www.sphinxsai.com

PharmTech

International Journal of PharmTech Research
CODEN (USA): IJPRIF ISSN : 0974-4304
Vol.1, No.4, pp 1485-1489, Oct-Dec 2009

Evaluation of Anticancer Activity of *Vitex negundo* in Experimental Animals: An *In Vitro* & *In Vivo* Study

Chitra V, Shrinivas Sharma*, Nandu Kayande
Department of Pharmacology,
SRM College of Pharmacy, SRM University, SRM Nagar,
Kattankulathur - 603203. Tamil Nadu, India.

***Corres. author: shrinivas.sharma29@gmail.com**
Tel.: +919962358436; Fax. 044-27455717

ABSTRACT: The antitumour activity of the ethanolic extract of leaves of *Vitex negundo* (EVN) has been evaluated against Dalton's ascitic lymphoma (DAL) in Swiss albino mice at the dose of 250 & 500 mg/kg, body weight. The experimental parameters used were tumour volume, tumour cell count, viable tumour cell count, mean survival time and increase in life span to assess antitumour activity. The extract administered orally for 14 consecutive days to tumor bearing group of animals. The extract increase the life span of DAL treated mice and restore the hematological parameters as compared with the DAL bearing mice in dose dependant manner. The study revealed that the EVN showed significant antitumour activity in tested animal models. The EVN was found to be cytotoxic to mouse lung fibroblast (L-929) cells in long term chemosensitive cytotoxic assay.
Keywords: Cytotoxic; Dalton's ascitic lymphoma; tumour volume; *Vitex negundo*

INTRODUCTION

A number of natural products have been studied for anticancer activity on various experimental models. This has resulted in the availability of nearly 30 effective anticancer drugs.[1] *Vitex negundo* Linn. (Family: Verbenaceae), known as Nirgundi in Hindi, is a large evergreen, climbing, much branched shrub and ascending up to an altitude 1100-1400 ft, is found almost throughout India.[2,3] Although all parts *V. negundo* are used as medicine in the indigenous system of medicine, the leaves are the most potent for medicinal use. It is used for treatment of eye-disease, toothache, inflammation, leucoderma, enlargement of the spleen, skin-ulcers, in catarrhal fever, rheumatoid arthritis, gonorrhoea and bronchitis. They are also used as tonics, vermifuge, lactagogue, emmenagogue, antibacterial, antipyretic and antihistaminic agents.[4,5,6] Its extract has also shown anticancer activity against Ehrlich ascites tumour cells.[7] The aim of the present study was to evaluate antitumour activity of ethanolic extract of leaves of *Vitex negundo* (EVN) on Dalton's ascitic lymphoma (DAL) in Swiss Albino Mice and to evaluate its *in vitro* cytotoxic property on mouse lung fibroblast (L-929) cells.

MATERIAL AND METHODS

Plant material and extraction

The leaves of *Vitex nigundo*, were collected from foot hills of Yercaud, Tamil Nadu, India in the month of September 2008 and identified and authenticated at Plant Anatomy Research Centre (PARC), Pharmacognosy Institute, Chennai, India. A voucher specimen (VI/Ph/2008/46) has been kept in our laboratory for future reference. The air-dried and coarsely powdered leaves (400 g) extracted successively with 1.5 L each of petroleum ether (60–80^0 C) and ethanol in a Soxhlet extractor for 72 h. The extracts were concentrated to dryness under reduced pressure and controlled temperature (40–50^0 C). The petroleum ether extract yielded a yellowish green sticky semisolid, weighing 3 g. The ethanol extracts yielded brown and semi-solid residues, weighing 7.0 g.

Animals

The experimental protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of

the SRM University, Kattankulathur, India. Mature male Swiss albino mice weighing 20-25g were housed in standard isolation cages (45×35×25 cm) under environmentally controlled conditions with 12-h light/12-h dark cycle. They were allowed free access to water, standard laboratory chow (Hindustan Liver Pvt. Ltd, Mumbai) given food and water *ad libitum*. After sufficient period of acclimatization, they were used to evaluate antitumour activity.

