

# Assessment of Anti-proliferative Effect of Extract Fractions of *Euphorbia neriifolia* Leaves in Human Prostate Adenocarcinoma DU-145 Cells

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## Mali *et al.*: Anti-proliferative Effect of *Euphorbia neriifolia* Leaves in DU-145 Cells

*Euphorbia neriifolia* Linn. Sp. Pl. (451.1753) belonging to family Euphorbiaceae has several ethnomedicinal uses. To authenticate these uses scientifically, we have assessed anti-proliferative effect of extract fractions of *Euphorbia neriifolia* leaves in human prostate adenocarcinoma DU-145 cells by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide cell proliferation assay. 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide cell proliferation assay was used for assessment anti-proliferative effect of extract fractions of *Euphorbia neriifolia* leaves in human prostate adenocarcinoma DU-145 and normal (Vero) cells. The cells were treated with series of concentrations of extract fractions (4.12-1000 µg/ml) for 48 h. Doxorubicin was considered as standard anti-cancer drug. In order to inhibition concentration and Selectivity Index results, extract fractions have been classified into 05 categories as described by Prayog *et al.* No extract fraction (EN1 to EN6) of *Euphorbia neriifolia* leaves was found in first 02 categories at the tested concentrations. Only doxorubicin standard drug sample was found in second category. It can be concluded that no other cells studies were documented yet on isolates and extract fractions of *Euphorbia neriifolia* by *in vitro* methods except murine B16F10 melanoma cells. Therefore, there is a need to carry forward these studies at higher concentrations of extract fractions (EN1 to EN6) in same and other adenocarcinoma cells. It can also be study by using other *in vivo* allograft and xenograft experimental models to confirm anti-proliferative or anti-cancer effects of these extract fractions.

**Key words:** Anti-proliferative effect, DU-145 cells, *Euphorbia neriifolia* Leaves, Extract fractions, MTT cell proliferation assay

*Euphorbia neriifolia* (*E. neriifolia*) is worldwide scattered in Baluchistan, Burma, India and Malaysian Islands. In India, it is found in rocky ground throughout Deccan Peninsula and Orissa. It is habitually cultivated for hedges in villages all over India<sup>[1,2]</sup>. *E. neriifolia*

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has several ethnomedicinal uses. The latex of *E. neriifolia* is an active ingredient of many Ayurvedic formulations like Abhaya lavana, Avittoladi bhasma, Citrakadi taila, Jatyadi varti, Snuhidugdhadi varti, Snuhi ghrta and Jalodarari ras. *E. neriifolia* has been traditionally indicated in Vatavyadhi, Gulma, Udara, Sula, Sotha, Arsas, Kusta and Medoroga<sup>[3,4]</sup>. Latex is acrid, laxative, pungent and good for treatment of tumors, abdominal troubles and leucoderma. It is also used as a purgative, rubefacient, carminative, expectorant, whooping cough, gonorrhea, leprosy, asthma, dyspepsia, and jaundice, enlargement of the spleen, colic and stone in the bladder. It is used to remove cutaneous eruptions and warts<sup>[1,5]</sup>. Leaves are brittle, heating, carminative, improve the appetite, good for treatment of tumors, pain, inflammation, abdominal swelling and bronchial infection<sup>[5]</sup>. The leaves of *E. neriifolia* extract and its isolates have been reported scientifically using various *in vivo* and *in vitro* experimental methods for anti-carcinogenic<sup>[6-8]</sup> and cytotoxicity<sup>[9,10]</sup> properties. The leaves of *E. neriifolia* is reported to have phytochemicals such as kaempferol<sup>[11]</sup>, taraxerol<sup>[12]</sup>, euphol<sup>[13]</sup>, quercetin and rutin<sup>[14]</sup>, (24R)-cycloartane-3 $\beta$ ,24,25-triol,5,4'-dihydroxy-3,7,3',5' tetramethoxyflavone, pachypodol, combretol<sup>[15]</sup>, 2-(3,4-dihydroxy-5-methoxy-phenyl)-3, 5-dihydroxy-6,7-dimethoxychromen-4-one<sup>[16,17]</sup>, etc. Prostate Carcinoma (PCa) is the 2<sup>nd</sup> most common cause of cancer and 6<sup>th</sup> leading cause of cancer death among men worldwide<sup>[18]</sup>. Molecular mechanisms underlying onset and progression of PCa was not fully understood, but the factors implicated in pathogenesis of this disease include age, race, diet, androgen secretion and metabolism and activated oncogenes<sup>[19-21]</sup>. The search for efficient mode of prevention is presently in progress. Recently studies have shown that the use of Pomegranate Juice (PJ) components consists of luteolin, ellagic acid and puniceic acid together inhibit the growth of hormone-dependent and hormone-refractory prostate cancer cells and inhibit their migration<sup>[22]</sup>. Additionally, Bhandari has reviewed the beneficial role of pomegranate in other disorders including prostate cancer<sup>[23]</sup>. In reference with these studies, the role of *E. neriifolia* leaves in human prostate adenocarcinoma DU-145 cells was not evaluated yet. Therefore, the present study was an effort to assess anti-proliferative effect of extract fractions of *E. neriifolia* leaves in human prostate adenocarcinoma DU-145 and normal (Vero) cells using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) cell proliferation assay. The *E. neriifolia* plant and its leaves (Fresh

