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Original Article

PHYTOCHEMICAL FINGERPRINT AND BIOLOGICAL ACTIVITIES OF THREE MALAYSIAN *FICUS DELTOIDEA* CULTIVARS.

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Abstract

Background

Ficus deltoidea (Moraceae), is a Malay Traditional Medicine locally known as Mas Cotek. Three varieties (*angustifolia*, *deltoidea* and *kunslerii*) has been indistinctly used.

Aims

We here aim to better understand their chemistry and bioactivities to inform future scientific and agronomic research.

Methods

We extracted and analyzed (HPTLC and HPLC-UV) samples from these varieties. The *in vitro* screening included the scavenging of DPPH and NO radicals, activity upon tyrosinase and cytotoxicity against three human prostate cancer cells (PC3, DU145 and LNCaP) using the sulforhodamine B proliferation assay and the MTT mitochondrial viability assay.

Results

We show that vitexin, orientin and isoorientin may act as intraspecific and interorgan phytomarkers. The biological activities of the extracts point out to the antioxidant value of extracts from the *deltoidea* and *kunslerii* varieties whilst the inhibition of tyrosinase is only present in the roots extract of the var. *deltoidea* which is also endowed with cytotoxic activity against prostate cancer cells.

Conclusion

We suggest that the three Malaysian *Ficus deltoidea* botanical varieties (*angustifolia*, *deltoidea* and *kunslerii*) can be considered chemovars. The most active extract was from the roots of var. *deltoidea* that shows antioxidant, antimelanogenic and cytotoxic potential.

INTRODUCTION

Ficus deltoidea, known as Mas Cotek in Malaysia and as Malaysian Mistletoe Fig internationally, is one of the species of fig tree from the Moraceae family, which is a traditional medicinal herb and has been widely used in postpartum medication among the Malays for a long time (Bunawan et al., 2014). The functions of this herb are thought to be capable of detoxifying the body, reducing cholesterol, restoring energy, improving blood circulation, repairing blood flow and the problems associated with blood flow (Huda Farhana et al., 2007). Thus, *Ficus deltoidea* also has been indicated in the treatments of wounds, rheumatism and sores. In addition, it has been applied to treat disorders related with the menstrual cycle, diabetic leucorrhoea, high blood pressure and gout (Burkill and Haniff, 1930). Its fruits are traditionally used to relieve toothache, cold and headache by means of chewing. Apart from this, its formulated products such as capsules, tea, and tonic tea are distributed throughout Malaysia. The herbal juices made from *Ficus deltoidea* are often used to improve health and beauty (Ramamurthy et al., 2014).



Figure 1. *Ficus deltoidea* Jack (Credits: Forest and Kim Starr, under CC-BY-3.0).

Ficus deltoidea is attracting many researchers' attention and some active compounds have already been isolated and determined from its leaves and figs. There are three major groups of constituents which are phenolic compounds (catechins, flavones, naringin, vitexin, isovitexin), tannins (ellagic acid and gallic acid) and phenylpropenes (caffeic acid, coumaric acid and ferulic acid (Ramamurthy et al., 2014). Apart from this, some researchers have now also isolated and studied active compounds contributing to its floral fragrances (aliphatic groups and terpenoids including terpenes, triterpenes and sesquiterpenes) and fruits of *Ficus deltoidea* which are rich in volatile compounds deriving from the shikimic acid pathway (Grison-Pig'e et al., 2002). According to this research, it has been found that these constituents are related to many antioxidant, anti-inflammatory, antibacterial, anti-diabetic, anti-nociceptive, anti-ulcer, anti-hypertensive and multiple cancer preventives as well as other activities (Bunawan et al., 2014).

Like other botanicals with a wide range of medicinal properties, much research and experiments have been conducted to explore its potential therapeutic values especially in anti-diabetic activity, anti-nociceptive activity, antioxidant and anticancer related properties (anti-melanogenic effects and anti-Human leukemic HL-60 cell line properties) (Norrizah et al., 2012). To elaborate and clarify the effectiveness and mechanisms of action of *Ficus deltoidea*, *in vitro* (cell, non-cell) studies, *in vivo* studies, research in animal

subjects been conducted, but also sophisticated analytical techniques (Lip et al., 2009; Omar et al., 2011) have been applied in research to determine the structures of related compounds. However, compared to other studies, the anti-melanogenic effects of this plant species have received little scientific research and little success. One recent study (Oh et al., 2011) was carried out to evaluate the anti-melanogenic activity of *Ficus deltoidea* by testing its extract with cultured B16F1 melanoma cells. In addition, α -MSH-induced melanin synthesis (α -MSH) assay, intracellular tyrosinase activity assay and the expression of microphthalmia-associated transcription factors (MITFs) assay were also carried out by the authors. According to the writers, they have drawn the conclusion that the abilities of *Ficus deltoidea*, down-regulation of cellular melanogenic components including tyrosinase, MITF and α -MSH, show that this plant can be a promising whitening cosmetic active ingredient. However, the related information about this plant still seems to be very limited. Moreover, although researchers have pointed out the mechanisms of antioxidant of *Ficus deltoidea* are concerned with its major compounds- flavonoids and tannins in many cases, the chemical variations of different varieties of *Ficus deltoidea* as well as their bioactivities are seldom evaluated or studied (Dzolin et al., 2010). Therefore, further studies in these aspects on *Ficus deltoidea* can help researchers to understand its potential therapeutic values better.

