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Isolation and Identification of Antioxidant Compounds in Methanolic Extract of Both Female and Male Plants of *Ficus deltoidea*

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Abstract **:** Antioxidants compounds that may present in the methanolic extract of the leaves and stems of female and male plants of *Ficus deltoidea* were isolated and characterised. The methanolic extracts of the plants were first fractionated by column chromatography. The fractions were then tested on their antioxidant activities by using 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay. The fractions that exhibited highest antioxidant activities were then analysed by liquid chromatography-mass spectrometry (LC-MS), phytochemical screening and Fourier-transform infrared spectroscopy (FTIR) studies in order to identify their antioxidant compounds. The DPPH results revealed that crude extracts of each leaves and stems of each female and male plants of *F. deltoidea* exhibited significant antioxidant activities. It was found that fraction-51 of the female leaf extract expressed the highest antioxidant activity, followed by fraction-8 of the female stem extract, fraction-35 of male leaf extract and lastly fraction-10 of the male stem extract. Possible phytoconstituents that might be in these fractions are terpenoids, lipids, carbonyl compounds, unsaturated hydrocarbons, tannins, phenols and alkaloids.

Keywords: *Ficus deltoidea* (Mas Cotek), antioxidants, methanolic extract, DPPH.

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Introduction

The genus *Ficus* is made up of about one thousand species [1,2]. *Ficus deltoidea* or better known as Mas Cotek, is the most popular *Ficus* species in Malaysia. It is used traditionally as post-partum medication, menstrual cycle regulator, abdominal muscles booster, anti-aging promoter and aphrodisiac for men and women [3,4]. Other traditional uses of the herb are to cure lung diseases, diabetes, high blood pressure and skin problems [3,5,6]. Recent studies have shown that *F. deltoidea* has strong antioxidant activities [7-10].

Antioxidants refers to a group of compounds that are able to protect human bodies from the oxidative damage of free radicals by neutralising and removing free radicals that present in biological systems, thus play an important role as a health-protecting factor [10]. Oxidative stress occurs when there is an imbalance of free radicals and antioxidants in the body. Oxidative stress significantly contributes to the pathogenesis of inflammatory disease, cardiovascular disease, cancer, diabetes, Alzheimer's disease, cataracts, autism and aging [11-16]. Plants usually contain rich source of antioxidants, such as flavonoid, tannins, polyphenols and proanthocyanin. There were some studies on the antioxidant activities of female leaves *F. deltoidea* [4,6,17]. However, scientific reports on the antioxidant properties and potential antioxidant compounds that may present in different parts of both female and male plants are still scarce. Therefore, the aim of this research was to isolate and identify the antioxidants that may present in the methanolic extract of the leaves and stems of female and male plants of *F. deltoidea*.

Experimental

Materials

Hexane, ethyl acetate, thin layer chromatography (TLC) silica gel F_{254} aluminium sheet 20 × 20 cm, silica gel 60 (0.015-0.040 nm), absolute ethanol and acetone were purchased from Merck. Ethanol 96%, methanol 99.8%, L-ascorbic acid powder, chloroform, sand, magnesium sulfate, cotton wool, hydrochloric acid, sodium hydroxide, distilled water, ultrapure water, vanillin, ninhydrin, concentrated sulfuric acid, potassium permanganate, ferric chloride, Ehrlich reagent, methanol of high performance liquid chromatography (HPLC) grade and acetonitrile HPLC grade were purchased from Fisher Scientific. DPPH and dimethyl sulfoxide (DMSO) 99.9% were purchased from Sigma-Aldrich Life Science. All chemicals and solvents used are of analytical grade.

Plant material

The plant materials were purchased from Sungai Buloh Nursery, Selangor, Malaysia. The plant material was identified by taxonomist from Institute Bioscience, University Putra Malaysia (UPM), Serdang, Selangor.

Extraction and fractionation

The leaves and stems of each female and male plant were sorted and blended into powder. The powdered leaves were extracted with methanol by successive soaking for three times at room temperature. The mixtures were gravity filtered. Methanol was then removed under vacuum using a rotary evaporator at 60°C. The dried extract obtained was stored at 4°C prior use. Suitable solvent system to be used for fractionation of the crude extract was developed by using TLC. Three solvents of different polarities such as methanol, ethyl acetate and n-hexane were mixed at different ratio and tested. The dried extract was then fractionated by column chromatography with suitable solvent system that has been determined previously by TLC. Fractions were collected at 10 mL intervals. All fractions were then subjected to TLC separation and fractions with similar retention factor (R_f) values were combined. A total number of 54 combined fractions of female leaves, 26 combined fractions of female stems, 37 combined fractions of male leaves and 10 combined fractions of male stems, were obtained, respectively. The combined fractions were then tested for their antioxidant activities using DPPH radical scavenging assay.

