COMPARATIVE ANTITUMOUR ACTIVITY OF CATHARANTHUS ROSEUS, PLUMBAGO ZEYLANICA PLANT EXTRACTS AND BOVINE URINE ON EHRLICH ASCITES CARCINOMA MOUSE MODEL

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Abstract:
The present study was conducted on the cow urine distillate (CUD) and ethanolic extracts of two plants of medicinal importance for exploring their therapeutic potential using the Ehrlich Ascites Carcinoma (EAC) mouse model. Ethanolic extracts of *Catharanthus roseus* (aerial parts) and *Plumbago zeylanica* (stem) were prepared and the presence of bioactive compounds was determined by spectrophotometric analysis and GC-MS profiling. The antiangiogenic activity was determined using the chicken chorioallantoic membrane (CAM) model and EAC tumour mouse models were used to investigate the antitumour activity of the two extracts and CUD. Polyphenolic flavonoid content was found to be highest in *P. zeylanica* extract. GC MS profiling has also revealed the presence of esters and different sterols in the same. Antioxidant assays revealed maximum free radical scavenging potential in the *C. roseus* extract. The CAM model showed the CUD as a better suppressor of blood vessel growth than the extract of two plants as well as ethanol-treated control eggs. Similarly, CUD has demonstrated significant antitumour activity determined by the reduction of tumour size, volume and weight when compared with the highest doses of both the plant extracts. This study demonstrates that CUD has antiangiogenic and anticancer activities which can be utilized as a remedy against various tumourigenic cancerous diseases.

Keywords: Antitumour activity, GC-MS, Antioxidant activity, Antiangiogenic activity, EAC Model, Cow urine diluent, Plant extracts, *Catharanthus roseus, Plumbago zeylanica*.

Abbreviations:
CAM, Chicken chorioallantoic membrane, EACST, Ehrlich Ascites Carcinoma Solid Tumor; EAC, Ehrlich Ascites Cells; 5-Flu, 5-Fluorouracil; FRAP, Ferric Reducing Antioxidant Power; GC-MS, Gas Chromatography coupled with mass spectrometry; TPTZ, 2,4,6-Tripyridyl-S-triazine.

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INTRODUCTION:

The present study was taken up to explore the therapeutic potential of two plants, *C. roseus* and *P. zeylanica*, known to have medicinal properties in addition to CUD, which has been reported in the texts/literature of Indian traditional medicine viz. Ayurveda possesses therapeutic properties [1]. Ayurveda signifies the science of life. Charaka Samhita and Sushruta Samhita are two major texts of Ayurveda. Sushruta Samhita deals primarily with different principles and theory of surgery, while Charaka Samhita is a book of internal medicine. Duration between the writing of these two texts is considered to be the period when basic concepts and principles were developed and enunciated evolving different formulations [1]. Indian healthcare is pluralistic and mainly comprises of Ayurveda, Siddha and Unani medicines, and are still popular among common folks for curing of diverse kind of chronic diseases [2]. Plants are integral part of Indian traditional medicine and they remain as the most important providers and easily available source of medicines to this day as they have various bioactive compounds which are utilised for curing of diverse types of diseases. About 2000 plants are mentioned in the literature of traditional alternative systems of medical practice and the vast majority of the global population still relies on drugs from plants [3][4].

The medicinal attributes of plants are found in different parts that are used as alterative, tonic, diuretic, blood purifier and antiphlogistic agents [3]. Secondary metabolites of the plants like polyphenolic flavonoids, condensed tannins, and alkaloids have demonstrated antioxidant and antimicrobial potential. Free radicals are formed as a consequence of various metabolic processes in the human body with respect to what is inhaled or ingested. Free radicals oxidize biomolecules like DNA, RNA, proteins, and cell membrane constituent lipids. These free radicals initiate and trigger cell damaging Fenton reactions triggering inflammatory immune response resulting in different diseases and conditions [5]. Numerous reports have demonstrated that dietary supplements of antioxidants guard our body against toxicity of free radicals. Plant extracts also possess enzymes like superoxide dismutase, glutathione S-transferase, catalase that are beneficial in scavenging of free radicals [6].

C. roseus (*Vinca rosea)* which is also known as 'Sadabahar' in Hindi and Periwinkle in English belongs to the family of Apocynaceae. It is native of Indian Ocean Island of Madagascar. This plant is an evergreen herb that attains the height of 1 m [7]. Various classes of phytochemicals have been reported in *C. roseus*, but active ingredients are differentially present in roots, leaves and stem. Like vinblastine, vincristine, and other similar alkaloids are present in aerial parts whereas ajmalicine, reserpine, catharanthine etc abound in roots and lower part of plant stem. Anticancer activity was found to be associated with the extracts of stem and leaves that have revealed the presence of anticancer compounds like vinblastine and vincristine. Antioxidant potential of ethanolic extracts of pink and white flowers has also been demonstrated [8]. *P. zeylanica* has been mentioned in Ayurveda as 'Chitrak' belongs to the super order of Caryophyllales and is comprised of two families, Plumbaginaceae and Limoniaceae [9]. It is a perennial soft woody shrub that grows especially in humid and moist conditions of Bengal, Uttar Pradesh, and southern peninsular India & Sri Lanka. Stems of *P. zeylanica* have shown the presence of phytochemicals such as plumbagin, zeylanone, isozeylanone, and a host of sterols, dihydroflavinol-plumbaginol, whereas flowers contain plumbagin, zeylanone, and glucose in them. Antimicrobial potential was detected in the extracts of *P. zeylanica* against *Salmonella, E. coli*, *Klebsiella* and other Gram negative bacteria [10]. Root extracts of *P. zeylanica* were found to have stimulatory action on central nervous system and dopaminergic activity in rats leading to improvement in their locomotor behaviour [11].

