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Article

GC–MS based metabolites profiling, *in vitro* antioxidant, anti-bacterial, and anti-cancer properties of different solvent extracts from leaves, stems, roots, and flowers of *Micromeria fruticosa* (Lamiaceae)

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Abstract: This study assesses the secondary metabolites, minerals, antimicrobial, antioxidant, and anticancer properties of *Micromeria fruticosa* plant different botanical parts (leaf, stem, root, flower) extracted with various solvents. The plant samples were sequentially obtained using different solvents (*n*-hexane, ethanol and water) through steeping. Then, each of the extracts was further analyzed by using gas chromatography–mass spectrometry (GC-MS). Moreover, the extracts were bioassayed to test their antioxidant, antibacterial, and anti-cancer activities. Quali-quantitative analysis of *M. fruticosa* crude extracts revealed the occurrence of 27 secondary metabolites were identified including mainly monoterpenes, sesquterpenes, and fatty acids, with varying quantities. Some of the major bioactive compounds included, Menthone (5.42-30.05%), Oleamide (3.40-32.20%), Pulegone (10.66-64.1%), and Menthol (3.61-100.0%), which were detected mostly in all plant parts with significant quantities. Several antioxidant minerals, mainly, Fe, Zn, and Mn, were detected with the highest amounts in the Micromeria water extracts. Results from antimicrobial assays showed that the water extract of leaves exhibited the highest DPPH scavenging activity (89.73%) followed by the water extract of flowers (80.07%) at a concentration of 100 µg/mL. The water extract of stems showed greater antimicrobial activity against all the tested gram negative and positive bacteria (*Methicillin-resistant Staphylococcus aureus, Staphylococcus aureus, Escherichia coli*, and *Shigella sonnie*). The leaves ethanolic and stem aqueous extracts had a strong antimicrobial activity against *E. coli*. and *C. albicans*. Flower aqueous extract demonstrated the highest cytostatic effect on the colon cell line by reducing viability up to 30.4%, followed by the leaf ethanol extract with 38.6% cell viability reduction at 1000 µg/mL. In conclusion, extraction solvents influenced the recovery of phytocompounds and the highest pharmacological activities of the different extracts could be correlated to the presence of additional bioactive compounds. Our results suggest that *M. fruticosa* plant is a promising source of natural products with antioxidant, anti-microbial and anti-cancer properties for potential nutraceutical, therapeutic, and functional food applications.

Keywords: *Micromeria fruticosa*; GC-MS; metabolites; anticancer activity; MTT; antioxidant activity; antimicrobial activity

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1. Introduction

Thousands of years ago, herbs and plant products were used in folk medicine in treating a wide spectrum of ailments and diseases. Folk remedies can be in different forms including poultices, powders, ointments, baths, decoctions, infusions and teas [1]. The interest in studying the biological effects of traditional medicinal plant or isolating their active components for treatment of illness has been increased all over the world and comprehensive screening programs have been established [2]. Currently, a great number of different medicinal plant products are available in markets including cosmetics and pharmaceuticals, which contain biologically active substances [1]. Recently, some products of plant origins have shown biological activity without side effects. Such results have attracted the attention of many scientists and encouraged them to screen vast number of plants to test their biological activities [3].

In the past, it was challenging, time consuming, and invaluable efforts to build such a library of purified natural products without applying improved technologies for separation, isolation, and identification of such natural products [4].

Herbal parts of Micromeria plant are fragrant with a lemon-mint flavor and is used mainly for making tea which provides sensation of coolness in hot summer, also used in seasonings of soups and foods. Micromeria is known as Thyme-leave savory, the plants' aerial parts (flower, roots, leaves, and stalk) are used in the traditional medicine [5]. *M. fruticosa* is a perennial plant habitant in rocky areas that has a height of 20-80 cm. The extracts of *M. fruticosa* have also been used for alleviation of respiratory system, chest, asthma, fever, skin infections, wounds, and eye inflammation. Numerous earlier studies performed on *M. fruticosa* extracts demonstrated anti-inflammatory effect. Micromeria also possesses insecticide, anticonvulsant, hepatoprotective, analgesic, and sedating effects [6].

Herbal parts of Micromeria plant are fragrant with a lemon-mint flavor and is used mainly for making tea which provides sensation of coolness in hot summer, also used in seasonings of soups and foods [7]. Micromeria is known as Thyme-leave savory, the plants' aerial parts (flower, roots, leaves, and stalk) are used in the traditional medicine. *M. fruticosa* has different uses in traditional medicine including treatment of respiratory system, eye infections, gastrointestinal ailments, heart disorders, urinary diseases, headaches, exhaustion, asthma, inflammation, diabetes and wounds [6]. The infusion of leaves is prepared as tea for colds and to relieve stomach pain as well as for weariness and exhaustion. Besides, the infusion of stalks and leaves in the folk medicine is used in treatment of diabetes, headaches, cough, and urinary diseases. The extracts of leaves have also been used for relief chest, respiratory system, asthma, fever, skin infections, wounds, and eye inflammation.[8] Several previous studies carried out on *M fruticosa* extracts showed anti-inflammatory effect. White *Micromeria* also exhibit insecticide, analgesic, hepatoprotective, anticonvulsants, and sedating effects [6].

