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Research paper

Ficus deltoidea: Effects of solvent polarity on antioxidant and anti-proliferative activities in breast and colon cancer cells

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ABSTRACT

Introduction: Ficus deltoiea (FD) is traditionally used to treat hyperlipidaemia, hypertension and diabetes. Limited studies are available on their anti-proliferative effects. The aim of this study was to evaluate the anti-oxidant activities of the hexane, ethyl acetate, methanol and water extracts of three varieties of FD and to evaluate their anti-proliferative effects on breast and colon cancer cells.

Methods: The plant extracts were analysed for polyphenolic and flavonoid content. Antioxidant activities were determined using DPPH, ABTS and $O_2^{\cdot -}$ radical scavenging assays, FRAP, Fe^{2+} chelating, and cellular anti-oxidant assays. The anti-proliferative activity and apoptotic effects of the extracts was analysed using breast (MCF-7, MDA-MB 231, HCC 1937) and colon cancer (HCT 116) cell lines. Gene expression analysis of selected extracts was investigated against HCT 116 cells.

Results: Methanol was the best solvent for extraction of antioxidants and had the highest polyphenolic content (70–100 mg GAE/g), FRAP (6.0–9.0 mmol Fe^{2+}/g), ABTS (2.0–3.0 mmol TE/g) and DPPH (EC_{50}:200-410 µg/mL) activities. The ethyl acetate extracts of FD variety A (FDA-EA) and B (FDB-EA) demonstrated anti-proliferative activities in MCF-7, MDA-MB 231, and HCT 116 (IC_{50} < 100 µg/mL) and moderate activities in HCC 1937 (IC_{50}:150-200 µg/mL) cells. The extracts also increased caspase 3/7 activities in HCT 116 and HCC 1937 cells. HCT 116 cells treated with FDA-EA showed upregulation of *Fas1*, *Bax*, *Cdk-1*, *TNF-α* and *Cdk-2* and downregulation of *Bcl-2* and *T*p53 genes, implying the induction of apoptosis.

Conclusion: FD especially FDA-EA is a promising source of antioxidants and anti-proliferative agents, especially against colon cancer.

1. Introduction

Plants have been used for centuries as traditional medicine and scientific evidence supports their traditional use. In addition, there are many emerging studies on their therapeutic and anticancer properties. In recent years, extensive research has been undertaken to explore plants with established ethno-medicinal properties in hopes of discovering novel biomolecules with anticancer properties that have possible life saving uses in the future.

Ficus deltoidea Jack or commonly known as mistletoe fig is an epiphytic shrub native to Southeast Asia [1] and can be classified into two subspecies based on their morphology: *F. deltoidea* subsp. *Deltoidea* and subsp. *Motleyana* [2]. Taxonomical classification classifies *F. deltoidea* into 13 varieties of which, eight are found in Malaysia; namely var. *angustifolia*, var. *bilobata*, var. *deltoidea*, var. *intermedia*, var. *kinabaluensis*, var. *kunstleri*, var. *motleyan*, and var. *trengganeusis* [3]. The leaves of the plant are traditionally used for the treatment of hyperlipidaemia, hypertension and diabetes [1]. Several studies have also reported on the anti-nociceptive [4], anti-cancer [5,6], anti-inflammatory [7], anti-bacterial [8] and neuroprotective [9,10] effects of this plant.

Antioxidants protect the cellular components against oxidative damage caused by reactive oxygen species (ROS). Flavonoids, phenolic acids, alkaloids, terpenoids, vitamins and other endogenous metabolites found in plants are rich in antioxidant activities [11]. Studies have revealed that many of these antioxidant compounds possess anti-inflammatory, anti-tumor, anti-mutagenic, anti-carcinogenic and have anti-viral capabilities [11]. The plant *Ficus* contains various phytochemicals with biological activities including polyphenols such as prenylated flavonoids, isoflavonoids, and lignans [12]. The antioxidant activities of *F. deltoidea* can be further investigated particularly when extracted with solvents of varying polarities. This approach can provide important information on the characteristics of antioxidants present in

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F. deltoidea which can be useful for future use as nutraceuticals or supplements.

Cancer is the primary cause of death in developed countries and the second cause of death in developing countries [13]. Lung cancer and breast cancer in males and females, respectively is the most commonly detected form of cancer as well as the main cause of death in developed countries [14]. In Malaysia, cancer contributed to 13.6% of all deaths in 2015 and it was the third most common cause of death in the country [15]. Colorectal cancer is the third most occurring cancer in males globally [16] while in Malaysia 16.3% of all cancer cases are colorectal cancer, making it the most frequent cancer in Malaysian males [17].

Anti-cancer studies involving *F. deltoidea* are relatively new with limited data available. The leaf extract of *F. deltoidea* showed anti-melanogenic effect when tested on B16F1 melanoma cells [18] but did not show anti-cancer activity on MCF-7 breast cancer cells [19]. Extracts of *F. deltoidea* have also been reported to be cytotoxic against human ovarian carcinoma cells [6]. A more recent study highlighted the anti-proliferative capacity of this plant on prostate cancer cells via intrinsic induction of apoptosis [20]. To date, there is a lack of scientific evidence on the effects of this plant on colorectal cancer as well as the mechanism of action. Hence, the aims of this study were to determine the antioxidant and anti-proliferative activity of extracts of three varieties of *F. deltoidea* on CRC and breast cancer, using cell culture approaches. The regulation of several apoptotic genes was studied to better understand the potential mechanism for the apoptotic effects caused by *F. deltoidea*.

2. Materials and method

2.1. Reagents and chemicals

The reagents and chemicals used in the in vitro antioxidant assays were of analytical grade while those used in in vivo cell based assays were of biological grade. The chemicals and reagents for the antioxidant assays were purchased from Fisher Scientific, Sigma-Aldrich and Merck Milipore. The cell lines were sourced from the American Type Culture Collection (ATCC, USA).

2.2. Preparation and extraction of the leaves of F. deltoidea

The leaves of three different varieties of *F. deltoidea; F. deltoidea* A (FDA), *F. deltoidea* B (FDB) and *F. deltoidea* C (FDC), were purchased from a farm located in the northern part of Malaysia. The species were confirmed by Dr. Sugumaran Manickam from the Institute of Biological Sciences, University of Malaya and a voucher specimen was deposited at Rimba Ilmu Herbarium, University Malaya (KLU 49465, KLU49240, KLU 49463). Three kilograms of each variety was used in this study and they were obtained from a single harvest and at the same time. As Malaysia is a tropical country and the weather is more or less the same throughout the year, the effect of the timing/season of harvest will be minimal. The leaves from each variety were separated, cleaned and dried in an oven at 40 °C until they were completely dried. The dried leaves from each variety were then crushed using a blender and the powder was weighed and kept at −20 °C for further use. The dried leaves were labelled FDA, *F. deltoidea* B FDB and *F. deltoidea* C FDC.

The dried leaf powder was extracted sequentially using solvents of increasing polarities, consisting of hexane, ethyl acetate, methanol and water [21–23]. The dried sample was mixed with solvent in a 1:10 ratio (weight:volume) and placed in an incubator at 40 °C with a shaker capability (145 rpm), for 8 h. The extracts were then centrifuged (2000 x g, 25 °C for 10 min), the supernatant was collected and the resulting residues were extracted again with the same solvent twice. The resulting supernatant was pooled, filtered (Filtres Fioroni no. 111) and subsequently dried using a rotary evaporator. The water extracts were lyophilised using a freeze dryer. The extracts were dissolved in 10% DMSO and stored at −20 °C for further analysis.