and well grown) were collected from local region of Bhopal, Madhya Pradesh, India. The taxonomist, Dr. Vijay V. Bhadane, Department of Botany, Pratap College, Amalner-425401, Maharashtra, India was authenticated the plant. Voucher specimen (No. PCA/Bot-P1637) was assigned and deposited in the same. The detailed procedure of extraction and fractionation of *E. neriifolia* leaves was mentioned in earlier report of Mali *et al.*<sup>[24]</sup>. The extract fractions obtained were concentrated, dried and designated as EN1 (Petroleum ether extract fraction), EN2 (Toluene extract fraction), EN3 (Chloroform extract fraction), EN4 (Ethyl acetate extract fraction), EN5 (n-butanol extract fraction) and EN6 (Aqueous extract fraction) use for further studies. The stock solutions of extract fractions was prepared by weighing accurately 10 mg of each extract fraction and dissolved into 1 ml of Dimethyl sulfoxide (DMSO) solution, i.e. 10 mg/ml and diluted with culture medium. The final working concentrations of each extract fractions were achieved 1000  $\mu$ g/ml. The human prostate adenocarcinoma (DU-145) and normal (Vero) epithelial cells (American Type Culture Collection, USA) were procured from the National Centre for Cell Science, Pune and Maharashtra, India. DU-145 and Vero cells was cultured in Dulbecco's modified eagle's media, fortified with 10 % fetal bovine serum and incubated under 5 % CO<sub>2</sub> humidified incubator at 37° until 70-80 % confluence reached. The cells were dissociated with 0.2 % trypsin, 0.02 % EDTA in phosphate buffer saline solution. The stock cultures were grown initially in 25 cm<sup>2</sup> tissue culture flasks, then in 75 cm<sup>2</sup>, and finally in 150 cm<sup>2</sup> tissue culture flask and cell proliferation assay was carried out 96 well microtiter plates. The cells were seeded in 96 well plates in DMEM supplemented with 10 % Fetal Bovine Serum (FBS). After 24 h of inoculation in humidified CO<sub>2</sub> (5 %) with air environment at 37°, when cells became 70-80 % confluent, then the cells were grown in 96 well plates for establishment of monolayer at density of 1 $\times$ 10<sup>5</sup> cells per well according to the instruction given in kit manual<sup>[25,26]</sup>. After achieving desired cell density, cells were treated with one third dilution series of various extract fractions (4.12, 12.35, 37.04, 111.1, 333.3 and 1000  $\mu$ g/ml) and incubated further for 24 h. Then after next day, 20  $\mu$ l of MTT solution (5 mg/ml) were added in each well and plate was re-incubated for 4 h. Finally, 100  $\mu$ l of DMSO:Isopropyl alcohol (IPA) (60:40) solubilizing mixture was added to dissolve formazan crystals. Then absorbance of the plate was measure at 570 nm by using a 96 well micro plate. Percentage cytotoxicity or

percentage cell growth inhibition was calculated using equation. Percentage viability= $(A_T - A_B)/(A_C - A_B) \times 100$

