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Original Article

ANTI-TUMOR POTENTIAL OF *ERVATAMIA DIVARICATA.* L. BURKILL AGAINST EHRLICH ASCITES CARCINOMA

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ABSTRACT

Objective: The present study aims to evaluate the anti-tumor potential of EEED (ethanolic extract of *Ervatamia divaricata.* L. Burkill) on EAC (Ehrlich ascites carcinoma).

Methods: The ethanolic extract of *Ervatamia divaricata.* L. Burkill was subjected to preliminary phytochemical screening and the antitumor effect of EEED was assessed by employing *in-vitro* methods. Compounds present in the ethanol extract of the plant were identified using GC-MS (Gas chromatography-mass spectrometry) and attempts were made to understand the mechanism of action using *insilico* methods.

Results: The results of the *in vitro* cytotoxicity assay and MTT assay revealed the anticancer potential of the ethanol extract of *Ervatamia divaricata*. When different concentrations of EEED were assayed, the dead cells were found to increase with increase in concentration of the extract. This proved that there was considerable damage of the cell membrane that leads to the blocking of the cell signaling in cancer cells.

Conclusion: The present study revealed that EEED possessed significant antitumor activity against EAC.

Keywords: *Ervatamia divaricata*, Ehrlich ascites carcinoma, *In vitro, In silico*.

INTRODUCTION

Malignant cancer disease is the second major killer disease in the world. As of 2013 GLOBACON statistical studies, the cancer burden rises to 4.2 million new cancer cases and 8.2 million cancer-related deaths in 2012 [1]. It is caused due to the disturbance in the homeostatic balance between normal cells and cancer cells. In practice, Synthetic drugs are widely used to kill cancer cells. Although synthetic drugs are effective in killing the cancer cells, the other side of it has serious life killing side effects also, because of the poor differentiating capability between the normal healthy cells and cancer cells [2]. This has ended-up in search of novel natural products in combating cancer cells. *Ervatamia,* a well known garden jasmine, is one of the genera of *Apocynaceae* family used since ancient times for its analgesic, anti infection, antioxidant, anti-tumor, antibacterial and anti-inflammatory properties [3, 4]. This prompted us to evaluate the anti-tumor potential of the *Ervatamia divaricata* using *in vitro* and *in silico* studies.

MATERIALS AND METHODS

Plant material

The leaves of *Ervatamia divaricata*. L. Burkill were obtained from the main campus of SASTRA University and the collected plant materials were identified and authenticated by Dr. P. Brindha, CARISM, SASTRA University.

Preparation of leaf powder and extraction

The leaves of *Ervatamia divaricata* were collected and shade dried. The dried leaves were mechanically powdered after keeping them at room temperature for 48 h. The powdered leaves were extracted with ethanol at room temperature. The ethanolic extract was filtered and evaporated to dryness using a distillation set-up. The concentrated extract was subjected to further analysis.

Preliminary Phytochemical analysis

The EEED was subjected to preliminary phytochemical analysis for the presence of tannins, terpenes, flavones, alkaloids, quinone, sterol, phenol, coumarin, proteins, reducing sugar, saponin, and gum [5].

Cells

EAC cells were obtained from Jiva Janthu Pariksha Kendra, Central animal facility at SASTRA University. The EAC cell lines have resemblance with human tumors and hence it is highly sensitive to chemotherapy. EAC cell lines remain undifferentiated and hyperdiploid, have 100% malignancy, no regression, shorter life span, and high transplantable capability [6].

Maintenance of cells

The carcinoma cells were maintained by intraperitoneal inoculation of 1 x 10^6cells/mouse weekly by the courtesy of Central animal facility, SASTRA University [7].

In vitro cytotoxicity assay

Short term *in vitro* cytotoxicity assay was done using EAC cell lines by incubating with four different concentrations of the EEED at 37 °C for 3 h. The carcinoma cells were aspirated from the peritoneal cavity of the mice using a 10 ml syringe and transferred to test tubes containing isotonic buffer solution. Then the cells were washed with normal saline and the cell number was determined using a hemocytometer and adjusted to 1 x 10^6cells/ml. The different concentrations of the EEED were added to each test tube and the final volume was made up to 1 ml using the phosphate buffer solution. The tubes were kept in incubation at 37 °C for 3 h. After incubation, the cells were stained with 0.1 ml of 0.4% trypan blue to check for viability. The dead cells usually take up the blue stain because of the disrupted cell membrane. The dead cells (stained) and viable cells (unstained) were then counted using a hemocytometer [8].

