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EVALUATION OF ANTI-CANCER ACTIVITY OF ETHANOL EXTRACT OF *ERYTHRINA VARIEGATA* LINN.

[*,1]Baskar N, [2]Parimala Devi B, [3]Jayakar B

[*,1] Thanthai roever college of pharmacy, Perambalur, Tamilnadu, India - 621 212.

[2] Prist University, Thanjavur, Tamilnadu, India - 613 403.

[3] Vinayaka missions college of pharmacy, Salem, Tamilnadu, India - 636 308.

Abstract

Ethanol Extract of *Erythrina variegata* was investigated for anticancer and *invitro* cytotoxic activities against transplantable tumors and human cell line. *Invitro* cycotoxicity studies were carried out using Hela and NIH3T3 cells by MTT assay and *in vivo* antitumor activity with Dalton's Ascites lymphoma (DLA) tumor bearing mice. Activity was measured by monitoring the Cancer Cell number, packed cell volume, increase in life span and haemetological parameters were evaluated and compared with the respective controls. The extract exhibited strong in vitro cytotoxicity against Hela cell line found to be safe with normal cell. EEEV with the doses 200 and 400 mg/kg. Significantly increase the life span and decrease in the cancer cell number and tumor weight ($P < 0.01$), exerted a protective effect on the hemopoietic system. Results showed significant antitumor and cytotoxics effects of EEEV against DLA and human Cancer Cell line which support the ethno medicinal use of EEEV in cancer therapy.

Keywords: *Erythrina variegata*, Dalton's Lymphoma, Anticancer activity, 5-fluorouracil.

Introduction

Over the past few years, cancer has remained a major cause of death and the number of individuals affected with cancer is continuing to expand. Reasons are many and increasing due to sedentary lifestyle, stress. Developing countries like India, due its heavy population and its economic crisis, looking for a better alternative to treat this drastic illness those who are poor. In connection with this, search of active medicinal molecule from plants are highly encouraging in recent days. Much of the resources of herbal origin is having wide range of therapeutic uses to treat cancer. Hence, a major portion of the current pharmacological research is devoted to anticancer drug design customized to fit new molecular targets[1]. Due to enormous propensity of plants, which synthesize a variety of structurally diverse bioactive compounds, the plant kingdom is a potential source of chemical constituents with antitumor and cytotoxic activities[2]. The rich and diverse plant sources of India are likely to provide effective anticancer agents. One of the

Author for Correspondence:
Baskar N,
Thanthai roever college of pharmacy,
Perambalur, Tamilnadu,
India - 621 212.
Email: baskar_333@yahoo.com

best approaches in the search of anticancer agents from plant sources is the selection of plants based on ethno medical leads[3].

The genus *Erythrina* (fabaceae) is distributed in the tropical and subtropical regions of the world and encompasses over 100 species. The antibacterial and anti inflammatory properties of *Erythrina variegata linn* are utilized in Chinese herbal medicine for the treatment of pyrexia, scabies and septicaemia. *Erythrina variegata* is a tall ornamental tree distributed throughout upper Gangatic plains of India and Nepal. The bark of the plant is astringent, febrifuge, anti-bilious and antihelmintic. It is also useful in opthalmia and skin diseases. The leaves are used in fever, inflammation and joint pain. The juice of the leaves is used to relieve ear ache and toothache[4]. The roots are used as bronchitis, febrifuge and as an insecticide. The roots are also used in the treatment of cancer, convulsions and used to treat pimples[5]. It has the reputation to stimulate lactation and menstruation and is used as laxative, diuretic and expectorant[6]. Although many compounds have been reported from the genus, *Erythrina*, previous phytochemical investigations with *E. variegata* revealed the occurrences of orientanol B, erycristagallin, cristacarpin, sigmoid in K, 2-(γ,γ-dimethylallyl)- 6a-hydroxyphaseollidin, erystagallin A[7], eryvarins A and B[8], bidwillon B[9], eryvarins F and G[10], alpinum isoflavone, isococculinine, decarbomethoxyerymelanthine, erysodienone, erythritol, erysodine[11], erysovine, stachydrine, sterols, fixed oils and fatty acids[12]. The present study is to evaluate the anticancer activity in the root bark extract of the plant *Erythrina variegata* linn.

