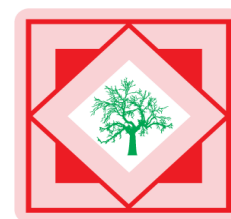




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Anticancer activity of *Tephrosia purpurea* root extracts against Ehrlich Ascites Carcinoma (EAC) cells in swiss albino mice

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ABSTRACT

The study aims to evaluate the anticancer properties of aqueous and ethanol extracts of *Tephrosia purpurea* (Linn) roots against Ehrlich Ascites Carcinoma (EAC) cell lines in Swiss albino mice. In case of EAC induced liquid tumor, after 24 hours of tumor inoculation, the extracts were administered daily for 14 days. On day 15 the mice were sacrificed for observation of antitumor activity. The effect of aqueous and ethanol extracts on the growth of transplantable murine tumor, lifespan of EAC bearing mice and simultaneous alterations in the hematological profile were estimated. The ethanol and aqueous extracts showed decrease in the tumor volume, tumor weight, viable cell count and increase in the mean survival time there by increasing the life span of EAC tumor bearing mice. Hematological profile was reverted to normal level in the extracts treated mice. From the present study the ethanol and aqueous extracts of *Tephrosia purpurea* roots exhibited the antitumor effect in a dose dependent manner comparable to that of standard drug, 5-Fluoro Uracil. Further the phytochemical investigations of aqueous and ethanol extracts revealed the presence of alkaloids, glycosides, flavonoids, phytosterols, phenolic compounds and tannins in ethanol extract and phytosterols and carbohydrates in aqueous extract. Hence the present investigation provides a scientific base to the ethno medicinal use of *Tephrosia purpurea* which is largely attributable to the additive or synergistic effect of their constituents.

Keywords: *Tephrosia purpurea*; EAC cell lines; Anticancer activity.

INTRODUCTION

Cancer continues to represent the largest cause of mortality in the world and claims over six million lives every year [1]. Most of the research carried worldwide focuses to find a way to prevent and treat the cancer. In the present day several methods exists for the treatment of cancer such as chemotherapy, radiotherapy and surgery. Among this chemotherapy is now considered as an efficient method for treatment of cancer. However, the most of the chemotherapeutic agents exhibit severe toxicity, resulting undesirable side effects [2]. Moreover many of the active molecules are highly expensive and teratogenic. Hence there is a need to find alternative drugs which are highly effective and nontoxic. 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 [3]. It is estimated that more than 50% of all the patients diagnosed with cancer explore

complementary and alternative medicine-especially herbal medicine [4]. EAC cells are experimental tumor models used worldwide in cancer research. In 1907, Paul Ehrlich discovered this tumor in the mammary gland of a white mouse, and the tumor was named after him [5]. The present form of EAC cells has been developed by Loewenthal and Jahn [6] from one of the several carcinoma lines [7].

Tephrosia purpurea (L). (Fabaceae), commonly known as “Sharapunkha” in Sanskrit, is a copiously branched, sub-erect, herbaceous perennial plant, which occurs throughout the India [8]. Whole plant has been used to cure tumors, ulcers, leprosy, allergic and inflammatory conditions such as rheumatism, asthma and bronchitis [9]. The aqueous extract of *Tephrosia purpurea* seeds has shown significant *in vivo* hypoglycemic activity in diabetic rabbits [10]. The flavanoids isolated from the plant has been reported to have antimicrobial activity [11]. It has also been reported to acquire hepatoprotective, mast cell stabilizing and erythrocyte membrane integrity enhancing effect in various animal models [12, 13]. Phytochemical investigations on *Tephrosia purpurea* have revealed the presence of various phytoactive constituents such as glycosides, rotenoids, isoflavone, flavanones, chalcones, flavanols, flavones and sterols [14].

Plant derived natural products such as flavonoids, terpenoids and steroids *etc* have received considerable attention in recent years due to their diverse pharmacological properties including antioxidant and anticancer activity [15, 16]. From this viewpoint present study was carried out to evaluate the anticancer activity of *Tephrosia purpurea* root extracts against Ehrlich Ascites Carcinoma (EAC) in Swiss albino mice.

MATERIALS AND METHODS

Chemicals

Trypan blue (Sigma, USA), dimethyl sulfoxide (Ranbaxy Fine Chemicals, Mumbai, India), 5-Fluoro Uracil, Ehrlich Ascites Carcinoma, (Amala Cancer research centre, Thrissur, India). All other chemicals and solvents were obtained from local firms (India) and were of highest purity and analytical grade.