This species presents a high regional variability with 2 subspecies, 13 varieties and 4 forms of the species have been recognized, the vast majority of them present in Malaysian forests: var. *bilobata*, var. *angustifolia*, var. *kunstleri*, var. *intermedia*, var. *motleyana*, var. *deltoidea*, var. *kinabaluensis* and var. *trengganuensis*. Local collectors of *F. deltoidea* identify them mainly based on the leaf and fruit morphology (Berg, 2003; Fatimah et al., 2012). Recently, the genetic basis for such variability have been studied and points towards a low intraspecific genetic diversity of *F. deltoidea* population in Malaysia (Zimisuhara et al., 2015).

We here aim to study the diversity in chemical constituents of *Ficus deltoidea* cultivars, by developing a HPTLC method to fingerprint these plant materials when extracted by different solvents (hexane extract, chloroform extract, water extract and ethyl acetate extract). The identity of characteristic compounds is achieved by the combination of HPTLC and HPLC-UV chromatography. Some basic biochemical properties such as scavenging against DPPH \cdot and NO \cdot radicals, inhibition of tyrosinase enzyme and cytotoxicity are also evaluated.

MATERIALS AND METHODS

Plant material

Plant materials used in this research were collected from Kedah, Malaysia over the period of September 2013 to February 2014. All the plant materials were authenticated by Mr Husnui Hanani Solb at Universiti Putra Malaysia, Malaysia. The vouchers were deposited at the Institute of Bioproduct Development, Universiti Teknologi Malaysia, Malaysia.

Table 1: Botanical characteristics of the *Ficus deltoidea* varieties.

| Cultivars | Part | Code | Voucher |
|---|--------|------|------------|
| <i>Ficus deltoidea</i> var. <i>angustifolia</i> (Miq.) Corner | Aerial | FDAa | SK 2309/13 |
| <i>Ficus deltoidea</i> Jack var. <i>deltoidea</i> | Aerial | FDDa | SK 2310/13 |
| <i>Ficus deltoidea</i> Jack var. <i>deltoidea</i> | Root | FDDr | SK 2311/13 |
| <i>Ficus deltoidea</i> var. <i>kunsleri</i> (King) Corner | Aerial | FDKa | SK 2312/13 |

Standards and other reagents

1,1-diphenyl-2-picrylhydrazyl, rutin, kaempferol, caffeic acid, quercetin, vitexin, *L*-tyrosine (reagent grade, $\geq 98\%$), and dimethyl sulfoxide were purchased from Sigma-Aldrich (Sigma-Aldrich, USA). Acetic acid (Fisher Scientific) and formic acid (for synthesis $\geq 97\%$, Alfa Aesar) were used. 0.5 g of diphenylboric acid aminoethyl ester was weighed and dissolved in 100 ml ethyl acetate ($\geq 99.9\%$, Fisher Scientific) as the natural product reagent for HPTLC. 2.5 g of polyethylene glycol-4000 (laboratory reagent) was weighed and dissolved in 50 ml absolute ethanol as the PEG reagent for HPTLC.

Preparation of plant extracts

50 grams of five plant samples were transferred into five Erlenmeyer flasks and 500 ml methanol ($\geq 99.9\%$ for HPLC, Sigma-Aldrich) was added in each of the flasks. Each flask was covered with aluminum foil and placed in a magnetic stirrer (KMO 2 basic, labortechnik, IKA). The samples were extracted with methanol for 24 hours. Then the solvent in each flask was collected and a fresh solvent was added to the plant samples. This procedure was repeated three times. Each extract was transferred into a round-bottom flask and the solvent was evaporated completely by a rotary evaporator (Laborota 4003 control, Heidolph). The dried plant samples were dissolved in 10% methanol (100 ml), and sonication bath (Grant Instruments Ltd) was applied to dissolve the plant samples in the solvent. Each 10% methanol extract was subjected to liquid-liquid partition with 100 ml hexane ($\geq 99.9\%$ for HPLC, Sigma-Aldrich) for 3 times and the hexane fraction was collected. The rest of the methanol extract was concentrated using a rotary evaporator to about 90 ml, and deionized water (Milli-Q water system, Millipore, Bedford, USA) was used to top-up the volume to 100 ml. Then the aqueous extract was sequentially partitioned with equal volume of chloroform ($\geq 99.9\%$ for HPLC, Sigma-Aldrich) and ethyl acetate ($\geq 99.9\%$ for HPLC, Sigma-Aldrich). In each partition step, the procedure was repeated 3 times. All of the chloroform fractions, aqueous fractions and ethyl acetate extracts were collected. Each fraction was transferred to a round-bottom flask. Then the solvents of each fraction (hexane, chloroform and ethyl acetate) were completely evaporated by a rotary evaporator whereas a freeze dryer was applied to eliminate the water in aqueous fractions. All the fractions were stored in a fridge ($-20\text{ }^{\circ}\text{C}$) until needed.

