

Antitumor Activity of *Dendrophthoe falcata* against Ehrlich Ascites Carcinoma in Swiss Albino Mice

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Abstract: The objective of the present study is to explore the anticancer activity of the ethanolic and aqueous extracts of the *Dendrophthoe falcata* in Swiss albino mice against Ehrlich Ascites Carcinoma (EAC) cell line. Anticancer activity of ethanolic and aqueous extracts of *D. falcata* was evaluated in EAC Swiss albino mice at the doses of 200 and 400 mg/kg body weight orally. Both extracts at both doses were administered for 13 consecutive days. After 24 h of the last dose and then eighteen hours of fasting, the mice were sacrificed and antitumor effect of ethanolic and aqueous extracts was assessed by evaluating tumor volume, viable and nonviable tumor cell count, tumor weight and hematological parameters of EAC bearing host. Ethanolic and aqueous extracts showed significant decrease in ($p < 0.0001$) tumor volume, viable cell count, tumor weight and elevated the life span of EAC tumor bearing mice. Haematological profiles such as red blood cell (RBC), haemoglobin, and white blood cell (WBC) count reverted to normal level in treated mice. The results demonstrated that the extract has potent dose dependent anticancer activity comparable to that of cisplatin. Aqueous extract at both doses (200 and 400 mg/kg) and ethanolic extract at 400 mg/kg dose showed potent anticancer activity.

Keywords: *Dendrophthoe falcata*, EAC cell line, anticancer activity, cisplatin.

INTRODUCTION

Cancer is one of the most dreaded diseases of the 20th century and spreading further continuously with increasing incidence in 21st century. Cancer is a group of more than 100 different diseases, characterized by uncontrolled cellular growth, local tissue invasion, and distant metastases [1]. Multidisciplinary scientific investigations are making best efforts to combat this disease, but the sure-shot, perfect cure is yet to be brought into world medicine. Cancer is caused by internal factors (tobacco, chemicals, radiations and infectious organisms) and external factors (mutation, hormones, and immune conditions) [2,3] and can be treated with surgery, radiation, chemotherapy, hormone therapy, and biological therapy. Plants have a long history of use in the treatment of cancer. Over 60% of currently used anti-cancer agents are derived in one-way or another from natural sources, including plants, marine organisms and microorganisms [4]. It is estimated that more than 50% of all the patients diagnosed with cancer explore complementary and alternative medicine – especially herbal medicine [5].

Dendrophthoe falcata (L.f.) Ettingsh. (known as mistletoe) is a perennial, climbing woody parasitic plant of the family Loranthaceae. It is indigenous to tropical regions especially in India, Srilanka, Thailand, China, Australia, Bangladesh, Malaysia, and Myanmar. In India, it is widely distributed and is commonly known as 'bandaa' and 'bandhulu'. The

medicinal properties of *D. falcata* are greatly influenced by the host plant. For example, when grown on *Calotropis gigantea* (L.) W.T. Alton (Asclepiadaceae), the parasitic plant is considered useful for improving cognitive function [6]. However, when it is grown on *Tamarindus indicus* L. (Fabaceae), it is used to treat impotence and on *Shorea robusta* Gaertn. f. (Dipterocarpaceae), it is used to treat paralysis by the tribes of the Bihar state of India [6]. In addition to its medicinal values, the fruits taste sweet and are consumed as a food [6]. The entire plant is used extensively in traditional medicine as an aphrodisiac, astringent, narcotic, diuretic, and for the treatment of pulmonary tuberculosis, asthma, menstrual disorders, swellings, wounds, ulcers, strangury, renal and vesical calculi [7]. The various uses of this plant were studied in different ethnic groups of Nepal. The leaf paste is reported to be used for skin diseases and is taken in abortion while its paste along with *Urtica doica* L. (Urticaceae) is used to treat bone fractures [8]. Bark juice/decoction is used in menstrual troubles and asthma while its paste is applied on boils, setting dislocated bones and extracting pus. The fruit is taken as flavour, astringent, narcotic and for curing wounds while its paste is applied on fractures for setting bones. The decoction of the whole plant is used by the Kokru tribe in Maharashtra to treat joint pain [9], and the Valaiyan community in Tamil Nadu uses leaf juice for relief from chest pain [10]. Nair and Krishnakumary (1989) isolated and reported different flavonoids, viz. quercetin, kaempferol, rutin, quercetrin etc., from *D. falcata* growing on six different host plants [11]. Three new triterpenes, 3 β -acetoxy-1 β -(2-hydroxy-2-propoxy)-11 α -hydroxyolean-12-ene, 3 β -acetoxy-11 α -ethoxy-1 β -hydroxy-olean-12-ene and 3 β -acetoxy-1 β -hydroxy-11 α -methoxyolean-12-ene were isolated along with nine known compounds, 3 β -acetoxy-1 β , 11 α -dihydroxy-

