ANTITUMOR EFFECT OF AMALAKYADI CHURNA ON EAC TUMOR BEARING MICE: A PRELIMINARY STUDY

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Summary

The antitumor efficacy of the ethanolic extract of Amalakyadi churna (Ayurvedic formulation) was evaluated against Ehrlich Ascites Carcinoma (EAC) tumor model in Swiss albino mice. The detailed analyses of the antitumour activity of Amalakyadi churna (AC) was assessed based on certain parameters such as, percentage increase in life span (% ILS), median survival time (MST), body weight and other morphological changes at doses of 40mg/kg for 10 days, 80, 105 mg/kg for 7 days, 160, 180mg/kg for 6 days and 210mg/kg b.wt. for 5 consecutive days intraperitoneally, exactly after 24 hours of tumor induction. The ethanolic extract (95%) of AC at 40, 80, 105 and 160 mg/kg b.wt. showed significantly increase in the survival time, life span and reduced body weight of the drug treated tumerous mice near to normal and there by reducing the rate of tumor incidence. But in case of higher doses at 180 and 210mg/kg were absolutely reduced the risk of tumor incidence, but due to drug toxicity, survival time was not increased significantly. The results of the current studies clearly demonstrated that, the AC possesses significant antitumor activity at milder and effective dose levels.

Key Words: Amalakyadi churna (AC)
Ehrlich Ascites Carcinoma (EAC)
Median Survival Time (MST)
Percentage Increased Life Span (%ILS)

Introduction

Cancer is now recognized as a global problem and it has become a leading cause of mortality next to heart attack ranks second, accounting for 23% of all deaths (1). However, it is a mysterious illness with no known cause and no known cure. However, during the past 20 years the situation has been transformed. Our understandings of the underlying disorders and effective treatments have been evolved for several kinds of cancer. The evidences from fossil remains bone sarcomata and the hike and of other osseous tumors bear witness to the antiquity of cancer and proved that it is by no means a modern disease although its incidence may be greatly enhanced by modern environmental conditions (2).

It is evident that the use of plants for various diseases since from Vedic period. However, a compilation of 1400 plant genera, which are used as drug for various malignant and simple tumors, was made by Hartwell (3). Despite the tremendous advancement in molecular biology and chemistry providing fast escalation of synthesized *de-novo* drugs, plants still remains as a traditional source of medicinal compounds. Up to 40% of modern drugs may directly/ indirectly be related to natural compounds. Several plant derived compounds have been approved as anticancer drugs, vinblastine, vincristine, etoposide, teniposide, taxol, taxotere, topotecan and irinotecan etc (4). Kalpaamrutha is one of the polyherbal formulation tested for anticancerous activity (5), Ayurvedic rasayanas in inhibition of lung metastasis produced by B16F10 melanoma cells (6). Much interest surrounds the potential for individual plant ingredients of Amalakyadi churna in cancer treatment. However, there are no published clinical trials or epidemiological data on this Amalakyadi churna.

By considering the important bioactivities, particularly immunomodulating properties and also widely used of the plant ingredients of Amalakyadi churna in traditional systems of medicine for various diseases, it is necessitated to explore the synergistic potential of all these above useful plants to get total therapeutic disease targets.

Materials and methods

Collection of Plant Materials: The plant materials of *Phyllanthus emblica* L., *Terminalia chebula* Retz., *Plumbago zeylanica* L,. from Sandur, *Piper longum* L. from the Agricultural University, GKVK, Bangalore, India in the month of October-November and authenticated at the herbarium of Department of Botany, Gulbarga University, Gulbarga. The rock salt was purchased from the local Ayurvedic shop, Gulbarga.

Preparation of Amalakyadi churna: The pre-cleaned dried powders of fruits of *Phyllanthus emblica, Piper longum, Terminalia chebula*, roots of *Plumbago zeylanica* and rock salt were taken in equal proportions and mixed well. This Amalakyadi churna was stored in an airtight container for further processing (7)

Preparation of the Extracts: The 100g of AC was extracted with 90% alcohol at 50 - 60°C in a soxhlet apparatus. The extract was concentrated to dryness in a flash evaporator (Buchi type) under reduced pressure with controlled temperature (40 - 50°C) and note down the yield of crude extract.

Animals: The mice (30 – 35g) were housed in polypropylene cages containing sterile paddy husk as bedding and maintained under controlled conditions of temperature (23±2°C), humidity (50 ± 5%) and light (10 : 14 hours of light and dark respectively). The mice were fed with balanced diet prepared in the laboratory and water adlibitum and maintained as per the norms and conditions laid by the Institutional ethical committee.

