

Anticarcinogenic effects of nothapodytes nimmoniana against Dalton's lymphoma ascites tumor model

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ABSTRACT

The aim of the present study was to explore the effect of ethanolic extract of *Nothapodytes Nimmoniana* heartwood and barks against Dalton's Ascitic Lymphoma (DAL) in Swiss mice. DAL cells were injected intraperitoneally 1×10^6 cell to the mice. Two days after cells injection the animals were treated with ethanolic extract heartwood and barks at dose of 200 mg/kg for 14 days. 5-fluorouracil (20 mg/kg) was used as reference drug. On day 11, cancer cell number, packed cell volume, decrease in tumour weight of the mice, increase in life span and haematological parameters were evaluated and compared with the same parameters in control. A significant increase in the life span and a decrease in the cancer cell number and tumour weight were noted in the tumour-induced mice after treatment with the extract. The hematological parameters were also normalized by the extract in tumour-induced mice. These observations are suggestive of the protective effect of ethanolic extract of heartwood and barks against Dalton's Ascitic Lymphoma (DAL).

Keywords: Nothapodytes Nimmoniana, Dalton's Ascitic Lymphoma, Anticancer agents

INTRODUCTION

Chemotherapy is now considered as the most effective method of cancer treatment. However, most of the anti-cancer agents severely affect the host normal cells [1]. Hence, the use of natural products now has been anticipated of extraordinary value in the control of cancer and its eradication program [2].

According to World health organisation, more than 80% of people in developing countries depend on traditional medicine for their primary health needs [3]. A survey reveals that usage of herbs as therapy by cancer patients for accounts more than 60% [4].

The proportion of increase of cancer occurrence and lack of anti-cancer drugs has enforced researchers to pharmacology and chemical evaluation of anticancer phyto constitutes from natural source. Emerging evidence suggest that a number of plants been accepted as the main source of cancer chemoprevention drug discovery and development due to their diverse pharmacological effects and cancer chemo preventive effects [5, 6].

Nothapodytes nimmoniana (Grah.) Mabb. (Synonyms, *Nothapodytes foetida* and *Mappia foetida*) belonging to family Icacinaceae, is an endangered medicinal tree is a shrubby small tree, 3-8 m tall, smooth, grey, wrinkled bark, about 5 mm thick with broad dark green leaves and flowers [7].

This plant is known to be distributed in the warmer regions like Indian sub-continent in Southern India, Sri Lanka, parts of eastern India in Assam, and in the Himalayan foothills in north India, Myanmar and Thailand [8].

Nothapodytes nimmoniana contains camptothecin as its active constituent which is used in the treatment of cancer. Camptothecin (CPT), a monoterpene indole alkaloid, is regarded as one of the most promising anticancer drug of the twenty first century [9, 10]. It is an excellent source of quinoline alkaloids, camptothecin (CPT) and 9-methoxy camptothecin (9-OMeCPT) which are used clinically or after derivatization as anti-cancer agents for the treatment of solid tumors [11].

Irinotecan [12] and topotecan [13] are two water-soluble derivatives of CPT, have been approved by the Food and Drug Administration (FDA) of the United States of America for treating colorectal and ovarian cancer [14, 15].

The faster increasing pharmaceutical market demand of the CPT is very high due to unavailability of synthetic CPT. Padmanabha et al (2006) has identified *Nothapodytes nimmoniana* Graham as alternate source of CPT in a survey. The projected global demand for CPT in 2002 was valued at US\$ 4045 million [10]. An estimated 20% of the population of this species is believed to have declined over the last decade. Recently, *Nothapodytes nimmoniana* has been assigned the threat status of 'Vulnerable' [16] and identified as is polygamous species in nature [17].

There are no investigation on the heartwood and bark of the plant are exposed for its activity against treatment for cancer and the present study was carried out to evaluate the antitumor activity of the ethanolic extract of heartwood and bark of the *Nothapodytes nimmoniana* against Dalton's Ascitic Lymphoma (DAL) in Swiss mice.

