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Cytotoxic Activity of Methanolic Extract of *Mentha longifolia* and *Ocimum basilicum* Against Human Breast Cancer

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Abstract: Labiatae family is represented in Saudi Arabia. The aim of the present study was to go insight to investigate the anticancer activity and antioxidative potentials of methanolic extracts of *Mentha longifolia* L. (ML) and *Ocimum basilicum* L. (OB) that grown in Madina province, western region, Saudi Arabia. OB exhibited the greater phenolic contents as mg gallic acid equivalent/g weight (mg GAE/g) for a value of 105±5.5 mg GAE/g. On the other hand, ML produced 29±3.12 mg GAE/g. The standard antioxidant vitamin E used in this experiment elicited a value of total phenolic contents equal 22±2.2 mg GAE/g. The percentage scavenging activity of against diphenylpicrylhydrazyl (DPPH) was 850 and 160% for OB and ML extracts, respectively. Vitamin E elicited% scavenging activity of against DPPH equal to 198%. Brine shrimp cytotoxic assay clearly indicated the cytotoxic effects of either ML or OB extract. The brine shrimp survival is inversely proportional to the concentration of either ML or OB extract used with LD_{50} 191.23 and 235.50 ppm, respectively. Toxic effects on brine shrimps indicated the anticancer potential of ML or OB extract. The ML or OB extract was unable to produce pbluescript (pBS) plasmid DNA damage, while the plasmid DNA treated with *EcoRI* produced a single band as a result of DNA damage. Also, both ML and OB extract exhibited marked cytotoxic activity against MCF-7 cells at various concentrations (20, 40, 80, 160 and 320 μg mL^{-1}). The 160 and 320 μg mL^{-1} showed more cytotoxic effect against MCF-7 cells. Based on results achieved, we can concluded that, OB and ML extracts have the potency to act as powerful antioxidants and protect against DNA damage and have cytotoxic activity against MCF-7 cell line.

Key words: *Mentha longifolia* L., *Ocimum basilicum* L., MCF-7 cell line, antioxidant, DNA damage, Brine shrimp assay, MTT assay

INTRODUCTION

Cancer is one of the leading causes of death worldwide. According to global cancer statistics released by the American cancer society, the total number of deaths from cancer in 2007 was 7.6 million, or about 20,000 deaths each day, with 38% in developed countries and 62% in developing countries. By 2050, 27 million new cancer cases and 17.5 million cancer deaths are projected to occur in the world (Dunham, 2007). Natural products from plants and microbes play an important role in amelioration of cancer (Cragg, 2005). According to (Al-Yahya *et al*., 1990) the Arabian peninsula is the birth place of herbal drugs and the use of folk medicine has existed there since time immemorial (Mossa *et al*., 1987). The Kingdom of Saudi Arabia is gifted with a wide range of flora, consisting of a large number of medicinal herbs, shrubs and trees. It is, however, estimated that the flora of Saudi Arabia has great medicinal species (Mossa *et al*., 2000).

Labiatae family is represented in Saudi Arabia. The Labiatae family (Lamiaceae) is one of the largest and most distinctive families of flowering plants, with about 220 genera and almost 4000 species worldwide. This family has an almost cosmopolitan distribution, with a great diversity (Hedge, 1986, 1992). Labiatae are best known for the essential oils common to many members of the family. This family is one of the major sources of culinary, vegetables and medicinal plants all over the world. Species of *Mentha* and *Ocimum* are used as food

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flavorings, vegetables and in industry. Also they are used in traditional and modern medicine (Rivera-Nunez and Obon de Gastro, 1992). *Mentha longifolia* L. (ML) also known as wild mint, is a fast growing aromatic perennial herb. It is widely used in herbal medicine and believed to be particularly beneficial in building the immune system and fighting secondary infections. ML is used for the treatment of cough, cold and influenza. Externally, wild mint is used to treat wounds and swollen glands (Van Wyk *et al*., 1997). The essential oil of this plant is partly responsible for the decongestant, antispasmodic and antibiotic and mosquito's repellent effects (Hutchings and Van Staden, 1994; Van Wyk *et al*., 2000). It has also been spread in granaries to keep rodents off the grain (Phillips and Foy, 1990). In Saudi Arabia ML known as horse or wild mint, is distributed in western mountain region. *Ocimum basilicum* L. (OB) family Lamiaceae has many medicinal properties and uses as carminative, expectorant, stimulant and diaphoretic. Leaf juice is used for the treatment of cough, fever, ringworms, internal piles, diarrhea and kidney disorders (Mossa *et al*., 1987, 2000). The essential oil of OB has been used extensively in food products, perfumery and dental products. Besides, it exhibit antimicrobial activity, insecticidal, antiviral and anticancer effects (Suppakul *et al*., 2003).

