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# Antimutagenic and Anticancer Activity of Al Madinah Alhasawy Mint (*Mentha longifolia*) Leaves Extract

[1]Khalil Al-Ali, [1]Mohamad Abdelrazik, [1]Abdulaziz Alghaithy, [1]Atef Diab,
[1]Hesham El-Beshbishy and [2]Hussam Baghdadi
[1]Department of Medical Laboratory Technology, College of Applied Medical Science,
[2]Departement of Biochemistry and Molecular Medicine, College of Medicine,
Taibah University, Madinah, P.O. Box 30001, Saudi Arabia

**Abstract:** *Mentha* is one of the genera of Lamiaceae family. The aim of the present study was to evaluate the antimutagenic and anticancer activity of the water and methanolic extract of Alhasawy mint (*Mentha longifolia*), that grown in Madina Province, western region, Saudi Arabia using three different bioassays namely; Brine shrimp bioassay, Ames mutagenicity bioassay using 3 Hist-*Salmonella typhimurium* strains of different mutations (TA98, TA97 and TA100) and 2 reference mutagenic drugs nitrosopiperidine (NP) and 2-amino-3-methylimidazo-quinolidine (IQ) and Mammalian cell lines bioassays using 2 different cell lines HepG2 and Vero cell lines. The plant extract showed an efficient antimutagenic activity against the studied bioassays in a directly proportional effect with concentration.

**Key words:** *Mentha longifolia*, antimutagenic activity, ames test, brine shrimp assay, HepG2 and Vero cell lines

## INTRODUCTION

Plants constitute an essential part of the benefit of human society since the beginning of life on the earth as used for nutrition and treatment. Medicinal plants are found in nature for the cure of several ailments of human communnities. In recent years, various studies dealing with the evaluation of plants capacity in healing various diseases was growing increasingly. The World Health Organization (WHO) stated that about three quarters of the world's population rely on traditional remedies (herbal remidies) for their health care (WHO, 1991). Recently, numerous studies have focused on the biological and antimicrobial properties of the herb extracts derived from several medicinal plants such as *Mentha* species. In fact, *Mentha* is one of the Lamiaceae family genera which comprise about 30 species spreading all over the world (Dorman *et al*., 2003).

Medicinal plants in Saudi Arabia constitute about 12% of its floral species while the total number of endogenous species may exceed 2300 species. These might include about 300 species belonging to 72 families which are considered as medicinal plants. The Labiatae, or the mint family (recently named Lamiaceae), include 76 species, of which 22 are considered to be medicinal plants. The genus *Mentha* includes 25-30 species distributed thought the world (Dorman *et al*., 2003). In Saudi Arabia, *Mentha longifolia* which is locally know as Al-Madina hasawy mint, is most common species of *Mentha* and is traditionally and commonly used as herbal tea either alone or mixed with normal tea having a characteristic aroma and flavor (Moreno *et al*., 2002). In addition, *Mentha* spp. has been commonly used in the folk remedy for treatment of various digestive ailments (Iscan *et al*., 2002). Also, several studies have been performed on the antioxidant activity of *Mentha* species in various parts of the world (Yumrutas and Saygideger, 2012).

Wild mint is an aromatic and nectariferous plant and the above-ground plant part possesses a characteristic, aromatic smell. It was commonly used in the pharmaceutical, tobacco and food industry and especially in cosmetology. It has carminative properties, improves digestion and alleviates the inflammation of respiratory organs. Mint was used internally in the form of infusers and externally for baths (Hajlaoui *et al*., 2009). Also, its antiviral activity has been shown, *Mentha longifolia* (L) Hudson (wild mint, English horsemint) belongs to the genus *Mentha*, Lamiaceae family and is considered as one of the medicinal and aromatic herbs (Al-Ali and El-Badry, 2010).

The aim of the present study was to evaluate the antimutagenic and anticancer activity of the water and

**Corresponding Author:** Khalil Al-Ali, Department of Medical Laboratory Technology, College of Applied Medical Sciences, Taibah University, Madinah, P.O. Box 30001, Saudi Arabia

methanolic extract of Alhasawy mint (*Mentha longifolia*) using three different bioassays.

## MATERIALS AND METHODS

**Collection of plant material:** The plant samples of *M. longifolia* (L.) Hudson subsp. *longifolia* were purchased from Almadina local market. The leaves were separated and washed under tap water then dried in the shade, grounded in a grinder with a mesh 2 mm in diameter. Five grams of dried mint leaves were refluxed using 100 mL of distilled water. The filtrate was separated and further filtered using Whatman filter paper.