Unlike conventional single drug, plant extracts or raw plants have a variety of phytochemicals and bioactive metabolites that may provide synergistic effects which allow for multitarget effect in the therapeutic mechanism of diseases [9]. The herbal medicine and their traditional use are considered one of the major approaches in developing new drug based on natural products. Secondary metabolites, including alkaloids, glycosides, flavonoids, phenols, steroids, saponins, tannins, and terpenoids. Those phytochemicals are important for healing diseases and are responsible for the therapeutic effect of plants. Previous works were focused on studying the essential oils in Micromeria species. In this sense, the investigation of the different parts of plant (leaves, roots, stems, and flowers) were extracted by using ethanol, water, hexane has been the main aim of this study as well, to identify their chemical composition and the potential biological activities of *M. fruticosa* growing in Palestine.

2. Materials and methods

2.1. Plant sample

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The leaves, stem, flower, and roots of Micromeria plant were collected in April 2017 from Tulkarm district (West Bank) in Palestine. The plant sample was botanically identified and deposited at Najah National University (NNU) under the voucher specimen code: Pharm-PCT-1575. The plant parts were shade-dried at room temperature. a portion of each part was soaked for 5 days in n-hexane, ethanol, and water; afterwards, the supernatants were collected filtered by suction filtration.

2.2. Extraction of crude extracts

The leaves, stem, flower, and roots of Micromeria plant were extracted by soaking sequentially and separately in hexane, ethanol, water for 5 days for each solvent and plant part sample. Approximately, 100 g of the dried parts samples (leaves, stems, flowers, and roots) was placed in a one liter round-bottom flask, about 300 mL of hexane, ethanol, water, separately and in a raw, were added, and the flask each time was placed in the dark at room temperature. This was repeated three times for each solvent and plant part. The resulting supernatant of each solvent were collected into separate clean and well closed glass bottles, and stored in the freezer at -20 °C until use.

2.3. DPPH• radical scavenging activity

The hydrogen atom or electron donation abilities of the corresponding compounds were measured from the bleaching of the purple-colored methanolic solution of DPPH (1, 1- Diphenyl -2-picryl-hydrazyl). The stable radical DPPH was used as a reagent in this spectrophotometric assay.

The DPPH (1,1-diphenyl-2-picrilhydrazyl) radical scavenging activity of the plant extracts was established using the method by Amessis-Ouchemoukh et al. (2014) [10] with some modifications. A stock solution of a concentration of 1mg/mL in methanol was initially prepared for plant extract. Stock solutions were used to prepare working solutions with the following concentrations (2, 5, 10, 30, 50, 80, 100 µg/mL) by using serial dilution in methanol. A solution of DPPH was freshly prepared at a concentration of 0.002% w/v. Then, it was mixed with methanol along with each of the working concentration in ratio of 1:1:1. Methanol was used as a blank solution and it was used to zero the spectrophotometer. The first solution of the series concentration was DPPH with methanol only. The solutions were kept at room temperature in a dark cabinet for 30 min. After that, their absorbances were measured by using the spectrophotometer at a wavelength of 517nm.

The percentage of antioxidant activity of plant extract were calculated the following formula:

DPPH inhibition activity (I %) = $(A_{blank}-A_{sample})/A_{blank}*100\%$.

2.4. Antimicrobial and antifungal activity tests

2.4.1. Preparation of samples for testing

Organic plant extracts (ethanol and hexane extracts) were dissolved in sterile 10% Dimethyl sulfoxide (DMSO) to obtain a concentration of 100 mg/mL, while aqueous extracts were dissolved in sterile distilled water to get a concentration of 100mg/mL. The dissolved extracts stored at 4°C for further assays.

2.4.2. Test Microorganisms

Microorganisms used in this study represent pathogenic species commonly associated with infections. The microorganisms were stored in the Microbiology research laboratory at An-Najah National University, Nablus-Palestine. These microorganisms consisted of 2 Gram-positive strains *Staphylococcus aureus* (S. *aureus*) (ATCC 25923), clinical isolate of methicillin resistant *staphylococcus aureus* (MRSA), two Gram-negative strains, *Shigella sonnie* (*S. sonnie*) (ATCC 25931) and multidrug clinical *Escherichia coli* (*E. coli*) isolate and one yeast strain *Candida albicans* (*C. albicans*) (ATCC 90028). All the bacterial strains were subcultured on Mueller-Hinton agar while *C. albicans* was sub-cultured on Sabouraud Dextrose Agar.

