivasan[23]. Cells were seeded at (1 X 10^5) in 100 µL of growth medium in each well in 96 well plates (NUNC, Roskide Denmark) and incubated overnight. Cells were treated with different concentration of the plant extract for 72 hours. The volume of 20 µL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (5mg/mL) was added to each well and incubated at 37˚C for 4 hours. After removal of supernatant, the colored crystals of produced formazan were dissolved in 100 µL of dimethyl sulfoxide (DMSO). The absorbance was measured using a microplate reader at a wavelength of 590 nm and reference 620 nm. All experiments were carried out in triplicates and the effect of plants extracts on cell viability was expressed using following formula:

$$\text{Percentage of Viability (\%)} = \frac{A_S}{A_C} \times 100$$

Where, A_S and A_C are the absorbance value of test compound and absorbance value of control.

Statistical Analysis

The experimental data were reported as mean ± standard deviation of three parallel measurements. Experimental results were further analyzed for Pearson's correlation coefficient test. All statistical analysis and correlations were analyzed using IBM SPSS version 19.

RESULTS AND DISCUSSION

Total Phenolic Content (TPC) and Total Flavonoid content

The amount of phenolic content was determined using Folin-Ciocalteu method. The calibration curve of gallic acid equivalent in Figure 1 (A) showed that the linearity for gallic acid was in the range of 25-500 µg/mL, with a correlation coefficient (r^2) of 0.9987. The total phenols were expressed as mg/g gallic acid equivalent using the standard curve equation: y = 0.0041 x + 0.1035 (Figure 1A). Meanwhile, the amount of total flavonoids was determined using aluminium chloride method. The calibration curve of quercetin equivalent in Figure 2 (A) showed that the linearity for quercetin was in the range of 25 - 200 µg/mL, with a correlation coefficient (r^2) of 0.999. The total flavonoids were expressed as mg/g quercetin equivalent using the standard curve equation: y = 0.0019 x + 0.0587 (Figure 2A). Result in Figure 1(B) and Table 1 shows the total phenolic content of extracts of FD varieties. It clearly showed that FD var. *deltoidea* (239.05±12.5 mg GAE/ g extract) had the highest total phenolic content and followed by FD var. *intermedia* (170.14±4.0 mg GAE/g extract), var. *borneensis* (144.93±8.9 mg GAE/g extract), var. *kunstleri* (92.54±9.8 mg GAE/g extract), var. *trengganuensis* (.31±12.87 mg GAE/g extract), var. *bilobata* (91, 74.83±10.1 mg GAE/g extract), var. *motleyana* (35.35±2.5 mg GAE/g) and var. *angustifolia* (27.83±6.2 mg GAE/100 g extract). The TPC values of the extracts were exhibited in the following order: FDD> FDI> FDBN> FDK> FDTG> FDB> FDM> FDA. Previous reports suggested that TPC in water extracts for FD var. *deltoidea* was higher than water extracts for FD var. *angustifolia*[15] as similar as the results in present study.

This finding was not consistent with the findings by Zunoliza et al.[6] as the higher TPC was found in FD var. *trengganuensis* as compared to FD var. *deltoidea*. Figure 2 (B) and Table 1 shows the contents of total flavonoid in eight varieties of *Ficus deltoidea*. TFC results showed that FD var. *angustifolia* (36.58±3.37 mg QE/g extract) had the highest total flavonoid content and followed by var. *bilobata* (33.09±3.22 mg QE/g extract), var. *kunstleri* (31.50±0.37 mg QE/g extract), var. *trengganuensis* (25.70±1.08 mg QE/g extract), var. *intermedia* (25.04±1.40 mg QE/g extract), var. *borneensis* (24.85±2.12 mg QE/g extract), and var. *deltoidea* (12.61±0.31 mg QE/g extract) whereas var. *motleyana* was found to be the lowest of total flavonoid content, with the mean value of 11.75±0.30 mg QE/g extract. This TFC results are found in contrast with the results of TPC, which are summarized in following order; FDA> FDB> FDK> FDTG> FDI> FDBN> FDD> FDM. The results of total flavonoid content showed that methanol extract of FD var. *angustifolia* was higher than FD var. *deltoidea*. These results therefore support previous studies, which showed that TFC in methanol extract of FD var. *angustifolia* was higher than that of FD var. *deltoidea* [6,15]. In contrast, the study reported by Dzolin et al. (2015) [24] have mentioned that water extract of FD var. *deltoidea* was the highest of TFC and followed by water extract of FD var. *intermedia*, var. *kunstleri* and var. *angustifolia*. According to Woon et al.[15], plants originating from different geographical locations may vary in their phytochemical profiles and compositions. The finding results of TPC and TFC was consistent with previous study reported by Zunoliza et al.[6] which, FD var. *angustifolia* was found to have the lowest TPC yet the highest TFC. Previous study demonstrated that different accessions of *Ficus deltoidea* possibly contain different types of phenolic compounds, which have different antioxidant activities[16]. Several studies have also reported the antioxidant activity of plant extracts and their relationship with the phenolic and flavonoid content[7,8,25]. The results of antioxidant activities in previous research suggested that phenolics and flavonoid content had stronger antioxidant activities with statistical analysis showed to have positive correlation between total antioxidant activity and phenolic content (*r* value: 0.993)[8]. Kazeem et al.[26] also reported that there were positive correlation between phenolic content of plants and their respective antidiabetic activities.