Acute Toxicity Studies: Swiss albino mice of either sex weighing 18-22g were randomly distributed to ten different groups of 10 animals each. The animals were fasted overnight. The 1^{st} group was given double distilled water (0.2ml/mouse), that is vehicle control. The 2^{nd}, 3^{rd}, 4^{th}, 5^{th} and 6^{th} groups were received acute doses of 200, 250, 300, 350 and 400mg/kg b.wt. churna extract intraperitoneally, respectively. Where as 7^{th}, 8^{th}, 9^{th} and 10^{th} groups were given orally at 500mg, 1g, 2g and 3g/kg b.wt. LD_{50} value was determined for 72 hours. The crude extract is weighed and dissolved in water and this aqueous suspension was administered intraperitoneally in all experiments. All treated mice were observed continuously for 6 hours and the LD_{50} value was determined in 72 hours by using standard method (8)

Tumor Cells: The tumor cells of EAC was obtained from the intraperitoneal cavity of the donor EAC tumor bearing mice in the ascites form and was maintained by serial transplantation. The EAC cells were maintained *in vivo* in Swiss albino mice serially by intraperitoneal transplantation of 10^6 cells/ mouse after every 7-10 days. Before tumor inoculation, a small portion of ascites fluid (100µl) was tested for bacterial contamination, tumor cell viability was determined by Trypan-Blue Exclusion test and cells were counted using hemocytometer.

Tumor cell viability test: The tumor cell suspension was diluted with 0.1% trypan blue in saline using WBC diluting pipette. It was mixed well and loaded into hemocytometer. The viable cells were counted using hemocytometer (9).

$$\text{Cell count} = \frac{\text{Number of cells X Dilution}}{\text{(Area X Thickness of fluid film)}}$$

Antitumor activity: The tumor cells were diluted by Dulbecco's Modified Eagle's Medium (DMEM), so as to get 10^6 cells / 0.2ml of the tumor cell suspension and injected into 70 mice, 0.2ml of 10^6 cells/ mouse. The AC (drug) treatment was started exactly after 24 hours of tumor cell induction. All the mice were weighed on the day of tumor inoculation. The first group of mice was given mainly 0.2 ml of 30% PEG-400 (vehicle control) for 10 days. The second groups of mice were given only 40mg/kg b.wt. of AC extract for 10 days continuously. The 3rd, 4th groups were given 80, and 105mg/kg b.wt. respectively, for 7 days continuously. The 5th and 6th groups of mice were received 160 and 180 mg/kg b.wt for 6 days and 7th group was given 210mg/kg b.wt for 5 consequent days. The body weight of and mortality was recorded throughout the observation period. The tumerous growth was assessed on the basis of the increased body weight of the mice (9).

Life Span: For EAC tumor growth was monitored by percent increase in life span which was considered to determine the antitumor efficacy and mortality was recorded for each group. The actuarial survival curves were drawn by Kaplan Meier method (10). The increase in life span was calculated by the formula.

$$\%ILS = \frac{(MST \text{ of treated group}) - (MST \text{ of Control group}) \times 100}{MST \text{ of Control group}}$$

$$\text{Median Survival Time (MST)} = \frac{\text{Day of } 1^{st} \text{ death} + \text{Day of last death}}{2}$$

The enhancement of life span by 25% or more over that of the control was considered as a effective antitumor response. The toxic effects of the crude extract (weight loss and mortality) was evaluated by comparing with a group of ten non tumor bearing mice which received the extract at the dosages as scheduled in the experiment groups. A weight loss of 20% per mouse or 20% of drug related deaths is considered as an index of excessive toxicity.

Statistical Analysis: All the data are expressed as mean ± S.E.M. (standard error of the mean). Significance level in different groups were analyzed using student 't' test. $P>0.05$ were considered to be statistically significant

Results

Acute toxicity: The LD_{50} of Amalakyadi churna was found to be 313mg/kg b wt. intraperitoneally. At acute dose of 400mg/kg b.wt of the drug dose and above, the animals posed toxic symptoms and were died with in 72 hours. At 350, 300, 250 and

200mg/kg b.wt. of the drug also posed mild toxic symptoms, but the mortality rate was reduced to 80%, 40%, 20% and 0% in these respective treatment groups at 72 hours. For acute dose of the 200mg/kg b.wt. ip of Amalakyadi churna was tolerated by mice without any apparent adverse manifestations. The oral administration of the drug up to 2g/kg b.wt. did not poses any threat / or toxic symptoms or mortality as compared to intraperitoneal administration.

Tumor Cell Viability Test: It showed that there were 100% viability of EAC tumor cells. It was confirmed through Trypan Blue Dye Exclusion test.

Microbial Contamination Test: The EAC tumor cell suspension was studied for microbial contamination test and found that they are completely free from the microbial contamination

Regression of Tumor Growth: The repeated administration of the Amalakyadi churna has pronouncedly inhibited the tumor growth in exponentially growing mice of EAC *in vivo* (Table-1). A total dose of 400 mg/kg for 10 fractions of 40mg/kg/day b.wt ip for 10 consecutive days, starting from 24 hours after tumor induction, produced 35.14% increase in the life span and also median survival time by 25 days. Similarly, 560 and 735 mg/kg b.wt. of the drug for 7 fractions of 80 and 105mg/kg respectively, for one week were produced 40.54 and 43.24% of ILS and also 26 and 26.5 days of MST respectively. Where as, 960mg/kg for 6 fractions of 160mg/kg produced maximum activity, which is 48.64% of ILS and 28.0 days of MST. However, the total dose of 1080mg/kg for 6 fractions of 180 mgkg and 1050 mg/kg in 5 fractions of 210mg/kg b.wt showed neither ILS nor MST (Table-1). This has happened might be due to drug toxicity. It was also confirmed by repeating the same experiment (ie., same dose composition of the drug) to non-tumerous mice and recorded the mortality rate (Table-3).