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olean-12-ene, 3 β -acetoxy-1 β ,11 α -dihydroxy-urs-12-ene, 3 β -acetoxy-urs-12-ene-11-one, 3 β -acetoxy-lup-20(29)-ene, 30-nor-lup-3 β -acetoxy-20-one, (20S)-3 β -acetoxy-lupan-29-oic acid, kaempferol-3-O- α -L-rhamnopyranoside, quercetin-3-O- α -L-rhamnopyranoside, and gallic acid from the methanolic extract of the fruits of *D. falcata* growing on *Shorea robusta* [12].

Pattanayak and Priyashree (2008) evaluated the hepatoprotective effect of ethanol and aqueous extracts of leaves of *D. falcata* using carbon tetrachloride induced liver damage in wistar albino rats and suggested the potential hepatoprotective activity of this plant [13]. Gupta and Kachhawa (2007) reported the potential contraceptive efficacy of methanolic extract of *D. falcata* stem in male albino rats [14]. Aleykutty et al. (1993) reported that the aqueous and alcoholic extract of *D. falcata* significantly increased the volume of urine and excretion of sodium, potassium and chloride ions and also caused a significant reduction in the weight of magnesium ammonium phosphate stones, thus indicating diuretic and antilithiatic activity [15]. The aqueous extract of leaves of *D. falcata* growing on *Shorea robusta* for the antitumor activity on 7, 12-Dimethylbenz[a]anthracene (DMBA) induced rat mammary tumor model and revealed 24.934% reduction in the tumor size at the dose level of 400 mg/kg, which was less than that of standard drug tamoxifen which exhibited reduction of 62.865% thus suggesting *D. falcata* as a source for anticancer drugs [16]. In the present study we evaluated antitumor activity of *D. falcata* against Ehrlich Ascites Carcinoma (EAC) in mice.

MATERIALS AND METHODS

Plant Collection and Extraction

Dendrophthoe falcata was collected from in and around Manipal, Karnataka, India, from mango (*Mangifera indica* L., Anacardiaceae) and jackfruit (*Artocarpus heterophyllus* Lam., Moraceae) as host plant in August 2009 and identified by Dr. Gopalakrishna Bhat, Taxonomist, Poorna Prajna College, Udipi, Karnataka. A voucher specimen (PP 564) has been deposited in the Department of Pharmacognosy, Manipal College of Pharmaceutical Sciences, Manipal University (Manipal, India). The shade dried, stems (500 g) of the plant were coarsely powdered, and then extracted by ethanol using soxhlet extraction apparatus for ethanolic extract and by a mixture of chloroform and water (1:99) using maceration method for aqueous extract. The extracts were concentrated to remove the solvent completely under reduced pressure and stored *in vacuo* till use. The percentage yield of ethanolic and aqueous extracts were calculated and found to be 9.93% w/w and 12.8 % w/w, respectively.

Chemicals

Sodium chloride, propylene glycol, trypan blue, methyl violet, sodium sulphate, methylene blue (Merck Limited, Mumbai, India), cisplatin (Sigma Aldrich, USA). All other chemicals and reagents used were of pure analytical grade.