Plant material

The roots of *Tephrosia purpurea* were collected in the month of January 2012 from Thalakona forest of Tirumala, Chittoore district, Andhrapradesh state, India. The samples were authenticated by Dr. Madhava chetty, Assistant Professor of Botany, S.V. University college, Tirupati, India. A herbarium specimen has been deposited at the college for further reference.

Preparation of plant extracts

The roots were dried in the shed and coarsely powdered. The powder was extracted with ethanol in a soxhlet apparatus for ethanol extract (EETP) and with a mixture of Chloroform: water (1:99) by maceration process for aqueous extract (AETP). The extracts were concentrated to remove the solvent completely under reduced pressure and stored *in vacuo* till use. The percentage yield of EETP and AETP were calculated and found to be 9.93% w/w and 12.8 % w/w, respectively.

Phytochemical screening

The ethanol extract (EETP) and aqueous extract (AETP) of *Tephrosia purpurea* roots were subjected to Phytochemical screening according to the phytochemical methods described by Harborne [17].

Experimental animals

Male Swiss albino mice (22 to 28 gm) were used for all the experiments in the present study. The animals were maintained under standard husbandry conditions in the animal house of the institute (temperature $25 \pm 2^\circ\text{C}$) in a natural light-dark cycle and fed with standard rodent diet and water *ad libitum*. Ethical committee clearance was obtained from IAE (Institutional Animal Ethics Committee) of CPCSEA (Ref. No./IAEC/XII/02/SIPS/2011-2012).

Acute toxicity studies

The acute toxicity of ethanol and aqueous extracts of *Tephrosia purpurea* roots were determined as per the OECD guideline no. 423 (Acute toxic class method) [18]. Based on the results obtained from this study, the dose for EETP was fixed to be 100 mg kg⁻¹ b.w. and 200 mg kg⁻¹ b.w. similarly the dose for AETP was fixed to be 250 mg kg⁻¹ b.w. and 500 mg kg⁻¹ b.w. for dose dependent study.

Transplantation of tumor

Ehrlich Ascites Carcinoma cells were supplied by Amala Cancer Research Centre, Trissur, Kerala, India. The cells were maintained *in vivo* in Swiss albino mice by intraperitoneal transplantation [19]. 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

Swiss albino mice were divided in to seven groups of six each. All the animals in six groups were injected with EAC cells (1×10^6 cells per mouse) intraperitoneally (*i.p.*) and the remaining one group is normal control group.

Group 1 served as the normal control, and *group 2* served as the tumor control; groups 1 and 2 receive normal diet and water.

Group 3 served as the positive control; treated with injection of 5-fluorouracil (5-FU) at 20 mg kg^{-1} b.w. *i.p.* [20].

The remaining groups were treated with EETP at 100 mg kg^{-1} b.w. and 200 mg kg^{-1} b.w. and AETP at 250 mg kg^{-1} b.w. and 500 mg kg^{-1} b.w. orally for dose dependent study. Drug treatments were given 24 hours after the inoculation, once daily for 14 days. After 24 hours of the last dose and then 18 hour of fasting, animals of each group were sacrificed by cervical dislocation to measure tumor volume, tumor weight, cell viability and haematological parameters.

Evaluation of the antitumor Activity**Tumor volume and tumor weight**

For the determination of tumor weight six mice from each group were sacrificed after 24 hrs of the last dose and weight was recorded before and after the collection of the ascitic fluid [21, 22]. The difference in weight before and after gives the tumor weight and is expressed in grams. For tumor volume, the ascitic fluid was collected from the peritoneal cavity and volume was measured with the help of graduated centrifuge tubes.

Determination of viable and non viable tumor cells

The ascitic fluid collected from the peritoneal cavity was taken in WBC diluting pipette and diluted up to 100 times with phosphate buffer saline [23]. A drop was placed on the Neubauer's chamber and number of cells in all 64 squares were counted. For the assessment of viable and non viable cells, Trypan blue dye (0.4 % in normal saline) was used as staining material. Viability of the cells was checked by using trypan blue dye, viable cells did not take the colour of the dyes (Trypan blue Exclusion test) and non viable cells respond to the blue stain of the dye [24].