MATERIALS AND METHODS

Collection of Plant Material:

The heartwood and barks of *Nothapodytes Nimmoniana* were collected from the botanical garden at the Campus of Padmavathi College of Pharmacy, Dharmapuri in June month, 2010. The plant was identified and authenticated by Dr. P. Jayaraman, Botanist, Director of Plant Anatomy Research Centre (PARC), Chennai, India. A voucher specimen (PARC/2011/0773) was deposited at the herbarium for future reference. The shade dried heartwood and barks were subjected to pulverization to get coarse powder. The coarsely powdered heartwood and barks of *Nothapodytes Nimmoniana* were used for extraction.

Preparation of ethanolic extract:

Briefly, 200gm of ground plant material was soaked in sufficient quantity of 70% ethanol by cold maceration at room temperature for 72 hrs after which the filtrate was collected through a piece of muslin cloth and then filter paper and the plant material was resoaked for twice. The filtrate was concentrated in a rotary evaporator at 40°C under reduced pressure to yield crude extract. This extract was stored at 4°C until use. The aqueous and alcoholic extracts were subjected for preliminary phytochemical analysis using standard methods [18].

Experimental animals

Swiss male albino mice of weighing between 20±5g were used. The animals were fed with sterile animal chow and water *ad libitum*. The mice were used after acclimatization under controlled conditions of temperature of 24 ± 2°C, humidity of 50 ± 5% and 10-12 h of light and dark cycles respectively for one week. The toxicity and anti-tumour experimental study were conducted after obtaining the approval of Institutional Animal Ethical Committee, Padmavathi College of Pharmacy (Approval ref. no IECA/CBCSEA/2011/014). Animal experiments were performed in accordance with the principles of good laboratory practices and CPCSEA guidelines of the Government of India.

Acute toxicity studies

Acute toxicity study was carried out on ethanolic extract of *Nothapodytes Nimmoniana* heart wood and bark as per OECD guidelines. The extract was found to be safe up to 2000 mg/kg of body weight.

Tumour cell lines

Dalton's ascitic lymphoma (DAL) and mouse lung fibroblast (L-929) cells were obtained through the courtesy of the Cancer Research Centre, Adyar, Chennai and National Institute of Virology, Pune, India respectively. DAL cells were maintained by weekly intraperitoneal (i.p.) inoculation of 1 x 10⁶ cells/mouse.

Determination of antitumour activity

Swiss male albino mice (20±5g) were randomly placed into six groups each of six animals and housed in separate cages. All the groups except group I were injected with DAL Cells (1×10⁶ cells/mouse.i.p.). This was taken as day 0.

Group I served as normal saline control (5 ml/kg, p.o.) and Group II served as DAL control. On day 1, the ethanolic extract of bark and heart wood at a dose of 200mg/kg body weight (Gp-III & IV) were administered orally and 20 mg/kg of 5-Fluorouracil treated group (Gp V) of six each and continued for 14 consecutive days. Control (Gp1) was reserved as cancer control, it was not treated with any extract but only with saline. On day 15, four mice of each group were sacrificed 24 h after the last dose and the rest were kept with food and water *ad libitum* to check the increase in the life span of the tumour hosts. The effect of ethanol extract on tumor growth and host's survival time were examined by studying the parameters like tumor volume, tumor cell count, viable tumor cell count, nonviable tumor cell count, mean survival time and increase in life span [19].

Body weight analysis:

All the mice were weighed for every five days, after tumour inoculation. Average gain in body weight was determined and % decrease in body weight was calculated by the formula.

% Decrease in body weight = (Decrease in weight/initial body weight) x 100.

Determination of Tumor cell count

The ascitic fluid was taken in a RBC pipette and diluted 1000 times. Then a drop of the diluted cell suspension was placed on the Neubauer counting chamber and the number of cells in 64 small squares was counted.