% dead cells (cytotoxicity) were calculated using the following formula,

$$\% \text{ Dead cells} = \frac{\text{Total cells counted} - \text{Total viable cells}}{\text{Total cells counted}} \times 100$$

MTT Assay

EAC cell lines were cultured in 96 well plates with growth medium RPMI1640 and 10% FCS. Increasing concentrations of EEED were

added to the cells and incubated at 37 °C for 14 h in a CO_2 incubator with 5 % CO_2. The media were replaced with a fresh growth medium along with 20 µl of MTT [(3-(4,5-dimethylthiazol-2yl)-2,5-diphennyltetrazoliumbromide]. Again, it was incubated for 4 h at 37 °C. After incubation, purple precipitate was clearly visible under the inverted microscope, then the growth medium was removed and 200 ml of 0.1% 0.1N acidic isopropyl alcohol was added to the cells to dissolve the MTT Formazan crystals. Then the covered plates were kept in the dark at 18-24 °C per overnight. The samples were drawn every 2 h and observed the reading at 570 nm. If the reading is low, the plate was returned for incubation. The plate cover was removed and the absorbance of the wells, including the blank at 570 nm. The absorbance was recorded at 570 nm immediately after the development of purple color. Each experiment was conducted in triplicate form. The average was calculated and compared with the control test samples [9].

The percentage growth inhibition was calculated using the following formula.

% Growth Inhibition = (Control OD–Treated OD) × 100/Control OD

Thin layer chromatography

Thin layer chromatography was performed on glass plates, pre-coated with Silica gel 60 F_{254} as stationary phase. Toluene: Ethyl acetate: Diethylamine (70:20:10) were used as mobile phase. 80 ml of ether, 20 ml of alcohol and 5 ml of dilute ammonia was added to 5 g of the extract and incubated for 1 h. The process was repeated until the colorless mixture was formed and filtered. 30 ml of 1N sulphuric acid was added to the filtrate and the bottom layer was collected in another separator. 25 ml of 3:1 alcohol and 0.5 N sulphuric acid was added and the acid layer was collected. 10 ml of chloroform was added to the acid layer and chloroform layer was collected and evaporated to dryness. The samples were loaded on the glass plates. The locations of the spots were determined under UV light at 254 nm and 366 nm.

GC-MS analysis

Perkin Elmer Clarus 500 GC-MS instrument was used to analyze the compounds present in the EEED. The capillary column was made of Elite 5ms (5% phenyl 95% dimethyl polysiloxane) of 30 m length was used. The oven program was fixed to 50ºC @ 8ºC/min to 220ºC (1 min) @ 7ºC/min to 280ºC (10 min). The split ratio was 1:10 and the carrier gas used was Helium at the flow rate of 1 ml/min. The samples were injected at 290 °C. The mass spectrometry was carried out with a mass range of 40-450 Daltons, the type of ionization was electron ionization (EI) and Gas-phase molecules enter the source through the heated probe or GC column. 1.12 x 10-[17] J electrons bombard molecules forming M+* ions that fragment in a unique, reproducible way to form a collection of fragment ions. The results were matched to the library NIST 2005 standards.

Insilico analysis

To support the anticancer activity, the insilico approaches have been implemented in which the docking software Autodock 4.2 was used. The target molecule was chosen to be Vascular Endothelial Growth Factor Receptor-2 (VEGFR-2) as it is a tyrosine kinase receptor protein of VEGF; an angiogenic growth factor belongs to the family of angiogenic regulating proteins [10]. Compounds obtained from GC-MS were docked with VEGFR-2 and the results were found to be significant.

RESULTS

The Preliminary Phytochemical screening of the EEED indicated the presence of alkaloids, flavones, steroids and reducing sugars.