Thin Layer Chromatography (TLC) analyses

The TLC analysis was performed on 10x20 silica gel 60 F254 TLC plate, aluminum sheet Analytical Chromatography, (Merck, Germany) for identifying flavonoids compounds in different plant ethyl acetate extracts. Approximately 10 μL of each sample (10 mg/ml) was applied on each TLC plate along with reference standards (1 mg/ml). All these samples were spotted on the TLC plate, 1 cm from the bottom of the plate, and then developed in a TLC chamber (24 cmx24 cm) saturated with the developing solvent system (ethyl acetate: acetic acid: formic acid: water (100:11:11:26) (Sherma and Fried, 1996; Shafaei et al., 2012). After the development, the natural product reagent and polyethylene glycol (PEG) 4000 were sprayed on each plate to enhance the fluorescence and viewed under UV light at white light, 254 nm and 365 nm by HPTLC (Camag, TLC Visualizer).

High-Performance Liquid Chromatography (HPLC) analyses

For HPLC analysis, extracts were mixed with methanol at 10 mg/ml and dispersed in an ultrasonic bath. A volume of 5 ml of each sample was filtered through a 0.45 μm filter before analysis. The rest were evaporated to dry and stored in a freezer. The filtered samples were injected (50 μL). The HPLC system was a Perkin Elmer series 200 EP Diode Array Detector combined with series 200 pump, Flexar LC autosampler and TotalChrom software (Perkin Elmer Company, USA), with an Agilent Aqua-C18 column (250mmx4.6mm i.d., 5 μm) was used for the extract analysis. All samples were eluted with a mobile phase consisting of formic acid solution (A, 0.1 % v/v) and acetonitrile (B, $\geq 99.9\%$ for HPLC, Fisher Scientific) using a linear gradient program (0%-25% B in 0–55 min, 25%-30% B in 55-65 min, 30%-40% B in 65-75 min, 40%-100% B in 75-77 min, 100% B in 77-79 min). The flow rate was 1.0 mL/min and the detector wavelength was 365nm. Each standard was dissolved in 1 mg/mL methanol and 10 μL injected.

1,1-Diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging assay

This assay measures the free radical scavenging potential of each crude extract. The method described by Sharma & Bhat (2009) and modified by Prieto et al. (2012) was used. Briefly, 100 μL of a 0.1 mM DPPH ethanolic solution was added to 100 μL of each diluted extract or reference standard antioxidant in 96-microwell plates. After 30 min of incubation in the darkness at room temperature the absorbance was measured at 517 nm against a blank.

$$\% \text{ radical scavenging activity} = [1 - ((\text{Abs Sample} + \text{DPPH}) - (\text{Abs Sample Blank})) / (\text{Abs Control})] \times 100$$

Nitric oxide scavenging assay

The experimental protocol is based on the Griess reaction as previously described (Sreejayan, 1997). A volume of 200µl of sodium nitroprusside (5mM) and 50µl of sample are mixed in microtiter plates (96 wells). At 1-hour intervals, pipet 50µl supernatant onto a second plate, add 50µl of Griess reagent (1% sulphanilamide, 0.1%). This was then incubated again at room temperature for an additional 15 minutes. The absorbance was read at 540 nm and the percentage of NO inhibition and total NO remaining in solution was calculated using a calibration curve built up with Sodium Nitrite.

$$\% \text{ NO scavenging} = [(conc. \text{ of control} - conc. \text{ of extract}) / conc. \text{ of control}] \times 100.$$

Cell Lines

The following tumour cell lines were used: the PC-3 cell line (ATCC Number: CRL-1435™) was kindly provided by Dr Cyrill Bussy (Centre for Drug Delivery Research, UCL School of Pharmacy, UK), the DU145 cell line (ATCC Number: HTB-81™) was purchased from Sigma Aldrich UK, and was obtained from the American Type Culture Collection (ATCC) and the LNCaP clone FGC cell line (ATCC Number: CRL-1740™) was purchased from Sigma Aldrich UK, and was obtained from the American Type Culture Collection (ATCC). All cell lines are adherent cells that tend to grow as monolayer and are classified as Biosafety Level 1. cells.

Cell Culture Protocols

Both PC-3 and LNCaP cell lines were grown in a cell culture flask (Nunc, UK), surface area 75cm² and maintained in RPMI-1640 (Roswell Park Memorial Institute medium) (Lonza, BE12-702F) containing L-glutamine. The media was supplemented with 10% of heat-inactivated fetal bovine serum (FBS) (Gibco, UK) and 1% penicillin-streptomycin antibiotics containing 10000 Units/ml of penicillin and 10000 µg/ml streptomycin (Gibco, UK) to prevent bacterial growth. DU145 cell line was grown in a cell culture flask (Nunc), surface area 75cm² and maintained in EMEM (Eagle's Minimum Essential Medium) (Sigma, M4655) containing Earle's salt, L-glutamine and Sodium bicarbonate in an incubator (NuAir Inc.) with humidified air of 5% CO₂ and atmosphere at 37°C (Freshney, 2005). The media was supplemented with 10% of heat-inactivated fetal bovine serum (Gibco, UK) and 1% penicillin-streptomycin antibiotics containing 10000 Units/ml of penicillin and 10000 µg/ml streptomycin (Gibco, UK) to prevent bacterial growth. All cells were maintained at 37°C in a humidified atmosphere of 5% CO₂. The prepared media was used to grow and seed the cells in a 96-well plate for cellular based assays and for plant extracts as well as fractions dilution.

