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Original Research

The antiproliferative effect of *Origanum majorana* on human hepatocarcinoma cell line: suppression of NF-κB

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Abstract: Hepatocellular carcinoma (HCC) is one of the most common cancer types with a high prevalence and itis the leading cause of cancer deaths worldwide. This study aimed to investigate the antiproliferative effect of aqueous and ethanol extracts of *Origanum majorana* leaf on human hepatocellular carcinoma (HepG2) cell line through incubation of various concentrations of *origanum majorana* extracts with HepG2 and at different time intervals. The effects of aqueous and ethanol extracts of *O. majorana L.* on HepG2 cell viability, nuclear factor kappa B (NF-kB) gene expression were examined. The results of the cell viability assays showed that aqueous and ethanol extracts exhibited a highly significant inhibitory effect on HepG2 cell proliferation which was evidenced by a reduction in viable cell count. The results were confirmed by microscopical examination of cell morphology. Furthermore, the *O. majorana L.* extracts suppressed the activity of NF-kB gene expression of HepG2 cells compared to the control.The conclusions from this study suggest that marjoram extracts exhibit anti-proliferative effect against HCC through suppressing the activity of NF-kB gene expression and high antioxidant activity.

Key words: HepG2, *Origanum majorana*, antiproliferative effect, hepatocellular carcinoma, nuclear factor kappa B.

Introduction

Liver cancer or hepatocellular carcinoma (HCC) is one of the most common cancer types worldwide, with a very survival rate of 3-4%. There are many factors play a role in the etiology of HCC, it can be caused by hepatitis C and B viruses and environmental factors(e.g. aflatoxin), other factors such as occupational exposure to chemicals as pesticides, cigarette smoking, and endemic infections in community, as schistosomiasis, may have additional roles in the etiology or progression of the disease (1).

HCC is a preventable disease rather than a curable one since there is no well-documented effective treatment modality until now. Recently, there has been a worldwide trend towards the use of various plants and many efforts are focused on the search for a potential source rich in biologically active compounds. It was reported that some plants, exercise various bioactivities, including anti-inflammatory, antioxidant, and anti- diabetic (2).

Phytochemicals exert their chemoprevention effect of carcinogenesis through several mechanisms. These include increased antioxidants and anti-inflammatory activity, modulation of cellular signaling pathways, inhibition of genotoxic effects, and altering gene expression to inhibit cell proliferation and/or induce apoptosis (3).

Origanum majorana (marjoram) is a herbaceous and perennial plant, native to the Mediterranean area and southern Europe. Marjoram plants are widely used to flavor food products and alcoholic beverages due to their variability in chemical and aroma composition.They are also used traditionally for their pharmacological properties, including antibacterial,antihyperglycemic andantithrombin activities (4).

Antioxidants play significant functions in eliminating free radicals, disintegrating peroxides, quenching singlet oxygen, donating hydrogen and chelating metal ion. These properties enable antioxidants to reduce lipid peroxidation, decrease DNA damage and inhibit cell proliferations (5).Several reports indicate that *O. majorana* is very rich in phenolic compounds. The high phenolics content in *O. majorana* has the capacity to scavenge free radicals and associated with the strong antioxidant activity (6). *O. majorana* contains flavonoids (diosmetin, luteolin, and apigenin), tannins, hydroquinone, phenolic terpenoids (thymol and carvacrol), phenolic glycosides (arbutin, methyl arbutin, orientin,vitexin, and thymonin) and triterpenoids (oleanolic acid and ursolic acid) (7).

Therefore, searching for the new natural compounds with the cytotoxic effects against HCC cells is of particular interest. The present study was undertaken to evaluate the potential anti-cancer effect and molecular mechanism of of aqueous and ethanol extracts of *Origanum mjorana. L.* on human hepatocellular carcinoma HepG2 cell line which is a well-differentiated transformed cell line closely related to HCC.

Materials and Methods

Chemicals

Phosphate-buffered saline (PBS), Dimethyl sulfoxide, and ethanol (high-performance liquid chromato-

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graphy, HPLC grade) were obtained from Fisher Scientific (USA). Fetal bovine serum, andtrypan blue stain (0.4%) were from Gibco BRL (USA). RPMI 1640 and amphotericin B were from Lonza Bio Whittaker.