Animals

Male Swiss albino mice weighing 22-28 g were used in the experiment. They were obtained from Manipal Central animal house and were acclimatized to the experimental

room having temperature 23 \pm 2 $^{\circ}$ C, controlled humidity conditions, and 12-h light - dark cycle. Animals were caged in poly acrylic cages (38 \times 23 \times 10 cm) with maximum of four animals per cage. The mice were fed with standard food pellets and water *ad libitum*. Before commencement of the experiment the mice were acclimatized to laboratory conditions for 7 days. All procedures described were reviewed and approved by the University Animal Ethical Committee and study was conducted after obtaining ethical committee clearance from the Institutional Animal Ethics Committee of KMC, Manipal (IAEC/KMC/75/2009-2010).

High-Performance Thin-Layer Chromatography (HPTLC)

Qualitative densitometric high-performance thin-layer chromatography (HPTLC) analysis was performed to develop the characteristic fingerprint profile for the methanolic extract of stem of *D. falcata*.

Preparation of Quercetin Standard Solution:

A stock solution of standard quercetin (1 mg/mL) was prepared by transferring 5 mg of quercetin (accurately weighed) into a 5 mL volumetric flask, dissolving in 2 mL methanol. It was then sonicated for 10 min and the final volume of the solutions was made up to 5 mL with methanol to get stock solutions containing 1 mg/mL.

Preparation of Sample Solution:

Accurately weighed 100 mg of dried methanolic extract of *D. falcata* was transferred to a 10 mL volumetric flask dissolving in 5 mL of methanol. It was then sonicated for 10 min and the contents of the flask were filtered through Whatman No. 1 paper (Merck, Mumbai, India). The final volume of the solution was made up to 10 mL with methanol to get stock solution of 10 mg/mL.

Instrumentation and Chromatographic Conditions:

HPTLC was performed on 20 cm \times 10 cm aluminum backed plates coated with silica gel 60F₂₅₄ (Merck, Mumbai, India). Standard solution of quercetin and sample solution were applied to the plates as bands on the same chromatographic plate by use of a Camag (Muttentz, Switzerland) Linomat V sample applicator equipped with a 100 μ L Hamilton (USA) syringe. Ascending development was performed at room temperature (28 \pm 2 $^{\circ}$ C), with Ethyl acetate: Formic acid: Glacial acetic acid: Water (100:11:11:26 v/v/v/v) as mobile phase, in a Camag glass twin-trough chamber previously saturated with mobile phase vapour for 20 min. After development, the plates were dried and then scanned at 366 nm with a Camag TLC Scanner-3.

Acute Toxicity Study

Acute toxicity study was carried out for ethanolic and aqueous extracts of *D. falcata* according to the method described by Litchfield and Wilcoxon, 1949 using male Swiss albino mice orally [17]. The LD₅₀ values were found to be 4 g/kg body weight respectively.

Transplantation of Tumor

The EAC induced mice were originally obtained from the animal house in Manipal. The EAC cells propagated for 12-

14 days were used in experiment. The ascitic fluid from mice was drawn using an 18 gauge needle into sterile syringe and was tested for microbial contamination. Tumor viability was determined by Trypan blue exclusion test and cells were counted using Haemocytometer. The ascitic fluid was suitably diluted in normal saline to get a concentration of 10×10^6 cells/mL of tumor cell suspension. From this stock suspension 0.25 mL (2.5×10^6 cells/mice) was injected intraperitoneally (i.p.) to obtain ascitic tumor.

Treatment Schedule

140 Swiss albino mice were used in the experiment, which were divided into seven groups (n=20), they were fed with food and water *ad libitum*. All the animals in each group received EAC Cells (2.5×10^6 cells/mouse i.p.) except Group-I. This was taken as day '0'. Group-I animals served as normal saline control (5 mL/kg i.p.) and group-II animals served as EAC control without any drugs. 24 h after EAC transplantation, groups-III and IV animals received ethanolic extract of *D. falcata* at a dose of 200 and 400 mg/kg orally, groups-V and VI animals received aqueous extract at a dose of 200 and 400 mg/kg orally for 13 alternative days, respectively. Group-VII animals received reference drug cisplatin (3.5 mg/kg i.p.) on the first day [18]. After 24 h of the last dose and then 18 h of fasting, ten animals of each group were sacrificed by cervical dislocation to measure tumor volume, tumor weight, cell viability and haematological parameters and the rest of the animals were kept with food and water *ad libitum* to check percentage increase in life span (% ILS) of the tumor host.