Materials and methods

Chemicals

5-Fluorouracil (5-FU) was obtained from Dabur pharmaceutical Ltd (New Delhi, India). Trypan blue, thiobarbituric acid, trichloro - acetic acid, ethylenediaminetetracetic acid (EDTA), RPMI-1640 media and 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltertazolium bromide (MTT) were procured from HiMedia (Mumbai, India). Dimethyl sulfoxide and methanol were obtained from Loba Chemie (Mumbai, India). All other chemicals used were of analytical grade.

Plant material and extraction

The root bark of *Erythrina variegate* Linn were collected from the surrounding areas of Trichy in the month of July. The plant was authenticated by Dr.S.Selvaraj, Annamalai university, tamil nadu,india. A voucher specimen is preserved in our laboratory for future reference (b.d.h. ref no 1011). The plant material was shade dried, pulverized and extracted (500 g) with 80% ethanol at room temperature for 72 hours. The extract was filtered and concentrated to dryness under reduced pressure and controlled temperature (40°C to 50°C) in a rotary vacuum evaporator. The extract was dark yellowish brown solid weighing 50.2g (yield, 10.4 %) and was preserved in a vacuum desiccator until further use.

Preliminary phytochemical screening

The extract was tested for various phytochemical constituents employing standard tests[13] revealed the presence of steroids, alkaloids, tannins, flavonoids, glycosides, etc.

Tumor cells and inoculation

Normal Mouse Embryonic Fibroblast (NIH 3t3), Human Cervical Cancer Cells (HeLA), were obtained from National Centre of Cell Sciences (Pune, India). The cultures were maintained in Dulbecco's modified eagles medium (DMEM) containing 10% inactivated calf serum and were grown in 25cm^2 tissue culture flasks (Tarson Products Ltd, Kolkatta, India). Until confluent and used for cytotoxic assays. DLA cells were supplied by Amala Cancer Research Centre (Trissur, Kerala, India). The cells were maintained *in vivo* in Swiss albino mice by intraperitoneal transplantation. Tumor cells aspirated form the peritoneal cavity of mice were washed with normal saline and were used for further studies.

Preparation of suspension and solutions

For cytotoxicity assays, the extract was dissolved in Dimethyl sulfoxide (DMSO) and the volume made up to 10ml to obtain a 1000µg/ml stock solution. Serial two-fold dilutions were made using DMSO to get lower concentration. MEEV was suspended in distilled water using sodium Carboxyl Methyl Cellulose (CMC, 0.3%) and administered orally to the animals with the help of an intragastric catheter to study *in vivo* antitumor activity.

Invitro cytotoxicity studies on human cancer cell lines

Stock cells of Normal Mouse Embryonic Fibroblast (NIH 3T3), Human Cervical Cancer Cells (HeLA), were cultured in RPMI-1640 and DMEM supplemented with 10% FETAL BOVINE serum, penicillin (100IU/ml) and streptomycin (100µg/ml) in a humidified atmosphere of 5% CO_2 at 37°C until confluent. The cells were dissociated with 0.2 % trypsin and 0.02% EDTA in PBS. The cytotoxic assay was carried out by adding 0.1 ml of cell suspension containing 10,000 cells to each well of a 96-well microtitire plate (Tarson, Kolkatta, India) and fresh medium containing various concentrations of extract was added at 24 h after seeding. Control cells were incubated without the extract and with DMSO. The microtitre plates were incubated at 37°C in a humidified atmosphere with 5 % CO_2 for a period of 72hours. The percentage cytotoxicity and IC_{50} were determined by the standard MTT assay method[14-17].

