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EVALUATION OF ANTIMICROBIAL, ANTIOXIDANT AND ANTICANCER ACTIVITIES OF *MENTHA LONGIFOLIA* EXTRACT GROWN IN SOUTHERN SAUDI ARABIA

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Abstract

Mentha longifolia of the family Lamiaceae is used in traditional herbal medicines. In this study, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) method was used to evaluate cytotoxic activity of *M. longifolia* extract prepared using methanol, hexane and acetone solvents on human liver cancer (HepG2) and human breast cancer (MCF 7) cell lines. In addition, Hoechst 33342 and propidium iodide (PI) stains were used, which have the ability to stain nuclei of membranes of both dead and living cells. As a result, cells were stained with PI, suggesting that some of cells had undergone necrosis. 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity technique was used to evaluate antioxidant properties of *M. longifolia* extract and it was observed that antioxidant properties increased with concentration. Maximum antioxidant effect (33%) was observed at the highest concentration (800 µg/ml). The antimicrobial properties of this plant were tested on 5 different microorganisms (*Candida albicans*, *Klebsiella pneumoniae*, *Escherichia coli*, *Bacillus megaterium* and *Staphylococcus aureus*) using well method. As a result of antimicrobial activity, it was found that *M. longifolia* extract prepared with methanol at a concentration of 400 mg/ml exhibited the highest antibacterial activity against *K. pneumoniae* with a zone diameter of 17±1.25 mm, while methanol extract with a concentration of 50 mg/ml showed the lowest activity against *B. megaterium* with a zone diameter of 7±1 mm. In conclusion, it is suggested that *M. longifolia* extract has a strong potential in terms of cytotoxic, antioxidant and antibacterial activity and may be a precursor for *In vivo* and *In vitro* experiments.

Key words: Cytotoxic activity, Antimicrobial activity, Antioxidant activity, *Mentha longifolia*, MCF 7 cell line, HepG2 cell line.

Abbreviations and notation: *M. longifolia: Mentha longifolia*, EO: Essential oil, DMSO: Dimethyl sulfoxide, PBS: Phosphate Buffered Saline, PI: Propidium Iodide, RPMI: Roswell Park Memorial Institute, MTT: (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide), DOXO: Doxorubicin, ELISA: Enzyme-Linked Immuno Sorbent Assay, SDA: Sabouraud dextrose agar, MIC: Determination of Minimum Inhibitory Concentration, MBC: Determination of Minimum Bactericidal Concentration, DPPH: 2,2'-Diphenyl-1-picrylhydrazyl.

Introduction

Throughout human history, plants have been vital to human culture. According to folklore, plants were a major source of food and spices as well as had a significant impact on health and medicine in ancient societies. Humans have discovered health benefits of plants and have used them therapeutically over thousands of years. In developing countries, traditional medicines are still widely used at a rate of 70-95% (Fridlender *et al.*, 2015; Niazi & Monib, 2024). Folk traditional medicines are home to many herbs that are known for their health benefits around world. It is known that about a quarter of modern medicines (about 25-28%) are derived from plants. This proportion underscores the significant contribution of plants to the field of medicine. These contributions are often realized through phytochemicals derived from plant materials. This approach, involves extracting plant materials to isolate for their active components. The enormous medicinal potential of plants is due to the chemical components they contain. These components in the structure of plants can have various effects on health (Dar *et al.*, 2023; Lichota & Gwozdzinski, 2018). *Mentha longifolia*, one of the extensively utilized medicinal plants, employed in traditional medicine for treating a range of ailments. *M. longifolia*, also known wild mint, is commonly found in Mediterranean regions. This perennial herb belongs to the Lamiaceae family and has a distinctive mint odour. It has straight to creeping stems and has a creeping root, typically 40 to 120 cm high. Leaves oblong-elliptic or lacerate and are finely to densely hairy. Upper part of the leaves is often green or grayish green, while the lower part is whiter. This herb is typically recommended for treating a wide range of health problems, including bronchitis, cough, nausea, headaches, asthma, digestive disorders, liver diseases and stomach upsets. Essential oil (EO) of *M. longifolia* is a significant component known to have various biological activities. With its anticancer qualities, this EO has capability to inhibit the proliferation of cancer cells. In addition it also has antimicrobial properties, it can also fight free radicals that damage cells by reducing cellular oxidative stress (Bai *et al.*, 2020; Mikaili *et al.*, 2013).

Among the biggest causes of death worldwide is cancer. Cancer cases are projected to rise by 47% cent in 2040 compared to 2020, reaching 28.4 million new cases. This increase shows that cancer is a major global health problem (Jain *et al.*, 2011; Sung *et al.*, 2021). In recent years, many medicinal plants have been used in the treatment of cancer. Plants are now widely used by people in many places due to their strong chemo-protective and anticarcinogenic properties. Moreover, the fact that 80% of the drugs produced worldwide are derived from plants clearly shows that plants can be used in treatments (Dalkılıç *et al.*, 2022; Fitzgerald *et al.*, 2020). Natural therapies based on plants are

attracting increasing interest in cancer treatment. This is because some plants are thought to have potential effects against cancer cells and may have fewer side effects than synthetic drugs (Regassa *et al.*, 2022).

M. longifolia is traditionally consumed for the treatment of various diseases. *M. longifolia* has various pharmacological activities, antimutagenic, high antioxidant activity due to polyphenols, chemoprotective and antimicrobial properties (Elansary *et al.*, 2020; Farzaei *et al.*, 2017). Due to these properties, this plant is of great importance especially in anticancer studies.

In this study, the cytotoxic, antimicrobial and antioxidant properties of *M. longifolia* extracts in different solvents were investigated. For this, the possible anticancer effects on human breast cancer cells (MCF 7) and human liver cancer cells (HepG2) as well as the effect on necrosis or apoptosis processes in these cells were investigated. In order to evaluate the antioxidant properties of this plant and its antibacterial effect on various microorganisms, it was aimed to examine the minimum bactericidal concentration (MBC) and minimum inhibitory concentration (MIC).