**Preparation of aqueous extract:** *Mentha longifolia* plant was purchased from the local market. Five grams of minced *Mentha longifolia* was infused in a thermos flask with 100 mL boiled distilled water. The flask was shaked every minute for duration of 10 min to obtain 5% of *Mentha* tea extract.

**Preparation of the methanol extract:** The air-dried leaves were finely ground; 5 g were separately extracted by stirring with 10 mL of pure methanol for 30 min. The extract was then kept for 24 h at 4°C, filtered through a Whatman filter paper, evaporated under vacuum to dryness and stored at 4°C until analyses.

**Brine shrimp bioassay:** Brine shrimp bioassay was performed as described by Xiong *et al*. (2004). In brief, brine shrimp eggs were hatched in a 1:2 mixture of sea water:de-ionized water. After 24 h, the hatched larvae suspension was left to stand for 1 h without aeration and the larvae were collected by pipetting from middle layer of solution in which most of the larvae were swimming. A 0.2 mL of brine shrimp saline solution, containing approximately 20-30 viable larvae, was pipetted with a multi-channel pipette into each well of 96-well tissue culture plate. Increased concentration of the tested plant extract was added. The larvae were incubated for 24 h at 30°C in the dark and dead larvae were there after counted under a stereo microscope (magnification X18). Viable larvae were subsequently killed by adding 10 µL of 37% formalin per well and the total number of dead larvae was counted. Each series of tests included negative controls (brine shrimp medium only) (Harwig and Scott, 1971):

$$\text{Mortality (\%)} = \frac{\text{No. of dead or stopped swimming brine shrimp}}{\text{Total No. of brine shrimps}} \times 100$$

**Ames mutagencity test:** In our current study the ames test (Maron and Ames, 1983) was achieved to evaluate the antimutagenic activity of the *Mentha longifolia* leaves water extract against some mutagenic compounds, particularly nitrosopiperidine (NP) and 2-amino-3-methylimidazo-quinolidine (IQ) with the aid of three different histidine negative strains of *Salmonella typhimurium* with three different mutation patterns of TA98, TA97 and TA100.

**Viability test:** Fresh bacterial culture suspension (0.1 mL) was added to 10 mL of steam sterilized nutrient liquid medium, thoroughly mixed by vortexing, then a 100 mL aliquot was poured to 10 mL of sterile nutrient medium. The 100 mL of the mixture was poured on to nutrient agar medium, containing 100 mL of L-histidine (0.1 M) and biotin (0.5 mM). The mixture was thoroughly mixed by vortexing, poured onto a minimal glucose agar medium and incubated for 24 h at 37°C. The grown colonies were counted with the determination of bacterial viability (the number of bacteria per mL of original culture). Bacterial spore suspension was adjusted to $3\times10^5$ bacterial cells per milliliter of fresh bacterial culture (Maron and Ames, 1983). Finally, the spontaneous reversion rate (number of colonies reverting from His-to His+ was recorded.

**Plate incorporation test:** Two milliliter of autoclaved nutrient agar medium 0.6% (w/v) was added to oven sterilized screw capped flasks containing 0.5% (w/v) NaCl containing 200 mL of low histidine/biotin (0.5 mM). histidine and 0.5 mM biotin), then was maintained at 45°C. Hundred milliliter of fresh bacterial suspension, 100 mL of the mutagen, 100 mL of *Mentha longifolia* leaf extract and 500 µL of S9 activation system were added. Then, mixed thoroughly with vortexing and finally poured onto oven sterilized plates contain nutrient agar medium. Plates were incubated at 37°C for 48 h. The grown (reverting) colonies were enumerated.

**Tissue culture (mammalian cell lines) bioassays:** Two different cell lines, obtained from the American type Culture Collection and the European Collection of Animal Cell Cultures, including one cancer cells namely human liver cancer (HepG2) and one normal (non tumor forming) cells namely Buffalo green monkey kidney (Vero), were used.