2.3. Analysis of antioxidant components

2.3.1. Polyphenolic analysis

The Folin-Ciocalteu (FC) reagent was used for analysis of polyphenolic content [24]. For the assay, 2 mg/mL of plant extract was thoroughly mixed with 100 µL of the FC reagent before the addition of 70 µL of 1 M Na_2CO_3. The mixture was incubated at room temperature for two hours before absorbance reading was taken at 765 nm. A gallic acid standard curve was plotted (0–250 mg/L) and polyphenolic content was presented as mg GAE/g dried extract.

2.3.2. Flavonoid analysis

Flavonoid analysis was performed following the method adapted from Liu et al. [25] with slight modifications. For the assay, 2 mg/mL of plant extract was mixed with equal volumes of aluminium trichloride (10% w/v) and potassium acetate before the addition of 200 µL of ethanol (30%). Absorbance of the mixture was read at a wavelength of 415 nm after 30 min of incubation at room temperature. A quercetin standard curve was plotted and flavonoid content was presented as µg QE/g dried extract.

2.4. Analysis of antioxidant activities

2.4.1. Ferric reducing activity (FRAP)

The original method described by Benzie and Strain was utilised for this assay [26]. The FRAP reagent consisted of acetate buffer (300 mM, pH 3.6), 2,3,6-tripyridyl-s-trazine (TPTZ, 10 mM) in 40 mM HCl, and $FeCl_3.H_2O$ (20 mM). The above solution was mixed in a 10:1:1 ratio, respectively and warmed at 37 °C for 5 min. For the assay, FRAP reagent (300 µL) was added to 10 µL of sample. Absorbance was then measured at a wavelength of 593 nm after 4 min incubation at room temperature. Results were expressed as mmol Fe^{2+}/g dried extract based on an iron sulphate ($FeSO_4$) (0–1 mM) standard curve and quercetin was used as positive control (0–2000 µg/mL).

2.4.2. 2, 2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS·) radical scavenging assay

This assay was conducted following the method described by Re et al. [27]. The ABTS·- radical cations was initially prepared by mixing 7 mM ABTS solution with 2.45 mM of potassium persulfate and left in the dark. The resulting solution was incubated in the dark at room temperature for 12–16 h. The subsequent absorbance was adjusted to 0.700 ± 0.02 at a wavelength of 734 nm, by diluting the solution with ethanol. For the assay, 10 µL of extracts (2000 µg/mL) was mixed with 200 µL of ABTS reagent and left for 15 min. The resulting absorbance was then read at 734 nm. Varying concentrations of Trolox was used to construct a Trolox calibration curve with quercetin as positive control (0-2000 µg/mL). ABTS radical scavenging activity was presented as mmol TE/g dried extract.

2.4.3. 1,1-diphenyl-2-picryl hydrazyl (DPPH) radical scavenging activity

The DPPH radical scavenging activity was measured following the method of Sharma & Bhat [28]. DPPH solution (100 µM) in methanol was initially prepared and stored in the dark for at least 30 min prior to its use. Twenty microlitres of plant extracts (0–2000 µg/mL) were added to 100 µl of DPPH solution and absorbance was read at 515 nm after 30 min. Quercetin was used as positive control (0–2000 µg/mL) and analysed simultaneously. Inhibition of the DPPH radicals was estimated using the following formula:

$$\text{Inhibition of DPPH radicals (\%)} = (A_c - A_s) / A_c \times 100$$

Where A_c and A_s represent absorbance of blank and sample/quercetin, respectively. Where applicable, the EC_{50} values was also calculated and expressed as µg/mL.

2.4.4. Superoxide anion (O2·-) radical scavenging activity

This assay was conducted based on the protocol described by Robak and Gryglewski [29] with minor modifications. For the assay, 50 µL of extracts (0–2000 µg/mL) was mixed with 150 µM nitroblue tetrazolium, 468 µM NADH and 60 M phenazine methosulphate in a ratio of 1:1:1. The mixture was left in the dark for 10 min before absorbance was read at a wavelength of 570 nm.

Various concentrations of quercetin (0–2000 µg/mL) were used as positive controls and subjected to the same treatment. Percentage inhibition of the $O_2^{\cdot -}$ radicals exhibited by the plant extracts was estimated. Where applicable, the EC_{50} value was calculated and expressed as µg/mL.

2.4.5. Ferrous (Fe^{2+}) ion chelating activity assay

This assay was conducted according to the method developed by Decker and Welch [30]. Fifty microlitres of plant extracts (0–2000 µg/mL) was mixed with 20 µL of 0.5 mM $FeCl_2$, 160 µL of distilled water and 20 µL of 2.5 mM ferrozine. The mixture was incubated for 10 min at room temperature after which formation of the ferrous iron-ferrozine complex was measured at a wavelength of 562 nm. EDTA was used as a positive control. The EC_{50} concentration was determined at which the chelating activity of the tested compound was at 50%.

2.4.6. Cellular antioxidant assay (CAA)

The protocol for the cellular antioxidant assay was adapted from Wolfe et al. [31]. HCT 116 cells were seeded at a density of 5×10^4 cells/well in a 96-well black microplate and incubated at 37 °C in 5% CO_2 for 24 h. Plant extracts (0–1500 µg/mL) and 25 µM dichloro-dihydro-fluorescein diacetate (DCFH-DA) dissolved in complete DMEM were added after the incubation period. After an hour of incubation, the media was removed and cells were washed with PBS before the addition of 600 µM of 2,2′-azobis(2-amidinopropane) dihydrochloride (ABAP) in 100 µL of Hank's Balanced Salt Solution (HBSS). Fluorescence intensity was measured every 5 min for 1 h using a multimode reader (Tecan Infinite M1000 Pro, Switzerland). Emission and excitation wavelengths of 538 nm and 485 nm, respectively were utilised for this assay.

A plot of fluorescence against time for the plant extracts was constructed and CAA was calculated according to the following formula:

$$\text{CAA unit} = 100 - (\int \text{SA} / \int \text{CA}) \times 100,$$

where ∫ SA and ∫ CA are area under the fluorescence versus time curve for the sample and control, respectively. Quercetin was used as a positive control (0–100 µg/mL) and results are expressed as EC_{50} (µg/mL). The EC_{50} was determined from the median effect plot of log (f_a/f_u) versus log (concentration), where f_a and f_u represent the fraction that is altered and unaltered, respectively by the treatment [31]. The EC_{50} value was obtained when f_a/f_u equals 1.

2.5. Cell culture

Four types of breast and colon cancer cell lines were used to investigate the anti-proliferative effects of the plant extracts. They consisted of three breast cancer cell lines HCC 1937, MCF 7 and MDA-MB 231 and a colon cancer cell line (HCT 116). Normal colon (CCD 841) and liver (WRL-68) epithelial cells were also used to determine the cytotoxicity of the extracts on normal cells. All cell lines, with the exception of HCC 1937, were grown in Dulbecco's Modified Eagle's Medium (DMEM) enriched with 10% (v/v) foetal bovine serum (FBS) and 1% (v/v) penicillin-streptomycin mixed solution. HCC1937 cells were grown in RPMI 1640 media enriched with 10% (v/v) FBS and 1% (v/v) penicillin-streptomycin mixed solution. All cells were cultured in T-25 tissue culture flasks and kept at 37 °C in a humidified atmosphere with 5% CO_2.