The main aim of the current study was to explore and demonstrate the therapeutic potential of plant extracts of the two plants that have reported medicinal value in the traditional as well modern literature of medicinal plants. Another objective was to explore the therapeutic value of cow urine that has found mention in the classical texts of India to possess curative properties for treating diverse health conditions. CUD was used to determine its composition using different techniques and tested for its antitumour activity along with the ethanolic extracts of the two plants, *C. roseus* and *P. zeylanica* using CAM and Ehrlich ascites Carcinoma (EAC) mouse model.

Materials and Methods

Chemicals: All the chemicals used in this study were of analytical grade. DPPH, TPTZ (2,4,6-tripyridyl-S-triazine), vincristine, plumbgin were procured from Sigma Chemical Co., USA and ascorbic acid, quercitin and Folin-Ciocalteu reagent were purchased from Fischer Scientific.

Collection of plant material and CUD:
Aerial parts (entire plant except roots i.e. stem, leaves and flowers was taken) of *C. roseus* and stem of *P. zeylanica* were collected from different locations of Khari Baoli, Kucha Challan, Chandni Chowk in Delhi, India. Both plants were identified and authenticated by Dr. Sunita Garg, (Emeritus Scientist, CSIR-NISCAIR) with Ref. No.- NISCAIR/RHMD/Consult/2020/3655-56-3, NISCAIR/RHMD/Consult/2020/3655-56-2.
Cow urine was collected from Mathura Gaoshala. Collected urine was distilled at 100℃ in a temperature regulated distillation unit. Fraction of the single distilled cow urine was acidified to pH < 2.0 using 85% orthophosphoric acid and was distilled again at 100℃ to remove ammonia present in the distillate. The collected distillate was hence forth known as cow urine distillate (CUD).

Culturing of EAC cells:
The Ehrlich Ascites Cells (EAC CCL-77 cells) were procured from National Centre of Cell Sciences, Pune, India. The frozen stock of cells was thawed and propagated as per supplier's guidelines [13].

Preparation of ethanolic extracts of *C. roseus* and *P. zeylanica:*
The collected plant parts were washed under running water to remove dirt and finally with distilled water to remove any residual soil particles and dried in shaded area. After drying, plant parts were grounded to fine powder using a grinder and used throughout the study. Ethanolic extract was prepared from 50 g of plant powder processed in Soxhlet apparatus and dried as described previously [13]. Extracts were dissolved in 50 % of ethanol and stored at 4^{o}C for further use.

Physicochemical evaluation of extract:
Parameters like total ash value, acid insoluble ash value, moisture content and alcoholic extractive value were determined and calculated according to the guidelines of World Health Organisation [12].

Quantification of polyphenolic flavonoids:
Total flavonoid content present in extract was determined spectrophotometrically by aluminum chloride method. Calibration curve was generated for determining of flavonoid content of the extract. Flavonoid concentration of extracts was calculated and expressed in mg Quercetin Equivalent g^{-1} of extract [13][14]. Standard calibration curve was generated for determining of polyphenolic content of the extracts and concentrations were expressed in mg Quercetin Equivalent of phenol g^{-1} of extract using Folin-Ciocalteau method [13].

GC-MS profiling:
Phytochemical composition was determined by employing gas chromatography coupled with mass spectrometry (GC-MS) to detect and identify various bioactive compounds present in the extracts. Samples were submitted to Advance Research & Analytical Services, Ghaziabad, Uttar Pradesh, India, for performing GC-MS. Samples were analysed on Thermo Scientific TSQ Series 9000 Triple Quadrupole Mass spectrometer and GC-MS report was generated.

Antioxidant assay:
Free radical scavenging assay using DPPH and FRAP assay were performed to determine antioxidant potential of the two ethanolic extracts of *C. roseus and P. zeylanica.*

DPPH Free Radical Scavenging: Free radical scavenging which is directly correlated with the antioxidant potential of the extracts was determined by colorimetric estimation at 520 nm. Diminishing absorbance of blue colour which is indicative of free radical scavenging activity or hydrogen ion donating ability of the extracts to DPPH (1, 1-diphenyl-2 picrylhydrazyl). DPPH scavenging assay was performed by following the standard protocol. Results were expressed as percentage of inhibition of DPPH which was calculated by applying the formula given below. The IC50 values of the extracts were plotted from standard calibration curve [15].

Percentage of inhibition = [(absorbance of control-absorbance of reaction mixture)/ absorbance of control] X 100 *FRAP Assay:* Ferric reducing antioxidant power assay utilizes the ability of antioxidants to donate electrons and reduce ferric (Fe^{3+}) to ferrous (Fe^{2+}) ions which are complexed to TPTZ yielding a Prussian blue Fe^{2+}–TPTZ complex which has the maximum absorption value of 593 nm. Antioxidant potential was determined by FRAP assay and the ability of the sample to reduce ferric ions was calculated from the linear calibration curve and expressed as mM Fe(II)/ g dry weight of extract as per the standardized protocol [16].

Antiangiogenic activity on CAM model:
Chicken chorioallantoic membrane (CAM) model was set up to evaluate *in vivo* antiangiogenic activity of the two extracts and CUD using standard laboratory protocol [17]. 50µl of 25mg syringe

filtered extracts and 50µl of CUD containing discs were implanted on 8-day old eggs by carving small window on the egg shell maintaining sterile conditions. Window was sealed using a laboratory film and eggs were continued to incubate for 72 hrs. After 72 hrs of incubation windows seal was removed to reopen the window to check antiangiogenic activity and determine the decrease in the number of vessels and their branching compared to the control group (Fig.3).

