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In vitro cytotoxic evaluation of some essential oils

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SUMMARY

Seven essential oils were tested for *in vitro* cytotoxicity against the cancerous cell lines A-549, HEp-2 and DLA and normal BRL-3A, NRK-49F and Vero cell lines using standard MTT, SRB and dye exclusion techniques. The A-549 cell line was found to be the most susceptible to all the essential oils. The essential oils of *A. nilagirica*, *A. calamus* and *O. sanctum* were found to be the more active against these cells with mean CTC_{50} values of 17.75, 19.00 and 24.37 μg/ml, respectively. The essential oil of *Acorus calamus* was found to be the most potent with low CTC_{50} values against the cancerous and comparatively higher CTC_{50} values against the normal cell lines. *Artemisia pellens* and *Pelargonium graveolens* oils also showed potent activity. These oils merit further investigation to identify the active principles and nature of the anti tumor activity in animal models.

Key words: Cytotoxicity; Essential oils; Terpenoids; Cell lines

INTRODUCTION

Natural products are known to provide lead compounds in the past and play a significant role in future in the treatment of cancer. Many research workers have evaluated the antitumor and anti carcinogenic potentials of essential oils (Belman, 1983; Zheng *et al.*, 1993; Aruna and Sivaramakrishnan, 1996). Essential oils obtained from *Helichrysum picardii* (De la Puerta *et al.*, 1993), *Myrcianthes* black fruit (Setzer *et al.*, 1999), lemon grass, galanga root (Zheng *et al.*, 1993), onion and garlic (Belman., 1983) have shown potent cytotoxic and antitumor properties. The major constituents of essential oils, terpenoids are also known to posses strong cytotoxic and anti tumor properties (Wang *et al.*, 1996; Chen *et al.*, 1999). In continuation of our studies to identify potent natural products for anti tumor activities, (Vijayan *et al.*, 2002; Vijayan *et al.*, 2003; Badami *et al.*, 2003), we have investigated the *in vitro* cytotoxic properties of seven essential oils against several cancerous and normal cell cultures using standard procedures. The oils were selected based on literature survey and traditional uses.

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MATERIALS AND METHODS

Plant materials

Acorus calamus rhizomes (Areaceae), *Artemisia nilagirica* leaves (Asteraceae), *Artemisia pellens* leaves and flowers (Asteraceae), *Pelargonium graveolens* entire plant (Geraniaceae), *Cymbopogon winterianus* seeds (Graminae), *Myristica fragrans* Kernels (Myristicaceae) and *Ocimum sanctum* aerial parts (Labiatae) were collected from the shola forests in and around Ootacamund, India and authenticated by Botanical Survey of India, Medicinal Plants Collection Unit, Government Arts College, Ootacamund, India. Voucher specimens are preserved in our laboratory for further reference.

Isolation of essential oils

Fresh plant materials were distilled in a Clavenger apparatus separately. The isolated oils were dried over anhydrous sodium sulphate and stored at 4-6°C. The essential oils were dissolved separately

in distilled Dimethyl Sulfoxide (DMSO) and the volume was made up to 10 ml with Dulbecco's Minimum Essential Medium (DMEM)/F-12 Coon's medium to obtain a stock solution of 1 mg/ml concentration and stored at -20°C until further use.

Chemicals

3-(4,5dimethyl thiazole-2 yl)-2,5-Diphenyl tetrazolium bromide (MTT), Sulphorhodamine B (SRB), Trypan blue and F-12 Coon's medium were obtained from Sigma Chemical Co., Mo, U.S.A. DMEM, phosphate buffered saline (PBS) and antibiotics used were obtained from Hi-media Ltd., Mumbai, India. Trichloro acetic acid (TCA) and tris buffer were obtained from SD fine chemicals Pvt. Ltd., Boisar, India. DMSO, glacial acetic acid and propanol were obtained from E.Merck Ltd., Mumbai, India.