The aim of the present study was to go insight to investigate the anticancer activity and antioxidative potentials of methanolic extracts of ML and OB that grown in Madina province, western region, Saudi Arabia.

MATERIALS AND METHODS

Chemicals: The breast cancer cell line MCF-7 was purchased from A.T.C.C., Rockville, MD, USA. The cells were maintained in DMEM medium (Invitrogen®, USA). RPMI medium was purchased from (Invitrogen®, USA). All other solvents and chemicals used in this study were of highest grade and purchased from Sigma-Aldrich® (USA) and Acros® (Belgium).

Plants extraction: ML and OB leaves were collected from Madinah, Saudi Arabia. The collected plants were identified by the staff of the Biology Department, Faculty of Science, Taibah University, Madinah, Saudi Arabia. Plant material was rinsed twice with water and then dried in shade. After shade drying plant material was pulverized using a domestic blender to powder form. Powdered plant material (100 g) was mixed with 1 L of 80% methanol and kept for 7 days in closed vessels at room temperature. After 7 days, mixtures was filtered through Whatman 41 filter paper. The extracted liquid was subjected to rotary evaporator. The water bath temperature was adjusted to 40°C. The semisolid extract produced was kept in open air for four days for complete evaporation of methanol.

Cell line preparation: The breast cancer cell line MCF-7 maintained in DMEM medium was supplemented with 10% fetal bovine serum. Cells were incubated at 37°C, 5% CO_2 and routinely sub-cultured when reaching 80% of confluency. Three days before experiments, the cells were incubated in phenol-red free RPMI medium instead of DMEM. Sterility test was done initially to check the plants extracts for the contamination. A 35 mm culture dish was plated with the MCF-7 cell suspension in 2 mL of DMEM media and allow the cells to adhere. ML or OB extract was added in the concentration of 0.1% v/v to the MCF-7 cells and incubated in 5% CO_2 incubator for 24 h. Cell density was seeded uniformly at 5×10^2 cells/mm^2 in all experiments (Gali *et al*., 2011).

Determination of total soluble phenolic contents: One mL of each plant extract solution, was mixed with 7.5 mL of Folin-Ciocalteu reagent which was diluted 10×with dist H_2O. After standing at room temperature for 5 min, 7.5 mL of 60 mg mL^{-1} of aqueous Na_2CO_3 solution were added. The mixture was kept at room temperature for 2 h and then the absorbance was measured at 725 nm spectrophotometerically. The results were expressed in Gallic Acid Equivalents (GAE)/g of dry weight from the calibration curve of gallic acid. The samples were analyzed in triplicates (Erkan *et al*., 2008).

DPPH free radical-scavenging assay: A 0.06 mm solution of DPPH in ethanol was prepared. The initial absorbance of the DPPH in ethanol was measured at 517 nm and did not change throughout the period of assay. A 0.5 mL solution of either ML or OB extract at concentration of 0.1 mg mL^{-1} was added to 3.5 mL of ethanolic DPPH solution. The change in absorbance at 517 nm was measured at 30 min and free radical scavenging activity was calculated as inhibition using following equation:

$$\text{DPPH radical scavenging activity (\%)} = 1 - \left[\frac{As}{Ac}\right] \times 100$$

where, A_s is absorbance of the DPPH solution containing samples. A_c is absorbance of the control solution without sample but with DPPH (Leong and Shui, 2002). The percentages of DPPH reduced were plotted against the samples. The experiment was also carried out using vitamin E (0.1 mg mL^{-1}) as a standard antioxidant. The samples were analyzed in triplicates.