***In vitro* cytotoxicity assay**

The percentage of dead cells increases as the concentration of the EEED increases against EAC cell lines as shown in table 1. Thus, the efficacy of the EEED in combating tumor cells has been proved.

Table 1: *In vitro* cytotoxicity assay of EEED on EAC cells

Concentration of EEED (µg/ml)	No. of viable cells	Viable cells (%)	No. of dead cells	Dead cells (%)
Control	91.67±0.58	96.84	3.67±1.53	4.21
150	60.33±3.21	66.67	29.33±0.58	32.22
300	40.67±0.58	45.56	49±3.61	54.44
500	26.67±1.52	26.21	76.67±4.51	74.76
1000	15.33±0.57	15.46	81.67±0.58	84.54

No. of experimental runs = 3; Results are expressed as mean±SD.

MTT assay

MTT assay also revealed that % Cytotoxicity was found to be increasing with an increase in concentration of EEED that shows the anticancer property of *Ervatamia divaricata* that is indicated in table 2.

Table 2: MTT assay of EEED on EAC cells

Conc of EEED (µg/ml)	% Cytotoxicity
Control	-
5	7.52
7.5	18.30
10	39.22
15	49.46
25	64.38

Thin layer chromatography

Thin layer chromatography was performed to spot out the alkaloids present in the ethanol extract of *Ervatamia divaricata* and the outcomes were shown in fig. 1(a) & 1(b) and R_f values were given in table 3.

GC-MS Analysis

The EEED was subjected to Gas chromatography-Mass spectrometry studies and the profile was shown in fig. 2.

The major compounds obtained from GC-MS analysis were 3-amino-2-oxazolidinone (Formula: $C_3H_6N_2O_2$ Molecular weight: 102.09 g/gmol), 2-cyclopenten-1-one,2-hydroxy-(Formula: $C_5H_6O_2$ Molecular weight: 98.09 g/gmol), 2,5-Dimethyl-4-hydroxy-3(2H)-furanone (Formula: $C_6H_8O_3$ Molecular weight: 128.12 g/gmol), Benzoic acid,2-hydroxy-6-methyl ester (Formula: $C_8H_8O_3$ Molecular weight: 152.14 g/gmol), Sucrose (Formula: $C_{12}H_{22}O_{11}$ Molecular weight: 286), D-Allose (Formula: $C_6H_{12}O_6$ Molecular weight: 120 g/gmol), 4,6-Di-O-methyl-a-D-galactose (Formula: $C_8H_{12}O_6$ Molecular weight: 204.1 g/gmol) and, 2,4-Diaminophenol (Formula: $C_{12}H_{14}N_2O_2$ Molecular weight: 250.5 g/gmol), Quinoline,3-ethyl-(Formula: $C_{11}H_{11}N$ Molecular weight: 157.2 g/gmol), Benzene-2(1,3-butadienyl)-1,3,5-trimethyl-(Formula: $C_{13}H_{16}$ Molecular weight: 172.26 g/gmol), Benzofuran,2,3-dihydro-(Formula: C_8H_8O Molecular weight: 120.1 g/gmol), 4H-pyran-4-one,2,3-dihydro-3,5-hydroxy-6-methyl-(Formula: $C_6H_8O_4$ Molecular weight: 144.1 g/gmol), Benzyl Chloride (Formula: C_7H_7Cl Molecular weight: 126.58 g/gmol), 5(2H)-Oxazolone,4-(phenylmethyl)-(Formula: $C_{10}H_9NO_2$ Molecular weight: 175.18 g/gmol).

***In silico* analysis**

The major compounds obtained from GC-MS analysis was docked with VEGFR-2. As a result of docking, table 3 listed out the binding energies and number of hydrogen bonds interacted with the compounds. The best result was considered based on the Binding energy and Polar interactions.

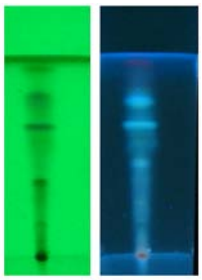


Fig. 1: Thin layer chromatography plate viewed at 254 nm (fig. 1a.) and 366 nm (fig. 1b.)

