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Comparative Antiproliferative activity of aerial parts of few Apocynaceae plants in HepG2, HT29 and SK-OV3 Human cancer cell lines

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ABSTRACT

Aims: Apocynaceae family is the 5th largest medicinal plant family rich in potent secondary metabolites such as Alkaloids, Cardiac glycosides, Terpenoids, irridoid/secoirridoids, flavonoids and Phenolic contents. The present study was aimed to evaluate and compare in-vitro antiproliferative activity of three plants of this family.

Methods: Aerial parts of Carissa carandas Linn. (C), Nerium indicum Mill. (N) and Wrightia tinctoria RBr. (W), were collected and dried. The powdered drugs were extracted in Ethanol (1), 60% Ethanol (2) and Water (3). Estimation of Phytoconstituents performed using standard methods. In-vitro cytotoxic activity performed using Sulphorhodamine B (SRB) assay in HepG2, HT29 and SKOV3 human cancer cell lines taking Adriamycin (ADR) as standard. For extracts, GI50 value ≤ 20µg/ml was considered to demonstrate activity.

Results: For HepG2 cell line graphs and photomicrographs showed GI50 value as ADR=39.79, C1=2.5, N2=66.3, N3<10 and C2=C3= N1=W1-3>80. Also TGI for C1>80. The extracts, C1, C2, N1, N2, and N3 were found to possess activity against HepG2.These extracts were screened on HT 29 and SKOV3cell lines. The GI50 value observed was<10 for C1, N2, N3 and ADR in HT 29 and <10 for N3 and ADR in SK OV3 cell lines. Thus it was found that aqueous extract of Nerium indicum (N3) and Ethanolic extract of Carissa carandas (C1) were most cytotoxic extracts against all three cell lines.

Conclusions: our study establishes that Apocynaceae family plants could be an important anticancer lead and could serve as Botanical drug for neoplasia.

Keywords: Apocynaceae, SRB Assay, Phytoconstituents, Anticancer drug screening models, Hep G2, HT 29, SK-OV3, HCC.

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1. INTRODUCTION

Cancer is among the prominent health problem after cardiovascular diseases in both developed and developing nations. Deaths due to cancer in India are 9% among all NCDs [1]. Cancer mortality in India has doubled from 1990 to 2016. With the advent of 2040, new cancer cases will increase by 29.5 million globally [2].

Public cancer facilities in India are woefully inadequate, and there is large presence of private cancer care facilities [3].

Cancer cells reproduce without restraint and colonize other tissues. Most cancers develop gradually from increasingly aberrant cells. Cancerous growth often depends on defective control of cell death, cell differentiation, or both. Cancer cells are usually altered in their responses to DNA damage and other forms of stress [4, 5]. Carcinogenesis is a result of many known and unknown factors biological, chemical, physical and environment factors [5, 7].

"Nature is the best healer". Remedy for all sort of illness is surmounted within Mother Nature. Aboriginals of the remotest places, disconnected from the central facilities survived and surviving even today by their skill of utilizing natural resources. Importance of Plants and their Phytoconstituents are being recognized globally for their pharmaceutical, neutraceutical and other livelihoods. Herbal drugs form an inseparable part of the allopathic drug as an adjuvant for management of various disorders [8].

Many important anticancer drugs like, the vinca alkaloids (vinblastine, vincristine and vindesine), the epipodophyllotoxins (etoposide and teniposide), the taxanes

(paclitaxel and docetaxel) and the camptothecin derivatives (camptotecin and irinotecan) etc. are derived from plants [9, 10].

"To date the best source of anticancer agents have come from toxic plants". Based on traditional claims, Arrow poisons are anticancer [11].

Apocynaceae family, the fifth valuable medicinal plant family among Angiosperms is also called as Dogbane or Arrow poisons family [12-20].Traditional uses and potential anticancer role of Apocynaceae family plants are widely documented [21-24].