Tyrosinase inhibitory activity

Quantitative inhibition was assessed according to the method described by Masuda et al. (2005). 2.5 mM L-tyrosine in buffer and the sample in DMSO are mixed in a total volume of 160 µL, to which 40 µL of mushroom tyrosinase (46 units/mL) is added. After 20 minutes the absorbance was measured for each well at 475 nm, then the % of Tyrosinase inhibition calculated against the control.

$$\% \text{ Tyrosinase activity} = \frac{\text{Absorbance (sample)} - \text{Absorbance (blank)}}{\text{Absorbance (vehicle control)} - \text{Absorbance (blank)}} \times 100$$

Sulforhodamine B assay (SRB) proliferation assay

The assay was performed independently in triplicate according to a previously described by Vichai & Kirtikara (2006). The cells were seeded at density of 10,000 cells/well in a 96-well plate (Thermo Scientific) and left overnight at 37°C to adhere. Afterwards, cells were treated with a serial dilution of the plant extracts (200, 100, 50, 25, 12.5, 6.25 µg/mL) at several time points. Upon completion of the incubation period, the cells were fixed with trichloroacetic acid solution for 1 h at 4°C. After washing with water, cellular protein was stained with SRB solution and left at room temperature for 1 h, followed by washing the plate four times with 1% acetic acid and flicking to remove unbound dye. Then, Tris base buffer solution was added to each well and the absorbance was measured at 510 nm. Cell growth was calculated using the following equation:

$$\% \text{ Cell growth} = \frac{\text{Absorbance (sample)} - \text{Absorbance (blank)}}{\text{Absorbance (vehicle control)} - \text{Absorbance (blank)}} \times 100$$

Mitochondrial viability Assay (MTT)

The MTT assay was performed independently in triplicate according to the previously described method by Mosmann (1983). Briefly, 10 μ L of the MTT solution (5 mg/ml dissolved in PBS) was added into all wells after the incubation period and then further incubated for 4 hours. After 4 hours in a humidified atmosphere at 37°C, both the cell media and the MTT solution were removed from the wells and 200 μ L of DMSO was added in each well in order to allow dissolution of the purple MTT-formazan crystals. The absorbance (optical density, OD) was measured at a wavelength of 570 nm and reference wavelength 630 nm with a microplate reader (Tecan Infinite® M200). The relative difference to control was determined by the following equation:

$$\text{Relative difference to control} = \frac{OD(\text{sample}) - OD(\text{Blank})}{OD(\text{Control}) - OD(\text{Blank})}$$

Statistics

The results present in this study were repeated three times (twice for the negative results). Data analysis was performed by Excel 2013 (Microsoft office, USA) and Graphpad prism version 6.0 (San Diego, USA). The results are given as Mean \pm SD.

RESULTS AND DISCUSSION

Yield of extracts

The yields of hexane extracts, chloroform extracts, water extracts and ethyl acetate extract of the three varieties of *F. deltoidea* were calculated and are presented in Table 2. Water soluble matter predominates in all extracts. The yield of the ethyl acetate soluble matter clearly differentiates *kunsleri* variety clearly from *angustifolia* and *deltoidea* varieties.

Table 2. Yield of the *Ficus deltoidea* chemovars

| Variety | Fractions | Yield (%) |
|-------------|---------------|-----------|
| FDAa | Hexane | 0.27 |
| | Chloroform | 0.96 |
| | Aqueous | 13.18 |
| | Hexane | 0.22 |
| FDDa | Chloroform | 0.65 |
| | Aqueous | 13.10 |
| | Ethyl Acetate | 1.95 |
| FDDr | Hexane | 0.24 |
| | Chloroform | 0.25 |
| | Aqueous | 6.92 |
| | Ethyl Acetate | 1.79 |
| FDKa | Hexane | 0.58 |
| | Chloroform | 0.60 |
| | Aqueous | 7.70 |
| | Ethyl Acetate | 4.30 |

HPTLC fingerprint of *Ficus deltoidea* varieties

A targeted chromatographic fingerprint using HPTLC was performed (Figure 2). The results of the HPTLC analyses (shown in Figure 2 and summarised in Table 3) clearly point out that these Malaysian *Ficus deltoidea* varieties are chemovars: orientin in the aerial parts can differentiate *kunslerii* (+) from the other two (-). Isoorientin differentiates *angustifolia* (-) from *deltoidea* (+). Vitexin also qualitatively differentiates *angustifolia* (+) from *deltoidea* (-). Moreover, the presence of vitexin and orienting differentiates the aerial parts from the roots of *deltoidea* variety.

