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Evaluation of anticancer activity of Andaman freeze dried powdered *Morinda citrifolia* L. fruit against Ehrlich Ascites Carcinoma (EAC) cell induced liquid and solid tumor in Swiss Albino mice.

Hariom Singh, Saswata Banerjee, Saumen Karan, Tapan Kumar Chatterjee*
Division of Pharmacology, Department of Pharmaceutical Technology, Jadavpur University, Kolkata 700032, India.

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ABSTRACT

Objective The objective of the study was to establish the antitumor effect of powdered freeze dried Noni against Swiss albino mice bearing liquid tumor in peritoneal cavity and solid tumor in hind limb induced by Ehrlich ascites carcinoma (EAC) cell lines. **Methods** For liquid tumor study Noni at doses 250 and 500 mg/kg body weight were administered to mice carrying EAC cell line up to 14 consecutive days. Half of the mice were sacrificed, for the assessment of antitumor activity by evaluating tumor parameters and hematological parameters and rest half were left for the survival studies by mean survival time (MST) and percent increase in life span (%ILS). For solid tumor study, tumor was induced by implanting EAC cell subcutaneously in the hind limb of mice. Same doses were used and mice were sacrificed after 21 days for evaluating tumor weight and tumor volume. Histopathological studies of tumor, liver and kidney were performed. **Results** In liquid tumor, it was found that Noni significantly ($p<0.01$) decreases tumor volume, tumor weight, viable tumor cell count and increases the life span of mice bearing EAC cell lines. Hematological parameters gained their normal values. In solid tumor, Noni at both doses decreased tumor weight and Noni 500mg/kg decreased tumor volume significantly ($p<0.05$). Histopathological findings revealed that Noni showed less proliferation with some necrosis and apoptosis along with hepatoprotective and renoprotective action on liver and kidney. **Conclusion** It was revealed from the experimental results that the powdered freeze dried Noni has potent antitumor activity.

Keywords Freeze dried Noni, EAC cell line, anticancer activity, 5-Flurouracil, Paclitaxel

INTRODUCTION

Cancer is a disease, involving uncontrolled multiplication and spreading of the body's own cells into abnormal forms. It is the most common cause of death secondary to the cardiovascular diseases (CVD), and occurs due to the failure of the normal controlled mechanism which regulates cell survival, cell proliferation, and cell differentiation. Cancer pathogenesis involves the activation of Proto-oncogene which leads to the loss of controlled cell division, differentiation and apoptosis and this results into uncontrolled proliferations of normal cells that gradually takes the form of tumor[1]. In United States, 1 out of 4 deaths are caused due to cancer. In 2011, approximately 5,71,950 deaths were reported due to cancer which is the second most common cause of death in the US, exceeded only by heart disease. The National Institutes of Health estimates the overall costs of cancer was $263.8 billion in 2010, out of which $102.8 billion for direct medical costs (total of all health expenditures), $20.9 billion for indirect morbidity costs (cost of lost productivity due to illness), and $140.1 billion for indirect mortality costs (cost of lost productivity due to premature death) [2,3].

Environmental exposure is probably the most important cause of cancer such as chronic exposure to ionizing radiation causes acute leukemia's, thyroid cancer, breast cancer, lung cancer etc. Several viruses were also found to be involved in the etiology of cancer such as HIV virus associated with Hodgkin and non Hodgkin lymphoma's, Human Papilloma Virus for Cervical Cancer and Ebstein-barr virus being responsible for Nasopharyngeal cancer[4]. Now in recent trend, use of herbal medicines with chemotherapy has been employed because of the anticancer effects of the herbal medicines. Alkaloids, flavanoids, phenolics and many other photochemical constituents of several medicinal plants confer activity against wide range of cancers[5].

***Corresponding author.**
Dr. Tapan Kumar Chatterjee,
Assosciate Professor,
Division of Pharmacology,
Department of Pharmaceutical Technology,
Jadavpur University, Kolkata 700032.

