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NOTE

In vitro anticancer activity of extracts of *Mentha* Spp. against human cancer cells

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In vitro anticancer potential of methanolic and aqueous extracts of whole plants of *Mentha arvensis, M. longifolia, M. spicata* and *M. viridis* at concentration of 100 µg/ml was evaluated against eight human cancer cell lines — A-549, COLO-205, HCT-116, MCF-7, NCI-H322, PC-3, THP-1 and U-87MG from six different origins (breast, colon, glioblastoma, lung, leukemia and prostate) using sulphorhodamine blue (SRB) assay. Methanolic extracts of above-mentioned *Mentha* Spp. displayed anti-proliferative effect in the range of 70-97% against four human cancer cell lines, namely COLO-205, MCF-7, NCI-H322 and THP-1; however, aqueous extracts were found to be active against HCT-116 and PC-3. The results indicate that *Mentha* Spp. contain certain constituents with cytotoxic properties which may find use in developing anticancer agents.

Keywords: *Mentha* Spp., Cancer cells, SRB assay

Mentha is a plant with worldwide distribution and contains several species which are used in traditional medicine, mainly for gastrointestinal disturbances. Cytotoxic and other pharmacological activities have also been reported from *Mentha* Spp.[1-3]. Methanolic extract and essential oils from six *Mentha* species *viz, M. piperita*, *M. spicata*, *M. pulegium*, *M. longifolia*, *M. aquatica* and *M. crispa* have shown cytotoxicity against HeLa and HEP-2 cancer cell lines[4]. Aqueous extract of *M. spicata* (pahari pudina), an important aromatic spice has also shown cytotoxic effect in mouse fibrosarcoma Wehi-64 and human monocytic U937 cells[5]. The cytotoxic effect of essential oil from *M. spicata* leaves on some cancer cell lines is also reported *in vitro*[6,7]. Chloroform and ethylacetate extracts of leaves of *M. piperita* (Gamathi pudina) has shown significant dose and time-dependent anticarcinogenic activity against HeLa, MCF-7, Jurkat, T24, HT-29 and MIAPaCa-2 cancer cell lines[8]. *M. arvensis*, commonly known as pudina is used in various symptoms of diseases, such as abdominal pain, vomiting, cough, loss of appetite, menstrual disorders, joint pain and in diseases of liver, spleen and asthma[9].

In the present study, anticancer potential of four *Mentha* Spp. *Viz., M. arvensis, M. longifolia, M. spicata* and *M. viridis* has been investigated against eight human cancer cell lines (A-549, COLO-205, HCT-116, MCF-7, NCI-H322, PC-3, THP-1 and U-87MG) of six different origins (breast, colon, glioblastoma, lung, leukemia and prostate).

Materials and Methods

Chemicals

RPMI-1640 medium, Dulbecco's minimum essential medium (DMEM), dimethyl sulfoxide (DMSO), EDTA, fetal calf serum (FCS), sulphorhodamine blue (SRB) dye, phosphate buffer saline (PBS), trypsin, gentamycin, penicillin and 5-flurouracil were purchased from Sigma Chemical Co., USA. All other chemicals were of high purity and obtained locally with the brand Sigma-Aldrich Chemicals Pvt. Ltd. and S.D. Fine Chemicals Pvt. Ltd.

Plant material and preparation of extracts

The whole plants of four *Mentha* Spp. *viz, M. arvensis, M. longifolia, M. spicata* and *M. viridis* were authenticated at site by Prof. M Saleem, Division of Agroforestry, SKUAST-Jammu and then collected in the month of May from Herbal Garden, Sher-e-Kashmir University of Agricultural Sciences and Technology of Jammu (SKUAST-Jammu), J&K, India. The freshly collected plant material was chopped, shade-dried and ground into powdered form and extracted with different solvents at room temperature to obtain extracts for bioevaluation. The methanolic extract was prepared by percolating the dried ground plant material (100 g) with 95% methanol and then concentrating it to dryness under reduced pressure The aqueous extract was obtained by boiling dried ground plant material (100 g) for 30 min in distilled water (300 ml) and freeze-dried. Stock

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solutions of 20 mg/ml were prepared by dissolving 95% methanolic extract in DMSO and aqueous extract in sterile water. Stock solutions were prepared at least one day in advance and were not filtered and the microbial contamination was controlled by addition of 1% gentamycin in complete growth medium *i.e.* used for dilution of stock solutions to make working test solutions of 200 μg/ml.

Cell lines and cultures

The human cancer cells — A-549, COLO-205, HCT-116, MCF-7, NCI-H322, PC-3, THP-1 and U-87MG were obtained from National Centre for Cell Science, Pune, India and National Cancer Institute, Frederick, USA. These human cancer cells were further grown and maintained in RPMI-1640 medium and DMEM. The media was supplemented with FCS (10%), penicillin (100 units/ml), streptomycin (100 μg/ml) and glutamine (2 mM).

