RESEARCH COMMUNICATION

Protective Effects of Ethanolic Extract Residue Isolated from the Bark of *Terminalia Arjuna* against DLA Tumour Cells

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Abstract

An ethanolic herbal extract residue was prepared from the seeds of premature fruits of *Terminalia arjuna* Linn. Different concentrations of it were tested against 1×10^6 million Dalton's Lymphoma Ascites (DLA) tumour cells. At a 200 μg/ml concentration it registered 90% toxicity. Then its effect on the lifespan of mice with DLA tumour cells was studied. At high and low dosages of 50 and 10 mg. b. wt. kg^{-1} of herbal extract residue, it exhibited 87.5% and 60.4% increase in the lifespan, respectively. Blood parameters such as percentage Hb, RBC and WBC counts were conducted with tail vein blood samples. Hb and RBC counts of treated mice were higher than that of tumour bearing mice, while WBC counts were lower. This is a good index of tumour recovery. Further studies were carried out on mice with solid tumours to record volumes, along with a lifespan study. Low dosage of the herbal extract residue was able to control the tumour volume 35.1% and 32.9% increase in the lifespan was noted both at high and low dosages, respectively.

Keywords: Dalton's Lymphoma Ascites - solid tumour - heamoglobin - RBC - WBC

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Introduction

Cancer is a group of diseases in which cells are aggressive, invasive, and sometimes metastatic. Cancer may affect people at all ages. Apart from humans, forms of cancer may affect other animals and plants (Tannock and Hilp, 2005). Widespread placement of cancers eliminates surgery and radiation as options, leaving chemotherapy as the preferred medical choice. Taxol, isolated from the stem bark of Taxus brevifolia was ranked as "the most important new drug in cancer for 15 years".

Yet adequate drugs are not currently available to treat major solid tumours such as those of the lung, breast, prostate, and colon. There is an urgent need to find a suitable treatment for the millions of patients who are affected (Fulder, 1996). Plants still remain a traditional source of medicinal compounds; up to 40% of modern drugs may directly or 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, just to name a few.

Hence an attempt was made to isolate herbal residue from the ethanolic extract of bark isolated from the Indian species, *Terminalia arjuna* (Roxb. ex DC) Wight & Arn. to test its antitumour activity.

Materials and Methods

Terminalia arjuna (Roxb. ex DC) Wight & Arn. is distributed throughout India. It is a large evergreen tree. Its barks have some medicinal value (Warrier et al., 1996). Barks of the *Terminalia arjuna* (Roxb. ex DC) Wight & Arn. were collected, shade and air dried and ground in to powder. 150-200 g of the powder was packed in filter paper packs and extracted with ethanol in a soxhlet apparatus continuously for about 6-10 h. Then the ethanolic extract was distilled off till semisolid substances were obtained. After evaporated to dryness, these substances were stored in a -20°C deep freezer until further analysis.

Cell lines

Dalton's Lymphoma Ascites (DLA) tumour cells were obtained through the courtesy of Amala Cancer Research Centre, Thrissur, Kerala, India.

In vitro cytotoxic assay

DLA was maintained by serial transplantation from mice to mice. The ascitic fluid of the DLA was drawn out from the donor mice carrying tumour for 7-9 days. The freshly drawn ascitic fluid from the peritoneal cavity was washed thrice with phosphate buffer saline (PBS, pH 7.4) and diluted in PBS to a concentration of 10^6 cells / ml. One million cells were incubated with various concentrations of the herbal extract residue (10 μg , 20 μg, 50 μg, 100 μg, 200 μg/ml) in a total volume of 1 ml for 3 hours at 37°C. 0.1% carboxy methyl cellulose (CMC) was used as control. After incubation the viability of the cells was determined by the tryphan blue exclusion method (Talwar, 1974).

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Effect of ethanolic herbal extract residues on ascites tumour bearing animals

BALB/c mice (20-25 g) were obtained from the Breeding section, Amala Cancer Research Centre, Thrissur. They were kept in groups of six in well ventilated cages in air controlled room, fed with normal mice chow (Sai Feeds, Bangalore, India) and water adlibitum. All animal experiments were conducted after permission from the Institutional Ethical Committee.