Morinda citrifolia

Morinda citrifolia L. (Rubiaceae), or Noni, is a tropical plant grown in South Pacific Asia and other tropical regions of the world. Noni, a traditional folk medicinal plant found its use in traditional medicine by Polynesians over 2000 years[6]. *Morinda citrifolia* L has been referred

to as an edible plant in a technical manual of edible and poisonous plants of the Pacific Islands, where the leaves and fruits could be used as emergency food[7]. Abbott also reported that Noni had been used as a food, drink, medicine, and colorful dye[8]. It is rich in chemical constituents, large number of compounds have been identified in the plant such as scopoletin alkaloids, anthraquinones (such as nordamnacanthal, morindone, rubiadin, and rubiadin-1-methyl ether, anthraquinone glycoside) octoanoic acid, potassium, vitamin C, terpenoids, β-sitosterol, vitamin A, flavone glycosides, carotene, linoleic acid, Alizarin, amino acids, acubin, L-asperuloside, caproic acid, caprylic acid, ursolic acid, rutin, and a proxeronine (precursor of xeronine) that converts into xeronine inside the body[9,10, 11].

MATERIALS AND METHODS

Plant material

Andaman Powdered freeze dried Noni (Salveo Lifesciences Ltd., Delhi, India) was used in the present study for the evaluation of antitumor activity.

Chemicals and reagents

5-Flurouracil (Biochem Pharmaceutical Industries Ltd., Mumbai, India), Trypan Blue dye (Loba Chemie Pvt. Ltd., Mumbai, India), Paclitaxel (Cipla Ltd., Mumbai, india)

Acute toxicity study

The LD50 value of Freeze dried Noni in Swiss albino mice was determined by adopting procedure in OECD guidelines 425 (OECD 2008)**[12].** Noni was found to be safe even at a dose >2 g/kg bw of mice thus, it was cleared that drug was non toxic to the animals. So, the effective dose was selected to be 250mg/kg and 500mg/kg b.w.

Animals

Swiss albino mice having weight between 20± 2 gm[13] were used throughout the experiment purchased from the Indian Institute of Chemical Biology (IICB), Kolkata, India. The animals were kept under standard laboratory conditions, temperature around 25±2 ^{0}C, 12 hour dark/light cycle[14] and relative humidity 55-60 % [15]. The animals were provided with the standard laboratory diet (Hindustan Uniliver Limited, Kolkata) and were also supplied with water *ad libitum*[13]. The mice were kept up to 7 days under standard laboratory condition for their adaptation to the laboratory condition[15]. All the explained procedures were carried out as per the Jadavpur University Animal Ethics Committee guidelines (147/1999/CPCSEA).

EAC (Ehlrich ascites carcinoma) cells

The EAC cell lines were collected from the Chittaranjan National Cancer Institute, Kolkata, India. The EAC cells were maintained and preserved *in vivo* by intraperitoneal administration of 2×10^6 viable cells/mouse and transferring of 2×10^6 cells/mouse from EAC cell lines bearing mice to the fresh mice every 9 days[15,16]. Ascitic liquid is of gray-white color, and sometimes appears as a light bloody type. 0.1 ml of ascitic fluid contains 10 million neoplastic cells (Aktas, 1996; Kaleoglu and Isli, 1977)[17]. Ehrlich ascite carcinoma is an undifferentiated tumor form which is hyperdiploid in nature, having transplantable capability, no-regression, rapid proliferation, short life span, 100% malignancy and also does not have tumour-specific transplantation antigen It is similar to human tumours that are responsive towards chemotherapy because of undifferentiated nature and rapid growth rate[18].

Induction of liquid tumor and solid tumor with treatment protocol for the evaluation of antitumor activity