Preparation of aqueous and ethanol extracts of *O. majorana L.*

Fresh marjoram (*Origanum majorana L.*) leaves were obtained from highest grade commercially available and identified by Dr. A. Abd El-Mogali, Senior Researcher in Flora &Phytotaxonomy Researchers Department, Agr. Museum, Dokki.*O. majorana* leaves were air dried in shade and ground to fine powder.

Preparation of the ethanol extract

The air dried powder leaves (400 gm) were macerated in 2L of 70% ethanol (EtOH) for 3 days and kept at 4°C in a refrigerator without stirring. This process is repeated till exhaustion. The filtration was done using a piece of cotton. The filtrate was evaporated till dryness under reduced pressure using a rotator evaporator (Heidolph, Germany) at 45°C and 90 rpm till a semisolid residue was obtained. The residue was converted to a lyophilized powder using a lyophilizer (CHRIST Alpha 1-2, UK) to give 16g extract/100g air dried powder leaves (8).

Preparation of water extract

The air dried powder leaves (400 gm) substance was macerated in 2L of distilled water and left at 60°C for 1 hour with continuous shaking, the mixture was cooled to room temperature. The filtration was done using a piece of cotton the filtrate was evaporated till dryness under reduced pressure using a rotatory evaporator (Heidolph, Germany) at 45°C and 90 rpm till the semisolid residue was obtained. The residue was left in lyophilizer (CHRIST Alpha 1-2, UK) until dried to give 18.5g extract/100g of dried powder leaves (9).

HepG2 Cell culture and treatment with *O. majorana L.*

Human hepatocellular carcinoma cells (HepG2) and human hepatocytes (positive control) were purchased from the holding company for biological products(VACSERA); in a semi-confluent 25 ml tissue culture flasks (T-25). HepG2 and human hepatocytes were maintained in RPMI-1640 medium supplemented with 100 units/ml penicillin, 100 µg/ml streptomycin10% heat inactivated fetal bovine serum (FBS), and 1% Amphotericin B in the laminar flow hood under completely sterile conditions, culture medium was changed three times per week. Then, T-25 flasks of completely confluent HepG2 cells were treated with each type of *O. majorana L.* extract which dissolved in DMSO (<0.5% in the culture media).Control cells were also subjected to DMSO only (<0.5% in the culture media) servedas control (10).

Concentration course study

This study was performed to obtain the concentration of both aqueous and ethanol extracts at which the HepG2 proliferation was significantly inhibited after 24 hrs. Both *O. majorana* extracts were dissolved in DMSO at concentrations of 50, 100, 150 µg/ml and diluted with tissue culture medium before use, as follows: 5ml of the above extracts (treatment) and 5ml of the tissue culture media were added to each flask, following the sterility rules. The treated flasks were then maintained at 37 °C humidified incubator with 95% air and 5% CO_2. After 24 hrs all flasks were trypsinized with 1 ml of trypsin EDTA and counted under the light microscope (100×) using trypan blue dye (0.04%) to count the number of viable cells.

Time course study

O. majorana extracts were each dissolved in dimethyl sulfoxide at a concentration of 150 µg/ml and diluted with tissue culture medium before use, as follows: 5 ml of the above extracts (treatment) and 5 ml of the tissue culture media were added to each flask, following the sterility rules. The treated flasks were then maintained at 37°C in a humidified incubator with 95% air and 5% CO_2. After 24 hrs all flasks were trypsinized with 1 ml of trypsin EDTA and counted under the light microscope (100×) using trypan blue dye (0.04%) to count the number of viable cells.

Nuclear factor kappa B gene expression using quantitative real-time PCR (RT-PCR) analysis

At 24, 48, 72 hrs after treatment total RNA has extracted from all of the above cell-lysates and using the RNeasy Mini kit (Qiagen, cat. No. 74104, USA) according to the manufacturer's instructions. In order to preserve RNA samples, and for avoiding the degradation at room temperature, total RNA was transcribed into cDNA using the reverse transcription system kit (High Capacity RNA-to-cDNA Master Mix, Applied Biosystems). The resultant cDNA was diluted 10 times in double distilled H_2O and kept at 20°C for RT-PCR. The relative expression levels of mRNA encoding NF-kB or β-actin was measured using the thermo Maxima SYBER Green/ROX qPCR Master mix (2×) (no. K0221), according to the manufacturer's protocol, and the results were computerized using PCR 7500 fast thermal cycler machine (Applied Bio-system, USA). Primer pairs for NF-kB and β-actin were designed by the primer design program (primer 3 software version 1.0). Forward and reverse primer sequences for the respective genes and their corresponding amplicon size are listed in Table1. The expression level of NF-kB was normalized to β-actin and presented as fold change relative to untreated control.