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2.4.3. Determination of Minimum Inhibitory concentration (MIC) against bacteria

MIC of organic plant extracts (ethanol and hexane) and aqueous plant extracts was determined by the broth microdilution method in sterile 96- wells microtiter plates according to the standard method described previously by the Clinical and Laboratory Standards Institute (CLSI) [11]. The organic extracts and aqueous extracts were dissolved in sterile 10% DMSO and sterile distilled water, respectively, to a final concentration of 100mg/mL. Both extracts organic and water and 10% DMSO (negative control) were two-fold-serially diluted in nutrient broth in the wells of the plates in a final volume of 100µL. After that, a bacterial inoculum size of 10^5 CFU/mL was added to each well. Other negative control wells containing either 100µL nutrient broth only, or organic plant extracts (or aqueous plant extracts) and nutrient broths without bacteria were included in these experiments. Each plant extract was run in duplicate. The microtiter plates were then covered and incubated at 37°C for 24 hours. The MIC was considered as the lowest concentration of the plant extract, which inhibited the bacterial growth.

2.4.4. Determination of Minimum Inhibitory concentration (MIC) against yeast

Minimum Inhibitory concentration (MIC) of organic plant extracts (ethanol and hexane extracts) and aqueous plant extracts was determined by the broth microdilution method in sterile 96- wells microtiter plates according to standard method described previously (CLSI, 2017) [12][56]. The organic extracts and aqueous extracts were dissolved in sterile 10% DMSO and sterile distilled water, respectively, to a final concentration 100 mg/mL. Both extracts organic and water and 10% DMSO (negative control) were two-fold-serially diluted in Mueller-Hinton broth in the wells of the plates in a final volume of 100µL. After that, a *C. albicans* inoculum size of 0.5×10^5 to 2.5×10^5 CFU/mL was added to each well. Other negative control wells containing either 100µL Mueller-Hinton broth only, or organic extracts (or aqueous extracts) and Mueller-Hinton broth without bacteria were included in these experiments. Each plant extract was run in duplicate. The microtiter plates were then covered and incubated at 37°C for 48 h. The MIC was considered as the lowest concentration of the plant extract, which inhibited the yeast growth.

2.5. Anti-tumer activity test

2.5.1. Preparation of Plant extracts

For the working concentartions, ten milligrams of the plant extract was dissolved in up to 1 mL fresh prepared media for the aqueous extract to have a stock solution of 10 mg/mL final concentration in 2 mL Eppendorf tubes. Then, the stock plant extracts were filtrated through a 0.25 µm membrane filter. Serial dilutions of the aqueous stock solutions (10 mg/mL) were prepared in fresh culture media (5, 2.5 and 1.25 mg/mL). From those prepared working plant extracts solutions, different concentrations obtained final treatment concentrations under study equal to 1000, 500, 250, and 125 µg/mL. All those preparations were carried out under aseptic conditions at the sterilizer biosafety cabinet.

2.5.2. Cell line and culture medium

Colon (human colon cancer) cell line under study was obtained from ATCC (American Type Culture Collection). The cells were cultured in liquid Roswell Park Memorial Institute (RPMI 1640) media in T25 cell culture flask. This media was freshly supplemented with 10% heat-inactivated fetal bovine serum (FBS), (1% v/v) of penicillin-streptomycin (antibacterial effect), (1% v/v) of amphotericin (antifungal effect), (1% v/v) of L-glutamine (amino acid as an energy source). Cells were incubated in CO_2 incubator (cell culture incubator) at 37 ^{O}C, 95% humidity, 5% CO_2 at dark. The cultured cells were observed routinely under an inverted microscope for checking their attachment to the media substratum in the culture flask, ensuring their confluence and if any contamination has occurred. Every three days' fresh culture medium was replaced until cell confluence was reached to 90%.

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2.5.3. Inverted microscopy visualization screening

For testing, the old media was removed, and the cells were washed by 5 mL phosphate buffer saline (PBS). Then cells were detached from T25 flask by Trypsinization using 1 mL Trypsin-EDTA. After that, cells were incubated for 10 to 15 minutes at a CO_2 incubator to increase the efficiency of detachment. Then, again cells were washed by 5 mL phosphate buffer saline (PBS). After that period, cells were harvested and centrifuged at 3000 rpm for 3 minutes. Later, the obtained cell pellet is dissolved in 1 mL fresh media, which were counted by Trypan blue exclusion in a hematocytometer to calculate the density of viable cells (living cells). Cells were then plated in 12 well plates at a concentration of 20,000 cells per well in a total volume of 1000 µL. They were treated with 100 µl of each of the plant species aqueous extract different concentrations under study separately (2.5, 5, and 10 mg/mL) to have final treatment concentrations equal to 250, 500, and 1000 µg/mL, respectively. Cells cultured in RPMI media only were used as normal control. Each treatment was carried out in duplicates. All plates were incubated at CO_2 incubator at 5% CO_2, 37^{O}C for 24 hrs. Cell viability, confluence and attachment were detected under the inverted microscope. Microscopic photographs were taken at the center of the wells at 10x.

2.5.4. MTT assay

This is a colometric and viability assay which uses reduction (conversion) of a yellow tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, or MTT) by NAD(P)H-dependent oxido-reductase enzymes in the viable cells which reduce the MTT reagent (MTT solvent) to an deep purple colored insoluble crystalline formazan. Formazan crystals are then dissolved using a solubilizing solution (MTT solution), and absorbance is measured at 500-600 nanometers using a plate-reader (ELISA reader). The higher recorded absorbance is, the higher the formazan concentration, which indicates the higher cell viability as indicated by higher metabolic activity.