Animals

Healthy male Swiss albino mice weighing 25±2g were obtained from Venkateshwara Enterprises, Bangalore, India. The mice were grouped and housed in polypropylene cages and maintained under standard conditions (25±2°C) with 12 h dark/light cycle. The animals were fed with standard animal pellet diet and water *ad libitum*. The experiment protocols were approved by the Institutional Animal Ethical Committee (IAEC) and CPCSEA, Chennai, India (Proposal No P.col/62/2011/IAEC/VMCP).

Acute toxicity studies

LD_{50} is the dose that is lethal to 50% of a population. LD_{50} of the extract was determined as per OECD guidelines. Healthy young adult albino mice of commonly used laboratory strains were employed. Females should be non pregnant. Each animal at the commencement of its dosing was between 8 and 12 weeks old and its weight was in an interval within ± 20% of the mean weight of any previously dosed

animals. The temperature in the experimental room was 22°C (±3°C). For feeding, conventional laboratory diets was used with an unlimited supply of drinking water. The animals are randomly selected marked enabling identification and kept in their cage for at least 5 days prior to dosing to allow for acclimatization to the laboratory conditions. The test substance was administered in a single dose by gavage using a stomach tube or a suitable intubation canula. These animals are used for each step. The dose level to be used as the starting dose was selected as 50 mg/kg body weight. The starting dose level should be that which is most likely to produce mortality in some of the dosed animals. The flow chart of Annex 2c(oecd423) describes the procedure that should be followed for each of the starting doses. The dose was administered to three animals .After 24 hrs none of the animals died. Hence the next dose at the level of 300mg/kg was attempted. Following this dose also none of the animals died .Hence the final dose tried was 2000mg/kg out of 3 animals 2 died within 24 hrs of administration. Hence the LD_{50} was concluded to be 2000 mg/kg.

Antitumor studies

The DLA cells maintained *in vivo* in Swiss albino mice by intraperitoneal transplantation. While transforming the tumor cells to the grouped animal the DLA cells were aspirated from peritoneal cavity of the mice using saline. The cell counts were done and further dilution were made so that total cell should be 1 x 10^6, this dilution was given intraperitonealy. Let the tumor grow in the mice for minimum seven days before starting treatments.

Treatment protocol

Swiss Albino mice were divided in to five group of six each. All the animals in four groups were injected with DLA cells (1 x 10^6 cells per mouse) intraperitonealy, and the remaining one group is normal control group. **Group 1** served as the normal control. **Group 2** served as the tumor control. Group 1 and 2 receives normal diet and Water. **Group 3** served as the positive control, was treated with injection fluorouracil at 20 Mg/kg body weight, Intra peritoneally. **Group 4** Served as a treatment control group and was administered Ethanolic Extract of *Erythrina variegata* (EEEV) at a dose of 200mg/kg through orally. (LD_{50} OECD Guidelines) **Group 5** Served as a treatment control group and was Administered Ethanolic Extract of *Erythrina variegata* (EEEV) at a dose of 400mg/kg through orally. **Treatment** In this study, drug treatment was given after the 24 hrs of inoculation, once daily for 14 days. On day 14, after the last dose, all mice from each group were sacrificed; the blood was withdrawn from each mouse by retro orbital plexus method and the following parameters were checked. Hematological parameters WBC count, RBC count, Hb content, Platelet count, packed cell volume.

Results and discussion

The preliminary phytochemical screening revealed that the extract contains glycosides, alkaloids, saponins, flavonoids, phenolilc compounds and terpenoids. The IC_{50} of EEEV was found to be 57 μg/ml for HELA cells and 66.9 μg/ml for NIH 3T3 cells. In MTT assay, the percentage cytotoxicity progressively increased in a concentration dependent manner. The $_{IC50}$ of EEEV was found to be less

than 100 µg/ml against all the human cancer cell lines used. However the $_{IC50}$ values against the normal mouse embryonic fibroblast (NIH3T3) were found to be very high when compared to that of cancer cell lines. This indicated that EEEV possess selective cytotoxicity against the cancerous cell lines, but is safer towards the normal cells (Table 1). In acute toxicity studies, animals treated with EEEV did not show any toxic symptoms or mortality when dosed up to 2000 mg/kg body weight by oral route. This indicated that the extract was found to be safe at the tested dose level. Hence $1/10^{th}$ (200mg/kg) and $1/5^{th}$ (400mg/kg) of this dose were selected for the *in vivo* studies.