Cell count = Number of cells x dilution factor / Area x thickness of liquid film

Assessment of mean survival time (MST)

MST of standard and extracts treated groups was noted by recording the mortality of the Swiss albino mice [25] and percent increase in life span was calculated by using MST values. MST was calculated by using following equation [26].

Mean survival time (MST)* = (First Death + Last Death)/ 2

*Time denoted by days.

% Increase in life span (%ILS)

The effect of EETP and AETP of *Tephrosia purpurea* on percentage increases in life span (% ILS) of the animals was calculated on the basis of mortality of the experimental mice [26].

$$\% \text{ ILS} = \frac{\text{Mean survival time (MST) of treated group} - \text{MST of control group}}{\text{MST of control group}} \times 100$$

Hematological studies

Blood was collected from the mouse by tail puncture method and used for the estimation of Hemoglobin (Hb) levels, red blood cell counts (RBC) [27] and white blood cell counts (WBC) [28]. WBC differential counts were determined using Leishman stained blood smear method [29].

Statistical Analysis

The experimental results were expressed as mean \pm S.E.M (n=6 mice per group). Results were analyzed by the one-way ANOVA followed by Tukey-kramer post hoc multiple comparison test using Graph pad InStat version 3.00. Where $p < 0.05$, $p < 0.01$ and $p < 0.001$ considered being significant, very significant and highly significant, respectively.

RESULTS**Phytochemical screening**

The preliminary phytochemical screening showed the presence of various Phyto constituents in the ethanol and aqueous extracts which are listed in Table 1.

Effect on tumor weight and tumor volume

It was found that oral administration of EETP leads to the reduction in tumor weight and tumor volume in EAC bearing Swiss albino mice which are shown in Table 2. Tumor weight of EAC control was found to be 4.15 gm and that of EETP treated mice, 2.05 gm (100 mg kg⁻¹ b.w.), 1.28 gm (200 mg kg⁻¹ b.w.) and AETP treated mice, 2.25 gm (250 mg kg⁻¹ b.w.), 1.85 gm (500 mg kg⁻¹ b.w.) respectively. In case of standard drug 5-fluorouracil (20 mg kg⁻¹ b.w., *i.p*) it was found to be 0.95 gm. Tumor volume of the EAC control group was 2.1 ml, and it was significantly reduced to 1.4 ml (100 mg kg⁻¹ b.w.), 0.8 ml (200 mg kg⁻¹ b.w.) in EETP treated mice and 1.69 ml (250 mg kg⁻¹ b.w.), 1.5 ml (500 mg kg⁻¹ b.w.) in AETP treated mice. Where as in the standard (5 FU, 20 mg kg⁻¹ b.w., *i.p*) treated mice it was found to be 1.2 ml.

Effect on mean survival time and % ILS

Effect of EETP and AETP on Mean survival time and % ILS of EAC bearing mice is shown in the Table 3. In the EAC control group the mean survival time was 17.5 days, while it increased to 29.5 (100 mg kg⁻¹ b.w) and 31 (200 mg kg⁻¹ b.w.) days respectively in EETP treated groups and it was found to be 26.8 (250 mg kg⁻¹ b.w.) and 28 (500 mg kg⁻¹ b.w.) days in AETP treated groups respectively. The group treated with standard drug 5-fluorouracil (20 mg kg⁻¹ b.w., *i.p*) showed the mean survival time of 33 days. Treatment with EETP at doses 100 and 200 mg kg⁻¹ b.w., AETP at doses 250 and 500 mg kg⁻¹ b.w. and the standard drug 5-fluorouracil (20 mg kg⁻¹ b.w., *i.p*) restored the % ILS values more or less similar to normal when compared with EAC control group.

Effect on viable and non viable cell count (cells $\times 10^7$ /ml)

The number of viable cells was found to be decreased, where as non viable cells number increased showed in Table 4. Viable cell count of EAC control group was found to be 7.9 \pm 0.19 and it was decreased to 4.0 \pm 0.51 (100 mg kg⁻¹ b.w.) and 2.2 \pm 0.11 (200 mg kg⁻¹ b.w.) respectively in case of EETP treated mice, and 2.95 \pm 0.25 (250 mg kg⁻¹ b.w.), 2.41 \pm 0.12 (500 mg kg⁻¹ b.w.) in AETP treated mice. In standard (5 FU, 20 mg kg⁻¹ b.w., *i.p*) treated mice it was found to be 5.3 \pm 0.37. Non viable cell count of EAC control group was 0.32 \pm 0.04 and that of treated groups were 1.4 \pm 0.08 (EETP, 100 mg kg⁻¹ b.w.), 1.99 \pm 0.07 (EETP, 200 mg kg⁻¹ b.w.), 2.5 \pm 0.03 (AETP, 250 mg kg⁻¹ b.w.), 2.1 \pm 0.09 (AETP, 500 mg kg⁻¹ b.w.) and 0.90 \pm 0.07 of 5-Fluorouracil (20 mg kg⁻¹ b.w., *i.p*) group respectively..