The animals were kept in large polypropylene cage, supplied with the standard diet and water *ad libitum* throughout the experiment. In case of liquid tumor five groups of the Swiss albino mice (20-22 g) were made, 12 animals in each group Animals of the entire groups were injected with the 2×10^6 EAC cells suspended in 0.2 ml of phosphate buffer saline[19] at pH 7.2-7.4 intraperitoneally except the animals in group 1 i.e. normal control group. Group 2 i.e EAC control did not receive any treatment, group 3 and group 4 were administered orally with 250 and 500 mg/kg body weight of Noni and animals of group 5 were given reference drug 5-flurouracil (20mg/kg/day) i.p. after 24 hrs of the tumor inoculation. Administration of Noni and 5-Flurouracil in alternate days was continued up to 14 days to their respective groups[16]. After 24 hrs of the last dose six animals from each group were sacrificed by cervical dislocation for the evaluation of anticancer activity by studying several parameters such as tumor weight, tumor volume, viable and non viable tumor cell count and rest six animals of each group were left as such for studying median survival time (MST) and percent increase in life span[20]. Now, for solid tumor induction five groups were made each having 12 animals, animals of all group were injected with 2.5 ml of 2×10^6 EAC cells in right hind limb subcutaneously for solid tumor implantation[21] except Group 1 receive normal saline group 2 receives only EAC cells. Treatment was started after 7 days of transplantation, group 3 and 4 receive 250 and 500 mg/kg b.w Noni once daily respectively, and group 5 receive paclitaxel 10mg/kg b.w at 4 day interval up to 21 days. Tumor volume was determined by using vernier caliper (Tricle Brand, Shanghai China) at every seven days, after twenty one days six animals of each group were sacrificed and solid form of tumors were removed from each group and weighed to evaluate the tumor weight for the assessment of anticancer activity.

LIQUID TUMOR PARAMETERS

Tumor volume and tumor weight

For the determination of tumor weight six mice from each group were sacrificed after 24 hrs of the last dose and weight was recorded before and after the collection of the ascitic fluid[22, 23]. The difference in weight before and after gives the tumor weight and is expressed in grams. For tumor volume, the ascitic fluid was collected from the peritoneal cavity and volume was measured with the help of graduated centrifuge tubes.

Determination of viable and non viable tumor cells

Tumor Cell number was found to decresed by Noni as compared to EAC control. The ascitic fluid collected from the peritoneal cavity was taken in WBC diluting pipette and diluted up to 100 times with

phosphate buffer saline[24]. A drop was placed on the Neubauer's chamber and number of cells in all 64 squares were counted[15]. For the assessment of viable and non viable cells, Trypan blue dye (0.4 % in normal saline) was used as staining material[25] viability of the cells was checked by using trypan blue dye[26] Viable cells did not take the colour of the dyes (Trypan blue Exclusion test)[27]and non viable cells respond to the blue stain of the dye[22, 23].

Cell count= (no. of cells ×dilution factor)/ (area× thickness of liquid film)

Assessment of survival parameters mean survival time (MST) and % increase in life span (%ILS)

MST of treated groups i.e. 2nd, 3rd, 4th was noted by recording the mortality of the Swiss albino mice[28] and percent increase in life span was calculated by using MST values. MST and % ILS was calculated by using following equation[16].

Mean survival time = [1st Death + Last Death] / 2

ILS (%) = [(Mean survival of treated group/ Mean survival of control group)-1] x100

Study of hematological parameters:

The blood was collected from the tail vein[29] and RBC count, WBC count and Haemoglobin content was determined by using Lieshman stain solution[30, 31].

SOLID TUMOR PARAMETERS

Tumor weight

Noni at doses 250mg and 500mg was given orally to the animals after 7 days of the tumor cell implanatation. Paclitaxel (10mg/kg) was taken as standard. On 21st day, six animals of each group were sacrificed by cervical dislocation and tumor was removed from the right hind limb and weight was taken[18].

Tumor volume

Tumor volume were measured on 7, 14 and 21 day by vernier caliper after implantation of EAC cells and the tumor volume of developed tumor was calculated by the following formulae[32]

Tumor volume (mm^3) = $4\pi (A/2)^2 \times (B/2)^2$

Where A is the minor tumor axis; B the major tumour axis π and equals to 3.14.

Histopatological findings

The animals were sacrificed by cervical dislocation and the tumor, liver; kidneys were removed and stored in 10 % formaline for the conduction of histopathology studies. Slides of each one is prepared by following standard methods using eosin and heamtotoxylin. Drug induced hepatotoxity, nephrotoxicity was studied and also the tumor histo section of noni (250mg/kg b.w and 500mg/kg b.w) group is compared with the EAC control.