2.5.1. Cell viability and MTT assay

Cells were grown in a 96 well plate with each well containing 5×10^3 cells and allowed to attach for 24 h prior to treatment. Upon attachment, cells were treated with varying concentrations of the plant extracts (0–500 µg/mL) and incubated at 37 °C for 48 h. Cell viability following treatment was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. MTT reagent (10 µL) was added to each well and the plate was incubated for 4 h. The growth media was subsequently removed and replaced with 100 µL of DMSO. The absorbance of the plate was measured at 595 nm using a microplate reader (Tecan Infinite M1000 Pro). Cell viability was calculated based on the following formula:

$$\text{Viability (\%)} = A_s / A_c \times 100$$

where A_s is the OD value of the sample and A_c is the OD value of the negative control.

A plot of cell viability (%) versus concentration was constructed and concentration of the plant extract that reduced cell viability by 50% (IC_{50}) was determined. Camptothecin was used as positive control for cell lines MCF-7, MDA-MB 231 and HCC 1937 while 5-FU was used against HCT 116 cells. Preliminary analyses of cell viability was performed on all cells using a high concentration (500 µg/mL) of the plant extracts.

2.5.2. Caspase 3/7 activation

The caspase 3/7 activation in treated HCT 116 and HCC 1937 cells was determined by the ApoTox-Glo™ Triplex Assay kit following the manufacturer's protocol (Promega, USA). Camptothecin was used as a positive control with HCC 1937 cells while 5-Fluorouracil was used with HCT 116 cells. The cells were seeded in white opaque plates (7×10^3 cells/well) and allowed to attach overnight. The cells were then treated with the IC_{50} concentration of the ethyl acetate extracts of FDA and FDB and incubated for 24 and 48 h. After the incubation period, 50 µL of Caspase-Glo® 3/7 reagent was added and further incubated at room temperature for 30 min. The luminescence was then measured with the Tecan Infinite M1000 Pro multimode reader. The caspase 3/7 activities were estimated by relating the luminescence of the treated samples against an untreated control and expressed as fold change.

2.5.3. Caspase inhibition with carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone (Z-VAD-FMK)

The pan caspase inhibitor, Z-VAD-FMK was used to inhibit the activation of caspase 3/7 in HCT 116 cells when treated with the ethyl acetate extract of FDA. Briefly, HCT 116 cells were seeded in a 96-well plate (10×10^3 cells/well) and incubated overnight at (37 °C, 5% CO_2) before treatment with the IC_{50} concentration of the ethyl acetate extract of FDA in the presence of 10 nM of ZVAD-FMK. MTT assay was performed after 24 h of incubation.

2.5.4. Reactive oxygen species (ROS)

For the determination of cellular ROS, HCT 116 cells were seeded in a black 96-well plate (5×10^4 cells/well) at 37 °C, 5% CO_2 overnight. The cells were then treated with the IC_{50} concentration of the ethyl acetate extract of FDA along with 25 µM of 2′, 7′-DCFH-DA. The plate was then incubated at 37 °C in the dark for 90 min after which fluorescence was measured at emission 538 nm and excitation 485 nm on a multimode reader (Tecan Infinite M1000 Pro, Switzerland). An untreated negative control was used and the fold change increase in fluorescence in the treated sample was calculated.

2.5.5. Treatment of HCT 116 cells for gene expression analysis

For the gene expression analysis, 2×10^6 HCT 116 cells were seeded in T75 flasks and left overnight at 37 °C for attachment. The cells were then treated with the IC_{50} concentration of the ethyl acetate

extract of FDA and incubated at 37 °C for 48 h. A separate set of untreated controls was incubated simultaneously with fresh DMEM. Trypsin was subsequently added to detach the cells and centrifuged at 300 x g for 5 min at room temperature. Collected cells were rinsed with PBS before extraction of total cellular RNA (tcRNA) from the cells.

2.5.6. Total cellular RNA extraction

The tcRNA from both treated and untreated HCT 116 cells was isolated using the RNeasy® Plus Mini Kit (Qiagen), following the protocol from the manufacturer. The quality and concentration of the tcRNA was measured using a NanoDrop™ 2000 spectrophotometer (Thermo Scientific, USA) at wavelengths 260 and 280 nm. An A260/A280 ratio above 1.8 indicated good RNA quality.

2.5.7. Synthesis of cDNA from tcRNA

Freshly extracted tcRNA from treated and untreated HCT 116 cells was reverse transcribed to single-stranded sense strand DNA (cDNA) using the Tetro cDNA synthesis kit (Bioline, USA). The cDNA synthesis was conducted following the guidelines from the manufacturer, at a total RNA concentration of 1000 ng.

2.5.8. Gene expression using qRT-PCR

The regulation of several apoptotic genes was investigated using qRT-PCR performed in a StepOne™ Real-Time PCR System (Applied BioSystems, USA). The forward and reverse primers used in this study are shown in Table 1. Regulation of gene expression was compared against *GAPDH*, a housekeeping gene, in untreated controls. The PCR amplification was carried out in 0.2 mL MicroAmp® Optical 8-tube strips with a final volume of 20 µL. The mixture contained 50 ng of cDNA, reverse and forward primers (200 nM), THUNDERBIRD® SYBR® qPCR Mix (Toyobo, Japan) and ROX passive reference dye. The PCR thermal profile consisted of 20 s Taq DNA Polymerase activation at 95 °C followed by 40 cycles of denaturation at 95 °C for 3 s and primer annealing at 60 °C for 30 s. Quantitation of gene expression was relatively performed using the Comparative C_T Method ($\Delta\Delta C_T$).

2.6. Statistical analysis

All analyses were conducted in triplicate and results expressed as mean ± standard deviation. The data were statistically analysed using the SPSS statistical software, version 23 (SPSS Inc., Chicago, Illinois, USA). One-way analysis of variance (ANOVA) and Tukey's Honestly Significance Difference test were used for comparisons of means among the different groups. The SPSS statistical software, version 23 (SPSS Inc., Illinois, USA) was utilized. The level of significance was set at $p < 0.05$. Paired sample *t*-test was used to analyse the gene expression data and the confidence interval percentage was set at 95%.

3. Results

3.1. Extraction yield, polyphenolic and flavonoid content

The leaves of the three varieties of *F. deltoidea* were extracted with solvents of different polarities. Results consistently demonstrated the methanolic extract to have the highest yield among the three varieties whereas the ethyl acetate extracts had the lowest yield (Table 2).

Table 2
Yield, polyphenolic and flavonoid content of the leaves of three varieties of *F. deltoidea* extracted with hexane, ethyl acetate, methanol and water.

	Extraction Solvent			
	Hexane	Ethyl acetate	Methanol	Water
Yield (%)				
FDA	12.18	1.76	27.96	8.40
FDB	3.85	1.24	23.80	8.88
FDC	3.64	2.84	8.04	6.88
Polyphenol (mg GAE/g)				
FDA	25.04 ± 0.63^a	31.23 ± 0.55^a	93.89 ± 3.30^b	77.27 ± 5.14^c
FDB	12.68 ± 0.25^a	21.68 ± 0.44^b	97.30 ± 4.39^c	55.10 ± 1.74^d
FDC	34.29 ± 0.85^a	57.38 ± 2.52^b	74.67 ± 4.48^c	45.58 ± 2.12^d
Flavonoid (mg QE/g)				
FDA	0.79 ± 0.01^a	0.21 ± 0.01^b	0.14 ± 0.01^c	0.09 ± 0.00^d
FDB	0.67 ± 0.00^a	0.15 ± 0.00^b	0.16 ± 0.00^c	0.07 ± 0.00^d
FDC	0.98 ± 0.00^a	0.13 ± 0.00^b	0.15 ± 0.01^c	0.05 ± 0.00^d

Results are expressed as means ± SD ($n = 3$).
Values with different lower case letters (^{a,b,c,d}) are significantly different at $p < 0.05$ within the same row.

