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Search towards an insight for comparative anti-tumour effects of *Wrightia tomentosa* leaf & bark in ehrlich ascites carcinoma bearing mice

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SUMMARY

In the present study, the ethanolic leaf and bark extract of *Wrightia tomentosa* were tested for comparative *in vivo* antitumour properties against Ehrlich ascites carcinoma (EAC) tumour bearing mice at 100 and 200 mg/kg body weight doses given orally once daily for 16 days. The EAC mice receiving 100 and 200 mg/kg ethanolic leaf and bark extract showed a dose dependent elevation in tumour – free survival and a highest number of survivors were observed at 200 mg/ kg for leaf extract of ethanol, which was considered as an optimum dose for its anti neoplastic action. The Median survival time for this dose was approximately 44 days when compared with 23 days of non-drug treated controls. The results indicate that the administration of leaf extract not only increased the survival of animals with ascites tumour and reduced packed cell volume and viable tissue cell count, but also altered many hematological parameters changed during tumour progression, indicating the potent antitumour nature of leaf extract than the bark extract. Statistical analysis also reveals that the leaf extract showed highly significant anti tumour potency ($p < 0.001$) when compared with control.

Key words: *Wrightia tomentosa*; Ethanol extract; Leaf, Bark; Ehrlich ascites carcinoma (EAC); antitumour

INTRODUCTION

Among the diseases of man, cancer is most feared and baffling. There are several hundred different types of cancer and these are associated with rapid and uncontrolled formation of abnormal cells in the body (Sammbamurty and Subrahmanyam, 1994). Because cancer can spread, it is important to find out as early as possible if a tumour is present (Ranajit, 2004) and if it is cancer.

Plant based medicines have definitely found a role in this type of treatment and the mechanism of interaction between many phytochemicals and cancer cells has been studied extensively (Harry *et al.,* 1999). Hence, a major portion of the current pharmacological research is devoted to anti tumour drug design customized to fit new molecular targets (Minami *et al.,* 2004).

There are examples of successful drugs obtained from plant sources which have had a profound

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impact in the field of cancer. World wide efforts are on to discover new antitumour agents from plants (Cragg *et al.,* 2000; Da Rocha *et al.,* 2000; Cragg and Newman, 2005).

Wrightia tomentosa is a small deciduous tree reaching 12 meter height, found throughout the warmer parts of India, ascending to an altitude of 600 m in the Himalayas and to 1,200 m in the Nilgiris (Anonymous, 1985). Various parts of this plant have been recognized to possess several medicinal properties in the folklore system of medicine. The stem and root bark are believed to be useful in snake bites and scorpion stings (Nadkarni and Nadkarni, 1976). The preparations from the bark are also given in menstrual and renal complaints (Basu and Kirtikar, 1973). A novel isoflavone, wrightiadione isolated from the plant possess cytotoxic activity against the murine P^{388} lymphocytic leukemia cell line (Geoffrey *et al.,* 1992). The butanol extract of *Wrightia tomentosa* bark and leaf were also reported to possess antibacterial properties (Nagarajan *et al.,* 2006). The ethanolic bark extract of *Wrightia tomentosa* was found to possess maximum antihyperglycemic activity in streptozotocin induced diabetic rats (Nagarajan *et al.,* 2008). The ethanolic bark & leaf extract of *Wrightia tomentosa* possesses significant anti-allodynic effects (Nagarajan *et al.,* 2007) with no observable signs of toxicity (Nagarajan *et al.,* 2007). The alcoholic extract of *Wrightia tomentosa* dried bark was reported to exhibit markedly high anti-oxidant activity (IC_{50} value of 75.0 mg/ml from DPPH radical scavenging assay), suitable for prevention of human disease (Nagarajan *et al.,* 2008). The leaf extract (100 mg) of *Wrightia tomentosa* has proved to be extremely useful against non-tuberculous mycobacterium infections (Nagarajan *et al.,* 2008), which are becoming a major concern for hospitals and medical clinics.

