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Research Article

Tumor inhibitory activity of methanolic and ethyl acetate soluble extracts of *Thuja occidentalis L.* on mice bearing Ehrlich ascites carcinoma

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Abstract

Thuja occidentalis (Cupressaceae) is an ornamental plant of European origin. It has been used in folk medicine for the treatment of cancer. Mice bearing Ehrlich Ascites Carcinoma (EAC mice) were treated with methanolic extract (165 mg/kg), ethyl acetate soluble fraction (30 mg/kg) and combination of both extracts of *Thuja occidentalis*. Inhibition of tumor growth, increase in survival time of animal with treatment, and hematological parameters were determined. Both extracts exerted tumor growth inhibitory activity in mice bearing EAC. Combination treatment of two extracts showed more pronounced effect. In conclusion, Methanolic and ethyl acetate soluble extracts of *Thuja occidentalis* exhibit anticancer activity against Ehrlich ascites carcinoma in mice. Thus, it has anticancer potential and should be further evaluated in higher models.
Keywords: *Thuja occidentalis, Ehrlich ascites carcinoma, antitumor, mice*

1. Introduction

Thuja occidentalis L. (Cupressaceae) is an ornamental plant of European origin. It has been used in folk medicine for the treatment of cancer.[1-4] Methanolic extract of leaves of *T. occidentalis* (TO) has shown antiangiogenic activity on tumor specific angiogenesis in B16F10 melanoma in C57BL/6 Mice as well as in vitro in rat aortic ring assay.[5] Angiogenesis has been established as a potential target for inhibition of neoplastic growth.[6] Ethanolic extract and thujone rich fraction of TO demonstrated apoptotic and antiproliferative potentials against A549 and A375 cell line in vitro respectively.[7,8] In addition to this, ethyl acetate soluble fraction (ESF) of TO is reported to inhibit malignant transformation of JB6 cells induced by tumor promoter TPA. ESF extract exerts this effect partly by cytotoxicity and partly by ornithine decarboxylase (ODC) inhibitory activity.[9] ODC is the enzyme involved in the synthesis of polyamines- putrescine, spermine, and spermindine. Putrescine and other polyamines are essential for cell proliferation. Deranged polyamine metabolism may be an important factor in carcinogenesis.[10] Depletion of polyamines inhibits growth of neoplastic cells in vitro and in animal models.[11] Thus, the polyamine-biosynthetic pathway represents an inviting target for the development of agents inhibiting carcinogenesis and tumor growth.[12] TO has shown to reduce tumor metastasis by stimulating cell-mediated immune system and decrease pro-inflammatory cytokines. [13]

Thus, several studies are done on antitumor role of TO including its antimetastatic potential, but no data is available regarding its direct effect on tumor load. In present study, we attempt to demonstrate its effect on tumor load in the primary screening model for antitumor activity i.e. Ehrlich ascites carcinoma in mice.

2. Materials and methods

2.1 Plant Material

Fresh leaves and twigs of TO were obtained from a garden at Baroda, Gujarat, and were authenticated by Dr. B. L. Pujani, P.G. Centre in botany, Smt S.M. Panchal Science College, Talod, Gujarat.

2.2 Preparation of plant extracts

The methanolic extract was prepared by exhaustive extraction of the plant material in a soxhlet extractor. For ethyl acetate soluble fraction, the plant material was extracted exhaustively by maceration with methanol-water. The resultant extract was partitioned with petroleum ether and ethyl acetate, respectively to obtain petroleum ether soluble and ethyl acetate soluble fraction (ESF).[9] Both the extracts were dried and dissolved in appropriate solvent to get desired concentrations.

2.3 Animals

Swiss albino mice, weighing 25-30 gm were used. The mice were maintained under standard laboratory conditions at 25±2°C, relative humidity 50+15% and normal photo period (12h dark/12 h light). Commercial pellet diet and water were provided *ad libitum*. The animal experiments were conducted according to protocol that was approved by the Institutional Animal Ethics Committee, Protocol No: KBIPER/0551.

2.4 Tumor cells

Ehrlich Ascites Carcinoma (EAC) cells were obtained as monolayer culture from National Centre for Cell Science (NCCS), Pune and were maintained by weekly intraperitoneal (i.p.) inoculation of 10^5cells/mouse.