Analysis of polyphenolic content showed the methanol extracts of all three varieties to contain the highest levels and the hexane extracts to contain the lowest (Table 2). Amongst the three varieties, FDB had the highest polyphenolic content, followed by FDA and FDC. In contrast, flavonoid content was highest in the hexane extracts of all three varieties whereas the water extracts had the lowest content. Amongst the three varieties, FDC had the highest flavonoid content (Table 2).

3.2. Antioxidant activities of F. deltoidea

To determine the antioxidant activity of the extracts, five antioxidant assays were performed. The highest ferric reducing activity was observed mainly in the methanol and water extracts of all three varieties (Table 3) with the methanol extract of FDB having the highest activity (8.54 ± 0.38 mmol Fe^{2+}/g extract). In the ABTS radical scavenging assay, with the water extract of FDA showed the highest activity (2.56 ± 0.02 mmol TE/g), followed by the methanol extract of FDB and the water extract of FDC (Table 3). The water extract of FDA showed more than 90% inhibition of the ABTS radicals at a concentration of 2000 µg/mL. In the DPPH radical scavenging assay, the methanol and water extracts of FDA as well as the methanol extract of FDB had EC_{50} values less than 230 µg/mL (Table 3), with the latter showing the highest DPPH radical scavenging activity.

The water extracts of FDA and FDB were the strongest in scavenging the superoxide anion radicals with EC_{50} values below 230 µg/mL (Table 3). All varieties of the hexane and ethyl acetate extracts as well as the methanol extracts of FDB and FDC failed to achieve 50% inhibition at the concentration range tested. On the contrary, the ethyl acetate and hexane extracts of the three varieties appeared to show negative inhibition of the radicals, implying the extracts may act cause

Table 1
Forward and reverse primer sequences used in qRT-PCR.

No.	Gene Name	Primer Sequence
1	Glyceraldehyde-3-phosphate (*Gapdh*)	Forward: 5'-TGCCTCCTGCACCACCAACTGC-3' Reverse: 5'- AATGCCAGCCCCAGCGTCAAAG-3'
2	BCL2 associated X, apoptosis regulator (*Bax*)	Forward: 5'-GGT TGC CCT CTT CTA CTTT-3' Reverse: 5'-AGC CAC CCT GGT CTT G-3'
3	B-cell lymphoma apoptosis regulator (*Bcl-2*)	Forward: 5'-ACT TTG CAG AGA TGT CCA GT-3' Reverse: 5'-CGG TTC AGG TAC TCA GCA T-3'
4	Tumor protein p53 (*TP53*)	Forward: 5'-GGG ACA GCC AAG TCT GTG A-3' Reverse: 5'-AAT CAA CCC ACA GCT GCA C-3'
5	Fas cell surface death receptor (*Fas1*)	Forward: 5'- AGC GGC CAT TTC CAT TGC CC -3' Reverse: 5'- CCA TGC CCA GAG GGT GGT TG -3'
6	cyclin dependent kinase 2 (*Cdk-2*)	Forward: 5'-ATG GGT GTA AGT ACG AAC AGG-3' Reverse: 5'-TTC TGC CAT TCT CAT CGG-3'
7	Cyclin dependent kinase 1 (*Cdk-1*)	Forward: 5'-AAG TGA AGA GGA AGG GGT TCC-3' Reverse: 5'-CCA AAA GCT CTG GCA AGG CC-3'
8	Tumor necrosis factor alpha (*TNF-α*)	Forward: 5'-GCT GTA CCT CAT CTA CTC CCA-3' Reverse: 5'-GCA ATT TCT AGG TGA GGT CTT C-3'

Table 3
Cellular and non-cellular antioxidant analyses of the leaves of three varieties of *F. deltoidea* extracted with hexane, ethyl acetate, methanol and water.

	Extraction Solvent			
	Hexane	Ethyl acetate	Methanol	Water
FRAP (mmol Fe^{2+}/g)				
FDA	4.02 ± 0.03[a]	2.55 ± 0.11[a]	6.14 ± 0.33[b]	4.46 ± 0.01[a]
FDB	1.86 ± 0.03[a]	1.02 ± 0.15[a]	6.54 ± 0.38[b]	3.36 ± 0.08[c]
FDC	3.59 ± 0.04[a]	3.92 ± 0.04[b]	4.71 ± 0.14[c]	1.90 ± 0.13[a]
Quercetin	9.54 ± 0.11			
ABTS radical scavenging activity (mmol TE/g)				
FDA	0.83 ± 0.01[a]	1.04 ± 0.09[b]	2.44 ± 0.03[c]	2.56 ± 0.02[d]
FDB	0.52 ± 0.03[a]	1.07 ± 0.02[b]	2.51 ± 0.01[c]	2.10 ± 0.10[d]
FDC	0.96 ± 0.05[a]	2.17 ± 0.05[b]	2.05 ± 0.05[c]	2.27 ± 0.04[d]
Quercetin	2.59 ± 0.00			
DPPH radical scavenging activity EC_{50} (μg/mL)				
FDA	978.40 ± 6.87[a]	1882.34 ± 13.02[b]	216.78 ± 2.95[c]	229.43 ± 2.05[d]
FDB	ND	1343.22 ± 38.78[a]	213.33 ± 2.88[b]	552.13 ± 11.00[c]
FDC	ND	337.83 ± 8.20[a]	409.42 ± 13.97[b]	1161.38 ± 15.52[c]
Quercetin	64.84 ± 0.33			
Superoxide anion radical scavenging activity EC_{50} (μg/mL)				
FDA	ND	ND	592.27 ± 57.72[a]	204.53 ± 39.23[b]
FDB	ND	ND	ND	223.45 ± 34.42
FDC	ND	ND	ND	1001.43 ± 99.90
Quercetin	163.80 ± 0.17			
Ferrous Ion Chelating Activity EC_{50} (μg/mL)				
FDA	ND	ND	ND	1408.67 ± 37.61
FDB	ND	ND	ND	1289.00 ± 22.63
FDC	ND	ND	ND	1572.83 ± 234.71
EDTA	63.39 ± 5.77			
CAA EC_{50} (μg/mL)				
FDA	23.17 ± 0.79[a]	9.49 ± 0.16[b]	ND	ND
FDB	1343.84 ± 148.88[a]	289.27 ± 9.74[b]	868.24 ± 8.80[c]	323.61 ± 39.10[d]
FDC	ND	82.32 ± 1.54[a]	146.90 ± 11.88[b]	ND
Quercetin	15.6 ± 0.84			

Results are expressed as means ± SD (n = 3).
Values with different lower case letters ([a,b,c,d]) are significantly different at $p < 0.05$ among the different solvents.
Abbreviations: ND not detected; ABTS 2, 2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging capacity, expressed as mmol trolox equivalent (TE)/g; CAA cellular antioxidant assay, expressed as EC50 (μg /mL); DPPH 1,1-diphenyl-2-picryl hydrazyl radical-scavenging activity and superoxide anion ($O2^-$) scavenging activity are expressed as EC50, concentration of the extracts (μg/mL) required to inhibit 50% of the radicals; FRAP ferric reducing antioxidant power, expressed as mmol Fe2 + /g.

oxidative effects.

