

EVALUATION OF *IN VITRO* CYTOTOXIC ACTIVITY OF *OCIMUM BASILICUM* AND *MENTHA SPICATA* EXTRACTS

JEGATHAMBIGAI RAMESHWAR NAIDU^{1,2*}, MANISH GUNJAN², YENG CHEN³, SREENIVASAN SASIDHARAN^{1*}

¹Institute of Research in Molecular Medicine, University Science Malaysia, 11800 Penang, Malaysia. ²Faculty of Medicine, Asia Metropolitan University, Johor Bahru, Malaysia. ³Faculty of Dentistry, Dental Research and Training Unit, and Oral Cancer Research and Coordinating Centre, University of Malaya, 50603 Kuala Lumpur, Malaysia. Email: srisasidharan@yahoo.com, drjegaramnaidu@gmail.com

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ABSTRACT

Objective: The objective of the current research was to evaluate the cytotoxicity of *Ocimum basilicum* (OB) and *Mentha spicata* (MS).

Methods: *In vitro* cytotoxic potential of OB and MS extracts was evaluated against human cancer HL60 cell lines using 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide assay. The treated cell morphology was observed by light microscope.

Results: The OB and MS extracts inhibited proliferation of HL60 cells in concentration dependent manner with IC₅₀ of 45.67 µg/ml and 98.1 µg/ml for 42 hrs, respectively. The morphology of the HL60 cells treated with OB and MS extracts at high concentrations (200 and 100 µg/ml) indicated cell shrinkage, clumping, and signs of apoptosis.

Conclusion: OB and MS extracts induced cell death in HL60 cell lines. Further, *in vivo* studies and identification of active components from OB extract, and their exact mechanism of action could be useful in designing new anticancer therapeutic agents.

Keywords: Cytotoxicity; HL60 cell; apoptosis; medicinal plant.

INTRODUCTION

The search of anticancer agents from plant sources started in earnest in the 1950's with the discovery of the vinca alkaloids, vinblastine, and vincristine and the isolation of the cytotoxic podophyllotoxins, colchicine, combretastatin. Chemoprevention by dietary agents has evolved as an effective strategy to control the incidence of oral cancer. Epidemiological studies have demonstrated a positive correlation between increased consumption of vegetables, fruits, and beverages with reduced risk of cancer [1,2]. The mechanisms responsible for this chemopreventive effect still remain largely unknown but are likely related to the presence of phytochemicals associated with fruits and vegetables.

The identification of fruits and vegetables containing phytochemicals with the highest anticancer properties as well as the characterization of the mechanisms by which these foods elicit their anticancer actions, thus represent an essential step for the development and implantation of dietary-based chemopreventive approaches. The main objective of the study was to determine the cytotoxic activity of *Ocimum basilicum* (OB) and *Mentha spicata* (MS) using 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide assay (MTT) against human cancer HL60 cell lines.

METHODS

Plant samples

Methanol extracts were prepared at 3-100 µg/ml (50% v/v methanol).

Cell culture

Cell culture media consisted of Roswell Park Memorial Institute (RPMI) 1640, fetal bovine serum (FBS), penicillin and streptomycin solutions were from Gibco, USA. Complete RPMI growth medium was prepared by adding 10% FBS, 5% penicillin, and 2% L-glutamine. The human promyelocytic leukemia cells (HL60) were obtained from Science cell, USA. They were grown in RPMI supplemented with 10% FBS and maintained in a humidified atmosphere of 5% CO₂ at 37°C. HL-60 cell lines were maintained in for 24 hrs to allow cell stabilization [3,4]. Cells

were trypsinized and plated in 96-well round-bottom tissue culture plates for cytotoxicity test.

MTT assay

MTT assay is based on the ability of a mitochondrial dehydrogenase enzyme present in the viable cells to reduce tetrazolium rings of the MTT (pale yellow) and form formazan crystals (dark blue) which are largely impermeable to cell membranes, thus resulting in the accumulation within the healthy cells. The crystals are solubilized by the addition of detergent to the cells. The number of surviving cells is directly proportional to the level of the formazan product [5]. Cytotoxicity was determined by using MTT as the cytotoxic indicator with some modifications [6,7]. HL-60 cells were diluted with complete RPMI medium to give a density of 1 × 10⁴ cells and 300 µl of this suspension were placed in a 96-well microplate and incubated for at least 24 hrs prior to treatment. To this, added 200 µl of the plant extracts at a concentration range of 3-100 µg/ml. Cells were incubated at 5% CO₂ at 37°C for 48 hrs. Dimethyl sulfoxide (DMSO) was used as a negative control and 5-fluorouracil was used as a positive control. All tested samples were performed in three replicates. After 48 h incubation, cell viability was determined by adding MTT (5 mg/ml, in phosphate buffered saline, 10 µl) to each well and following 5 minutes shaking at 150 rpm and then the plates were incubated for 3 h. About 100 µl of acidified propanol was added to wells to dissolve the formazan crystals, and the plates were shaken for 20 min at 150 rpm. Finally, the absorbance was read at 590 nm with a scanning multi-well spectrophotometer (Thermolab system 354, Finland). Cells were observed in inverted microscope. Cytotoxicity index (CI) percentage was calculated by using the following equation:

$$\%CI = \left[\frac{1 - OD_{590 \text{ treatment}}}{OD_{590 \text{ control}}} \right] \times 100 [3] \text{ (Treatment-plant extract treated, control - negative control)}$$

Morphological changes

The cell morphology was observed by a light microscope, to note the presence of cell shrinkage and signs of apoptosis [8,9].

Statistical analysis

The IC₅₀ value for the % CI was calculated by regression analysis using the slope equation. One-way ANOVA was done by using IBM SPSS 20 software to compare the CI of the plants investigated and the different doses employed.

RESULTS

MTT assay

The cytotoxic activity of OB and MS were determined by MTT assay. Cytotoxicity assay indicated that there was a significant inhibition of percentage cell viability of HL60 cell lines treated with OB, MS extracts. The percentage inhibition of cell viability expressed as CI of OB and MS extract treated cells was found to be 50 % and 39%, respectively. There was a significant increase ($p < 0.05$) in the CI value when compared to the negative control treated cells. The CI was found to be significantly different ($p < 0.05$) between OB, MS extracts at 3, 6, 12, 25, and 50 $\mu\text{g/ml}$. However, there was no difference in percentage inhibition of OB and MS at 100 $\mu\text{g/ml}$. The CI was found to increase with increasing dose tested in the plant extracts and significant differences ($p < 0.05$) were observed between the all the concentrations tested for the plants investigated. The IC₅₀ values were calculated by using the slope equation. Values indicated the concentration required to bring 50% inhibition. The IC₅₀ for cytotoxicity was found to be the lowest for the cells treated with OB (45.67 $\mu\text{g/ml}$) being the most potent inhibitor among the tested plants. MS treated cells indicated the IC₅₀ value of 98.1 $\mu\text{g/ml}$. However, the CI was found to be significantly lower ($p < 0.05$) for all the plants when compared to the standard drug 5-fluorouracil. Hence, the IC₅₀ values of the tested plants were found to be higher when compared to the IC₅₀ (5.9 $\mu\text{g/ml}$) of the standard (Table 1).

CI percentage was calculated by using the equation shown in Table 1.

Morphology of HL-60 cell lines treated with plant extracts

The control cells were spheroid in shape and the cytoplasm restricted to the periphery of the cell (Fig. 1). The morphology of the plant extract treated cells generally showed cell shrinkage clumping and apoptosis. The morphology of the HL60 cells treated with OB, MS extracts at high concentrations (200, 100 $\mu\text{g/ml}$) indicated cell shrinkage and clumping, signs of apoptosis, macrophage differentiation accompanied by cell adhesion and spreading on tissue culture plastic, and inhibited cell growth. Fewer vacuolated cells were also noticed in the MS treated cells (Figs. 2 and 3).

DISCUSSION

Cytotoxicity potential of OB and MS, extracts were determined using MTT assay against Human cancerous cell lines (HL60-promyelocytic blood leukemia cells). A significant increase ($p < 0.05$) in the CI value of the OB, MS, treated cells were noted when compared to the negative control cells. The CI was found to increase with increasing dose tested in the plant extracts and a significant difference ($p < 0.05$) was observed between the all the concentrations tested for the plants investigated. The IC₅₀ for cytotoxicity was found to be the lowest for the cells treated