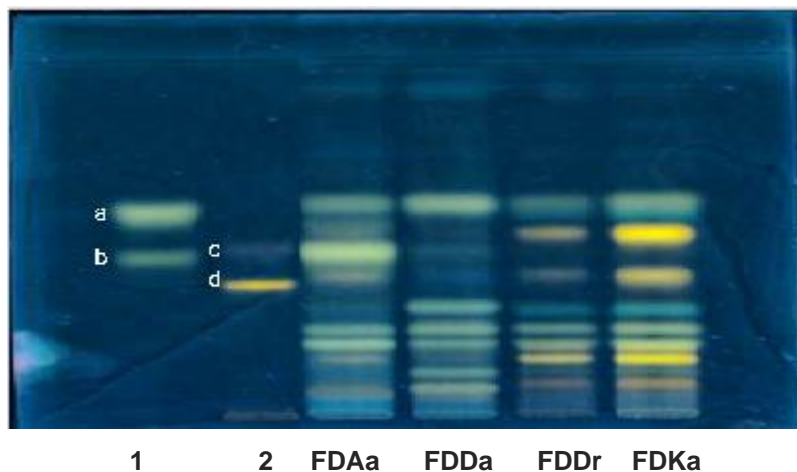


Figure 2. HPTLC fingerprint of extracts of Malaysian *Ficus deltoidea* varieties. Lane 1: Isovitexin (a) and vitexin (b); Lane 2: Isoorientin (c) and orientin (d).

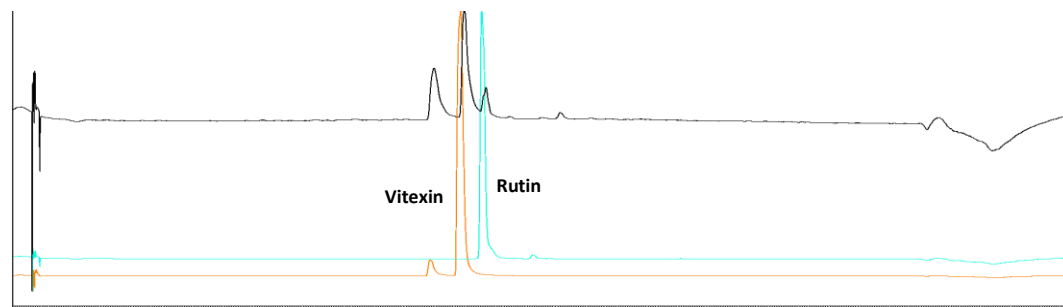
Table 3. Presence of phytochemicals according HPTLC analyses.

| | FDAa | FDDa | FDDr | FDKa |
|-------------|------|------|------|------|
| Isovitexin | + | + | + | + |
| Vitexin | + | + | - | - |
| Orientin | - | - | + | + |
| Isoorientin | - | + | + | + |

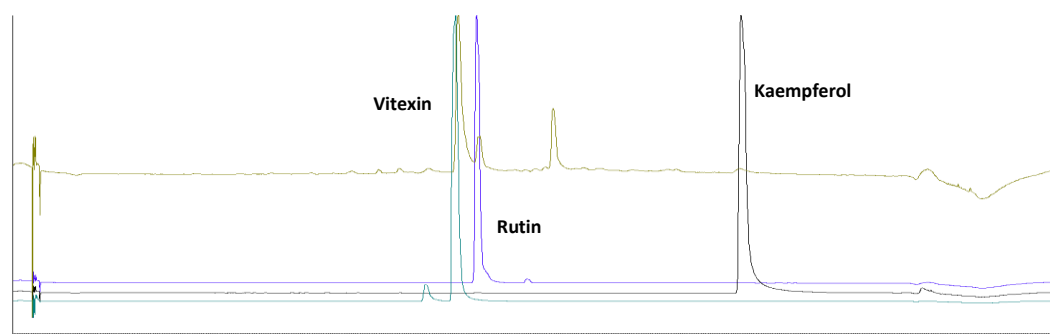
HPLC fingerprint of *Ficus deltoidea* varieties

The HPLC analyses suggested the presence of both vitexin and small traces of rutin in all samples, although with some small Rf and colour variations. We decided to corroborate these findings with HPLC-UV. Figure 3 shows the presence of these phytochemicals in all varieties as well as suggesting that traces of kaempferol are also present in var. *deltoidea*.

(A) *Ficus deltoidea* var. *angustifolia* aerial parts.



(B) *Ficus deltoidea* var. *deltoidea* aerial parts.



(C) *Ficus deltoidea* var. *kunsleri* aerial parts.

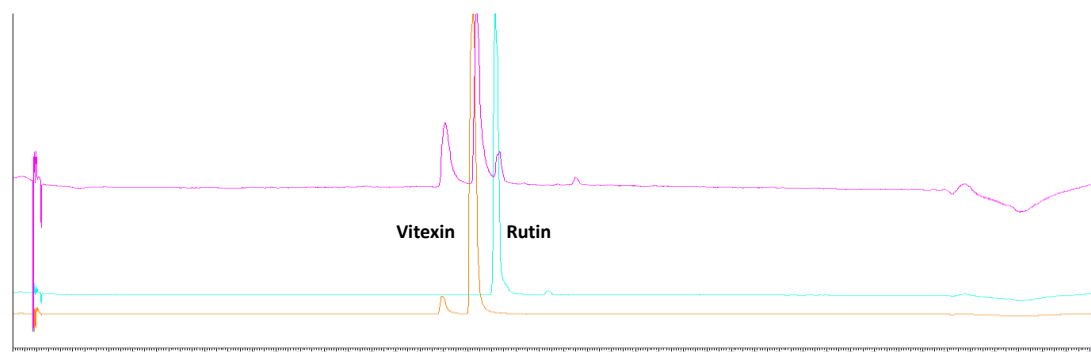


Figure 3. HPLC of ethyl acetate extracts (upper trace) overlapped with standards (lower traces).