DLA cells were aspirated from the peritoneal cavity and washed three times with PBS. Ascites tumour cells were then inoculated to three groups (6 animals / groups) of BALB / c mice by injecting one million cells in to their peritoneal cavity. After 24 h of tumour inoculation two different dosages of herbal extract residues were given and continued for ten consecutive days.

Group I : Untreated control for DLA

Group II : DLA cells + 10 mg/kg b.wt of

T. arjuna herbal extract residue in 0.1% CMC (ip) – low dosage

Group III : DLA cells +50 mg/kg b.wt of

T. arjuna herbal extract residue in 0.1% CMC (ip) – high dosage

The death pattern of animals due to tumour burden was noted every day and the percentage of increase in life span was calculated using the formula, T-C/C X 100 where 'T' and 'C' are the number of days the treated and control animals survived respectively (Ramnath and Kuttan, 2000).

Haematological parameters

On the 15th day post tumour inoculation, blood was collected in heparinzed tubes from the respective animals by tail vein and the haematological parameters such as white blood cell count, red blood cell count and percentage of haemoglobin were determined by following the standard procedures (Ramnath and Kuttan, 2000).

Tumour cell count

0.1 ml of the ascitic fluid was aseptically withdrawn on the 15th day post tumour inoculation using a 1 ml syringe and diluted with 0.9 ml of PBS (pH 7.4) to adjust the cell count to $1x10^6$ cells. From the stock 0.1 ml was taken and mixed with 0.8 ml of PBS and then incubated for 3 hours at 37 0C. 0.1 ml of tryphan blue was then added to this. Then a drop of the resulting solution was loaded on the heamocytometer and the number of tumour cells randomly in every 100 cells was counted (Ramnath and Kuttan, 2000).

Estimation of SGPT and SGOT

On 15th day post tumour inoculation, blood from the tail vein of treated and control mice was withdrawn individually into test tubes which were then centrifuged at low speed for about 10-15 min to collect the serum for enzyme analysis following the method of Reitman and Frankel (1957).

Effect of herbal extact residues on the solid tumour

Female Swiss albino mice (20-25 g) were obtained from the Breeding section, Amala Cancer Research Centre, Thrissur. They were kept in groups of six in well ventilated cages in air controlled room, fed with normal mice chow (Sai Feeds, Bangalore, India) and water adlibitum. All animal experiments were conducted after permission from the Institutional Ethical Committee.

DLA tumour cells ($1x10^6$ cells/animal) were injected subcutaneously to the right hind limb of three groups of (6 animals / group) Swiss albino mice.

Group1: Untreated control for DLA

Group2: DLA cells + 2 mg/kg b.wt of T. arjuna extract residue in 0.1%

CMC (ip) - low dosage

Group3: DLA cells + 10 mg/kg b.wt of T. arjuna extract residue in 0.1%

CMC (ip) - high dosage

After 24 h of tumour inoculation different dosages of herbal extract residue were given and continued for 10 consecutive days. Initial diameter of the hind limb was noted using vernier calipers. From the 7th day onwards the tumour diameter was measured every 3rd day and recorded up to 40 days. The tumour volume was calculated using the formula, $V=4/3 \times \pi r1^2 \times r2$ (Ramanath and Kuttan 2000). The survival of the animal was also recorded up to 60 days.

Statistical analysis

Results were expressed as mean ± standard deviation and Student's 't' test was used.

Table 1. Cytotoxic Effects of Ethanolic Herbal Extract Residue on DLA Tumour Cells

S. No.	Concentration of herbal extract residue (µg)	% of cytotoxicity	
		Control (CMC 0.1%)	T. arjuna
1	10	0	7
2	20	0	12
3	50	0	24
4	100	0	72
5	200	0	90

One million cells were incubated with various concentrations of the herbal extract residue (10μg , 20μg, 50μg, 100μg, 200μg/ml) in a total volume of 1 ml for 3 h at 37°C. After incubation the % viability of the cells was determined by the tryphan blue exclusion method. Duplicates were carried out