Analyses of ferrous ion chelating activity demonstrated that only the water extracts of all three varieties showed ferrous chelating capacity with FDB having the lowest EC_{50} value (Table 3).

3.3. Cellular antioxidant assay

The cellular antioxidant activity of the plant extracts was determined by treating HCT 116 cells with the hexane, ethyl acetate, methanol and water extracts of FDA, FDB and FDC. This assay is based on the ability of antioxidants in the plant extracts to inhibit the formation of peroxyl radicals, whereby the latter is generated by the addition of ABAP. Treatment of the cells with the plant extracts prior to the assay was able to inhibit the formation of peroxyl radicals, in a concentration dependent manner (Fig. 1). This is evident with reduction in fluorescence signals. The ethyl acetate extracts generally showed better antioxidant activities in all the three varieties compared to other solvents, with the exception of the hexane extract of FDA. Amongst the three varieties, the ethyl acetate extract of FDA had the lowest EC_{50} value which was significantly lower than quercetin (Table 3). The hexane extract of FDA also showed high CAA capacity with an EC_{50} of 23.17 ± 0.79 μg/mL.

3.4. Cell viability and MTT assay

A preliminary cell viability analysis was performed whereby the different cancer cells were treated with a high concentration (500 μg/mL) of the hexane, ethyl acetate, methanol and water extracts of the three varieties of *F. deltoidea*. The extracts which displayed more than 50% cell death were selected for further cytotoxicity analyses. Among the extracts, only the ethyl acetate extracts of FDA and FDB demonstrated significant anti-proliferative activities whereas the remaining extracts were non-cytotoxic against the cell lines tested. Both the ethyl acetate extracts of FDA and FDB demonstrated cytotoxicity against the four cancer cell lines with IC_{50} values in the range of 35–190 μg/mL (Table 4, Fig. 2). The ethyl acetate extract of FDA and FDB showed stronger cytotoxicity against MCF7 and MDA MB 231 cells. Both extracts showed higher cell viability at a concentration of 21.9 μg/mL in HCT116 cells compared to untreated cells. It was reported that polyphenols present in plants may protect cells against mitochondrial injury or alter the activity of succinate hydrogenase, hence increasing cell viability [32]. The fact that this was only observed in HCT116 cells and at a low concentration suggest that this may be concentration- and cell-specific.

3.5. Caspase 3/7 and cellular reactive oxygen species (ROS) analyses

The effect of the ethyl acetate extracts of FDA and FDB on the activity of caspase 3/7 in HCT 116 and HCC 1937 cells was determined to ascertain if the anti-proliferative effects were due to apoptosis. Results indicated a significant increase in caspase 3/7 activation after 48 h of treatment of HCT 116 cells with the ethyl acetate extract of FDA (Fig. 3(A)). The same extract also significantly increased caspase 3/7 activity in HCC 1937 cells (Fig. 3(B)) after 24 and 48 h of treatment. The ethyl acetate extract of FDB significantly activated the caspases in HCT 116 cells at 24 and 48 h and HCC 1937 cells at 48 h (Fig. 3(A) and (B)).

Based on these results as well as the cellular antioxidant assay data,

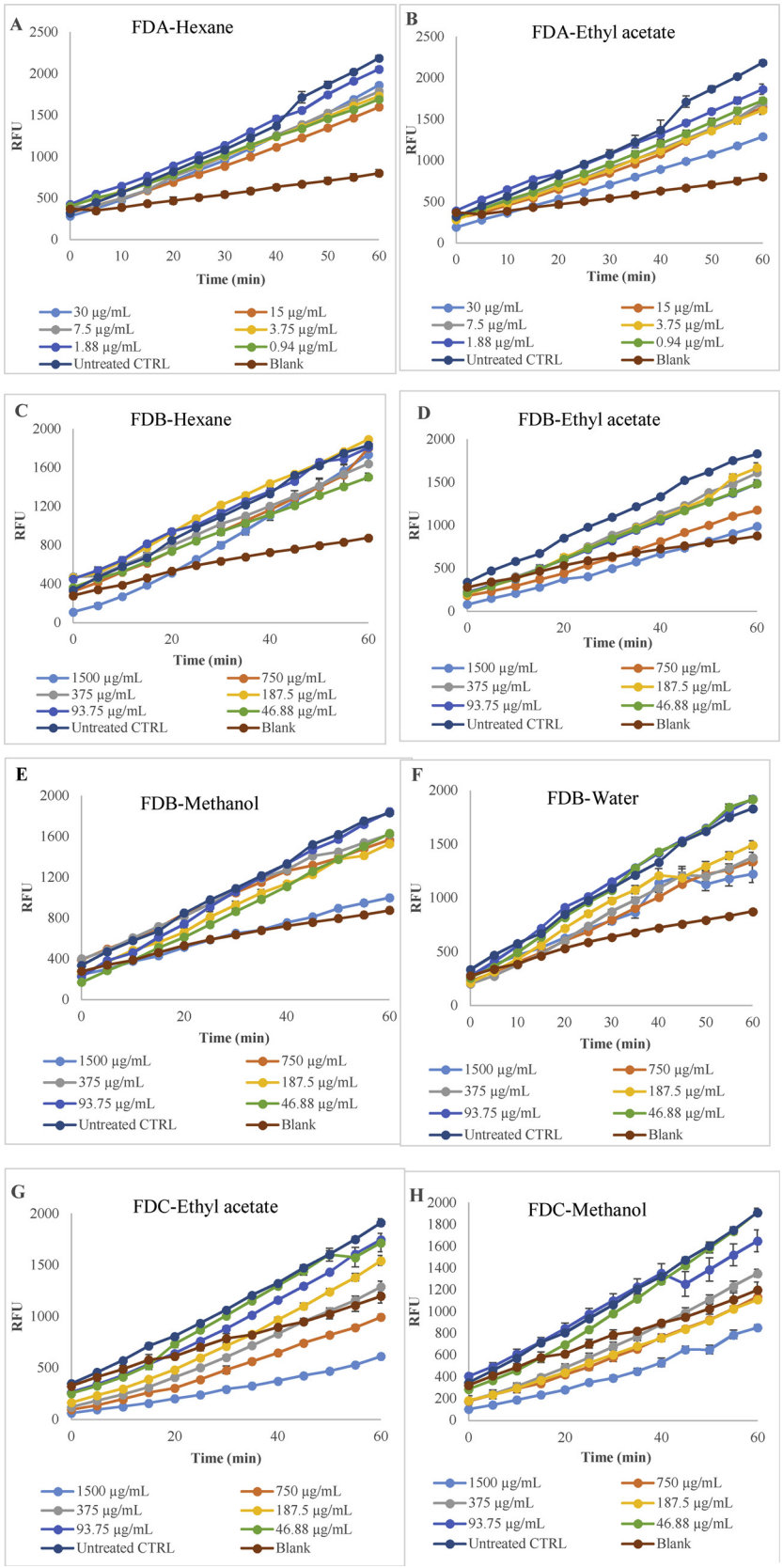

Fig. 1. Peroxyl radical-induced oxidation of DCFH to DCF in HCT 116 cells and the inhibition of oxidation by FDA-hexane (A), FDA-ethyl acetate (B), FDB-hexane (C), FDB-ethyl acetate (D), FDB-methanol (E), FDB-water (F), FDC-ethyl acetate (G) and FDC-methanol (H). Abbreviations: RFU: Relative Fluorescence Unit.

