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Original Article

EVALUATION OF *IN VITRO* CYTOTOXIC ACTIVITY OF PETROLEUM ETHER AND METHANOL EXTRACT OF *MENTHA ARVENSIS* (WHOLE PLANT) ON HUMAN CANCER CELL LINES

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ABSTRACT

Objective: *Mentha arvensis* (MA) commonaly known as Mint or Pudina, belongs to the Lamiaceae family. It is an aromatic herb traditionally used as an antiseptic, antihelmintic, diuretic, digestive, expectorant and cardio tonic. The objective of the present investigation was to examine *in vitro* cytotoxic activity of crude whole plant extracts of MA.

Methods: Crude extracts were prepared from whole dried plant of MA by Soxhlet apparatus, using solvents petroleum ether (60°- 80°) and methanol successively. *In vitro* cytotoxic activity of crude extracts was evaluated by Sulforhodamine B assay on three human cancer cell-lines of different tissues i.e. A-549 (lung), MCF-7 (breast) and COLO-205 (colon). Hemolytic activity of crude extracts of MA on human RBCs was also checked.

Results: Methanol extract of MA was observed to be significantly more cytotoxic in dose dependent manner than Petroleum ether extract of MA with IC_{50} ranging from 120-165µg/ml for selected cell lines. Methanol and petroleum ether extracts of MA were found to have no hemolytic effect on RBCs suggesting membrane destabilization is not the mechanism of action for their cytotoxic activity.

Conclusion: This study suggests potential anti-tumor activity of *Mentha arvensis* and a need for further studies to identify the active component/s and to understand their mechanism/s of action.

Keywords: *Mentha arvensis*, Antitumor Drug Screening Assays, A549, MCF-7, COLO-205.

INTRODUCTION

Cancer is a leading cause of death worldwide. Around 5,56,400 deaths occurred in 2010 due to cancer in India. In age group of 30-69; oral, stomach and lung cancer (for men) and cervical, stomach, breast and oral cancers (for women) were most fatal cancers [1]. Existing chemotherapy and treatment leads to painful side effects. Hence there is a need to explore new alternative and complementary medicine with anticancer activity. It has been observed that ethanomedicinal plants frequently serve as a source of new drugs with little or no side effects.

Mentha arvensis (Family- Lamiaceae) is an erect branched, strongly aromatic herb that stems up to 75 cm long. Leaves are elliptic to oblong-ovate, 5 cm long, short-petioled, toothed margins, rounded or blunt tipped. Plant has hairy lilac to light blue axillary flowers [2]. It is commonly known as 'Pudina', 'Mint', 'Wild mint' or 'Corn mint'. Though mainly found in western Himalayas, it is cultivated throughout India. According to Indian Ayurvedic medicine, *Mentha arvensis* (MA) is used as antiseptic, anthelmintic, diuretic, antispasmodic, stimulant, stomachic, carminative, digestive, emmenagogue, expectorant and cardio tonic. It is useful in ulcers, wounds, cough, asthma, skin diseases, liver diseases, fever and general weakness [3, 4]. It is reported to exhibit antioxidant [5], anti-inflammatory and sedative-hypnotic activity [6]. It also possesses antimicrobial [7], antifungal [8], antimycotic [9] and antifertility activity [10]. 95% ethanol extract of *Mentha arvensis* has shown significant brine shrimp cytotoxic activity [7]. This activity can be extrapolated for cell-line toxicity and probable antitumor activity of MA.

For the present study the cytotoxic activity of petroleum ether and methanol extract of *Mentha arvensis* whole plant on three human cancer cell lines (A-549 lung adenocarcinoma, MCF-7 breast adenocarcinoma, and COLO-205 colorectal adenocarcinoma) was assessed.

MATERIALS AND METHODS

Plant Material

Whole plant of *Mentha arvensis* was purchased from the local market at Mumbai and authenticated (Voucher specimen no. 2010/01) by Dr. Ganesh Iyer, Ruia College, Mumbai. Shade dried plant material was ground, passed through mesh No. 12 and collected on Mesh size 40.

Preparation of Crude Extracts

The Dried whole plant powder was first subjected to Soxhlet extraction with petroleum ether (60-80° C) to get petroleum ether extract (P.E.-MA) and successively by methanol for methanol extract (M.E.-MA). Extracts were concentrated in rotary evaporator and then evaporated till dryness to remove traces of solvents. Both the extracts of Mentha arvensis were subjected to preliminary phytochemical evaluation using qualitative chemical tests for detecting the presence of the phytoconstituents like alkaloids, glycosides, tannins, phenolic compounds, phytosterols, carbohydrates, proteins and amino acids [11-13].

