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Evaluation of the anticancer potentials of *Origanum marjorana* on fibrosarcoma (HT-1080) cell line

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PEER REVIEW

Peer reviewer

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Comments

This is a good study in which the authors evaluated the anticancer potential of *O. marjorana* extracts on fibrosarcoma cell line and its safety on humans was checked by testing on normal human lymphocytes. The results are encouraging for further identification of the active component for future animal studies and anticancer drug development.

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ABSTRACT

Objective: To evaluate the potential anticancer activity of *Origanum majorana* (marjoram) (*O. marjorana*) on the fibrosarcoma cancer cell line HT-1080 through various *in-vitro* assays.

Methods: Crude extracts were prepared from *O. marjorana* using ethanol, methanol and water as the solvents following standard protocols. Cytotoxicity was assessed using MTT assay, trypan blue dye exclusion, AO/EB staining and fluorescence microscopical analysis and DNA fragmentation analysis.

Results: Ethanol extract has shown significant cytotoxicity ($P < 0.001$) to fibrosarcoma and least toxicity to normal human lymphocytes when compared to the controls.

Conclusions: This study shows that *O. marjorana* ethanol extract has anticancer potentials and can be explored further for active component isolation, identification and characterization.

KEYWORDS

Origanum majorana, Anticancer, Cytotoxic, Ethanol extract, Fibrosarcoma, Lymphocytes

1. Introduction

Cancer is one of the leading causes of death in the US and around the world. Several chemotherapeutic, cytotoxic and immunomodulating agents are available in Western medicine to treat cancer. Besides being enormously expensive, these drugs are associated with serious side effects and morbidity. Still, the search continues for an ideal treatment that has minimal side effects and is cost-effective[1]. Medicinal plants are frequently used

by traditional healers to treat a variety of ailments and symptoms including diabetes and cancer. Over 50% of drugs in clinical trials for anti cancer activity were isolated from natural plant sources[2].

Origanum majorana (marjoram) (*O. majorana*) is a herbaceous and perennial plant native to southern Europe and the Mediterranean area. Due to the variability in chemical and aroma composition, marjoram plants are widely used to flavor food products and alcoholic beverages. They are also used traditionally

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for their pharmacological properties, including antibacterial, antithrombin and antihyperglycemic activities^[3]. It is one of the most important aromatic plants that contain major antioxidants like flavonoids and triterpenoids^[4]. Marjoram contains phenolic terpenoids (thymol and carvacrol), flavonoids (diosmetin, luteolin, and apigenin), tannins, hydroquinone, phenolic glycosides (arbutin, methyl arbutin, vitexin, orientin, and thymonin) and triterpenoids (ursolic acid and oleanolic acid)^[5]. Natural antioxidants are extensively studied for their ability to scavenge free radicals and to protect cells from different diseases such as from cancer. Aqueous and ethanolic extracts from marjoram played a role in preventing carcinogenesis and oncogenic mutations^[6]. *O. majorana*, is a widely used plant in folk medicine. Marjoram tea (extract of its leaves and flowers) has been prescribed in folkloric medicine for relieving the symptoms of hay fever, sinus congestion, indigestion, asthma, stomach pain, headache, dizziness, colds, coughs, and nervous disorders. The plant extract contains mainly terpinene, aroma-active compounds, carvacrol and thymol, alkaloids, flavonoids, and essential oils^[7–9]. The anticancer potential of *O. majorana* has not been studied on fibrosarcoma cell lines. In the present study we tried to evaluate the potential anti-cancer effects of *O. majorana* ethanol, aqueous and methanol extracts on fibrosarcoma HT-1080 cell line and also to evaluate their effects on human peripheral lymphocytes.

2. Materials and methods

2.1. Medicinal plant selected

O. majorana (marjoram) was collected during the month of August 2009, from Lalbagh, Bangalore, India, and identified by Prof. S. B. Sullia, Botanist and Microbiologist of Jain University. Specimen samples were kept in the herbarium of Jain University for future reference (voucher no. JUH-115).