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 10^6 cells/mouse.[8]

***In Vitro* cytotoxicity**

Long term cytotoxicity of the EVN to L-929 was determined by seeding 1 x 10^5 cells (L-929) in a culture bottle containing 10 ml minimum essential medium (MEM) supplemented with 10% heat inactivated goat serum and 100 mg streptomycin. After 24 h incubation at 37°C, the cells were exposed to different (1–100µg) concentration of the extract or camptothecin (a reference drug). Inhibition of the cell proliferation was assessed after 6 days by trypsinising and counting the cells with haemocytometer.[9]

Antitumour activity in mice

After acclimatization, mature male Swiss albino mice divided into four groups (n=10) and given food and water *ad libitum*. All the groups (Table 1) except group I were injected with DAL Cells (1×10^6 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 EVN at a dose of 250 and 500 mg/kg body weight (Gr-III & IV) were administered orally and continued for 14 consecutive days. The dose of EVN was selected based on previous study on hepatoprotective activity.[10] On day 15, five 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 tumor 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.[8, 11]

Determination of tumor volume

The mice were dissected and the ascitic fluid was collected from the peritoneal cavity. The volume was measured by taking it in a graduated centrifuge tube and packed cell volume determined by centrifuging at 1000 g for 5 min.

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

Recording the mortality monitored the effect of the EVN on tumor growth and percentage increase in life span (ILS %) were calculated.[12]

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

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

Hematological studies

The effect of EVN 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 conc. was estimated by Lowry's method and packed cell volume (PCV) was determined by the method described by Docie et al.[13, 14]

Statistical analysis

The experimental results were expressed as the mean ± 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

Results of the preliminary phytochemical analysis carried out on the crude ethanol extract indicated the presence of alkaloids, glycosides, lignin, flavonoids and saponins. In long term chemosensitive cytotoxic assay, 65 µg/ml of the extract produced 50% death of L-929 cells whereas 2 µg/ml camptothecin produced the same result. The effect of EVN on the survival of tumour bearing mice showed MST (Table 1) for the tumour control group (DAL treated) to be 21.40±1.41 days, while it was 30.81±1.02 days (43.50%) and 35.64±1.05 days (66.35%) for the group treated with EVN at the dose of 250 & 500 mg/kg respectively. The average number of tumour volume (Table

1) in DAL treated animals was found to be 3.68±0.11. EVN treatment at both dose level significantly (P<0.05) reduced tumour volume which was found to be 2.40±0.11 and 1.01±0.04 respectively. Viable cell count of the tumor bearing mice was significantly decreased while non- viable cell count were increased in EVN treated groups in dose dependant fashion when compared with DAL treated group. Moreover, hematological parameters of (Table 2) tumour bearing mice on day 15 were found to be significantly altered from normal group. The total WBC count, protein and PCV were found to be increased with a reduction of the haemoglobin and RBC. In a differential count of WBC, the percent of neutrophils increased while the lymphocyte count decreased. At the same time interval, EVN treatment could change those altered parameters to near normal.

DISCUSSION

In the above results, the *in vitro* cytotoxic activity was more pronounced in long term exposure of fibroblasts (L-929) to the EVN. Although the ctyotoxicity of camptothecin to L-929 cells was more than that of the extract, it should be noted that camptothecin is a pure compound whereas the extract is a crude one containing numerous compounds. The reliable criterion for judging the value of any anticancer drug is the prolongation of lifespan of the animal and disappearance of WBC from blood.[15,16] The above results demonstrated the antitumour effect of EVN against DAL is Swiss albino mice. A significant (P<0.05) enhancement of MST and non-viable cell count in peritoneal exudates (P<0.05) was observed due to EVN treatment. To evaluate whether EVN treatment indirectly inhibited tumour cell growth, the effect of EVN treatment was examined on the viable & non-viable cell counts against tumour bearing mice. Normally, each mouse contains about 5 x 10^6 intraperitoneal cells, 50% of which are macrophage. EVN treatment was found to enhance non-viable cell counts in peritoneal exudates and decrease the viable cell count. It might be due to the absorption of EVN by viable cells which leads to lysis of cell through to the activation of macrophages or some cytokine production in peritoneal cavity. Usually, in cancer chemotherapy, the major problems that are being encountered are of myelosuppression and anaemia[17,18] but the results have clearly shown that EVN has not only brought back hemoglobin content to normal but also the RBC count to normal. Analysis of the other hematological parameters showed minimum toxic effect in the mice which were treated with EVN. After 14 days of transplantation, EVN-treated groups were able to reverse the changes in the haemotological parameters consequent to tumour inoculation. All these data point to the possibility of developing an ethanolic extract of leaves of *Vitex nigundo* as a novel, potential agent in the area of cancer chemotherapy. The phytochemical study indicated the presence of flavonoids, Alkaloids and terpenoids in EVN. Flavonoids have been shown to possess antimutagenic and antimalignant effects.[19, 20] Further more, flavonoids have a chemopreventive role in cancer through their effects on signal transduction in cell proliferation[21] and angiogenesis.[22] According to the previous reports, *Vitex nigundo* possess antioxidant ability.[23] Thus, antitumour effect produced by the EVN may be due to its flavonoids as well as its antioxidant potential. The ethanolic extract of *Vitex nigundo* restore the mean survival time, decrease tumor volume count in treated mice. Thus our present study suggests that EVN possess potent anticancer activity and increase life span. Further studies to characterize the active principle and elucidate the mechanism of action of EVN are in progress using different cell lines.