DPPH radical scavenging assay

Concentration of 100 µg/mL of crude extract and fractions of the leaves and stems of each female and male plant were mixed with 50 µL of DMSO containing DPPH radicals (0.45 mM). Ascorbic acid was used as a positive control, whereas water was used as a negative control in this study. Absorbance at 540 nm was measured by ELISA microplate reader with ultraviolet-visible (UV/Vis) spectroscopy after 45 min of incubation at room temperature in the dark condition. The percentage of inhibition (% of IB) was calculated using the following equation (Eq 1) :

$$\text{Percentage of inhibition } \% = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100 \quad \text{(Eq 1)}$$

The fraction with the highest antioxidant activity was recorded and subjected for dose dependence DPPH radical scavenging assay. This was done by serial dilution of this fraction into different concentrations of 12.5 µg/mL, 25 µg/mL, 50 µg/mL, 100 µg/mL, 200 µg/mL, and 400 µg/mL. About 50 µL of each concentration was mixed with 50 µL of DMSO containing DPPH radicals (0.45 mM) and then subjected to the same UV/Vis measurement as mentioned above. The experiments were performed in triplicate. The mean of percentages of inhibition of different concentrations were calculated and the curve of percentage of inhibition versus concentration of fraction was plotted.

Liquid chromatography-mass spectrometry (LC-MS) analysis

Chemical composition of the active fractions with the highest antioxidant activities was determined by LC-MS, using Dionex UltiMate 3000 HPLC and Rapid Separation Diode Array Detector (DAD-3000RS) coupled with Bruker Daltonics micrOTOF-Q ESI-Q-q-TOF mass spectrometer. The fraction components were separated by using reversed phase Perkin Elmer Carbon-18 column (RP C_{18}). The mobile phase was delivered in gradient mode as shown in Table 1. Injection volume of the plant sample was 20 µL, with the flow rate of 1 mL/min at room temperature. Eluted components were ionised and the generated ions were detected by the Time of Flight (ToF) detector of the MS, in m/z range of 200-800 Da.

Fourier Transform Infrared (FTIR) analysis

The FTIR spectra of the fractions with the highest antioxidant activities were recorded on a Shimadzu FTIR-8400S instrument at 16 scans from 4000 to 600 cm^{-1} with resolution of 2.0. The cast film method was employed, where films of the fractions were cast from their solution directly on sodium chloride cell by evaporating off the solvent using a hot air blower.

Table 1 : Gradient profile of mobile phase in HPLC separation

Step	Time (minute)	Flow rate (ml/min)	Acetonitrile (%)	Ultrapurified water (%)
0	5	1.0	50	50
1	6	1.0	0	100
2	3	1.0	20	80
3	3	1.0	50	50
4	3	1.0	80	20
5	3	1.0	90	10
6	12	1.0	100	0

Phytochemical Screening

Phytochemical analysis for major phytoconstituents of the fractions with the highest antioxidant activities was done by using standard qualitative methods described by Awad *et al.* [1,18]. The chemical reagents that were used to stain the active fraction on the TLC plates were vanillin, ninhydrin, concentrated sulfuric acid, potassium permanganate, ferric chloride and Ehrlich reagent. The colour changes was observed and recorded.

Statistical analysis

All data collected from DPPH results were expressed as mean ± standard deviation. The data were subjected to appropriate statistical analysis using analysis of variance (ANOVA), performed using SPSS 18.0 programme. A significant difference will be considered at level of p-value of less than 0.05.

Results and discussion

Determination of the antioxidant activity

DPPH assay is an antiradical assay that was applied for the investigation of antioxidant activity in various studies [19-21]. The principle of this antiradical assay is based on the reduction of DPPH radical by antioxidants. DPPH is a stable nitrogen centred free radical and their colour change from violet to yellow when is reduced by either the process of hydrogen donation or electron donation [22] (Fig. 1). DPPH method is recommended by many authors because this method is repeatable and provides an accurate assay for measuring the antioxidant activity [20,21]. Besides, DPPH assay has many advantages as compared with other methods, such as good stability, sensitivity, simplicity and feasibility [20,23].

In our study, the ability of all the fractions and crude extracts from the leaves and stems of both female and male plants of *F. deltoidea* to act as donors of hydrogen or electron was investigated by DPPH assay with result given in Table 2.

From Table 2, the DPPH results revealed that crude extracts of each leaves and stems of each female and male plants of *F. deltoidea* demonstrated ability to scavenge DPPH radicals (p<0.001). This was in agreement that the *Ficus* species contains rich source of polyphenolic compounds which are responsible for strong antioxidant properties [7,9,10,24,25]. In line with that, previous findings also suggested that crude extracts from the leaves of *F. deltoidea* possesses stronger antioxidant activity than other parts of the plant, such as stem, fruit and root [7,10]. This was in agreement with our findings that overall crude extracts from leaves exhibited higher percentage of scavenging as compared to the crude extracts from stems for both female and male plants. From Table 2, it was also notable that F51 of female leave exhibited the highest antioxidant activity, followed by F8 of female stem, F35 of male leave, F10 of male stem with percentage scavenging of 62.22%, 35.71%, 27.31%, 21.25%, respectively. This suggested that female plants generally possess stronger antioxidant activity than male plants.

When these fractions were further subjected to dose dependent antioxidant activity study, it was found that the percentage of inhibition increased as the concentration of fractions increased from 12.5 to 400 µg/ml. Table 3 indicated that all F51, F8, F35, F10 demonstrated dose dependent radical scavenging ability. This implies that reducing property of these fractions is capable of donating hydrogen or electron to DPPH radicals.