Estimation of Viable tumor cell count

The cells were then stained with Trypan blue (0.4% in normal saline) dye. The cells that did not take up the dye were viable and those that took the stain were nonviable. These viable and nonviable cells were counted.

Cell count = (No of cells x dilution) / (Area x Thickness of liquid film)

Percentage increase life span

The mortality was monitored by recording the effect of the ethanolic extract on tumor growth and percentage increase in life span were calculated [20].

Hematological studies

The effect of ethanolic extract of *Nothapodytes Nimmoniana* heartwood and barks on peripheral blood was investigated. RBC, WBC counts and estimation of hemoglobin were done by standard procedures from freely flowing tail vein blood. Serum protein concentration was estimated by Lowry's method and packed cell volume (PCV) were determined [21].

Determination of hematological parameters

Apart from the above mentioned parameters, the effects of ethanolic extract on hematological parameters were also studied in the mice of all the groups. Blood was collected from all groups of animals by retro-orbital puncture and counted for RBC and WBC. For comparison a normal control group (G4) was used which was neither inoculated with cancer cells nor treated [22].

Statistical analysis

The experimental results were expressed as the mean \pm S.E.M. Data were assessed by the method of One-way ANOVA followed by Dunnett post hoc test. *P* value of <0.05 was considered as statistically significant.

RESULTS AND DISCUSSION

Ethanol extract of *Nothapodytes Nimmoniana* heart wood and bark were studied for oral acute toxicity at dose of 2000mg/kg. The extract was found devoid of neither mortality of the animals nor any visible clinical signs of general weakness in the animals. Hence, 2000mg /kg was considered as LD₅₀ cut-off value. So the dose of 200 mg/kg (1/10th of 2000 mg/kg) was selected for extracts of heart wood and bark based on fixed dose method of OECD guidelines.

The results from the preliminary qualitative phytochemical analysis of the ethanolic extract of *Nothapodytes Nimmoniana* heartwood and barks revealed the presence of glycosides, phytosterols, alkaloids, flavonoids, tannins, saponins, and terpenoids.

The intraperitoneal inoculation of DAL cells in the mice produces increased proliferation of cells in group I. In the case of tumor growth response study, treatment with 200 mg/ kg of ethanol extract of heartwood showed significant (*p*<0.01) reduction in tumor volume (Table 2).

The effect of ethanol extract of heart wood and bark on life span, viable cell count and nonviable cell count were depicted in table 1. It revealed that there was increase in mean survival time. Administration of both ethanol extracts appreciably decreases the viable cell count compared to DAL bearing mice.

Ethanol extract of heart wood and bark treated mice survived upto 30.26 ± 1.02 and 38.34 ± 0.67 days respectively whereas the DAL control mice survived upto 21.60 ± 0.44 only. The percentage increase in lifespan of heart wood and bark of ethanol extract treated mice increased by 71% and 90% respectively (Table 1). Ethanol extract of heart wood treatment reduces the tumour volume to greater extent compared with DAL control and bark ethanol extract treated groups and hence increased the life span of 38.34 days in group III (Figure 1).

In case of the haematological parameters, DAL control mice showed reduced RBC count but increase in WBC count than normal group. The treatment with heart wood and bark of ethanol extract also raised the RBC count significantly to 5.66 ± 0.31 million/ mm^3 and 7.33 ± 0.34 million/ mm^3 respectively. Similarly both extracts restored the WBC value to $12.33 \pm 0.44 \times 10^3$ / mm^3 and $11.66 \pm 0.33^a \times 10^3$ / mm^3 respectively. Haemoglobin content in DAL control mice decreased significantly when compared with normal group. But, the heart wood and bark of ethanol extract increased haemoglobin content to 10.63 ± 0.55 gm/dl, 10.90 ± 0.47 gm/dl respectively.