2.5 Tumor load in animal

2 X 10^5 EAC cells were inoculated into 4 groups of mice (12 in each) on day 0. Group 1 received 0.5ml of saline i.p. Group 2 and 3 were treated with methanolic extract (165 mg/kg) and ESF (30 mg/kg) i.p. respectively. The fourth group was treated with both extracts. Treatment was started on day 1 and was continued for 4 days. On 5th day 6 animals from each group were sacrificed. Ascites fluid containing tumor cells was collected from the peritoneal cavity of the animal. Remaining tumor cells were collected by repeated washing with known volume of saline.[14] Viable tumor cells were counted by trypan blue test using a hemocytometer. Results were expressed as number of tumor cells per ml.[15] Tumor load in the animal was calculated by multiplying number of viable tumor cells per ml with volume of ascites fluid (ml) for each animal.

2.6 Survival time assay

Treatment to rest of the animals in group was continued as before. Survival time of animals was recorded. Average of *life span of animals* in each group gives Mean Survival Time (MST) for that treatment group. Survival time of treated group was compared with that of control group using the following calculation[16].

$$\% \text{ increase in survival time} = \left(\frac{MST\ of\ treated\ group \times 100}{MST\ of control\ group}\right) - 100$$

2.7 Hematological Studies

In order to detect the influence of TO treatment on the hematological status of EAC bearing mice, total and differential WBC counts were determined on day 5 of tumor inoculation and were compared with normal mice. Cell counts were made using hemocytometer. Differential WBC count was performed using Leishman's stain.

2.8 Statistical analysis

Results are expressed as mean ± SEM. Significant differences among the groups were determined by one-way ANOVA followed by Dunnett's test using 12th version of SPSS. Differences were considered significant if $P < 0.05$.

3. Results

3.1 Tumor load in animal

Treatment with both the extracts (methanolic and ESF) of TO resulted in a significant reduction in tumor load in animals as compared to control group (Table 1). Tumor load is a product of Tumor cell density (cells per ml) and volume of ascites (ml). All the three treatments showed a significant reduction in tumor cell density as compared to control group. Methanolic extract treatment and combination treatment also resulted in a significant decrease in volume of ascites tumor. (Table 2)

Table 1: Effect of TO treatment on tumor load

Group (N=6)	Number of tumor cells per animal (x10^6 cells)	% Reduction in tumor load
Control	3.36 ± 0.20	-
Methanolic Extract treatment	2.29 ± 0.26*	31.84
ESF treatment	2.14 ± 0.13*	36.30
Combination treatment	1.4 ± 0.1*	58.33

Results are expressed as Mean ± SEM. N is number of animals in each group.
* Values significantly different from control group, $p < 0.05$.

Table 2: Effect of TO treatment on Tumor cell density and Volume of Ascites tumor

Group (N=6)	Tumor Cell Density (x10^6 cells/ml)	Volume of Ascites tumor (ml)
Control	4.96 ± 0.13	6.97 ± 0.17
Methanolic Extract treatment	4.23 ± 0.18*	4.77 ± 0.33*
ESF treatment	3.52 ± 0.19*	6.07 ± 0.22
Combination treatment	2.47 ± 0.20*	5.72 ± 0.29*

Results are expressed as Mean ± SEM. N is number of animals in each group.
* Values significantly different from control group, $p < 0.05$.

3.2 Survival Time Assay

All the three treatments increased the survival time of mice, however a more notable increase was observed with ESF treatment and combination treatment.

Table 3: Effect of TO treatment on Mean survival time of animal

Group (N=6)	Mean Survival time (Days)	% increase in survival time
Control	17.16 ± 0.87	-
Methanolic Extract treatment	22.5 ± 0.56*	31.11
ESF treatment	25 ± 1.86*	45.68
Combination treatment	27 ± 1.91*	57.34

Results are expressed as Mean ± SEM. N is number of animals in each group.
* Values significantly different from control group, $p < 0.05$.

3.3 Hematological Studies

Hematological parameters of tumor bearing mice on day 5 were found to be significantly altered from normal group. The total WBC count was found to be increased. In differential count of WBC, the percent of neutrophils increased while the lymphocyte count decreased. Treatment with TO extracts could prevent this disturbance in WBC counts (Table 4).