Preparation of positive controls

Positive controls like adriamycin and 5-fluorouracil were prepared in distilled water, while paclitaxel was prepared in DMSO. These were further diluted in gentamycin medium to obtain desired concentrations of 2×10^{-5} M and 2×10^{-6} M.

***In vitro* assay for cytotoxic activity**

Extracts were subjected to *in vitro* anticancer activity against various human cancer cell lines[10]. In brief, the cells were grown in tissue culture flasks in growth medium at 37°C in an atmosphere of 5% CO_2 and 90% relative humidity in a CO_2 incubator (Hera Cell; Heraeus; Asheville, NCI, USA). The cells at sub-confluent stage were harvested from the flask by treatment with trypsin (0.05% trypsin in PBS containing 0.02% EDTA) and suspended in growth medium. Cells with more than 97% viability (trypan blue exclusion) were used for determination of cytotoxicity. An aliquot of 100 μl of cells (10^5 cells/ml) was transferred to a well of 96-well tissue culture plate. The cells were allowed to grow for 24 h. Extracts (100 μl/well) were then added to the wells and cells were further allowed to grow for another 48 h.

The anti-proliferative SRB assay which estimates cell number indirectly by staining total cellular protein with the dye SRB was performed to assess growth inhibition. The SRB staining method is simpler, faster and provides better linearity with cell number. It is less sensitive to environmental fluctuations and does not require a time sensitive measurement of initial reaction velocity[11]. In brief, the cell growth was stopped by gently layering 50 μl of 50% (ice cold) trichloroacetic acid on the top of growth medium in all the wells. The plates were incubated at 4°C for 1 h to fix the cells attached to the bottom of the wells. Liquid of all the wells was then gently pipetted out and discarded. The plates were washed five-times with distilled water and air-dried. SRB 100 μl (0.4% in 1% acetic acid) was added to each well and the plates were incubated at room temperature for 30 min.

The unbound SRB was quickly removed by washing the cells five-times with 1% acetic acid. Plates were air-dried, tris buffer (100 μl, 0.01 M, pH 10.5) was added to all the wells to solubilize the dye and then plates were gently stirred for 5 min on a mechanical stirrer. The optical density (OD) was recorded on ELSIA reader at 540 nm. Suitable blanks (growth medium and DMSO) and positive controls (prepared in DMSO and distilled water) were also included. Each test was done in triplicate and the values reported were mean values of three experiments.

The cell growth was determined by subtracting average absorbance value of respective blank from the average absorbance value of experimental set. Percent growth in presence of test material was calculated as under:

- OD Change in presence of control = Mean OD of control – Mean OD of blank
- OD Change in presence of test sample = Mean OD of test sample – Mean OD of blank
- % Growth in presence of control = 100/OD change in presence of control
- % Growth in presence of test sample = % Growth in presence of control × OD change in presence of test sample
- % Inhibition by test sample = 100 – % Growth in presence of test sample

The growth inhibition of 70% or above was considered active while testing extracts, but in testing of active ingredients at different molar concentrations, the growth inhibition of 50% or above was the criteria of activity.

Results and Discussion

The *in vitro* cytotoxic activity of whole plants of *Mentha* Spp. is summarized in Table 1. The methanolic extract of *M. arvensis* showed *in vitro* cytotoxic effect against four human cancer cell lines from four different tissues. Maximum growth inhibition (92%) was observed

Table 1—Growth inhibitory effect of extracts of whole plants from *Mentha* Spp. with appropriate positive controls against human cancer cell lines

Plant	Extract	Conc. (µg/ml)	Lung A-549	Colon COLO-205	Colon HCT-116	Breast MCF-7	Lung NCI-H322	Prostate PC-3	Leukemia THP-1	Glioblastoma U-87MG
			Growth inhibition (%)							
M. arvensis	Methanolic	100	58	**86**	23	**76**	**85**	0	**92**	36
	Aqueous	100	46	21	**97**	63	42	**75**	3	9
M. longifolia	Methanolic	100	58	**92**	20	**84**	**75**	0	**92**	58
	Aqueous	100	56	14	**81**	57	31	3	7	2
M. spicata	Methanolic	100	53	**81**	53	**75**	**71**	0	**97**	38
	Aqueous	100	42	22	68	61	34	**85**	18	16
M. viridis	Methanolic	100	61	**88**	7	**70**	**71**	0	**94**	54
	Aqueous	100	48	24	**70**	59	39	**71**	9	12
Positive controls		Conc. (Molar)								
5-Flurouracil		2×10^{-5}	-	51	68	-	-	-	73	60
Paclitaxel		1×10^{-6}	79	-	-	-	52	-	-	-
Adriamycin		1×10^{-6}	-	-	-	60	-	59	-	-

Growth inhibition of 70% or above is indicated in bold.