Table 4
Cytotoxicity analyses of the ethyl acetate extracts of *Ficus deltoidea* variety A (FDA-EA) and *Ficus deltoidea* variety B (FDB-EA) against breast cancer cells lines; MCF-7, MDA-MB 231, HCC1937 and colon cancer cell line; HCT 116.

Sample	IC_{50} (μg/mL)			
	MCF 7	MDA-MB 231	HCC1937	HCT 116
FDA-EA	52.82 ± 0.43	37.39 ± 0.59	190.83 ± 0.71	71.86 ± 0.40
FDB-EA	46.32 ± 0.24	40.71 ± 2.05	177.64 ± 1.80	54.89 ± 0.49
5-FU	NA	NA	NA	14.55 ± 0.84
Camptothecin	23.65 ± 0.58	21.84 ± 0.49	41.77 ± 1.23	NA

Results are expressed as means ± SD (n = 3).
NA: not analysed.

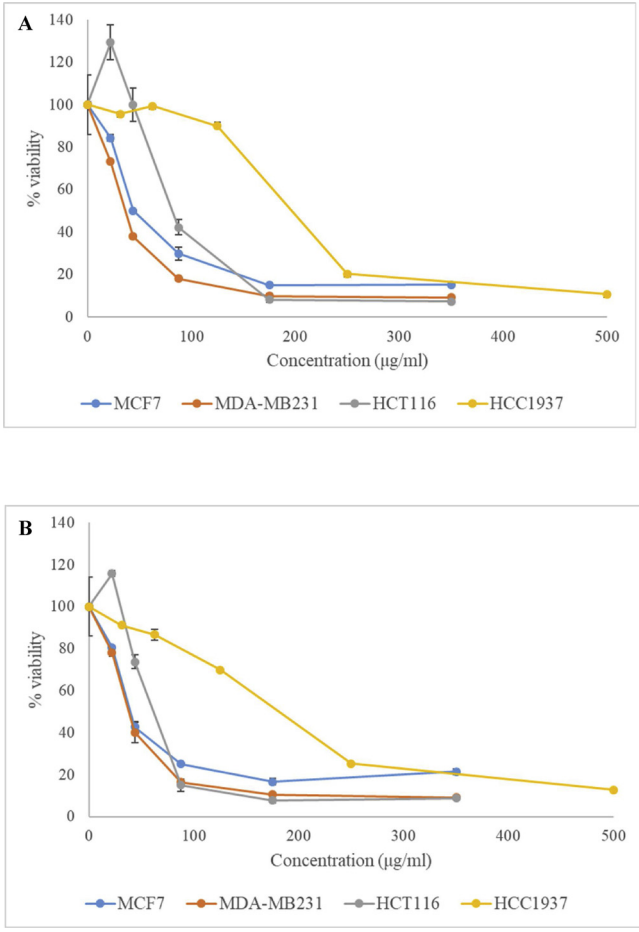

Fig. 2. The effect of ethyl acetate extracts of *F. deltoidea* variety A (A) and *F. deltoidea* variety B (B) on the proliferation of MCF-7, MDA-MB231, HCT 116 and HCC 1937 cells.

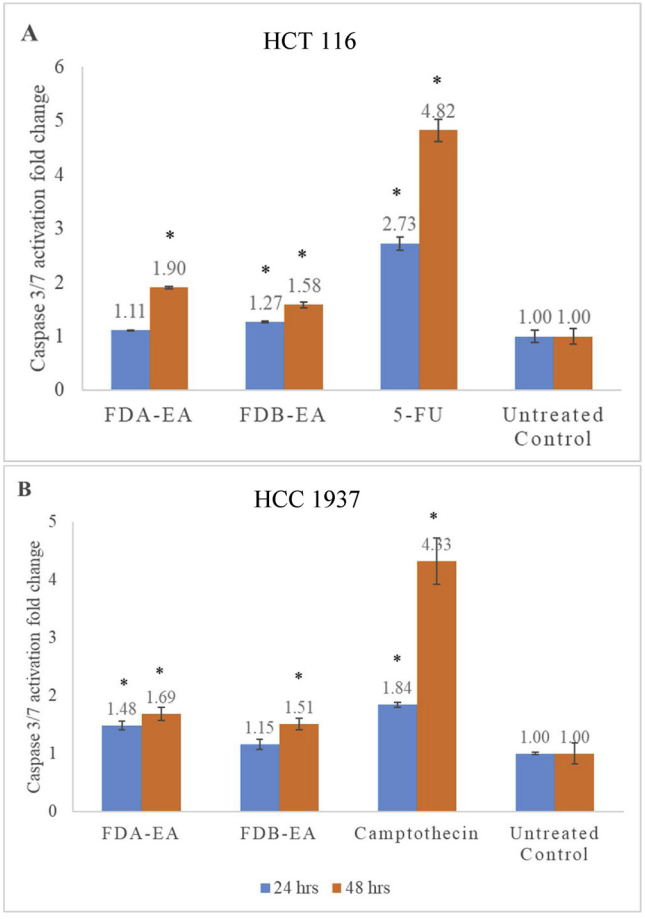

Fig. 3. Caspase 3/7 activation in (A) HCT 116 cells and (B) HCC 1937 cells treated with the ethyl acetate extracts of FDA and FDB for 24 h and 48 h. * indicates values are significantly different compared to untreated control at the respective time points ($p < 0.05$). Abbreviations: 5-FU: Fluorouracil, EA: ethyl acetate.

the ethyl acetate extract of FDA was selected for further analyses. In addition, further analyses were conducted only on HCT 116 cells as data on the anti-proliferative effects of *F. deltoidea* on colon cancer cells are not available. Cytotoxicity analysis showed that the ethyl acetate extract of FDA was also non-toxic against normal CCD841 colon cells (IC_{50} = 352.50 ± 26.19 μg/mL).

The activation of caspases 3/7 by the ethyl acetate extract of FDA was further validated with the use of caspase inhibitor z-VAD.fmk, with HCT 116 cells. The results obtained indicated that viability improved when the cells were simultaneously treated with the extract and the inhibitor, indicating the inhibition of these caspases (Fig. 4 (A)).

To determine if the high antioxidant activities of the ethyl acetate extract of FDA could influence the anti-proliferative effects, production of ROS in HCT116 cells treated with the IC_{50} concentration of the extract was measured. There was no significant difference in ROS levels in treated and untreated cells after one hour of treatment (Fig. 4 (B)).

3.6. Gene expression analyses

The expression of seven genes involved in apoptosis was studied using HCT116 cells treated with the ethyl acetate extract of FDA. *GAPDH* was used as the reference gene (Fig. 5). Significant change in expression of the genes studied was observed. The upregulated genes were *Fas1, Bax, Cdk-1, TNF-α* while the downregulated genes were *Cdk-2* while *Bcl-2* and *Tp53*. The highest fold change was observed in *Cdk-1* followed by *TNF-α* and *Fas1*.