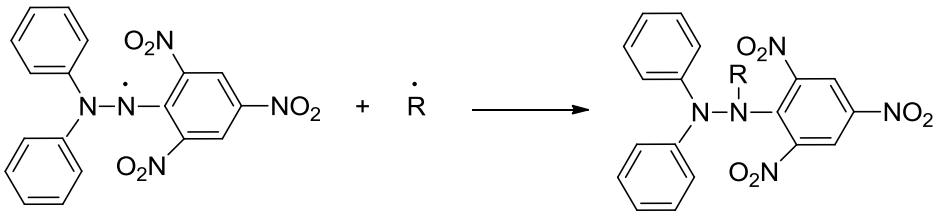

Figure 1 : Reaction of DPPH radical with an antioxidant (R●)

Table 2 : DPPH radical scavenging ability of different parts of *F. deltoidea* plants

Ficus deltoidea		Crude extract/ Fractions exhibited highest antioxidant activity	Percentage of scavenging, %
Plant	Parts of plant		(Mean ± SD)
Female	Leave	Crude extract	39.11 ± 4.30***
		Fraction-51 (F51)	[a]62.22 ± 3.00***
	Stem	Crude extract	10.67 ± 2.38*
		Fraction-8Fraction-8 (F8)	35.71 ± 2.25*
Male	Leave	Crude extract	16.49 ± 5.19*
		Fraction-35Fraction-35 (F35)	27.31 ± 9.29**
	Stem	Crude extract	9.81 ± 1.66
		Fraction-10Fraction-10Fraction-10 (F10)	21.25 ± 1.55*
Positive control	-	-	92.43 ± 0.53***

Table 3 : DPPH radical scavenging ability of *Ficus deltoidea* fractions at different concentrations

Concentration (µg/ml)	Percentage inhibition (Mean ± SD)			
	F51	F8	F35	F10
0	0	0	0	0
12.5	20.12 ± 0.38	5.71 ± 1.03	5.10 ± 0.88	1.24 ± 0.53
25	39.90 ± 0.73***	10.73 ± 1.59***	9.09 ± 2.22	3.30 ± 1.09
50	53.69 ± 1.47***	23.27 ± 0.67***	22.81 ± 1.67***	10.95 ± 1.22***
100	63.48 ± 0.43***	37.16 ± 2.55***	34.30 ± 2.89***	24.54 ± 2.34***
200	74.01 ± 2.67***	51.18 ± 1.98***	50.22 ± 3.72***	32.28 ± 0.65***
400	78.24 ± 0.27***	71.08 ± 0.93***	52.03 ± 3.54***	39.96 ± 1.78***

Note: All fractions were collected using column chromatography separation technique.
Ascorbic acid was used as a positive control for this study.
Each value is the mean of six triplicate experiments.
Values were subjected for analysis of variance one way ANOVA.
Value with superscript letter "a" means 50% inhibition was achieved.
Values with superscript * indicate $p < 0.05$, as compared to control.
Values with superscript **indicate $p < 0.01$, as compared to control.
Values with superscript ***indicate $p < 0.001$ as compared to control.

Identification of the antioxidant compounds

F51, F8, F35 and F10 were then analysed by LC-MS, phytochemical screening and FTIR in order to indicate and identify their antioxidant compounds. LC-MS study showed that these fractions were mixtures of 20, 16, 21 and 15 antioxidant compounds that present in F51, F8, F35, F10, respectively.

Table 4 exhibits the results of phytochemical screening of F51, F8, F35 and F10. From ninhydrin test, it was found that amino acid, amino sugar and amine were absent in all fractions. However, as indicated by positive results from the concentrated sulfuric acid and potassium permanganate tests, lipids, carbonyl compounds, unsaturated hydrocarbons were found present in all fractions. It was found that the possible phytoconstituents present in female leaves (F51) were similar to those present in female stems (F8) and from the vanillin test, terpenoids were only available in female plant. From Ehrlich reagent test, alkaloids were found present in all fractions except F10 of male stem.

Antioxidant compounds present in F51 of female leaves of Ficus deltoidea

From the LC-MS, FTIR, phytochemical screening studies, F51 contained four possible classes of antioxidant compounds named carotenoids, phytosterols terpenoids, together with another known class of compound called tannins that were reported in the previous studies [9,24]. As illustrated in Fig. 2, the possible antioxidant compounds that might present in F51 were lupeol, stigmasterols, lutein, fistulosine and proanthocyanidins [9,26-29].

Lupeol was reported found in the fresh leaves of *Ficus thumbergii* [26]. This compound belongs to the class of terpenoids. From Table 4, vanillin test in phytochemical screening showed the presence of terpenoids in *F. deltoidea*. From our FTIR analysis as shown in Fig. 3, absorption bands at 1451, 3356, 1613, 2924 and 2855 cm^{-1} indicated the presence of cycloalkanes, hydroxyl group, C=C bond and methylene group. The –OH group and the C=C bonds that present in the structure of lupeol exhibited antioxidant activity through hydrogen and electron donation respectively.