Materials and Methods

Obtaining of material and extract preparation: In May 2021, *M. longifolia* leaves were collected from Abha city, located in the southern region of Saudi Arabia. It is ground into powder in a porcelain mortar. 1 gram of each powder was taken and measured on a precision scale, and 10 ml of methanol, hexane and acetone were added respectively. Three separate extracts were obtained. These three extracts were processed in a shaking oven (Nücleon NCI55). It was left for 72-96 hours of incubation. It was then filtered through Whatman No.1 filter paper and the solvents were evaporated by passing it through a rotary evaporator at 40-60˚C for 4-6 hours (Buchi R100). The resulting extract was calculated as % yield. The remaining extracts were scraped, and 10 ml of dimethyl sulfoxide (DMSO) was added to each of them, thus the main stock was prepared. Since polyphenols are extracted from plant tissues with great efficiency using polar solvents, polar solvents including acetone, methanol, and hexane were selected during the extraction process (Fig. 1.).

$$\text{Percent efficiency (w/w)} = \frac{\text{(Weight of dried extract)}}{\text{(Weight of dry seed material before extraction)}} \times 100$$

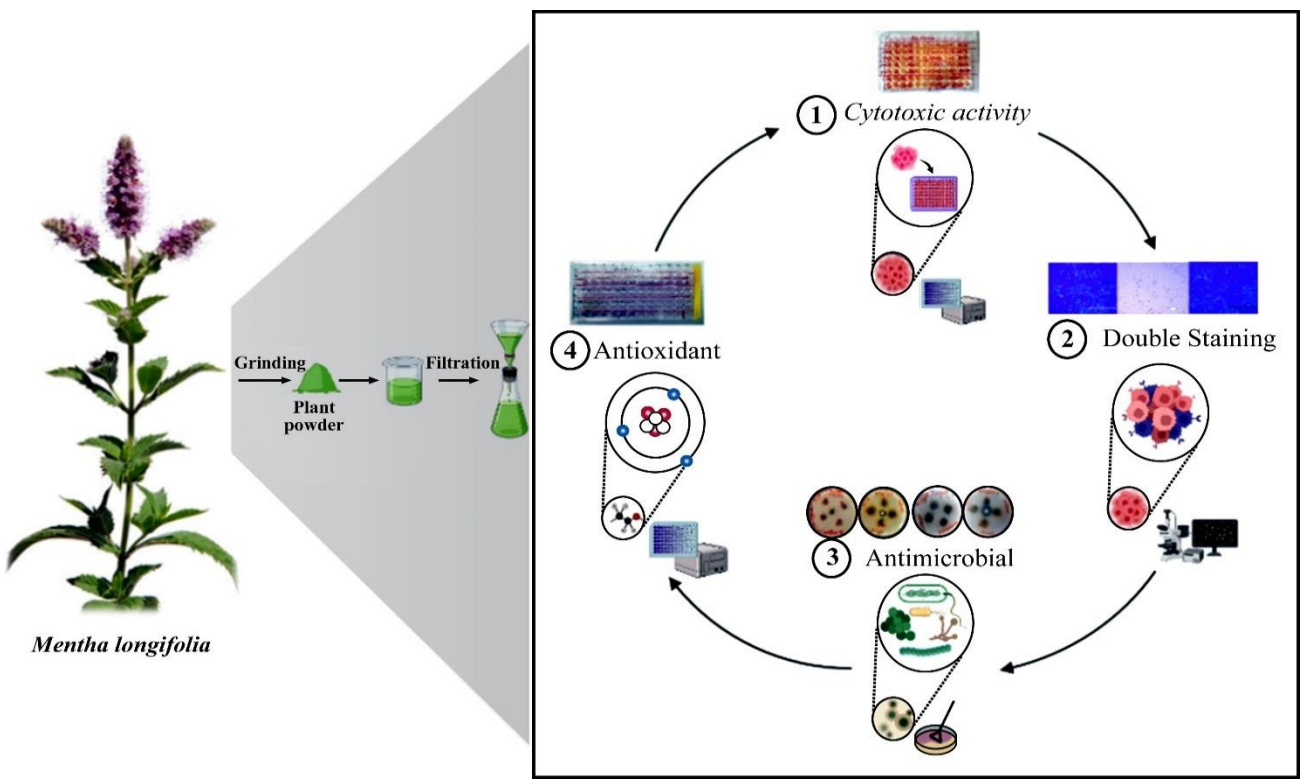

Fig. 1. Experimental flow diagram of this study.

Determination of cytotoxic activity: MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] involves the conversion of a water-soluble yellow dye to an insoluble purple formazan by the action of mitochondrial reductase. The substrate is converted into the chromogenic product by living cells (Kumar *et al*., 2018). HepG2 and MCF 7 cell lines were cultured in 75 cm^2 flasks containing RPMI (1% penicillin streptomycin, 10% fetal bovine serum, 25 mM L-glutamine) at 37˚C and 5% CO_2 atmosphere. In 75 cm^2 flasks, 90% confluent cells were plated in 96-well plates at a density of 5×10^3 cells per well. Plates were then incubated overnight. After incubation, the medium was withdrawn, and the positive control was treated with varying doses of *M. longifolia* extract (100-200-400-800 µg/ml) and 2.5 µg/ml doxorubicin (DOXO). The first row of 96-well wells contained only RPMI (blank), the second row contained negative control (RPMI+cell line) and the third row contained positive control (RPMI+cell line+ DOXO). Starting from the fourth row, the activity of *M. longifolia* extract in different solvents and concentrations was

examined in 6 replicates. After the first 72 hours of incubation, 10 µl of MTT solution (5 mg/ml) was added to each well and cells were incubated at 37°C for 4 hours without light. After this incubation period, the plate was shaken to dissolve the MTT and an ELISA (Enzyme-Linked ImmunoSorbent Assay) was used to measure absorbance using a microplate at a wavelength of 517 nm. Cytotoxicity was calculated in this way (Dalkılıç *et al.,* 2022).