Effect on hematological parameters

In order to detect the influence of *Tephrosia purpurea* extracts on the hematological status of EAC bearing mice, a comparison was made among different groups of animals on the 14 th day after inoculation. The hematological profile showed significant changes when compared with the normal animals (Table 5).The total WBC counts were found to be increased with a reduction in the hemoglobin content of RBC. The differential count of WBC showed that the percentage of neutrophils increased while that of lymphocytes decreased. At the same time interval, treatment with different extracts of *Tephrosia purpurea* brought these altered parameters to near normal.

DISCUSSION

The present study revealed that both ethanol and aqueous extracts of *Tephrosia purpurea* roots at both dose levels 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 [30]. The ethanol and aqueous extracts delayed the cell division, thereby suggesting the reduction in EAC volume and increased survival time in mice.

Aqueous extract (250 and 500 mg kg⁻¹ b.w.) and ethanol extract (100 and 200 mg kg⁻¹ b.w.) 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 [31] and the extracts of this plant were able to meet this criterion. Myelosuppression and anemia (reduced haemoglobin) have been frequently observed in ascites carcinoma [32, 33]. Anemia encountered in ascites carcinoma mainly due to iron deficiency, either by haemolytic or myelopathic conditions which finally lead to reduced RBC number [34]. In this study, elevated WBC count, reduced haemoglobin and RBC count were observed in EAC control mice, and the oral administration of *Tephrosia Purpurea* 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 alkaloids, glycosides, flavonoids, phytosterols, phenolic compounds and tannins in ethanol extract and phytosterols and carbohydrates in aqueous extract. 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 [35]. The cytotoxicity and anticancer activity of ethanol 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 membrane 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 [36].

Table 1: Phytochemical screening of ethanol and aqueous extracts

S.No.	Test	Ethanol	Aqueous
1	Alkaloids	+	-
2	Carbohydrates	-	+
3	Phytosterols	+	+
4	Fixed oils and fats	-	-
5	Saponins	-	-
6	Phenolic compounds and tannins	+	-
7	Proteins and amino acids	-	-
8	Gums and mucilages	-	-
9	Volatile oil	-	-
10	Flavanoids	+	-
11	Glycosides	+	-

+ denotes the presence of the respective class of compounds.

- denotes the absence of the respective class of compounds.

Table 2: Effect of EETP, AETP and 5-Fluorouracil on Tumor growth in EAC inoculated Mice

Groups	Dose (mg/kg)	Tumor weight (gm)	Tumor volume (ml)
Control	-	4.15 ± 0.15	2.1 ± 0.12
Standard	20	0.95 ± 0.25***	1.2 ± 0.05***
EETP 100	100	2.05 ± 0.12***	1.4 ± 0.11***
EETP 200	200	1.28 ± 0.26***	0.8 ± 0.09***
AETP 250	250	2.25 ± 0.16***	1.69 ± 0.02*
AETP 500	500	1.85 ± 0.33***	1.5 ± 0.13**

Standard: 5-Flouro Uracil (20 mg kg⁻¹ b.w.i.p.), EETP 100: Ethanol Extract at dose 100 mg kg⁻¹ b.w., EETP 200: Ethanol Extract at dose 200 mg kg⁻¹ b.w., AETP 250: Aqueous Extract at dose 250 mg kg⁻¹ b.w., AETP 500: Aqueous Extract at dose 500 mg kg⁻¹ b.w. Each value is the Mean ± S.E.M for 6 rats. *P<0.05, **P<0.01, ***P<0.001 compared with control.