Stigmasterol, lipid oriented antioxidants, was reported found in the leaves of *Ficus benjamina* and *Ficus ulmifolia* [27,28]. This antioxidant compound was postulated to be present in F51 as the phytochemical analysis (Table 4) has indicated the presence of C=C bonds and lipid (phytosterols). This was further confirmed by the FTIR analysis (Fig. 3) with the presence of absorption bands at 3356, 1451, 1613 cm^{-1} which corresponded to the presence of hydroxyl, cycloalkanes and C=C bonds. The antioxidant activity of stigmasterol was attributed to the –OH group and C=C bonds that present in the structure.

Table 4 : Results of phytochemical screening of F51, F8, F35 and F10

Staining test	F51	F8	F35	F10
	(Female leave)	(Female stem)	(Male leave)	(Male stem)
Vanillin	+	+	-	-
Ninhydrin	-	-	-	-
Conc. sulfuric acid	+	+	+	+
Potassium permanganate	+	+	+	+
Ferric chloride	+	+	+	-
Ehrlich reagent	+	+	+	-
Possible phytoconstituents present	Terpenoids, lipids, carbonyl compounds, unsaturated hydrocarbons, tannins, phenols, alkaloids.	Terpenoids, lipids, carbonyl compounds, unsaturated hydrocarbons, tannins, phenols, alkaloids.	Lipids, carbonyl compounds, unsaturated hydrocarbons, tannins, phenols, alkaloids.	Lipids, carbonyl compounds, unsaturated hydrocarbons.

Note: "+" indicates positive result; "-" indicates negative result.

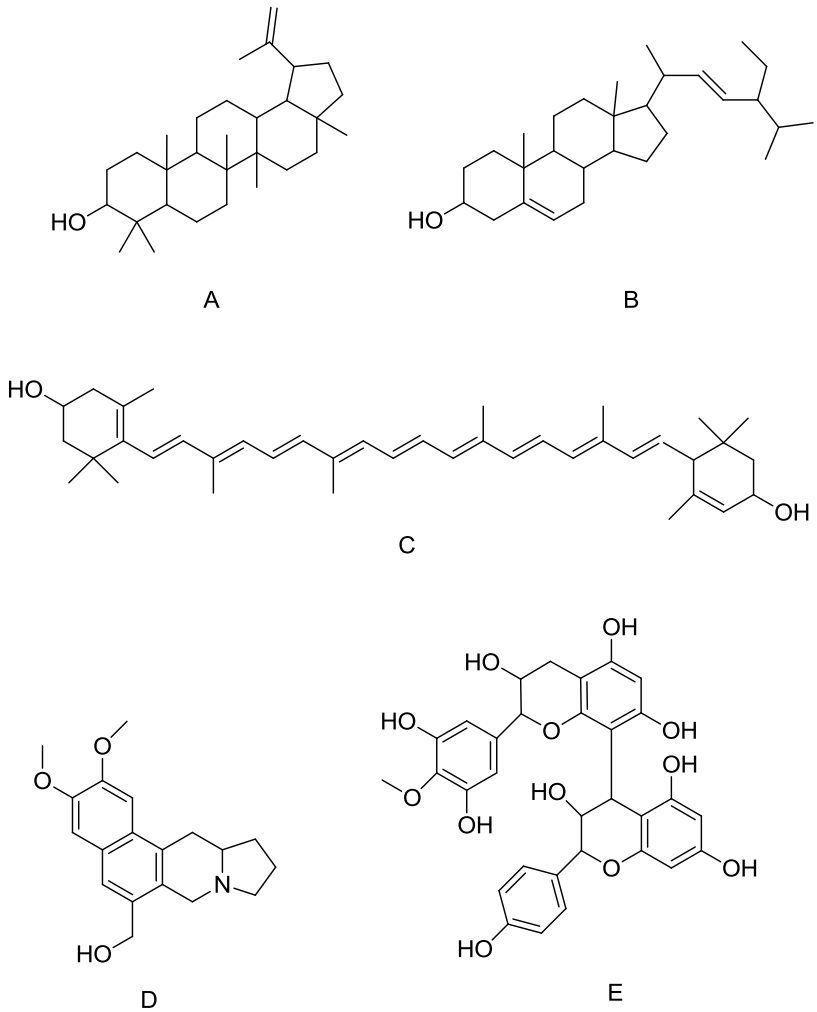

Figure 2 : Structures of postulated compounds present in F51 of female leaves of *Ficus deltoidea* (A: lupeol; B: stigmasterol; C: Lutein; D: fistulosine; E: proanthocyanidins)

Lutein, from the class of carotenoids, was reported found in the leaves of *Ficus ulmifolia* [29]. Phytochemical screening has shown the presence of unsaturated hydrocarbons in F51. As illustrated in Fig. 3, FTIR analysis showed two absorption bands at 3356 and 1613 cm^{-1} which suggested the presence of hydroxyl and C=C bonds. The chemical structure of lutein is predominant with the C=C conjugations which served as the excellent source of electrons which aggressively sought by the free radicals.

