

Original Research Article

Antiangiogenic Effect of *Ficus deltoidea* Jack Standardised Leaf Extracts

Armaghan Shafaei¹, Nahdzatul Syima Muslim², Zeyad D Nassar^{2,3}, Abdalrahim FA Aisha^{1,2}, Amin Malik Shah Abdul Majid² and Zhari Ismail^{1*}

¹Department of Pharmaceutical Chemistry, ²EMAN Testing & Research Laboratory, Department of Pharmacology, School of Pharmaceutical Sciences, Universiti Sains Malaysia, Minden 11800, Pulau Pinang, Malaysia, ³School of Pharmacy, University of Queensland, Brisbane, QLD, Australia

*For correspondence: **Email:** ismailzhari@gmail.com; **Tel:** +604-653-2242

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Abstract

Purpose: To standardise the methanol and aqueous extracts of *Ficus deltoidea* leaf by developing a reverse phase high performance liquid chromatography (RP-HPLC) for determination of the ursolic acid content and to investigate their antiangiogenic activity.

Methods: To prepare the water extract (FD-A), the powder of the plant was extracted with water under reflux for 24 h at 50 °C. The methanol extract (FD-M) was prepared using Soxhlet extractor for 24 h at 50 °C. The extracts were standardised for ursolic acid content by reverse phase high performance liquid chromatography (RP-HPLC), as well as by total phenolic and flavonoid contents. Antiangiogenic activity was studied using *ex vivo* rat aortic rings and *in vivo* chick chorioallantoic membrane angiogenesis models. The anti-proliferative effect of the extracts against normal human endothelial cells and two cancer cell lines was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay.

Results: In rat aortic rings, methanol and water extracts inhibited the outgrowth of microvessels with IC₅₀ values of 48.2 ± 1.1 and 62.7 ± 1.4 µg ml⁻¹, respectively. Methanol and water extracts at doses of 100 µg disc⁻¹ also inhibited vascularisation of chick embryo chorioallantoic membrane with inhibition values of 62.0 ± 6.3 and 43.3 ± 4.8 %, respectively. In addition, both extracts showed potent cytotoxicity against breast and colon cancer cells while demonstrating non-cytotoxic activity against normal endothelial cells. Furthermore, the two extracts showed relatively high concentration of ursolic acid, total phenolics and flavonoids associated with potent antiangiogenic activity.

Conclusion: The inhibition of angiogenesis by *F. deltoidea* extracts may be attributed to the relatively high ursolic acid content as well as the presence of antioxidant compounds of phenolics and flavonoids in the extracts.

Keywords: *Ficus deltoidea*, Antiangiogenesis, Ursolic acid, Cytotoxicity, Antioxidant

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INTRODUCTION

Medicinal plants have been used for centuries in folk medicine in the treatment of various diseases and today, they still play an important role in health. *Ficus deltoidea* (FD) Jack (Moraceae) is a traditional medicinal plant widely

distributed in Southeast Asia. In Malaysia, FD is known as “mas cotek” [1,2]. Traditionally, the plant has been utilised in treating gout, high blood pressure, pneumonia, diarrhoea, skin infections, diabetes and to improve blood circulation [3]. Previous studies have shown that FD leaves possess antinociceptive, wound

healing and antioxidant properties [1,3,4]. Previous research on FD showed the presence of relatively high content of polyphenols, flavonoids, which are known as antioxidant compounds, amino acids, vitamins, carbohydrates, and purine alkaloids. Presence of a number of antioxidant compounds is known to possess various pharmacological activities including antiangiogenic and cytotoxic effects on a number of human cancer cell lines [1-3].

Angiogenesis, the formation of new blood vessels, plays a critical role in pathogenesis of various human diseases such as cancer, psoriasis, arterial plaque formation, ocular neovascularization, gastrointestinal ulcers, rheumatoid arthritis, and diabetic retinopathy [5]. Therefore, inhibition of angiogenesis may provide promising therapies for several human life threatening ailments.