Quantification of Marker Compounds, vitexin and isovitexin by HPLC

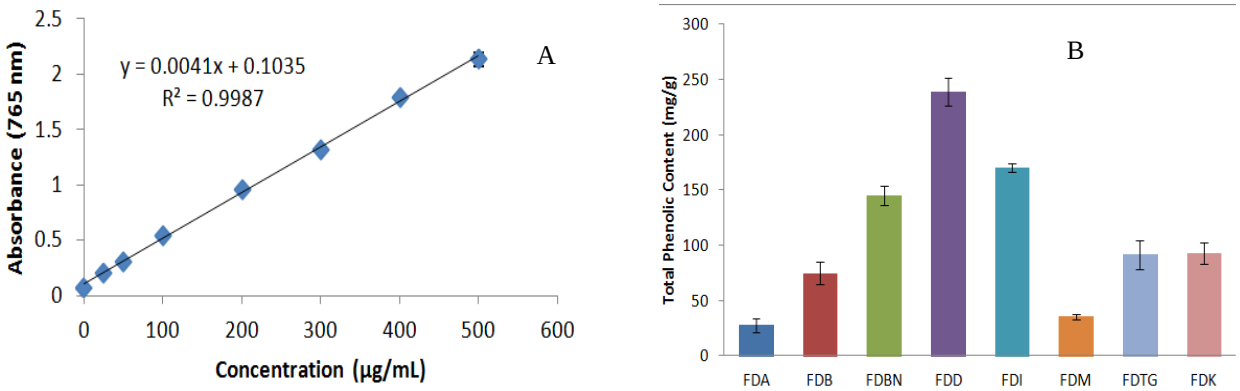


Figure 1: Calibration curve of gallic acid equivalent at concentrations of 0, 25, 50, 100, 200, 300, 400 and 500 µg/ mL (A). Absorbance detection was at 765 nm. Total phenolic content of *Ficus deltoidea* varieties determined by the Folin-Ciocalteu assay and calculated as GAE in mg·g⁻1 extract based on dry weight (B). Results are the average of triplicates ± SD.

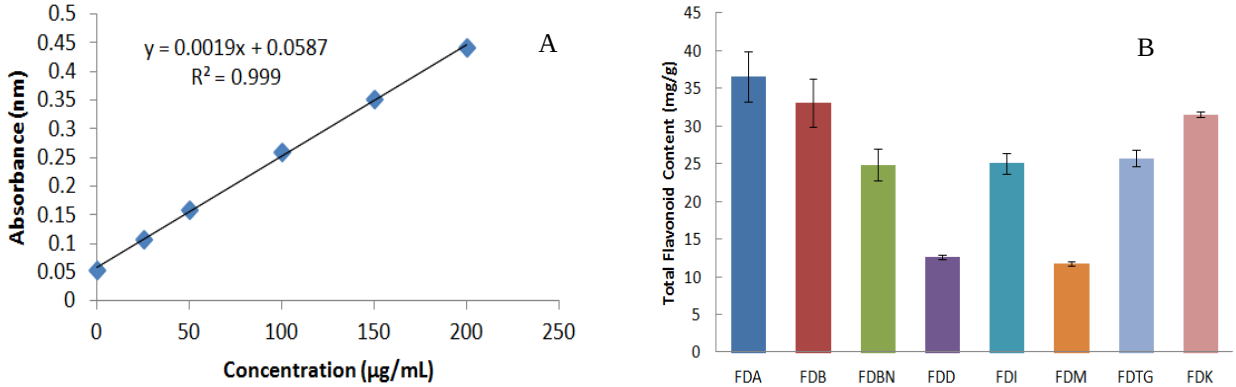


Figure 2: Calibration curve of quercetin equivalent at concentrations of 0, 25, 50, 100, 150, 200 µg/ mL (A). Absorbance detection was at 415 nm. Total flavonoid content of FD varieties determined by the aluminium chloride ($AlCl_3$) assay and calculated as QE in mg·g⁻1 extract based on dry weight (B). Results are the average of triplicates ± SD.