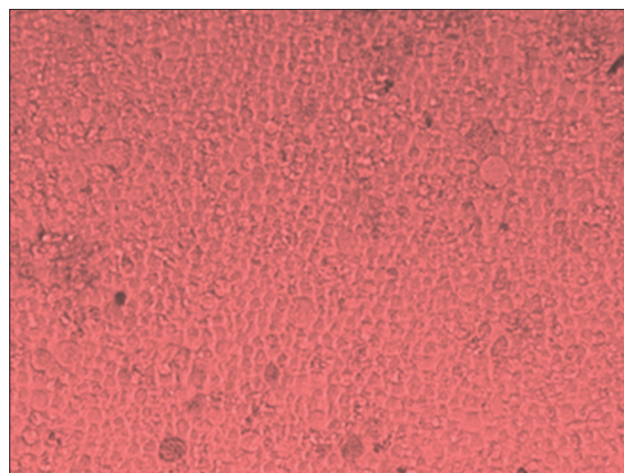


Fig. 1: Morphology of HL60 cells treated with negative control. Cells are treated with dimethyl sulfoxide for 48 hrs in Roswell Park Memorial Institute, medium; cells are spheroid in shape when grown in suspension, dense normal cells, $\times 20$

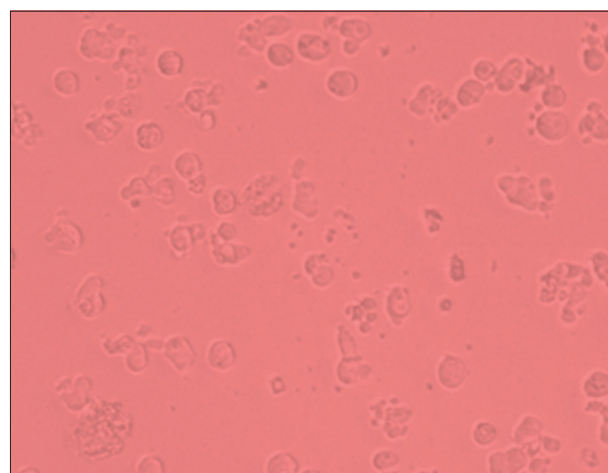


Fig. 2: Morphology of HL60 cells treated with *Mentha spicata* (100 $\mu\text{g/ml}$), cell shrinkage and clumping, signs of apoptosis, macrophage differentiation, accompanied by cell adhesion and spreading on tissue culture plastic were noticed and cell growth was inhibited, $\times 20$

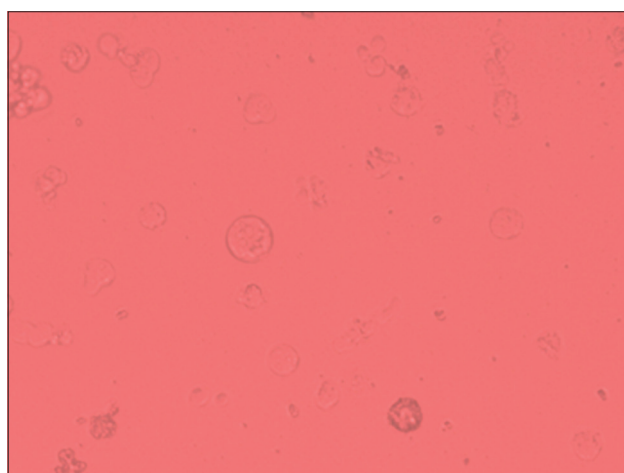


Fig. 3: Morphology of HL60 cells treated with *Ocimum basilicum*, (100 $\mu\text{g/ml}$), cell growth inhibition, cell shrinkage and clumping, signs of apoptosis accompanied by cell adhesion, $\times 20$

Table 1: Cytotoxicity index - % Inhibition of OB and MS

Concentration ($\mu\text{g/ml}$)	Standard	OB	MS
3	43 \pm 3	31 \pm 3 ^a	19 \pm 3 ^b
6	49 \pm 5	41 \pm 1 ^a	20 \pm 3 ^b
12	57 \pm 1	47 \pm 1 ^a	25 \pm 3 ^b
25	72 \pm 2	48 \pm 3 ^a	28 \pm 4 ^b
50	88 \pm 4	49 \pm 1 ^a	35 \pm 6 ^b
100	91 \pm 1	52 \pm 1 ^a	50 \pm 6 ^b
IC ₅₀ ($\mu\text{g/ml}$)	5.9	45.67	98.1

OB: *Ocimum basilicum*, MS: *Mentha spicata*, % CI = $(1 - \text{OD } 590 \text{ treatment} / \text{OD } 590 \text{ control}) \times 100$. The plant extracts were tested at range of 3, 6, 9, 12, 25, 50, 100 $\mu\text{g/ml}$. Values represent mean \pm SD, n=3 replicates. Values with different superscripts (a, b, c) in rows differ significantly ($p < 0.05$). CI: Cytotoxicity index, SD: Standard deviation

with OB (45.67 µg/ml) being the most potent inhibitor. MS treated cells indicated an IC₅₀ value of 98.1 µg/ml. However, the percentage inhibition of cytotoxicity was found to be lower for both the plants investigated when compared to the standard, 5-fluorouracil. In addition, the IC50 values were found to be higher when compared to the standard (5.9 µg/ml). The morphology of the HL60 cells treated with OB, MS extracts at high concentrations (200, 100 µg/ml) indicated cell shrinkage and clumping, signs of apoptosis, macrophage differentiation accompanied by cell adhesion and spreading on tissue culture plastic, and inhibited cell growth.