Statistical analysis of data

All data were expressed as means ± S.E.M. and statistically analyzed using SPSS 17.0 for Windows (SPSS

Table 1. Sequence of NF-kB and β-actin primers.

Primer name	Forward	Reverse	Size (bp)
NF-kB	5'-GCGTACACATTCTGGGGAGT-3'	5'-CCGAAGCAGGAGCTATCAAC-3'	178
β-Actin	5'-AGCCATGTACGTAGCCATCC-3'	5'-GCTGTGGTGGTGAAGCTGTA-3'	222

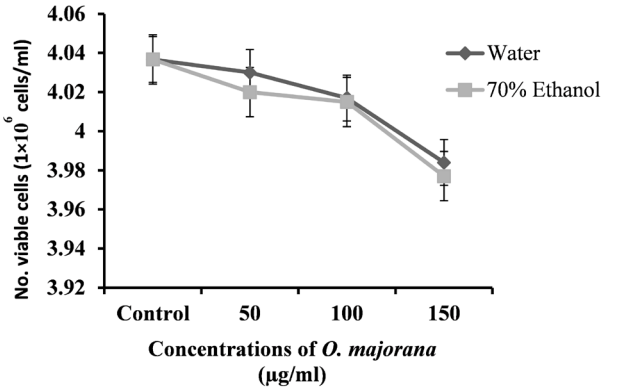

Figure 1. Effect of *O.majorana* extracts on viability of normal human hepatocytes. Values are expressed as means ± the standard error of the mean in each group. Statistically significant difference (p> 0.05) compared to the control group.

Inc, Chicago, IL). Statistical significance of differences among different study groups was evaluated by one-way analysis of variance (ANOVA). Post hoc testing was performed for intergroup comparisons using the Least Significant Difference (LSD) test, and a P value <0.05 was considered significant.

Results

Effect on normal hepatocyte

When the different concentrations (50,100 and 150 µg/ml) of water and ethanol extracts were added to normal hepatocytes for 72 hrs, and the cells counted under the light microscope (100×) using trypan blue dye (0.04%) to count the number of viable cells. It was observed from Figure 1, that water and ethanol extracts have not shown significant cytotoxic effects on the normal hepatocytes (P>0.05), this mean that the ethanol and water extracts were safe to the normal hepatocytes and shown cytotoxic effect on cancer cell line.

Effect of *O. majorana L.* extracts on growth and morphological changes in HepG2 cells

As shown in Table 2, the pilot study indicated that the number of viable cells decreased in proportional with increasing the concentration of 70% EtOH and water extracts, and150 µg/ml of *O. majorana L.* extracts were found to be the most potent concentration for significantly inhibited the proliferation of HepG2 cells using, 70% EtOH ((p=0.000, p<0.001) and water (p= 0.006, p<0.01) extracts, so this concentration used in the time course study.

The results of the time course study indicated that *O. majorana L.* extracts was significantly inhibited the proliferation of HepG2 cells in a time-dependent manner at 24, 48, and 72 hrs post-treatment using 70% EtOH and water extracts at concentration of 150 µg/ml, as the no. of viable cellswas significantly decreased with the progress of time compared with the untreated control cells and the ethanol extract has a more inhibitory effect than aqueous extract after 24, 48, and 72 hrs post-treatment as shown in Figure 2.

Microscopic observations of cell morphology show that *O. majorana L.* extracts induced morphological changes in HepG2 cells toward more mature forms of hepatocytes (using an inverted light microscope) which showed that the ethanol extract has more morphological changes on HepG2 than aqueous extract (Figure 3A-C).

Effect of *O. majorana L.* extracts on nuclear factor kappa B gene expression of HepG2 cells.