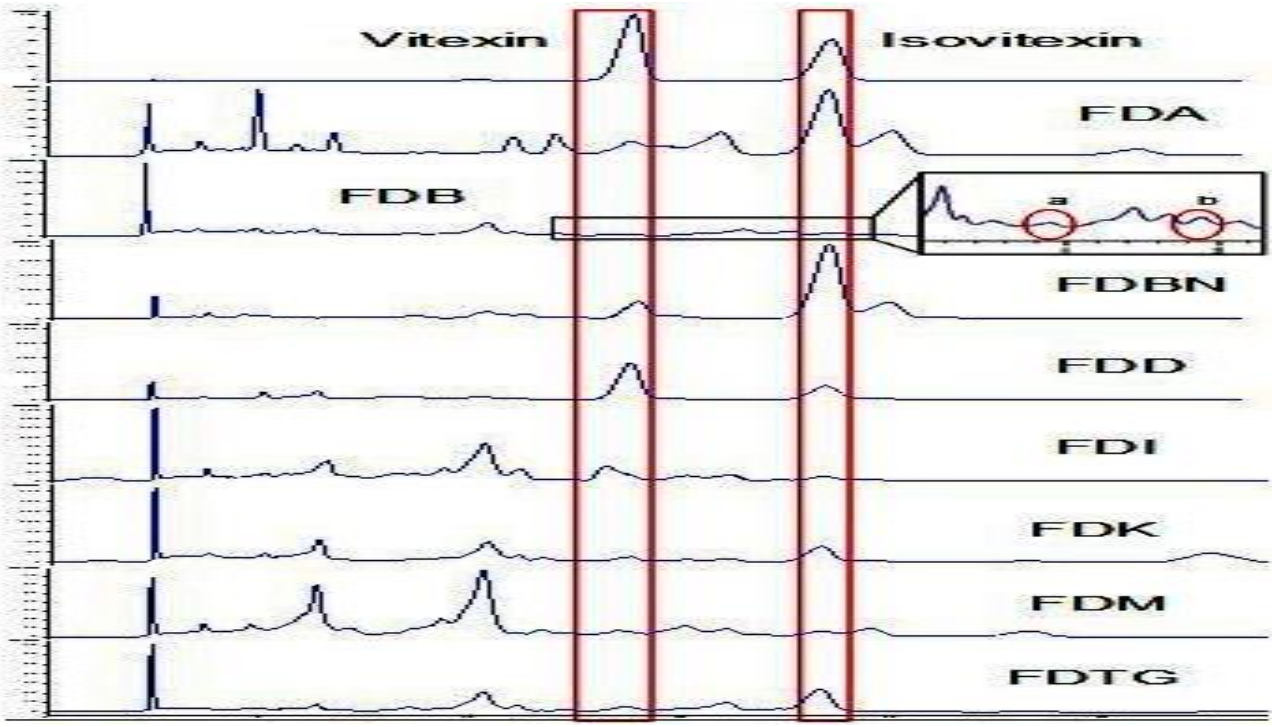


Figure 3: HPLC profiling of marker compounds in eight varieties of *Ficus deltoidea* in methanol extract. (a) vitexin, (b) isovitexin

Table 1: Total phenolic content and total flavonoid content of *Ficus deltoidea* varieties.

Samples	TPC (GAE in mg·g⁻1 ± SD)	TFC (QE in mg·g⁻1 ± SD)
FDA	27.83±6.2	36.58±3.37
FDB	92.54±9.8	33.09±3.22
FDBN	144.93±8.9	24.85±2.12
FDD	239.05±12.5	12.61±0.31
FDI	170.14±4.0	25.04±1.40
FDK	91.31±12.87	31.50±0.37
FDM	74.83±10.1	11.75±0.30
FDTG	35.35±2.5	25.70±1.08

Each value in the table was obtained by calculating the average of three experiments ± standard deviation (S.D)

Table 2: The content of vitexin and isovitexin in FD varieties by validated HPLC method.

Varieties		Vitexin	Isovitexin
		µg/mg ± SD	µg/mg ± SD
Methanol extract	FDA	0.68 ± 0.01	7.95 ± 1.63
	FDB	0.50 ± 0.01	0.86 ± 0.02
	FDBN	8.46 ± 0.17	46.57± 0.03
	FDD	15.30 ± 0.03	9.02± 0.02
	FDI	4.31 ± 0.06	1.43 ± 0.002
	FDK	1.36 ± 0.04	6.36 ± 0.08
	FDM	0.67 ± 0.02	0.70 ± 0.004
	FDTG	0.70 ± 0.01	3.92 ± 0.01

Figure 3 shows the HPLC profiles of methanol extract of FD varieties. The identification of vitexin and isovitexin in all samples were confirmed by comparing the retention times of the chromatographic peaks of the analytes in the samples extracts with corresponding both marker compounds, vitexin and isovitexin as well as the spiking of the standard markers in the samples due to little shifting of the peaks. HPLC chromatogram showed a good separation of vitexin and isovitexin in standard mixture and in the extracts samples. From the HPLC chromatogram, FD var. *deltoidea* were observed to have the highest chromatographic peaks of vitexin while FD var. *borneensis* was the highest of that of isovitexin. Meanwhile, FDB and FDM have shown the lowest peaks for both standard markers. For quantitative analysis, calibration curves of two marker compounds were plotted by using peak areas of five levels concentration of vitexin and six level concentration of isovitexin against its concentration. Those levels selected with the percentage errors were less than 3%. The calibration curves of vitexin and isovitexin had shown good linearity in the range of 15.63- 500 µg/mL which gives correlation coefficient (r^2) of 0.999 for their standard curves and linear regression equation for vitexin and isovitexin were Y= 32.33x – 64.07 and Y= 24.37x – 38.87, respectively (Figure 4). The marker compounds in the samples extract were quantified and the results, which expressed in term of µg marker compound per mg extract and the percentage of weight of marker compound were tabulated and summarized in Table 2 and Figure 5, respectively. Figure 5 showed that FD var. *deltoidea* have the highest content of vitexin while FD var. *borneensis* shows the highest amount of isovitexin. The sequence of marker compounds' content in methanol extract decreased in the order of, vitexin; FDD> FDBN> FDI> FDK> FDTG> FDM> FDB> FDA and isovitexin; FDBN> FDD> FDA> FDK> FDTG> FDI> FDB> FDM.