Another postulated antioxidant compound found in F51 was fistulosine. Fistulosine was reported found in the bark of *Ficus fistulosa* [29]. This compound belongs to the class of alkaloids. From Table 4, phytochemical screening has indicated the presence of alkaloid in F51. Alkaloids are compounds with nitrogen as part of its heterocyclic ring structure and the availability of the lone pair electrons on the nitrogen atom is able to contribute in the radical scavenging ability through electron donation. FTIR spectrum has further confirmed the presence of alkaloids by showing absorption bands at 3356, 1724 and 1238 cm^{-1} which were assigned to hydroxyl, substituted benzene and ether (Fig. 3).

According to Maisuthisakul *et. al*, phenolics compounds and its derivatives are strongly correlated with antioxidant activities due to the presence of hydroxyl groups, which function as hydrogen donor [21]. The -OH groups that attached to the benzene rings can contribute to radical-scavenging activity by donating hydrogen atoms to combine with the DPPH radicals. In addition, phenolics are the most common and widely distributed compounds in the *Ficus* species [30-32]. The polyphenolics compound found in F51 was proanthocyanidins which belongs to the class of tannins. The presence of tannins in F51 was supported by the Ferric chloride test in phytochemical screening. FTIR spectrum in Fig. 3 has shown absorption bands at 3356, 1238 and 1724 cm^{-1} suggested the presence of phenols, hydroxyl, ether and aromatic groups. The hydrogen atoms of the multiple –OH group in tannins are able to react with free radicals through hydrogen donations.

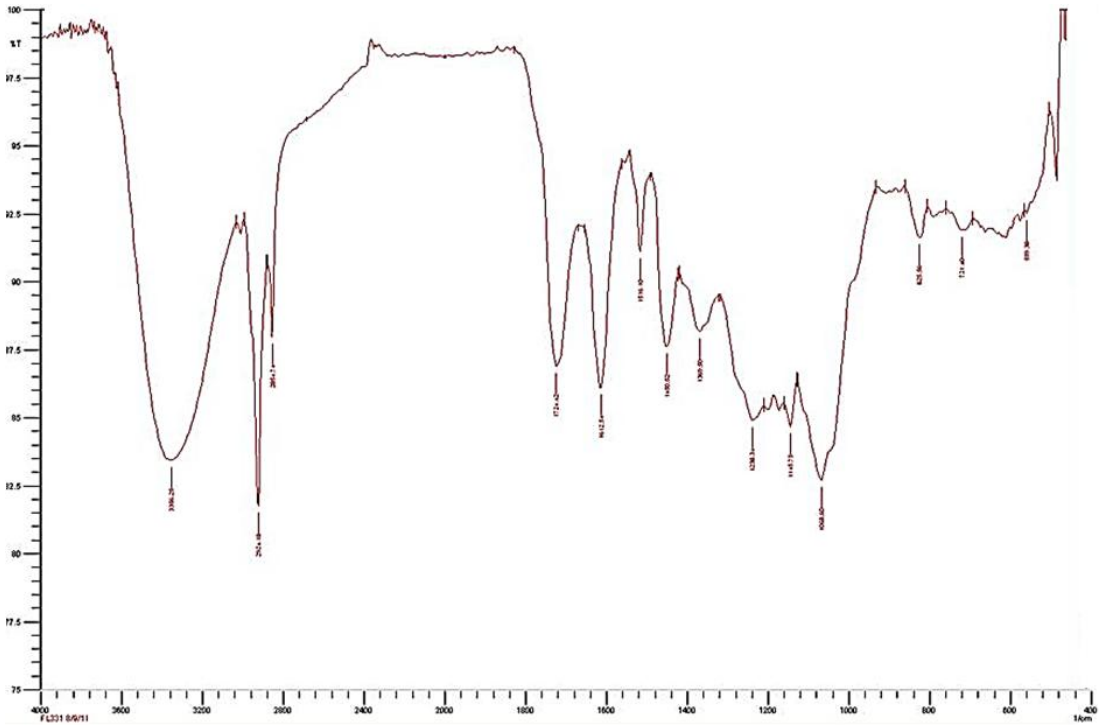

Figure. 3 : FTIR spectrum of F51 of female leaves of *Ficus deltoidea*

Antioxidant compounds present in F8 of female stems of Ficus deltoidea

For F8, LC-MS study showed that there were sixteen compounds present in this fraction and these compounds may be attributed to the presence of naringenin, oleanolic acid, caffeic acid, cinnamic acid, lycopene and hexadecanoic acid. According to the hypothesis that allied species may contain similar antioxidant compounds [34,35], these possible compounds found in other *Ficus* species were likely to be present in FS8 as they were further supported by our phytochemical screening and FTIR studies. The chemical structures of naringenin, oleanolic acid, caffeic acid, cinnamic acid, hexadecanoic acid and lycopene were shown in Fig. 4.

From Fig. 5, IR peaks at 3360 cm^{-1} (-OH stretching), 2932 cm^{-1} (C-H stretching), 1709 cm^{-1} (lactone ring) and 1207 cm^{-1} (C-O stretching) indicated the presence of lactone rings which were usually found in flavonoids. Naringenin, a strong flavonoid antioxidant, might present in F8 as its fragmented ion was detected at m/z 273 in the LC-MS spectrum [33]. This antioxidant was also reported found in *Ficus benjamina* [25,27]. Besides, it was used as an antioxidant marker in a study carried out by Ong *et. al* (2011) on *Ficus deltoidea* [33].