Table 2 showed that hematological parameters of tumor bearing mice on day 15 were found to be significantly different as compared to the extracts treated groups. In tumor bearing mice, it was found that there was increase in WBC count, and decrease in Haemoglobin content and RBC count.

In the current investigation, intraperitoneal inoculation of DAL cells in the mice produced an enormous increase in the cancer cell count, which indicated that there is progression of cancer in the animals. The decrease in the cancer cell number observed in the ethanolic extract treated (i.e., Group II and III) animals proves that the tested extract is having significant inhibitory effect on the tumour cell proliferation.

The increase in tumour weight of Group II animals may be due to accumulation of peritoneal fluid as an abnormal enlargement of peritoneal cavity was observed in tumour-induced mice. Treatment with ethanolic extract reduced the tumour weight and hence increased the lifespan.

DISCUSSION

When DAL is induced in animals, the cancer cell count in the peritoneal fluid has been used as the standard marker to confirm the proliferation of cells. In this study, increased cell count after 10 days confirmed the proliferation of cells in the group II control group animals.

The ethanolic extract of *Nothapodytes Nimmoniana* treated animals at the doses of 200 mg/ kg (group III, IV) and 5-Fluorouracil (5-Fu) at the dose of 20 mg/kg (group V) significantly inhibited the tumor volume, tumour cell count, and restore back the hematological parameters to more or less normal levels.

Myelosuppression and anaemia is the foremost difficulties encountered in the cancer chemotherapy [23, 24]. But the results have evidently shown that both ethanolic extracts have restored back haemoglobin content and RBC count to normal. After 14 days of transplantation, ethanolic extract treated groups II & III were able to reverse the changes in the haematological parameters consequential to tumour inoculation. An increase in RBC count and a decrease in elevated WBC count were reported as confirmatory indicators for the protection against DAL [24]. However from the above observations on other hematological parameters it can be concluded that the plant possesses activity against DAL.

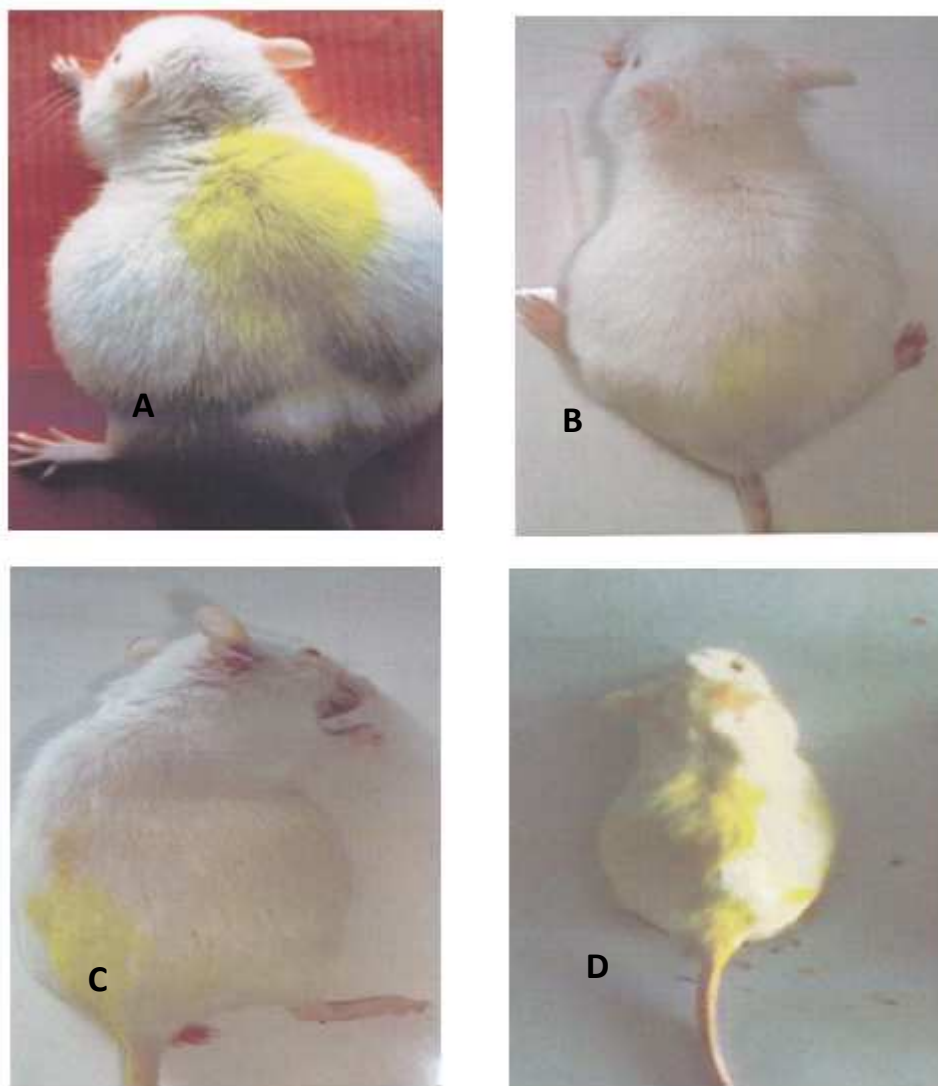
Nothapodytes Nimmoniana extract treatment was found to enhance nonviable cell counts in peritoneal exudates and decrease the viable cell count. It might be due to the absorption of ethanolic extract by viable cells which leads to lysis of cell through to the activation of macrophages or some cytokine production in peritoneal cavity. Viable cell count of the tumour bearing mice was significantly decreased while non- viable cell count were increased in ethanolic extract treated groups when compared with DAL treated group.

