

Research Article

Evaluation of Antitumor Activity of *Cuscuta Reflexa* Roxb (Cuscutaceae) Against Ehrlich Ascites Carcinoma in Swiss Albino Mice

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Abstract

Purpose: The aim of this study was to investigate the antitumor effect of the chloroform and ethanol extract of the whole plant of *Cuscuta reflexa* Roxb. (Cuscutaceae) in Swiss albino mice against Ehrlich Ascites Carcinoma (EAC) cell line.

Methods: The antitumor activity of the chloroform and ethanol extracts of *Cuscuta reflexa* was evaluated against Ehrlich ascites carcinoma (EAC) tumor in mice at doses of 200 and 400 mg/kg body weight orally, respectively, while acute oral toxicity studies were performed to determine the safety of the extracts. Briefly, the EAC cells were injected (i.p.) into ninety six mice (divided into 6 numerically equal groups), and after a one-day incubation period, the extracts were administered to the mice daily for 16 days. On day 21, six animals in each group were sacrificed for observation of antitumor activity and the remaining animals were observed to determine host the life span. Antitumor effect was determined by evaluating tumor volume, viable and nonviable tumor cell count and hematological parameters of the host. The standard antitumor used was 5-fluorouracil.

Results: Administration of the extracts resulted in a significant ($p < 0.05$) decrease in tumor volume and viable cell count, but increased non-viable cell count and mean survival time, thereby increasing the life span of the tumor-bearing mice. Restoration of hematological parameters - red blood cells (RBC), hemoglobin, white blood cells (WBC) and lymphocyte count - to normal levels in extract-treated mice was also observed.

Conclusion: The results suggest that the chloroform and ethanol extracts of *C. reflexa* exhibit significant antitumor activity in EAC-bearing mice that is comparable to that of the reference standard, 5-fluorouracil.

Keywords: *Cuscuta reflexa*, Ehrlich ascites carcinoma, 5-Fluorouracil, Tumor volume, Viable cell count.

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INTRODUCTION

Malignancy is one of the most serious diseases afflicting mankind today and indeed, the second most deadly disease after heart disease. According to WHO estimates, more than 7 million people died of cancer in 2005[1]. There exists a close relationship between the occurrence, growth and decline of tumor. In recent times, the demand for more effective and safer therapeutic agents for the chemoprevention of human cancer has increased. Natural products produced by plants and their synthetic derivatives are expected to play an important role in the development of innovative agents to inhibit the onset of cancer [1]. There is a growing interest in the pharmacological evaluation of various plants used in the Indian traditional system of medicine.

Cuscuta reflexa (Cuscutaceae), known in Hindi as Amarbela, is a phanerogamic stem parasite, and is distributed worldwide. The species are rootless, leafless twining annual parasites with yellowish stems, distributed across tropical and temperate regions, and in India about 6 species are found. It grows on thorny or other shrubs, sometimes completely covering bushes and trees [2]. *C. reflexa* spread from one host to another, and on each victim, they twine and cling tightly with special branching organs called houstoria, penetrating the host and connecting to the host xylem as well as to the host phloem, and absorbing from it both water and elaborated food stuffs such as sugars and amino acids [3]. Various parts of this plant are used in tribal medicine for the diseases such as impotence, premature ejaculation, sperm leakage, frequent urination, ringing in the ears, lower back pain, sore knees, leucorrhea, dry eyes, blurred vision, and tired eyes.

Cuscuta is one of nine herbs included in the manufacture of Equiguard, a Chinese herbal medicine recommended for kidney and prostate disorders. Research performed at New York Medical College indicates that the

combination of ingredients in Equiguard may well be effective in the treatment of prostate cancer. The preparation inhibited the growth of cancer cells, increased the rate of self-destruction (apoptosis) of cancer cells, and prevented the surviving cells from forming colonies [4]. Phytochemical investigation of *Cuscuta reflexa* indicates the presence of kaempferol-3-O-glucoside, astragallic acid [5], myricetin, benzopyrones [6], glucopyranosides [7], propenamide, flavonols [8], quercetin and quercetin-3-O-glucoside, β -sitosterol, and bergenin [9].

The objective of the present study was to evaluate the antitumor effect of *Cuscuta reflexa* against Ehrlich's Ascites Carcinoma (EAC) in Swiss albino mice.

EXPERIMENTAL

Plant materials

Whole plants were collected from PNT Square, Jawahar Chowk, Bhopal (M.P.) India, during the months of January and February 2007. The species was identified by the local people during the time of collection and later authenticated by Dr Pradeep Tiwari, taxonomist, Department of Botany, Dr. H.S. Gour University, Sagar (M.P.), India. A voucher specimen (no. BOT/412/123) was prepared and preserved along with the crude drug sample at the herbarium of Department of Botany, Dr. H.S. Gour University, Sagar (M.P.), India. The whole plant was shade-dried, reduced to coarse powder and stored in an airtight container until further use.