Table 2. Effects of Ethanolic Herbal Extract Residue on DLA Tumour Cell Counts

Control/Herbal extract residue treated animal groups	Dosage	Cells(10^6/ml) Mean ± SD
Control		21.27 ± 0.31
Terminalia arjuna	10mg.b.wt.kg^{-1}	11.6 ± 0.30
treated	50mg.b.wt.kg^{-1}	8.60 ± 0.36

BALB/c mice were segregated into three groups (6 animals/group) and induced with ascites tumour by injecting $1x10^6$ cells into the peritoneal cavity of the test animals. After 24 h of tumour inoculation, different dosages of drugs were given and continued for ten consecutive days. Tumour cell count was done on the 15th day post tumour inoculation by drawing 0.1 ml of the ascitic fluid from the animals treated with herbal extract residues and from the control. Tryphan blue exclusion method was followed

Table 3. % of Hb, RBC and WBC Counts of the Test Animals Treated with Low and High Dosage of Ethonolic Herbal Extract Residue after Tumour Induction

Control/Herbal extract residue treated animal groups	Dosage	% Haemoglobin Mean ± SD	RBC Counts (Cells / mm^3) Mean ± SD	WBC Counts (Cells / mm^3) Mean ± SD
Normal		19.77 ± 0.70	12.64 ± 1.25	13300 ± 1389
Tumour bearing		12.45 ± 1.43	6.8 ± 0.17	68666 ± 3762
Terminalia arjuna treated	10 mg.b.wt.kg^{-1}	13.68 ± 1.24	7.03 ± 0.51	67433 ± 3082
	50 mg.b.wt.kg^{-1}	15.56 ± 1.47	9.08 ± 1.0	35683 ± 1701

BALB/c mice were segregated into three groups (6 animals/group) and induced with ascites tumour by injecting $1x10^6$ cells into the peritoneal cavity of the test animals. After 24 h of tumour inoculation, different dosages of drugs were given and continued for ten consecutive days. On the 15th day blood from the tail vein of drug treated and control mice was withdrawn individually under aseptic condition and collected in heparinized tubes. Haemoglobin, RBC and WBC content were estimated

Table 4. Serum Glutamate Pyruvate Transaminase and Serum Glutamate Oxaloacetate Transaminase Activity of Test Animals treated with Low and High Dosage of Ethanolic Herbal Extract Residue after Tumour Induction

Test animal	Dosage	SGPT enzyme activity (IU/L)	SGOT enzyme activity (IU/L)
Normal		150	170
Tumour bearing		230	240
Terminalia arjuna treated	10 mg. b. wt. kg-1	70	85
	50 mg. b. wt. kg-1	140	160

BALB/c mice were segregated into 3 groups (6 animals/ group) and induced with ascites tumour by injecting $1x10^6$ cells into the peritoneal cavity of the test animals. After 24 h of tumour inoculation, different dosages of drugs were given and continued for ten consecutive days. On the 15th day, blood from the tail vein of treated and control mice was withdrawn individually into test tubes which were then centrifuged at low speed for about 10-15 min to collect the serum for enzyme analysis following the method of Reitman and Frankel (1957). Duplicates were carried out

Table 5. Effect of Ethanolic Herbal Extract Residue on the Increased Lifespan of Animals with Ascites Tumour

Control/Herbal extract residue treated animal groups	Dosage	Mean Survival Time (Days) Mean ± SD	% of increase in lifespan
Control		16.00 ± 0.89	
Terminalia arjuna treated	10 mg.b.wt.kg-1	25.70 ± 1.5	60.42
	50 mg.b.wt.kg-1	30.00 ± 2.54	87.50

BALB/c mice were segregated into three groups (6 animals/ group) and induced with ascites tumour by injecting $1x10^6$ cells into the peritoneal cavity of the test animals. After 24 h of tumour inoculation, different dosages of drugs were given and continued for ten consecutive days. 0.1% carboxy methyl cellulose was used as a vehicle for the delivery of herbal extract residues. The entire experiment was carried out for 45 days

Results

Results of the in vitro cytotoxicity test were presented in Table 1. T. arjuna extract residue showed 90% cytotoxicity at 200 µg concentration and at 100 µg concentration also, it registered the toxicity of 72%. With regard to tumour cell count, T. arjuna extract residue both at low and high dosage levels led to the decreased formation of 11.6 ± 0.3 x 10^6 tumour cells and 8.60 ± 0.36 x10^6 tumour cells in the peritoneal cativity of the mice (Table 2) respectively.