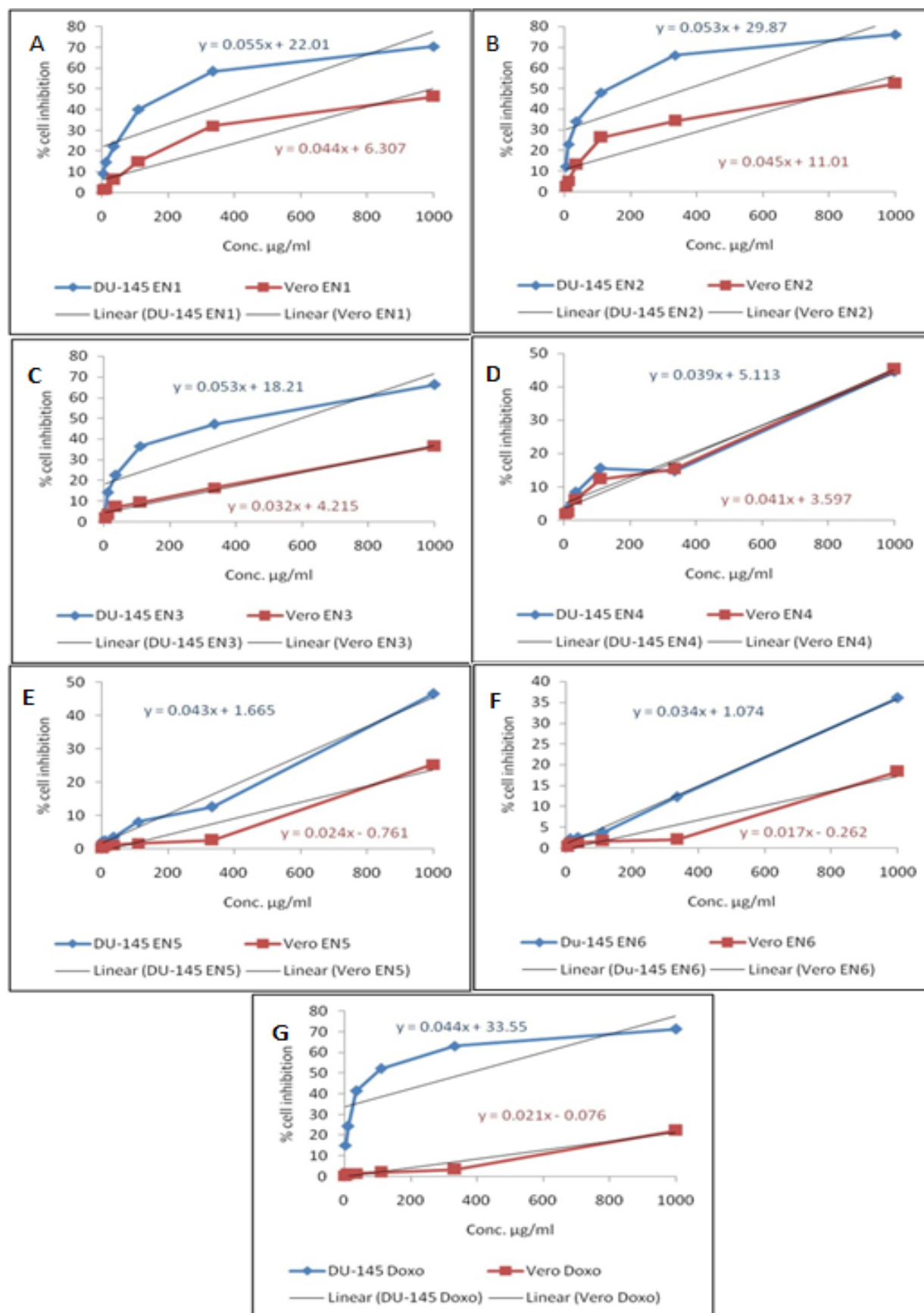
Where,  $A_T$ =Absorbance of treated cells (drug);  $A_B$ =Absorbance of blank (only media);  $A_C$ =Absorbance of control (untreated). Therefore, percentage cytotoxicity or percentage cell growth inhibition=100-percentage cell survival. The plot of percentage cell inhibition versus sample concentration was used to calculate the 50 % Inhibition Concentration ( $IC_{50}$ ). Selectivity Index (SI) was also calculated from the ratio of  $IC_{50}$  of Vero cells versus cancerous (DU-145) cells. SI values indicate selectivity of extract fraction in tested cells. Extract fractions with  $SI > 3$  were measured to have high selectivity<sup>[27-29]</sup>. Data of percent cell growth inhibition were expressed as means of three independent observations ( $n=3$ ). The  $IC_{50}$  was determined by interpolating concentration (X-axis) vs. percentage cell inhibition (Y-axis) by linear regression equation using Microsoft Excel, 2007, Microsoft Corporation, USA. Cytotoxicity (*In vitro*) analysis has become an essential part of drug discovery process because it is convenient, cost effective and predictive means of characterizing toxic effects of new chemical entities. The early and routine implementation of this analysis is evidence to its prognostic importance for humans<sup>[30]</sup>. *In vitro* cell line model is of at least equal concern to the human xenograft model<sup>[31]</sup>. Herbal medicine comprise a widespread substitute for cancer prevention and treatment worldwide<sup>[32-35]</sup>. Around sixty percent of anticancer medicines presently have isolated from medicinal plants. Recently, more than three thousand plants globally reported to have anticancer effects<sup>[36]</sup>. In emphasized with this, our result of percentage cell inhibition,  $IC_{50}$  and SI of extract fractions (EN1 to