Anticancer activity of plant extracts and CUD: Animal studies

Five to six weeks old, female Swiss albino mice, weighing 20-30 g were used in the present study. The animals were kept in standard polycarbonate cages and fed on standard pellet diet (golden feed) under standard laboratory conditions (26 ± 1 ◦ C, 12-h light: 12-h dark cycle) with food and water ad-libitum. The animals were procured from the institute's animal house and kept in laboratory to acclimatize for 7 days before starting of the experiment. Protocols for the present study were approved by the Institutional Animal Ethics Committee (CPCSEA/IAEC/AIP/2020/08/04/29).

Ehrlich Ascites Carcinoma model

Transplantation of tumor cells and induction of EAC in Swiss albino mice

EAC CCL-77 cells were cultured and propagated. 5×10^6 CCL-77 cells/mouse were administered to induce EAC in mice via peritoneal cavity. After 15 days of inoculation of EAC CC-77 cells, mice were selected for harvesting EAC cells for passaging into new set of mice for further study. Peritoneal cavity was punctured to collect the fluid containing EAC cells from the peritoneal cavity of mice which were then centrifuged and washed with saline. The cells were injected intraperitoneallly (2×10^6 cells/ mouse) in fresh mice to induce ascitic tumor formation every 10 days and it was used for further *in vivo* studies [18].

The injected tumor cells undergo free multiplication and proliferation within the peritoneal cavity of experimental mice. The cells are collected from peritoneal cavity of mice using fresh sterile disposable syringe and diluted with PBS [19]. Animals (n=6) were divided into 8 groups.

Group 1: Diseased Control (Tumor Induced)
Group 2: Vehicle control (50% ethanol)
Group 3: Received 0.25 ml of CUD
Group 4: Orally received low dose of the extract of aerial parts of *C. roseus* i.e. 50mg/kg body weight
Group 5: Orally received high dose of the extract of aerial parts of *C. roseus* i.e. 500mg/kg body weight
Group 6: Orally received low dose of *P. zeylanica* stem extract i.e. 50mg/ kg body weight per orally
Group 7: Orally received high dose of *P. zeylanica* stem extract i.e. 500mg/ kg body weight
Group 8: Orally received 5-Flu i.e. 10mg/kg body weight

The first group of mice was orally administered with normal saline from day 1 to 13 and marked as the tumor bearing control; while two-seventh groups received oral treatment of different doses of extracts. The group treated with 5-Flu was used as standard drug control and the drug was administered orally. On 14^{th} day of the experiment, ascitic fluid was drained and collected through a measuring tube from the peritoneal cavity of each slaughtered mouse and the volume was noted for the evaluation of tumor growth. It was then centrifuged at 1000 rpm for 5 min and the packed cell volume was determined [20].

2.9.3 Ehrlich Ascites Carcinoma Solid Tumor Model

EAC collected from ascitic fluid harboring 8-10^{th} day old ascitic tumor. A fixed number (2×10^7) of viable cells were injected subcutaneously in right thigh on day zero of the experiment. Next day animals were randomly divided into seven groups. Animals (n=6) were divided into 8 groups as followed with treatment:

Group 1: Diseased Control (Tumor Induced)
Group 2: Vehicle control
Group 3: Received 0.25 ml of CUD
Group 4: Orally received low dose of the extract of aerial parts of *C. roseus* i.e. 50mg/kg body weight
Group 5: Orally received high dose of the extract of aerial parts of *C. roseus* i.e. 500mg/kg body weight
Group 6: Orally received low dose of *P. zeylanica* stem extract i.e. 50mg/ kg body weight
Group 7: Orally received high dose of *P. zeylanica* stem extract i.e. 500mg/ kg body weight
Group 8: Orally received 5-Flu i.e. 10mg/kg body weight,

The first group was orally administered with normal saline from day 1 to 13 and categorized as the tumor bearing control; whereas two-seventh

groups were orally administered with different doses of extracts. 5-Flu was used as standard and administrated orally. All the animals were weighed on the day of inoculation and after the post inoculation period once in 3 days. Inhibition of tumor growth was determined on day 14 by comparing of the average values obtained from the treated groups against tumor bearing control group [18] [20].

Statistical analysis:
Statistical analysis of data was carried out using Microsoft Excel 2010. All the experiments were performed in triplicate; experimental data are expressed as Mean ± SD (n=3).