The major difference between the IR spectra of F51 (female leaves) (Fig. 3) and F8 (female stems) (Fig. 5) was the presence of broad –OH stretching at 3360 cm^{-1} in Fig. 5. Oleanolic acid (OA), caffeic acid (CA) and cinnamic acid (CI) which contain carboxylic groups and aromatic rings might be present in F8. This can be supported by the presence of IR peaks at 3360 cm^{-1} (-OH stretching), 1207 cm^{-1} (C-O stretching), 1516 cm^{-1} and 1408 cm^{-1} (aromatic C=C stretching). From literature review, oleanolic acid was a pentacyclic triterpene reported in *Ficus microcarpa L. fil* and *Ficus cordata.*[25,36,37], whereas caffeic acid and cinnamic acid were polyphenolic antioxidants reported in *Ficus benjamina* [25,27].

In addition, hexadecanoic acid, a saturated fatty acid which has long carbon chain and a COOH group, might also be in F8. This compound was reported to be in *Ficus hitral vahl* and *Ficus hispida* [29,30].

Lycopene, a carotenoids tetraterpene assembled from eight isoprene units, was reported found in the fruits of *Ficus carica* [38]. This antioxidant might also be present in F8 as indicated by IR peaks at 1640 cm^{-1} and 1613 cm^{-1} due to C=C conjugated stretching (Fig. 5).

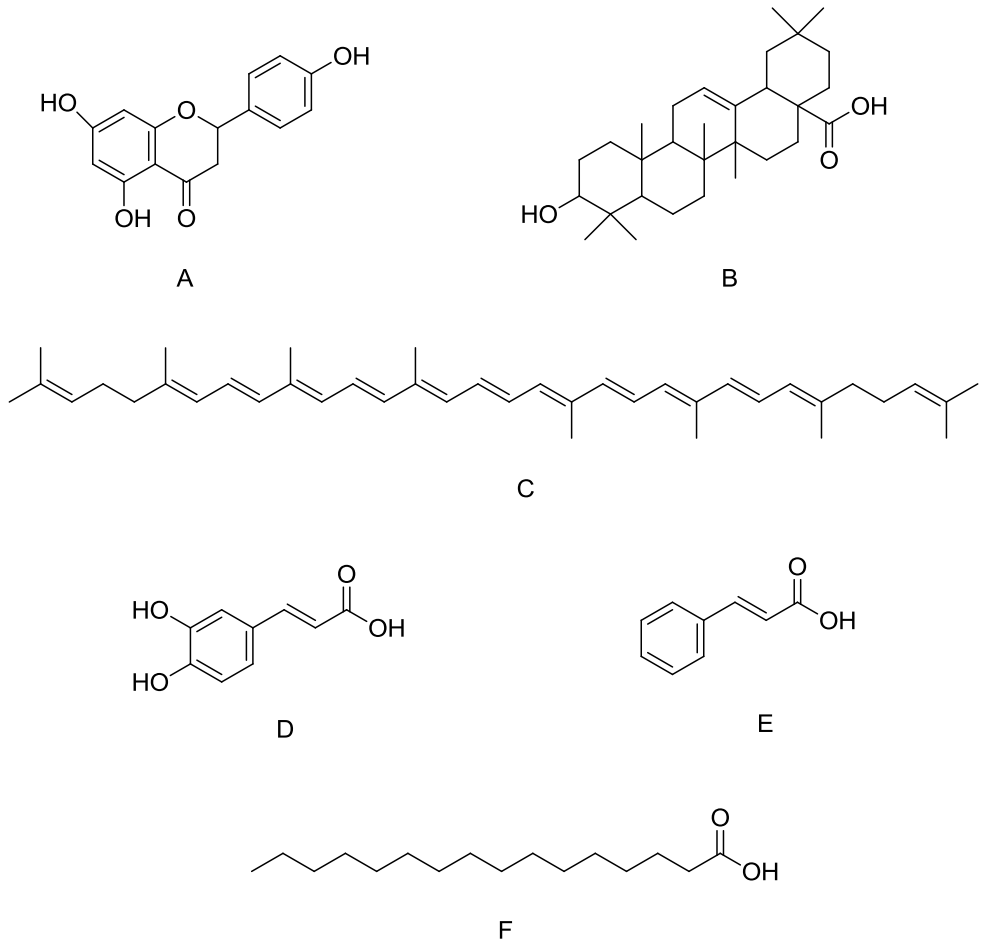

Figure 4 : Structures of postulated compounds present in F8 of female stems of *Ficus deltoidea* (A: naringenin; B: oleanolic acid; C: Lycopene; D: Caffeic acid; E: Cinnamic acid; F: Hexadecanoic acid)