Evaluation of the Antitumor Activity

The antitumor activity of the ethanolic and aqueous extracts of *D. falcata* was measured in EAC animals using the following parameters: (1) Tumor volume: The ascitic fluid was collected from the peritoneal cavity of Swiss albino mice and the volume was measured by using a graduated centrifuge tube. (2) Tumor weight: The tumor weight was measured by taking the weight of the mice before and after the collection of the ascitic fluid from peritoneal cavity. (3) Percentage increase in life span: The effect of ethanolic and aqueous extracts of *D. falcata* on percentage increases in life span (% ILS) of the animals was calculated on the basis of mortality of the experimental mice [19]. %ILS = Mean survival time (MST) of treated group- MST of control group / MST of control group \times 100, MST= Mean survival time, Mean survival time* = [First Death + Last Death]/2, *Time denoted by days. (4) Tumor cell count: The ascitic fluid withdrawn from the peritoneal cavity of the mice was taken with a WBC pipette and diluted 100 times with normal saline. A drop of the diluted cell suspension was placed on the Neubauer's counting chamber and the numbers of cells in the 64 squares were counted. (5) Viable/ nonviable tumor cell count by trypan blue assay: The viability and nonviability of the cells were checked by trypan blue assay. The cells were stained with trypan blue (0.4 % in normal saline) dye. Upon staining, the viable cells did not take the stain while the non viable cells were stained blue. Cell count = Number of cells \times dilution factor / Area \times thickness of liquid film. (6) Blood parameters: At the end of the experimental period, the next day after an overnight fasting, blood was

withdrawn from the retro-orbital plexus and used for the estimation of haemoglobin (Hb) content, red blood cell (RBC) count and white blood cell (WBC) count by using an automatic analyzer (ERMA INC Tokyo, - PEC-21 OVET).

Statistical Analysis

Statistical significance (*p*) was calculated by one-way ANOVA between the treated groups and the EAC control group followed by Dunnett's post hoc test of significance where, $p < 0.05$, $p < 0.01$ and $p < 0.0001$ considered being significant, very significant and highly significant, respectively. All data are expressed as mean \pm S.E.M (n=10 mice per group).

RESULTS

The ethanolic and aqueous extracts of *D. falcata* at the doses of 200 and 400 mg/kg body weight, when administered orally, elevated the MST and life span of EAC tumor bearing mice (ILS) (Table 1 and 2). The haematological profile such as RBC count and haemoglobin content increased (Fig. 1A and 1C) but the WBC count was decreased as compared to that of EAC control (Fig. 1B). The extracts showed increased nonviable cell count (Fig. 2B) and decreased viable cell count (Fig. 2A), tumor volume (Fig. 3) and tumor weight (Table 3) when compared to that of EAC control mice.

Co-chromatography of *D. falcata* extract along with quercetin as a marker revealed the presence of quercetin in the extract, with an Rf value of 0.33 (Fig. 4, 5 and 6).

Table 1. Effect of Various Extracts on Mean Survival Time (MST) in EAC Inoculated Mice (Mean \pm S.E.M)

Treatment	MST (Days)
Control	16.16
Cisplatin (3.5 mg/kg)	40.66
Ethanolic (200 mg/kg)	21.83
Ethanolic (400 mg/kg)	24.00
Aqueous (200 mg/kg)	26.80
Aqueous (400 mg/kg)	30.16

Table 2. Effect of Various Extracts on Percent Increase in Life Span

Treatment	%ILS
Control	-
Cisplatin (3.5 mg/kg)	151.60
Ethanolic (200 mg/kg)	35.08
Ethanolic (400 mg/kg)	48.51
Aqueous (200 mg/kg)	65.84
Aqueous (400 mg/kg)	86.63