Results and Discussion
Indian traditional medicine has been in use for long in preventing various diseases and conditions such as Parkinson, Alzheimer's, cancer and various microbial diseases. Extracts from plants have demonstrated antibacterial, antioxidant and anticancer activities. These activities are because of the localization of bioactive compounds in different parts of plants. This study was taken up to explore and elucidate the antitumour potential of the extracts of two plants and CUD, all of which have been extensively reported to harbour therapeutic potential, against Ehrlich ascite carcinomas . Physicochemical parameters of extracts show the yield of extract and ash value in percentage (w/w) (Table 1). Quantification of flavonoid content of the extracts was carried out spectrophometrically; $y = 0.010x$, $r^2 = 0.984$, where x is the concentration and y is the absorbance of quercetin (µg/ml) expressed in mg Quercetin Equivalent/g of extract as shown in (Fig.1 Graph 1). Flavonoid content of the aerial parts of *C. roseus* ethanolic extract was determined as 21.8±0.012 mg QEq/g of extract, against the leaves of *C. roseus* 17.50mg/100g [21]. From calibration curve i.e. $y = 0.015x$ $r^2 = 0.994$ (where x is concentration and y is the absorbance of quercetin (µg/mL)), phenolic content was 127.3±0.261 mg Q Eq/g of extract observed in the aerial part of *C. roseus* (Fig.1 Graph 2), whereas with respect to the previous studies, phenolic content observed to be slightly higher in the leaves i.e. 422.56 mg/ 100g [21]. Flavonoid and phenolic content in the stem of *P. zeylanica* has been revealed as 34±1.47 mg QEq/g of extract and 46.6±0.334 mg Q Eq/ g of extract, respectively as obtained from calibration curve, where as compared to the previous studies flavonoid and phenolic content was less in the methanolic extract of stem [22]. Phytoconstituent composition of aerial parts *C. roseus* and stem of *P. zeylanica* was determined through GC-MS and the profile is depicted in Fig.2. Main constituents identified in the extracts of *C. roseus* were esters whereas with respect to the previous studies 50% methanolic extract of *C. roseus* has revealed the presence of methyl 8,11,14-heptadecatrienoate, 9,12,15-octadecatrienoic acid, 9-octadecynoic acid, pentadecanoic acid, 1,2,3,5-cyclohexanetetrol, methyl ester, muco-inositol and sucrose in pink morphotype, and 5-hydroxymethylfurfural, 9,12,15-octadecatrienoic acid, and phytol is notable for their presence in white morphotypes [23]. Previous studies of stem of *P. zeylanica* have shown three sterol compounds i.e. γ-sitosterol, taraxasterol, and lanosterol [17], where as in the present study compounds other than sterols has been identified as listed in Table 3. Antioxidant potential of the two extracts were determined using DPPH which has shown 50% of inhibition at 135±0.298 µg/ml of ethanolic extract of aerial parts of *C. roseus* and stem of *P. zeylanica* has shown 50% of inhibition at 191±0.732µg/ml as shown in Figure 1. Leaves of *C. roseus* has shown IC50 in range of 08.53-31.67 µg/ml in methanolic extracts based on samples from different regions [24], whereas stem of *P. zeylanica* has shown IC50= 18.06µg/ml in ethanolic extract prepared by cold extraction method [25].

Antioxidant potential in FRAP assay was determined by standard calibration curve generated by $FeSO_4$ using concentrations of 100-1000 µM i.e. $y = 0.001x$, $r^2 = 0.919$ where x is denotes concentration and absorbance is denoted by y which shows the e potential to reduce ferric (III) iron to ferrous (II) iron. Ferric reducing potential in FRAP assay was determined to be 7975.90 ±0.458mM Fe(II)/ g dry wt of extract and 6156±0.947 mM Fe(II)/ g dry wt of extract in the ethanolic extracts of *C. roseus* and *P. zeylanica*, respectively (Fig. 1 Graph. 2).

Antiangiogenic activity of extracts and CUD was determined by evaluation of the amount inhibition of blood vessel formation in chick CAM model. After the exposure of embryos to the extracts and CUD, it was found that both the extracts of *C. roseus* and *P. zeylanica* have shown much less formation of blood vessels as compared to the control eggs. With respect to the results of extracts, CUD has also shown its inhibitory efficacy against blood vessel formation, which signifies the antiangiogenic activity of CUD as shown in Figure 3.

Antitumour activity in EAC and EACST albino mouse was tested to confirm the inhibitory efficacy of the extracts and CUD. After determining of tumour volume and tumour weight it could be inferred from the results that each of the tested samples had significant inhibitory effect on tumour size, weight and volume with the doses that were given to each group. This is a clear indication that the two extracts and CUD are effective in mitigating the tumours generated in the studied carcinoma model. The results obtained are also comparable to 5Flu, the standard reference drug that was used as a control drug to compare the results (Fig.4). CUD was found to be most effective among the tested samples and their tested doses. Previous reports on anticancer activities of medicinal plants using extracts of root nodules of *Premna herbacea* Roxb has also demonstrated effectiveness against EAC cells generated solid tumours. [20].

Tumour initiation and its growth along with angiogenesis are critical indicators of tumour progression. Newly formed and growing cells leading to tumour formation require good nourishment in the form good blood supply. It is to cater to this demand that the process of angiogenesis is initiated and to block tumour growth it is essential to cut the blood supply of a growing tumour. In order to inhibit tumour growth, tumour blocking and antiangiogenic drugs/agents are required. The current study is an attempt to evaluate and understand the bases of the therapeutic and antitumour potential of the ethanolic extracts of *C. roseus* and *P. zeylanica* in addition to CUD. EAC and EACST are one of the widely used experimentally induced cancers to study various aspects of cancer and anticancer drug therapy/discovery. Inoculation of EAC cells results in carcinomatous peritonitis which also produces ascitic fluid rich in neoplastic/cancerous cells [26]. In the current study, this model was used along with the CAM model to study the effects of two plant extracts and CUD. CUD and the ethanolic extracts of *C. roseus* and *P. zeylanica* have significant antioxidant activity as demonstrated by free radical scavenging assays. Also, the data from the flavonoid content assay successfully demonstrate the presence of polyphenolic flavonoids that might be responsible for the reduction of oxidative stress and inflammatory reactions and thus prevent cancerous tumours by blocking angiogenesis and other yet unknown mechanisms.

In many ancient traditional practices around the world in places like India, Egypt, China and the empires in Central and South Americas, Greece, Rome etc, consuming of urine of some animals like cows, camels, and others or self is considered to have a healing effect on many types of diseases. Also, many scientific studies have been carried out to explore the basis of these claims. Some of the studies have performed chemical analyses employing different techniques of FTIR, GC-MS etc and analysed the data thus obtained to explain the therapeutic nature of these excretory fluids. Mammalian urine of ruminants, camels and humans although have bodily waste products but it also possesses numerous compounds of biological and non-biological origin that may have pharmacological value. GC-MS analysis of camel and cow urine has shown to have some common features with differences in concentrations of some of the compounds. Urines of both the animals are rich in enzymes and hormones. It may be due to the presence of these compounds that the healing properties of urine are attributed to [28].