Development of anticancer drug, pre-clinical and clinical trials to compare its benefits to risks ratio with the conventional drugs is need of an hour [25-27].This research article evaluates the anticancer potential of randomly selected three plants of Apocynaceae after an exhaustive literature review. Most active aqueous extract of *Nerium indicum* (N3) was further evaluated for its anti-inflammatory, antimicrobial, immunomodulatory and in Hepatic damage induced DEN and CCl4 model used to simulate Hepatocellular carcinoma (HCC). This kind of study and its inferences are unique of its kind.

2. MATERIAL AND METHODS

2.1 Collection and authentication of plant

Fresh parts of Carissa carandas linn., *Nerium indicum* mill. and *Wrightia tinctoria* RBr, were collected in the month of March-April 2015, from Jhansi region of Bundelkhand (Figure 1-3). The Plants were identified and authenticated by Dr. Mudailiya, taxonomist NVARI, Jhansi, UP India with accession no. 24380, 24381 and 24382 respectively for future reference.

2.2 Plant extracts preparation

The collected aerial parts of the plants were washed thoroughly, shade dried, powdered and sieved Extractive yield of plants performed by extracting the plant through maceration process. 5g powdered drug added with 50 ml of solvents polarity wise taken in 250 ml conical flasks. Intermittent shaking of flasks done in 24 hrs maceration period. Afterwards filtration, concentration and drying of extract done and percentage yield calculated using the formula

%yield= (W1/W2) x 100

Soxhlet extraction of powdered drug was performed in water, 60% Ethanol and Ethanol separately [26, 27].The extracts were filtered, concentrated to dryness in Rotary evaporator and Lyophilizer. Percentage yield calculated and stored in deep freezer for future use.

2.3 Phytochemical study

Qualitative estimation of Phytoconstituents performed according to standard method [28- 30].

2.4 Anticancer screening

For in vitro study, the cancer cell line culture (Hep G2, HT 29 and SK OV3) and media used in this study and standard drug Adriamycin (ADR) were arranged at Advanced Centre for Treatment Research and Education in Cancer (ACTREC), Kharghar, Navi Mumbai. Other studies were conducted at Bundelkhand University, Jhansi and Pinnacle Biomedical research institute, Bhopal.

In vitro Study

SRB Assay procedure as per ACDSF (ACTREC), Mumbai [31, 32].

The cell lines were grown in RPMI 1640 medium containing 10% fetal bovine serum and 2 mM L-glutamine. Cells were inoculated into 96 well microtiter plates in 100 µL at plating densities, depending on the doubling time of individual cell lines. After cell inoculation, the microtiter plates were incubated at 37°C, 5% CO2, 95% air and 100% relative humidity for 24 h prior to addition of experimental drugs.

Extracts were solubilized in dimethyl sulfoxide at 100mg/ml and diluted to 1mg/ml using water and stored frozen prior to use. At the time of drug addition, an aliquote of frozen concentrate (1mg/ml) was thawed and diluted to 100 µg/ml, 200 µg/ml, 400 µg/ml and 800 µg/ml with complete medium containing test article. Aliquots of 10 µl of these different drug dilutions were added to the appropriate microtiter wells already containing 90 µl of medium, resulting in the required final drug concentrations i.e.10 µg/ml, 20 µg/ml, 40 µg/ml, 80 µg/ml. then plates were incubated for 48 hours and assay was terminated by the addition of cold TCA. Cells were fixed in situ by the gentle addition of 50 µl of cold 30 % (w/v) TCA (final concentration, 10 % TCA) and incubated for 60 minutes at 4°C. The supernatant was discarded; the plates were washed five times with tap water and air dried. Sulforhodamine B solution (50 µl) at 0.4 % (w/v) in 1 % acetic acid was added to each of the wells, and plates were incubated for 20 minutes at room temperature. After staining, unbound dye was recovered and the residual dye was removed by washing five times with 1 % acetic acid. The plates were air dried. Bound stain was subsequently eluted with 10 mM trizma base, and the absorbance was read on a plate reader at a wavelength of 540 nm with 690 nm reference wavelength.

Percent growth was calculated on a plate-by-plate basis for test wells relative to control wells. Percent Growth was expressed as the ratio of average absorbance of the test well to the average absorbance of the control wells * 100.