Antioxidant activity of the plant extracts

The free radical scavenging activity of the active extracts of the *Ficus deltoidea* varieties are presented in Table 5. The hexane and chloroform extracts were inactive a below 100 µg/ml. The *angustifolia* variety is the less active. The *kunsleri* variety is as antioxidant as *deltoidea* variety and the later concentrates its water-soluble antioxidants in the roots. None of the extract were active against the nitric oxide radical (data not shown). We used Caffeic acid as reference (EC₅₀=1.7 µg/ml).

Table 5. DPPH radical scavenging activity (EC₅₀, µg/ml) of *Ficus deltoidea* extracts.

| | FDAa | FDDa | FDDr | FDKa |
|------------------------------|------|------|------|------|
| Ethyl acetate extract | 52±1 | 15±2 | 14±1 | 11±2 |
| Water extract | >100 | 72±3 | 16±2 | 25±3 |

Reference: Caffeic acid (EC₅₀=1.7 µg/ml).

Tyrosinase inhibition assay

The inhibition of tyrosinase inhibition activity by each extract are presented in Table 6. Only the root extract of the *deltoidea* variety (IC₅₀, 124±8 µg/ml) is significantly most active than the rest. The hexane and chloroform extracts were inactive a below 100 µg/ml. We used kojic acid as reference (IC₅₀=1.0 µg/ml).

Table 6: Tyrosinase inhibition of *Ficus deltoidea* extracts.

| | FDAa | FDDa | FDDr | FDKa |
|------------------------------|-------|-------|-------|------|
| Water extract | > 200 | > 200 | 124±8 | (-) |
| Ethyl acetate extract | (-) | > 500 | > 500 | (-) |

(-) Interference of the extract; Reference: kojic acid (IC₅₀=1.0 µg/ml).

Cytotoxic activity of the plant extracts

The cytotoxic activities of *F. deltoidea* var *angustifolia* and var. *deltoidea* were previously reported (Hanafi et al., 2017). The authors favoured the study of the aerial parts but pointed out to the roots of the later as a potentially interesting organ to follow up. In table 7 we show the results of different fractions of increasing polarity Overall, it follows the same trend as the aerial parts of the plant. The correlation between Inhibitory concentration 50% (IC₅₀) in the MTT assay and Growth Inhibition 50% (GI₅₀) in the SRB assay may indicate a cytotoxic mechanism mediated by inhibition of mitochondrial viability.

Table 7. Cytotoxicity of *Ficus deltoidea* var. *deltoidea* root extracts (µg/ml) against a panel of human prostate cancer cells (MTT and SRB assays at 72 hours).

| Treatment | Extract | IC ₅₀ - MTT | | | GI ₅₀ - SRB | |
|--------------|------------------|------------------------|--------|------|------------------------|-------|
| | | DU145 | LNCaP | PC3 | DU145 | LNCaP |
| FDAr (µg/ml) | <i>n</i> -Hexane | >200 | 175±12 | >200 | >200 | 175±9 |
| | Chloroform | 28±5 | 26±2 | 34±3 | 30±5 | 29±2 |
| | Aqueous | >200 | 48±3 | >200 | >200 | 51±6 |

Reference drug: Paclitaxel (IC₅₀ ≤ 0.01 µM for all lines).

DISCUSSION

The results from HPTLC fingerprints indicated that all tested *Ficus deltoidea* samples significantly differed from each other in their phytochemical constituents. The condition used allows the establishment of a simple, rapid and effective method for the accurate identification of some flavonoids in the selected Malaysian *Ficus* varieties. We believe they may be reproduced in normal TLC plates as long as the analyst operates with the necessary manual skills.

The results mutually support that these botanical varieties here studied are also chemical varieties (chemovars) and that there is an evident interorgan chemical metabolic differentiation in the case of *F. deltoidea* var. *deltoidea*. HPTLC (and perhaps the careful use of TLC).

Orientin in the aerial parts is specific for var. *kunslerii* whilst isoorientin differentiates var. *angustifolia* from var. *deltoidea*. Vitexin also qualitatively differentiates var. *angustifolia* from var. *deltoidea*. Moreover, the presence of vitexin and orienting differentiates the aerial parts from the roots of the *deltoidea* variety (Figure 4). These results support the chemotaxonomical value of C-glycoflavones in the genus *Ficus* (Kuijt & Hansen, 2015). Rutin and kaempferol traces also show in the HPLC analyses.