2.5.4. MTT procedure

Cells were detached and counted as mentioned previously; cells were then inoculated in 96 – well microtiter plate at a density of 20,000 cells/100 µL total volume/well (cytotoxic test). While in (cytostatic test) the density was 5,000 cells/100 µL total volume/well. Cells were treated with 10 µL of the different concentrations of water plant extract under study equal to 1.25 - 10 mg/mL to have a final concentration in each well equal to 125-1000 µg/mL, respectively. Cells cultured in RPMI media only were used as normal control. Each type of treatment in each well was carried out in duplicates. Later, cultured plates were incubated at CO_2 incubator at 5% CO_2 and 37 ˚C for 24 hrs and 72 hrs for cytotoxic test and cytostatic tes, resepcetively. After the required incubation period (24 or 72 hrs) the media was removed from each well with subsequent washing with PBS. Then the cultured cells under study were re-cultured in 100 µL serum-free RPMI media to which 10 µL MTT solution (0.5 mg/mL) was added to each well and incubated for 4 hours at CO_2 incubator. After that, the media was removed, washed, and cells were incubated for 15 minutes with 100 µL of acidic isopropanol (0.08N HCL) to dissolve the formazan crystals. The absorbance of MTT formazan was determined at 570 nm in an ELISA reader. Cell viability was calculated as percentage of absorbance of treated cells to absorbance of normal control (untreated cells).

2.5.5. The GC–MS analysis

The chemical composition of the extracts of *M. fruticosa* was identified by using utilizing a Perkin Elmer Clarus 500 Gas Chromatography coupled with a Perkin Elmer Clarus 560D mass spectrometer was utilized in the current examination equipped with Elite-5MS fused silica column (0.25µm thickness, 30m length) and interfaced with a detector of the variant ion trap. The method used was described by Šamec and Zeljkovic (2020) [13] with some modifications. The temperature transfer line was set at 290°C and 280°C,

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respectively. The temperature of the injector was 260°C with an initial temperature of 60°C, initial hold 5 min, and ramp 5.0°C/min to 280°C. The transfer line and injector temperatures were set at 290 and 280°C, respectively. The Carrier gas flow rate (helium) was kept at 1mL/min with a linear velocity of 31.0 cm/s. The split ratio was 1/60, electron ionization energy was 70 eV, scan time was 1s and the mass range was from 50 to 400 m/z. Aliquots of 20µL Diluted samples (10mg/mL) of the organic extracts (ethanol and hexane ones) of plant parts were injected in the GC-MS system. The mass spectrometry data center of the national institute of standards and technology (NIST) was used as a reference to identify the chemical components of the extracts by comparing their MS spectra with data of NIST. The quantitative data were obtained electronically from integrated peaks, and based on area percentages without the use of correction factor.

2.6. ICP-MS Analysis

ICP-MS was used for quali/quanttitive measurements of a total of 19 elements in the *M. fruticosa* water extracts. Samples are decomposed to neutral elements in high-temperature argon plasma and analyzed based on their *m/z* masses to charge ratios using a mass spectrometer system. Elements can be analyzed at the parts-per-million (PPM) to parts-per-trillion (PPT) concentration levels. ICP-MS is also capable of monitoring isotopic specification for the ions of choice.

3. Results and discussion

According to the reports of the World Health Organization (WHO), herbal and traditional medicine are considered as the major source of primary health care for residents living in developing countries. Moreover, they form a major source of medicinal preparations discovered mainly from traditionally prepared herbal extracts comprising high quality and quantity of active components.

3.1. GC-MS analysis of the phytochemical compenents

The GC–MS apparatus was utilized to characterize qualitatively and quantitatively of the ethanol, hexane, and water extracts of *M. fruticosa* leaves, stems, and flowers. Table 1. show the data of the quali-quantitative analysis of the Micromeria extracts/parts. A total of 27 compounds were identified in different extracts. These components were classified mainly into terpenes, sesquiterpenes, and fatty acids identified in all three extracts and botanical parts under study. Detailed results of the components, % area, retention time and index, molecular mass and formula, are represented in Table 1. The most abundant compounds in all samples and extracts were Menthone, Menthol, and Pulegone. Figure 1 shows the chemical structure of the main bioactive comounds identified in *M. fruticosa* extracts/parts. These components were already reported in *M. fruticosa* essential oils [14] with various percentages which agree with the obtained results in our present work.