Normal animals had the Hb content of 19.77 ± 0.70% whereas the tumour bearing animals had that of 12.45 ± 1.43%. *T. arjuna* treated animals showed only about 15.56 ± 1.47% and 13.68 ± 1.24% of Hb both at high and low dosages. RBC counts of normal and tumour bearing animals were 12.64 ± 1.25 cells/mm^3, 6.8 ± 0.17 cells/ mm^3. T. arjuna extract residue treated animals showed the values of 9.08 ± 1.00 cells/mm^3 and 7.03 ± 0.51 cells/mm^3 both at high and low dosages respectively. Normal and tumour bearing animals had the WBC counts of 13300 ± 1389 cells/ mm^3 and 68666 ± 3762 cells/ mm^3 respectively whereas T. arjuna treated ones registered the WBC counts of 35683 ± 1701 cells/ mm^3 and 67433 ± 3082 cells/ mm^3 both at high and low dosages respectively (Table 3). Serum glutamate pyruvate transaminase (SGPT) and serum glutamate oxaloacetate transaminase (SGOT) enzyme activities of test animals were found to be decreased than that of tumour bearing animals (Table 4). T. arjuna extract residue exhibited 87.50% and 60.42% increase in the life span of animals induced with DLA at 50 mg/Kg b. wt and 10 mg/Kg b. wt dosages respectively (Table 5).

Both at high and low dosage levels, T. arjuna extract residue was able to keep the tumour volume between the ranges 0.32 ± 0.24 mm^3 and 0.33 ± 0.17 mm^3, when compared to the controls' tumour volume of 0.75 ± 0.63 mm^3 (Table 6). There was no much difference between these two dosage effects. Concurrently percentage of increase in the life span of animals induced with solid tumour was studied up to 60 days. T. arjuna extract residue exhibited 35.13% and 32.88% of increase in the life span of animals induced with solid tumour at high and low dosages respectively (Table 7).

Discussion

At 200µg concentration, T. arjuna herbal extract residue showed 90% of cytotoxicity. Hence it was decided to carryout further studies on mice after tumour inoculation. Preethi et al., (2005) reported that *Ruta graveolens* was cytotoxic towards DLA and Ehrlich ascites carcinoma (EAC) only at higher concentration. The 100% cytotoxicity was attained only at 16 mg/ml. Jelly et al., (2005) investigated that the 70% methanolic extract of *Holostemma adakodien* shows 50% cytotoxicity

Table 6. Measurement of Tumour Volume of Animals treated with Ethanolic Herbal Extract Residue

Days	Control (0.1% CMC) Mean ± SD	*T. arjuna* LD Mean ±SD	*T. arjuna* HD Mean ± SD
1	0.05 ± 0.02	0.05 ± 0.01	0.05 ± 0.00
7	0.09 ± 0.01	0.05 ± 0.02	0.06 ± 0.02
10	0.11 ± 0.03	0.07 ± 0.01	0.07 ± 0.01
13	0.12 ± 0.03	0.08 ± 0.02	0.09 ± 0.02
16	0.14 ± 0.04	0.10 ± 0.02	0.10 ± 0.03
19	0.16 ± 0.05	0.11 ± 0.03	0.11 ± 0.03
22	0.19 ± 0.06	0.13 ± 0.03	0.13 ± 0.02
25	0.20 ± 0.07	0.14 ± 0.05	0.14 ± 0.05
28	0.24 ± 0.09	0.17 ± 0.06	0.15 ± 0.05
31	0.29 ± 0.01	0.18 ± 0.06	0.17 ± 0.08
33	0.4 ± 0.03	0.21 ± 0.07	0.21 ± 0.09
36	0.54 ± 0.05	0.24 ± 0.08	0.26 ± 0.1
40	0.75 ± 0.06	0.33 ± 0.17	0.32 ± 0.12
		P<0.01	P<0.01