Cell lines and culture medium

A-549 (Human epithelial small cell lung carcinoma), BRL-3A (Normal, rat liver), HEp2 (Caucasian male larynx epithelium carcinoma) and NRK-49F (Normal, rat kidney) cell cultures were obtained from National Center for Cell Sciences, Pune, India. DLA (Daltonís Lymphoma Ascites) cell line was obtained from Amala Cancer Institute, Trissur, India. Vero (Normal, African green monkey kidney) cell culture was obtained from Pasteur Institute of India, Coonoor, India. Stock cells of A-549, DLA, HEp2, NRK-49F and Vero cell lines were cultured in DMEM and BRL-3A cell line was cultured in F-12 Coon's medium supplemented with 10% inactivated new born calf serum, penicillin (100 IU/ml), streptomycin (100 µg/ml) and amphotericin B (5 µg/ml) in a humidified atmosphere of 5% CO_2 at 37°C until confluent. The cells were dissociated with TPVG solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS). The stock cultures were grown in 110 ml flat bottles and all experiments were carried out in 96 well microtitre plates (Tarson India Pvt. Ltd., Kolkata, India). DLA cells used were maintained in peritoneal cavity of Swiss albino mice.

Cytotoxicity assay

The cytotoxic assays were carried out using 0.1 ml of cell suspension, containing 10,000 cells seeded in each well of a 96 well microtitre plate. Fresh medium containing different concentrations of the essential oils was added to the wells 24 h after seeding. Control cells were incubated without the test solutions and with DMSO solvent. The very little percentage of DMSO present in the wells (maximal 0.2%) was proved not to affect the experiment. The microtitre plates were incubated at 37°C in a humidified incubator with 5% CO_2 for a period of 3 days. Eight wells were used for each concentration of the essential oil. Morphological changes were recorded using an inverted microscope. The cells were observed at different time intervals after incubation in the presence or absence of the test solutions. Cellular viability was determined by using the standard MTT (Francis and Rita, 1986) and SRB (Philip *et al.*, 1990) assays from the treated culture of 4 wells of each concentration. The percentage inhibition was plotted against concentration and CTC_{50} (concentration required to reduce viability by 50%) value for each cell line was calculated.

Short term antitumor property of the essential oils was assayed by determining the percentage viability of DLA cells using Trypan blue dye exclusion technique (Moldeus *et al.*, 1978). The percentage viability and CTC_{50} were calculated.

RESULTS

Among the six cell lines used for cytotoxicity of seven essential oils tested, *A. calamus*, *A. pellens*, *P. graveolens* and *O. sanctum* were found to be more potent against the cancerous cell lines. The cancerous A-549 cell line was found to be most susceptible to all the essential oils. The essential oils of *A. nilagirica*, *A. calamus* and *O. sanctum* were found to be more active against these cells with mean CTC_{50} values of 17.75, 19.00 and 24.37 µg/ml, respectively. However *A. calamus* and *A. pellens* essential oils were found to be more potent against HEp-2 cell line with low CTC_{50} values. The essential oils of *A. calamus*, *A. pellens*, *O. sanctum and C. winterianus* showed potent activities against most of the normal cell lines.

DISCUSSION

In the present study, seven essential oils were evaluated for *in vitro* cytotoxicity against three normal and three cancerous cell lines. The essential

Table 1. Cytotoxic effect of some essential oils on cancerous and normal cell lines after 72 h. (Number of independent experiments=3, 4 replicates, mean±SEM)