Statistical analysis

The results obtained were expressed as Mean±SEM (standard error of mean). Graph pad prism 5.0 was used for statistical analysis. Statistical analysis was done using one way ANOVA (analysis of variance) followed by Dunnett's post- hoc test. P value less than 0.01($p<0.01$) was considered as statistically significant.

RESULTS

The present study reveals that Freeze dried Noni at the doses of 250 and 500 mg/ kg b.w decreases the total tumor cell count (figure 1,2,3) tumor weight, viable cells, tumor volume of EAC cell line bearing mice in comparison to EAC control. It was revealed that Noni increases non viable cell count and % ILS. It was also found that Noni restored the hematological parameters (RBC, WBC, hemoglobin). The WBC count was found to be decreased whereas the RBC count and hemoglobin were found to be increased.

LIQUID TUMOR

Effect on mean survival time (MST) and % ILS

The MST of EAC control was 25.83±0.87 and that of the treated groups of freeze dried Noni 250mg, 500mg/kg b.w and 5-flurouracil (20mg/kg/day) was found out to be 33.00±1.03, 40.83±0.98 and 46.67±0.55 respectively. Significant increase ($P<0.01$) in the MST of treated group was found when compared with tumor control group. The % ILS of the treated group was 27.75% (250mg Noni), 58% (500mg Noni) and 80.68% of 5-flurouracil (20mg/kg/day). The parameters were summarized in Table 1.

Table 1.Effect on the survival time of EAC bearing mice:

Sl.No	Groups	Mean survival time(days)	% Increase in life span
1	EAC control	25.83±0.87	0
2	Freeze dried Noni (250mg/kg, p.o)	33.00±1.03*	27.75
3	Freeze dried Noni (500mg/kg, p.o)	40.83±0.98*	58
4	5-flurouracil (20mg/kg, i.p)	46.67±0.55*	80.68

*n = 6 animals in each group, *p<0.01 vs EAC control*

Effect on tumor weight and tumor volume

It was found that oral administration of Noni leads to the reduction in tumor volume and tumor weight in EAC bearing Swiss albino mice ($P<0.01$). Tumor volume of the EAC control group was 3.86±0.25ml, and that of treated groups 2.26±0.31 (250mg Noni), 1.68±0.19ml (500 mg Noni), 0.94±0.11ml (20mg/kg/day 5-flurouracil) respectively. Tumor weight of EAC control was found to be 3.95±0.16g and that of freeze dried Noni 1.76±0.19g (250 mg), 1.15±0.11g (500 mg) and 0.80±0.13g of 5-flurouracil respectively. The parameters were showed in Table 2.

Table 2. Effect on tumor weight and tumor volume

Sl.No	Groups	Tumor weight(g)	Tumor volume(ml)
1	EAC control	3.95±0.16	3.86±0.25
2	Freeze dried Noni (250mg/kg, p.o)	1.76±0.19*	2.26±0.31
3	Freeze dried Noni (500mg/kg, p.o)	1.15±0.11*	1.68±0.19*
4	5-flurouracil (20mg/kg, i.p)	0.80±0.13*	0.94±0.11*

*n = 6 animals in each group *p<0.01 vs EAC control*

Effect on viable and non viable cell count (cells ×10^7/ml)

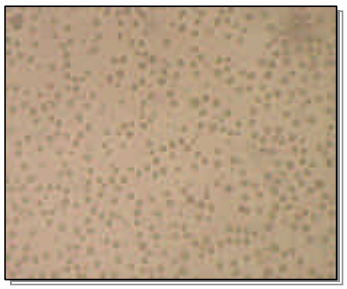
Number of viable cells was found to be decreased where as in case of non viable cells there number increased (P<0.01) showed in Table 3. Viable cell count of EAC control group was found to be 8.9±0.17 and that of treated groups were 3.15±0.22 (250mg), 1.39±0.13 (500mg) and 0.89±0.13 of 5- Flurouracil respectively. Non viable cell count of EAC control group was 0.41±0.06 and that of treated groups were 1.20±0.08 (250mg), 1.98±0.15 (500mg) and 2.96±0.15 of 5 –Flurouracil group respectively. Comparison of tumor cell number between EAC control and treated group was showed in Fig 1(A-C).