In vitro α-glucosidase inhibitory assay

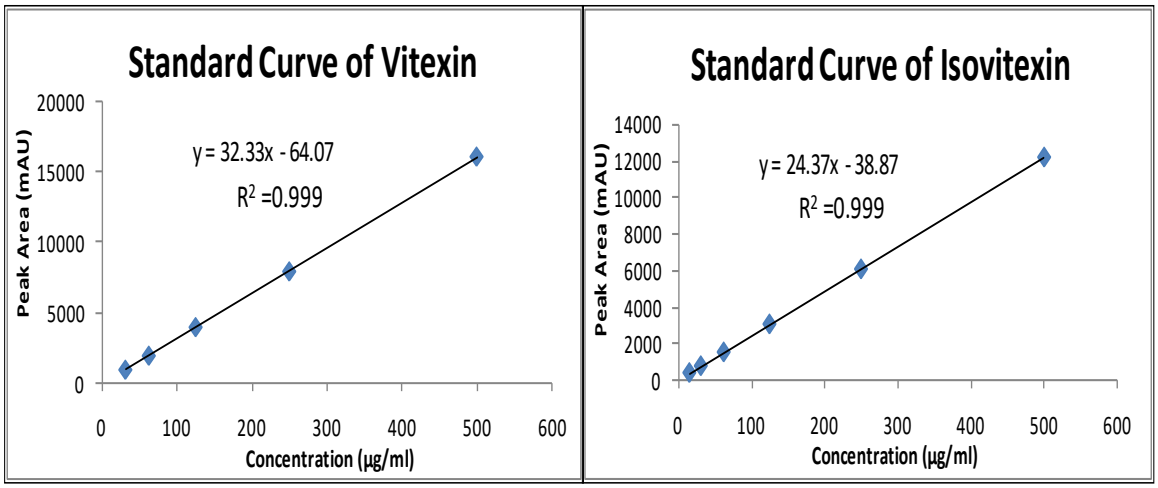


Figure 4: The calibration standard curve of vitexin and isovitexin by validated HPLC method.

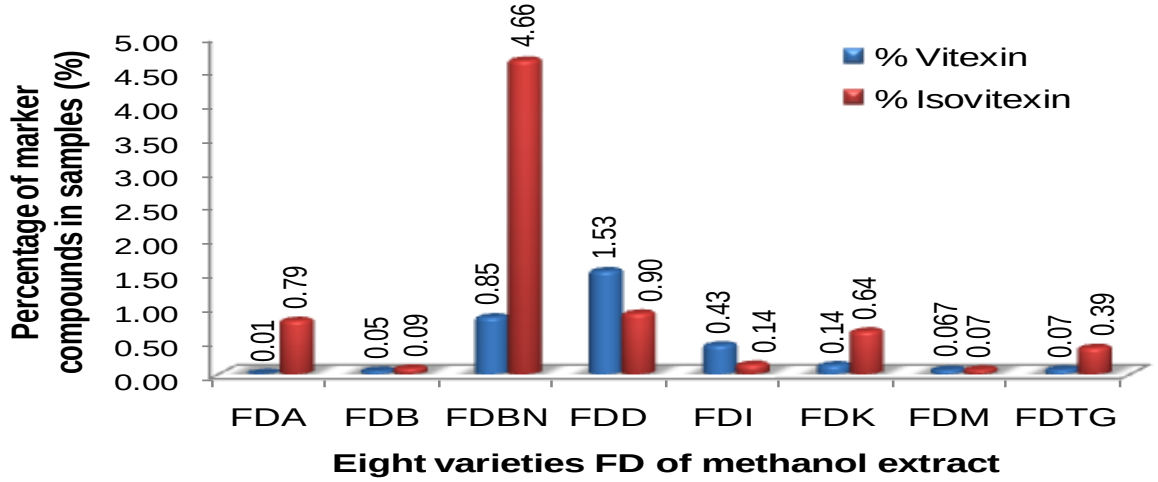


Figure 5: The summary of percentage of weight for vitexin and isovitexin in methanol extract of eight FD varieties by validated HPLC method.

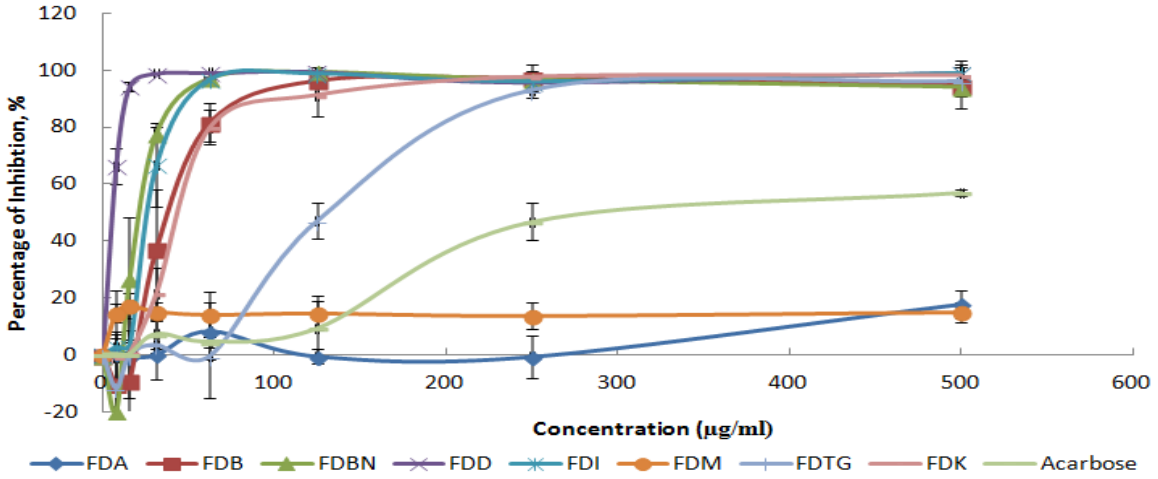


Figure 6: Percentages of inhibition of α-glucosidase enzymes for methanol extract of eight varieties of *Ficus deltoidea* and acarbose was used as positive control (value are expressed as mean ± SD, n=3).