Ascetic fluid is the direct nutritional source for tumour cells and a quick increase in ascetic fluid with tumour development would be a means to meet the nutritional requirement of tumour cells [26]. Prolongation of life span of animals is the trustworthy standard criteria for judging the potency of anticancer drug [27]. It can be inferred that ethanol extract of *Nothapodytes Nimmoniana* increased the life span of DAL bearing mice may be due to decrease the ascetic fluid volume and delay the cell division [20].

The phytochemical study indicated the presence of flavonoids, alkaloids and terpenoids in ethanol extract. Flavonoids have been shown to possess antimutagenic and antimalignant effects [28, 29]. Furthermore, flavonoids have a chemopreventive role in cancer through their effects on signal transduction in cell proliferation [30] and angiogenesis [31].

Terpenoids, steroids and phenolic compounds have a chemo preventive role in cancer through their effects on signal transduction in cell proliferation and angiogenesis has been reported [32]. Thus, antitumour effect produced by the ethanol extract of *Nothapodytes Nimmoniana* may be due to presence of terpenoids and flavonoids as well as its antioxidant potential [7].

Figure 1: Tumour Photographs showing (A) DAL Control (Group II) (B) ethanolic extract of bark (Group III) (C) ethanolic extract of heartwood (Group IV) (D) 5-fluorouracil treatment (Group V)



The cytotoxicity and anticancer activity of ethanolic extract of *Nothapodytes Nimmoniana* are probably due to presence of Camptothecin, isoquinoline alkaloid [33]. Water-soluble form of camptothecin has been reported in treatment of colorectal and ovarian cancer [34].

Thus, anti-tumour effect produced by the both the extracts may be due to its flavonoids as well as its antioxidant potential. The ethanolic extract of *Nothapodytes Nimmoniana* restore the mean survival time, decrease tumor volume count in treated mice. Thus our present study suggests that ethanolic extract of heartwood possess potent anticancer activity against Dalton's Ascitic Lymphoma (DAL).

Therefore in conclusion, the present investigation showed a decrease in cancer cell count, tumour volume, RBC count as a confirmatory evidence for protection against DAL. Consequently increased WBC counts, life span, haemoglobin content were observed with both the extracts of *Nothapodytes Nimmoniana* extract treated mice.