Cell Lines

Cell lines such as human lung carcinoma cell line (A549), human breast adenocarcinoma cell line (MCF7) and human colorectal adenocarcinoma cell line (COLO-205) were procured from NCCS, Pune. Cell lines were maintained in respective nutrient media (HiMedia ®) such as F-12 K (Kaighn's modification of Ham's F-12 media) for A549, MEM (Eagle's Minimum Essential Media) for MCF-7 and RPMI-1640 for COLO-205. Media were supplemented with 10% Fetal Bovine Serum (HiMedia®) and 40 µg/ml Gentamycin; Genticyn® by Piramal Health Care. Cell lines were cultured in Nunc T-25 flasks at 37 °C in 5% CO_2 environment (Nuairre) and subcultured by trypsinization (Trypsin Phosphate Versene Glucose, HiMedia®) depending on the confluency of the cells. Before every assay the cell count was done using haemocytometer and kept constant. Cell viability was determined by trypan-blue dye exclusion technique.

Cytotoxic Assay by Sulforhodamine B Method

To evaluate *in vitro* cytotoxic activity, to each well of the 96 well flat bottom microtitre plate approximately 10,000 viable cells were added and kept for incubation in CO_2 incubator for 24 hrs at 37°C. Later, test extracts were added to wells in broad concentration range (31.25 to 2000 µg/ml) and incubated for 48 hours (72 hours for MCF-7 cells). After incubation time, Sulforhodamine B (SRB) assay was performed using standard procedure to check the viable cells [14, 15]. Fixation was carried out using 50µl of 50% trichloroacetic acid (Himedia®) at 4°C for one hour. Then plates were stained with 100µl 0.04% SRB (Sigma Aldrich) in dark for 30 minutes at room temperature (RT). The excess of dye was removed by washing four times with 1% acetic acid. 100µl of 10mM Tris base (Himedia®) (pH 10.4) was added to each well and the absorbance was measured using microplate reader (BioTec Instrument) at a wavelength of 540 nm. Percent cell viability was calculated using following formula,

$$\text{Percent cell viable} = \frac{\text{OD of treated cells}}{\text{OD of control cells}} \times 100 \text{ (Equation 1)}$$

Statistical Analysis- All the experiments were performed in triplicate. IC_{50} (Mean ± SD) were calculated by non-linear regression analysis using GraphPad Prism version 5.00 for Windows, Graph Pad Software, San Diego California USA. Statistical significance with P value < 0.05 was determined by One-way ANOVA with Dunnett's post test as compared to control cells using the same software.

Hemolytic Assay

In vitro hemolytic activity of MA extracts was evaluated [16], to check the biocompatibility of extracts. Human blood sample (2 ml) was collected from a healthy volunteer. Blood sample was diluted 1:1 with sterile Phosphate Buffered Saline (PBS, pH 7.4). Red blood cells (RBCs) were separated by centrifugation (1000g for 5 min) and resuspended in PBS. This procedure was repeated three times.

A final 2% RBCs suspension (v/v) was prepared by suspending RBCs in PBS. Hemolytic assay was conducted in 96-well flat bottom microtitre plates (Tarsons). For positive control 100µl sterile Distilled water (D/W) was used. Test extract's stocks (as made for SRB assay) were serially diluted in PBS. 100ul of 2% RBCs suspension were added to all wells of the plate. PBS and RBCs alone served as the 0% hemolysis negative control. Experiment was performed in triplicate. Plates were incubated for 3 h at room temperature. Liberated hemoglobin was measured by reading well optical density at 450 nm using Microplate Reader (Biotek Instrument). Increase in optical density as compared with negative control is known to be associated with RBCs lysis. The percentage of hemolysis was calculated and IC_{50} was determined by GraphPad software by non-linear regression analysis.

Residual Solvent Analysis

A gas chromatograph (Agilent Technologies 7890 A) equipped with FID (Flame ionization detectors) connected to Agilent G1888 Headspace sampler and a data processor Agilent technologies EZChrome Elite software version 3.2.1 was employed. Under the standard conditions, a 30 m long HP-1 column, 0.53 mm in inner diameter and 1.8 µ in film thickness (Agilent Technologies, USA) was used. The injection ports were heated at 200°C while the temperature of detector was maintained at 250 °C. The sample was introduced in the column in a split mode with split ratio, 1:5. Nitrogen, the carrier gas was allowed to flow at a rate of 1.0 ml per minute. The column (oven) temperature was kept at 80 °C for 2 min followed with an increase in the temperature at a rate of 10°C per minute to 150°C. Headspace equilibration temperature was kept at 80°C for 45 min. The reference solution contained 1000 ppm of Methanol (HPLC grade) in Dimethyl Sulphoxide (DMSO, Extrapure AR grade). Sample contained accurately weighed 100mg of M.E.-MA extract in 10ml of DMSO.