Table 3.Effect on viable and non viable cell count

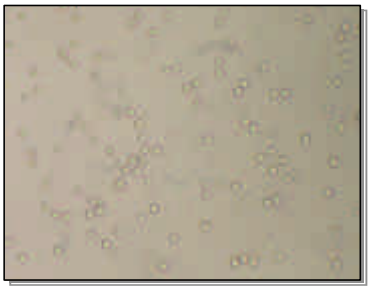
Sl.No	Groups	Viable cells×107/ml	Non viable cell×107/ml
1	EAC control	8.90±0.17	0.41±0.06
2	Freeze dried Noni (250mg/kg, p.o)	3.15±0.22*	1.20±0.08*
3	Freeze dried Noni (500mg/kg, p.o)	1.39±0.13*	1.98±0.15*
4	5-flurouracil (20mg/kg, i.p)	0.89±0.13*	2.96±0.15*

*n= 6 animals in each group *p<0.01 vs EAC control*

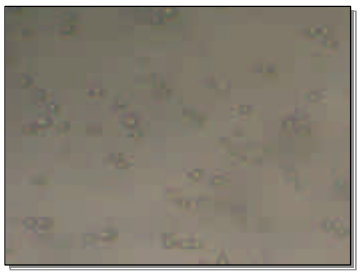
Fig.1. (A-C) Effect of NONI on tumor cell population as comparison to EAC control in liquid tumor



A.:Tumor cell number in EAC control



B. Tumor cell number get decreased in NONI (250mg/kg/day)



C. Tumor cell number decreased in NONI (500mg/kg/day)

Effect on hematological parameters

Hemoglobin level, RBC count, lymphocyte cont in the EAC control group was found to be significantly (P < 0.01) decreased as compared to the normal group. Treatment with freeze dried Noni at the dose of 250 and 500 mg/ kg significantly (P < 0.01) increased the hemoglobin level, RBC count more or less to their normal ranges and lymphocytes count also increased showed in Table 4 The total WBC counts was found to be increased significantly in the EAC control group when compared with the normal group. Administration of freeze dried Noni drug at the dose of 250mg and 500 mg/kg in EAC-bearing mice significantly (P<0.01) reduced the WBC count when compared with the EAC control.

Table 4.Effect on hematological parameters

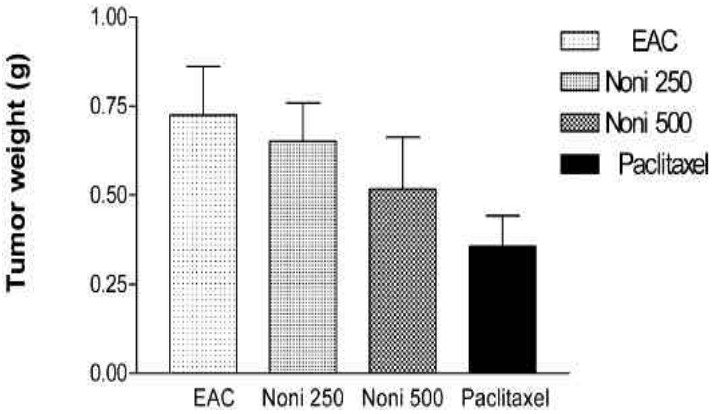
Sl.No	Parameters	Normal control	EAC Control	Freeze dried Noni (250mg/kg, p.o)	Freeze dried Noni (500mg/kg, p.o)	5-Flurouracil (20mg/kg, i.p)
1	Hemoglobin (g/dl)	13.62±0.24	9.33±0.32*	11.01±0.25**	12.41±0.29**	13.27±0.34**
2	RBC(million/mm^3)	5.90±0.20	3.27±0.22*	4.21±0.25**	5.07±0.26**	5.51±0.27**
3	WBC (million/mm^3)	6.36±0.19	12.75±0.38*	9.15±0.38**	7.14±0.34**	5.72±0.26**
4	lymphocytes%	71.33±0.49	36.17±0.60*	54.17±0.70**	61.50±0.67**	68.67±0.49**

*n = 6 animals in each group, *p<0.01vs normal control, **p<0.01 vs EAC control*

SOLID TUMOR

Tumor weight

It was found that oral administration of Noni leads to the reduction in tumor weight in EAC cell induced solid tumor Swiss albino mice (Graph 1).