Using the six absorbance measurements [time zero (Tz), control growth (C), and test growth in the presence of drug at the four concentration levels (Ti)]; the percentage growth was calculated at each of the drug concentration levels. Percentage growth inhibition was calculated as:

[Ti/C] x 100 %

Statistical analysis of Data

All values are expressed as Mean ± SD (mean and standard deviation) of three replicated experiments. The analysis was performed using Microsoft excel 2007 and SPSS statistical package for WINDOWS (version 16.0; SPSS, Inc., Chicago, IL, USA).

3. RESULTS AND DISCUSSION

Extraction

Extraction of phytoconstituents performed and obtained percentage yield (Table 1)

In Vitro Anticancer activity

Results are shown in Table 4a-4b and 5a-5b. Photomicrograph of the cells predicts the cell density (Figure 4-29). Adriamycin was taken as standard to validate the studies. Extracts of *Wrightia tinctoria* (W1, W2 and W3) was found totally inactive in Hep G2 and SK OV3 cell lines. Other extracts except C1 and N3 tested did not show any significant activity.

Index of antiproliferative activity was calculated from graph and expressed in terms of LC50, GI50 and TGI. Plant extract with GI 50 value ≤ 20µg/ml is considered to demonstrate activity. In Hep G2 cell line the order of activity was as: C1 (2.5)> N3 (<10)> N2 (66.3)> C2=C3 (>80). Antiproliferative effect of these extracts in HT 29 has GI 50 value as C1, N2, N3<10 and N3 is the highly active with GI50<10 µg/ml in SK OV3 cell line. Thus Aqueous extract of *Nerium indicum* Mill. (N3) is most active extract in all three cancer cell lines. The Concentration of drug causing total inhibition of cell growth (TGI) value <10 for N3 and 36.8 for C1 in HT 29 cell line. The Concentration of drug causing 50% cell kill (LC50) value for N3 is 56.8 and it is Non-evaluable in other extracts.

Table 1 Extractive Yield

Solvents with decreasing polarity	% yield w/w= W1/W2x100		
	Carissa	Nerium	Wrightia
n-Hexane	1.182	3.814	1.631
Petroleum Ether	0.64	4.21	3.32
Ethyl Acetate	0.77	1.45	2.81
chloroform	4.43	1.439	2.50
Ethanol	7.27	10.86	7.86
Water	11.28	3.78	5.11

Table 2 Qualitative phytoconstituents Analysis

S.NO.	Test	*Carissa carandas*			*Nerium indicum*			*Wrightia tinctoria*		
Tests	Extracts	C1	C2	C3	N1	N2	N3	W1	W2	W3
Glycosides-cardiac/anthraquinone	Borntrager's test	+++	+++	+++	++	++	+/-	+	+	+
Saponins	Foam test	++	++	++	-	-	++	++	++	++
Oils and fat	Spot test	+	+	+	+++	++	+	+	+	+
Phlobatannins/ Chalcones	HCl test/spot test	-	-	-	-	-	-	+	-	-
Flavonoids	$AlCl_3$ test/ alkaline reagent test/lead acetate test	+++	+++	+++	+++	+++	+++	+++	+++	+++
Tannins/Phenolic compound	$FeCl_3$ test/ Lead acetate test	+++	+++	+++	+++	+++	+++	+++	+++	+++
Alkaloids	wagner's test/mayer's test/hagers/dragendorffs	++++	++++	++++	++++	++++	++++	++++	++++	++++
Protein/Amino acids	ninhydrin/ xanthoproteic	++	++	++	++	++	++	++	++	++
Steroids	salkowski's test/ Liebermann buchard	++	+	-	-	-	-	+	-	-
Phytosterols	sulphuric acid test	+	-	-	+	-	-	+	-	-
Carbohydrates/ sugar	molish's test/ Benedict's test	+	+	-	+	-	+	+	+	+
Coumarins	fluorescens test	+	-	-	+	-	-	+	-	