$$\text{Cytotoxic activity (\%)} = \frac{\text{(Sample Absorbance)}}{\text{(Control Absorbance)}} \text{ x } 100$$

Determination of apoptotic/necrotic activitiy: This stage; Apoptotic and necrotic activity was tested using the double staining (Hoechst 33342 and propidium iodide) technique, these fluorescent dyes bind to DNA, resulting in the visualization of chromatin and therefore the cell nucleus. Hoechst 33342 (MedChemExpress, 23491-45-3, NJ. USA) is a dye that binds to DNA and can also cross cell membranes. Since propidium iodide (PI- MedChemExpress, 25535-16-4, NJ. USA) dye is absorbed only by cells with reduced membrane integration, it is possible to identify late apoptotic/necrotic cells. Compared to normal cells, apoptotic cells are characterized by a smaller nucleus (pyknotic) and/or fragmentation, while necrotic cells have a slightly larger nucleus and show less staining (Baran *et al.,* 2017; Dalkılıç *et al.,* 2023). Hoechst stain is blue, PI stain is red. To apply this double staining method, firstly, 2 ml each of HepG2 and MCF 7 cells were added to 6-well plates, with $10x10^3$ cells in each well. The plates were then incubated in an oven containing 5% CO^2 at 37°C for 24 hours. After the incubation, the medium in the wells was emptied and 2 ml of the medium containing the methanol extract of *M. longifolia* at the maximum dose (800 µg/ml) was added and incubated again for 48 hours. Then, 1 ml of the solution prepared with 7.5 µg/ml Hoechst and 1 µg/ml PI dyes (the dye solution was prepared in 1x PBS (Ca/Mg-free)) was added to the wells. The plate was then incubated under the same conditions and the cell morphologies were compared with the control groups (negative control: untreated cells, positive control: cells treated with 2.5 µg/ml DOXO) and evaluated under the Fluorescence Inverted Microscope (Evos FLc) (Dalkılıç *et al.,* 2023).

Determination of antimicrobial activity: Four bacterial and one fungal strains were used as test microorganisms. As gram negative; *Esherichia coli* ATCC 25322 and *Klebsiella pneumoniae* ATCC 700603 are gram positive; *Bacillus megaterium* ATCC DSM32, *Staphylococcus aureus* ATCC 25923, and *Candida albicans* FMC17 were used as the fungal strain. All microorganisms were clinical isolates obtained from the central laboratory of Fethi Sekin City Hospital.

Nutrient broth and Sabouraud dextrose agar (SDA) were used for growth of microorganism suspensions. Bacterial strains were grown in the medium at 37±0.1°C for 24 hours and fresh cultures were diluted to a McFarland density of 10^8 cfu/ml. *C. albicans* were inoculated aseptically into petri dishes containing autoclaved, cooled and settled SDA medium. Petri dishes were incubated at 31°C for 48 hours. These were aseptically sub cultured and yeast colonies were then sterilized, suspended in 0.9% sodium chloride solution (normal saline) and compared with McFarland's solution. Incubated at 45°C to reach a concentration of $2x10^7$ cells/ml (Al-Bayati 2009). Mueller-Hinton agar (Merck Lot: VM779137) and Nutrient Broth (Biolife Lot: HE2602) were used for bacterial growth.100 µl of nutrient agar microbial cell suspension was taken and was inoculated into 20 ml of medium, and after spreading with Drigalski, wells were opened with a cork borer. 100 µl of *M. longifolia* produced in four concentrations (25, 50, 75 and 100 mg/ml) was added to each well and kept in the incubator at 37±0.1°C for 24 hours. *C. albicans*; Incubated at 31°C for 48 hours. The inhibition zones formed on the agar surface at the end of the incubation periods were measured and evaluated in mm (millimeters). Clindamycin (2 mcg -Lot 171127A) antibiotic discs were used as positive control, and 100% DMSO (Merc-Lot K51154243 948) was used as negative control (Aytar *et al.,* 2019; Nalawade *et al.,* 2016).

Determination of Minimum Inhibitory Concentration (MIC): MIC of an antimicrobial agent, measured in µg/ml, is the concentration at which bacteria cannot visibly grow. Add 100 µl of nutrient broth to all wells of a sterile 96-well plate and then 100 µl of *M. longifolia* extract (100 mg/ml concentration) was added to the first well and mixed, followed by serial dilution. Then, bacteria were incubated at 37°C for 24 hours, and fungi were incubated at 31°C for 48 hours (Gummuluri *et al.,* 2019).

Determination of minimum bactericidal concentration (MBC): MBC is defined as the minimum concentration of medium that inhibits bacterial growth in a culture. To perform the MBC assay, 2 µl of Nutrient Broth and bacteria mixture from each MIC plate well was used. The mixture was then placed on Mueller-Hinton agar plates and left in the incubator at 37°C for one day. The minimum concentration without detectable bacterial growth was determined using MBC data (Gummuluri *et al.,* 2019).

Determination of antioxidant activity: Radical Scavenging Test 2,2'-Diphenyl-1-picrylhydrazyl (DPPH) free radical test was performed to measure free radical scavenging activity (Hussain AI *et al.,* 2011). Briefly, concentrations of *M. longifolia* extract (100-800µg/ml) were mixed with 0.25 mM DPPH radical solution (0.5 ml). After the mixture was shaken well and incubated in the dark for 30 minutes, the absorbance at 540 nm was calculated. The following formula was used to estimate the DPPH radical scavenging capacity. The scavenging (%) was calculated by the following formula:

$$\text{Antioxidant activity (\%)} = \frac{\text{Control Absorbance-Sample Absorbance}}{\text{Control Absorbance}} \text{ x } 100$$

Statistical analysis: All experiments were performed in 6 replicates and data are presented as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was used to statistically assess the data from the test extracts, and least significant difference (LSD) test was used to evaluate the results at a significance level of $p<0.005$. These statistical activities were carried out using the SPSS statistical software for Windows (Version 22, SPSS Inc., Chicago, IL, USA).