As shown in Figure 4**,** there is a time dependent downregulation in the level of NF-kB mRNA expression of HepG2 cells treated with 70% EtOH and water extracts of *O. majorana L.*as compared to control cells,

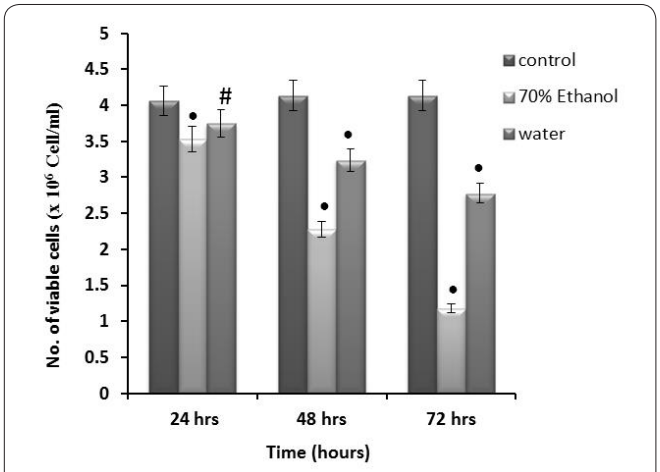

Figure 2. The time course responses of *O. majorana* extracts on HepG2 cells survival rate. Values are expressed as means ± the standard error of the mean in each group. #Statistically significant difference (p< 0.01) compared to the control group.• Statistically significant difference (p< 0.001) compared to the control group.

Table 2. The effect of different concentrations of *O. majorana* extracts on HepG2 cell viability.

	Control	70% Ethanol extract			Water		
Conc. of *O. majorana* extracts (µg/ml)	0	50	100	150	50	100	150
Number of viable cells (x10⁶cells/ml)	4.036± 0.032	3.97± 0.01	3.943± 0.015	3.54± 0.03	4.01± 0.08	3.98± 0.025	3.94± 0.015
P value Significant		0.038 P < 0.05	0.006 P<0.01	0.000 P<0.001	0.378 P>0.05	0.058 P>0.05	0.006 P<0.01

Values are expressed as mean ± standard error

P > 0.05, compared to the control group was non-significant.

P < 0.05, compared to the control group was weakly significant.

P < 0.01, compared to the control group was moderately significant.

P < 0.001, compared to the control group was highly significant.

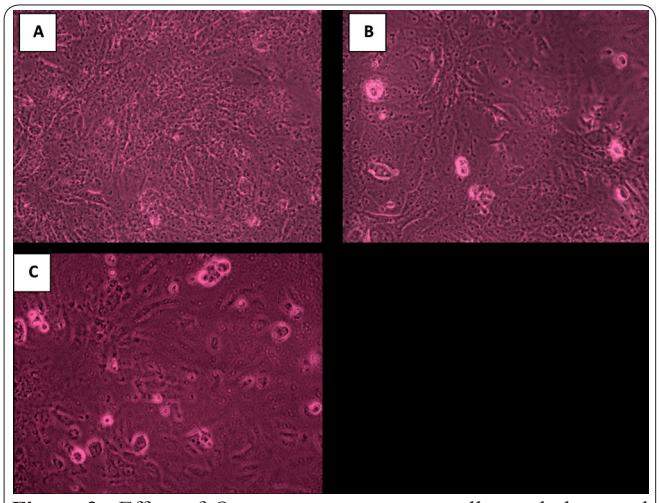

Figure 3. Effect of *O.majorana* extracts on cell morphology and viability of HepG2 cells (A): the control HepG2 cells as observed under inverted light microscope; (B): HepG2 cells treated with the aqueous extract of *O. Majorana* (150 µg/ml) for 72 hrs; (C): HepG2 cells treated with the ethanol extract of *O. Majorana* (150 µg/ml) for 72 hrs.

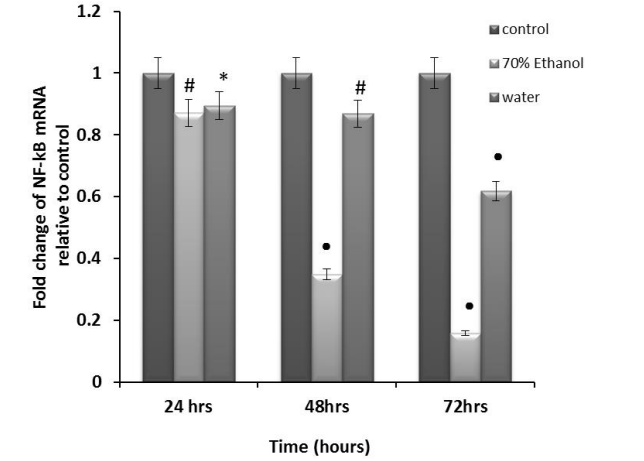

Figure 4. The effects of *O. majorana* extracts on nuclear factor kappa B gene expression. Values are expressed as means ± the standard error of the mean in each group. * Statistically significant difference ($p < 0.05$) compared to the control group. #Statistically significant difference ($p < 0.01$) compared to the control group.• Statistically significant difference ($p< 0.001$) compared to the control group.

which was evident by depression in the fold change of NF-kB gene compared with the control.