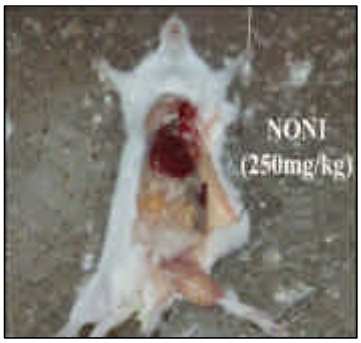
Graph 1: Effect of Noni on EAC induced solid tumor weight in Hind limb.

Tumor volume and tumor size

Noni reduces the rate of tumor growth and tumor size Fig 2(A-D) when compared to the EAC control group and also the tumor volume at dose 500mg/kg b.w significantly (p<0.05) in mice bearing EAC cell induced solid tumor implantations (Graph 2).

Fig.2. (A – D) Effect of NONI on EAC induced solid tumor as compared to EAC control.

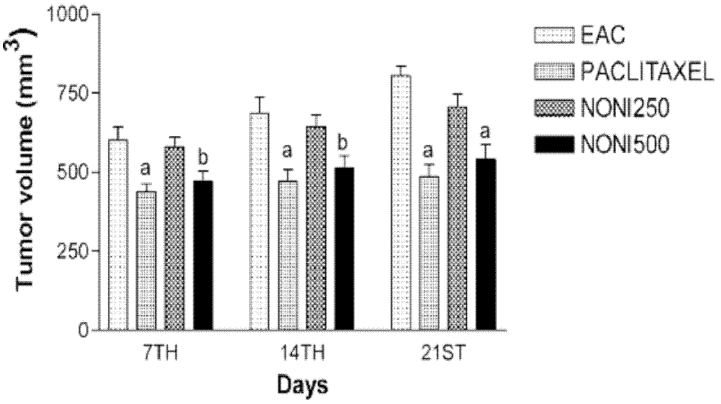
A



B

C

D



a represents P<0.01 vs EAC control and b represents P<0.05 vs EAC control

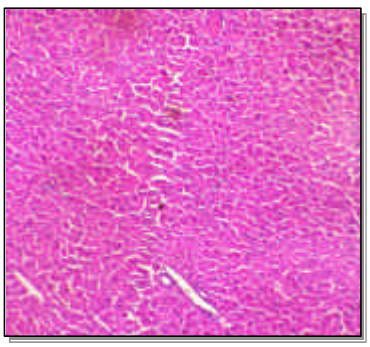
Graph 2: Effect of Noni on the tumor volume on 7th 14th and 21st day in Swiss albino mice.

Histopathological findings

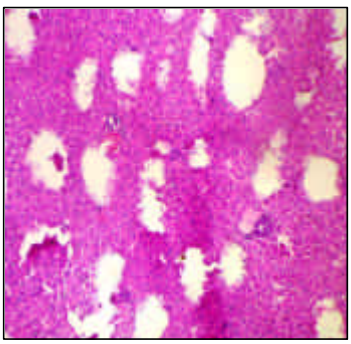
Liver histopathology

Histopathological section of liver of normal mice shows no abnormality (Fig 3A). EAC control shows well differentiated hepatocellular carcinoma with marked infilteration, central vein dialation and necrosis (Fig 3B). NONI 250mg shows very liitle necrosis, infilteration and no central vein dialation (Fig 3C). NONI 500mg showed no infilteration and central vein dialation (Fig3D). Standard group (Paclitaxel 10mg) shows dysplastic and benign hepatocytes in sheet and cord around hepatic vein. Portal tract show mild lymphocytic infilteration (Fig 3E).