2.2. Extraction using ethanol

The dried leaves of the plant (50 g) were crushed, soaked in 75 mL of ethanol (80% v/v) for 24 h and then percolated (5 h, 30 drops/min). The extract obtained was concentrated by a rotary evaporator and dried in oven at 40 °C. Then 20 mg of solid residue was dissolved in 1 mL of dimethyl sulfoxide (DMSO) and was diluted to 100 mL with distilled water. This was filtered through 0.22 micro filters. The dilution was continued so that final concentrations of extract were 40, 80 and 120 µg/mL^[10].

2.3. Extraction using methanol

Dry leaf samples were ground using an electric blender.

2 g of ground material was soaked in 25 mL of methanol for 24 h and was filtered using filter paper. The material was again mixed with 25 mL of fresh methanol and filtered after 24 h. This was repeated (25 mL×3 times). Then the filtrate was transferred into vials and allowed to dry. Dry extract was resuspended in 2 mL of DMSO. The concentration of extract was 1 mg/mL^[11]. The dilution was continued so that final concentrations of extracts used were 40, 80 and 120 µg/mL.

2.4. Aqueous extraction

Dried plant (50 g) was ground and mixed with 1 liter boiling distilled water for 1 h. The mixture was filtered twice through a funnel by using suction pump. The extract was evaporated under reduced pressure till it dried by using a lyophilizer. Extract (1 mg/mL) was prepared by dilution of the stock with sterile distilled water^[12].

2.5. Cell lines selected

Human peripheral lymphocytes were isolated in the laboratory and fibrosarcoma (HT 1080) cell line was procured from National Center for Cell Sciences (NCCS), Pune, India. Fibrosarcoma cells were grown in complete DMEM medium (2 mmol/L L-glutamine, 100 g/mL of streptomycin, and 100 U/mL of penicillin) supplemented with 10% fetal bovine serum (HIMEDIA) and maintained in a 5% CO₂ humidified incubator at 37 °C. Cells were seeded at a density of 1×10⁵ cells/mL, except where otherwise indicated.

2.6. Isolation of lymphocytes

Lymphocytes were obtained from the blood of five healthy male and female individuals, about 20 years of age, apparently free from infection by pathogenic agents and had not been under any treatment for the last six months. HiSep medium (HIMEDIA, India) was used for the isolation. Cells were suspended in complete RPMI 1640 medium supplemented with 10% fetal bovine serum (HIMEDIA, India), 5 g/mL phytohemagglutinin and maintained at 37 °C in a 5% CO₂ humidified incubator. Lymphocytes were used as control cells to assess the cytotoxicity of plant extracts.

2.7. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay

MTT assay was performed to assess the cytotoxicity of the plant extracts. (MTT is a yellow colored dye which is reduced into purple colored formazan crystals by the activity of mitochondrial succinate dehydrogenase enzyme in viable cells). Cells were cultured in 96 well microtitre plates. Cells were treated with varying concentrations of *O. majorana* extracts for 24 h (9 wells per each concentration) and incubated. At the end of treatment period, to each well 20 µL

of MTT was added. After the addition of MTT, the plates were incubated for 3 h in dark chamber. Then 100 μ L of DMSO was added to dissolve the formazan crystals. The absorbance was read at 540 nm using ELISA reader^[13].

2.8. Determination of cell concentration and viability by trypan blue dye exclusion

At the end of treatment period, the cells were counted with the aid of a haemocytometer and cell viability was determined by trypan blue dye exclusion method. Trypan blue was prepared at a concentration of 0.4% in phosphate buffered saline^[14].

2.9. Analysis of DNA fragmentation

In a medium containing 10% fetal bovine serum, 1.0×10^5 cells were incubated for 24 h. After 24 h, cells were treated with *O. marjorana* leaf extracts (aqueous, methanol and ethanol) at different concentrations. After 24 h, cells were collected by trypsinization, cells from different wells of the same concentration were pooled and rinsed twice in cold phosphate buffered saline (pH 7.4). Genomic DNA was extracted from HT-1080 cells as described earlier^[15]. Briefly cells were re-suspended twice in a lysis buffer containing 1% Nonidet-P40, 20 mmol/L EDTA and 50 mmol/L Tris-HCl, pH 8. The cells were centrifuged at 1600 g for 10 min, recovered supernatant were combined and incubated with 0.5% SDS and 0.5 mg/mL RNase A (Bangalore Genei, India) at 56 °C for 2 h and thereafter treated with 1 mg/mL proteinase K (Bangalore Genei, India) at 37 °C for 4 h. The DNA was precipitated by the addition of 1/10 volume of 7.5 mol/L ammonium acetate and two volumes of ethanol and analyzed by agarose gel electrophoresis.