Brine shrimp cytotoxic assay: A rectangular dish (22×32 cm) was divided into 2 unequal halves with plastic divider of 2 mm with several holes and filled with artificial seawater (28 g sea salt/L). Approximately 25 mg eggs (*Artemia salina* Sera) were sprinkled in the larger piece, which was darkened, while the smaller piece was illuminated. After 24 h, phototropic nauplii (brine shrimp larvae) were collected by pipette from the lightened side. 0.5 mL of 100, 1000 and 10,000 ppm concentrations of the extract prepared in methanol was poured in vials and kept at room temperature to evaporate methanol. Then, about 2 mL of sea water was added and 10 shrimps were transferred to each vial. The vials were placed under illumination at room temperature. Then, the number of survivors was counted and LD_{50} was calculated after 24 h (Finney, 1971; Meyer *et al*., 1982).

DNA damage analysis: 10 μg pBluescript (pBS) plasmid DNA was treated with either 10 μL ML or OB methanolic extract. These reaction mixtures were kept for 2 h at 37°C for the complete digestion of pBS DNA. Then, pBS DNA was visualized on 2% agarose gel electrophoresis. The pBS was digested with 1U *EcoR1* as positive control (De Carvalho *et al*., 2003).

***In vitro* assay for cytotoxic activity (MTT assay):** The MTT assay is a laboratory test which measures changes in colour for measuring the activity of enzyme that reduce MTT to formazan, giving a purple colour. Yellow MTT [3-(4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, a tetrazole) is reduced to purple formazan in living cells (Mosmann, 1983). MCF-7 cell line cells (3×10^3 per well) were plated in 100 μL of medium/well in 96-well plates and incubated overnight. Then, ML or OB extract was added in various concentrations (20, 40, 80, 160 and 320 μg mL^{-1}) 5 wells were included in each concentration. After treatment with ML or OB extract for 1, 2, 3, 4 and 5 days, 20 μL of 5 mg mL^{-1} MTT (pH 4.7) was added per well and cultivated for another 4 h, the supernatant fluid was removed, 100 μL DMSO was added per well and shaken for 15 min. The absorbance at 570 nm was measured with a microplate reader. All experiments were performed in triplicate. The effect of ML or OB extracts on the proliferation of MCF-7 cells was expressed as the percentage cytoviability, using the following formula (Selvakumaran *et al*., 2003):

$$\text{Cytoviability (\%)} = \frac{\text{Absorbance of treated cells}}{\text{Absorbance of control cells}} \times 100\%$$

Data analysis: Data are reported as the mean±S.E. of 3 measurements. The standard error was calculated and were presented using the GraphPad Prism software version 4.0 (San Diego, USA).

RESULTS

Total soluble phenolic contents and reducing power: The determination of total phenolics based on the absorbance values of ML or OB extracts that react with Folin-Ciocalteu reagent and followed by comparing with the standard solution of gallic acid equivalents. The standard curve of gallic acid (Fig. 1a) was done by using gallic acid concentration ranging from 0.01-0.30 mg mL^{-1}. The following equation expressed the absorbance of gallic acid standard solution as a function of concentration: A = 10.071 C+0.314; whereas A: absorbance at 765 nm and C: Gallic acid concentration (mg mL^{-1}). OB exhibited the greater phenolic contents as mg gallic acid equivalent/g weight (mg GAE/g) for a value of 105±5.5 mg GAE/g (Fig. 1b). On the other hand, ML