Standardisation of herbal extracts and preparations provides an important tool to study the chemical profile and to evaluate reproducibility of extraction procedures, pharmacological effects, and provides the markers for stability and pharmacokinetics studies.

In this context, this study sought to standardise FD leaf extracts and to investigate their antiangiogenic activity due to their high content of phytochemical components as well as local use of FD leaf for curing diseases.

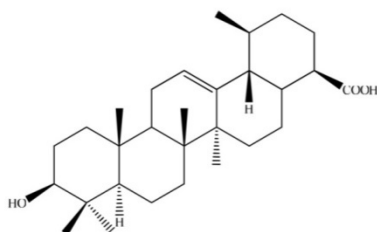


Figure 1: Structure of ursolic acid.

EXPERIMENT

Chemicals and reagents

Earles' salt M199 medium, trypsin, Dulbecco's modified eagle medium (DMEM), minimum essential medium (MEM), fibrinogen and foetal bovine serum (FBS) were obtained from Bio-Diagnostics (Petaling Jaya, Selangor, Malaysia). Aprotinin, L-glutamine, thrombin, sodium chloride, amphotericin B, gentamycin, 6-aminocaproic acid, suramin, dimethylsulfoxide (DMSO), phosphate buffered saline (PBS), 3-(4,5-dimethylthiazol-2-yl-2,5-diphenyl)tetrazolium bromide (MTT), agarose, ursolic acid, quercetin, gallic acid, Folin-Ciocalteu reagent, sodium

carbonate, aluminium chloride and potassium acetate were purchased from Sigma-Aldrich (Subang Jaya, Selangor, Malaysia). Human umbilical vein endothelial cells (HUVECs), human hormone resistant breast cancer cells (MDA-MB-231) and human colorectal carcinoma cells (HCT 116) were purchased from ATCC (Manassas, Virginia). Endothelial cell medium (ECM) was obtained from Team Medical Scientific (Shah Alam, Selangor, Malaysia). HPLC-grade of acetonitrile and orthophosphoric acid were purchased from Merck (Petaling Jaya, Selangor, Malaysia). Deionised water for HPLC was prepared using Ultra pure water purifier system (Elgastat, Bucks, UK).

Plant material and extraction

The leaves of the plant (*Ficus deltoidea*) were purchased from Herbagus Sdn. Bhd. Penang-Malaysia and identified by Mr. Shunmugam of the School of Biological Sciences, University Sains Malaysia. A voucher specimen (no. 11204) was deposited at the herbarium of School of Biological Sciences, University Sains Malaysia. The batch of leaves for the current study was collected at the end of July and early in August, 2010, oven-dried at 40°C and then pulverised into fine powder using a milling machine (Retsch GmbH, Germany). To prepare the water extract (FD-A), 100 g powder was extracted with 1000 mL water under reflux for 24 h at 50 °C. The methanol extract (FD-M) was prepared using Soxhlet extractor for 24 h at 50 °C at the same solvent to plant powder ratio. After cooling, the extracts were filtered using Whatman filter paper no. 1, concentrated at 50 °C under vacuum using a rotary evaporator (RE121 Buchi, Switzerland), and dried using a freeze-dryer (Labconco, USA).

Determination of ursolic acid content by RP-HPLC

Agilent 1100 HPLC system equipped with a degasser, a quaternary pump, auto sampler, a column oven and UV detector was used. Separation was performed on Zorbax Eclipse C₁₈ SB-Aq column (250 × 4.6 mm × 5 μm) fitted with analytical guard column (4.6 × 12.5 mm, 5 μm) (Agilent Technologies, USA). Elution was performed with an isocratic mobile phase comprising of acetonitrile (65%) and 1.25 % H₃PO₄ (35 %), flow rate was 1.2 mL min⁻¹, injection volume was 20 μL, column temperature was 25°C, detection was carried out at 210 nm and data acquisition was performed by Chemstation A.08.03.