OB indicated an IC₅₀ value of 45.67 µg/ml. The results are in the similar range and in accordance with the reports from a recent study, where OB (sweet basil) oil showed potent cytotoxic effect with an IC₅₀ value of 36.2 µg/ml in murine leukemia cell lines (P388) and hairy basil oil and holy basil oil indicated IC₅₀ values of 51.8 and 84.8 µg/ml, respectively [3]. Sweet basil and hairy basil have been used in Thai traditional medicine and Thai dishes. Essential oil derived from Lamiaceae species are composed of mono- and ses-terpenes. *Ocimum* spp. essential oils are composed of monoterpene derivatives such as camphor, limonene, thymol, citral, geraniol, and linalool [10]. Phenolic compounds such as cinnamic acid, caffeic acid, sinapic acid, ferulic acid, and rosmarinic acid have been reported in OB [11]. These compounds are potent antioxidants, free radical scavengers, and metal chelators. Basil has shown antioxidant, antimicrobial, and antitumor activities due to its phenolic acids and aromatic compounds [12,13]. These beneficial substances can act as antioxidants and electrophile scavengers, stimulate the immune system, inhibit nitrosation and the formation of DNA adducts with carcinogens, and induce detoxification enzymes [14]. In the present investigation, preliminary phytochemical/chemical characterization of OB extracts has confirmed the presence of polyphenols including quercetin, rutin, kaempferol, caffeic acid, and terpenoids and terpenoid alcohols. However, the extracts were proven to have potent antioxidant property causing free radical quenching effect. Hence, the reported cytotoxic activity of OB may be due to the presence of polyphenolic compounds and antioxidant potential of the extracts.

The results for the MS extracts were found to be in accordance with a recent report which indicated that six *mentha* species extracts and essential oils exerted cytotoxic activity against Vero, HeLa, and HepG2 cell lines at different concentrations (IC₅₀ 28.1-166.2 µg/ml), in HepG2 the IC₅₀ value was found to be 94.3 µg/ml [15]. Preliminary phytochemical/chemical characterization have confirmed the presence of polyphenols including quercetin, rutin, kaempferol, caffeic acid, and terpenoids and terpenoid alcohols in MS extracts in the present study. Further, the MS extracts were proven to have potent antioxidant property causing free radical quenching effect. Hence, the reported cytotoxic activity of MS may be due to the presence of polyphenolic compounds and antioxidant potential of the extracts.

Morphological changes such as cell shrinkage and clumping, vacuolation, distorted cells, cell adhesion, and cell growth inhibition were observed in the HL60 cells treated with OB and MS extracts indicating the signs of apoptosis [9]. Important features of apoptotic cell death are cell shrinkage, chromatin condensation, DNA fragmentation, and apoptotic bodies [16]. Differences in the cytotoxicity potential may be attributed to the differences in the extraction procedures and the natural variability of the plants [17]. Chemoprevention is defined as the use of specific chemical substance (natural or synthetic) to suppress the process of carcinogenesis and is one of the novel approaches to control cancer alternative to therapy that has its own limitations. Flavonoids come under the category of blocking agent which inhibit the metabolic activation of procarcinogen and interaction of cellular DNA [18]. OB and MS indicated IC₅₀ values <125 µg/ml and can be potential candidates for further development to cancer therapeutic agent. However, according to the American National Cancer Institute guidelines, plant extracts are considered to be antiproliferative with IC50 values <30 µg/ml after 72 hrs treatment [19], and they are considered to be highly cytotoxic with IC₅₀ value <20 µg/ml [20].

CONCLUSIONS

Hence, OB, MS, and *Centella asiatica* were found to possess moderate cytotoxic potential. The reported cytotoxic activity of the plant extracts in the present study may be due to the presence of phenolic and flavonoid constituents. This indicates the possibility of the plant extracts investigated, for further development to cancer therapeutic agent and warrants further studies to understand the mechanisms of cytotoxic activity and purification studies to streamline the bioactive principle responsible for the reported activity.

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