Results

Extract efficiency: Percent (%) yield of the extracts were calculated and found to be between 80-91% (Table 1) (Ateşşahin *et al.*, 2023).

Cytotoxic activity: Results of the most significant cytotoxic activity of *M. longifolia* against HepG2 and MCF 7 cell lines are shown in Fig. 2 and Fig. 3. The extract made in methanol showed maximum cytotoxic activity against HepG2 cells with 26% at the lowest concentration (100 µg/ml). In comparison, the hexane extract showed the lowest cytotoxic activity at a concentration of 200 µg/ml with 54% viable cell number. The extract in acetone showed the highest cytotoxic effect on MCF 7 cells with up to 54% reduction in viable cells at a concentration of 200 µg/ml.

Half maximum inhibitory concentration (IC_{50}) values were calculated for *M. longifolia* (Table 2). Looking at the calculated values, it was determined that the acetone extract was 199.9 µg/ml in HepG2 cell line, and the methanol extract was 224.4 µg/ml in MCF 7 cell line. These values indicate which extract has the lowest effective concentrations in HepG2 and MCF 7.

Double staining: In HepG2 and MCF 7 cell lines, Hoechst stains cells in the negative (-) control group without any treatment. There are more cells in the negative control group than before, indicating that the membrane integrity of healthy cells is preserved. When the DOXO-treated positive (+) control group is examined, it can be seen that the number of cells is reduced, and the membrane integrity of more cell membranes is damaged when stained with PI. This shows that DOXO causes necrosis that destroys cells. Although the positive control group is more necrotic, MCF 7 cells have pyknosis, but HepG2 cells have disorganized nuclei. The MCF 7 cell line exhibited cells with dispersed nuclei and ruptured membranes. As a result, cells may be secondary necrotic or early apoptotic (Fig. 4).

Table 1. Data to determine the percentage of efficiency.

Extracts	Dry leaf material before extraction (gr)	Extract after extraction (gr)	Yield efficiency percentage (%)
Hexane	1	0.86	86
Methanol	1	0.8	80
Acetone	1	0.91	91

* No reduction in the extracts % efficiency

Table 2. Cytotoxic activity's half inhibition (IC_{50}) value on cells.

	Methanol (µg/ml)	Hexane (µg/ml)	Acetone (µg/ml)
HepG2 Cell line	224.4	389.6	199.9
MCF 7 Cell line	224.4	945.3	368.5

*Hoechst (+)/PI (-) cells: Absence of pyknotic and/or fragmented nuclei: Live.
Hoechst (+)/PI (-) cells: In the presence of pyknotic and/or fragmented nuclei: Early apoptotic.
Hoechst (+)/PI (+) cells: In the presence of pyknotic and/or fragmented nuclei: Late apoptotic or secondary necrotic.
Hoechst (+)/PI (+) cells: In the absence of pyknotic and/or fragmented nuclei: Necrotic (Dalkılıç *et al.*, 2022).

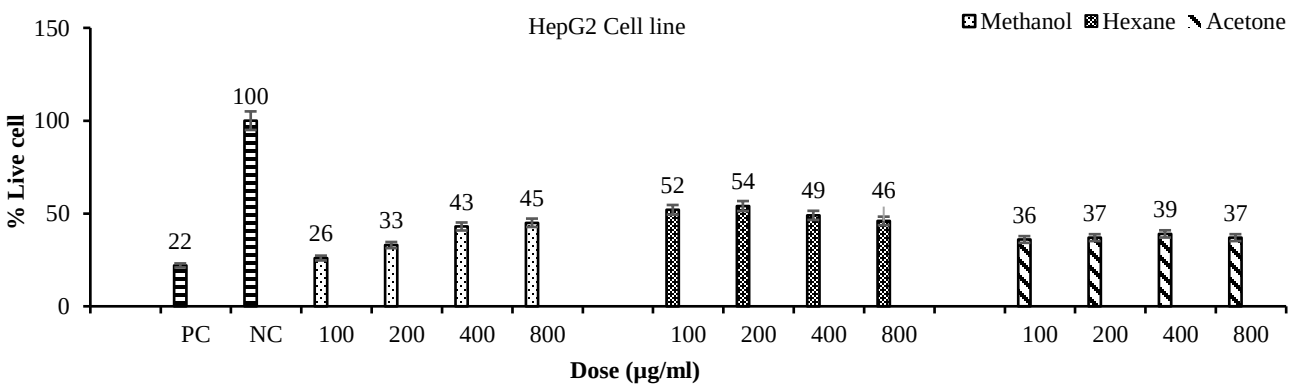

Fig. 2. The MTT test result of *M. longifolia* extracts on the HepG2 cell is shown as % viable cells.
[*PC = Positive control (DOXO 2.5 µg/ml); *NC = Negative control (untreated cells); *Cell lines: HepG2 (human hepatocellular carcinoma); *Extractions: hexane, methanol and aceton; *Concentration: 100-200-400-800 µg/ml].