2.10. Fluorescence microscopy

To determine the live apoptotic cells, cells treated for 24 h were stained with acridine orange: ethidium bromide (AO/EB). After 24 h, the control and treated cells were stained with acridine orange (50 μ g/mL) and ethidium bromide (50 μ g/mL) mixture^[16]. Then the cells were analyzed under a fluorescence microscope (Labomed, Germany) and representative photographs were taken for further quantitative analysis.

2.11. Statistical analysis

All experiments were carried out in triplicates. The results were calculated as mean \pm standard error (SE) values. Statistical significance was calculated using One-way analysis of variance (ANOVA) and Student's *t*-test. A value of $P < 0.05$ was taken as statistically significant.

3. Results

3.1. Cytotoxicity by MTT assay

When *O. marjorana* aqueous extract was administered to fibrosarcoma cells, at 40 and 80 μ g/mL concentrations, induced significant cell proliferation ($P < 0.05$) rather than inhibition (Figure 1). The ethanol extract was highly cytotoxic, reducing the cell viability to less than 50% at 120 μ g/mL concentration. The reduction in cell viability observed at 120 μ g/mL extract treated fibrosarcoma cells was found to be highly significant ($P < 0.001$) when compared to that of the controls (Figure 1). The IC_{50} value was found to be 110 μ g/mL from the graph. As the concentration of the extract increased, cell viability decreased. The methanol extract was not cytotoxic at 40, 80 and 120 μ g/mL concentrations.

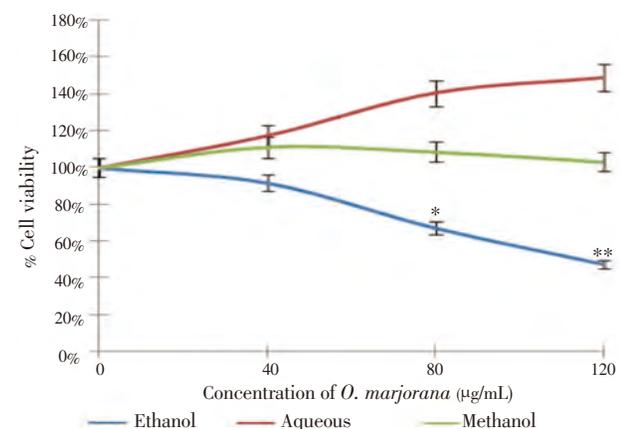


Figure 1. Effect of *O. marjorana* on the percentage viability of fibrosarcoma (HT-1080) cells as measured by MTT assay.

* $P < 0.05$ compared with control; **Indicate high significance with $P < 0.01$.

3.2. Total cell count by trypan blue dye exclusion

By trypan blue dye exclusion method cell concentration as well as cell viability were determined with a haemocytometer (Figure 2). The ethanol extract (120 μ g/mL) had reduced the cell count from 1×10^4 cells/mL in these control flasks to only 0.4×10^4 cells/mL.

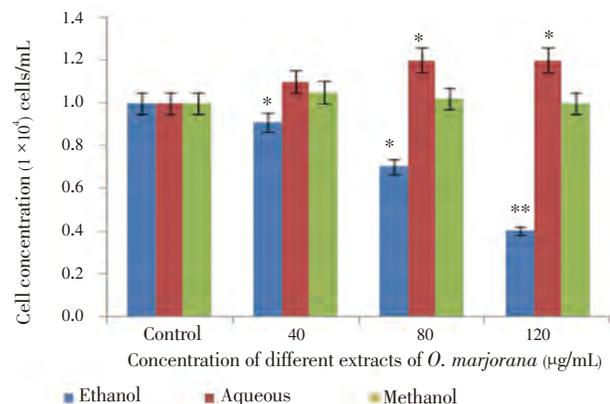


Figure 2. Effect of *O. marjorana* on the total cell count of fibrosarcoma (HT-1080) cells by trypan blue assay.