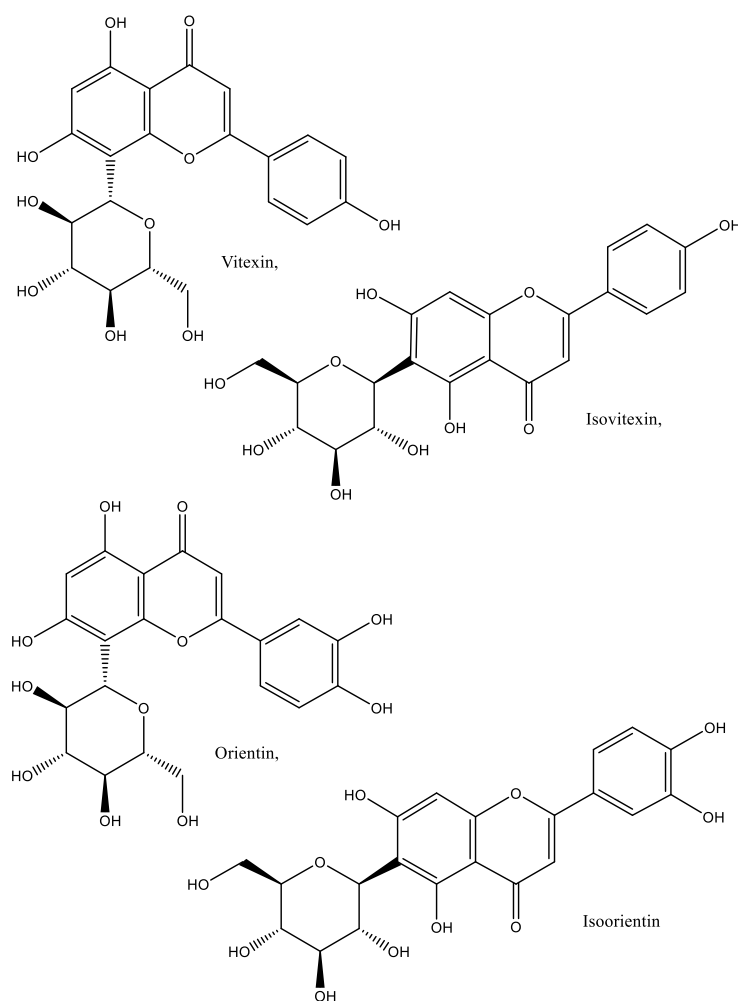


Figure 4. Vitexin, Isovitexin, Orientin, and Isoorientin.

The water extracts and ethyl acetate extracts of almost all these plants showed high antioxidant activity, while only *Ficus deltoidea* roots may contain some tyrosinase inhibitors. The compounds related to these activities, antioxidant activity and tyrosinase inhibition activity, from these plants on melanogenesis as well as melanoma cells may need to be further researched in future.

The three prostate cancer cell lines present different characteristics: LNCaP cells have androgen receptors that are functional enabling them to be androgen sensitive and these cells also secrete prostate-specific antigen (PSA). Both PC3 and DU145 cells are androgen independent but PC3 cells are highly invasive with strong metastatic potential as compared to DU145. The ethyl acetate extract of roots of *F. deltoidea* var. *deltoidea* only shown similar IC₅₀ for all of them in both proliferation and viability assays, suggesting a hormone-independent mechanism of action targeting the mitochondria. This is in line with our previous report (Hanafi et al., 2017) where the active plant extracts of two farmed varieties (var. *angustifolia* and var. *deltoidea*) induced apoptosis in PC3 cells via the intrinsic pathway, as evidenced by a significant activation of caspases 3 and 7 and their ability to affect the gene expression of proteins such as Bcl-2, and Smac/DIABLO. Smac/DIABLO is a novel mitochondria-derived pro-apoptotic protein that plays an important role in sensitizing tumor cells to die by apoptosis (Du et al., 2000; Verhagen et al., 2000).

CONCLUSION

We here achieve to analytically differentiate three Malaysian *Ficus deltoidea* botanical varieties / cultivars (*angustifolia*, *deltoidea* and *kunslerii*) and suggest that they are also chemovars. Their biological activities point out to the antioxidant value of extracts from the *deltoidea* and *kunslerii* varieties whilst the inhibition of tyrosinase is only present in the roots extract of the var. *deltoidea* which is also endowed with cytotoxic activity against prostate cancer cells.

Conflicts of Interest

The authors declare no personal or financial conflict of interest related to this work.

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Authors contribution

(MMM) Investigation; (O.S.O.A.) Investigation; (L.G.) Investigation; (H.Y.) Resources, Funding; (JMP) Conceptualization, Writing, Supervision.