Table 3a Percent control growth in Hep G2 cancer cell line

sample	Human Hepatoma Cell Line Hep-G2															
	% Control Growth															
	Drug Concentrations (µg/ml)															
	Experiment 1				Experiment 2				Experiment 3				Average Values			
	10	20	40	80	10	20	40	80	10	20	40	80	10	20	40	80
C1	51.3	46.5	28.7	23.2	44.8	37.4	24.9	8.4	48.9	48.3	27.4	19.7	48.3	44.1	27.0	17.1
C2	134.9	140	144.6	133.9	132.6	140.1	141.3	136.5	125.8	137.4	137.8	155.2	131.1	139.2	141.2	141.9
C3	138.5	155.1	152.3	145.8	136.8	139.2	141.1	151.5	130.0	147.2	146.5	159.4	135.1	147.2	146.6	152.2
N1	132.5	143.5	151.5	136.0	130.0	150.8	146.7	144.9	123.1	133.4	143.4	133.9	128.5	142.6	147.2	138.3
N2	76.2	59.1	68.1	42.0	70.0	59.2	63.8	42.0	80.2	59.6	60.2	48.6	75.5	59.3	64.0	44.2
N3	14.8	22.3	17.5	23.5	15.8	20.2	15.0	15.0	20.7	15.1	3.9	5.9	17.1	19.2	12.1	14.8
WI	129.2	135.2	143.0	123.8	134.7	139.3	133.4	135.2	136.1	146.2	120.7	119.9	133.3	140.2	132.4	126.3
W2	129.8	141.0	125.1	123.4	140.7	144.1	127.3	98.6	143.2	159.3	140.2	98.3	137.9	148.1	130.9	106.8
W3	135.6	164.6	176.9	177.5	151.4	158.3	168.2	172.0	136.1	160.0	158.5	160.1	141.0	161.0	167.9	169.9
ADR	180.0	134.3	63.6	-27.7	67.2	139.9	-9.5	-38.7	123.3	105.8	-39.4	-32.3	123.5	126.7	4.9	-32.9

Table 4a Percent control growth in HT 29 cancer cell line

	Human Colon Cancer Cell Line HT-29, % Control Growth															
	Drug Concentrations (µg/ml)															
	Experiment 1				Experiment 2				Experiment 3				Average Values			
	10	20	40	80	10	20	40	80	10	20	40	80	10	20	40	80
C 1	20.2	14.7	5.7	-42.5	22.4	14.6	4.8	-53.4	20.1	15.6	4.9	-35.4	20.9	14.9	5.1	-43.7
C 2	107.1	108.9	115.4	112.7	104.2	106.1	106.8	106.7	106.4	117.5	115.8	109.3	105.9	110.8	112.7	109.6
N 1	100.8	106.7	123.9	131.9	106.0	106.5	119.4	120.3	105.7	118.1	117.7	121.5	104.2	110.5	120.3	124.6
N 2	37.0	23.0	28.2	20.3	34.1	23.8	29.2	18.6	31.2	24.2	25.1	18.5	34.1	23.7	27.5	19.1
N 3	6.5	-34.3	-50.4	-53.9	3.6	-35.1	-48.2	-51.4	-11.9	-53.2	-69.4	-60.5	-0.6	-40.9	-56.0	-55.3
A DR	-2.9	-6.1	-9.1	-11.3	1.6	-9.2	-3.2	-9.1	-6.3	-18.3	-18.3	-8.2	-2.5	-11.2	-10.2	-9.3