Practitioners of Ayurveda advocate urine therapy as a treatment for various maladies like asthma, arthritis, allergies, acne, cancer, infertility and numerous other conditions. Nautiyal and Dubey identified the various bioactive compounds, using TLC, FTIR and GC-MS, responsible for antioxidant and antibacterial activity in the urine of Badri cow [29]. GC-MS analysis of a fraction showed the presence of 1-heneicosanol as the main compound among 12 identified compounds most of which were fatty alcohols. Fatty alcohols have been demonstrated to possess antioxidant and antimicrobial activity [30]. Fatty alcohols are bacterially derived metabolites present in the rumen of cattle. Comparative analysis of cow and camel urine has shown similarities in composition although varying concentrations of the constituents and presence of long-chain fatty acids have been confirmed [31][32]. Fatty acids along with fatty alcohols and their derivatives exhibit antiproliferative and anticancer activity [33] [34]. These compounds along with the action of certain enzymes might be responsible for synergistic antitumour effects of CUD.

C. roseus is among the most widely studied plants for its medicinal importance and value. It is known to possess more than 100 different types of alkaloids having diverse actions of these alkaloids vinblastine and vincristine are known anticancer agents and were among the first plant-derived drugs to get approved as anticancer trial drugs. Recently, several alkaloids from *C. roseus* have been shown to successfully inhibit cancer cells in

vitro. Vinblastine and vincristine inhibit the growth of cancer cells by binding to the mitotic spindle, thus disrupting the process of cell division and tumour growth. Besides the alkaloids, flavonoids might also play a decisive role in mitigating the effects of cancer and tumour formation [35].

P. zeylanica is a plant of high medicinal value and has found mention in the ancient texts of Ayurveda. It is reported to work against chronic rheumatoid arthritis, skin ailments It also has antiatherogenic, hepatoprotective, cardiotonic and neuroprotective properties [36]. Its anti-inflammatory and analgesic properties have been studied. Plumbgin found in *P. zeylanica* is a naphthaquinone known to inhibit various types of cancers. It inhibits proliferation and survival of eosophageal cancer cells (Esophageal Squamous Cell Carcinoma-ESCC)-one of the most fatal form of cancers. It induces mitotic arrest and potentiates massive apoptosis in cancer cells to exert its effects. At molecular level it inactivates STAT3 and down regulates PLK1 and AKT expression thus, abrogating STAT3-PLK1-AKT signaling. Besides, it shows anticancer activity towards lung, breast, colon, ovarian and prostrate cancers through different mechanisms viz., inhibition of growth and metastasis and antiangiogenesis [37-42].

Angiogenesis is one of the hallmarks of cancerous tumours and is a crucial therapeutic target. Blood vessel formation or the process of angiogenesis is the result of VEGF activity which is an important regulator and promoter of angiogenesis. Inhibitors of angiogenesis act by blocking VEGF receptors especially VEGF-R2. VEGF receptors are characterized as tyrosine kinases and their inhibitors as tyrosine kinase inhibitors (TKI). TKIs not only block angiogenesis but also block cell migration, metastasis and hence prevent epithelial-mesenchymal transition of tumour cells. Many known flavonoids like, quercitin, kempferol, curcumin are TKIs [43-48]. The observed antitumour activity in EAC and EACST models may be due to the presence of various compounds and the diverse types of effects produced as the result of their targeted action on their cellular or molecular targets. These actions may be antiproliferative as a result of cell cycle arrest, apoptosis-induction, modulation of reactive oxygen species by free radical scavenging as antioxidants, and a host of other actions. These effects are the results of the various bioactive compounds flavonoids, fatty acids and their derivatives, various enzymes involved in redox reactions.

The study carried out on ethanolic extracts of aerial parts of *C. roseus* and stem of *P. zeylanica* has revealed the presence of different bioactive compounds determined by GC-MS. Antioxidant potential was found to be high, which might be due to the presence of polyphenolic compounds like flavonoids of the two plants. Significant amount of comparable antiangiogenic activity was observed in the plant extracts and CUD using CAM model. Reduction in tumour size, weight and volume in EAC and EACST mice models demonstrated that CUD has significant antitumour activity along with the two extracts. Conclusively, the current study clearly demonstrates that bioactive compounds present in *C. roseus, P. zeylanica* have antioxidant, antiangiogenic and antitumour activities that can be utilized as herbal remedy for various tumourigenic diseases. Similarly CUD also possesses antiangiogenic and antitumour potential and could be utilized as remedy for conditions that are similar to carcinoma and result in tumour formation.

Further studies may be required to confirm and identify bioactive components having antitumour and anticancer potential in two plant extracts and CUD to further establish the bases of therapeutic value and also to explore their potential targets. This study further opens new vistas to explore and identify the potential bioactive constituents present in the two extracts and CUD.

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Conflict of Interest

We wish to confirm that there are no known conflicts of interest associated with this publication.

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Table 1

S/No	*Test Parameters (in % w/w)*	*Catharanthus roseus*	*Plumbago zeylanica*
1	*Total Ash*	*10.04*	*8.07*
2	*Acid Insoluble Ash*	*0.40*	*0.34*
3	*Water soluble ash*	*1.23*	*1.03*
4	*Loss on Drying*	*5.04*	*4.06*
5	*Ethanolic Extract*	*39.52*	*28.44*