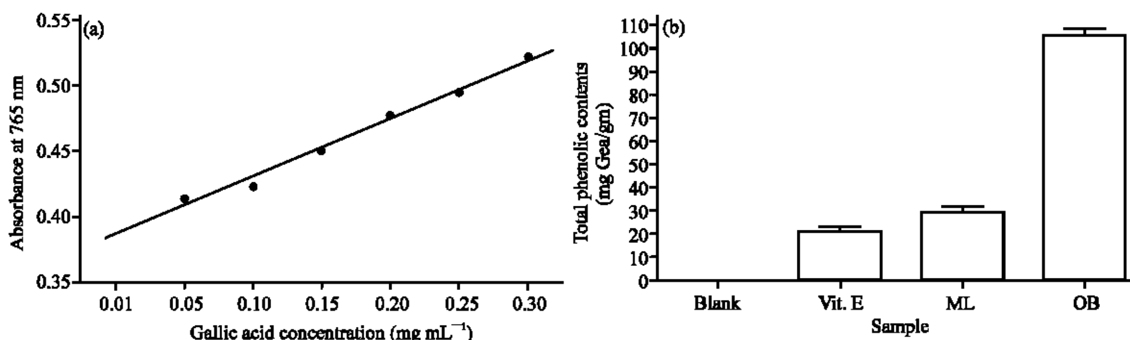

Fig. 1(a-b): (a) Gallic acid standard curve using gallic acid concentrations ranging from 0.01-0.30 mg mL^{-1} (b) Total soluble phenolic contents of ML and OB methanolic extracts determined by Folin-Ciocalteu reagent expressed as Gallic Acid Equivalents (GAE) utilizing absorbance versus concentration curve for gallic acid. Vitamin E was used as reference antioxidant. The samples were analyzed in triplicates. Values were expressed as Mean±SE

produced 29±3.12 mg GAE/g. The standard antioxidant vitamin E used in this experiment elicited a value of total phenolic contents equal 22±2.2 mg GAE/g.

DPPH free radical-scavenging assay: Free radical scavenging activity of either ML or OB extract was tested using the DPPH method and the results were showed in Fig. 2. In this study, the free radical scavenging activity of each sample was evaluated through recording the change of absorbance produced by the reduction of DPPH. The percentage scavenging activity of against DPPH. were 850 and 160% for OB and ML extracts, respectively. The standard antioxidant vitamin E used in this experiment elicited percentage scavenging activity of against DPPH equal to 198%.

Brine shrimp cytotoxic assay: Brine shrimp bioassay results clearly indicated the cytotoxic effects of either ML or OB extract (Fig. 3). Our results showed that, the brine shrimp survival is inversely proportional to the concentration of either ML or OB extract used with LD_{50} 191.23 and 235.50 ppm, respectively (Table 1). Toxic effects on brine shrimps indicated the anticancer potential of ML or OB extract.

DNA damage analysis: DNA damage assay was carried out by using pbluescript (pBS) plasmid DNA. Results showed that methanolic extract of either ML or OB was unable to produce DNA damage, while the plasmid DNA treated with *EcoRI* produced a single band as a result of DNA damage (Fig. 4).

***In vitro* assay for cytotoxic activity (MTT assay):** Both ML and OB extract exhibited marked cytotoxic activity against MCF-7 cells at various concentrations (20, 40, 80, 160 and 320 μg mL^{-1}). The 160 and 320 μg mL^{-1} showed more cytotoxic effect against MCF-7 cells as indicated by the lower cytoviability percentage of MCF-7 cells (Table 2). Figure 3, illustrated the inhibition of MCF-7 cells treated with 160 μg mL^{-1} extract. The inhibition rate was higher in case of OB compared to ML extract.

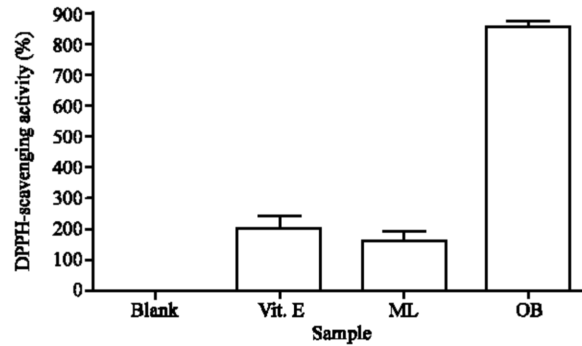

Fig. 2: DPPH free radical scavenging activity of ML and OB extracts determined using diphenyl picrylhydrazyl radical (DPPH) and expressed as (%) inhibition of DPPH radical. Vitamin E (0.1 mg mL^{-1}) was used as reference antioxidant. The samples were analyzed in triplicates. Values were expressed as mean±SE

Table 1: Effect of methanolic extract of either ML or OB methanolic extract on brine shrimps (*Artemia salina*)

	Concentration used (ppm)			
Effect	10	100	1000	Ld_{50}(ppm)
OB				
No. of shrimps used	30	30	30	
No. of shrimps killed	2	11	21	191.23
ML				
No. of shrimps used	30	30	30	
No. of shrimps killed	10	20	25	235.50