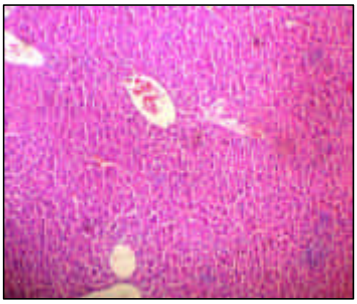
Fig.3. (A-E) Liver histopathology



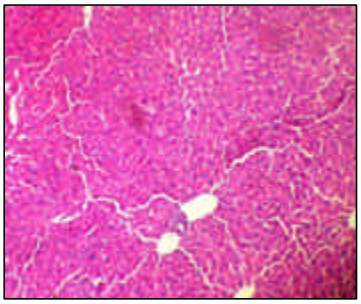
A) Normal control



(B) EAC control



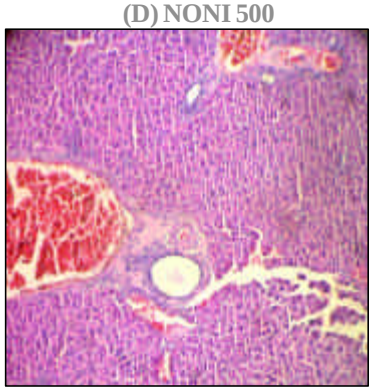
C) NONI 250



(D) NONI 500

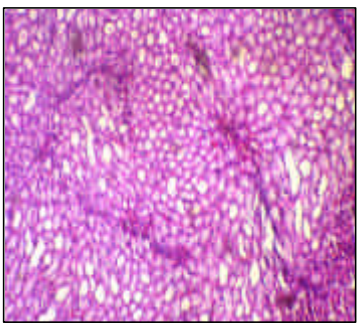
Kidney histopathology

Section of Normal mice kidney shows no abnormality and reveals normal histology (Fig 4A). EAC control shows glomerular infilteration (Fig 4B). NONI 250 mg shows little infilteration and some glomerular congestion (Fig 4C). NONI 500mg shows some dialated tubules with no major changes (Fig 4D). Paclitaxel group showed glomerulus congestion with atrophic changes in renal tubule and glomeruli (Fig 4E).