Further studies to characterize the active principle and elucidate the mechanism of action of ethanolic extract of *Nothapodytes Nimmoniana* heart wood and bark are in progress using different cell lines. All these data point to the possibility of developing an ethanolic extract of *Nothapodytes Nimmoniana* heart wood as a novel, potential phytochemical in the field of cancer management.

Table 1: Effect of the ethonolic extract on Nothapodytes Nimmoniana on tumour volume, Body weight analysis, viable and Non-Viable Cell Count in mice

Treatment group	Tumour volume (ml)	Body weight analysis % decrease in body weight	Mean survival time (Days)	Viable Cell Count	Non-Viable Cell Count
Group II: DAL control	4.96±0.102	-	21.60±0.44	9.78±0.18	0.85±0.11
Group III: DAL + ethanolic extract of bark (200mg/kg p.o)	4.33±0.227 ^b	22.33	30.26±1.02 ^b	4.51±0.23 ^a	4.60±0.27 ^a
Group IV: DAL + ethonolic extract of heartwood (200mg/kg p.o)	2.482±0.134 ^a	28.47	38.34±0.67 ^a	2.90±0.10 ^a	5.23±0.084 ^a
Group V: DAL+5-FU Standard drug	1.117±0.095 ^a	39.55	42.51±0.77 ^a	2.29±0.8 ^a	5.76±0.08 ^a

Values are expressed as the mean ± S.E.M., (n=6) on day 14 of the experiment; Statistical significance (p) calculate by one way ANOVA followed by dunnett's ^aP<0.001, ^bp<0.01, ^cp<0.05, NS- non significant calculated by comparing treated group with DAL control
EL-Extract of leave, DAL-Dalton's ascites lymphoma

Table 2: Effect of the ethonolic extract on Nothapodytes Nimmoniana on heamtological parameter in mice

Treatment group	Total WBC Count (TWBC) (1X10 ³ /mm ³)	Red blood cells (million/mm ³)	Haemoglobin Hb(g/dl)	Serum Glutamate Oxaloacetate Transaminase (SGOT) SGOT(u/l)	Serum Glutamate Pyruvate Transaminase (SGPT) SGPT(u/l)	Triglycerides (TGL)	Creatinine (CR)
Group I: Normal control	10.2±0.34	10.6±0.34	13.8±0.38	87.42±1.8	25.67±1.17	123.6±5.6	1.05±0.04
Group II: DAL control	14.66±0.46	4.23±0.14	8.63±0.36	76.67±3.64 ^s	26.49±1.36	191.34±5.93	1.30 ± 0.04
Group III: DAL + ethanolic extract of bark (200mg/kg p.o)	12.33±0.44 ^b	5.66±0.31 ^{ns}	10.63±0.55 ^b	69.41±2.54 ^{ns}	26.29±0.99 ^{ns}	148.17±6.086 ^b	1.09 ± 0.02 ^b
Group IV: DAL + ethonolic extract of heartwood (200mg/kg p.o)	11.66±0.33 ^a	7.33±0.34 ^b	10.90±0.47 ^b	93.167±5.245 ^{ns}	25.07±1.11 ^{ns}	161.±7.788 ^c	1.03 ± 0.02 ^a
Group V: DAL+5-FU Standard drug	11.33±0.7 ^a	10.0±0.57 ^a	12.66±0.74 ^a	78.17±1.38 ^{ns}	28.51±0.65 ^{ns}	140.34±3.15	1.09 ± 0.03 ^b

Values are expressed as the mean ± S.E.M., (n=6) on day 14 of the experiment; Statistical significance (P) calculated by one way ANOVA followed by dunnett's ^aP<0.001, ^bP<0.01, ^cP<0.05, NS-non significant calculated by comparing treated group with DAL control.

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