The α-glucosidase enzymes in the intestinal lumen and in the brush border membrane play main roles in carbohydrate digestion to degrade starch and oligosaccharides to monosaccharide before they can be absorbed[27] and delay the absorption of carbohydrates from the small intestine and thus have a lowering effect on postprandial blood glucose and insulin levels. Several inhibitors including acarbose, voglibose and miglitol are clinically used for treatment but clinical side effects occur[28,29]. In this study, 4-nitrophenyl α-D-glucopyranoside (*p*NPG) been used as substrate and the abilities of FD varieties extracts against α-glucosidase

inhibitor were measured. The enzymatic hydrolysis of substrate was monitored by the amount of yellow coloured of ρ-nitrophenol released and the results were expressed as IC_{50} values (µg/mL) for comparison purposes and acarbose standard reference was used as positive control[18,30]. The α-glucosidase inhibitory activities of eight varieties of *Ficus deltoidea* extracts were evaluated and results were shown in Figure 6 and Tables 3. Table 3 revealed that only FD var. *deltoidea*, var. *borneensis*, var. *intermedia*, var. *bilobata*, var. *kunstleri, and* var. *trengganuensis* give the IC_{50} values for their α-glucosidase inhibition. FD var. *deltoidea* showed to have the highest α-glucosidase

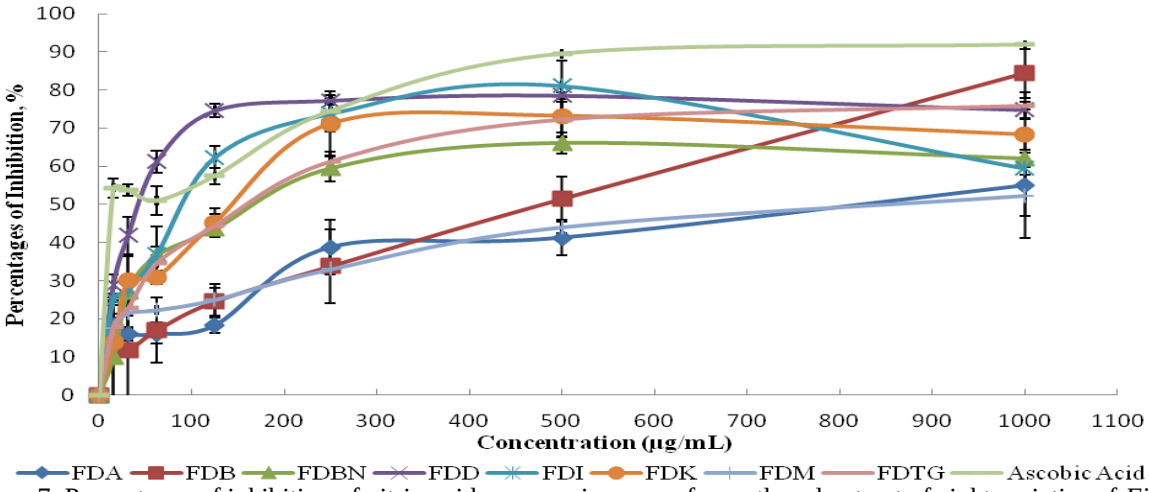


Figure 7: Percentages of inhibition of nitric oxide scavenging assay for methanol extract of eight varieties of *Ficus deltoidea* and acarbose was used as positive control (value are expressed as mean ± SD, n=3).

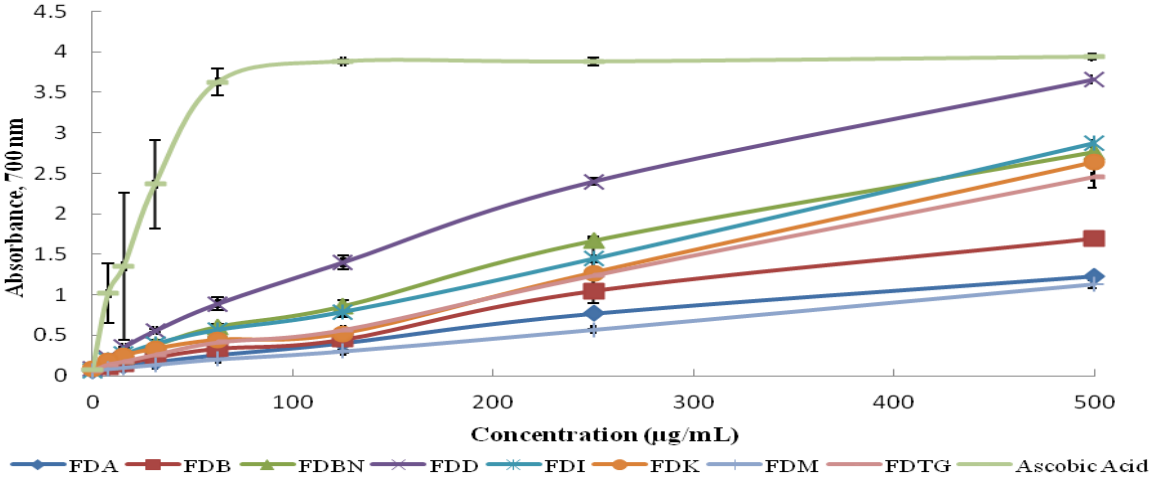


Figure 8: Reducing power of eight varieties of *Ficus deltoidea*. Ascorbic acid was used as control (value are expressed as mean ± SD, n=3).