Preparation of extract

The powdered drug (1 kg) was packed in a Soxhlet apparatus and extracted for 24 h with petroleum ether (60 - 80 °C) to defat the drug. The defatted material was then extracted with chloroform (2.5 L). The chloroform extract was separated while the marc was extracted with ethanol (2.5 L, 95 %). The solvent, in each case, was removed by evaporation and the residual solvent

eliminated under reduced pressure. Separate solutions of the chloroform and ethanol extracts were prepared in two doses - 200 and 400 mg/kg - for the antitumor test.

Experimental animals

Swiss albino mice, weighing (23 – 25 g), were used in this study. They were maintained in a well-ventilated room at a temperature of $25 \pm 1^\circ \text{C}$ with 12/12 h light/dark cycle in polypropylene cages. Standard pellet feed and tap water were provided *ad libitum* throughout the experimentation period. The animals were acclimatized to laboratory conditions for 10 days prior to initiation of experiments. The project proposal was approved by the Institutional Animal Ethical Committee (ref. no. IAEC/A/500/2001).

Acute oral toxicity study

Acute oral toxicity test was performed by following OECD guideline – 420 [10]. Increasing doses of the extracts, up to 2000 mg/kg body wt, were administered to the mice.

Antitumor studies

Ehrlich ascites carcinoma (EAC) cells were obtained from the National Centre for Cell Science, Pune, India and maintained *in vivo* in Swiss albino mice, by intraperitoneal (i.p.) transplantation of 1×10^6 cells/mouse every 10 days. The mice were divided into six groups of 16 animals each. EAC cells were collected from the donor mice and suspended in sterile isotonic saline (0.9 % NaCl). The viable EAC cells were counted (with the aid of trypan blue staining) under the microscope and were adjusted to 10^6 cells/mL. An amount (0.1 mL) of these cells per 10 g body weight of the animals was injected (i.p.) into the animals on day 0. One day of incubation was allowed for multiplication of the cells.

Ninety six animals were used for study and they were divided into six groups of 16

animals each. Group I served as control mice and was administered 0.9 % NaCl solution for 16 days. Group II EAC mice administered orally chloroform extract (200 mg/kg). Group III received orally chloroform extract (400 mg/kg); Group IV ethanol extract (200 mg/kg, orally); Group V ethanol extract (400 mg/kg, orally); and Group VI the standard drug, 5-fluorouracil (20 mg/kg, i.p.). The treatments continued daily for 16 days. On day 21, six animals from each group were sacrificed and the remaining animals were kept for further observation for 3? weeks to determine host life span.

Determination of antitumor parameters

Tumor volume: The mice were dissected and the ascitic fluid was collected from the peritoneal cavity. The fluid volume was measured in a graduated centrifuge tube and packed cell volume was determined after centrifuging (REMI, R4C and REMI Group) at 1000 rpm for 5 min.

Viable/non-viable tumor cell count: The cells from the preceding test were stained with trypan blue (0.4 % in normal saline) dye. The cells that took up the dye were non-viable while those that did not were viable. Viable and nonviable cells were counted and cell count computed as in Eq 1.

$$\text{Cell count} = C/(A \times T) \dots\dots\dots (1)$$

where C is the number of cells (viable or nonviable) multiplied by the dilution factor, A is the area occupied by the liquid film and T is the thickness of the liquid film.

Increase in life span: The effect of extract on tumor growth was monitored by recording mice mortality daily for a period of 6 weeks from the starting day and the percent increase in life span (ILS) was calculated for each animal group with respect to the control [15-18].

Hematological parameters: At the end of the experimental period, all the mice were sacrificed by cervical dislocation. Blood was collected from a freely flowing tail vein and used for the estimation hemoglobin (Hb) content, as well as for red blood cell (RBC)

and white blood cell (WBC) counts. WBC differential count was carried out using Leishman stained blood smears [11-17].

Statistical analysis

The results of estimation of biochemical and functional parameters are reported as mean value \pm standard error of mean (SEM). Variation within a set of data assessed by one-way analysis of variance (ANOVA) while individual comparison of group mean values by Dunnett's test (Sigma Stat 3.5). *P* values < 0.05 were considered statistically significant.

RESULTS

Antitumor activity

The extracts elicited tumor growth response with respect to packed cell volume, viable and non-viable cell counts and increase in life span. Table 1 indicates that the extracts, at doses of 200 and 400 mg/kg, respectively, as well as the reference standard drug, 5-fluorouracil, reduced significantly ($p < 0.05$) tumor volume, when compared with the control group. A similar finding was made for viable cell count. The number of non-viable cell count significantly ($p < 0.05$) increased in ethanol extract-treated (at a dose of 400 mg/kg) and reference standard groups, but no significant increase was observed in other extract-treated groups.