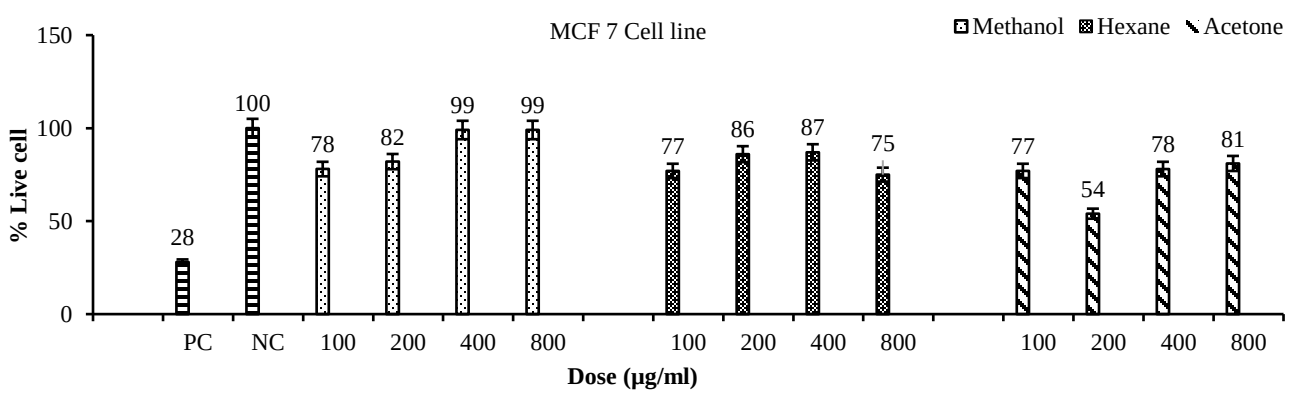

Fig. 3. The MTT test result of *M. longifolia* extracts on the MCF 7 is shown as % viable cells.
[*PC = Positive control (DOXO 2.5 µg/ml); *NC = Negative control (untreated cells); *Cell lines: MCF 7 (human breast cancer); *Extractions: hexane, methanol and aceton; *Concentration: 100-200-400-800 µg/ml]

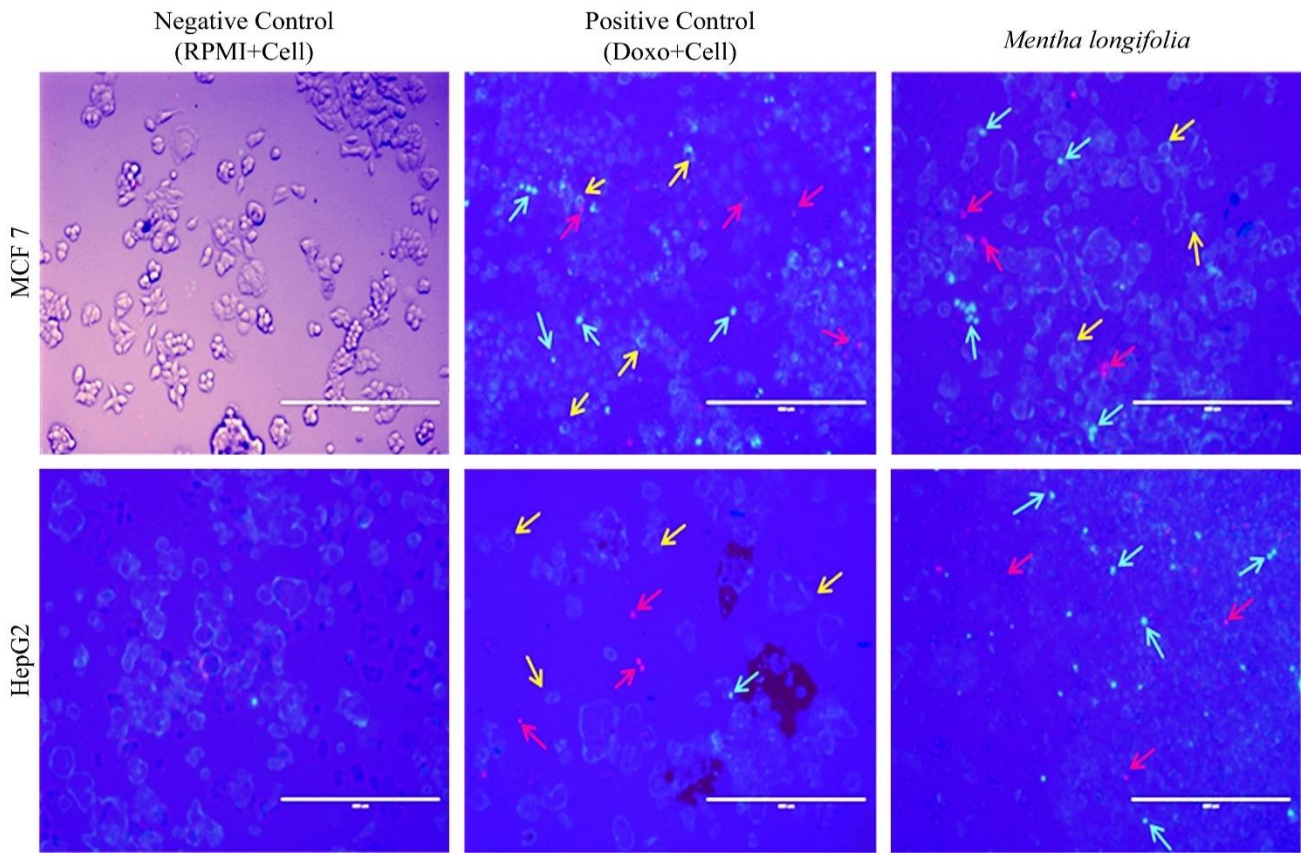

Fig. 2. After 48 hours of treatment, pictures of *M. longifolia* methanol extracted at a dosage of 800 µg/ml were obtained from the HepG 2 and MCF 7 cell lines. [*A Fluorescence Inverted Microscope with a 20x magnification was used to capture the images. To see cell death, cells were labeled with PI (red) and Hoechst (blue). Necrosis is indicated by red arrows, chromatin condensation by green arrows, and apoptosis by yellow arrows. (negative control: non-treated cells; positive control = DOXO 2.5 µg/ml)].