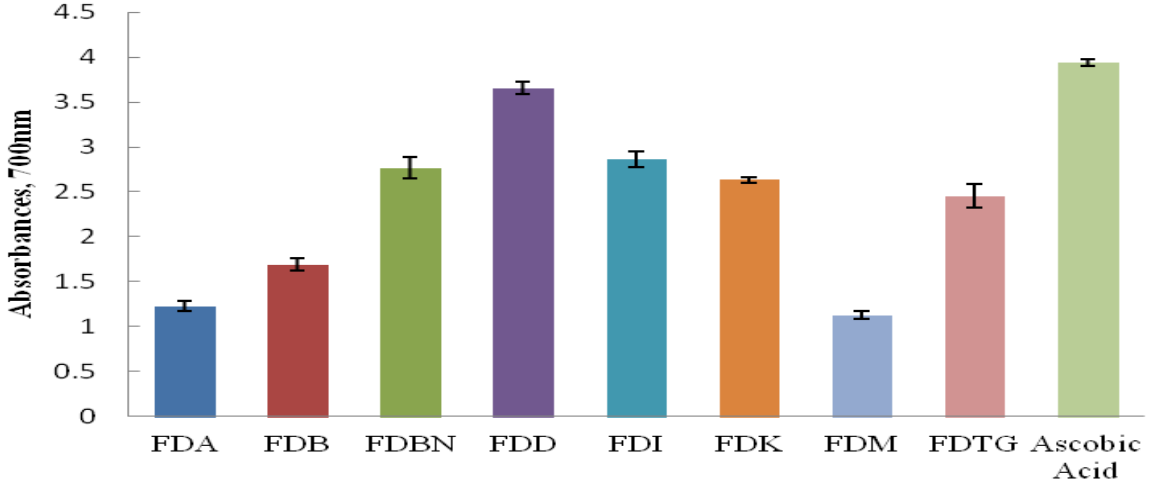


Figure 9: Absorbance value reducing power of eight varieties of *Ficus deltoidea* at concentration 500 µg/mL. Ascorbic acid was used as control (value are expressed as mean ± SD, n=3).

Table 3: The IC_{50} values of α-glucosidase inhibition by methanol extract of eight varieties of *Ficus deltoidea* (FD).

Samples	IC_{50} values (µg/mL)
FDA	CBM
FDB	36.5
FDBN	20
FDD	6
FDI	26
FDK	44
FDM	CBM
FDTG	130
Acarbose	286

CBM = Cannot Be Measured

Table 4: The IC_{50} values of inhibition of nitric oxide scavenging by methanol extract of eight varieties of *Ficus deltoidea* (FD).

Samples	IC_{50} values (µg/mL)
FDA	824
FDB	460
FDBN	166
FDD	42
FDI	88
FDK	143
FDM	850
FDTG	160
Ascorbic Acid	13

activity as compared with other varieties. Meanwhile, FD var. *angustifolia* and var. *motleyana* were found to have the lowest glucosidase inhibitory effects, as their IC_{50} values cannot be measured. The results suggested that α-glucosidase inhibitory activities of the different FD varieties varied possibly due to the phenolic contents in the extracts. However, the result of acarbose as a standard references in this study was found to exert little inhibition on α-glucosidase activity with IC_{50} value of 286 µg/ mL. This was in agreement with other previous studies, which stated that little inhibition or no inhibitory activity of acarbose[7,31]. From the same study, Misbah et al. (2013)[7] showed that α-glucosidase inhibitory activities of fruit of FD var. *kunstleri* in aqueous extract showed higher activity than that of FD var. *angustifolia*.