As Table 3 shows, median survival time and life span of the mice increased following administration of the extracts at both doses (200 and 400 mg/kg), thus indicating that the extracts exhibited a remarkable capacity to inhibit the growth of solid tumor induced by EAC cell line in a dose-dependent manner in experimental animals.

Acute toxicity

The extracts of *C. reflexa* did not provoke any gross behavioral changes or manifestations of toxic symptoms in the animals, such as weight loss, increased motor activity, tremors,

spasticity, loss of right reflex, decreased motor activity, ataxia, sedation, muscle relaxation, hypnosis, arching and rolling, lacrimation, salivation, watery diarrhea, writhing and urination, over an observation period of 24 h. The extracts were non-lethal even at the maximum single oral dose of 2000 mg/kg.

Table 1: Antitumor activity of *Cuscuta reflexa* extracts with regard to tumor volume, viable tumor cells count and nonviable tumor cells count (mean \pm SEM, n=6)

Treatment	Tumor volume (ml)	Viable tumor cells count (10^6 cells/mouse)	Non-viable tumor cells count (10^6 cells/mouse)
Control	5.51 \pm 0.38	12.12 \pm 0.45	0.38 \pm 0.10
CHCl ₃ extract (200 mg/kg)	5.11 \pm 0.32	9.60 \pm 0.25*	0.43 \pm 0.05
CHCl ₃ extract (400 mg/kg)	3.98 \pm 0.41*	5.48 \pm 0.41*	0.62 \pm 0.03
Ethanol extract (200 mg/kg)	4.20 \pm 0.30*	8.13 \pm 0.44*	0.56 \pm 0.04
Ethanol extract (400 mg/kg)	2.5 \pm 0.03*	3.06 \pm 0.51*	0.96 \pm 0.11*
5-fluorouracil (20 mg/kg)	2.31 \pm 0.21*	2.55 \pm 0.34*	0.83 \pm 0.07*

**P* < 0.05 compared to control group

Hematological effects of the extracts

Table 3 shows that after 16 days of treatment, the hematological parameters of the mice were significantly ($p < 0.05$) altered, compared to the control group. While total WBC count increased and Hb content of RBC decreased in the control group, RBC count and hemoglobin content increased in extract-treated groups. Furthermore, there was decrease in WBC count, as well as counts of neutrophils and monocytes in extract- and reference standard-treated groups, while lymphocyte count rose for these groups.

Table 2: Antitumor activity of *Cuscuta reflexa* extracts with regard to median survival time and increase in life span (mean±SEM, n=10)

Treatment	Median Survival time (days)	Increase in life span (%)
Control	28.32±2.51	100
CHCl ₃ extract (200 mg/kg)	34.62±1.14	121
CHCl ₃ extract (400 mg/kg)	37.40±2.73	132
Ethanol extract (200 mg/kg)	37.11±1.58	131
Ethanol extract (400 mg/kg)	43.26±3.10	152
5-fluorouracil (20 mg/kg)	46.59±2.43	164

DISCUSSION

The results of the present study indicate the tumor inhibitory activity of both the chloroform and ethanol extracts of *Cuscuta reflexa* against EAC cells. Usually, the parameters for evaluating the antitumor activity of a drug are prolongation of life span, reduction in tumor volume and improvement in the hematological parameters of the host.

Ascitic tumor implantation promotes local inflammatory reactions leading to increase in vascular permeability, and results in intense edema formation, cellular migration and progressive ascitic fluid formation. Ascitic fluid is essential to tumor growth, since it constitutes the direct nutritional source for tumor cells. Decrease in viable cell count and increase in non-viable cell count in tumor-bearing mice suggest antitumor activity against EAC cells in mice [18, 19].

Biological agents, such as interferons and interleukins, provide non-specific active immunity (indirect cytotoxic action), whereas monoclonal antibodies provide passive immunity (direct cytotoxic action). Interferons are the small proteins synthesized by the immune cells in response to various stimuli, such as viral infections and cytokines, and inhibit viral replication and promote cellular (T cell) immune response. Interferons act via specific cellular receptors linked with JAK-STAT (Janus Kinase-Signal Transducer and Activator of Transcription) pathway to stimulate the formation of specific proteins which mediate their actions. Their antitumor action is complex, and includes antiproliferative effects, promotion of differentiation, immunomodulation, alteration

Table 3: Effect of *Cuscuta reflexa* extracts on hematological parameters of Swiss albino mice (mean ± SEM, n = 6)