Essential oils	Cell line	CTC_{50} in μg/ml			
		MTT	SRB	Mean	Dye exclusion
Acorus calamus	A-549	18.00 ± 0.81	20.00 ± 1.24	19.00	
	HEp-2	52.75 ± 0.50	47.00 ± 1.36	49.87	
	DLA	-	-	-	69 ± 2.69
	BRL-3A	85.66 ± 3.65	57.25 ± 1.91	71.45	
	NRK-49F	57.00 ± 2.78	91.37 ± 0.36	74.18	
	VERO	74.75 ± 2.98	77.75 ± 3.62	76.25	
Artemisia nilagirica	A-549	23.00 ± 1.30	12.50 ± 0.23	17.75	
	HEp-2	175.00 ± 8.32	180.50 ± 3.70	177.75	
	DLA	-	-	-	165 ± 7.21
	BRL-3A	54.75 ± 1.61	160.50 ± 5.55	107.62	
	NRK-49F	268.25 ± 10.11	250.00 ± 6.38	259.12	
	VERO	177.00 ± 2.05	122.78 ± 2.21	149.87	
Artemisia pellens	A-549	49.25 ± 0.99	41.50 ± 1.50	45.37	
	HEp-2	55.00 ± 0.86	61.50 ± 2.67	58.25	
	DLA	-	-	-	86 ± 4.27
	BRL-3A	50.75 ± 1.81	57.12 ± 0.72	53.93	
	NRK-49F	68.00 ± 4.75	95.62 ± 6.02	81.81	
	VERO	305.00 ± 4.27	81.75 ± 1.42	193.62	
Cymbopogon winterianus	A-549	42.00 ± 0.72	167.00 ± 2.76	104.50	
	HEp-2	153.50 ± 7.04	152.00 ± 3.06	152.75	
	DLA	-	-	-	44 ± 1.72
	BRL-3A	41.75 ± 0.91	69.87 ± 3.93	55.81	
	NRK-49F	92.00 ± 2.09	86.25 ± 0.99	89.12	
	VERO	226.50 ± 5.00	408.75 ± 5.76	317.62	
Myristica fragrans	A-549	82.25 ± 2.39	94.50 ± 2.64	88.37	
	HEp-2	151.50 ± 10.03	188.25 ± 6.00	169.87	
	DLA	-	-	-	600 ± 28.81
	BRL-3A	151.75 ± 10.79	73.25 ± 1.12	112.50	
	NRK-49F	87.75 ± 1.77	78.62 ± 1.01	83.18	
	VERO	154.00 ± 8.05	433.25 ± 3.24	293.62	
Ocimum sanctum	A-549	24.75 ± 1.25	24.00 ± 1.18	24.37	
	HEp-2	118.25 ± 1.77	139.50 ± 3.15	128.87	
	DLA	-	-	-	36 ± 1.82
	BRL-3A	29.25 ± 1.85	55.25 ± 1.70	42.25	
	NRK-49F	75.50 ± 1.85	81.12 ± 1.97	78.31	
	VERO	122.50 ± 7.67	86.75 ± 2.34	104.62	
Pelargonium graveolens	A-549	59.25 ± 2.29	34.50 ± 1.37	46.87	
	HEp-2	95.00 ± 0.90	90.75 ± 2.92	92.87	
	DLA	-	-	-	27 ± 0.82
	BRL-3A	78.75 ± 3.22	76.25 ± 2.59	77.50	
	NRK-49F	94.00 ± 1.58	66.50 ± 1.40	80.25	
	VERO	131.00 ± 12.60	208.75 ± 3.65	169.87	

oils of *A. calamus*, *A. pellens*, *P. graveolens* and *O. sanctum* were found to be potent against the cancerous cell lines. Among these *A. calamus* essential oil was found to be more promising with low CTC_{50} values against the cancerous and comparatively higher CTC_{50} values against the normal cell lines. *A. pellens* and *P. graveolens* oils also showed similar results. A decoction of the dried entire plant or rhizome of *A. calamus* is given orally to human adults traditionally to treat cancer and phantom tumor in China and India (Motley, 1994; Lama and Santra, 1979; Duke and Ayensu, 1985). The present study gives an indication towards its anti tumor property.

Asarone (up to 82%) and davanone are important constituents of *A. calamus* oil and *A. pellens oil*, respectively. Similarly geraniol and citronellol are found to be the important constituents of *P. graveolens* (Anonymus, 1995). Hence, the observed cytotoxic activity of these oils may be due to the presence of these constituents. Hwang et al., (1999) reported the *in vivo* anti tumor activity of *Artemisia princes*. The anti cancer activity of *Ocimum sanctum* was also reported (Karthikeyan *et al.*, 1999; Prakash *et al.*, 1999). Our study also supports the cytotoxic properties of these and other commonly used essential oils. These results assume significance and importance, since these oils are widely used in India for flavoring of many food preparations, cosmetics and also in Indian System of Medicine (Satyavathy *et al.*, 1987). The essential oils of *A. calamus, A. pellens, P. graveolens* and *O. sanctum* merit further investigation to identify the active principles and the nature of the antitumor activity in animal models.

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