**Maintenance of cell lines:** Stock cell line flasks were examined for confluencey and viability using inverted microscope. The trypsin solution 0.25% was used as a proteolytic enzyme for the dissociation of cell lines confluent sheets from the surfaces of cell culture flask with varying times according to every cell type. Cells were

cultured onto cell culture broth medium enriched with 10% fetal calf serum. Various cells were enumerated with the aid of haemocytometer, then were adjusted to concentration of $3\times10^5$ mL$^{-1}$. In sterilized conditions, the MEM Earl's diluting fluid medium with 2% calf serum was prepared then tubes were maintained in ice. About 0.2 mL of $3\times10^5$ mL$^{-1}$ of the prepared cell lines (HepG2 and Vero) were cultured individually in 96 well cell culture plates. Kept for growing at 37°C for 24 h in 5% $CO_2$ incubator. Then *Mentha longifolia* leaves water and methanol extracts with increasing concentrations (50, 100, 200, 300, 400 and 500 μg mL$^{-1}$) and the reference anticancer drug vincristine sulphate at conc. of 0.25, 0.5, 0.75, 1.0, 2.0 and 5.0 μg mL$^{-1}$ were then added in triplicates to the microtitre plates. The 10% DMSO was tested for anticancer effect and phosphate buffered saline solution was used as a control. All pates were incubated in 5% humidified $CO_2$ for 48 h. Then, 1 mL of trypsin-EDTA solution was added. The relative residual living cells count was determined relatively to the optical density of residual stained cells using ELISA reader. Absorbance at 570 nm of the maximum was detected by a Power Wave X Microplate ELISA Reader. The absorbance for DMSO treated cells was considered as 100%.

## RESULTS

**Brine shrimp bioassay:** The brine shrimp bioassay results clearly indicated the cytotoxic effect of both water and methanol extract to brine shrimp larvae (Table 1). Also, the results confirmed that the brine shrimp survival reversely proportional to the tested concentrations of *Mentha longifolia* either water or methanol extract, thus indicating its anticancer potential.

**Ames mutagencity test:** *Mentha longifolia* aqueous extract, prepared in a natural manner exactly as traditionally consumed. *Mentha longifolia* aqueous extract, prepared in a concentration of 5% (w/v) and was examined for mutagenicity and antimutagenicity using the ames test with three different bacterial strains (TA 97, TA 98 and TA 100). All strains tested did not reveal any mutagenic activity for *Mentha longifolia* aqueous extract upon adding to bacterial cultures, confirming the absence of any mutagenic effect by its own.

However, upon using a combination of the *Mentha longifolia* extract with the known and reported tested chemical mutagens of various chemical classes, such as nitrosopiperidine (NP) and 2-amino-3-methylimidazo-quinolidine (IQ) caused a remarkable and a concentration directly proportional antimutagenic activities against the 2 tested chemical mutagens in the presence of 10% (w/v) hepatic S9 preparation from Aroclor 1254-induced rates, as an activation system (Table 2).

**Tissue culture (mammalian cell lines) bioassays:** The viable cell counts obtained following exposure of cancer and normal cell lines to different concentration of water and methanol extracts of *Mentha longifolia* are given in Table 3. The effect of water extract on HepG2 cancer cells and Vero non-tumor forming cells showed that concentration above 50 μg L$^{-1}$ of both water and methanol *Mentha longifolia* extracts resulted in a minimal reduction in cancer cell number with a minimal exponential decline in cancer cell count and viability in a directly proportional dose manner. On the other hand, almost there was no marked effect on count and viability of vero normal cell lines. Of the two extracts the methanol extract showed slightly higher cytotoxic effect on cancer cells than was demonstrated by the water extract. However, both water and methanol extracts of *Mentha longifolia* showed no cytotoxic effect on normal cell lines even at the highest tested concentration.

The anticancer drug (vincristine sulphate) used as a positive control in our study: The viable count of the three cell types after 48 h drug treatment showed a more pronounced cytotoxic effect on both cancer and normal studied cell lines. However, these cells were not killed completely as a few numbers of cells were still obtained even at the higher concentration tested.

Table 1: Effect of *Mentha longifolia* leaves water extract on the viability of brine shrimp (Larvae *Artemia salina* )

| *Mentha* extract conc. (μg mL$^{-1}$) | Shrimps larvae mortality (%) | |
|---|---|---|
| | Water extract | Methanol extract |
| Control | 0 | 0 |
| 100 μL/well | 21 | 34 |
| 200 μL/well | 41 | 53 |
| 300 μL/well | 63 | 76 |
| 400 μL/well | 87 | 91 |
| 500 μL/well | 100 | 100 |