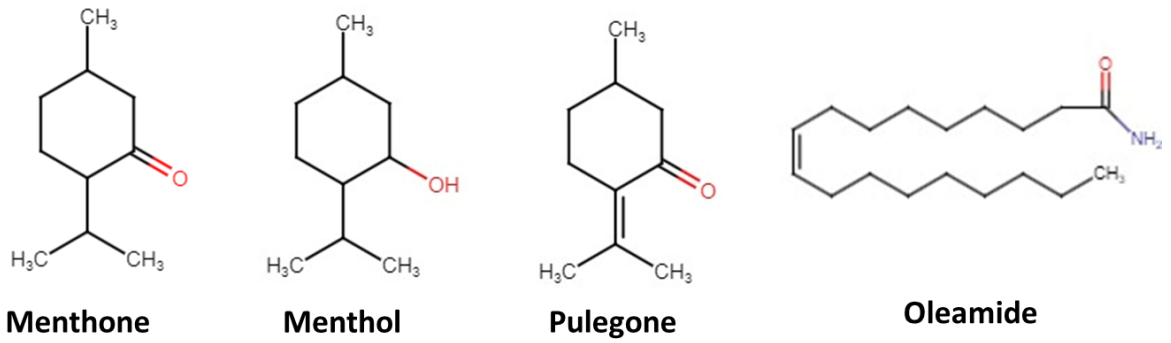

Figure 1. Chemical structure of the main bioactives in *Micromeria fruticosa* extracts.

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The flower/hexane extract was found to have a total of 11 compenents, including Menthol (46.40%) and Ethyl 13-methyl-tetradecanoate (24.10%) as the major compounds. In the leaves/hexane extract, a total of 10 compounds were detected, including Oleamide (32.19%), and Menthol (17.82%) with the highest amounts. Otherwise, Pulegone (31.79%), Menthone (30.05%), and Menthol (21.22%) were found as the major phytocompounds in the flower/ethanol extract which gave rise for 9 bioactive compounds. The leave/ethanol, stem/hexane, stem/ethanol extracts were found with a total of 6, 3, 1 bioactives, respectively. These detected components were classified mainly into terpenes, sesquiterpenes, and fatty acids in all three extracts and botanical parts (Table 1). The three major compounds found abundantly in all the samples and extracts were menthone, menthol, and pulegone. Terpinolene is a monoterpene, was solely found in the hexane extract of Micromeria leaves (2.56%). This compound has an antioxidant, and antibacterial activities [15]. Terpinolene was described in essential oils of *M. fruticosa* but with smaller amounts [16].

The ethanolic extract of Micromeria flower found to have the bioactive compounds namely, Pulegone (31.8%), Menthone (30%) and Menthol (21.2%). Otherwise, the hexane extract of Micromeria flower had no Pulegone, but Menthone (5.4%), Ethyl 13-methyl tetradecanoate (24%) and Menthol (46.4%) with the higheist percentage (46.4%).

The ethanolic extract of Micromeria leaves showed that Pulegone (64.1%), Menthone (14.6%) and cryophyllene (12%) as the major compounds. While the hexane extract of leaves possessed Menthol (17.8%), Pulegone (10.7%), and Menthone (9.1%). The ethanolic extract of Micromeria stems showed to extract the maximum amount of Menthol (100%). However, the hexane extract showed 66.61% of Menthol. β-caryophyllene is a sesquiterpene compound has merely detected in the ethanolic extract of flowers of *M. fruticosa* (4.14%). These compounds were described previously in *Micromeria fruticosa* [16] but not in the solvent extracts from different botanical parts.

Table 1. Phytochemical compounds identified in the extracts and plant parts of *Micromeria fruticosa.*

Extract type	R.T. (min.)	R.I.	Identity	Class	%Area	M.F.	M.W.	Biological Studies
Flower Ethanol	18.44	796	Menthone	Monoterpene	30.05	$C_{10}H_{18}O$	154.25	Antimicrobial activity, antibiofilm, antitumor activities.
	19.2	866	Menthol	Monoterpene	21.22	$C_{10}H_{20}O$	156.27	Antibacterial, antifungal, antipruritic, anticancer, antioxidant, cooling effects
	20.82	816	Pulegone	Monoterpene	31.79	$C_{10}H_{16}O$	152.23	Antimicrobial, antioxidant,
	25.12	614	4,6-Decadiene	-	0.86	$C_{10}H_{18}$	138.25	Antioxidant, antiasthmatics,
	27.08	794	β-caryophyllene	Sesquiterpene	4.14	$C_{15}H_{2}$	204.3	Anti-inflammatory, antioxidant,
	29.03	725	β-Cubebene	Sesquiterpene	1.44	$C_{15}H_{24}$	204.36	Antioxidant activity