The objective of the present investigation is to assess the comparative anti-tumour potency *in vivo* in Ehrlich ascites tumour cells bearing mice with leaf and bark extract of ethanolic fractions of *Wrightia tomentosa.*

MATERIALS AND METHODS

Plant material and extraction

The dried wood bark and leaves of *Wrightia tomentosa* (Family: Apocynaceae) were obtained from Yercaud hills, India. The plant materials were identified (Basu, 1991; Matthew, 1982) & authenticated as leaves and wood bark of *Wrightia tomentosa* by Mr. Dhiravia Das of the Botany department, Bharathidasan University, Trichy. Both the leaves and bark were extracted successively with petroleum ether (60 - 80 °C) and ethanol using soxhlet's extractor till the materials were exhausted as indicated by a blank thin layer chromatography. The extracts were concentrated under vacuum & dried, preserved in a refrigerator at 4 °C until further use.

Drug and chemicals

5-Fluorouracil (5-FU) was obtained from Ranbaxy Ltd., Gurgaon, India. Tryphan blue was obtained from Hi-Media Laboratories Ltd., Mumbai, India. All the chemicals used were of analytical grade.

Tumour cells

Ehrlich ascites carcinoma (EAC) cells were supplied by Amala Cancer Research Centre, Trissur, Kerala, India. The cells were maintained *in vivo* in swiss albino mice by intra peritoneal transplantation. EAC cells aspirated from the peritoneal cavity of mice were washed with saline and given intra peritoneally to develop ascitic tumour.

Animals

Healthy Swiss Albino mice of both sex weighing between 18 - 25 g were obtained from the animal house, Periyar College of Pharmaceutical Sciences for Girls, Trichy, India. The mice were grouped and housed in polyacrylic cages and maintained under standard conditions (25 ± 2 °C) with 12 ± 1 h dark/light cycle. The animals were fed with rat pellet feed supplied by Hindustan Lever Ltd.,

Bangalore, India and water ad libitum. All the procedures were reviewed and approved by CPCSEA, Chennai, India (No. PCPSG/IAEC/265A).

Tumour transplantation procedure

EAC in ascitic form was selected for the present study, where the tumour cell was known to grow as uniform cell suspension in the abdominal cavity of the host. EAC being maintained in the ascitic form in swiss mice was carried out by serial transplantation of tumour cells intraperitoneally in 5 - 6 weeks old mice. Very well developed ascitic tumour contains large number of neo plastic mono nuclear cells produced around the 10 - 14th day of tumour transplantation. The ascitic fluid of EAC were drawn out from donor mice, diluted in saline P^H (0.9%) and aliquot (1×10^6 cells/ml) of the diluted solution were injected in to the mice.

***In vivo* tumour growth inhibition and survival assays**

The extracts of leaf & bark were evaluated for their anti-tumour activity (Akamanchi *et al.,* 1991) in accordance with the U.S. National Cancer Institute standard protocols (Anonymous, 1978) for primary screening.

Swiss albino mice were divided in to seven groups (n = 15). All the animals were injected with EAC cells (1×10^6 cells/mouse) intraperitoneally except for the normal group. This was taken as day zero. Group I served as the normal control and group II served as the tumour control. These two groups received sodium chloride solution (0.9% w/v). Group III, which served as the positive control, was treated with the solution of 5-FU at 20 mg/kg body weight. Groups IV, V, VI and VII were treated with ethanolic leaf extract at 100 and 200 mg/kg and with ethanolic bark extract at 100 and 200 mg/kg body weight doses, respectively. All these treatments were given 24 h after the tumour inoculation, once daily for 16 days.