Swiss albino mice were segregated into three groups (6 animals/group) and induced with ascites tumour by injecting $1x10^6$ cells into the right hind limb of the test animals. After 24 h of tumour inoculation, different dosages of drugs were given by keeping one group each for low dosage and high dosage and continued for ten consecutive days. Initial diameter of the hind limb was noted using vernier calipers. From the 7th day onwards the tumour diameter was measured every 3rd day and recorded up to 40 days. The tumour volume was calculated using the formula, $V=4/3 \times \pi r1^2 \times r2$

Table 7. Effect of Ethanolic Herbal Extract Residue on the Percentage of Increased Lifespan of Animals with Solid Tumour

Control/Herbal extract residue treated animal groups	Dosage	Mean survival time (days) Mean ± SD	% of increase in life span
Control		44.4 ± 0.89	
Terminalia	2 mg.b.wt.kg-1	55.0 ± 2.75	32.88
arjuna treated	10 mg.b.wt.kg-1	60	35.13

Swiss albino mice were segregated into three groups (6 animals/group) and induced with ascites tumour by injecting $1x10^6$ cells into the right hind limb of the test animals. After 24 h of tumour inoculation, different dosages of drugs were given by keeping one group each for low dosage and high dosage and continued for ten consecutive days. The survival of the animal was also recorded up to 60 days

at a concentration of 750 µg/ml against DLA cells. The alcoholic Boerhavia diffusa extract was 100% cytotoxic towards DLA at a concentration of 500 µg (Lini and Kuttan, 2000). Methanolic extract of Piper longum was toxic at 250 µg /ml to DLA cells and 100 µg/ml to EAC cells. The aqueous extract was non toxic to both DLA and EAC cells even at a concentration of 500 µg /ml. Babu et al., (2001) published that 70% ethanolic extract of Cuscuta reflexa was toxic to DLA and EAC cells at a concentration of 1.2 mg/ml and 800 µg/ml respectively for causing 50% tumour cell death.

In the present investigation, in vitro cytotoxic studies against the DLA cells with the extract residue of *T. arjuna* are found to concide with the results of already reported studies and we also observed that the concentrations required to cause >90% toxicity are lesser than that required in the earlier reports.

Effect of herbal extract residue on the increased life span of animals with DLA was studied upto 45 days. At high and low dosage levels, *T. arjuna* herbal extract residue registered 87.50% and 60.42% increase in the life span of the animals induced with DLA respectively. As far as Hb estimation and RBC counts are concerned, all the values of *T. arjuna* herbal extract residue treated mice were generally higher than that of tumour bearing animals. This clearly shows that the extract residue has no side effects as far as blood parameters are concerned. Regarding WBC counts, all the values were lower than that of tumour bearing animals. Low WBC counts are good index of tumour recovery (Obiling and Guerin, 1954; Clarkson and Burchenal, 1965). SGPT and SGOT enzyme activities of test animals were found to be decreased than that of tumour bearing animals and also found to be dosage dependent.

Life span of DLA bearing animals was increased by methanolic extract of *R. graveolens*. At 400 mg/Kg b. wt and at 200 mg/Kg b. wt dosages, the increase in life span was 21.6% and 43.2% respectively (Preethi et al., 2005). Likely the life span of DLA bearing mice treated with alcoholic extract of Boerhaavia diffusa was significantly increased with an increase in life span of 64% (Lini and Kuttan, 2000).

Animals administered orally with NDEA (0.02%, 5 days a weak for 20 weeks) developed visible liver tumours by the end of the 20th week and the liver weight was raised. Only 11% of the animals treated with Emblica officinalis polyphenolic fraction (EOP) (60 mg/Kg 5 days a week for 20 weeks) orally developed visible liver tumours by this period and the liver weights were reduced. Elevated levels of serum ALP, GPT, bilirubin, GST and GSH in the NDEA administered group were significantly reduced by EOP treatment. The EOP was found to scavenge superoxide and hydroxyl radicals and inhibited lipid peroxidation in vitro (Kumar and Kuttan, 2001).

Serum and tissue levels of LPO, ALP and GPT which were elevated by CTX was reduced by Emblica officinails (EO) and Chayavana prash (CHY) treatment (Joy and Kuttan, 1998).