Table -1 Effect of Amalakyadi churna on the life span of Ehrlich Ascites Carcinoma tumor bearing mice.

Sl. No	Treatment (mg/kg b. wt)	Number of Mice	Schedule days	Median Survival Time	Percentage of Increased Life Span
01	Control (ddw)	10	10	18.5±1.2	0%
02	40	10	10	25.0±1.5 a	35.14
03	80	10	7	26.0±1.0 a	40.54
04	105	10	7	26.5±1.1 a	43.24
05	160	10	6	28.0±1.2 a	48.64
06	180	10	6	22.5±1.5	21.62
07	210	10	5	22.0±1.8	18.91

Note: a= statistically significant (P>0.05%) when compared the Amalakyadi churna treated groups of mice with non-drug treated vehicle control group

Body weight: Table - 2 Showed that there was significant increase in the average body weight of only EAC tumor bearing control group of mice. But slight reduction in the body weight of tumor infected mice was noticed in 40 and 80mg/kg b.wt. of treated groups of mice. Where as, in case of 105 and 160mg/kg of drug was significantly inhibited the decrease in the body weight, there by reducing tumor incidence rate, meanwhile, %ILS and MST also increased, but at 180 and 210 mg/kg b.wt treated groups of tumor infected mice did not show any increase in their body weight than that of the normal mice. It clearly indicates that, the growth rate of tumor in these groups of mice was totally arrested, but due to drug toxicity, mice could not get total recovery, meanwhile the survival rate was also reduced

Table-2 Average body weight changes and increase in the survival days of the Amalakyadi churna drug treated EAC tumor bearing mice for every 5 days

Treatment (mg/kg b. wt)	Schedule days	Mean body weights of EAC tumor bearing mice (in gm)							
		1 day	5^{th} day	10^{th} day	15^{th} day	20^{th} day	25^{th} day	30^{th} day	34^{th} day
Control (ddw)	10	33.37	35.63	41.99	49.89	56.42	--	--	--
40	10	35.78	35.49	39.14	48.14	45.95	55.30	56.01	57.05
80	7	34.17	34.55	38.93	46.44	43.29	51.50	--	--
105	7	33.92	29.46	37.53	42.35	45.37	45.36	--	--
160	6	34.89	32.27	35.32	39.43	34.46	33.36	--	--
180	6	29.04	25.57	27.15	28.21	27.86	--	--	--
210	5	34.25	33.16	33.73	35.04	37.69	--	--	--

Table-3 The toxic effect of the drug studied against Mortality rate of animals (non tumor bearing mice)

Treatment (mg/kg b. wt)	Number of mice	Schedule days	I-Week	II-Week	III-Week	IV-Week	Percentage of death in 4 weeks
	10		--	--	--	--	--
Control (ddw)	10	10	--	--	--	--	--
40	10	10	--	--	--	--	--
80	10	7	--	--	--	--	--
105	10	7	--	--	--	--	--
160	10	6	--	--	1	--	10%
180	10	6	--	1	1	--	20%
210	10	5	--	2	1	--	30%

Note: ddw – double distilled water

Discussion

The above results clearly demonstrated that, Amalakyadi churna acts as a one of the potent anticancer agent against EAC mouse tumor model. However, the present phytochemical studies, as well as survey of literature indicated the presence of alkaloids, flavonoids, tannins, saponins, terpenoids, steroids, etc. in Amalakyadi churna. Several of such compounds like plumbagin, piperine and piplartine have been reported to show cytotoxicity activity towards several tumor cell lines in both *in vivo* and *in vitro* studies, also possess antioxidant and immunomodulatory activities, these chemicals are possibly either directly or indirectly involved in controlling of several cancers (11- 14). In view of anticipated interest in Ascites tumor, it was believed that tests against the EAC of compounds of demonstrated or suggested activity against other tumors would be useful as a base-line for future studies with this tumor (15).

Conclusion

The different doses of Amalakyadi churna extract showed potent antitumor activity by increase in the life span, as well as median survival time and also reduction in the total body weight of the tumerous mice. The 40, 80, 105 and 160mg/kg drug doses were found to be effective in significant reduction in the body weight, increased the median survival time and as well as percent increase in life span. But, 180 and 210 mg/kg dose levels were capable of arresting the tumor incidence but due to the drug toxicity, the animals could not show percent increase in the life span. Based on these findings, it is confirmed that, Amalakyadi churna is one of the potent anticancerous agent it holds enormous therapeutic potentials and provide rich pool of novel efficacious agents and perform multifunctional activity for treating cancer and its related complications. The phytochemical analysis clearly indicated the presence of alkaloids, phenols, tannins, flavonoids, steroids and saponins. Perhaps the combined effect of these principal chemical groups synergistically exhibited the antitumor activity and also provides evidence, that these compounds responsible for the folk anticancer uses of this plant.

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