EN6) of *E. neriifolia* leaves for both cells were shown in Table 1 and fig. 1. In order to  $IC_{50}$  and SI results, extract fractions have been classified into 05 categories as described by Prayog *et al.*<sup>[28]</sup>. Firstly, potentially cytotoxic ( $IC_{50}$  in DU-145 < 100  $\mu\text{g/ml}$ ) and high SI ( $SI \geq 3$ ), no sample was found in this category. Secondly, moderate cytotoxic (100  $\mu\text{g/ml} < IC_{50}$  in DU-145 < 1000  $\mu\text{g/ml}$ ) and high SI ( $SI \geq 3$ ), only Doxo sample was found. Thirdly, moderate cytotoxic (100  $\mu\text{g/ml} < IC_{50}$  in DU-145  $\leq 1000 \mu\text{g/ml}$ ) and less SI ( $SI < 3$ ), the EN1, EN2 and EN3 samples were found. Fourthly, toxic to only Vero cells and it was EN1 and EN2. Lastly, non-toxic in DU-145 and Vero cells ( $IC_{50} > 1000 \mu\text{g/ml}$ ) and they were EN4, EN5 and EN6. Extract fractions in first 02 categories could be taken for further bioassay guided experimentation and highest SI indicate its ultimate potential for biopharmaceutical use among the tested extract fractions<sup>[28]</sup>. In line with above statement, no extract fraction was found in first 02 categories at the tested concentrations. Only doxorubicin standard drug sample was found in second category. As per earlier evidences on *in vitro* cytotoxicity studies on *E. neriifolia* isolates and extracts which suggested that studies were performed using murine B16F10 melanoma cell line only. These studies includes *in vitro* cytotoxicity of euphol isolated from triterpenoidal sapogenin fraction of *E. neriifolia* leaf was assessed using murine F10B16 melanoma cell line. Results of assay revealed that 50 % inhibition concentration was 173.78  $\text{mg/ml}$ <sup>[13]</sup>. Anticancer activity of methanolic extracts of *E. neriifolia*, *Euphorbia hirta* (*E. hirta*) and *Euphorbia tirucalli* (*E. tirucalli*) against B16F10 melanoma cancer cell line was performed. These plants showed significant cytotoxicity against B16F10

**TABLE 1: PERCENTAGE CELL INHIBITION,  $IC_{50}$  AND SI OF EXTRACT FRACTIONS OF *E. NERRIFOLIA* AGAINST DU-145 AND VERO CELLS**