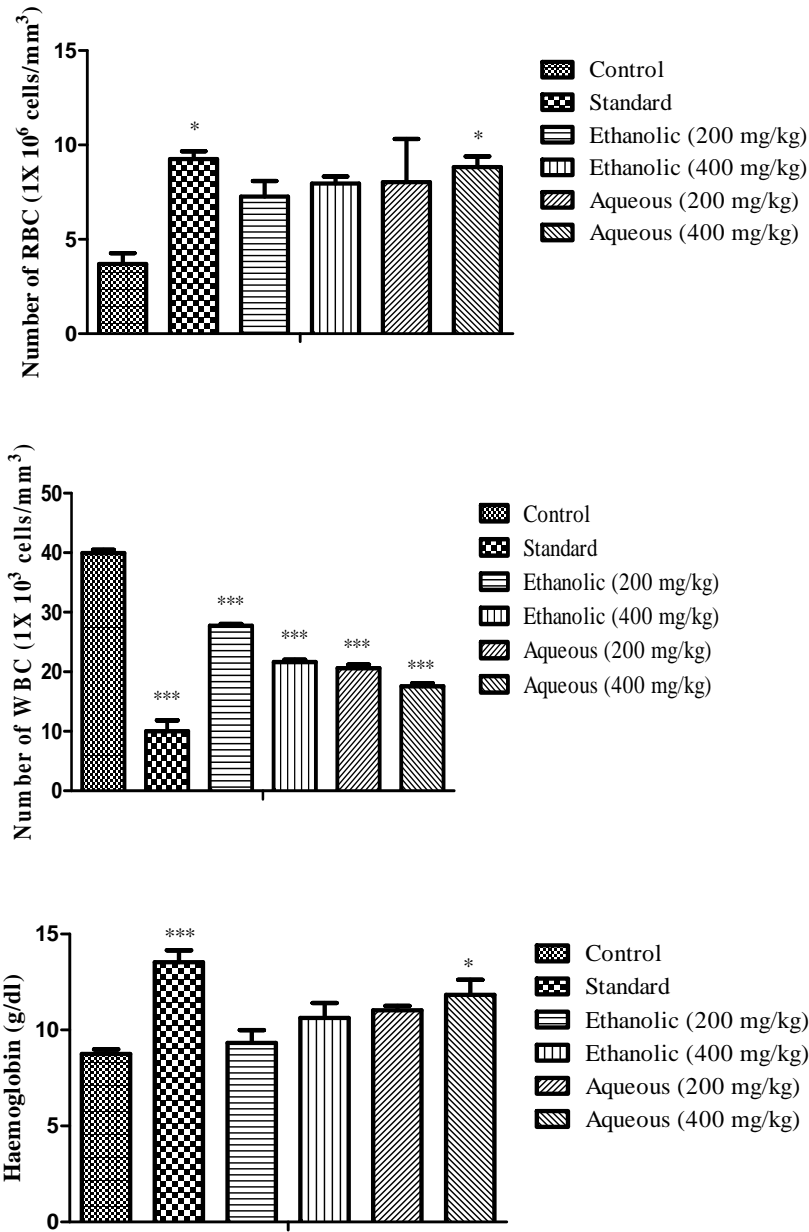


Fig. (1). Effects of various extracts on blood parameters. (A) RBC count; (B) WBC count; (C) Haemoglobin level. Each point represents the mean \pm S.E.M (n=10 mice per groups). *p<0.05, **p<0.01 and ***p<0.0001 when treated is compared with control.