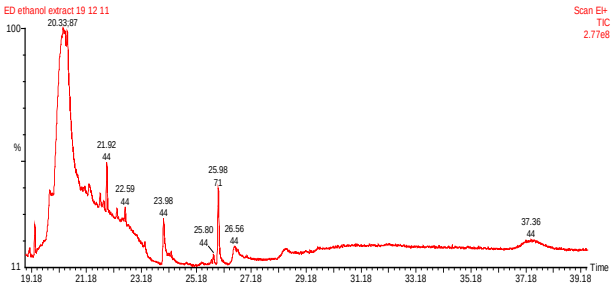


Fig. 2: GC-MS profile of Ethanolic extract of *Ervatamia divaricata*. L

Table 3: R_f values of 9 spots viewed at 254 nm and 366 nm

S. No.	R_f value at 254 nm	R_f value at 366 nm
1	0.09	0.09
2	0.18	0.18
3	0.27	0.27
4	0.4	0.4
5	0.52	0.52
6	0.63	0.63
7	0.67	0.67
8	0.81	0.81
9	0.98	0.98

In fig. 3(a) and 3(b), the docking of sucrose and D-Allose with VEGFR-2 was shown respectively. The binding energies were found to be-25.32 kJ/mol and-22.64 kJ/mol and the no. of hydrogen atoms interacted were found to be 5 and 6 for Sucrose and D-Allose respectively.

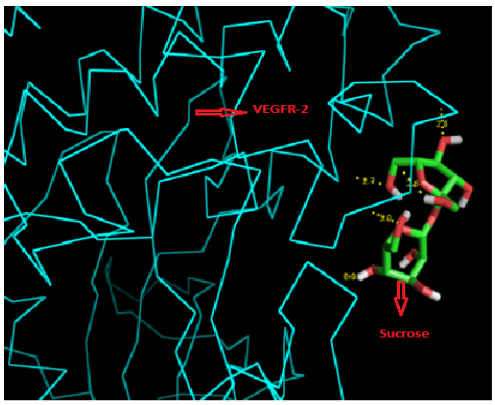


Fig. 3(a): Docking of Sucrose with VEGFR-2

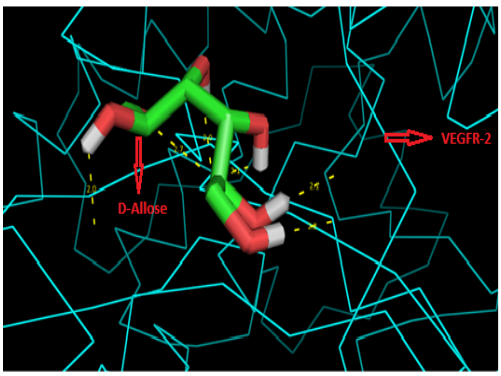
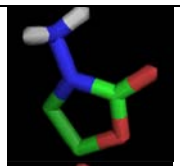
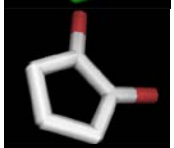
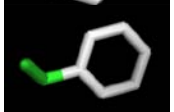
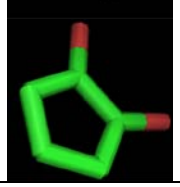
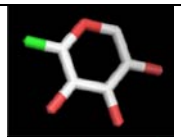
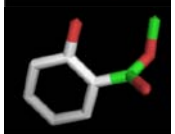
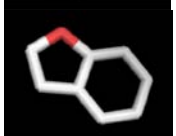
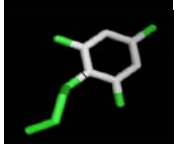
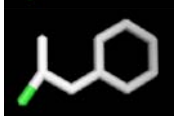
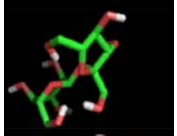
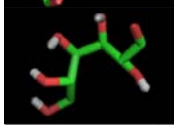
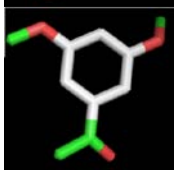
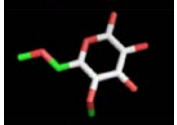
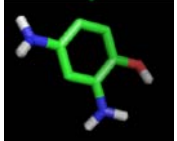
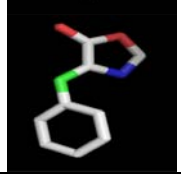