Conc.	DU-145 cells						Vero cells							
	EN1	EN2	EN3	EN4	EN5	EN6	Doxo	EN1	EN2	EN3	EN4	EN5	EN6	Doxo
$\mu\text{g/ml}$														
4.12	9.15	12.12	2.26	2.25	2.26	1.55	15.24	1.64	2.31	1.67	1.98	0.35	0.35	0.65
12.35	14.67	23.02	14.15	3.65	2.43	2.23	24.35	2.06	5.34	3.41	2.45	0.98	0.98	1.14
37.04	22.36	34.13	22.56	8.45	3.56	2.65	41.37	6.34	13.05	7.23	6.34	1.22	1.23	1.57
111.11	40.14	48.07	36.48	15.54	8.23	3.75	52.2	15.24	26.34	9.34	12.34	1.65	1.65	2.13
333.33	58.5	66.15	47.18	14.65	12.65	12.24	63.1	32.21	34.24	16.34	15.32	2.65	2.04	3.62
1000	70.39	76.09	66.28	44.55	46.45	36.05	71.24	46.21	52.31	36.35	45.34	25.34	18.32	22.31
$IC_{50} \mu\text{g/ml}$	508.9	379.8	599.8	1150 <sup>A</sup>	1124 <sup>A</sup>	1439 <sup>A</sup>	376.8	993	866.4	1430 <sup>A</sup>	1131 <sup>A</sup>	2115 <sup>A</sup>	2956 <sup>A</sup>	2384 <sup>A</sup>
<b>Selectivity Index (S.I)</b>														
Samples	EN1	EN2	EN3	EN4	EN5	EN6	Doxo							
S.I	1.95	2.28	2.38	0.98	1.88	2.05	>6.32 <sup>B</sup>							

DU-145: Human prostate adenocarcinoma cells; Vero: normal African green monkey kidney cells; EN1: Petroleum ether extract fraction; EN2: Toluene extract fraction; EN3: Chloroform extract fraction; EN4: Ethyl acetate extract fraction; EN5: n-butanol extract fraction; EN6: Aqueous extract fraction; Conc.: Concentration ( $\mu\text{g/ml}$ ); Doxo: Doxorubicin;  $IC_{50}$ : 50 % inhibition concentration; Data of percent cell growth inhibition were expressed as the means of three independent observations ( $n=3$ ).  $SI = IC_{50}$  of Vero cells/ $IC_{50}$  of DU-145 cells; <sup>A</sup> $IC_{50} > 1000 \mu\text{g/ml}$  is considered to inactive; <sup>B</sup>SI referred to SI, when SI value > 3 indicates high selectivity.



**Fig. 1: Percentage cell inhibition of extract fractions of *E. neriifolia* against DU-145 and Vero cells. A: Percentage cell inhibition of EN1 extract fraction; B: Percentage cell inhibition of EN2 extract fraction; C: Percentage cell inhibition of EN3 extract fraction; D: Percentage cell inhibition of EN4 extract fraction; E: Percentage cell inhibition of EN5 extract fraction; F: Percentage cell inhibition of EN6 extract fraction; G: Percentage cell inhibition of doxorubicin**

melanoma cell line in concentration range 10-1000 ml using Sulforhodamine B (SRB) and MTT assay. Fifty percent inhibition concentration of methanolic extract of *E. neriifolia*, *E. hirta* and *E. tirucalli* was 198.26, 185.41 and 20.10 by SRB assay and 212.78, 240.98 and 237.07 by MTT assay. Methanolic extract of all these three plants showed significant activity against B16F10 melanoma cells<sup>[9]</sup>. Based on reported biological activities, the *E. neriifolia* extracts and isolates can be explored for their therapeutic potential by use of modern assay methods. Molecular mechanisms should be established for therapeutic applications<sup>[37]</sup>. It can be concluded that no other cells studies were documented yet on isolates and extract fractions of *E. neriifolia* by *in vitro* methods except murine B16F10 melanoma cells. Therefore, there is a need to carry forward these studies at higher concentrations of extract fractions (EN1 to EN6) in human prostrate adenocarcinoma (DU-145) and normal (Vero) cells. It can also be study by using other *in vivo* allograft and xenograft experimental animal models to confirm anti-proliferative or anti-cancer effects of these extract fractions.

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### Conflict of interests:

The authors declared no conflict of interest.

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