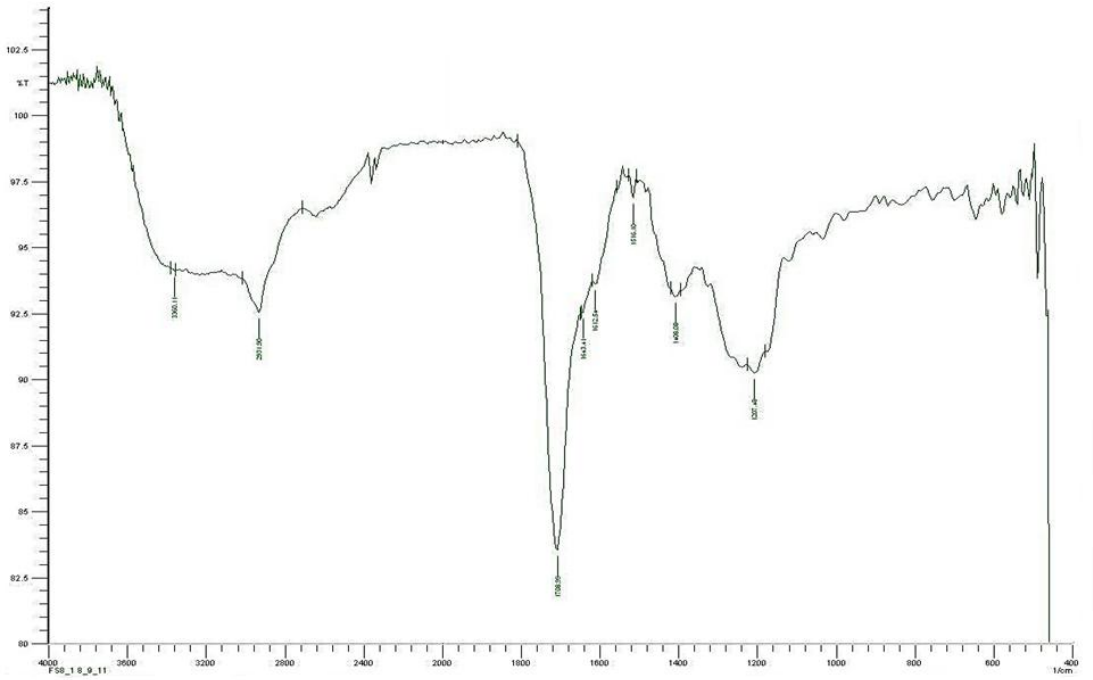

Figure 5 : FTIR spectrum of F8 of female stems of *Ficus deltoidea*

Antioxidant compounds present in F35 of male leaves of Ficus deltoidea

LC-MS study illustrated there were twenty one compounds present in F35. From Table 4, phytochemical screening of F35 showed the presence of lipid, alkaloids, tannin and other polyphenol compounds that might be flavonoid, gallocathecin and proanthocyanidin. From FTIR study, one peak was observed at bending region around 700 cm^{-1}. This might be attributed to the presence of 1,3,5-trisubstituted benzene ring which could be observed in tannins [25]. Another strong peak was observed at 3352 cm^{-1} indicating the presence of hydroxyl group. From the literature review, both -OH group and benzene ring are the most common functional groups that can be found in all polyphenol compounds such as tannins, flavonoids, proanthocyanidins, and gallecathecin which present in the methanolic extracts of some *Ficus* species [9,25,30,31,41]. In addition, both absorbances at 1107 cm^{-1} (C-O stretching) and 3352 cm^{-1} (hydrogen-bonded –OH stretching) indicated the presence of saturated secondary alcohols which are commonly found in the structure of flavonoids, gallocathecins and proanthocyanidins [9,30,31]. In addition, C-O bond could also be found in the structure of tannin and proanthocyanidin [9,25].

Antioxidant compounds present in F10 of male stems of Ficus deltoidea

LC-MS study illustrated there were fifteen compounds present in F10. From Table 4, phytochemical screening showed the presence of lipids, carbonyl compounds and unsaturated hydrocarbons in F10. FTIR spectrum of F10 was very similar to F35, except there was no significant peak observed at bending region around 700 cm^{-1}. There was rarely any literature reported on the methanolic extracts of male stems of *F. deltoidea*, based on the findings of other *Ficus* species, the possible compounds that might contribute towards the antioxidant activity of the plant were flavonoid glycosides, phytosterols and sterol derivatives [7,9,10,24,25,30].