Table 4: Effect of TO treatment on Hematological parameters

Group (N=6)	WBC count (x10^6 cells/ml)	Lymphocytes (%)	Segmented granulocytes (%)	Monocytes (%)
Normal	8.5 ± 0.76	70.3 ± 1.2	28.0 ± 1.52	1.6 ± 0.33
Control	18.1 ± 0.79	46.6 ± 0.88	52.6 ± 0.88	0.6 ± 0.1
Methanolic Extract treatment	14.2 ± 0.55*	56.8 ± 2.13*	43.5 ± 1.34*	1.0 ± 0.05*
ESF treatment	13.5 ± 0.68*	59.2 ± 1.91*	41.8 ± 2.4*	1.1 ± 0.07*
Combination treatment	12.4 ± 0.7*	63.6 ± 2.6*	34.6 ± 3.48*	1.6 ± 0.09*

Results are expressed as Mean ± SEM. N is number of animals in each group.
* Values significantly different from control group, $p < 0.05$.
Normal group: Saline treated animals with no tumor

4. Discussion

Tumor load in the animal is an expression of volume of ascites tumor and tumor cell density in animal. Increased volume of ascites fluid in the tumor bearing mice is a result of angiogenesis and/or increased vascular permeability. Methanolic extract of TO shows a more pronounced effect on volume of ascites tumor, which indicates that inhibition of angiogenesis and/ or prevention of increase in vascular permeability may be its probable mode of antitumor action. The antiangiogenic activity of methanolic extract of TO has been demonstrated earlier on tumor specific angiogenesis in B16F10 melanoma in C57BL/6 mice as in vitro on rat aortic ring assay.[5] In this study methanolic extract of TO was able to reduce serum titers of proinflammatory cytokines, namely IL 1, IL 6, TNF-α, and GM-CSF. These cytokines have proangiogenic activity and are known to increase vascular permeability.[17] Thus, decrease in ascitic fluid volume observed with methanolic extract treatment might be due to reduction in cytokine titres.

All the three treatments show a significant reduction in tumor cell density. This may reflect the cytotoxic potential of the drug. The observed results are in confirmation with earlier reports showing the inhibitory effect of ESF treatment on cell transformation and tumor cell cytotoxicity.[9] Of the six different constituents identified from ESF of TO, (1S,2S,3R)-(+)-isopicrodeoxypodophyllotoxin, (-)-deoxypodophyllotoxin, and (-)-deoxypodorhizone are reported to produce in vitro cytotoxicity against KB cells. While other four compounds (+)-7-oxo-13-epi-pimara-14,15-dien-18-oic acid, (+)-7-oxo-13-epi-pimara-8,15-dien-18-oic acid, (+)-isopimaric acid, (1S,2S,3R)-(+)-isopicrodeoxypodophyllotoxin are shown to inhibit ODC activity in ME-108 cells. Expression of Ornithine decarboxylase is transiently increased upon stimulation by growth factors, but becomes constitutively activated during cell transformation induced by carcinogens, viruses or oncogenes. Another study shows that the gene encoding ODC is a proto-oncogene which becomes activated in many types of cancers.[18] Polyamine-biosynthetic pathway mediated by ODC represents an inviting target for the development of agents inhibiting carcinogenesis and tumor growth.[10] Thus, reduction in tumor load due to ESF treatment may be mainly mediated through its action on ODC.

The total WBC count was found to be increased with a reduction in lymphocyte count in tumor bearing mice. The combination treatment with TO extracts was able to normalize the levels significantly. Perturbation of hematological parameters in tumor bearing animals is partly responsible for the toxic effects produced in them. In addition, myelosuppression in cancer chemotherapy is a common phenomenon which is responsible for poor prognosis.[19] This improvement of haematological parameter may reflect its potential to give better prognosis on treatment.

5. Conclusion

The methanolic extract and ESF treatment shows decrease in tumor load and increase in survival time of animals. This indicates that it has antitumor potential. Angiogenesis inhibitory activity, reduction in cytokine titers and cytotoxic action against tumor cells may be the possible modes of action. However, it needs further study for complete evaluation. Improvement of hematologic parameters adds assets to its antitumor action.

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