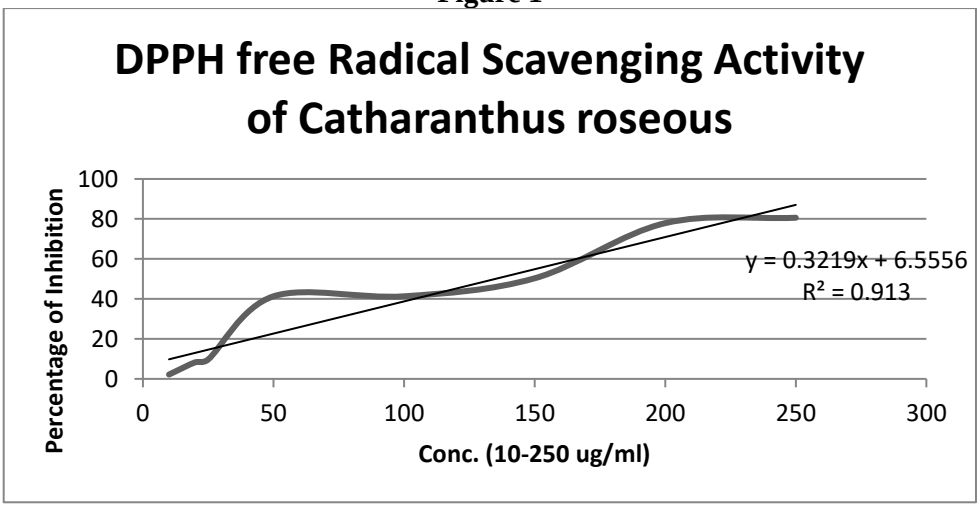
Table 2

S. No	RT	Possible Compound	Formula	MW
1	6.28	4,5'-Dibenzamido-1,1'-iminodianthraquinone	$C_{42}H_{25}N_3O_6$	667.7 g/mol
2	11.24	2,2-Dimethyl-propyl `	$C_{42}H_{70}F_6N_8O_{12}$	993 g/mol
3	18.11	Tetradecanoic acid, 10,13-dimethyl-, methyl ester	$C_{17}H_{34}O_2$	270.5 g/mol
4	31.13	Phthalic acid, di(2-propylpentyl) ester	$C_{24}H_{38}O_4$	390.6 g/mol

Table 3

S. No	RT	Possible Compound	Formula	MW
1	5.21	Spiro[3.4]octan-1-one, 5-methyl-, cis-	$C_9H_{14}O$	138.21g/mol
2	7.38	Butane, 2-cyclopropyl-	C_7H_{14}	98.19 g/mol
3	8.03	1,6:3,4-Dianhydro-2-deoxy-beta-d-ribo-hexopyranose	$C_6H_8O_3$	128.13 g/mol
4	9.31	2-Dodecanol	$C_{12}H_{26}O$	186.33 g/mol
5	10.6	3-Ethyl-4-nonanol	$C_{11}H_{24}O$	172.31 g/mol
6	12.72	1-Hexyl-2-nitrocyclohexane	$C_{12}H_{23}NO_2$	213.32g/mol
8	30.24	Cyclobutanone, 2-tetradecyl-	$C_{18}H_{34}O$	266.5 g/mol
9	31.13	2-(1-amino-2-1h-imidazol-1-ylethyl)-4-amino-6-dimethylamino-s-triazine	$C_{10}H_{16}N_8$	248.29 g/mol
10	37.15	Cholest-5-en-3-ol (3á)-, trifluoroacetate	$C_{43}H_{71}F_6N_5O_7$	884 g/mol
11	40.38	5,10-Pentadecadiyne, 1-chloro-	$C_{15}H_{23}Cl$	238.79 g/mol

Figure 1



(a)