The method was validated for linearity, accuracy, precision, limits of detection (LOD), and

quantification (LOQ) according to a previously described method [6]. Linearity was determined as the regression coefficient (R^2) of calibration curve constructed at 8 concentrations. Accuracy was evaluated as the percentage recovery of reference compound added to extracts. Precision of peak area was determined as a relative standard deviation (%RSD) of intraday and interday data ($n = 6$). LOD and LOQ were established at signal to noise ratio (S/N) of 3 and 10, respectively. The ursolic acid content in FD extracts was calculated by using the calibration equation of reference compound.

Determination of total phenolics

Total phenolic content was determined as described previously [7]. Briefly, 20 μ l extract (4 mg mL^{-1}) or gallic acid (0.0625 - 4 mg mL^{-1}) was added to 1.58 ml deionised water and 100 μ l folin-ciocalteu reagent, mixed thoroughly and incubated at room temperature (rt) for 8 min. Subsequently, 300 μ l of 20 % Na_2CO_3 was added, incubated for 2 h, and absorbance was measured at 765 nm. The results are expressed as average mg gallic acid equivalents per gram of the extract ($n = 3$).

Determination of total flavonoids

Total flavonoid content determined using a previously described method [8]. Aluminium chloride (0.1 mL, 10 %w/v), 0.1 mL of 1M potassium acetate solution, 1.5 mL methanol, and 2.8 mL of distilled deionised water were added to 500 μ L of extract (4 mg mL^{-1}) or quercetin (0.0078 - 4 mg mL^{-1}), mixed, and incubated at room temperature (RT) for 30 min. Absorbance was measured at 415 nm. Aluminium chloride was substituted by water for the blank. Total flavonoid content was determined, as mg quercetin equivalents per gram of the extract ($n = 3$), by applying quercetin calibration equation.

Experimental animals

The 8 to 12 weeks old Sprague Dawley male rats were obtained from the Animal House Unit, Universiti Sains Malaysia. The animals were kept for two weeks in transient animal house before carrying out the experiment. Moreover, well ventilated cages with 12 h of light as well as food and water were provided for the animals. The animals were sacrificed by suffocated under carbon dioxide (CO_2).

Rat aortic ring assay

The assay was performed following previously described protocols [9]. In brief, 1-mm thick rings of thoracic aortas were seeded in fibrin clots, treated at various concentrations of the extracts (5 - 100 $\mu\text{g mL}^{-1}$) and incubated at 37 °C in a humidified CO_2 incubator for 5 days. The second layer medium was replaced with a fresh one on day 4, and the outgrowth of blood vessels in the explants was quantified on day 5 using an inverted light microscope supplied with Leica QWin imaging software [10]. Suramin (100 $\mu\text{g/ml}$) and 1.0% dimethyl sulfoxide (DMSO) were used as positive and negative controls respectively.

Cell viability assay

The effect of the extracts on cell viability was evaluated by MTT assay. The cells were treated with extract or 1.0 % DMSO for 48 h, following a previously described method [11]. The absorbance was taken at 570 nm using a microtitre plate reader (Tecan, Switzerland), and the results are presented as % cell viability relative to negative control.

In vivo chorioallantoic membrane (CAM) assay

The antiangiogenic effect of the extracts was screened *in vivo* using a modified fertilised chicken embryos assay [12]. Briefly, 5-day old fertilised eggs were obtained from a local hatchery and 5 mL albumin was withdrawn. The samples were prepared as discs in 1.2 % of agarose at 100 $\mu\text{g disc}^{-1}$. Discs containing the vehicle (ethanol 1 %) were used as negative control. Treatments were applied on CAMs through a small window opened in the shell, and incubated for 24 h. CAMs were then photographed under a dissecting microscope ($n = 6$), and blood vessels counted. The results are expressed as mean inhibition \pm SD (%), $n = 6$.