RESULTS AND DISCUSSION

Sulforhodamine B (SRB) assay is a high-throughput and sensitive method for evaluating cytotoxic activity against cancer and non-cancerous cell lines. It has several advantages over other contemporary cytotoxicity assays; because SRB assay is independent of cell metabolic activity, not interfered by test compounds and easy to perform [15]. The half maximal inhibitory concentration (IC_{50}) of MA extracts was determined for three cancer cell lines by SRB assay. IC_{50} values of M.E.-MA were found to be statistically significant and lower than that of P.E.-MA as shown in Table 1. M.E.-MA was observed to be cytotoxic in dose dependent manner (Figure 1) on all three cancer cell lines. The cytotoxic ability of crude extracts can be attributed to their phytochemical constitution, which also explains difference in level of activity of M.E.-MA and P.E.-MA, as solvents used for their extraction are of different polarities.

Table 1: IC_{50} values of petroleum ether and methanol extracts of *Mentha arvensis* for human cancer cell lines

Test Extracts	IC_{50} (µg/ml)		
	A549	MCF7	COLO-205
P.E.-MA	287.17 ± 1.85	82.77 ± 4.26 *	186.33 ± 6.8
M.E.-MA	165 ± 2.97**	124.63 ± 6.31**	158.57 ± 15.80**

IC_{50} is calculated as mean ± SD (n=3). P value < 0.05 vs negative control (nutrient media + cells) using one way ANOVA followed by post hoc Dunnett's Test

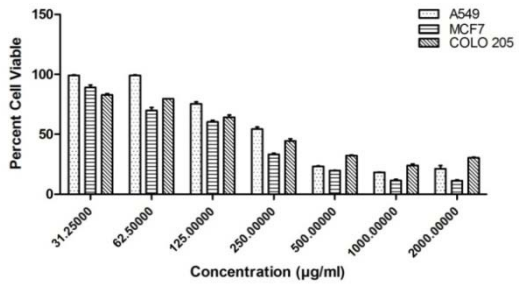


Fig. 1: *In vitro* cytotoxic activity of M.E.-MA on three human cancer cell lines in dose dependent manner

Percent cell viable expressed as Mean ± SEM (n=3) vs. Concentration of M.E. – MA (µg/ml) for three cancer cell lines.

In preliminary phytochemical analysis, carbohydrate, glycosides, flavonoids, phenolic compound and tannins were found to be present in M.E.–MA (Table 2). Flavonoids, such as curcumin, genistein, quercetin, luteolin, kaempferol, are known to have cell line specific antiproliferative and apoptosis inducing activity [17]. It has been postulated that flavonoids possess anticancer activity by several mechanisms including inhibition of DNA topoisomerase I/II activity, decrease of ROS, modulation of signaling pathways and down regulation of nuclear transcription factor kappa B (NF-κB) [18]. For MCF-7 cell line, Petroleum ether extract was found to be more growth inhibitory than M.E.-MA with IC_{50} 82.77 ± 4.26 µg/ml. P.E.-MA showed presence of tepenoids and steroids prominently. Many plant derived terpenoids are known to possess potential anti-inflammatory and anti-cancer activity by inhibiting NF-k/beta signaling [19]. In general, mint plants contain the flavonoids such as acacetin, chrysoeriol, diosmin, eriocitrin (eriodictoyl-7-o-rutinoside), hesperidin, hesperidoside, isorhoifolin, linarin, luteolin, menthoside, methyl rosmarinate, rutin, tilianine, narirutin, and

nodifloretin [4], but specific reports on phytoconstituents present in MA are not existing.

Table 2: Qualitative Phytochemical Analysis of Crude Extracts of MA

Phytochemical Test/ Reagent	P.E.-MA	M.E.-MA
Carbohydrates		
Molish's test	-	+
Test for reducing sugar		
Fehling's Test	-	+
Benedict's Test	-	+
Test for monosacharides		
Barfoed's Test	-	+
Test for Hexose Sugars		
Tollen's phloroglucinol test for galactose	-	-
Cobalt –chloride test for glucose and/or fructose	-	+
Iodine test for Starch	-	-
Test for Gums	-	-
Test for Mucilage	-	-
Glycosides		
Molish's Test after Hydolysis	-	+
PROTEINS		
Biuret Test (General Test)	-	-
Fixed Oils and Fats		
Filter paper spot test	+	-
SAPONINS		
Foam Test	-	+
Steroids		
a. Salkowski reaction	+	-
b. Liebermann-Burchard Reaction	+	-
Terpenoids	**+**	**-**
Flavonoids		
Shinoda Test	-	+
Lead Acetate Test	-	+
Sodium Hydroxide Test	-	+
Alkaloids		
Dragendorff's Test	-	-
Hager's Test	-	-
Tannins and phenolic compounds		
5% $FeCl_3$ Test	-	+
Lead Acetate Test	-	+