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REFERENCES

- Berg, C.C. (2003) Flora Malesiana precursor for the treatment of Moraceae 3: *Ficus* subgenus *Ficus*. *Blumea*, 48, 529–550.
- Bunawan H., Amin N.M., and Bunawan S.N. et al. (2014) *Ficus deltoidea* Jack: A Review on Its Phytochemical and Pharmacological Importance. Evidence-Based Complementary and Alternative Medicine. Vol. 2014: Article ID 902734, 8 pages.
- Burkill I.H. and Haniff M. (1930) Malay village medicine. *Garden's Bulletin*. Vol. 6(2):167–332.
- Du, C., Fang, M., Li, Y., Li, L., and Wang, X. (2000). Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. *Cell* 102, 33–42.
- Dzolin S., Syed Aris S.R., Ahmad R. et al. (2010) Radical Scavenging and Neurotoxicity of Four Varieties of *Ficus deltoidea*. *2010 International Conference on Science and Social Research (CSSR 2010)*:11-15.
- Grison-Pigé, L., Hossaert-Mckey, M., Greeff, J.M., and Bessièrè, J.-M. (2002). Fig volatile compounds—a first comparative study. *Phytochemistry* 61, 61-71.

- Fatihah, H.N.N.; Mat, N.; Zaimah, A.R.; Zuhailah, M.N.; Norhaslinda, H. (2012) Morphological phylogenetic analysis of seven varieties of *Ficus deltoidea* Jack from the Malay Peninsula of Malaysia. *PLoS ONE*, 7, e52441.
- Hanafi, M. M. M., Afzan, A., Yaakob, H., Aziz, R., Sarmidi, M. R., Wolfender, J. L., & Prieto, J. M. (2017). *In Vitro* Pro-apoptotic and Anti-migratory Effects of *Ficus deltoidea* L. Plant Extracts on the Human Prostate Cancer Cell Lines PC3. *Front Pharmacol*, 8, 895. doi:10.3389/fphar.2017.00895
- Kuijt, J., Hansen, B. (2015). Chemosystematics. In: Flowering Plants. Eudicots. The Families and Genera of Vascular Plants, vol 12. Springer, Cham.
- Lip, J. M., Hisham, D. N. and Zaidi, J. A et al. (2009) Isolation and identification of moretenol from *Ficus deltoidea* leaves. *Journal of Tropical Agriculture and Food Science*. Vol. 37(2):195–201.
- Masuda, T., Yamashita, D., Takeda, Y., & Yonemori, S. (2005). Screening for tyrosinase inhibitors among extracts of seashore plants and identification of potent inhibitors from *Garcinia subelliptica*. *Bioscience, biotechnology, and biochemistry*, 69(1), 197-201.
- Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of immunological methods*, 65(1), 55-63.
- Norriah J.S., Norizan A., and Sharipha Ruzaina S.A. et al. (2012) Cytotoxicity Activity and Reproductive Profiles of Male Rats Treated with Methanolic extracts of *Ficus deltoidea*. *Research Journal of Medicinal Plant*. Vol. 6(2):197-202.
- Oh M.J., Hamid M.A., and Ngadiran S. et al. (2011) *Ficus deltoidea* (Mas cotek) extract exerted anti-melanogenic activity by preventing tyrosinase activity *in vitro* and by suppressing tyrosinase gene expression in B16F1 melanoma cells. *Archives of Dermatological Research*. Vol. 303(3):161-170.
- Omar M.H., Mullen W., and Crozier A. (2011) Identification of proanthocyanidin dimers and trimers, flavone C-glycosides, and antioxidants in *Ficus deltoidea*, a malaysian herbal tea. *Journal of Agricultural and Food Chemistry*. Vol. 59(4): 1363–1369.
- Prieto J M, Ogunsina M O, Novak A, Joshi A, Kokai J, Rocha I C, Santayana M P. (2012) Comparative study of the *in vitro* bioactivities of *Bupleurum rigidum* and *B. fruticosens*. *Natural Product Communications* 7(6):757-60.
- Ramamurthy S., Kumarappan C., and Dharmalingam S.R. et al. (2014) Phytochemical, Pharmacological and Toxicological Properties of *Ficus deltoidea*: A Review of a Recent Research. *Annual Research and Review in Biology*. Vol. 4(14): 2357-2371.
- Shafaei, A., Farsi, E., Ismail, Z., Asmawi, M. Z. 2012 Quantitative high performance thin-layer chromatography method for analysis of vitexin and isovitexin in extracts of leaves of *Ficus deltoidea*. *Asian Journal of Chemistry*, 24(5), 2283-2286.
- Sharma, O. P., & Bhat, T. K. (2009). DPPH antioxidant assay revisited. *Food chemistry*, 113(4), 1202-1205.
- Sreejayan, R. M. N. A. (1997). Nitric Oxide Scavenging by Curcuminoids. *Journal of Pharmacy and Pharmacology*, 49(1), 105–107.
- Verhagen, A. M., Ekert, P. G., Pakusch, M., Silke, J., Connolly, L. M., Reid, G. E., et al. (2000). Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins. *Cell* 102, 43–53.
- Vichai, V., & Kirtikara, K. (2006). Sulforhodamine B colorimetric assay for cytotoxicity screening. *Nature protocols*, 1(3), 1112-1116.
- Zimisuhara, B., Valdiani, A., Shaharuddin, N.A., Qamaruzzaman, F., and Maziah, M. (2015). Structure and Principal Components Analyses Reveal an Intervarietal Fusion in Malaysian Mistletoe Fig (*Ficus deltoidea* Jack) Populations. *International Journal of Molecular Sciences* 16, 14369-14394.