Ethical approval

All the procedures were carried out according to the Animal Ethics Guidelines of Universiti Sains Malaysia which is based on standard protocols for the use of laboratory animals [13] and with approval reference no. USM/Animal Ethics Approval/2011/(66)(302).

Statistical analysis

The results are presented as mean \pm SD. Differences between groups were compared by

one-way ANOVA and differences were considered significant at $p < 0.05$. Statistical analysis was carried out using SPSS version 16.0 (SPSS, Chicago, Illinois).

RESULTS

Phytochemical profile

HPLC chromatograms of ursolic acid are shown in Figure 2. The peak corresponding to ursolic acid in the extracts was assigned by comparing its retention time with that of ursolic acid reference compound. Good linearity ($R^2 = 0.9999$) was obtained in the concentration range ($0.1 - 1000 \mu\text{g mL}^{-1}$). The linear regression equation was $y = 7.2265x - 14.754$. LOD and LOQ were 1.0 and $5.0 \mu\text{g mL}^{-1}$, respectively. Precision, as %RSD of the peak area, was $< 3\%$ (Table 1). Accuracy, as % recovery of ursolic acid, was in the range $98.3 - 102.5\%$ with %RSD $< 3\%$, which indicates good reproducibility of the method (Table 2). Ursolic acid content of FD extracts, as mg g^{-1} extract, was 4.3 ± 0.2 and 2.5 ± 0.03 for methanol (FD-M) and aqueous (FD-A) extracts, respectively.

Table 1: Intra-day and inter-day precision of ursolic acid (based on HPLC method, $n = 6$).

Concentration ($\mu\text{g mL}^{-1}$)	Intra-day (n=6)		Inter-day (n=6)	
	Mean (%)	RSD (%)	Mean (%)	RSD (%)
50	92.9	3.0	94.8	2.9
100	107.2	0.1	101.9	2.0
200	101.6	0.2	103.1	3.0

The results are shown as % recovery of ursolic acid added to methanol (FD-M) and water (FD-A) extracts of *F. deltoidea*.

Table 2: Accuracy of the HPLC method

Concentration ($\mu\text{g mL}^{-1}$)	FD-M		FD-A	
	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
55	98.3	1.2	100.1	1.8
100	102.5	0.7	101.9	3.0
180	100.5	0.3	99.5	1.5

The total phenolic and flavonoid contents of FD-M extract was 118.1 ± 2.6 and $469.6 \pm 5.6 \text{ mg g}^{-1}$, while for FD-A extract the values were 12.6 ± 1.0 and $16.8 \pm 0.3 \text{ mg g}^{-1}$, respectively.