* $P < 0.05$ compared with control; **Indicate high significance with $P < 0.01$.

3.3. Effect on normal lymphocytes

When different concentrations of *O. marjorana* were added to normal lymphocytes, the aqueous extract had decreased their viability to 98.7% at 40 µg/mL concentration, 93.6% at 80 µg/mL concentration, and to 89.36% at 120 µg/mL concentration (Figure 3). The inhibitions at 40 and 80 µg/mL concentrations were not significant ($P>0.05$) only at 120 µg/mL concentration it was significant. The ethanol extract had not significantly inhibited the viability of the lymphocytes at 40 and 80 µg/mL concentration with the percentage viabilities being 99% and 94% respectively. At 120 µg/mL concentration, the viability had slightly reduced to 82.5% of that of the controls. Methanol extract was showing slight cytotoxicity when compared to the other two extracts. The ethanol extract was safe to the normal human lymphocytes and has shown cytotoxicity to the cancer cell line. When cell count was taken by trypan blue staining and haemocytometry, it was observed that (Figure 4), the ethanol extract and aqueous extracts have not shown significant cytotoxic effects on the peripheral blood lymphocytes ($P>0.05$), whereas the methanol extract was slightly toxic at 120 µg/mL ($P<0.05$).

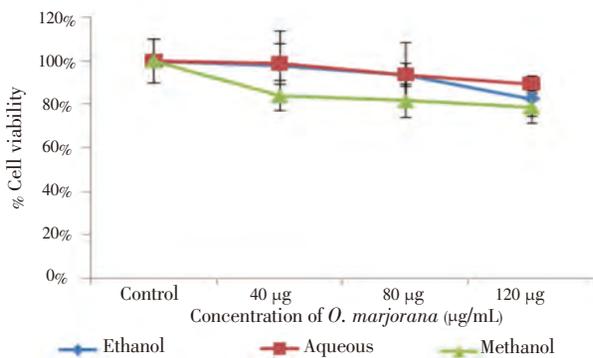


Figure 3. Effect of *O. marjorana* on the percentage viability of normal lymphocytes as measured by MTT assay.

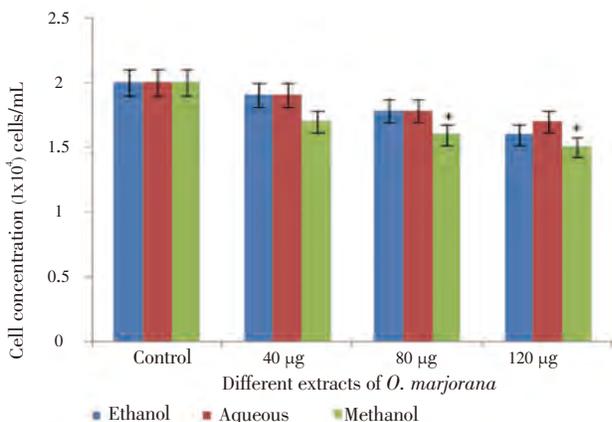


Figure 4. Effect of *O. marjorana* on the total cell count of lymphocytes by trypan blue assay.

* $P<0.05$ compared with control.

3.4. DNA fragmentation analysis

DNA fragmentation was tested by agarose gel

electrophoresis. Figure 5 indicates a significant increase in inter-nucleosomal DNA fragmentation of HT-1080 cells. When the DNA isolated from *O. marjorana* treated cells was subjected to agarose gel electrophoresis, a DNA ladder characteristic of apoptotic DNA was observed in the cells treated with different concentrations of the ethanol extract.