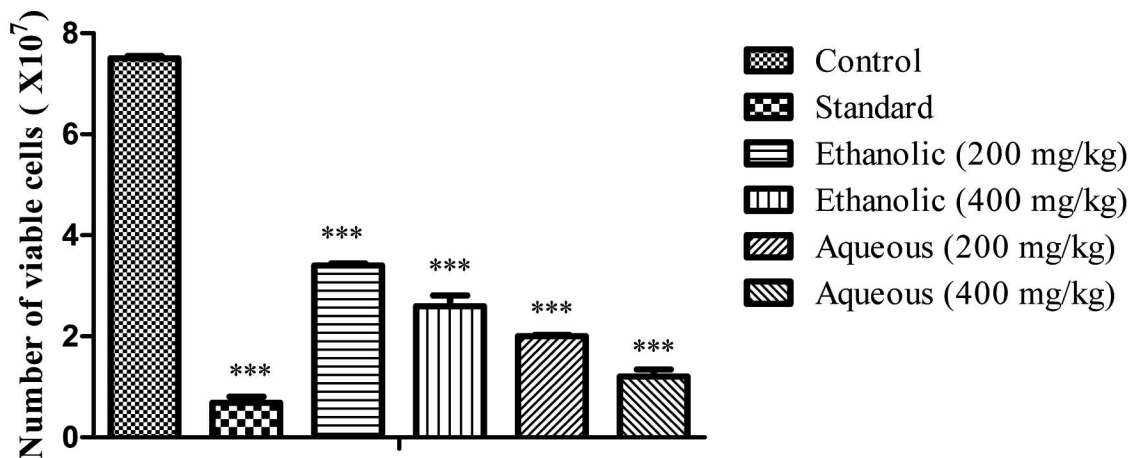


Fig. (2). Contd....

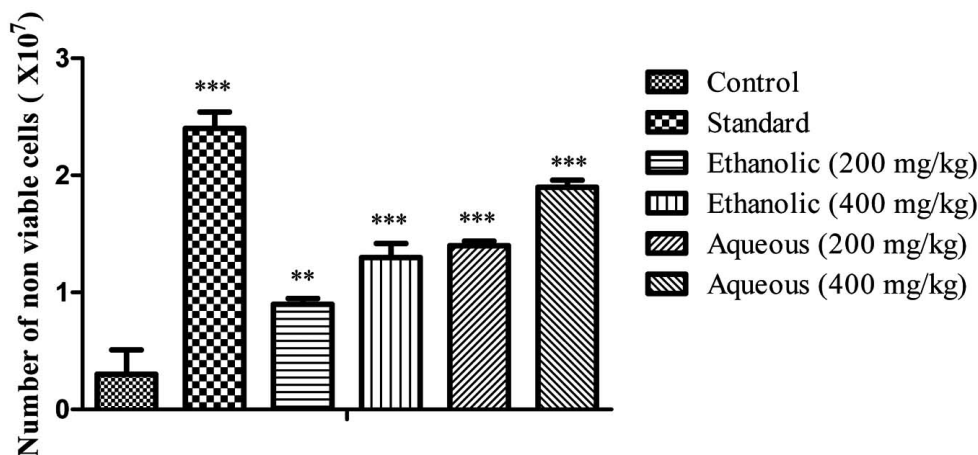


Fig. (2). Effects of various extracts on viability of cells. (A) Viable cells ; (B) Non-viable cells. Each point represents the mean ± S.E.M (n=10 mice per groups). *p<0.05, **p<0.01 and ***p<0.0001 when treated is compared with control.

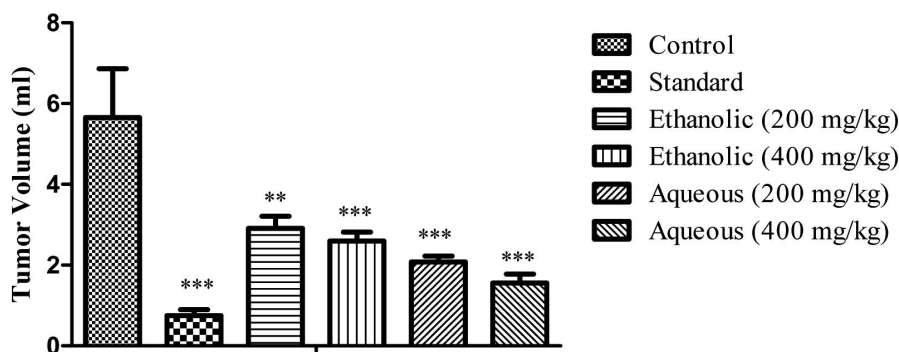


Fig. (3). Effects of various extracts on tumor volume.