Discussion

Nowadays, one of the main methods of modern cancer treatment is chemotherapy. Most chemotherapeutic agents for cancer have different substantial short and long term side effects. Thus, in recent years major research has been focused on components isolated from herbs and plants which have been considered for being nontoxic and for the prevention and treatment of certain types of cancer. Recently, natural plants have received much attention as sources of biological active substances including antioxidants, antimutagens, and anticarcinogens (11).

In the present study the cell viability assays showed that 70% EtOH and water extracts exhibited a highly statistically significant inhibition on HepG2 cell proliferation which was evidenced by a reduction in the viable cell count. The ethanol extract had much stronger inhibitory activity than the water extract after 24, 48 and 72 hrs. Therefore, the 70% EtOH extract of *O. majorana L.* had the most effective inhibiting effect on the growth of HepG2 cells. These results are in agreement with the previous work on breast cancer cell line (MDA-MB231 cells), which showed that the *O. majorana* extract could inhibit cell proliferation by inducing cell cycle arrest at G2/M phase, furthermore, the current light microscopy results in line with this previous study, which reported the morphological changes in MDA-MB231 cells characterized by a loss of their epithelial morphology visible after 24 hrs of treatment with 150, 300, and 600 µg/ml *O. majorana* extract (8). Also the current results consistent with prior reports demonstrating the anti-proliferative effect of *O.majorana* on C6 (rat brain tumor) cell lines (12) and human lymphoblastic leukemia cell lines (13).

In the present study the ethanol extract exhibit a higher inhibition on HepG2 cell proliferation than water extract, this observation were in agreement with Rusaczonek et al. (14), who reported that the content of polyphenol compounds and antioxidant capacity of herbal plants differs depending on the extraction method. Using alcohol extraction enables achieving a considerably higher concentration of polyphenol compounds and, consequently, antioxidant capacity, in comparison with the values obtained in the case of water.

NF-kB is a transcription nuclear factor that could promote tumorigenesis, and is linked to cell invasion and metastasis. Suppression of NF-kB activation is effective in the prevention and treatment of cancer. NF-kB activation is a frequent and early event in human liver cancers of viral or nonviral etiologies and has been associated with the acquisition of a transformed phenotype during hepatocarcinogenesis (15). A number of different factors may trigger activation of NF-kB during hepatocarcinogenesis in HBV-induced HCC, due to the suggestion that the oncogenic HBV-X protein activates the NF-kB signaling pathway (16).

In the present study RT-PCR analysis showed that the *O. majorana L.* extracts suppressed the of NF-kB gene expression level of HepG2 cells with the treatment of water and 70% ethanol extracts which effectively suppresses tumor growth when compared to the control.

These results were in agreement with Al Dhaheri et al. (8), who demonstrated that the antiproliferative effect of *O.majorana* on other type of cell lines was due to inhibition of I kB phosphorylation and reduction the protein level of nuclear NF-kB suggesting that *O.majorana* might negatively regulate the activity of NF-kB possibly by affecting the activity of the IKK complex. And thus, *O. majorana* significantly reduced the expression of several NF-kB downstream target genes involved in tumor metastasis.

Marjoram has high antioxidant capacity due to its high polyphenolic content.The *O. majorana* plant extract contains mainly terpinenes, aroma-active compounds, carvacrol and thymol, alkloids, flavonoids, and essential oils (17). It was reported that polyphenol-rich plant extracts exerted different antioxidant and

anti-inflammatory effect, through down-regulated ROS production, increased SOD antioxidant enzyme gene expression and reduced mRNA levels of NF-κB pro-inflammatory transcription factor. These antioxidant and anti-inflammatory properties of polyphenols may depend on their chemical nature, dose and ability to target cellsaccording to their bio-accessibility extent (18).

In conclusion, this study clearly demonstrated that the ethanol extract of *O. majorana* processes more inhibitory effect than water extract on HepG2 hepatocellular carcinoma cell line, and also both extracts suppressed the activity of NF-kB gene expression in parallel with increasing the time of exposure to *O. majorana* extracts. These results suggest that marjoram extracts exhibit anti-proliferative effect via their ability to augument cellular antioxidant defences by suppressing the activity of NF-kB gene expression. The demonstrated antiproliferative effect of *O. majorana* make them good candidates for future use in treating HCC.

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