Table 5a Percent control growth in SK-OV3 cancer cell line

	Human Ovarian Cancer Cell Line SK-OV-3, % Control Growth															
	Drug Concentrations (µg/ml)															
sample	Experiment 1				Experiment 2				Experiment 3				Average Values			
	10	20	40	80	10	20	40	80	10	20	40	80	10	20	40	80
C1	93.2	66.7	65.4	63.2	83.7	68.0	62.0	72.1	87.0	103.6	111.4	69.1	88.0	79.4	79.6	68.1
C2	104.0	104.6	109.5	111.8	116.6	132.8	121.7	145.0	130.2	122.7	143.4	123.3	116.9	120.1	124.9	126.7
N1	106.2	101.2	113.5	122.0	122.0	113.9	130.1	134.7	135.9	119.8	131.4	136.9	121.4	111.6	125.0	131.2
N2	103.7	77.4	94.8	71.7	101.3	82.4	92.4	77.5	106.9	110.9	122.4	93.3	103.9	90.2	103.2	80.8
N3	76.0	62.2	65.4	58.6	70.0	59.5	54.1	75.2	70.1	77.3	94.5	68.5	72.0	66.3	71.3	67.4
ADR	-39.8	-47.4	-44.2	-31.4	-58.6	-60.6	-61.3	-40.7	-58.2	-60.1	-55.3	-23.7	-52.2	-56.0	-53.6	-31.9

Table 6b Percent control growth in Hep G2 cancer cell line

	Human Hepatoma Cell Line Hep G2- % Growth									
	Drug concentrations (µg/ml) calculated from graph									
	C1	C2	C3	N1	N2	N3	W1	W2	W3	ADR
LC 50	NE	NE	NE	NE	NE	NE	NE	NE	NE	80.9
TGI	>80	NE	NE	NE	NE	NE	NE	NE	NE	60.3
GI50*	2.5	>80	>80	>80	66.3	<10	>80	>80	>80	39.79

Table 5b-7b Percent control growth in HT 29 and SK-OV3 cancer cell line

	Human Cancer Cell Line % Growth											
	Drug concentrations (µg/ml) calculated from graph											
	Human Colon Cancer Cell Line- HT 29						Human Ovarian Cell Lines SK OV 3					
	C1	C2	N1	N2	N3	ADR	C1	C2	N1	N2	N3	ADR
LC 50	NE	NE	NE	NE	56.8	NE	NE	NE	NE	NE	NE	32.6
TGI	36.8	NE	NE	NE	<10	NE	NE	NE	NE	NE	NE	NE
GI50*	<10	>80	>80	<10	<10	<10	>80	>80	>80	>80	<10	<10

** LC50 = Concentration of drug causing 50% cell kill. GI50 = Concentration of drug causing 50% inhibition of cell growth. TGI = Concentration of drug causing total inhibition of cell growth. ADR = Adriamycin, Positive control compound. NE = Non- evaluable data. Experiment needs to be repeated using different set of drug concentrations. GI50 value of ≤ 10^-6 molar (i.e. 1 µmolar) or ≤ 10µg/ml is considered to demonstrate activity in case of pure compounds. For extracts, GI50 value ≤ 20µg/ml is considered to demonstrate activity. Yellow highlighted test values under GI50 column indicate activity

Figure 1 *Carissa carandas* Linn. (C)

Figure 2 *Nerium indicum* Mill.(N)

Figure 3 *Wrightia tinctoria* R.Br.(W)