+ Indicates present, - Indicates absent

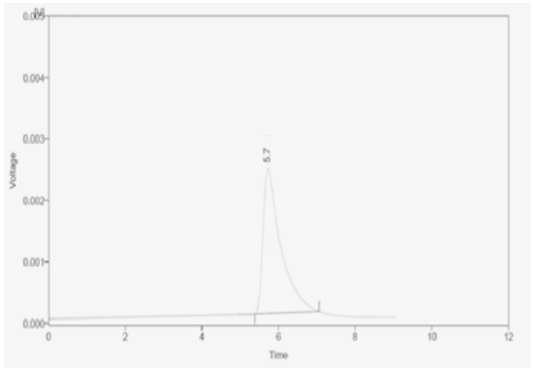
In vitro hemolytic activity on erythrocytes was also evaluated for M.E.-MA and P.E. -MA. Total hemolysis was observed by 100µl of sterile D/W after three hours of incubation at room temperature. In a hypotonic environment provided by distilled water; an influx of water occurs that leads to swelling of cells and disruption of the integrity of cell membranes; thus causing osmotic hemolysis of RBCs. As shown in Table 3, test extracts did not possess any hemolytic activity against erythrocytes. Lack of hemolytic activity suggests that methanol and petroleum ether extracts of MA are protecting biological membrane and hence membrane destabilization is not the mechanism of killing cancer cells [20].

Table 3: *In vitro* Hemolytic assay of different extracts of *Mentha arvensis*

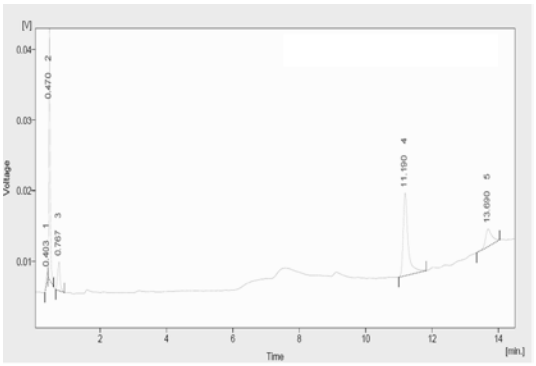
Extracts	Human Erythrocytes IC_{50} (µg/ml)
M.E.-MA	>1000
P.E.-MA	>1000

All the experiments were performed in triplicate. IC_{50} (Mean ± SD) were calculated by non-linear regression analysis using Graph Pad Prism. Sterile distilled water was used as positive control which resulted in 100 % hemolysis, whereas phosphate buffered saline was used as negative control and 0 % hemolysis was observed.

As M.E.-MA was found to be significantly active against cancer cell lines, residual solvent analysis of M.E.- MA was carried out by Headspace GC method. Reference solvent methanol (1000ppm) showed peak at 5.7 min (Area 100.85 mV.s) as depicted in fig. 2A. Chromatogram of M.E.-MA did not show any peak at 5.7 min suggesting absence of methanol in M.E.-MA (fig. 2B). Hence crude extract M.E.-MA does not contain traces of the solvent, thus eliminating any possibility of interference by the solvent in the activity of ME.MA.



A



B

Fig. 2: Headspace Gas Chromatography to detect residual solvent in M.E.-MA. (A- 1000 ppm Methanol; B- 10 mg/ml M.E.-MA)

Husain et al. have already reported cytotoxic activity of MA essential oil on MCF-7 and Lan-CAP cell lines [21]. Present study reports significant dose-dependent *in vitro* cytotoxic activity of successive methanol extract of MA on cancer cell lines suggesting potential antitumor activity of MA. Similar findings have not been observed till date. Hence further studies are essential for identification of bioactive anticancer compounds from *Mentha arvensis* and understanding their mechanism/s of action.

CONCLUSIONS

Crude whole plant extracts of *Mentha arvensis* were prepared and evaluated for *in vitro* cytotoxic and hemolytic activity. Both extracts, P.E.-MA and M.E.-MA did not show hemolytic activity suggesting their biocompatibility. Methanol extract of MA was found to be significantly cytotoxic on cancer cell lines in dose dependent manner implying potential antitumor activity of *Mentha arvensis* and scope for further studies.

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CONFLICT OF INTERESTS

None

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