Table 2: Inhibition of MCF-7 cell growth by either ML or OB extract. Cells were loaded onto 96-well plate at 3×10^3 per well and were treated with either ML or OB extract at various concentrations (20, 40, 80, 160, 320 μg mL^{-1}) and percentage of cell viability was determined by the MTT assay after 1-5 days

Day	20 μg mL^{-1}	40 μg mL^{-1}	80 μg mL^{-1}	160 μg mL^{-1}	320μgmL^{-1}
OB					
1	81±2.56	75±2.21	62±1.10	63±1.21	62±1.23
2	71±3.78	63±1.61	42±1.10	42±1.21	40±0.92
3	60±2.61	58±0.53	28±0.91	24±0.42	23±0.62
4	60±3.10	50±0.52	25±0.45	21±0.41	22±0.71
5	60±2.70	45±0.92	22±0.41	16±0.21	15±0.23
ML					
1	80±3.37	72±2.19	60±2.10	60±2.23	60±2.14
2	75±3.18	60±1.31	48±0.71	51±0.62	50±2.15
3	65±2.10	55±2.31	35±1.31	45±2.10	39±2.31
4	65±2.84	50±1.94	28±1.10	35±0.97	30±0.34
5	65±2.49	50±1.83	25±0.21	29±0.41	25±0.27

Results were expressed as Mean±SD in triplicates

DISCUSSION

Since ancient times, mankind has used plants to treat common diseases and some of these traditional medicines are still included as part of the habitual treatments of various maladies (Rios and Recios, 2005). In general, the antiradical and antioxidant activities of plant extracts are associated to their phenolic contents (Baydar *et al*., 2006). In the present study, methanolic extract of either OB or ML collected from Madina were examined for their antioxidant and antitumor activities. In our study, the OB and ML extracts elicited high total phenolic contents. It was reported from other study that, ML contain the flavonoid, quercetin-3-O-glycoside which possesses antioxidant activity (Akroum *et al*., 2009). The data presented in this study indicated that, the marked

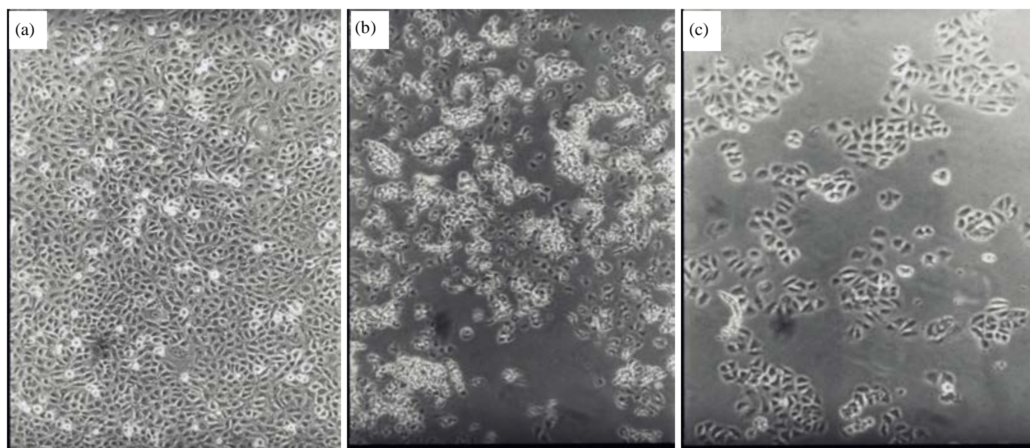

Fig. 3(a-c): Effect of ML or OB methanolic extract on inhibition of MCF-7 cell growth (a) Control untreated cells, (b) MCF-7 treated with 160 μg mL^{-1} ML and (c) MCF-7 treated with 160 μg mL^{-1} OB