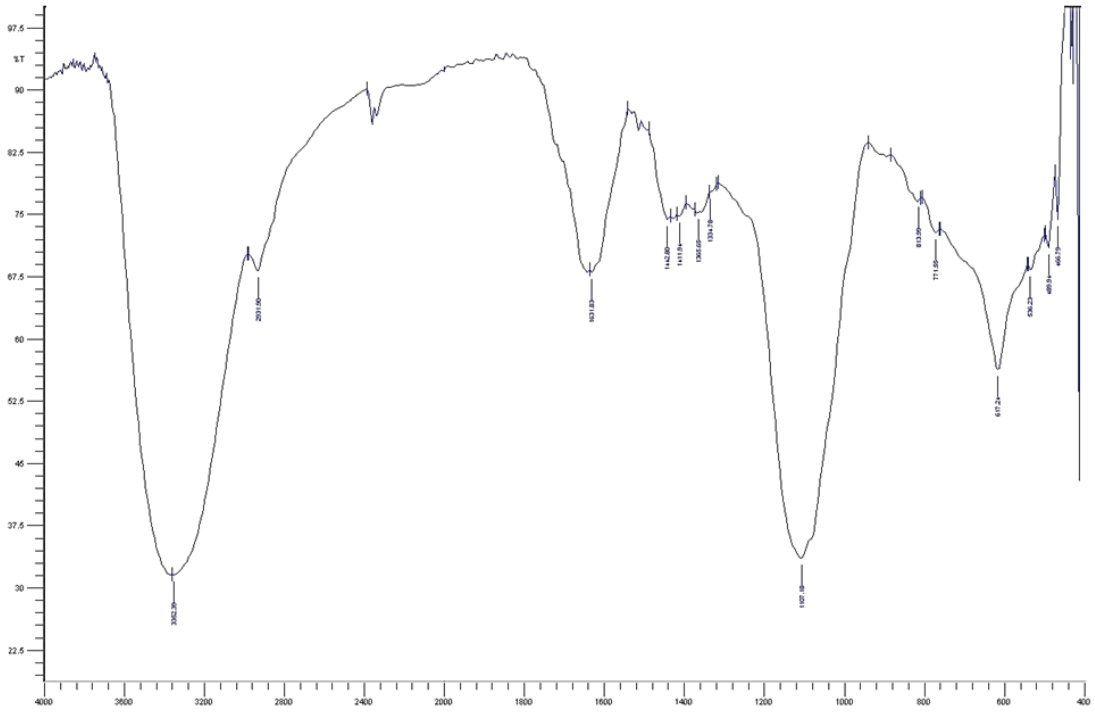

Figure 6 : FTIR spectrum of F35 of male leaves of *Ficus deltoidea*

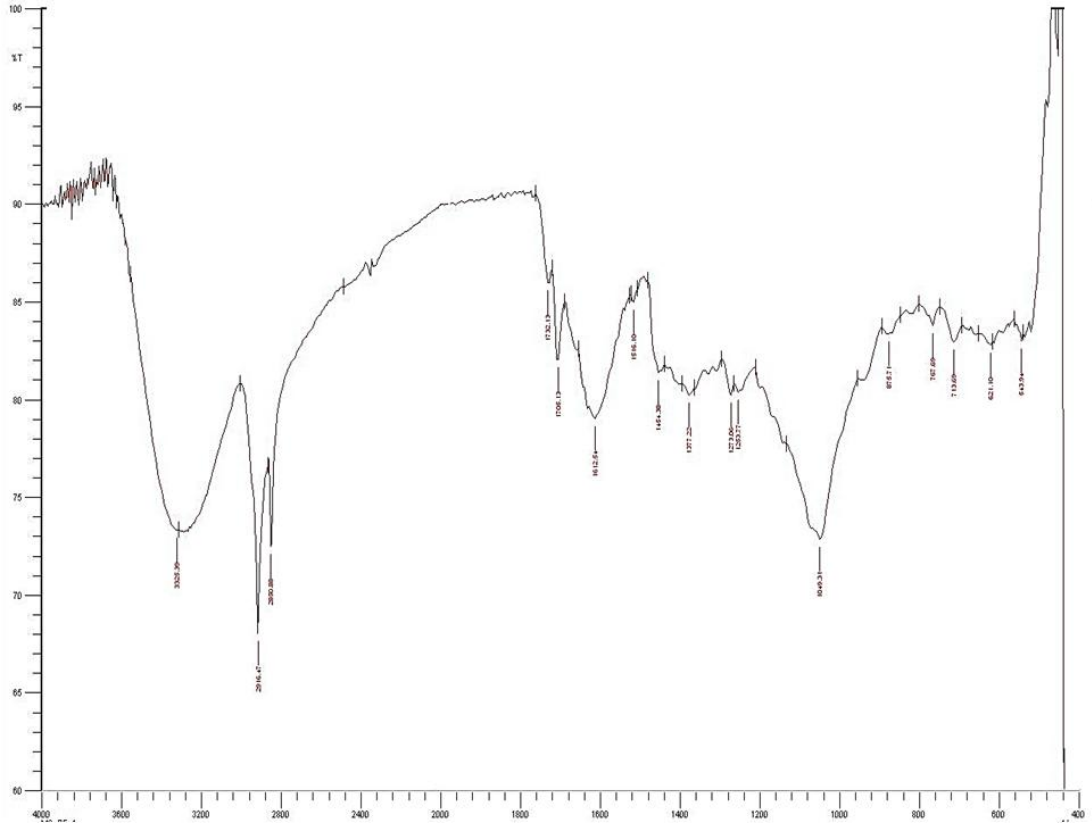

Figure 7 : FTIR spectrum of F10 of male stems of *Ficus deltoidea*

Conclusion

The results from DPPH radical scavenging assay revealed that both female and male plants of *F. deltoidea* showed significant antioxidant activities. The possible classes of compounds that responsible for the antioxidant activity of the leaves and stems of female and male plants of *Ficus deltoidea* were identified from LC-MS analysis, phytochemical screening and FTIR study. These results showed that female plant exhibited higher antioxidant activities than male plants of *F. deltoidea*. Therefore, *F. deltoidea*, especially female leaves, can be considered as one of the potential new sources of natural antioxidants for nutraceutical products. Further work such as F51 could be re-chromatography to collect pure compound for further structural elucidation and identification.

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