Table 2: Histidine reverting colonies of *Salmonella typhimurium* strains treated with the mutagen NP and IQ and gradually increased concentrations of *Mentha longifolia* leaves extract

| *Mentha* extract conc./plate | Histidine-revertent *Salmonella* colonies per plate | | |
|---|---|---|---|
| | TA97 | TA98 | TA100 |
| Mutagen 1 (NP) | 480 | 463 | 371 |
| Mutagen 1+100 μL/plate | 356 | 379 | 269 |
| Mutagen 1+200 μL/plate | 293 | 281 | 199 |
| Mutagen 1+300 μL/plate | 213 | 211 | 136 |
| Mutagen 1+400 μL/plate | 179 | 173 | 113 |
| Mutagen 1+500 μL/plate | 73 | 91 | 83 |
| Mutagen 2 (IQ) | 351 | 317 | 321 |
| Mutagen 2+100 μL/plate | 311 | 275 | 270 |
| Mutagen 2+200 μL/plate | 219 | 213 | 193 |
| Mutagen 2+300 μL/plate | 177 | 281 | 133 |
| Mutagen 2+400 μL/plate | 103 | 103 | 96 |
| Mutagen 2+500 μL/plate | 56 | 63 | 41 |

Table 3: Viable cell counts of HepG2 and Vero cell lines after 48 h exposure to different concentrations of water and methanol extract of *Mentha longifolia* leaves compared with the standard anticancer drug vincristine

| | Effect of *Mentha* water and methanol extract | | | | | | |
|---|---|---|---|---|---|---|---|
| | HepG2 | | Vero | | Effect of anticancer drug | | |
| *Mentha* extract conc. (μg mL$^{-1}$) | Water extract | Methanol extract | Water extract | Methanol extract | Drug conc. (μg mL$^{-1}$) | HepG2 | Vero |
| Cells at 0 | 3.0×10$^5$ | 3.0×10$^5$ | 3×10$^5$ | 3.0×10$^5$ | | 3.00×10$^5$ | 3.00×10$^5$ |
| 50 | 3.0×10$^5$ | 3.0×10$^5$ | 3×10$^5$ | 3.0×10$^5$ | 0.25 | 2.30×10$^5$ | 1.30×10$^5$ |
| 100 | 2.9×10$^5$ | 2.9×10$^5$ | 3×10$^5$ | 3.0×10$^5$ | 0.50 | 1.19×10$^5$ | 1.10×10$^5$ |
| 200 | 2.9×10$^5$ | 2.8×10$^5$ | 3×10$^5$ | 3.0×10$^5$ | 0.75 | 1.11×10$^5$ | 0.93×10$^5$ |
| 300 | 2.8×10$^5$ | 2.7×10$^5$ | 3×10$^5$ | 2.9×10$^5$ | 1.0 | 0.69×10$^5$ | 0.71×10$^5$ |
| 400 | 2.7×10$^5$ | 2.7×10$^5$ | 3×10$^5$ | 2.9×10$^5$ | 2.0 | 0.43×10$^5$ | 0.52×10$^5$ |
| 500 | 2.7×10$^5$ | 2.6×10$^5$ | 3×10$^5$ | 2.8×10$^5$ | 5.0 | 0.31×10$^5$ | 0.43×10$^5$ |

## DISCUSSION

**Use of brine shrimp:** Cytotoxic assay has been documented in various bioassay systems (Meyer *et al*., 1982; Ratnayake *et al*., 2009) as it is a quick, easy and reliable test. It was commonly used for the anticancer activity evaluation of plant extracts. It was successfully used for the preliminary screening and the detection of antitumor active ingredients (Meyer *et al*., 1982). It was documented, testing the carcinogens inhibitory effects on the larvae hatching (Harwig and Scott, 1971). There was a confirmed correlation between the toxicity of brine shrimp larvae and the Hanuman nasopharyngeal carcinoma. Also, reliability and efficiency of the assay has been confirmed as a prescreen of a six human solid tumor cell lines at the cell cultures Lab of Purdue cancer center. More than 300 novel antitumor natural products have been detected with the aid of brine shrimp bioassay (Meyer *et al*., 1982; Ratnayake *et al*., 2009). Our current study confirmed that both water and methanol extract of *Mentha longifolia* leaves exhibited marked cytotoxic activity against brine shrimps larvae at various concentrations. The toxicity on brine shrimps larvae was evident in a directly proportional manner with the concentration indicating the anticancer potential of both water and methanol extract of *Mentha longifolia* leaves.