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	36.71	770	Trans-1,2-diphenylcyclobutane	Cyclobutane	0.56	$C_{16}H_{16}$	208.3	Antitumor activity
	54.65	558	(2,3-diphenylcyclopropyl)methyl phenyl sulfoxide	Stilbene	0.74	$C_{22}H_{20}OS$	332.46	Anticandidal and
	59.01	559	Oleamide	Fatty amide	9.2	$C_{18}H_{35}NO$	281.48	Anti-inflammatory, antibacterial, and antioxidant activities
Flower Hexane	18.14	849	Menthone	Monoterpene	5.42	$C_{10}H_{18}O$	154.25	Antimicrobial activity, antitumor activities.
	19.14	872	Menthol	Monoterpene	46.4	$C_{10}H_{20}O$	156.27	Antibacterial, antifungal, antipruritic, anticancer, antioxidant activities
	22.63	777	8-Hydroxy-p-menth-4-en-3-one	Terpene-like	8.96	$C_{10}H_{16}O_2$	168.23	No activity was recorded
	25.49	702	4-Pentyl-1-(trans-4-propylcyclohexyl)cyclohexene	-	1.35	$C_{20}H_{36}$	276.51	No activity was recorded
	29.73	722	Ethyl 14-methyl-hexadecanoate	Fatty acid	1.95	$C_{18}H_{36}O_2$	284	-
	32.1	846	1-Formyl-2,2-Dimethyl-3-Trans-(3-Methyl-But-2-Enyl)-6-Methylidene-Cyclohexane	-	1.76	$C_{15}H_{24}O$	220.36	Antifungal, insecticidal and larvicidal agent.
	42.12	808	Palmitic acid	Fatty acid	3.63	$C_{16}H_{32}O_2$	256.4	Antioxidant, anticancer,and anti-inflammation.
	42.87	890	Ethyl 13-methyl-tetradecanoate	Fatty acid	24.1	$C_{17}H_{34}O_2$	270.46	Antioxidant, β-glucuronidase inhibitory and anticancer activities
	47.48	867	Ethyl 10-bromodecanoate	Fatty acid derivative	2.43	$C_{12}H_{23}BrO_2$	279.21	Antidiabetic activity

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	51.72	841	Methyl 17-methyl-octadecanoate	Fatty acid	0.6	$C_{20}H_{40}O_2$	312.54	Antibacterial agents, antioxidants, anticancer activities
	58.95	781	Oleamide	Fatty amide	3.39	$C_{18}H_{35}NO$	281.48	Anti-inflammatory,antibacterial, and antioxidant Activities
Leave Ethanol	18.44	966	Menthone	Monoterpene	14.59	$C_{10}H_{18}O$	154.25	Antimicrobial, antibiofilm, antitumor activities
	20.87	870	Pulegone	Monoterpene	64.11	$C_{10}H_{16}O$	152.23	Antimicrobial, antioxidant, anti-inflammatory, anti-ulcer, insecticidal properties.
	27.08	905	Caryophllene	Sesquiterpene	11.99	$C_{15}H_{24}$	204.36	Anti-inflammatory, antioxidant, antinociceptive, neuroprotective, anxiolytic, anti-depressent, anti-microbial, anti dermatitic, anti-acne, anti-asthmatic, anti-ulcer, gastro-protective, sedative, and anti-spasmodic activities.
	29.03	865	β-Cubebene	Sesquiterpene	3.96	$C_{15}H_{24}$	204.35	Antibacterial activity
	36.7	863	Trans-1,2-diphenylcyclobutane	Cyclobutane	1.03	$C_{16}H_{16}$	208.3	Agonist activity
	46.26	889	Methyl 8, 11, 14-heptadecatrienoate	Fatty acid derivative	4.32	$C_{18}H_{30}O_2$	278.44	No activity was recorded
Leave Hexane	7.14	898	Styrene	-	5.55	C_8H_8	104.15	Agonist activity
	17.61	717	Terpinolene	Monoterpene	2.56	$C_{10}H_{16}$	136.23	Antioxidant, antiasthmatics, antibacterial activities

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	18.14	920	Menthone	Monoterpene	9.1	$C_{10}H_{18}O.$	154.25	Antimicrobial activity, antibiofilm, antitumor activities
	19.19	853	Menthol	Monoterpene	17.82	$C_{10}H_{20}O$	156.27	Antibacterial, antifungal, antipruritic, anticancer, antioxidant activities
	20.88	823	Pulegone	Monoterpene	10.66	$C_{10}H_{16}O$	152.23	Antimicrobial, Antioxidant, anti-inflammatory, anti-ulcer, insecticidal properties.
	22.67	701	8-Hydroxy-p-menth-4-en-3-one	Terpene-like	4.03	$C_{10}H_{16}O_2$	168.23	No activity was recorded
	36.69	858	Trans-1,2-diphenylcyclobutane	Cyclobutane	4.17	$C_{16}H_{16}$	208.3	Agonist activity
	42.1	585	Palmitic acid	Fatty acid	6.33	$C_{16}H_{32}O_2$	256.4	Antioxidant, anticancer, food additive, anti-inflammation.
	46.25	847	Methyl-hydroxylinolenate	Fatty acid	7.59	$C_{19}H_{32}O_3$	308.46	No activity was recorded
	58.94	771	Oleamide	Fatty amide	32.19	$C_{18}H_{35}NO$	281.48	Anti-inflammatory activity, antibacterial and antioxidant Activities.
Stem Hexane	19.2	880	Menthol	Monoterpene	66.61	$C_{10}H_{20}O$	156.27	Antibacterial, antifungal, antipruritic, anticancer, antioxidant activities
	54.75	887	Hentriacontane	Alkane hydrocarbon	4.4	$C_{31}H_{64}$	436.85	Antifungal, antioxidant, antitumor and antibacterial activities.
	58.93	766	Oleamide	Fatty amide	28.99	$C_{18}H_{35}NO$	281.48	Anti-inflammatory, antibacterial, and antioxidant activities.
Stem Ethanol	19.21	864	Menthol	Monoterpene	100	$C_{10}H_{20}O$	156.27	Antibacterial, antifungal, antipruritic, anticancer, cooling effects, Antioxidant.