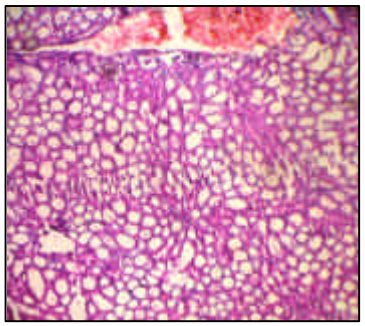


(E) Paclitaxel

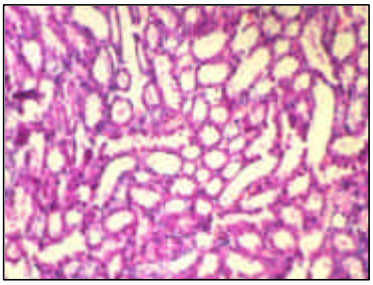
Fig.4. (A-E) Kidney histopathology



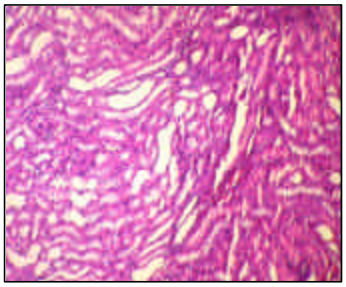
A) Normal control



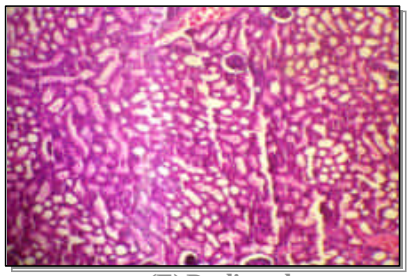
(B) EAC control



C) NONI 250



(D) NONI 500

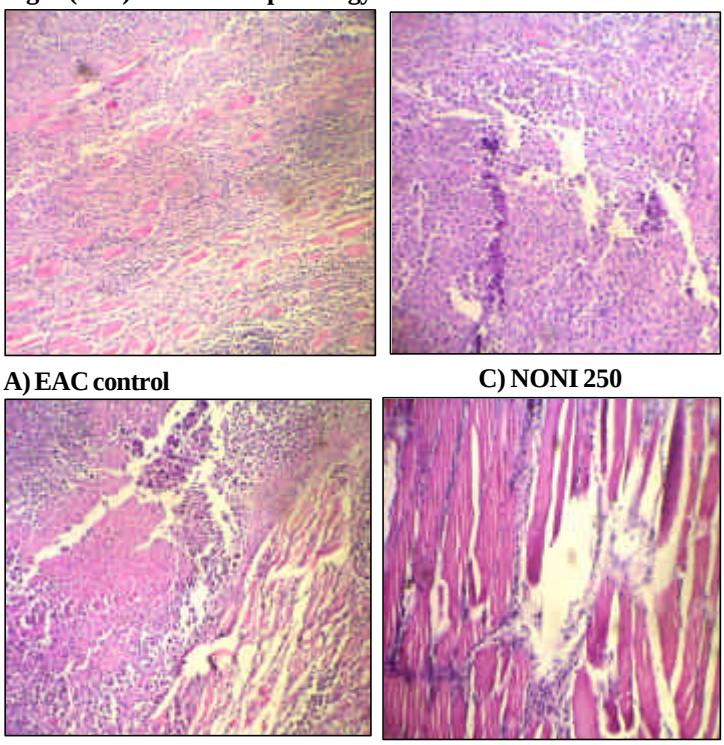


(E) Paclitaxel

Tumor tissue histopathology

EAC control solid tumor shows proliferation and sheets of tumor cells of different shapes (Fig 5A). Noni 250mg shows less proliferation with some necrosis and apoptosis (Fig 5B). NONI 500mg more apoptosis and necrosis(Fig 5C). Palitaxel group shows almost no proliferation, fibroskeletal muscle fibre and lymphoid aggregation show some malignant cell infilteration (Fig 5D).

Fig.5. (A-D) Tumor histopathology



A) EAC control **C) NONI 250**

(D) NONI 500 **(E) Paclitaxel**

DISCUSSION

It was found Swiss albino mice having EAC-cell lines showed rapid and regular increase in ascites fluid. Ascitic fluid is the nutritional requirement for the growing tumor cells and a sharp increase in tumor fluid with tumor growth would be necessary to meet the nutritional demand of the developing tumor[33]. It was concluded that treatment with the freeze dried Noni at dose 250 and 500mg/kg up to 14 days significantly decreased the tumor volume, tumor weight, viable cells and increased the percentage of Trypan blue stained cells i.e. non viable cells. The most reliable parameter for conferring the anticancer activity of any drug is the increase in the life span of animals[34]. It was further noticed that Noni decreases the volume of ascitic fluid and thereby retarding the growth of tumor increase the life span of EAC bearing mice. Cancer chemotherapy leads to the major problems such as myelosuppression and anemia[35, 36]. Anaemia in EAC cell bearing mice is just because of the decrease in Hb content and RBC count and this occur because of iron deficiency or because of myelopathic condition[37]. Morever, administration of freeze dried Noni restores the haemoglobin level, RBC, and WBC count more or less to normal levels and this confers that the freeze dried Noni possess protective action on the haematopoietic system. The above *invivo* study reveals that the freeze dried Noni has potent anticancer activity. Staining with Trypan blue reveals dose dependent increase in the number of non viable cells in treated groups of Noni reveals the cytotoxic action of Noni against cancerous cells. Solid tumor implantation by EAC cells in swiss albino micer cells lead to morphological and metabolic changes as structural basis, reduced number of mitochondria, nucleotides loss such as purines and pyrimidines, nucleosides and bases, decreased DNA and RNA synthesis, fall in turnover and pool of ATP, protein synthesis, glutathione concentration also decreases and triglycerides, cholesterol esters and free fatty acids gets increased[38]. It was found that Noni reduces tumor weight, tumor volume and retards the tumor growth when compared to EAC control. Histopathological findings revealed that no Noni at both doses shows tumor necrosis, apoptosis and less cell proliferation when compared to the EAC control. Furthermore slides of liver and kidney reveals that Noni has hepatoprotective effect and less nephrotoxic as compared to Paclitaxel and EAC control. Further research is going on for the isolation of compounds and establishment of mode of action that were actively responsible for the anticancer activity of Noni.

CONCLUSION

The above study shows that Freeze dried powdered Noni possess potent anticancer activity against EAC induced solid tumor as well as liquid tumor. Staining with trypan blue also reveals that Noni has cytotoxic action.

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