Ames mutagencity test results showed that *Mentha longifolia* extract can counteract the process of mutagenesis and provide protection against genotoxicity. This protective effect can be explained biochemically. The antimutagenic effect of *Mentha longifolia* extract might be acting via its effect on the enzyme activities modulation which play a definite mediating role in the process of metabolic activation for the acting chemical carcinogens (Katiyar *et al*., 1993). This biochemical modulation mechanism might be responsible for the *Mentha longifolia* extract antimutagenic and anticarcinogenic activities. Also, this effect might be achieved via altering the promutagens and procarcinogens metabolic pathways which efficiently affect their detoxication and excretion effects (Qari, 2008). It is strongly believed that the cytochrome P450 enzymes are the major biotransforming systems involved in the metabolism of more than 90% of known chemical carcinogens (Rendic and Di Carlo, 1997). Accordingly, *Mentha longifolia* extract effect might be achieved via its active effect on the P450 enzymatic activity modification (Christou *et al*., 1995; Ioannides, 1999).

*Mentha longifolia* extract has shown the ability to counteract the IQ mutagenic effect. It was reported that IQ metabolic activation effects are mediated by CYP1A2 via the N-oxidation reaction (Ioannides and Parke, 1993) Thus, *Mentha longifolia* effect might be interpreted as it works via the interference with IQ metabolic activation mediated N-oxidation reaction. Similarly, the mutagenic bioactivities effect of NP was reported to work via a pathway mediated by CYP1A1 (Ioannides and Parke, 1993). This was effectively inhibited by *Mentha longifolia* extract in a dose dependent manner.

The current study strongly suggests that *in vitro* antimutagenic activity of the Saudi Arabian *Mentha longifolia* leaf extract could be extended to be active in protecting against many food and environmentally borne mutagens and carcinogens when used as an herbal drink. Also, it might have efficient protective effect against the mutagenesis of several dietary-borne mutagens and environmental genotoxicants. This protective effect apparently might be achieved via its ability to interfere with the metabolic activation of these mutagens and carcinogens thus prohibiting its effect. Also, medical evidence of tobacco smoke has been accumulating for 200 years. Tobacco smoke which is very common in our society can cause cancers of the lung, bladder, head and neck (Alexandrov *et al*., 1992). *Mentha longifolia* might be useful as a protective herbal drink against human cancer incidence among smokers as a sort of chemical carcinogen.

Humans are permanently or often subjected to environmental carcinogens through their lifestyle and their various kinds of canned or manufactured foods. It is reported that human cancer could be initiated if not established via the uptake of many of the entrusted

manufactured food items which play a vital role in the initiation of human cancer. Also, the way of food cooking might be responsible for the production and the release of different complexes of mutagenic substances which are greatly generated from foods containing higher protein percentages (Atiqur Rahman *et al*., 2004).

The human hepatoma cell line HepG2 was used to differentiate among the cytotoxicities of *Mentha longifolia* extract and the anticancer drug (vincristine sulphate). The HepG2 are characterized by maintaining an elevated level of indigenous enzymatic metabolizing capacity (Babich and Borenfreund, 1991). Vincristine sulphate was obviously more cytotoxic to the HepG2 cells than were the *Mentha longifolia* extract, (Table 2). This differential level of cytotoxicity was similar to that noted with the green monkey Vero cell line as the toxicity bioindicator. Our results showed that the vincristine sulphate compound appeared to be more toxic than *Mentha longifolia* extract. The reference anticancer drug used in the present study for comparison was highly purified as compared to the crude extract of *Mentha longifolia* plant leaves extract. Thus, the present study suggest that both the pronounced effects of the plant water extract against brine shrimp larvae and the anti mutagenic effect on *Salmonella* (ames test) strongly confirm its antimutagenic activity. The anticancer drug vincristine sulphate showed a remarkable cytotoxic and killing effect for both cancer and normal mammalian cells.

While, the anticancer drug vincristine sulphate showed a remarkable cytotoxic and killing effect for both cancer and normal mammalian cells compared to minimal direct killing activity of *Mentha longifolia* against cancer cells which confirm its anticarcinogenic effect which should be considered for further investigation. Besides, the confirmed absence of carcinogenic or cytotoxic nature against normal cells as reported in our previous study (Al-Ali *et al*., 2013a, b). Accordingly, this study confirms that both the aqueous and methanol extracts of *Mentha longofolia* is not a carcinogenic in nature.

## CONCLUSION

*Mentha longifolia* extract possesses pronounced and efficient antimutagenic characteristics which can counteract the mutagenic and carcinogenic effects of various chemicals used in the present study. This protective effect might be via its reactive inhibition of the metabolic activation of chemical carcinogens. Further studies are still needed for the complete chemical characterization and determination of the most active components of *Mentha longifolia* leaves, acting as active antimutagenic and anticarcinogenic.

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