Earlier reports clearly state that herbal extracts which have potent antioxidant activities enhance the percentage of increase in the life span of animals induced with DLA tumour cells and in some cases induced with carcinogens. In the present investigation too, mice bearing DLA and treated with herbal extract residue showed the percentage of increase in the life span. This might be due to its good antioxidant activities. In this way, results of the present study stand in good agreement with the already reported studies in general. Variations in the percentage of increase in the life span of animals bearing tumour might be due to variations in the type of extracts used and the degree of antioxidant activities elicited.

Effect of herbal extract residues on solid tumour development was studied for 40 days. Based on the results, it was found that *T. arjuna* extract residue was able to keep the tumour volume under check. No much difference was noticed between the low and high dosages.

Percentage of increase in the life span of animals

induced with solid tumour by DLA cells was also studied concurrently up to 60 days. High and low dosages of *T. arjuna* exhibited the 35.13% and 32.88% increase in the life span of animals induced with solid tumour by DLA cells respectively.

Jelly et al., (2005) reported that the tumour volume in untreated control mice was 4.84 ± 1.23 mm^3 on 30th day and this was reduced to 0.68 ± 0.39 mm^3 by the administration of methanolic extract of *H. adakodien* root at concentration of 25 mg/Kg b. wt. The ethanolic extract of *L. dicholamellatus* showed profound antitumour activity in a dosage dependent manner. The extract decreased the tumour volume by 56.62% and 67.94% respectively when administered at concentrations of 250 and 500 mg/kg body weights to animals after implantation of solid tumour by DLA cell line (Nitha et al., 2005).

Oral administration of the berberine hydrochloride (5, 2.5 and 0.5 mg/Kg b.wt) was found to inhibit the sarcoma development by 60%, 53% and 33% respectively in female Wistar rats of 120-150 gm b. wt (Anis et al., 2000). Oral administration of methanolic extract of *Emilia sonchifolia* could reduce significantly the solid tumour induced with DLA in Swiss Albino mice. On the 30th day the tumour volume of the control animal was 4.25 mm^3 and that of drug treated (100 mg/Kg b.wt) was 1.25 mm^3 (Shylesh and Padikkala, 1999). On the 40th day oral administration of 75% methanolic extract of *P. kurroa* (750 mg/Kg b.wt) retarded the development of solid tumours induced with DLA by 44% in male Swiss albino mice (Joy and Kuttan, 1998).

In linewith already studied reports, *T. arjuna* was able to keep the tumour volume between the ranges 56-57.3%. T. arjuna herbal extract residue showed considerable tumouricidal activity i.e., >50% thereby reducing the tumour volume to significant level. Encouraging results were therefore noticed in the percentage of increase in the lifespan of the solid tumour bearing animals by 35.13 and 32.88 % both at high and low dosages when compared to that of control animals.

Data of Chander et al. (2004) showed that both ethanol and solvent ether fractions of *T. arjuna* exerted lipid lowering activity in vivo. This suggests that arjunic acid as well as its derivatives when undergo biotransformation through hepatic drug metabolizing cascade, produce common active molecules which may be responsible for lipid lowering activity *in vivo*. The quantity of arjunic acid in solvent ether fraction was comparatively very less than those of its derivatives in ethanolic fraction and due to this, at the same dosages ethanolic fraction was more effective than solvent ether fraction. More work on drug metabolism and to assess the biological activity in vivo and in vitro of *T. arjuna* fractions is under progress to substantiate the findings.

In the present investigation too we strongly belive that arjunic acid present in the ethanolic extraction (Chander et al. 2004) may be responsible for the antitumour activity of the ethanolic herbal residue isolated from the bark of *T. arjuna*. The present study emphasizes the scientific validation for the herbal extract residues which are in common use among the tribals and find place in the literature of Indian medicine based on the traditional knowledge. Out of the nine herbals tested in the current study, only four could show the encouraging results in controlling the ascites as well as solid tumour in the animal models. Hence the present investigation strongly recommends scientific validation for all supposed to be anticancer herbs by carrying out invitro as well as *in vivo* studies on animal models because of the different behaviour of the cancer cells against the same testing drugs in these two different systems and also the very fact that animals as well as human bodies are not merely pool of cells.

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