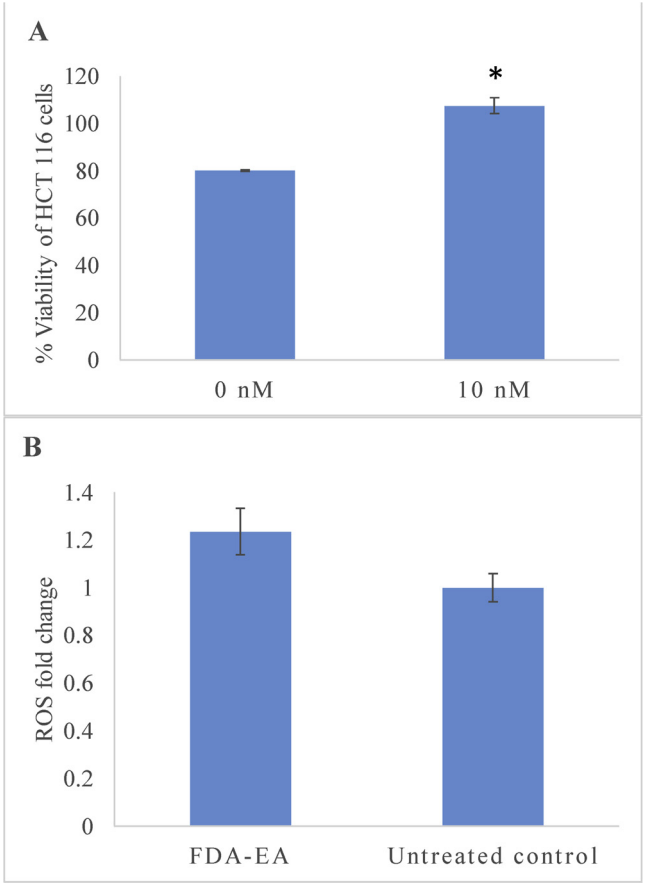

Fig. 4. (A) Percentage viability of HCT 116 cells treated with the ethyl acetate extract of FDA and pan-caspase inhibitor zVAD.FMK for 24 h. (B) ROS determination in HCT 116 cells treated with the ethyl acetate extract of FDA for one hour. Abbreviations: EA: ethyl acetate.

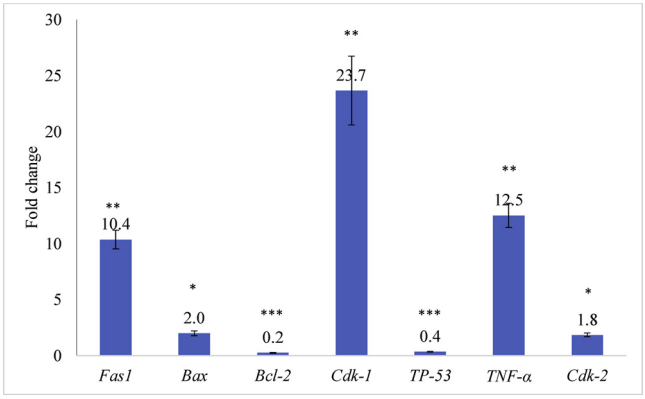

Fig. 5. Gene expression analyses of HCT 116 cells treated with the ethyl acetate extract of FDA. The bar chart shows the gene expression patterns (expressed as fold change) of selected genes involved in apoptosis. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

4. Discussion

Phytochemicals in the three varieties of *F. deltoidea* were separated based on their polarities, using sequential extraction. The use of solvents of varying polarities are useful for separation and estimation of phytochemicals according to polarity, which in turn can help in the determination of the most optimal solvent for extraction of antioxidant polyphenols. The highest yield shown by the methanol extracts followed by the water extracts implies that a majority of phytochemicals in *F. deltoidea* are polar whereas the remaining compounds are semi-polar and non-polar. Similar results were reported in a previous study on *F. deltoidea*, suggesting the presence of more chemical constituents in the methanol extracts [33]. The methanol extracts of all three varieties also contained the highest amount of polyphenolic content compared to other solvent extracts, and the polyphenolic content was found to be higher in relation to previously published studies [34]. The high polyphenolic content in the methanol extracts is suggestive of the presence of the glycosidic forms of polyphenols. Variation in the polyphenolic content could be due to different growth conditions and locations as well as extraction methods. The sequential and exhaustive extraction method could have resulted in higher extraction of polyphenolic compounds which in turn contributed to the high antioxidant activities [33]. Polyphenolic compounds are secondary plant metabolites that are of high importance in human health. In addition to their well-known antioxidant capabilities, various biological functions are attributed to these compounds including anti-bacterial, anti-allergic, and anti-inflammatory activities [35].

Similarly, we found that a majority of the methanol extracts of the three varieties of *F. deltoidea* showed strong antioxidant activities as compared to previously published data [19,36,37] and this may likely be due to the high polyphenolic content detected in these extracts. Several polyphenols have been detected in *F. deltoidea* including flavan-3-ol monomers, proanthocyanidins such as epicatechin [38] and flavone C-glycosides such as vitexin and isovitexin [1].

Flavonoids were mostly detected in the hexane extracts of all the three varieties indicating they are present mainly as aglycones and are less polar. The flavonoid content detected in the hexane extracts was much lower than that detected in the crude water extracts and ethyl acetate fractions of the fruits of *F. deltoidea* [36]. The same group also reported no correlation between flavonoid content and antioxidant activities in *F. deltoidea*.

Antioxidants can act via several mechanisms, either enzymatic or non-enzymatic. Therefore, the use of various assays that measure different mechanisms of the antioxidant action could provide a better understanding of the true potential of the extract as biological antioxidants. In this study, only the water extracts of *F. deltoidea* and the methanol extract of FDA were able to inhibit the production of superoxide anion radicals. The superoxide anion radical scavenging effects in this study was about 2-fold lower than that reported for the fruits of *F. deltoidea* [39]. Superoxide anion is a reactive radical that is produced in the body as a response to the immune system. Hydroxyl radicals and singlet oxygen can be generated from superoxide anions which can contribute to oxidative stress [40]. Superoxide anion and its derivatives may cause damage to DNA and cell membrane and induce illnesses such as cancers, arthritis and Alzheimer's disease [41,42]. Negative inhibition of the superoxide anion radicals at higher concentrations has been reported before [23,43,44]. The pro-oxidant effect of flavonoids is dependent on several factors such as their chemical structures and the concentration used [23].

F. deltoidea was shown to possess stronger antioxidant effects compared to other plants such as *Andrographis paniculate* and *Morinda citrifolia* [45]. Other *Ficus* species have also demonstrated strong antioxidant activity such as *F. microcarpa* [46]. The data obtained also demonstrated that *F. deltoidea* showed higher antioxidant potential as compared to other popular herbs such as ginseng [47].

In addition to the biochemical antioxidant assays, a cellular antioxidant assay was also included for validation purposes. Non-cellular antioxidant assays provide very useful information on the antioxidant activities of potential plant extracts; however, these assays do have their limitations and do not take into account parameters such as bioavailability and metabolism. Biological systems are complex and antioxidant compounds may operate via multiple mechanisms and pathways hence potentially producing variable results from that observed in non-cellular chemical assays [48]. Cell culture model provides an alternative to measuring cellular antioxidant activities, in addition

to being cost-effective, relatively fast and providing information on uptake, distribution and metabolism of the antioxidant compounds. Antioxidant activities were estimated through the changes in intracellular fluorescence following addition of the plant extracts. In this study, HCT 116 cells were used for the CAA assay as these cells are a part of the intestinal system and are therefore a good candidate for intestinal absorption studies. The ethyl acetate extracts, which had moderate antioxidant activities in the non-cellular assays exhibited strong cellular antioxidant capacity. The ethyl acetate extracts contain antioxidants of semi-polar nature implying the possibility that these compounds are able to act effectively as inhibitors of lipid peroxidation.