Antioxidant

Nitric Oxide scavenging assay

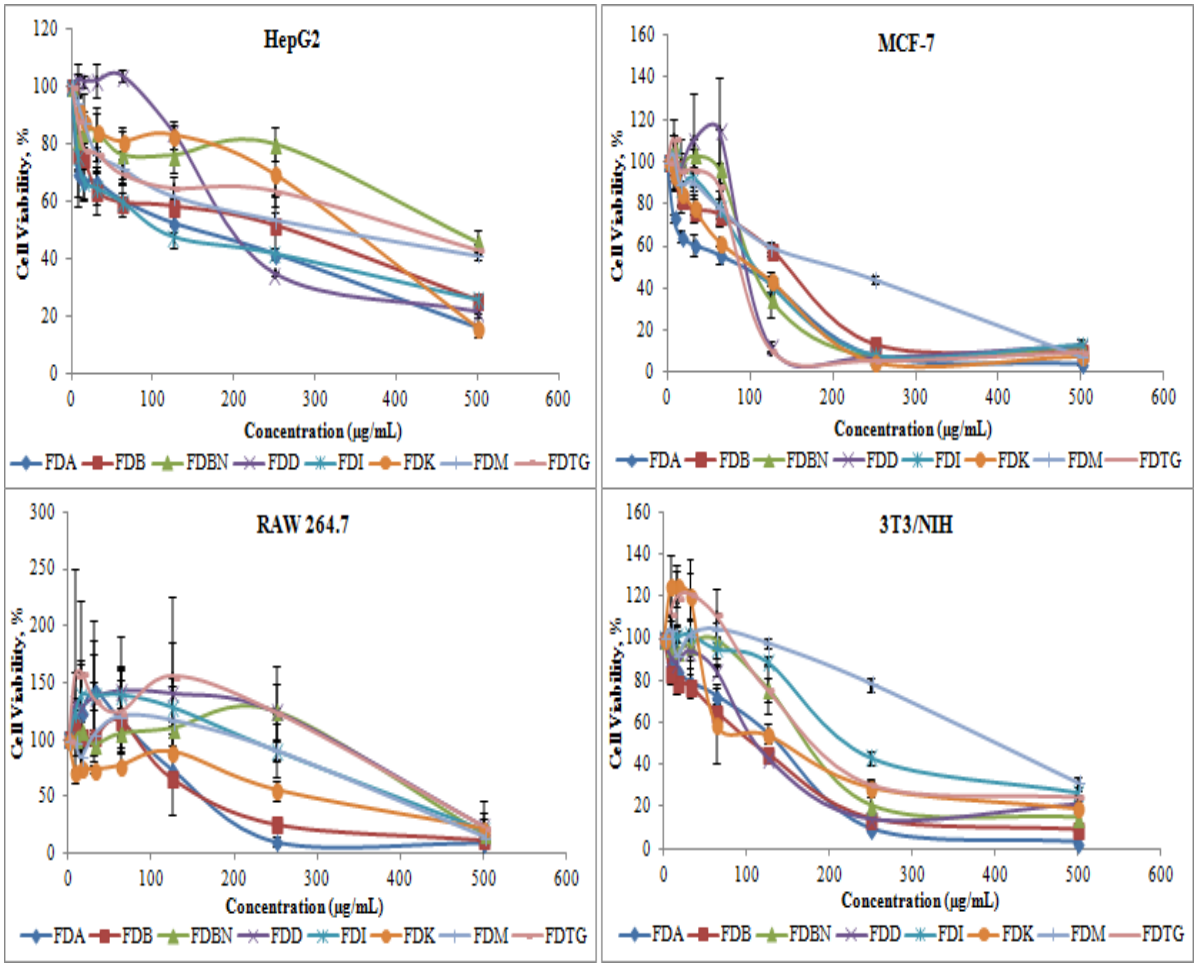
Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions that can be estimated by use of Griess reagent scavengers[32], which can be measured at 546 nm. In addition to reactive oxygen species, nitric oxide is also implicated in inflammation, cancer and other pathological conditions. Nitric oxide is a very unstable species under the aerobic condition and formed during their reaction with oxygen or with superoxide, such as NO_2, N_2O_4, N_3O_4, NO_3 while NO_2 was very reactive. These compounds are responsible for altering the structural and functional behavior of many cellular components[33]. The results are shown as percentage of inhibition of NO scavenging assay in Figure 7 and Table 4. The standard reference used in this study as a positive control was ascorbic acid. The results of NO scavenging activity were expressed in terms of IC_{50} values. All eight FD varieties showed to have the NO radical scavenging activity significantly lower than ascorbic acid with IC_{50} value of 13 µg/ mL. Overall, FD var. *deltoidea* showed the highest NO radical scavenging activity with IC_{50} value of 42 µg/ mL as compared to other varieties. This study was the first attempt in evaluating the ability of the eight varieties of *Ficus deltoidea* to act as antioxidant agents using nitric oxide scavenging assay method. In contrast, previous study showed that methanol extract of FD var. *deltoidea* was significantly lower than FD var. *trengganuensis* as compared with the finding in this work, which FD var. *deltoidea* showed higher activity than FD var. *trengganuensis*[6].

Ferric (Fe^{3+}) reducing antioxidant power (FRAP) assay

In the reducing power assay, the presence of antioxidants in the samples would result in the reducing of Fe^{3+}- Fe^{2+} by donating an electron, which is an important mechanism of phenolic antioxidant action[34]. Reducing power was measured by the intensity of the resultant Prussian blue colour complex at 700 nm. In this assay, the yellow colour of the test solution changes to green and blue depending on the reducing power of test sample. The higher absorbance at high concentration indicates the strong reducing capacity[35]. Figure 8 shows dose-response curves for the reducing powers of eight varieties of *Ficus deltoidea* as compared to that of ascorbic acid. The reducing power capacity of the extracts may serve as an indicator of its potential antioxidant activity. All the samples increased their reducing ability when the concentration of extracts was increased. Figure 9 shows the value of reducing power of all varieties at concentration 500 µg/mL in the following order: FDD> FDI > FDBN > FDK> FDTG> FDB> FDA > FDM. These results in the present study was in agreement with other studies [6], which methanol extract of FD var. *deltoidea* showed higher reducing power than that of FD var. *angustifolia* but in contrast with the results of methanol extract of FD var. *trengganuensis* which showed its reducing power higher than that of FD var. *deltoidea*.