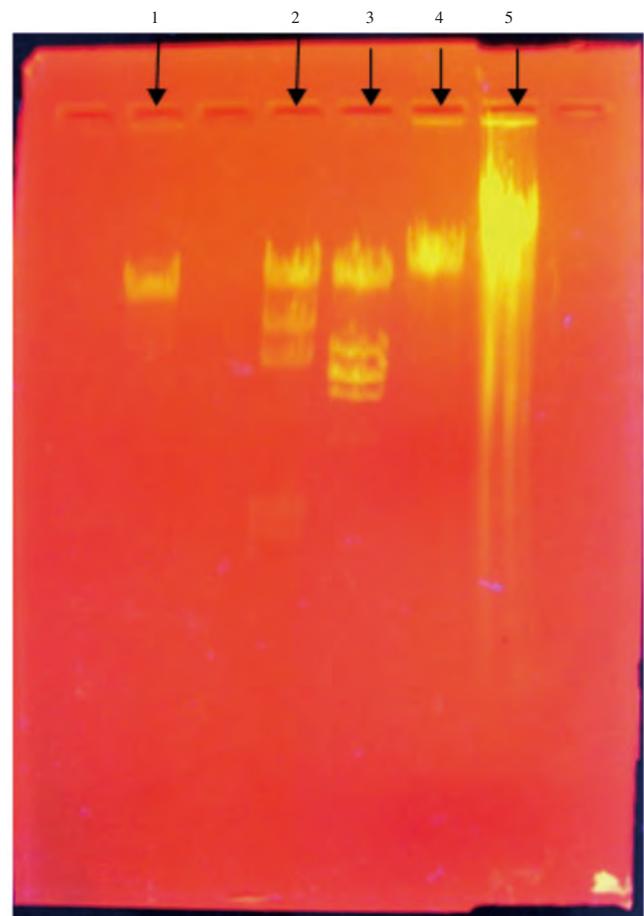


Figure 5. DNA fragmentation analysis of fibrosarcoma cells treated with different extracts of *O. marjorana*.

1: Control; 2: *O. marjorana* aqueous extract (80 µg/mL); 3: *O. marjorana* methanol extract (120 µg/mL); 4: *O. marjorana* ethanol extract (80 µg/mL); 5: *O. marjorana* ethanol extract (120 µg/mL).

3.5. Ethidium bromide–acridine orange staining

Staining with AO/EB of the samples treated with different concentrations of the ethanol extracts of *O. marjorana* (Figures 6), non-viable cells had bright orange chromatin when observed under the fluorescence microscope. Viable cells in the control flasks were green in color. Apoptosis was demonstrated by the appearance of cell shrinkage with condensation and breaking up of the nuclei.

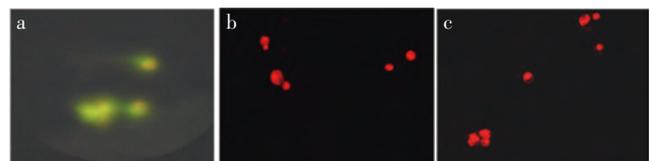


Figure 6. a: Control Fibrosarcoma (HT-1080) cells as observed under the fluorescence microscope; b: Fibrosarcoma cells treated with the ethanol extract of *O. marjorana* (80 µg/mL) for 24 h; c: Fibrosarcoma cells treated with the ethanol extract of *O. marjorana* (120 µg/mL) for 24 h.

4. Discussion

This study shows that ethanol extract of *O. marjorana* could induce apoptosis of human cancer cell line fibrosarcoma. Cell proliferation was significantly inhibited by ethanol extract of *O. marjorana* (80 and 120 µg/mL) after 24 h of culture. The dose-dependent growth inhibitory effect of the ethanol extract was significantly different from that of methanol and water extracts. In addition, numerous tumor giant cells which were vacuolized, many cells detached from the substrate, undergoing chromatin condensation and with apoptotic bodies (observed by trypan blue staining, AO/EB staining and fluorescence microscopical observation) were observed after treatment with ethanol extract of *O. marjorana*. It could induce cell death by apoptosis in the cancer cell line fibrosarcoma as evidenced by fluorescence microscopical analysis and DNA fragmentation analysis, but these effects were not observed in the normal human lymphocytes after 24 h of treatment with the ethanol extract of *O. marjorana*.