After the last dose, six mice from each group were sacrificed at the end of 21st day and tumour volume was measured with vernier calipers. The tumour volumes were estimated from two- dimensional measurements: tumour volume (mm^3) = (axb^2)/2, where a & b are the tumour length and width (mm), respectively (Kokura *et al.,* 1999). For the survival analysis, results are expressed as percent of mean survival time of treated animals (T) over mean survival time of the control (Ahluwalia *et al.,* 1984) group (C). The percentage of increased life span (ILS%) was calculated according to the formula: ILS% = (T-C)/C × 100. By NCI criteria, a T/C exceeding 125% and an ILS exceeding 25% indicate that the drug has significant antitumour acitivity (Alley *et al.,* 1995). Median survival times (MST) for each group was noticed. The antitumour efficacy of plant extracts were compared with 5-FU (20 mg/kg/day) for 16 days (Dilip and Pratima, 1994). MST was noted with reference to control.

Ascites fluid is a direct nutritional source to tumour cells, and the faster increase in ascites fluid with tumour growth could possibly be means to meet more nutritional requirements of tumour cells (Giri and Prasad, 1994). Viability of ascitic cells were checked by trypan blue (0.4% in normal saline) dye exclusion test (Talwar, 1974; Fleischer *et al.,* 1978) and the count was taken in Neubauer counting chamber.

Hematological studies

Blood was obtained from tail vein, 24 h after the last dose. For total count, blood was drawn in to RBC or WBC pipettes in proper dilution and counted in Neubauer counting chamber. Hemoglobin concentration was determined by Sahli's Hemoglobinometer method (Dacie, 1958). Differential count of leukocytes was done on freshly drawn blood film using Leishman's stain.

Statistical analysis

Results are expressed as means ± S.E. of the representative experiment; experiments were repeated at least twice. Statistical significance was evaluated using the student's *t* test (Kobayashi and Pillai, 2003). $P < 0.001$ were considered significant.

RESULTS

The effect of ethanolic leaf and bark extract of the test drug on the survival time of the tumour bearing mice showed the MST. The MST for the Control group was 23 days, while it was 33 days and 44 days for the groups treated with 100 mg & 200 mg/kg/day i.p. of ethanolic leaf extract of *Wrightia tomentosa*. Mean while, the MST were 29 days and 39 days for the groups treated with 100 mg and 200 mg/kg/day i.p of ethanolic bark extract of *Wrightia tomentosa*. MST was compared with standard drug 5-Fluoro uracil (20 mg/kg/ day i.p) which is having 49 days (Table 3).

Cytological studies of Leishman stained cell smears have revealed a decrease in the number of mitotic cells followed by the leaf & bark extract drug treatment when compared with that of control. The leaf extract (200 mg/kg) was found to have maximum antitumour acitivity as the tumour volume have been reduced from 4.8 ml to 2.3 ml at the end of treatment. This tumour volume shows that there was a negative change over compared to control (-52.08%), whereas the standard drug 5-Fluoro uracil represents the % decrease in tumour volume as compared to control (-64.58%). Among the extracts in different doses tested, higher dose of leaf extract (200 mg/kg) of *Wrightia tomentosa* was found to have better therapeutic effect in reducing the tumour volume (-52.08%), which was slightly lesser than the standard 5-FU (-64.58%).

In addition to the above results, the viable tumour cells count (1×10^7 cells) also decreased drastically and shows promising effect (-ve change over control; -69.23%) with higher dose of the leaf extract (200 mg/kg) and it was almost similar with that of standard drug, 5-FU (-ve change over control; -74.03%). Meanwhile, the non-viable tumour cells count (1×10^7 cells) for leaf extract has been increased as positive change with respect to control (+75%), whereas the non-viable tumour cells count using 5-FU has been increased remarkably high as twice as the leaf extract (200 mg/kg) with positive change over control (+125%) (Table 1).