Cytotoxicity assay

The cytotoxicity effect of the methanol extract of eight varieties of *Ficus deltoidea* were investigated through *in vitro* using MTT assay in order to screen for possible cytotoxicity activity against two cancer cell line; Human liver hepatocellular carcinoma (HepG2) and human breast adenocarcinoma (MCF7) as well as normal cell lines; RAW264.7 macrophages and normal mouse fibroblast cell line (3T3-NIH) in a dose-dependent manner after 72 h of treatment. MTT assay measures the reducing potential of the cell using a colorimetric reaction based on the reduction of yellow tetrazolium MTT to a purple formazan dye mitochondrial dehydrogenase enzyme. Figures 10 showed that the percentage of cell viability of methanol extract of eight varieties of *Ficus deltoidea* onto four cell lines (HepG2, MCF-7, RAW264.7 and 3T3-NIH) and all the extracts in varied concentrations possessed cytotoxicity



Figures 10: Percentages of cell viability of methanol extract of eight varieties of *Ficus deltoidea* onto four cell lines (HepG2, MCF-7, RAW264.7and 3T3-NIH).

activity in a concentration dependent manner. In this present study, IC_{50} values of cells viability were higher than the value of 80 µg/ mL for all eight FD varieties in all cells lines. According to the standard National Cancer Institute, the criteria for significant cytotoxicity effect was IC_{50} value which should be less than 20 µg/ mL that considered active against the tested cancer cells[36]. MTT results showed that methanol extract of eight varieties of *Ficus deltoidea* possessed no cytotoxicity effect against all studies cell line. From previous study, toxicological study on *Ficus deltoidea* was reported that this plant does not contain toxic component. Nor Azurah et al. (2011) [37] also reported that *Ficus deltoidea* does not show cytoxicity against cell lines tested.

Correlation analysis

Pearson's correlation coefficient was positively high if $0.61 \leq r \leq 0.97$ and negatively high if $-0.61 \leq r \leq -0.97$. Meanwhile, coefficient of determination (r^2) was measured on how well the regression line represents the data. Statistical analysis showed that there are no correlation between phenolic and flavonoid content of the eight varieties *Ficus deltoidea* extracts ($r^2 < 0.192$). The results therefore reported by Misbah et al. (2013)[7], which no correlation between phenolic and flavonoid content of the extract and fractions of the different fruits of *Ficus deltoidea* varieties with $r^2 < 0.0216$. The correlations between the IC_{50} of α-glucosidase, IC_{50} of Nitric oxide scavenging, FRAP, TPC, TFC and marker contents (vitexin and isovitexin) of *Ficus deltoidea* varieties are presented in Table 5. As shown in this table, our findings showed that a negative weak correlation between IC_{50} of α-glucosidase, TPC, vitexin and isovitexin contents (r = -0.376, r = -0.319 and r = -0.149, respectively), coefficient of determination (r^2 = 0.141, r^2 = 0.102 and, r^2 = 0.022, respectively) and showed positive weak correlation between IC_{50} of α-glucosidase and TFC (r = 0.101, r^2 = 0.010). The results showed a negative high correlation between IC_{50} of Nitric oxide scavenging and TPC with correlation coefficient (r = -0.620), coefficient of determination (r^2 = 0.385). The result correlation for IC_{50} of Nitric oxide scavenging and TPC was positive weak correlation (r = 0.101, r^2 = 0.010). The result also showed negative weak correlation between IC_{50} of nitric oxide scavenging, vitexin and isovitexin content (r = -0.553 and r = -0.253, respectively), coefficient of determination (r^2 = 0.306 and, r^2 = 0.064, respectively). In this study, we also established that the FRAP have positive high correlation (r = 0.791, r = 0.784) with TPC ($P<0.05$) and vitexin content ($P<0.05$), coefficient of determination (r^2 = 0.626, r^2 = 0.615). However, in different with correlation results between FRAP, TFC and isovitexin content, we obtained a negative weak correlation between FRAP and TFC (r =

Table 5: Correlations between the IC_{50} value of α-Glucosidase, IC_{50} value of Nitric oxide scavenging, FRAP assay and marker contents (vitexin and isovitexin) of *Ficus deltoidea* varieties.

Assays	TPC		TFC		Vitexin		Isovitexin	
	r	r^2	*r*	r^2	*r*	r^2	*r*	r^2
IC_{50} α-Glucosidase	-0.376	0.141	0.208	0.043	-0.319	0.102	-0.149	0.022
IC_{50} Nitric oxide scavenging	-0.620	0.385	0.101	0.010	-0.553	0.306	-0.253	0.064
Reducing power (FRAP)	0.791*	0.626	-0.295	0.087	0.784*	0.615	0.292	0.085

*Correlation is significant at the 0.05 level (2-tailed)

-0.295, r^2 = 0.087) as well as positive weak correlation between the FRAP and isovitexin content (r = 0.292 and r^2 = 0.085).

CONCLUSION

The obtained results demonstrate that there are variations in total phenolic, total flavonoid content as well as α-glucosidase inhibition activity, nitric oxide scavenging activity and reducing power of FD varieties. Data also showed that phenolic compounds might play significant role in inhibitory activity of FD as compared to flavonoid content. Accordingly, FD var. *deltoidea* was shown to have the highest total phenolic content, vitexin content, α-glucosidase activity, nitric oxide and reducing power activity as compared to other varieties but had lower total flavonoid content. This finding also shows that all eight varieties of *Ficus deltoidea* do not have potential as cytotoxic agent. The data that were gathered from this study will be the platform of the development of botanical drug for *Ficus deltoidea* Jack.

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