Fig. 3(b): Docking of D-Allose with VEGFR-2

Table 3: Binding energy, no. of hydrogen bonds interacted with the compounds

S. No.	Ligand's Name	Structure	Binding energy(G) (KJ/mol)	No. of hydrogen bonds interacted with the compound
1	3-amino-2-oxazolidinone		-17.45	3
2	2-cyclopenten-1-one,2-hydroxy-		-73.04	4
3	Benzyl Chloride		-12.93	0
4	2,5-Dimethyl-4-hydroxy-3(2H)-furanone		-14.85	4

5	4H-pyran-4-one,2,3-dihydro-3,5-hydroxy-6-methyl-		-15.73	1
6	Benzoic acid,2-hydroxy-6-methyl ester		-16.99	3
7	Benzofuran,2,3-dihydro-		-16.07	1
8	Benzene-2(1,3-butadienyl)-1,3,5-trimethyl-		-24.94	0
9	Quinoline,3-ethyl-		-23.48	0
10	Sucrose		-25.32	5
11	D-Allose		-22.64	6
12	3',5'-Dimethoxyacetophenone		-21.84	1
13	4,6-Di-O-methyl-a-D-galactose		-16.03	4
14	2,4-Diaminophenol		-19.21	2
15	5(2H)-Oxazolone,4-(phenylmethyl)-		-17.91	1

DISCUSSION

Being the second major killer disease in the world, the plant kingdom is an excellent resource for the discovery of many anti-cancer drugs. Because of its "natural" origin, they are inherently safe and non-toxic to normal cells. They can act on multiple targets unlike synthetically produced drug molecules. The EEED has the ability to inhibit angiogenic pathways of tumor cells since angiogenesis is one of the most important criteria for tumor growth [11].

Earlier reports [12] stated that Mallikarjuna G *et al.,* 2013 evaluated the ethanolic extract of *Sida cordifolia* against AFB_1 (Aflotoxin B_1) induced HCC (hepatocellular carcinoma) in winstar rats (250µg/kg/dose). The results showed a significant restoration of abnormal serum and tissues indicating the protective effect [13]. The results of cytotoxic activity on hela cells treated with *Sida cordifolia* extracts showed cells with uncontrolled growth has been arrested and there is decline level of cancerous cells was studied by Joseph *et al.,* 2011 [14]. The results of the present study with regard to *in vitro* cytotoxicity assay and MTT assay revealed the anticancer potential of the ethanol extract of *Ervatamia divaricata*. When different concentrations of EEED were assayed, the dead cells were found to increase with increase in concentration of the extract. The number of dead cells at 1000 µg/ml concentration of EEED was found to be 81.67 (table 1) which proves that the cancer cells were destroyed by the EEED. MTT assay also exhibited that the % cytotoxicity was found to be increasing with an increase in concentration of the plant extract. It was observed that 25 µg/ml of EEED showed 64.38% cytotoxicity (table 2). This proved that there

was considerable damage of the cell membrane that leads to the blocking of the cell signaling in cancer cells.

The *in silico* analysis was done to study the anti-tumor activity of the active compounds present in this plant in detail. The structures obtained from GC-MS analysis were docked with the VEGFR-2 by which the molecular mechanism of action was studied. Among the major compounds, Sucrose and D-Allose were found to be the best to dock with the VEGFR-2 by means of polar interactions with binding energies of-25.32 kJ/mol and-22.64 kJ/mol respectively.

CONCLUSION

The present *in vitro* and *in silico* studies vividly show that the ethanol extract of *Ervatamia divaricata* possesses significant anti-tumor activity. Hence the bioactive compounds in the plant can be developed as pharmacologically safe chemopreventive agents against cancer after carrying out thorough pre-clinical and clinical trials.

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CONFLICT OF INTERESTS

Declared None

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