The bark extract (200 mg/kg) have shown good

Table 1. Effect of Leaf extract treatment on tumor growth

Groups	EAC control	Leaf Extract (100 mg/kg)	Change over Control (%)	Leaf Extract (200 mg/kg)	Change over Control (%)	5 FU (20 mg/kg)	Change over Control (%)
Tumour volume (ml)	4.8 ± 0.68	3.4 ± 0.89	-29.17	2.3** ± 0.09	-52.08	1.7** ± 0.52	-64.58
Viable Tumor cells count × 10^7	10.4 ± 0.56	5.4* ± 0.23	-48.07	3.2** ± 0.06	-69.23	2.7** ± 0.14	-74.03
Non viable Tumour cells count ×10^7	0.4 ± 0.03	0.6 ± 0.03	+50	0.7 ± 0.03	+75	0.9 ± 0.03	+125

Results are represented as mean ± S.E.
*P < 0.01 vs Control + Positive change over compared to control
**P < 0.001 vs Control by Students 't' test. – Negative change over compared to control

Table 2. Effect of Bark extract treatment on tumor growth

Groups	EAC control	Extract (100 mg/kg)	Change over Control (%)	Extract (200 mg/kg)	Change over Control (%)	5 FU (20 mg/kg)	Change over Control. (%)
Tumour volume (ml)	4.8 ± 0.68	3.6 ± 0.93	-25	2.7** ± 0.1	-43.75	1.7** ± 0.52	-64.58
Viable Tumor cells count × 10^7	10.4 ± 0.56	5.8* ± 0.05	-44.23	4.2** ± 0.08	-59.61	2.7** ± 0.14	-74.03
Non viable Tumour cells count × 10^7	0.4 ± 0.03	0.5 ± 0.03	+25	0.8 ± 0.04	+100	0.9 ± 0.03	+125

Results are represented as mean ± S.E.
*P < 0.01 vs Control + Positive change over compared to control
**P < 0.001 vs Control by Students 't' test. – Negative change over compared to control

Table 3. Anti tumour activity of the given plant extract Effect on Median Survival time of mice

Treatment	Median Survival Time (days)	Life span (%)
EAC + Control	23.2 ± 1.51	100
EAC + Leaf (100 mg/kg)	32.5 ± 3.3	140.08
EAC + Leaf (200 mg /kg)	43.6 ± 3.9	187.93
EAC + Bark (100 mg/kg)	29.3 ± 2.3	126.29
EAC + Bark (200 mg/kg)	38.8 ± 3.6	167.24
EAC + 5 - Fluoro uracil (20 mg/kg)	48.5 ± 4.1	209.05

Data are represented as mean ± S.E.; n = 10.

antitumour effect with respect to parameter groups like tumour volume (-ve change over control; -43.75%) and counting of viable tumour cells (-ve change over control; -59.61%). The bark extract at higher dose (200 mg/kg) showed increase in non-viable cell count (1×10^7 cells) as compared with EAC control (+100%). This result specifically suggested that the ethanolic bark extract of *Wrightia tomentosa* at higher dose (200 mg/kg) exhibited antitumour effect by augmenting the positive change (in %) four times over the respective control (Table 2). The bark extract (100 mg/kg) was found to have negligible antitumour potency as the volume reduced from 4.8 ml to 3.6 ml only. Degenerative changes in the treated tumour cells have been observed in the form of membrane blebbing, vacuolated cytoplasm and a reduction in the staining intensity.

Among the extracts tested with different parameter groups (Table 1, 2), the leaf extract of *Wrightia tomentosa* was found to have much more promising antitumour potency at higher dose (200 mg/kg) than the bark extracts tested at different doses.

Hematological parameters of tumour bearing mice (Table 4) was found to be significant and altered from the normal group. The total WBC count was found to increase from 6.9 to 13 cells/ml $\times 10^6$ with the subsequent reduction of hemoglobin content (16 to 13 gram %) of RBC tumour bearing mice. The total number of RBC had shown a modest change. In differential count of WBC, the presence of neutrophil increases (28 to 76%), while lymphocyte count decreases (66 to 22%) in tumour bearing mice. At the same interval, ethanolic leaf extract of *Wrightia tomentosa* (200 mg/kg/day) have shown predominant antitumour activity by restoring those altered parameters to near normal (Hb = 16.2 gram%; Total RBC = 1.37 cells/ml $\times 10^{10}$;