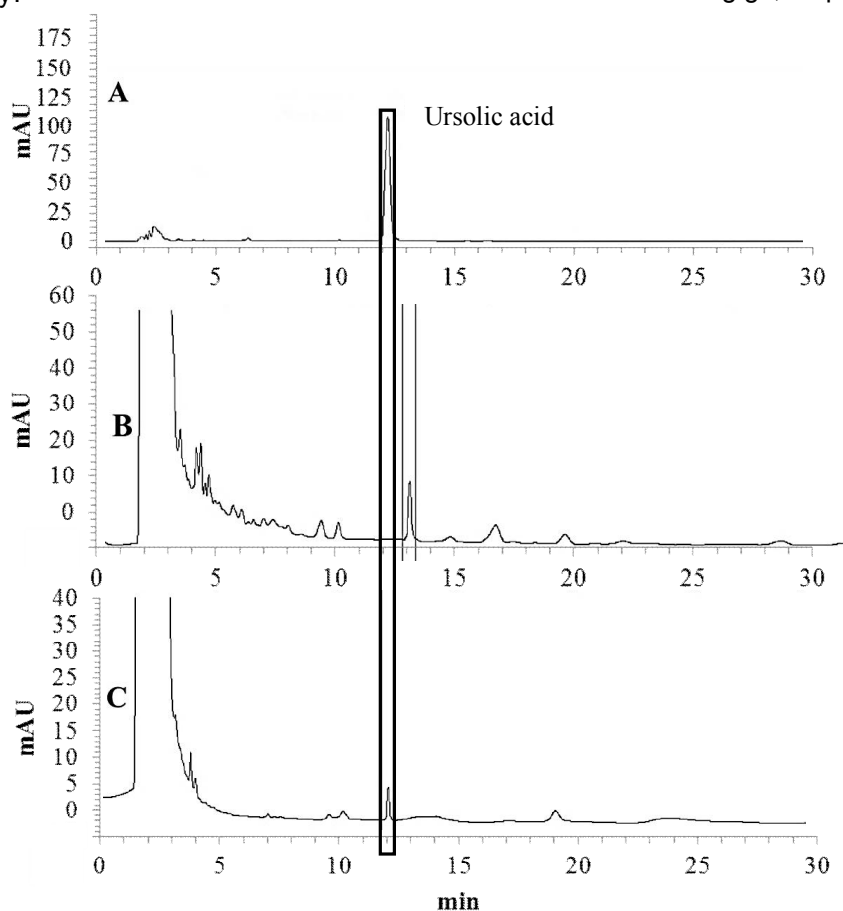


Figure 2: HPLC chromatograms of *F. deltoidea* extracts and ursolic acid. **Note:** Ursolic acid (A) standard, FD-M extract (B), and FD-A extract (C).

Antiangiogenic effect of extracts

The antiangiogenic effect of the extracts was confirmed by rat aortic ring assay data. Figure 3 shows normal microvessels outgrowth from the untreated aortic rings. On the other hand, aortic rings treated with FD-M and FD-A extracts exhibited outgrowth reduction with IC_{50} of 48.2 ± 1.1

and $62.7 \pm 1.4 \mu\text{g mL}^{-1}$, respectively. Both extracts showed significant dose-dependent inhibition ($p < 0.05$ and $p < 0.001$) compared with untreated aortic rings. Suramin (standard, $100 \mu\text{g mL}^{-1}$) showed 100 % inhibition of microvessels outgrowth.