Antimicrobial activity: The antimicrobial effects of *M. longifolia* extract on five different microorganisms were tested and the diameter of the inhibited zone was measured (mm) and DMSO was used as negative control and clindamycin was used as positive control (Table 3).

The zone diameters formed at the lowest concentration of 50 mg/ml methanol extract were between 7±1 and 15±0.7 mm. At 400 mg/ml concentration, these values ranged between 9±3 mm and 17±1.2 mm. The highest zone diameter of 17±1.2 mm was found against *K. pneumoniae*. Zone diameters in hexane ranged between 7±2.2 and 12±0.5 at the lowest concentration. *E. coli* showed a maximum zone diameter of 16 mm at a concentration of 400 mg/ml. The table shows that the hexane extract gave the best result against *E. coli*. It showed the least activity against *C. albicans*. Overall, it showed the strongest antibacterial effect against *K. pneumoniae* and the weakest activity against *B. megaterium* (Table 3 &Fig. 5).

MIC results showed that methanol and hexane extracts exhibited an inhibitory capacity of 1/32 µg/ml against *B. megaterium*, *E. coli*, *S. aureus* and *C. albicans*. In *K. pneumonia*, the hexane extract (1/16 µg/ml) was found to have a higher inhibition ability compared to methanol. In MBC findings, the value for *E. coli* methanol and hexane extracts was 1/4 µg/ml. For *E. coli*, methanol and hexane extracts had the same MBC value, indicating that both solvents have a similar lethal effect against this pathogen. In contrast, for *K. pneumonia*, the methanol extract had a lower MBC value (1/32 µg/ml), indicating that its ability to destroy the microorganism was more effective compared to the hexane extract. The methanol extract showed the most effective defense against *K. pneumonia* (1/256 µg/ml). The MBC value for *S. aureus* (1/4 µg/ml) was the best effect of the methanol extract (Table 4).

Antioxidant activity: DPPH radical inhibition percentage was calculated according to 100, 200, 400, 800 µg/ml concentrations of extract *M. longifola*. They showed the best antioxidant percentage with 33% and the lowest antioxidant effect with 4.1% (Table 5).

Table 3. *M. longifolia* extract's antibacterial activity (zone diameters in millimeters).

Microorganism	Concentrations (mg/ml) zone diameters (mm)								
	Methanol				Hexane				Clindamycin (Positive control)
	50	100	200	400	50	100	200	400	(2 mcg)
B. megaterium	7 ± 1	8 ± 2	*	9 ± 3	7 ± 4,2	11 ± 0,2	13 ± 1,7	14 ± 2,7	22 ± 0,4
E. coli	9 ± 3	12	13 ± 1	14 ± 2	10 ± 3,7	14 ± 0,2	15 ± 1,2	16 ± 0,7	24 ± 1,6
S. aureus	*	11 ± 2	12 ± 3	13 ± 4	12 ± 0,5	12 ± 0,5	13 ± 0,5	13 ± 0,5	23 ± 0,6
C. albicans	10 ± 0,7	10 ± 0,7	10 ± 0,7	13 ± 2,2	7 ± 2.2	8 ± 1,2	11 ± 1,7	11 ± 1,7	20 ± 2.4
K. pneumoniae	15 ± 0,7	16 ± 0,2	15 ± 0,7	17 ± 1,2	*	13 ± 3,2	13 ± 3,2	13 ± 3,2	23 ± 0,6

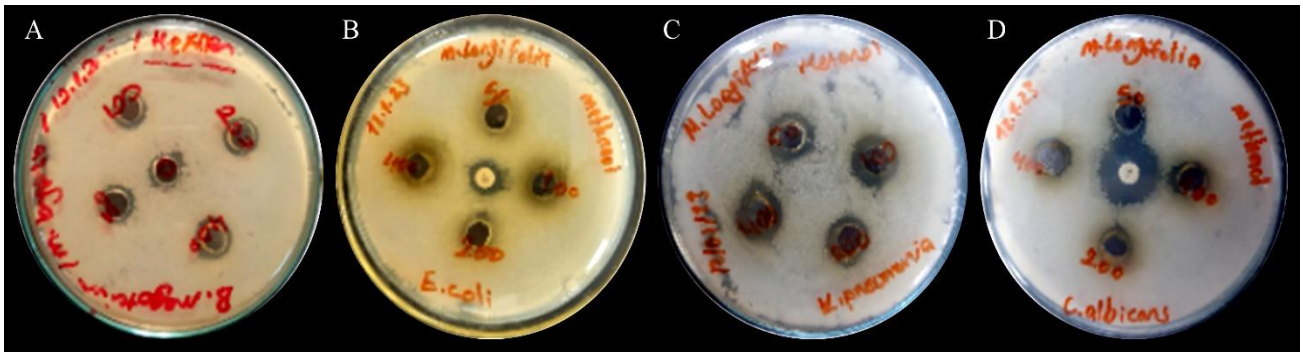

Fig. 5. Zones of inhibition (mm) of methanol and hexane extracts of *M. longifolia* against *B. megaterium*: A, *E. coli*; B, *K. pneumoniae*; C & D, *C. albicans*.

Table 1. MIC and MBC values of *M. longifolia* on 5 different microorganism strains.

Organism	*E. coli*		*K. pneumoniae*		*S. aureus*		*B. megaterium*		*C. albicans*	
Concentration (µg/ml)	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Methanol	1/32	1/4	1/32	1/32	1/32	1/4	1/32	1/8	1/32	1/8
Hexane	1/32	1/4	1/16	1/256	1/32	1/8	1/32	1/32	1/32	1/32

Table 2. Percent change in the amount of DPPH free radical scavenging activity inhibited by *M. longifolia* methanol extract.