ACKNOWLEDGEMENT

The authors are grateful for the financial assistance by Tamil Nadu Pharmaceutical Welfare Trust, Chennai, Tamil Nadu, India.

Table 1. Effect of ethanolic extract of *Vitex nigundo* on survival time, life span, tumor volume, viable and non-viable cell count in dal bearing mice.

Treatment group	Servival time (Days)	Increase of life span (%)	Tumour volume (ml)	Viable cell count x 10^6 cells/ml	Non-viable cell count x 10^6 cells/ml
Normal saline (5 ml/kg p.o)	-	-	-	-	-
DAL control (1 x 10^6 cell)	21.40±1.41	-	3.68±0.11	9.42±0.14	3.41±0.21
DAL (1 x 10^6 cell) + EVN (250 mg/kg p.o)	30.81±1.02*	43.50	2.40±0.11*	3.89±0.04*	1.91±0.09*
DAL (1 x 10^6 cell) + EVN (500 mg/kg p.o)	35.64±1.05*	66.35	1.01±0.04*	2.62±0.09*	2.14±0.19*

Statistical significance (p) calculated by one-way ANOVA followed by Dunnett's test.
*p < 0.01 calculated by comparing treated groups with DAL control group.

Table 2. Effect of ethanolic extract of *Vitex nigundo* on hematological parameters in dal bearing mice.

Treatment group	Hb (g %)	RBC (million/mm^3)	WBC (10^3cells/mm^3)	Proteins (g %)	PCV (mm)	Differential count %		
						Lymphocytes	Neutrophils	Monocytes
Normal saline (5 ml/kg)	14.5±0.2	6.5±0.2	7.2±0.2	8.5±0.2	17.8±0.7	70.2±1.31	29.8±1.1	2.2±0.4
DAL control (1 x 10^6 cell)	7.8±0.6^{a}**	3.8±0.1 ^{a}**	15.2±1.3 ^{a}**	14.6±1.4 ^{a}**	27.5±0.4 ^{a}**	30.3±0.4 ^{a}**	68.6±1.6 ^{a}**	3.8±0.5^{a}*
DAL (1 x 10^6 cell) + EVN (250 mg/kg p.o)	10.2±0.6^{b}**	5.1±0.5 ^{b}**	11.2±0.7 ^{b}*	11.8±0.1 ^{b}*	21.4±0.4 ^{b}**	55.8±1.1 ^{b}**	42.1±1.3 ^{b}**	2.9±0.4^{ns}
DAL (1 x 10^6 cell) + EVN (500 mg/kg p.o)	12.4±0.4 ^{b}**	5.8±0.3 ^{b}**	8.6±0.7 ^{b}**	9.2±0.1 ^{b}**	18.4±0.1 ^{b}**	67.3±2.1 ^{b}**	30.1±2.2 ^{b}**	2.8±0.3^{ns}

Statistical significance (p) calculated by one-way ANOVA followed by Dunnett's test.
*P < 0.05, **P < 0.01, ^{ns}P > 0.05; a *vs*. Normal group, b *vs*. DAL control. n = 5.