The cytotoxicity of the three varieties *F. deltoidea* extracts was evaluated using breast cancer and colon cancer cell lines with the ethyl acetate extracts of FDA and FDB showing strong cytotoxicity against these cells. Many drugs currently used for cancer therapeutics are isolated from natural products [49]. Polyphenols such as quercetin, catechin, kaempferol, myricetin, rutin and malvidin contributed largely in combatting cancers including colorectal cancer [50]. These biomolecules induce apoptosis via extrinsic and intrinsic signaling pathways. The extrinsic pathway is activated by ligation of death receptors which is followed by the activation of caspase-8 while cellular stress stimuli and activation of caspase-9 triggers the intrinsic pathway [51]. Caspases are proteases that are involved in inflammation and apoptosis. Activation of caspases triggered cellular changes that leads to apoptosis [52]. Caspase 3 and 7 are crucial mediators of mitochondrial events leading to apoptosis [53] hence determination of their activity can shed light into the mode of action of the plant extracts in inducing apoptosis. The tissue-selective caspase-3 is a protease which is frequently activated in mammalian cell apoptosis [52]. However, this protease is not activated in MCF-7 breast carcinoma cell line due to functional deletion of *CASP*-3 gene as a result of genomic mutation introducing a premature stop codon in the mRNA [54]. Thus, this cell line was not used in the determination of Caspase 3 and 7 in this study. The activation of caspase-3 and -7 in HCT 116, and HCC1937 cells by the ethyl acetate extracts of FDA and FDB indicated that the extracts were able to induce programmed cell death. This was further validated when their action was inhibited by zVAD.fmk, thus protecting the cells from cytotoxicity. ZVAD.fmk inhibits caspase by irreversibly binding to the catalytic site of caspase, thus inhibiting apoptosis.

The ethyl acetate extract of FDA is an efficient cellular antioxidant, capable of quenching ROS produced in the cells. This was further confirmed by measurements of ROS levels which showed no significant increase following treatment of HCT 116 cells with the ethyl acetate extract of FDA. Increased concentrations of ROS can cause oxidative stress and are thus important mediators for damage to cell structures, nucleic acids, lipids and proteins [55]. Studies have emerged on the apoptotic-inducing effects of ROS and plants with this capability have potential use in cancer therapeutics. An increase in cellular ROS triggers apoptotic signaling pathways that leads to programmed cell death [56]. Examples include the ROS-mediated activation of *Bax* and *p53* apoptotic signaling pathways [57,58]. However, the gene expression studies showed a significant decrease in the expression of *TP53* indicating the non-involvement of ROS in inducing apoptosis. In addition, the upregulation of *Bax* in this study could be a result of other mode of action of the plant extract and not via ROS induction.

Fibroblast associated antigen (*Fas*) and tumor necrosis factor (*TNF*) are typical death receptors present in the cell membrane which are activated by extracellular ligands [51]. This signaling pathway increases the sensitivity of tumor cells to Fas-mediated apoptosis, hence making them prone to cytotoxic chemotherapy that possess tumor suppressing ability [59]. Binding of respective ligands to Fas and TNF receptors activates the death-inducing signalling complex (DISC) including Fas-associated death domain protein (FADD), caspase 8 and caspase 10. Activation of these caspases initiates further downstream caspase cascade (caspase 3, 6 and 7), subsequently triggering apoptosis. In addition to activating the DISC and caspases, binding of TNF-α to the TNF receptor 1 (TNF-R1) can also activate the MAP kinases signal transduction pathways including the JNK pathway which is pro-apoptotic [60].

Bax and *Bcl-2* encode for proteins in the BCL-2 protein family and function as apoptotic regulators via the intrinsic or mitochondrial pathway. The BCL-2 protein family consists of anti-apoptotic proteins such as BCL-2 and MCL-1 and pro-apoptotic proteins such as BAX and BAK. The intrinsic pathway is initiated by the release of cytochrome c from the mitochondria, as a result of activation of the TNF death receptors [51]. This subsequently triggers the activity of caspase 3. *Bcl-2* which is anti-apoptotic prevents the release of cytochrome c by binding to *Bax* and *Bak* and inhibiting their action [51]. Hence, when the intrinsic pathway is activated, the gene expression of *Bax* and *Bcl-2* will increase and decrease, respectively, triggering the release of cytochrome c from mitochondria and ultimately apoptosis.

The gene expression analysis of the HCT 116 cells when treated with the ethyl acetate extract of FDA indicates that apoptosis is activated via different pathways. The expression of *Bax* is 8 times higher than *Bcl-2* in the HCT 116 cells, suggesting the involvement of the intrinsic apoptotic pathway as well. The induction of apoptosis by *F. deltoidea* via the intrinsic mitochondrial pathway in prostate cancer cells was recently reported by Hanafi [20]. The study also reported on the activation of caspase 3 and 7, upregulation of *Bax* and down-regulation of *Bcl-2* with increased depolarisation of mitochondrial membrane potential [20].

The activity of cyclin-dependent kinase 1 (*Cdk-1*) determines the cell cycle timing of mitosis. Activation of the Cdk1/cyclin B1 complex drives the progression of mitosis from G2 to M phase. The activation of p53 negatively affect transcription of *Cdk-1* and *cyclin B1*. CDK-1 has been proposed as a pro-apoptosis mediator whereby its activation was reported as a result of Fas-induced apoptosis [61]. The pro-apoptotic ability of Cdk-1 was observed in microtubule inhibitors such as paclitaxel [61]. In this study, the significant increase in expression of *Cdk-1* in HCT116 cells could be the result of the pro-apoptotic expression of this gene. Cdk-1 could also activate apoptosis through the *Bcl-2* family proteins [61].

Flavones and flavan-3-ol monomers such as apigenin, catechin and epicatechin have been identified in the leaves of *F. deltoidea* [38]. A number of studies have demonstrated the antiproliferative activities of these compounds in colorectal cancer. They caused apoptosis, cell cycle arrest and DNA damage in colon cancer cell lines [50].

This initial study highlights the distribution and characteristics of polyphenols in the different varieties of *F. deltoidea* and demonstrates the potential anti-proliferative effects of the ethyl acetate extract. Although polyphenols that are present in polar solvents are expected to exert better biological activities, nevertheless, various studies have also used less polar solvents such as ethyl acetate or methanol for the exhaustive extraction of phytochemicals from *F. deltoidea* and to study their biological effects [20,62]. Additionally, the methanol extracts of *F. deltoidea* have also been used in animal studies involving rodent models, with positive outcomes [63,64]. Future studies employing in vivo models including measuring absorption of the bioactive compounds in biological fluids/tissues post-treatment would give a better indication of their potential. In addition, polyphenols in the polar extracts could also be tested for possible biological activities.

5. Conclusion

This study shows that different solvent extracts of *F. deltoidea* influence the antioxidant and antiproliferative capacities of this plant. The methanolic extracts show better antioxidant potential whereas the ethyl acetate extracts have higher antiproliferative activities. The ethyl acetate extract of FDA induced cell death via apoptosis. The activation and upregulation of genes may have involved both the extrinsic and intrinsic pathways and may indicate a multi-dimensional approach by this extract in inducing apoptosis in the cancer cells. Data obtained in this study highlights this plant as a potential antioxidant and anti-

cancer agents, particularly for colon cancer. Further investigations are needed in identifying and isolating the active compounds responsible and the molecular mechanisms involved in inducing apoptosis in colon cancer cells.

Declaration

All research were done by the authors, the manuscript was drafted and edited by all authors.

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Conflict of interest

The authors declare they have no conflicts of interest.

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