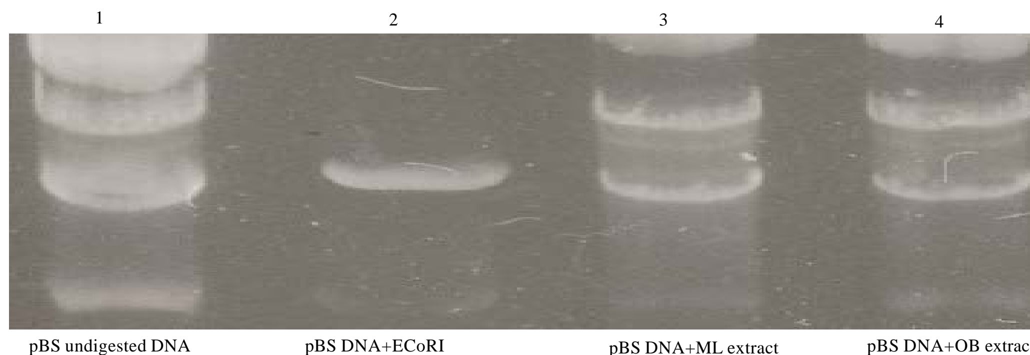

Fig. 4: Effect of either ML or OB methanolic extract on plasmid bluescript (pBS) DNA. A 2% agarose gel electrophoresis was stained with ethidium bromide and the DNA was visualized under illluminescence. Lane 1: 10 μg pBS DNA, Lane 2: pBS DNA+*EcoR*I, Lane 3: pBS DNA+ 10 μL ML extract and Lane 4: 10 μL OB extract

antioxidant activity of OB and ML extracts seemed to be as a result of their reducing power due to the presence of flavonoids (Gordon, 1990).

Plants play a significant role in health promotion through free radical scavenging activity (Sanchez-Moreno, 2002). In DPPH assay, the antioxidant were able to reduce the stable DPPH radical to the yellow-colored DPPH (Frankle and Meyer, 2000). The results obtained from this study indicated that, OB extract yielded high percentage of DPPH scavenging activity compared to the ML extract that may be attributed to the high total phenolic contents of OB extract (Singh *et al*., 2007). Several investigations attributed the medicinal activity of plant extract to the presence of phenolic compounds (Khan *et al*., 2011). The OB and ML extracts will be subjected for further fractionation and these fractions were re-evaluated biologically.

Brine shrimp cytotoxic assay considered as a preliminary screening for the presence of antitumour compounds and used to determine the plant extract toxicity (Meyer *et al*., 1982). Brine shrimp cytotoxic assay is a rapid and inexpensive test used for the evaluation anticancer activity of plant extracts (Jayasuriya *et al*., 1989). Brine shrimp assay utilized previously in various bioassay systems (Meyer *et al*., 1982; Ratnayake *et al*., 1992). Among these applications, organophosphates (Rao *et al*., 2007) mycotoxins (Harwig and Scott, 1971) anaesthetics (Robinson *et al*., 1965) morphine-like compounds (Granada *et al*., 1976) antibacterial and antifungal (Sanchez *et al*., 1993; Chohan *et al*., 2005) and active compounds from marine environments (El-Masry *et al*., 1995). The present study indicated that, the brine shrimp survival is inversely proportional to the concentration of either ML or OB extract used with LD_{50} 191.23 and 235.50 ppm, respectively. Toxic effects on brine shrimps indicated the anticancer potential of ML or OB extract. The present study showed that both ML and OB extract exhibited marked cytotoxic activity against

MCF-7 cells at various concentrations (20, 40, 80, 160 and 320 μg mL^{-1}). The 160 and 320 μg mL^{-1} showed more cytotoxic effect against MCF-7 cells as indicated by the lower cytoviability percentage of MCF-7 cells. These results showed the cyotoxic activity of either ML or OB extract against breast cancer cell line MCF-7 cells with more profound effect in case of OB higher than in ML. These results were accompanied with the protection against DNA damage against pBS DNA. The antioxidant and antimutagenic activity of some ML species was reported (Orhan *et al*., 2012). Also, the antigenotoxic potential of OB derivatives could be attributed to their antioxidative properties (Beric *et al*., 2008). It was reported that, OB contains individual phenolic compounds that influencing its antioxidant capacity (Kwee and Niemeyer, 2011).

CONCLUSION

Based on results achieved, we can concluded that, OB and ML extracts have the potency to act as powerful antioxidants and protect against DNA damage and have cytotoxic activity against MCF-7 cell line.

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