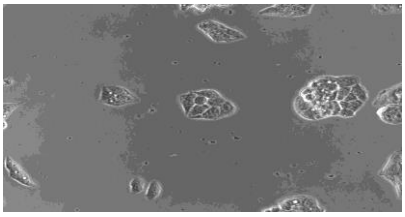

Figure 4 HEP G2 Control

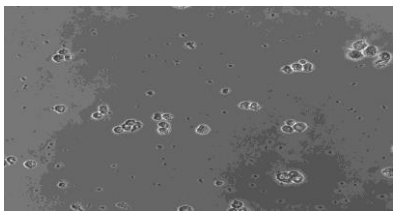

Figure 5 HEP - G2 positive control

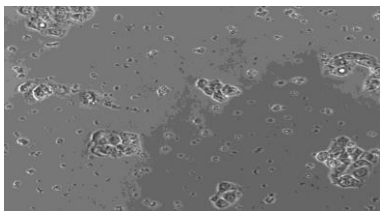

Figure 6 HEP - G2 C1

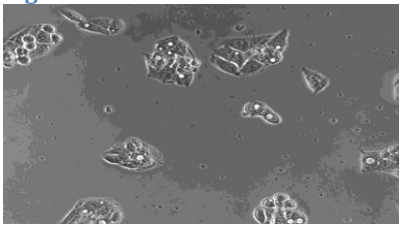

Figure 7 HEP G2 C2

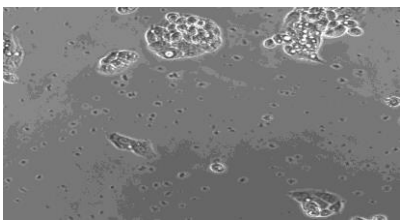

Figure 8: HEP G2 C3

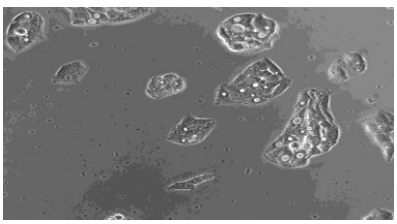

Figure 9 HEP - G2 N1

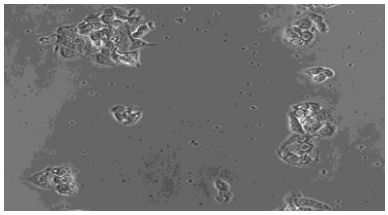

Figure 10 HEP G2 N2

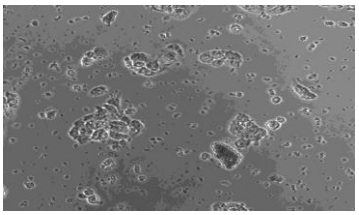

Figure 11 HEP - G2 N3

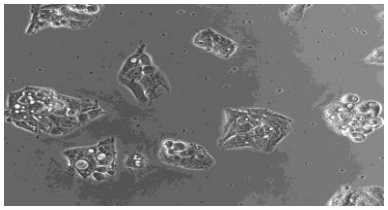

Figure 12 HEP - G2 W1

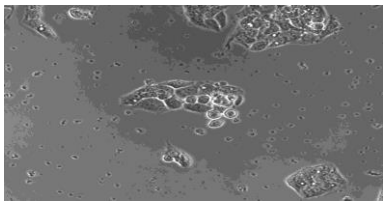

Figure 13 HEP - G2 W2

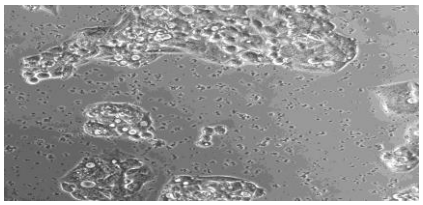

Figure 14 HEP - G2 W3

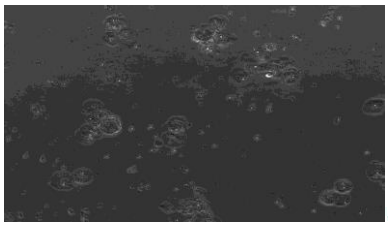

Figure 17 HT 29 C1

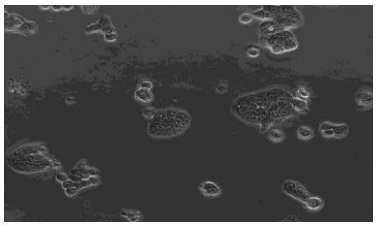

Figure 18 HT 29 C2

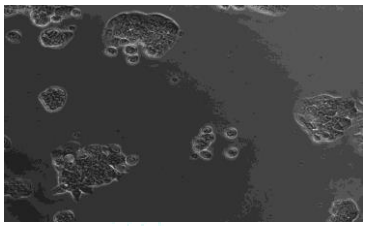

Figure 19 HT 29 N1

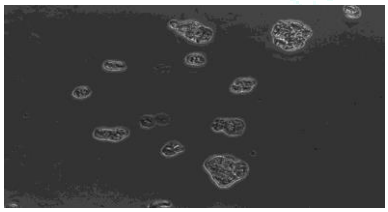

Figure 20 HT 29 N2

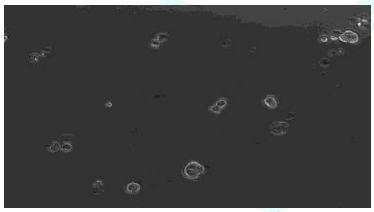

Figure 22 HT 29 N3

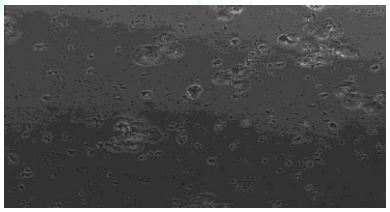

Figure 15 HT 29 Positive Control