Table 3: Effect of EETP, AETP and 5-Fluorouracil on survival time and % ILS, in EAC inoculated Mice

Groups	Dose (mg/kg)	Mean survival time (days)	%ILS
Control	-	17.5 ± 0.22	
Standard	20	33.0 ± 0.25***	89
EETP 100	100	29.5 ± 0.33***	69
EETP 200	200	31.0 ± 0.16***	77
AETP 250	250	26.8 ± 0.33***	53
AETP 500	500	28.0 ± 0.19***	60

Standard: 5-Fluorouracil (20 mg kg⁻¹ b.w.i.p.), EETP 100: Ethanol Extract at dose 100 mg kg⁻¹ b.w., EETP 200: Ethanol Extract at dose 200 mg kg⁻¹ b.w., AETP 250: Aqueous Extract at dose 250 mg kg⁻¹ b.w., AETP 500: Aqueous Extract at dose 500 mg kg⁻¹ b.w. Each value is the Mean ± S.E.M for 6 rats. ***P<0.001 compared with control.

Table 4: Effect of EETP, AETP and 5-Fluorouracil on viable and non viable cell count

Groups	Dose (mg/kg)	Viable cells	Non viable cells
Control	-	7.9±0.19	0.32±0.04
Standard	20	5.3±0.37***	0.90±0.07***
EETP 100	100	4.0±0.51***	1.4±0.08***
EETP 200	200	2.2±0.11***	1.99±0.07***
AETP 250	250	2.95±0.25***	2.5 ±0.03***
AETP 500	500	2.41 ±0.12***	2.1 ±0.09***

Standard: 5-Fluorouracil (20 mg kg⁻¹ b.w.i.p.), EETP 100: Ethanol Extract at dose 100 mg kg⁻¹ b.w., EETP 200: Ethanol Extract at dose 200 mg kg⁻¹ b.w., AETP 250: Aqueous Extract at dose 250 mg kg⁻¹ b.w., AETP 500: Aqueous Extract at dose 500 mg kg⁻¹ b.w. Each value is the Mean ± S.E.M for 6 rats. ***P<0.001 compared with control.

Table 5: Effect of EETP, AETP and 5-Fluorouracil on hematological parameters in EAC inoculated Mice

Groups	W.B.C count (10 ³ cells /mm ³)	R.B.C Count (10 ⁶ cells /mm ³)	Hb (g %)	Lymphocytes (%)	Neutrophils (%)	Mono cytes (%)
Normal mice	8.62 ± 0.03	5.02 ± 0.09	16.23 ± 0.59	75 ± 0.92	28.5 ± 0.56	1 ± 1.95
Control	15.2 ± 0.35	2.85 ± 0.12	9.73 ± 0.49	33.5 ± 1.56	64 ± 1.95	2 ± 0.95
Standard	9.05 ± 0.36**	4.35 ± 0.08***	16.03 ± 0.26***	70.52 ± 1.52***	35 ± 0.79***	1 ± 0.65***
EETP 100	12.30 ± 0.20**	3.72 ± 0.02***	13.62 ± 0.21***	59.92 ± 2.16***	41.21 ± 0.98***	2 ± 0.85
EETP 200	11.05 ± 0.15**	3.98 ± 0.05***	13.98 ± 0.28***	62.15 ± 1.45***	39.92 ± 0.67***	1 ± 0.19***
AETP 250	12.59 ± 0.35**	3.5 ± 0.15*	12.85 ± 0.33***	56.15 ± 2.5***	45.3 ± 1.7***	2 ± 0.59
AETP 500	12.90 ± 0.29**	3.61 ± 0.23**	12.99 ± 0.35***	59.25 ± 2.23***	43.17 ± 0.92***	1 ± 0.36***

Standard: 5-Fluorouracil (20 mg kg⁻¹ b.w.i.p.), EETP 100: Ethanol Extract at dose 100 mg kg⁻¹ b.w., EETP 200: Ethanol Extract at dose 200 mg kg⁻¹ b.w., AETP 250: Aqueous Extract at dose 250 mg kg⁻¹ b.w., AETP 500: Aqueous Extract at dose 500 mg kg⁻¹ b.w. Each value is the Mean ± S.E.M for 6 rats. *P<0.05, **P<0.01, ***P<0.001 compared with control.

CONCLUSION

Ethanol and aqueous extracts of *Tephrosia purpurea* roots showed significant anti cancer activity, which may be due to the additive and synergistic activity of its phytochemicals. Further investigations to identify the active principles involved in this antitumor activity and the establishment of mode of action that was actively responsible for the anti cancer activity may prove to be worthwhile.

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