Table 3. Effect of Various Extracts on Tumor Weight

Treatment	Tumor Weight (g)
Control	6.79 ± 0.132
Cisplatin (3.5 mg/kg)	0.90 ± 0.242**
Ethanolic (200 mg/kg)	3.49 ± 1.385*
Ethanolic (400 mg/kg)	2.72 ± 0.740**
Aqueous (200 mg/kg)	2.19 ± 0.194**
Aqueous (400 mg/kg)	1.96 ± 0.920***

Each point represents the mean ± S.E.M (n=10 mice per groups). *p<0.05, **p<0.01 and ***p<0.0001 when treated is compared with control.

DISCUSSION AND CONCLUSION

The present study revealed that both ethanolic and aqueous extracts of *D. falcata* at the dose of 200 and 400 mg/kg significantly increased the life span of the mice when compared to the EAC control. The steadfast criteria for judging the potency of any anticancer drug are prolongation of life span and decrease in WBC [20]. The ethanolic and aqueous extracts delayed the cell division, thereby suggesting the reduction in EAC volume and increased survival time in mice.

Aqueous extract at both the doses (200 and 400 mg/kg) and ethanolic extract (400 mg/kg) significantly improved the

MST in tumor bearing mice. No visible sign of toxicity and changes in vital functions were observed in any of treated animals. The prolongation of life span is a reliable criterion for judging efficacy of anticancer drugs [21] and the extracts of this plant were able to meet this criterion. Myelosuppression and anemia (reduced haemoglobin) have been frequently observed in ascites carcinoma [22, 23]. Anemia encountered in ascites carcinoma mainly due to iron deficiency, either by haemolytic or myelopathic conditions which finally lead to reduced RBC number [24]. In this study, elevated WBC count, reduced haemoglobin and RBC count were observed in EAC control mice, and the oral administration of *D. falcata* restored haemoglobin content and maintained normal values of RBC and WBC, thus supporting its haematopoietic protecting activity without inducing myelotoxicity, the most common side effects of cancer chemotherapy.

Preliminary phytochemical study indicated the presence of flavonoid, phytosterols and tannins in ethanolic extract and phytosterols, tannins and carbohydrates in aqueous extract [25]. Flavonoids such as quercetin, kaemferol and their glycosides have been shown to possess antimutagenic and antimalignant effect. Furthermore, flavonoids have a chemopreventive role in cancer through their effect on signal transduction in cell proliferation and angiogenesis [26]. The cytotoxicity and anticancer activity of ethanolic extract is probably due to the presence of these flavonoids. Aqueous extracts reported the presence of phytosterols. Phytosterols are able to be incorporated into the cell membrane, alter mem-

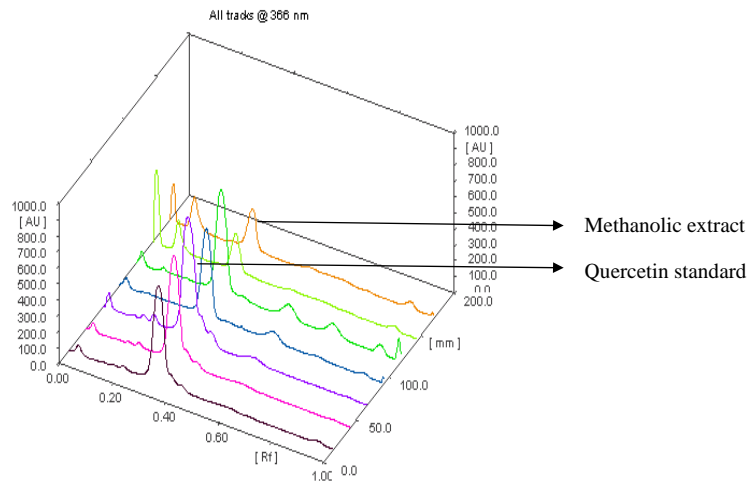


Fig. (4). 3-D chromatogram of Quercetin and *Dendrophthoe falcata* extract scanned at 366 nm.