Table 4. Effect of *Wrightia tomentosa* extract on Hematological Parameters

Treatment	Hb (gram %)	Total RBC Cells/m1 $\times 10^{10}$	Total WBC Cells /ml $\times 10^6$	Differential Count %		
				Lymphocyte	Neutrophil	Monocyte
Normal mice	16.1 ± 0.2	1.38 ± 0.06	6.9 ± 0.2	66 ± 4.2	28 ± 1.7	2 ± 0.1
Tumour bearing mice	13.0 ± 1.2	1.23 ± 0.06	13 ± 0.7	22 ± 1.2	76 ± 4.2	3 ± 0.2
Wrightia tomentosa leaf extract (100 mg/kg)	14.8 ± 0.9	1.28 ± 0.02	5.0 ± 0.9	43 ± 4.3	47 ± 0.3	2.9 ± 0.03
Wrightia tomentosa leaf extract (200 mg/kg)	16.2 ± 1.3	1.37 ± 0.04	5.8 ± 0.9	58 ± 2.4	31 ± 1.3	4 ± 0.02
Wrightia tomentosa Bark extract (100 mg/kg)	14.2 ± 0.4	1.24 ± 0.04	4.3 ± 0.03	40 ± 0.2	50 ± 3.8	2.5 ± 0.04
Wrightia tomentosa Bark extract (200 mg/kg)	15.8 ± 0.4	1.32 ± 0.6	5.4 ± 0.04	49 ± 3.2	42 ± 2.6	3 ± 0.02

Data are expressed as mean ± S.E., n = 6

Total WBC = 5.8 cells/ml × 10^6; Lymphocyte = 58%; Neutrophil = 31%).

The student's *t*-test, used to compare two groups using the distribution of measured values & clearly indicates that the test drug (leaf & bark) showed significant antitumour acitivity ($p < 0.001$) when compared with the control (Table 1, 2).

DISCUSSION

The greatest recent impact of plant derived drugs is observed in the area of antitumour research, where compounds such as taxol, vinblastine, vincristine, and camptothecin have dramatically improved the effectiveness of chemotherapy against some of the dreaded cancers (Rates, 2001). Earlier studies have shown cytotoxic properties of *W. tomentosa* in murine P388 lymphocytic leukemia cell line (Geoffrey *et al.,* 1992). Ehrlich tumour is a rapidly growing carcinoma with very aggressive behaviour (Barbero *et al.,* 2000). It is able to grow in almost all strains of mice. The Ehrlich ascitic tumour implantation induces per se a local inflammatory reaction, with increasing vascular permeability, which results in an intense edema formation, cellular migration and a progressive ascitic fluid formation (Fecchio *et al.,* 1990). Based on these observations, in the present study, the ethanol leaf and bark extract of *W. tomentosa* were evaluated for its comparative *in vivo* antitumour properties.

The results of the present study clearly demonstrate the higher tumour inhibitory activity of the leaf than the bark extract against transplantable ehrlich ascites tumour cell line. The mechanism by which these compounds mediate its antitumour effect is still to be elucidated.

In comparing the leaf and bark extract phytoconstituents, leaf extract possess large quantities of flavanoids (Nagarajan *et al.,* 2006). The presence of flavanoids and triterpenoids are known to possess antitumour properties (Kintzios, 2006). The effect of leaf extract on cell proliferation is yet unknown but certain structurally – related 4' 5, 7 – trihydroxy flavone (apigenin) is reported to inhibit cell proliferation by arresting the cell cycle at the G2/M phase (Matsukawa *et al.,* 1994; Czyz *et al.,* 2004). Inhibition of growth through cell cycle arrest and induction of apoptosis appear to be related to induction of p53 (Brandner *et al.,* 1996; Kao *et al.,* 2005). Like wise, the action of the leaf extract could also be mediated via cell cycle arrest at G2/M phase due to the presence of flavanoids as predominant active constituent. In conclusion, the present study demonstrates the potent antitumour properties of the ethanolic leaf extract than the bark extract and the plant merits further investigation in isolating its active constituents.

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