Effect on tumor growth

In the DLA tumor control group, the average life span of animal was found to be52% where as EEEV at the dose of 200 mg/kg and 400mg/kg body weight increase the life span to 74% and 78% respectively. These values were significant. However the average life span of 5- FU treatment was found to be 90%, indicating its potent antitumor nature. The antitumor nature of EEEV was evidenced by the significant reduction in percent increase in body weight of animal treated with EEEV at the dose of 200 mg/kg and 400mg/kg body weight when compared to DLA tumor bearing mice. It was also supported by the significant reduction in packed cell volume and viable Tumor cell count in both the extent of treatment when compared to the DLA tumor control. (Table No .2).

Table No. 1: *In vitro* cytotoxicity Studies of Ethanol Extract of *Erythrina variegata* against Human Cancer Cell Lines by MTT Assay

S.No	Cell Lines Studied	IC50 (µg/ml)*
1.	HeLa (Human Cervical Cancer)	57(µg/ml)
2.	NIH 3T3 (Normal Mouse Embryonic Fibroblast)	66.9 (µg/ml)

Table No. 2: Effect of EEEV on the life span, body weight and cancer cell count of tumor induced mice.

Treatment	Number of animals	% ILS Life span	Increase in Body weight grams	Cancer cell count ml X 10^6
G_1	6	>30 days	2.22±0.50	-
G_2	6	52%	$7.85\pm1.10^{a**}$	$2.62\pm0.32^{a**}$
G_3	6	90%	$3.70\pm0.55^{b**}$	$1.37\pm0.31^{b**}$
G4	6	74%	$6.10\pm0.90^{b*}$	$1.95\pm0.620^{b*}$
G5	6	78%	$6.10\pm0.90^{b*}$	$1.80\pm0.52^{b*}$

All values are expressed as mean ± SEM for 6 animals in each group.

**a – Values are significantly different from control (G_1) at $P < 0.001$

*b – Values are significantly different from cancer control (G_2) at $P < 0.01$

**b – Values are significantly different from cancer control (G_2) at $P < 0.001$

Effect on hematological parameters

As shown in (Table No.3) RBC, Hgb, Platelets were decreased and WBC count was significantly increased in the DLA control group compared to the normal control group. Treatment with EEEV at the dose of 200 mg/kg and 400mg/kg significantly increases the Hgb content, RBC, Platelets and significantly decreased the WBC count to about normal level. All these results suggest the anticancer nature of the EEEV. However, the standard 5-FU at the dose of 20 mg/kg body weight produced better result in all these parameters.

Table No. 3: Effect of EEEV on Hematological parameters

Treatment	Total Wbc Cells /Mlx10^3	Rbc Count Mill/Cumm	Hb Gm/Dl	Pcv %	Platelets Lakhs/cumm
G1	9.98 ±1.30	4.24±0.99	12.35 ±2.30	14.60±2.70	3.20±0.74
G2	14.25 ±2.60^{a**}	2.40±0.45^{a**}	7.30 ±0.88^{a**}	31.50±3.60^{a**}	1.65±0.60^{a**}
G3	11.45 ±1.90^{b**}	3.98±0.88^{b**}	11.3 ±1.75^{b**}	19.30±2.55^{b**}	2.68±0.55^{b**}
G4	12.55 ±2.40^{b*}	3.30±0.80^{b*}	10.77±1.70^{b*}	22.20±1.55^{b*}	2.30 ±0.70^{b*}
G5	12.10 ±1.92^{b*}	3.48±0.67^{b*}	10.87±1.82^{b*}	21.18±2.52^{b*}	2.42 ±0.92^{b*}

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