Concentrations (µg/ml)	Positive control (Ascorbic acid)	Methanol % DPPH Reduction
100	80	4.1 ± 1.3
200		8.2 ± 1.2
400		16.5 ±1.1
800		33 ± 1.6

* Positive control: Ascorbic acid, Negative control: Methanol

Discussion

M. longifolia grown in Taif, Saudi Arabia was extracted using microwave-assisted hydrodistillation, solvent-free microwave extraction and hydrodistillation methods. Antimicrobial activity and anticancer activity (on HepG2, MCF 7 and A549) of the extracted EOs was tested. The results showed that all EOs showed high antibacterial and mild anticancer activity *In vitro* (Abdel-Hameed *et al.*, 2018). In our study, low concentration of methanol extract of *M. longifolia* plant showed an effect on HepG2 cell line almost close to DOXO, which is a positive control. Using GC-MS analysis, the EOs from *Mentha pulegium* and *M. longifolia* were identified. Nanoparticles were then synthesized, and their anticancer effects were evaluated using the MTT assay against MDA-MB-468, MCF 7 breast cancer cell lines, as well as melanoma cell line A-375. The results revealed that *M. longifolia* at a 1200 µg/ml concentration (the highest concentration) showed the best cytotoxicity against MDA-MB-468 cell line, *M. longifolia* synthesized with nanoparticles against A-375 cell line, *M. pulegium* against A-375 cell line and *M. pulegium* synthesized with nanoparticles against A-375 cell line (Kelidari *et al.*, 2022). According to our results, the best cytotoxic activity in MCF 7 was found at a 200 µg/ml acetone extract concentration.

The dry form of *M. longifolia*, which grows in Wadi Bana-Abb region of Yemen, was extracted by hydrodistillation method to obtain EO and EO contents and compositions were studied. The antibacterial activity was tested on *Pseudomonas aeruginosa*, *S. aureus*, *E. coli* and *Streptococcus pyogens* microorganisms by agar disk diffusion method. The radical scavenging activity was evaluated by using DPPH radical. As a result of the antibacterial activity determination, it was stated that it had the best effect against *S. pyogens* bacteria with 41 mm inhibition zone. As a result of DPPH antioxidant determination, 93.62%. The radical scavenging ratio was determined as the best antioxidant effect (Ahmeda *et al.*, 2018). *M. longifolia* EO, hexane, dichloromethane and methanol extracts were prepared at a concentration of 1-100 µg/ml. DPPH activity was found range of 6.7 and 33.3 µg/ml. In this study, *M. longifolia* extracts were prepared at a concentration of 100-800 mg/ml and DPPH activity was found range of 4.1 and 33 µg/ml. Although the best antimicrobial impact of the EO was found to be on *S. pyogens*, a gram-positive bacterium, in our study, the best effect was found on *K. pneumoniae*, a gram-negative bacterium. At the highest concentrations of 400 µg/ml of the extract of *M. longifolia* methanol, the diameter of the zone formed in *K. pneumoniae* was measured as 17 mm. *E. coli*, which is gram-negative, gave the second-best effect shows that it has a high antimicrobial effect on this bacterium. This effect on *E. coli* was observed at a 400 µg/ml concentration of the extract prepared in hexane. *S. aureus* didn't form a zone diameter only at the lowest methanol extract *M. longifolia* concentration of 100 µg/ml, while a zone diameter was measured in other wells, and it was found to have antimicrobial activity. Furthermore, *M. longifolia* EO possesses a high antioxidant capacity. In our study, hexane extract exhibited the strongest antioxidant activity with 34%.

Dry leaves of *M. longifolia* and *Mentha piperita* from the city of Riyadh in northern Saudi Arabia were extracted with methonol by sonication. The extracts were analyzed using diode array detection on a Merck-Hitachi liquid chromatograph (HPLC-DAD). Using T-cell lymphoblast-like (Jurkat) cells, MCF 7, colon adenocarcinoma (HT-29), normal human cells (HEK-293), and cervical adenocarcinoma (HeLa) cell lines, the cytotoxic and antiproliferative properties of leaf extracts were investigated using the MTT method. Leaf extracts were analyzed using β-carotene bleaching, ferric reducing antioxidant power (FRAP) and DPPH assays.

Antimicrobial, MIC and MBC tests of *S. aureus*, *Aspergillus ochraceus*, *E. coli*, *Bacillus cereus*, *P. aeruginosa*, *Micrococcus flavus*, *Penicillium ochrochloron*, *C. albicans*, *Aspergillus niger*, *Aspergillus flavus*, *L. monocytogenes* (clinical isolate) and *Penicillium funiculosum* were pwerformed. Several polyphenols were detected in *M. piperita* and *M. longifolia*. As a result of cytotoxic activity, *M. piperita* and *M. longifolia* had the best effect against the HT-29 cell line with 44% and 58% cytotoxicity, respectively. The majority of the phenols exhibited antioxidant properties and were linked to the leaf extracts antioxidant properties. Most of the bacteria were sensitive to both *M. piperita* and *M. longifolia* extracts, while *M. piperita* had a higher activity than *M. longifolia* against fungi (Elansary *et al.,* 2020). Antimicrobial activity of menthol compound isolated from *M. longifolia* leaf oil in Mosul and Nineveh cities of Iraq was evaluated against *S. pyogenis*, *P. aeruginosa*, *S. aureus*, *Streptococcus mutans*, *Streptococcus faecalis*, *Lactobacillus acidophilus* and *C. albicans* microorganisms by disc diffusion method at concentrations of 1:1, 1:5, 1:10, 1:20 µg/ml followed by MIC test. The antimicrobial activity assay revealed that all concentrations of menthol were active against all tested bacteria except *P. aeruginosa* and showed the best activity against *S. mutans* at a concentration of 1:1 µg/ml with a zone diameter of 25 mm and the lowest activity against *S. pyogenis* at a concentration of 1:20 µg/ml with a zone diameter of 10.0 mm. The strongest MIC value was 15.6 µg/ml against *S. aureus* and *S. mutans* (Al-Bayati 2009). The antibacterial activity of EOs obtained from *M. longifolia* and *Rosmarinus officinalis* plants grown in Baluchestan region of Iran was analysed against *Salmonella typhi*, *E. coli*, *P. aeruginosa*, *L. monocytogenes*, *B. cereus*, *Bacillus licheniformis* and *S. aureus* using MIC and MBC tests. In the determination of antimicrobial activity, *M. longifolia* EO showed the best effect against *P. aeruginosa* and *S. aureus* with 156.25 µg/ml in MIC and MBC tests, while *R. officinalis* EO showed the best effect against *S. aureus* with 312.5 µg/ml (Mohkami *et al.,* 2014). Although the high antimicrobial properties of *C. albicans* and *S. aureus* microorganisms were emphasised in these two studies, *C. albicans* was found to have the lowest antimicrobial activity in our study. In MIC results, *K. pneumoniae* gave the best effect, not *S. aureus*. *S. aureus* had the same inhibition ability as the other microorganisms we tested (1/32 µg/ml). Furthermore, MBC results showed that *E. coli* had the best killing effect against the pathogen in both solvents. In addition, the fact that *M. longifolia* has the same killing effect against *S. aureus* and *E. coli* is in parallel with the previous studies.