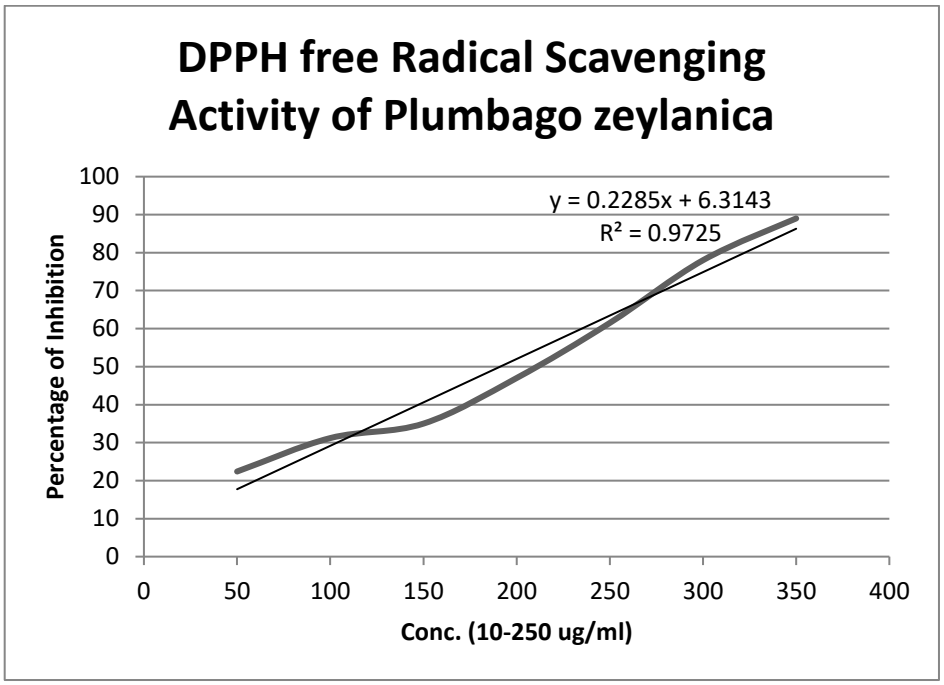


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Figure 2

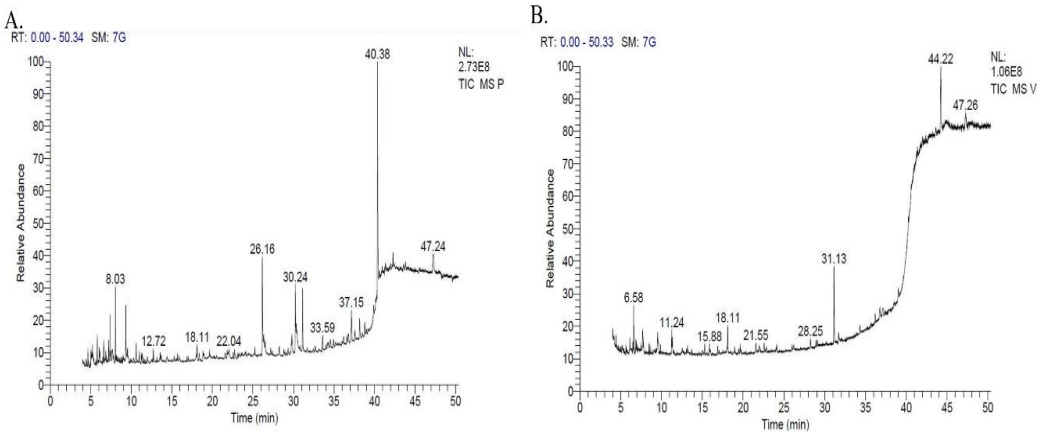
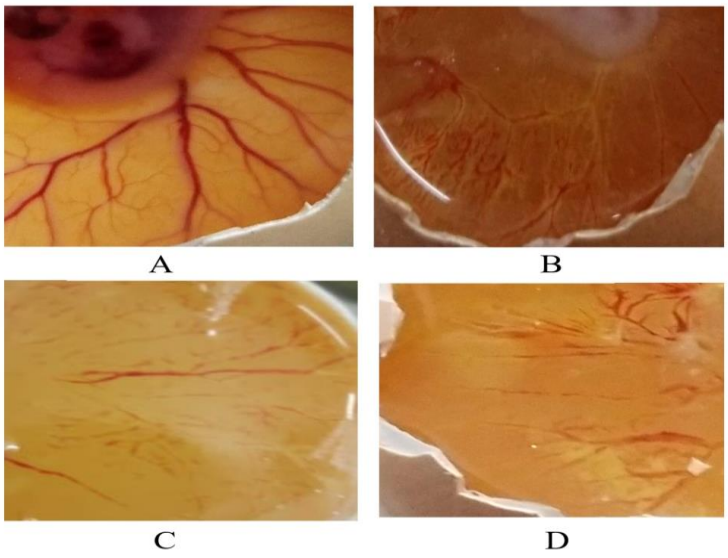


Figure 3



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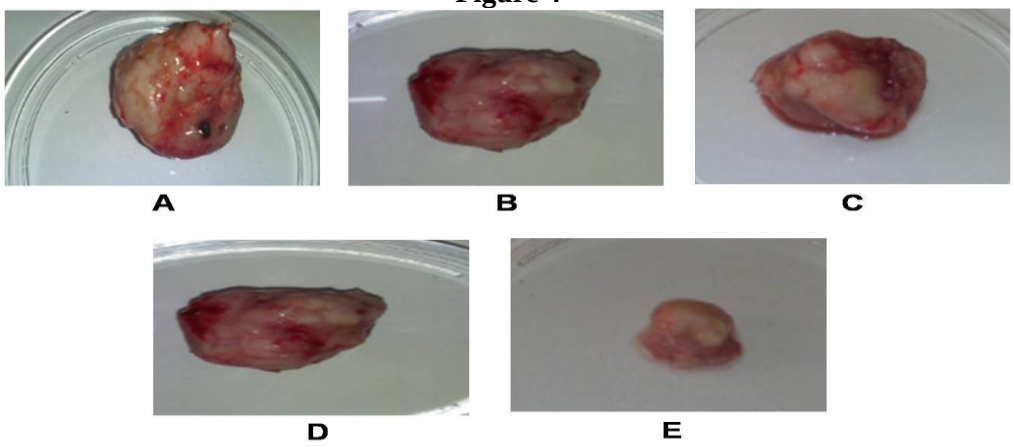

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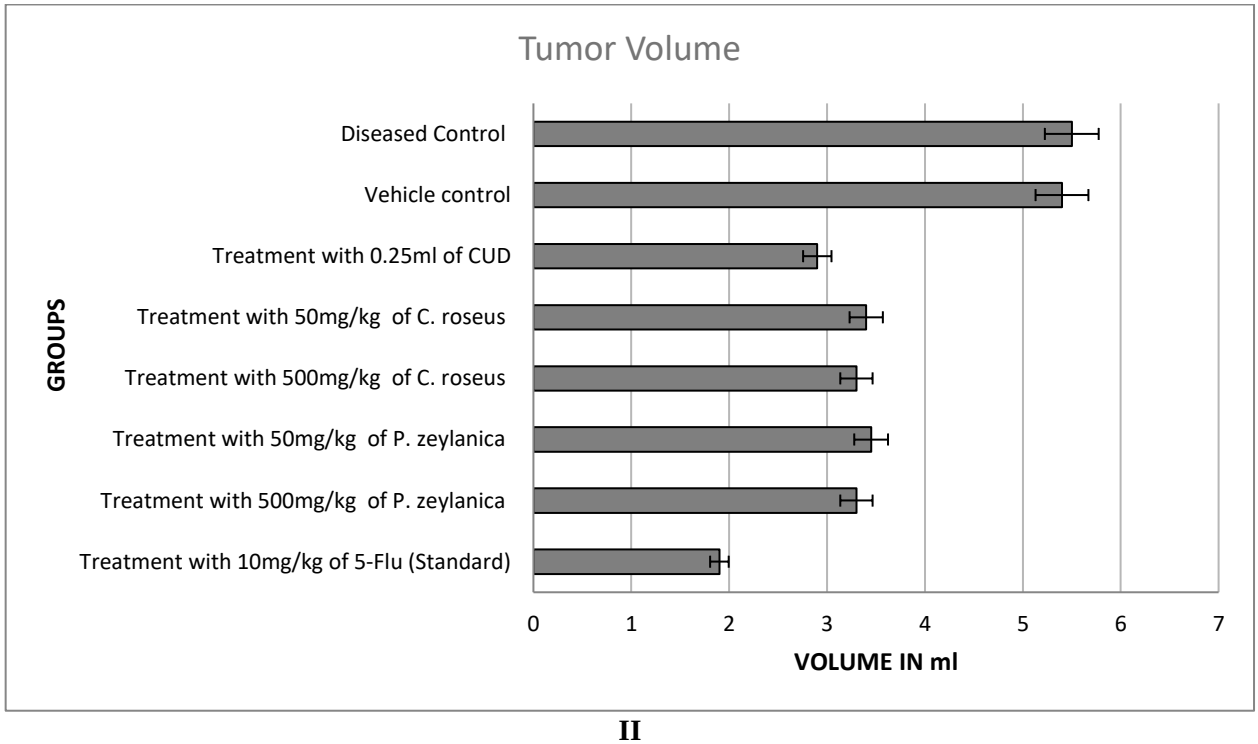