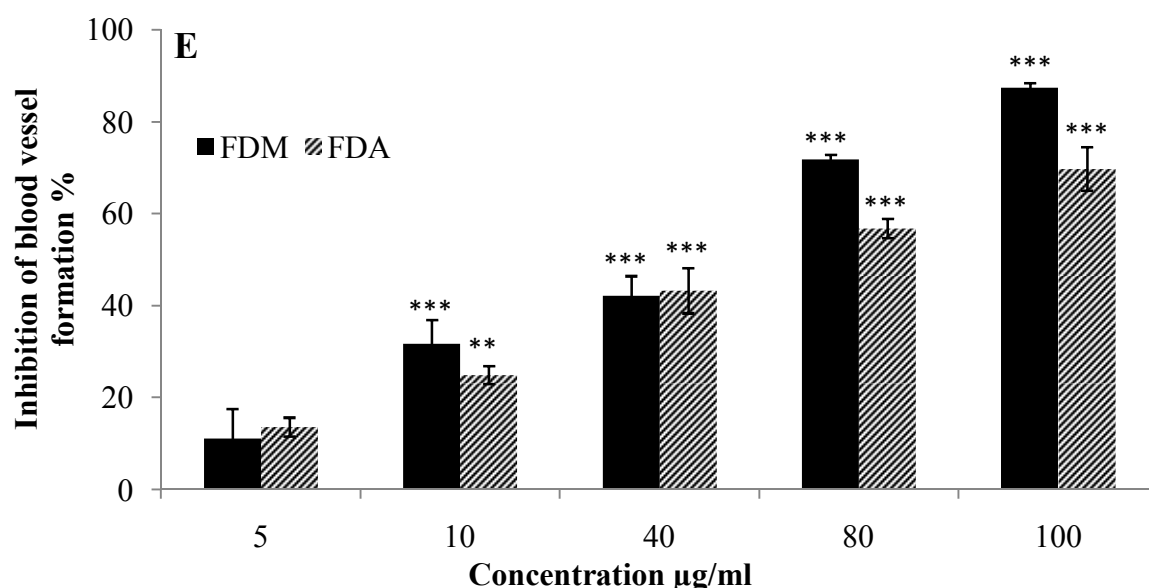
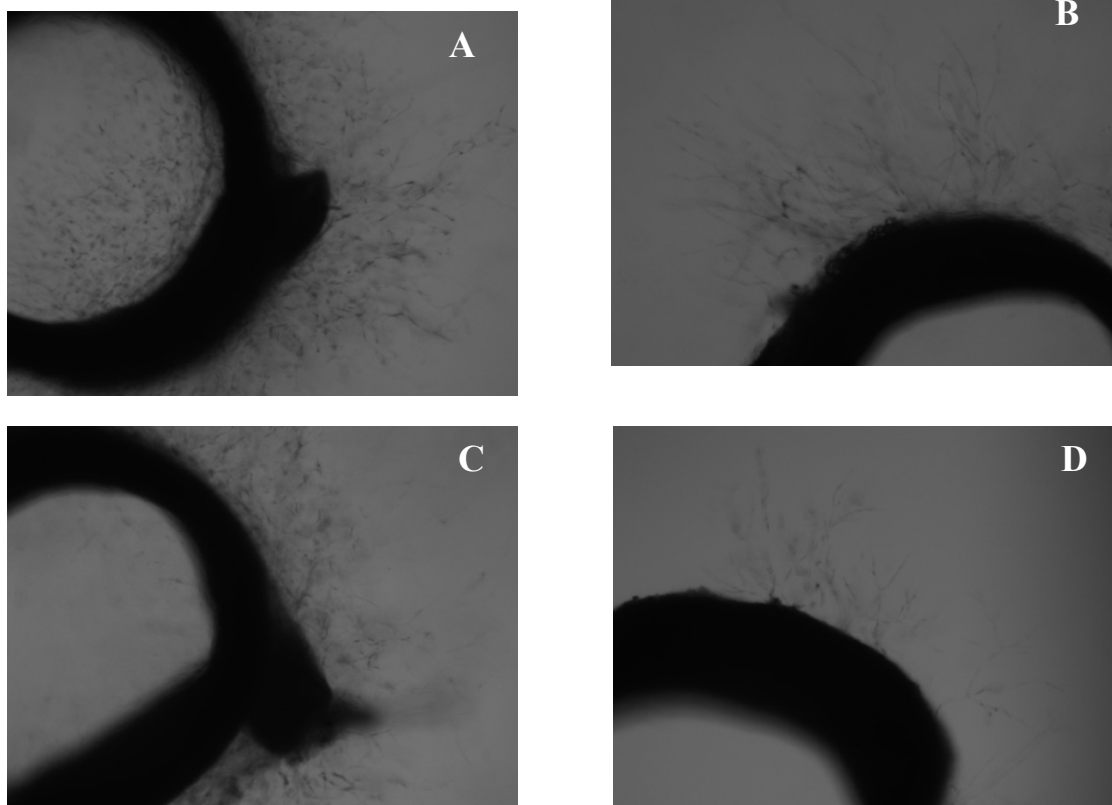


Figure 3: Effect of *F. deltoidea* leaf extracts on microvessels outgrowth in rat aortic rings. **Note:** 1 % DMSO (A), FD-M extract (B), FD-A extract (C), Suramin $100 \mu\text{g mL}^{-1}$ (D), and dose dependent relationship of FD extracts on the microvessels outgrowth (E). Data are presented as mean \pm SD (n = 3); ** $p < 0.05$ and *** $p < 0.001$