REFERENCES

1. Ramakrishna Y, Manohor A.I, Mamata P, Shreekant K.G. Plants and novel antitumour agents: A review. Indian Drugs 1984, 21, 173-85.
2. Chopra R.N, Nayar S.L, Chopra I.C. Glossary of Indian medicinal plants. 1956. New Delhi: CISR: p. 256.
3. Anonymous, Wealth of India, Raw Materials. Vol. V, 2001. New Delhi: Publication and Information Directorate (CSIR); India: p. 522.
4. Chaturvedi G.N and Singh R.H. Indian Journal of Medical Research, 1965, 53, 71.
5. Hansal R, Leuckert C, Rimpler H, Schaaf K.D. Phytochemistry, 1965, 4, 19.
6. Sirait L.M, Rimpler H, Haensal R. Experientia, 1962, 18, 72.
7. Banerji A, Chadha M.S, Malshet V.G. Phytochemistry, 1969, 8, 511.
8. Gothoskar S.V, Ranadive K.J. Anticancer screening of SAN-AB; an extract of marking nut, *Semicarpus anacardium*, Indian Journal of Experimental Biology, 1971, 9, 372-375.
9. Babu T.D, Kuttan G, Padikkala J. Cytotoxic and antitumor property of certain taxa of Umbelliferae with special reference to *Centella asiatica* (L.) Urban. Journal of Ethnopharmacology, 1995, 48, 53–57.
10. Tandon V.R, Khajuria V, Kapoor B, Kour D, Gupta S. Hepatoprotective activity of Vitex negundo leaf extract against anti-tubercular drugs induced hepatotoxicity. Fitoterapia, 2008, 79, 533–538.
11. Mazumder U.K, Gupta M, Maiti S, Mukherjee M. Antitumor activity of *Gygrophila spinosa* on Ehrlich ascites carcinoma and sarcoma-180 induced mice. Indian Journal of Experimental Biology, 1997, 35, 473-477.
12. Sur P and Ganguly D.K. Tea plant root extract (TRE) as an antineoplastic agent. Planta Med, 1994**,** 60, 106-109.
13. Dacie JV, Lewis SM. Practical Hematology. 2nd ed. J and A Churchill, London. 1958. p. 38-48.
14. D'Armour F.E, Blood F.R and Belden D.A. (The Manual for Laboratory Work in Mammalian Physiology. 3rd ed. The University of Chicago Press, Illinois. 1965. p. 4-6.
15. Clarkson B.D, Burchenal J.H., Preliminary screening of antineoplastic drugs. Progress in Clinical Cancer, 1965, 1, 625-629.
16. Obiling C and Guerin M. The Role of Viruses in the Production of Cancer. Advances in Cancer Research II . Academic Press, New York. 1954. p. 406-411.
17. Price V.E, Greenfield R.E. Anemia in cancer. In: Grenstein, J.P., Haddow, A. (Eds.), Advance in Cancer Research, vol. 5. Academic Press, New York. p. 199-200.
18. Hogland H.C. Hematological complications of cancer chemotherapy. Sem Oncology, 1982, 9, 95–102.
19. Brown J.P. A review of the genetic effect of naturally occurring flavonoids, anthraquinones and related compounds. Mutation Research, 1980, 75, 243-247.
20. Hirano T, Oka K, Akiba M. Antiproliferative effect of synthetic and naturally occurring flavonoids on tumor cells of human breast carcinoma cell lines, ZR-75-1. Research in Community Pathology and Pharmacology, 1989, 64, 69-78.
21. Weber G, Shen F, Prajda N, Yeh Y.A, Yang H. Increased signal transduction activity and down regulation in human cancer cells. Anticancer Research, 1996, 16, 3271-3282.
22. Fotsis T, Pepper M.S, Aktas E, Breit S, Rasku S, Adlercreutz H. Flavonoids, dietary-derived inhibitors of cell proliferation and in vitro angiogenesis. Cancer Research, 1997, 57, 2916-2921.
23. Tiwari O.P, Tripathi Y.B. Antioxidant properties of different fractions of Vitex negundo Linn. Food Chemistry, 2007, 100, 1170–1176.

*****