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Roots Ethanol	37.902	824	Cis-1-Chloro-9-Octadecene	-	8.3	$C_{18}H_{35}Cl$	286.92	-
	42.929	884	Ethyl 14-Methyl-Hexadecanoate	-	26.82	$C_{18}H_{36}O_2$	284.48	-
Roots Hexane	19.204	801	Menthol	Monoterpene	3.61	$C_{10}H_{20}O$	156.27	Antibacterial, antifungal, antipruritic, anticancer, and antioxidant activities
	42.159	814	Palmitic acid	Fatty acid	8.24	$C_{16}H_{32}O_2$	256.4	Antioxidant, anticancer, food additive, anti-inflammation.
	42.934	902	Ethyl 13-Methyl-Tetradecanoate	Fatty acid	47.52	$C_{17}H_{34}O_2$	270.46	-
	54.433	823	Diisooctyl phthalate		6.25	$C_{24}H_{38}O_4$	390.56	Antioxidant, antimicrobial, and antifungal activties
	58.965	750	Oleamide	Fatty amide	14.61	$C_{18}H_{35}NO$	281.48	Anti-inflammatory, antibacterial, and antioxidant activities.
	61.036	926	Hentriacontane	Alkane hydrocarbon	19.77	$C_{31}H_{64}$	436.85	Antifungal, antioxidant, antitumor and antibacterial activities

Another sesquiterpene (β-Cubebene), was identified in the ethanolic extract of both leaves and flowers of *M. fruticosa* with percentage area, 3.96 and 1.44%, respectively. This compound was reported with anti-bacterial activity [17].

The monoterpene, Menthol, was found to be in higher percentages in *M. fruticosa*, especially for the ethanolic extract of the stem parts, which showed a 100% area of the total ion chromatogram. Hexane extract, on the other hand, showed 66.61% for the stem part. Hexane and ethanol extracts of the flower parts showed 46.40 and 21.22% area, respectively. Its worth mentioning that this compound has been reported to have several bioactivities such as, antioxidant, antibacterial, antifungal, antipruritic, and anticancer properties [18]. Oleamide, a fatty amide has been detected and reported for the first time in Micromeria species with the following percentage areas: 32.19% (leave/hexane extract), 29% (stem/hexane extract), 9.20% (flower/ethanol extract), and 3.39% (flower/hexane extract). Oleamide was reported with various bioactivities including, anti-inflammatory, antibacterial, and antioxidant properties [19].

A stilbene compound, (2,3-diphenylcyclopropyl)methyl phenyl sulfoxide, has been detected in ethanolic extract of Micromeria flower in tiny percentage (0.74%). Interestingly, this compound was described to have an anticandidal and antioxidant activities [20].

3.2. ICP-MS Analysis

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The analysis of elements by using ICP-MS in the different extracts and parts of *M. fruticosa* is illustrated in Table 2. They were present at higher concentrations in leaves' aqueous extract in comparison to other Micromeria plant parts. These elements were Fe, Zn, Mn, and Sr (346, 85.8, 81.7, and 67.7 ppb), respectively. Fe, Zn, Cu, and Mn are considered as important antioxidant minerals. Zn may act as antioxidant, either on its own or by contributing to the antioxidant proteins such as metalliothionin and superoxide dismutase. On the other hand, Fe and Mn, which have two valency states in nature, can participate in redox reactions which may have antioxidant effects [21].

Table 2. Concentration of various elements from aqueous extract of different parts of *Micromeria fruticosa* plant using ICP-MS spectrometer.

Element	Concentration (µg/L or ppb)			
	Leave WE*	Stem WE	Flower WE	Root WE
Ag	0.286	0.043	0.145	0.204
Al	33.514	17.991	27.264	65.201
Ba^{-1}	21.494	14.663	15.640	19.218
Cd	0.067	0.257	0.086	0.136
Cr	7.783	4.977	5.891	6.972
Co	0.344	0.194	0.441	1.643
Cs	0.020	0.041	0.062	0.024
Cu	8.969	6.882	18.716	11.910
Fe	346.751	165.348	238.593	228.406
Ga	0.174	0.115	0.123	0.174
Li	1.305	1.006	0.859	0.496
Mn	81.700	23.311	43.494	48.519
Mo	1.245	0.607	0.305	1.102
Ni	10.784	3.492	9.320	6.977
Pb	1.066	1.494	1.210	2.270
Rb	13.949	14.384	0.908	12.532
Sr	67.704	23.018	38.965	25.692
V	0.181	0.095	0.091	1.170
Zn	85.881	77.144	58.116	25.926

* WE, stands for water extract.