MIC and MBC antimicrobial tests of *M. longifolia* EO were performed against *L. monocytogenes* and *S. aureus* bacteria at different pH and temperatures using broth microdilution susceptibility method. MIC and MBC showed that *M. longifolia* EO showed a wide range of activity on *L. monocytogenes*, the best activities were observed at pH 6 and 8°C, MIC was 150 µg/ml and MBC was 600 µg/ml (Mahmoudi *et al.,* 2016). The antibacterial properties of *M. longifolia* EO on cariogenic microorganisms such as *Lactobacillus*, *S. mutans* and *Streptococcus sobrinus* were investigated. MBC and MIC values of *M. longifolia* EO were tested using broth microdilution method. The corresponding MIC and MBC ratios for *S. mutans*, *S. sobrinus* and *Lactobacillus* were 3.12% and 6.25%, 6.25% and 12.5% and 3.12% and 6.25%, respectively. EO inhibits antibacterial growth in all three species (Shazdehahmadi *et al.,* 2023). Methanolic and aqueous extracts of *M. longifolia* at a concentration of 100 µg/ml were evaluated against eight human cancer cell lines (A-549, COLO-205, HCT-116, MCF 7, NCI-H322, PC-3, THP1 and U-87MG). *Mentha spp*. It was found that methanolic extracts showed anti-proliferative effects in the range of 70-97% against COLO-205, MCF 7, NCI-H322 and THP-1 cell lines, but aqueous extracts were active against HCT-116 and PC-3 (Sharma *et al.,* 2014). In order to test the antibacterial activity of EO obtained from *M. longifolia*, 4 different bacteria, *P. aeruginosa*, *E. coli*, *S. aureus*, *B. subtilis*, were used and the measured zone diameters were found to be 24.5 - 7.5 - 7.5 - 7.5 and 16 mm, respectively (Tourabi *et al.*, 2023b). To evaluate the cytotoxic activity, the IC_{50} value of *M. longifolia* EO against A549 cell line calculated for 24 hours was 195.7 µg/ml, while the results for 48 hours were reported to be 218.4 µg/ml (Kilinç *et al.,* 2022). In our study, *M. longifolia* methanol, hexane and acetone extracts had IC_{50} values of 224.4 - 389.6 - 199.9 µg/ml, respectively, against the HepG2 cell line. It is against MCF 7 cell line; It was observed that it had an IC_{50} value of 224.4 - 945.3 - 368.5 µg/ml. It was reported that *M. longifolia* at various concentrations (20, 40, 80, 160 and 320 µg/ml) showed significant cytotoxic activity against MCF 7 cells. 160 and 320 µg/ml showed more cytotoxic effect against MCF 7 cells (Al-Ali *et al.,* 2013). It has been observed that *M. longifolia* kills 22% - 46% of viable cells against the MCF 7 cell line at a concentration of 100-200 µg/ml. The antioxidant potential and antibacterial capacity of hydroethanolic, acetonic and aqueous extracts of *M. longifolia* leaves were studied. The hydroethanolic extract showed the best antioxidant capacity (74.40 ± 1.34). While the MBC value of *E. coli* and *S. aureus* bacteria was found to be 25 mg/ml. In our study it was found to be 1/4 in methanol extract and 1/8 in hexane extract, respectively. Acetone extract showed the highest MIC value against *E. coli* with a value of 6.25 ± 0.00 mg/ml, while acetone extract showed the highest MIC value against *S. aures* with a value of 2.34 (Tourabi *et al.*, 2023a). In our study, the MIC value of these two bacteria was 1/32 in both methanol and hexane extracts.

Conclusions

Anticancer, antioxidant and antimicrobial activities of *M. longifolia* grown in southern Saudi Arabia were evaluated. The tested concentrations of all extracts were found to have strong cytotoxic activity on HepG2 cell line. Especially the methanol extract used at low concentrations was found to have a better cytotoxic effect on HepG2 cell line than other concentrations. Furthermore, methanol extract showed apoptotic/necrotic cell death patterns in double staining. Methanol extract showed a partial antioxidant effect. In antimicrobial activity, the strongest inhibitory effect was on *K. pneumoniae*, while the least inhibitory effect was on *B. megaterium*. These results indicate that the compounds in *M. longifolia* extracts have the potential to be used as adjuvants in cancer treatment and can be evaluated as antioxidants. In addition, the remarkable antimicrobial effect of the plant increases its importance. It is thought that more extensive *In vitro* and *In vivo* studies are needed to reveal the true potential and safety of *M. longifolia* extracts in treatment.

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