The mark (-) indicates that particular human cancer cell line was not treated with that particular positive control.

against THP-1 (leukemia). The extract showed 86%, 85% and 76% growth inhibition against colon cancer cells (COLO-205), lung cancer cells (NCI-H322) and breast cancer cells (MCF-7), respectively. Whereas the aqueous extract of *M. arvensis* suppressed 97% and 75% proliferation of HCT-116 (colon) and PC-3 (prostate), respectively.

The methanolic extract from *M. longifolia* suppressed the proliferation in the range of 75-92% against four human cancer cell lines from colon, breast, lung and leukemia origin. Its aqueous extract showed maximum growth inhibitory effect (81%) against colon cancer cells (HCT-116), which was considered significant. The methanolic extract of *M. spicata* displayed *in vitro* anticancer efficacy the range of 71-97% against THP-1, COLO-205, MCF-7 and NCI-H322. However, its aqueous extract showed *in vitro* cytotoxicity only against PC-3 (85%). Similarly, methanolic extract from *M. viridis* exhibited *in vitro* cytotoxic efficiency against COLO-205, MCF-7, NCI-H322, THP-1 in the range of 71-94%, but its aqueous extract was found active against only HCT-116 (70%) and PC-3 (71%).

Most of the drugs used in cancer chemotherapy exhibit cell toxicity and can induce genotoxic, carcinogenic and teratogenic effects in non-tumor cells. Therefore, there is a need for alternative drugs of natural origin that are less toxic, endowed with fewer side effects and more potent in their mechanism of action. Recently, we have reported that methanolic extract from the leaves of *Nardostachys jatamansi* (commonly known as muskroot) exhibits *in vitro* anticancer effect against five human cancer cell lines *viz*., NCI-H23, HeLa, SK-N-MC, SW-620 and COLO-205 in the range of 70-93%[12].

Similarly, the methanolic extract from the fruit part of 'Kamala tree' (*Mallotus philippinensis*) has displayed significant cytotoxic effect against fourteen human cancer cell lines — A-549, COLO-205, DU-145, HEP-2, HeLa, IMR-32, KB, MCF-7, NCI-H23, OVCAR-5, SiHa, SK-N-MC, SW-620 and ZR-75-1[13]. Also, the methanolic extract from the stem-leaves of *Calotropis procera* has shown 70% growth inhibition of colon cancer cells (HCT-15)[14] and the seed part of *Apium graveolens* is observed to be active against COLO-205, HeLa, KB, SK-N-MC[15].

The strong anti-proliferative effect on a range of human cancer cell lines is also displayed by the methanolic extract from the fruit part of *Momordica charantia;* this particular extract has been observed to be cytotoxic to a wide spectrum of cancer cells (A-549, COLO-205, MCF-7, NCI-H322, PC-3, THP-1 and U-87MG) with the growth inhibition ranging between 75-100%. The extract has shown a

high degree of growth inhibition against NCI-H322 (lung, 100%), MCF-7 (breast, 99%) and COLO-205 (colon, 97%). Interestingly, the extract has exhibited the cytotoxic effect significantly higher than 5-flurouracil, adriamycin and paclitaxel used as positive controls[16].

Moreover, potent cytotoxic effect of 80% methanolic extract and chloroform fractions of *Mentha spicata* has been reported against HeLa, HEP-2 and PC-3 cancer cell lines[1,4]. Aqueous extract from *M. spicata* has also shown cytotoxic effect on U-937 human monocytic leukemia cells[5]. *M. longifolia* has also shown cytotoxicity of IC_{50} at a concentration of 119 µg/ml and 89.9 µg/ml against HeLa and HEP-2 human cancer cells[4]. The oral administration of aqueous extract from *M. piperita* leaves has exhibited a significant reduction in number of lung tumors from an incidence of 67.92% in animals given only benzo[a]pyrene to 26.31%[17]. *M. piperita* has a chemopreventive effect against the tumorigenicity of shamma which could be due to anti-mutagenic properties[18]. Essential oil from *M. pulegium* is also found to be a cytotoxic agent against human ovary adenocarcinoma SK-OV-3, human malignant cervical adenocarcinoma HeLa and human lung carcinoma A-549 cell lines[19].

To conclude, *Mentha* Spp. possess *in vitro* cytotoxic effect against COLO-205, HCT-116, MCF-7, NCI-H322, PC-3 and THP-1 cancer cells. However, further studies are required for the isolation of active ingredient(s) which may serve as lead molecule(s) in the development of anticancer agents, especially for colon, breast, lung cancer and leukemia carcinoma.

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