Effect on cell viability

The anti-proliferative effects of the extracts on HUVECs, HCT 116 and MDA-MB-231 cells are shown in Fig 4. The extracts showed a dose dependent anti-proliferative activity after 48 h of treatment compared to negative control in this study. IC₅₀s was deduced from the logarithm regression equations obtained by plotting % cell viability versus concentration. The extracts were non-cytotoxic against HUVECs, but showed potent cytotoxic effect on the hormone-resistant breast cancer (MDA-MB-231) and colon cancer (HCT 116) cells (Table 3).

Table 3: Median inhibitory concentrations (IC₅₀) of *F. deltoidea* extracts on human cancer and normal cell lines. Results are shown as mean ± SD (n = 3).

Extract	IC ₅₀ (µg mL ⁻¹)		
	HUVECs	MDA-MB-	HCT 116
FD-M	377.2 ± 5.6	22.2 ± 1.2	36.6 ± 2.2
FD-A	387.5 ± 10.0	22.6 ± 0.6	35.5 ± 0.7

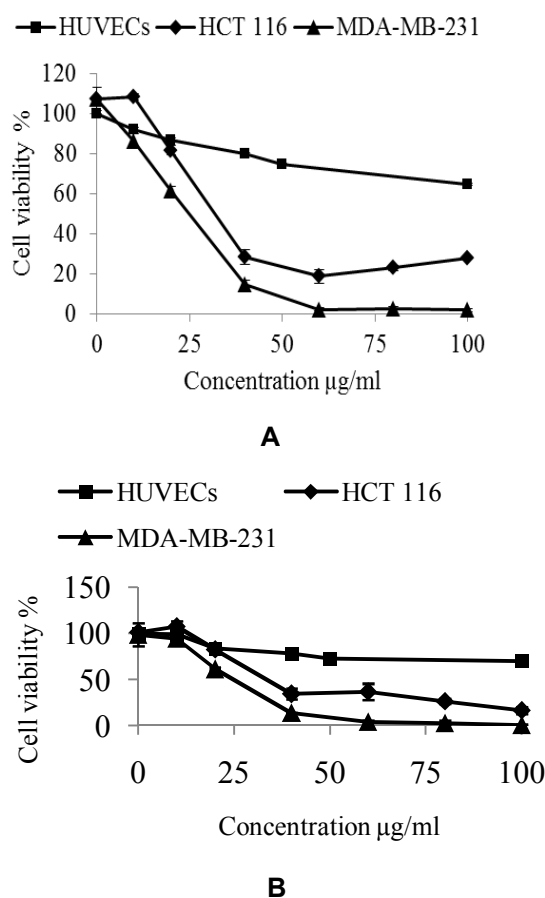


Figure 4: Anti-proliferative effect of (A) FD-A and (B) FD-M extracts against HUVECs, HCT 116 and MDA-MB-231 cell lines. Values are shown as mean ± SD (n = 3).

In vivo antiangiogenic effect of the extracts on neovascularisation

Vascularisation in the fertilised chick embryos was significantly inhibited at 100 µg disc⁻¹ of FD-M and FD-A extracts. Figure 5 shows normal vasculature pattern obtained in untreated CAMs with primary, secondary and tertiary vessels and with dendritic branching pattern, whereas the CAMs treated with the extracts showed inhibition of new blood vessels growth as well as distortion in the existing vasculature architecture. Inhibition was 62.0 ± 6.3 (FD-M) and 43.3 ± 4.8 % (FD-A).