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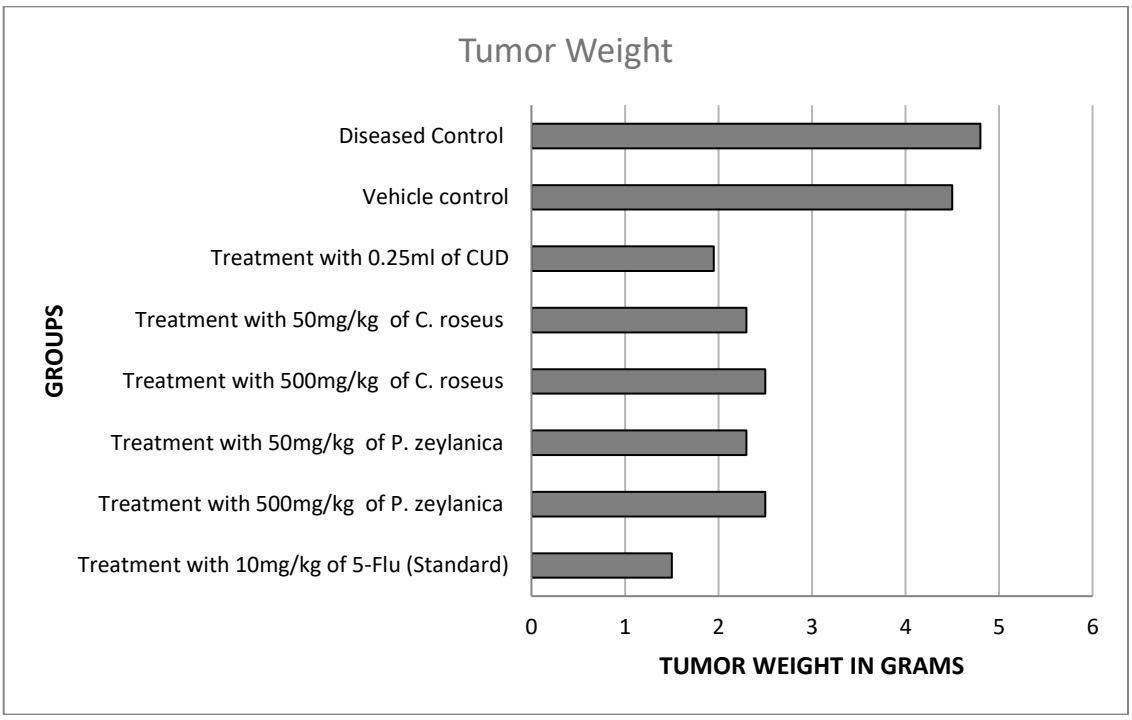

III

Legends for Tables

Table 1 General physico-chemical properties of extracts of *C. roseus and P. zeylanica*

Table 2 Major compounds identified in ethanolic extracts of aerial parts of *C. roseus* by GC-MS analysis; retention times (RT), formula and molecular weight (MW)

Table 3 Major compounds identified by GC-MS in ethanolic extracts of stem of *P. zeylanica*; retention times (RT),

Legends for Figures

Figure 1 DPPH Free Radical Scavenging assay and IC_{50} of ethanolic extracts of **(a)** aerial parts of *C. roseus* (10-250 µg/ml) and **(b)** stem of *P. zeylanica s* (50-350µg/ml).

Figure 2*:* GC-MS profile of (A) ethanolic extract of stem of *P. zeylanica and (B)* ethanolic extract of aerial parts of *C. roseus,* RT: Retention time.

Figure 3 Antiangiogenic activity of extracts of *C. roseus, P. zeylanica* and CUD on chick chorioallantoic membrane. Embryos were given different treatments, (A) represents control egg treated with 50% ethanol as negative control, (B) represents embryo treated with 25 mg of ethanolic extract of aerial parts of *C. roseus,* (C) is embryo treated with 25mg of ethanolic extracts of stem of *P. zeylanica* and (D) 50µl of CUD.

Figure 4 (I.) Effect of different treatments on tumour size, weight and volume in EAC and EAST Swiss albino mouse models. (A) Control tumor bearing mice, (B) tumor bearing mice treated with 50mg/kg *P. zeylanica, (C)* tumor bearing mice treated with 50mg/kg *C. roseus*, (D) tumor bearing mice treated with CUD and (E) tumor bearing mice treated with standard drug i.e. 5-Flu. Graphs depicting (II) and (III) antitumour activity of different doses of *C. roseus, P. zeylanica,* CUD and Standard drug i.e. 5-Flu with regards to tumor volume and tumor weight on EAC and EAST Swiss albino mouse models