Marjoram leaves were used in folkloric medicine as a poultice for the rheumatic pain and for sprains. The oil from the leaves is used to relieve toothache pain. As a tea, marjoram aids in digestion and helps prevent motion sickness, relieves flatulence, colds and headaches, calms nerves, and promotes menstruation. The unsweetened tea can also be used as a mouthwash or gargle. Many studies suggest that *O. majorana* has a potent antioxidant and antimicrobial activities [17–20], and the other study suggest that anti-hepatoma activity by *O. majorana* extract on five human liver-cancer cell lines[21]. The anti-genotoxicity of *O. majorana* was reported in one study by El-Ashmawy et al.[5] which illustrated the ability of *O. majorana* extracts to significantly reduce the rate of micronucleus, number of aberrant cells and different kinds of chromosomal aberrations which were induced by lead toxicity in mice. Abdel-Massih et al[22], have reported the antiproliferative effects of *O. majorana* extracts on human leukemia cells. To our knowledge, there was no previous mechanistic study on the apoptosis inducing ability of the ethanol extract of *O. majorana* against the cancer cell line fibrosarcoma (HT-1080). This is the first report and also our study indicates that the extract has no toxicity on human lymphocytes as indicated by MTT assay and trypan blue dye exclusion results. Although the efficacy of *O. majorana* ethanol extract has been presently tested against *in-vitro* cancer cell line, it is likely that the result can be extrapolated to animal or human systems. Since our results indicated that the key bioactive component was in the ethanol fraction, future studies can be focused towards bioactivity guided fractionation, purification, isolation and identification of the active compound which has the potential to be developed

into an anticancer agent.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

The authors are grateful to the management of Jain Group of Institutions for the financial support and infrastructural facilities provided to carry out the work.

Comments

Background

Cancer is one of the leading causes of death in the US and around the world. Medicinal plants are frequently used by traditional healers to treat a variety of ailments and symptoms including diabetes and cancer. *O. marjorana* (marjoram) is a herbaceous and perennial plant native to southern Europe and the Mediterranean area. It contains major antioxidants like flavonoids and triterpenoids. Natural antioxidants are extensively studied for their ability to scavenge free radicals and to protect cells from different diseases such as from cancer. *O. marjorana*, is a widely used plant in folk medicine.

Research frontiers

The anticancer potential of *O. marjorana* has not been explored on fibrosarcoma cell lines. In the present study the authors evaluated the potential anti-cancer effects of *O. marjorana* ethanol, aqueous and methanol extracts on fibrosarcoma HT-1080 cell line and also to evaluate their effects on human peripheral lymphocytes.

Related reports

Many studies suggested that *O. majorana* has a potent antioxidant and antimicrobial activities and the other study suggested about the anti-hepatoma activity by *O. majorana* extract. This study shows that ethanol extract of *O. marjorana* could induce apoptosis of human cancer cell line fibrosarcoma. Cell proliferation was significantly inhibited by ethanol extract of *O. marjorana* (80 and 120 µg/mL) after 24 hrs of culture. It could induce cell death by apoptosis in the cancer cell line fibrosarcoma as evidenced by fluorescence microscopical analysis and DNA fragmentation analysis.

Innovations & breakthroughs

There has been no previous mechanistic study on the

apoptosis inducing ability of the ethanol extract of *O. majorana* against the cancer cell line fibrosarcoma(HT-1080). This is the first report and also this study indicates that the extract has no toxicity on human lymphocytes.

Applications

The efficacy of *O. majorana* ethanol extract has been presently demonstrated against *in-vitro* cancer cell line, it is likely that the result can be extrapolated to animal and further to human systems. It has the potential for purification and identification of active component for further development of a safe anticancer drug with least side effects and cost-effectiveness.

Peer review

This is a good study in which the authors evaluated the anticancer potential of *O. majorana* extracts on fibrosarcoma cell line and its safety on humans was checked by testing on normal human lymphocytes. The results are encouraging for further identification of the active component for future animal studies and anticancer drug development.

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