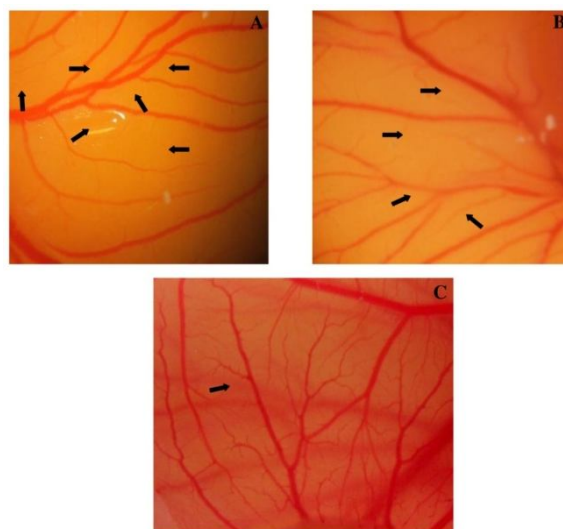


Figure 5: Effect of *F. deltoidea* extracts on neovascularisation in chick chorioallantoic membrane. CAM treated with FD-M extract at 100 µg disc⁻¹ (A), FD-A extract at 100 µg disc⁻¹ (B), and 1 % ethanol (C).

DISCUSSION

Phytochemical analysis of FD extracts reveals the presence of relatively high content of ursolic acid, total phenolics and flavonoids with the highest concentration obtained in FD-M extract. Ursolic acid is a triterpene distributed widely in the plant kingdom. The compound is gaining more and more interest due to its wide range of pharmacological properties such as liver protection [14], anti-inflammatory [15], antiangiogenic [16], chemopreventive [17], anticancer, and antioxidant effects [18]. The RP-HPLC method for quantification of ursolic acid in FD reported is rapid, reproducible, and precise.

FD extracts potently inhibited the outgrowth of microvessels in rat aortic rings in a dose-dependent manner. In order to test if the antiangiogenic activity observed is due to direct cytotoxic effect on endothelial cells or due to inhibition of other angiogenesis cascade mechanisms, the cytotoxicity of FD extracts was

tested against HUVECs and two cancer cell lines (HCT 116 and MDA-MB-231). Cell viability results show that FD extracts are not cytotoxic to HUVECs; according to the National Cancer Institute (NCI) extracts with $IC_{50} > 20 \mu\text{g mL}^{-1}$ are not considered cytotoxic [19]. However, the extracts showed higher cytotoxicity towards the colon cancer (HCT 116) and breast cancer (MDA-MB-231) cells. In order to illustrate the antiangiogenic potential of FD *in vivo*, the extracts were tested on chick CAMs as an *in vivo* angiogenesis model [20]. The results showed that FD extracts significantly inhibited the new blood vessels formation and distorted the existing vasculature. This result further supports the antiangiogenic activity observed *in vitro*.

Angiogenesis plays an important role in pathogenesis of various human diseases such as cancer, psoriasis, and diabetic retinopathy [5]. Therefore, *F. deltoidea* extracts, as a natural angiogenesis inhibitor, may provide new candidates for the treatment of these diseases. Previous studies showed that ursolic acid may inhibit the different steps of the angiogenesis process using bovine aortic endothelial cells, notably down regulation of VEGF expression level. In support of the our findings, extensive studies have shown antiangiogenic effect of ursolic acid, phenolics and flavonoids compounds [16]. Therefore, the antiangiogenic activity of FD extracts may likely be due to the presence of ursolic acid, phenolics and flavonoids.

The selective and potent anti-proliferative effect obtained against the cancerous cells, but not on normal cells, highlights FD as a potential source of new anti-cancer candidates.

CONCLUSION

Taken together, the inhibition of angiogenesis observed in rat aortic ring and CAM assays is not due to direct cytotoxicity on endothelial cells, but to inhibition of other vital steps in angiogenesis cascade which needs further investigation. The antiangiogenic effect of FD extract may be related to the presence of relatively high contents of ursolic acid, phenolics and flavonoids. The selective cytotoxicity towards colon and breast cancer cell lines, and anti-angiogenic effect indicates the potential anti-cancer effect of FD extracts.

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