Treatment	Hb content	Total RBC (cells/ml×10 ⁶)	Total WBC (cells/ml×10 ⁶)	Differential count		
				Lymphocyte (%)	Neutrophil (%)	Monocyte (%)
Control	12.1±0.3	1.29±0.01	17.5±0.28	20.1±3.2	65.2±7.3	4.2±0.5
CHCl ₃ extract (200mg/kg)	12.6±0.2	1.31±0.03	15.17±0.30*	39.5±4.2	53.7±6.2	3.9±0.2
CHCl ₃ extract (400mg/kg)	14.3±0.2*	1.35±0.02	11.2±0.35*	50.6±4.8	32.4±5.8*	3.1±0.4
Ethanol extract (200mg/kg)	13.5±0.2*	1.36±0.04	12.67±0.27*	43.2±5.1*	42.6±5.4*	3.4±0.5
Ethanol extract (400mg/kg)	15.9±0.3*	1.45±0.05*	7.03±0.24*	66.6±4.6*	24.5±3.8*	2.8±0.3*
5-fluorouracil (20 mg/kg)	16.1±0.1*	1.42±0.05*	7.31±0.26*	62.5±3.7*	26.7±7.6*	2.6±0.6*

*P <0.05 compared to control group

in tumor cell surface antigen expression, inhibition of oncogene activation, and angiogenesis. The cytokines produced in the body by the lymphocytes are known as interleukins and they mediate cytotoxic actions through the cell surface receptors in relevant target cells. Interleukins stimulate the growth and activity of immune cells, which target cancer cells. It acts as an antitumor agent by increasing the cytolytic activity of antigen-specific cytotoxic T lymphocytes and natural killer (NK) cells and by increasing the gene expression responsible for encoding the lytic component of cytotoxic granules, that is, perforin and granzymes.

Monoclonal antibodies are the clones of similar antibodies that are directed against specific target antigens. Tumor cells express a wide variety of antigens that are attractive targets for monoclonal antibody-based therapy. These antibodies can activate the immune functions and facilitate the destruction of malignant cells by complement-dependent cytotoxicity and antibody-dependent cell-mediated cytotoxicity.

Reduction in viable cell count and increased non-viable cell count towards normal in tumor host suggested that extracts stimulate the growth and activity of immune cells by the production of Interleukins, which target tumor cells and cause lysis of the tumor cells by indirect cytotoxic mechanism. Furthermore, the reduced volume of EAC and increased survival time of the mice suggest that the extracts might have exerted a delay in vascular permeability to the cells [20]. While the standard drug, 5-fluorouracil as a pyrimidine analogue, is transformed inside the cell into different cytotoxic metabolites which are then incorporated into DNA and RNA, eventually inducing cell cycle arrest and apoptosis by inhibiting the cell's ability to synthesize DNA. The standard drug activity thus indicates a gene expression mechanism, which is indirect cytotoxicity. The antitumor

activity of the extracts appear to also follow this mechanism.

Preliminary phytochemical screening indicated the presence of flavonoid, triterpenoids, alkaloids and tannins in the ethanol extract, and phytosterols, triterpenoids and alkaloids in the chloroform extract. It has been reported that flavonoids possess antimutagenic and antimalignant effects. Moreover, flavonoids have a chemopreventive role in cancer through their effects on signal transduction in cell proliferation and angiogenesis. The antitumor properties of the extracts may be due to these compounds. Phytosterols are able to be incorporated into the cell membrane, where they 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 [21,22]. This supports the indirect cytotoxic mechanism of both extracts.

The results also revealed that the ethanol extract showed significantly higher antitumor activity than the chloroform extract; this may be due to the presence of triterpenoids, alkaloids and tannins which might have enhanced the antitumor property of the flavonoids in the extract. On the other hand, triterpenoids and alkaloids in the chloroform extract appear not to have enhanced the antitumor property of phytosterols in the extract.

Carcinogenesis or tumor development generates highly toxic and diffusible reactive oxygen species that cause extensive cell damage or adducts on the biomolecules, contributing to malignant transformation. Erythrocytes membranes are particularly susceptible to oxidative stress due to its high content of polyunsaturated fatty acids, which are more vulnerable to oxidative damages. The erythrocytes are particularly susceptible to peroxide stress due to high content of iron, a potent catalyst for the production of reactive oxygen species and due to continuous

challenge with high oxygen tension [23]. Susceptibility of erythrocytes to peroxides was confirmed by the elevated WBC count, reduced hemoglobin and RBC count, as observed in the control group. The constituents of the extracts probably facilitated improvement in hematological parameters by scavenging reactive oxygen species and improving the antioxidant defense system, thus indicating that the extracts exerted haematopoietic protective activity as well as antioxidant property [24,25].

CONCLUSION

The chloroform and ethanol extract of *Cuscuta reflexa* possess significant antitumor activity and increased the life span of tumor-bearing mice. Further investigations are in progress in our laboratory to identify the active principles involved in this antitumor activity.

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