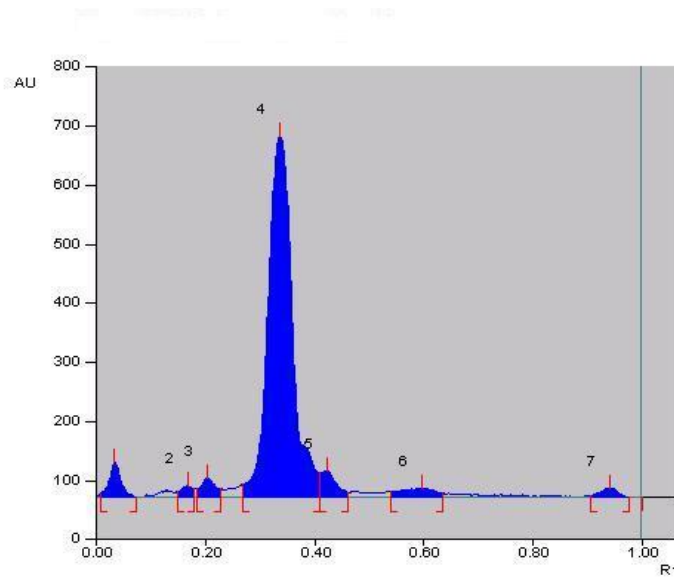


Fig. (5). HPTLC chromatogram of Quercetin.

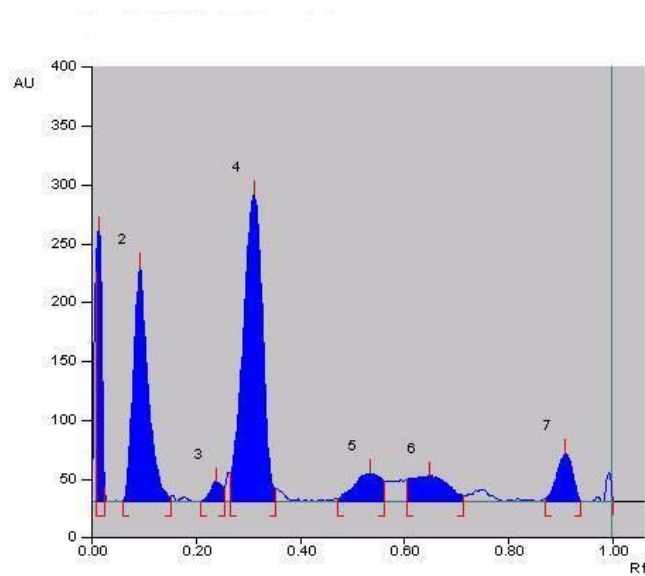


Fig. (6). HPTLC chromatogram of methanolic extract of *Dendrophthoe falcata*.

brane fluidity and the activity of membrane-bound enzymes. They also alter signal transduction in pathways leading to tumor growth and stimulate apoptosis in tumor cell lines. They also have been shown to enhance *in-vitro* human peripheral blood lymphocyte and T-cell proliferation *in vitro*, which suggests a possible stimulation of the immune system function [27]. Oleanolic acid, a triterpenoid is reported to be present in this plant extract [28] and known for its antitumor effect thus suggesting its possible role as antitumor agent. Therefore, further investigation in order to explore the potential of the aqueous extracts of *D. falcata* in tumor treatment may prove to be worthwhile.

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ABBREVIATIONS

DMBA	=	7, 12-Dimethylbenz[a]anthracene
EAC	=	Ehrlich Ascites Carcinoma
HPTLC	=	High-performance thin-layer chromatography
KMC	=	Kasturba Medical College
LD50	=	Lethal dose
MST	=	Mean survival time
RBC	=	Red blood cell
WBC	=	White blood cell
% ILS	=	Percentage increases in life span

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