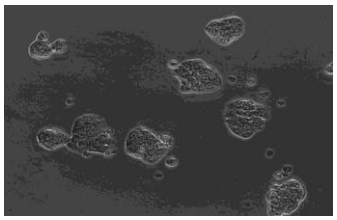

Figure 16 HT 29 Control

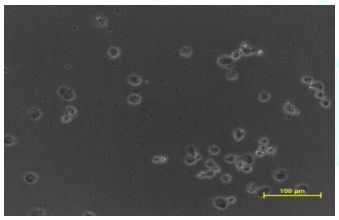

Figure 23 SK OV3 Positive control

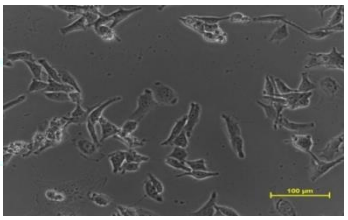

Figure 24 SK OV3 Control

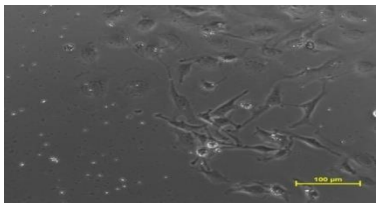

Figure 25 SK OV3 C1

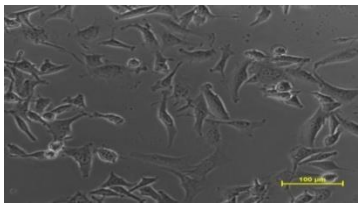

Figure 26 SK OV3 C2

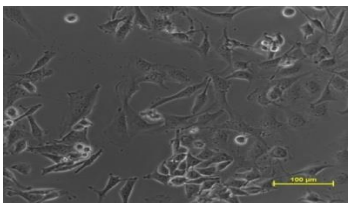

Figure 27 SK OV3 N1

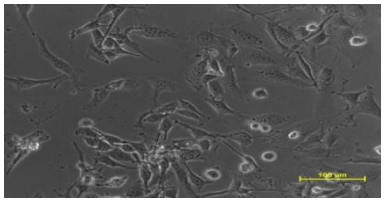

Figure 28 SK OV3 N2

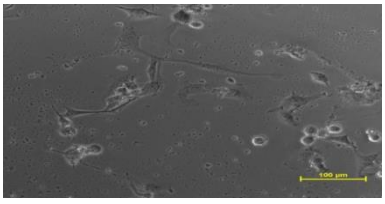

Figure 29 SK OV3 N3

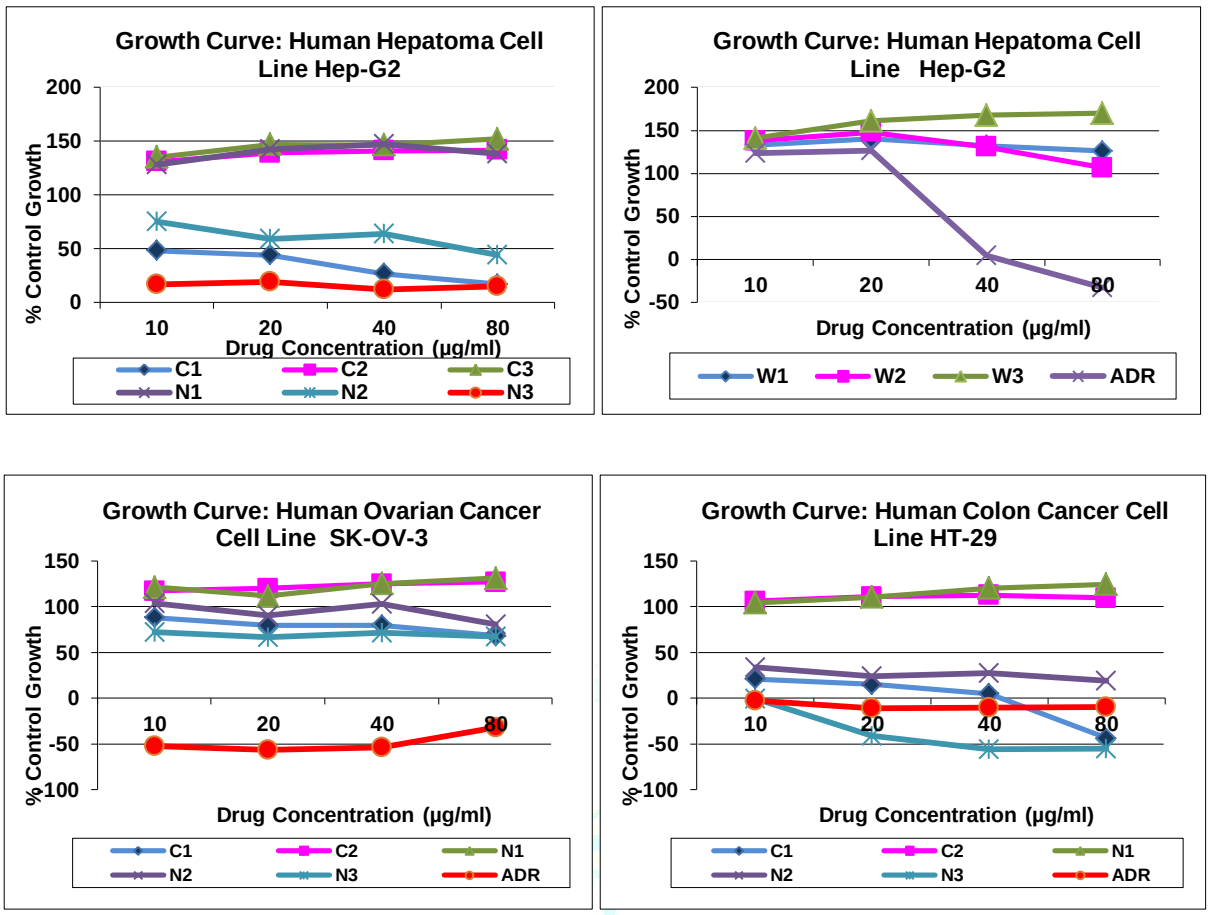

Figure 30 Growth Curve of various cell lines

CONCLUSION

Since three decades a vast majority of plants are being screened for their cytotoxic actions. A plethora of preclinical data is available in reviews, research and reports. Abundance of traditional knowledge from herb practitioners (tribal, villagers and Ayurvedachaarya etc.) has made possible to screen cytotoxic plants [33, 34]. These successes have resulted from testing of some extracts or derived compounds, with little regard to the possible metabolism of constituents in vivo or to activities other than cytotoxicity that might reduce carcinogenesis. In vitro assays are easy tools and provide large data at time. This study is the re-investigation of extracts based on their traditional claims to possess cytotoxicity. Therefore this is a novel approach to complement standard cytotoxic screening procedures for research on previously uninvestigated material. The Results clearly indicate that Carissa carandas, Nerium indicum and *Wrightia Tinctoria* belonging to Apocynaceae family could be an important lead in cancer remediation. Thus, the current work clearly indicates that carvacrol could be a potent anti-tumor molecule Antineoplastic mechanism of these plants should be further studied for its possible mechanism and through different models in vivo models. Isolation of active phytoconstituents and their separate screening would further validate the for identification and authentication of plants and to provide the zones of distribution of these plants in Bundelkhand region.

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