Thus, the aqueous extract of *M. fruticosa* leaves was found with the highest concentrations of Fe (346.75 ppb), Mn (81.70 ppb), and Zn (85.88 ppb). The aqueous extract of flower was found with high amounts of the antioxidant mineral Cu. Nevertheless, Al (65 ppb) was found with the highest concentration in the root aqueous extract of *M. fruticosa*. The iron was reported to be the major element with the highest amount in other Micromeria species (*Micromeria pseudocroatica*) [22]. Its worth mentioning that the elemental analysis from *Micromeria fruticosa* extracts is reported here in our work for the first time.

3.3. Antioxidant activity by DPPH method

The percent of inhibition values of the *Micromeria fruticosa* plant extracts/samples are presented in **Figure 2**. In water and hexane extracts, leaves showed a high inhibition percentage at low concentration, while root extract exhibited a minimum pecent of inhibition. Yet, the ethanol extract of roots showed the top quantity as percentage of inhibition at low concentration (**Figure 2**). At the concentration of 100 µg/mL for the ethanol extracts, our results showing the same range of inhibition (48-65 %), are in agreement with the previously cited bibliography [23].

In aqueous and hexane extracts, the leaf displayed functional inhibition at low concentration 2µg/mL. Similarly, the ethanolic root extract showed a high inhibition percentage (44.56%) at the same concentration.

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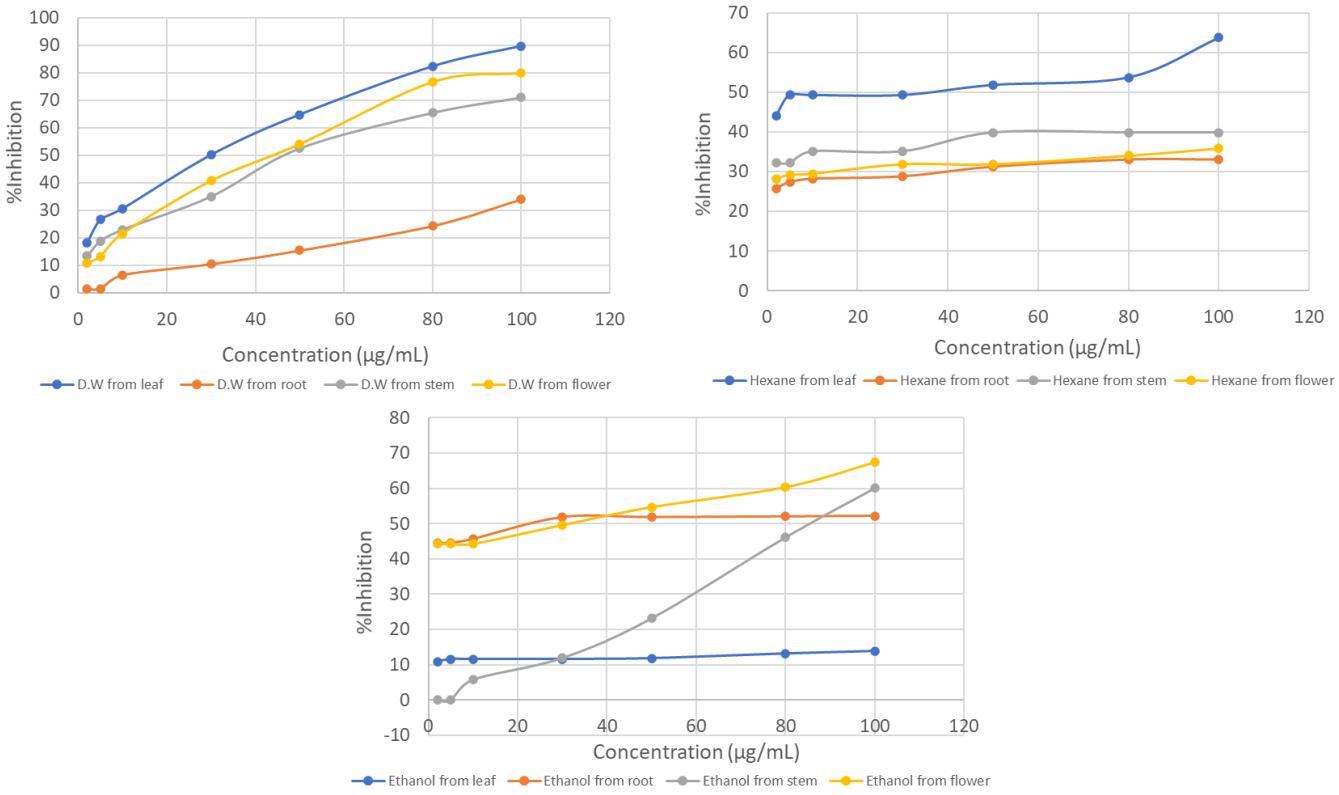

Figure 2. Antioxidant % Inhibition vs. concentration (µg/mL) of Water extracts (A), Hexane extracts (B), and Ethanol extracts (C) of the Micromeria botanical parts (Leaf, root, stem and flower).

3.4. Anti-bacterial activities

The antibacterial activities of the extracts/plant parts obtained from the *Micromeria Fruticosa* under study by the broth microdilution method against different pathogens are shown in **Figure 3**.

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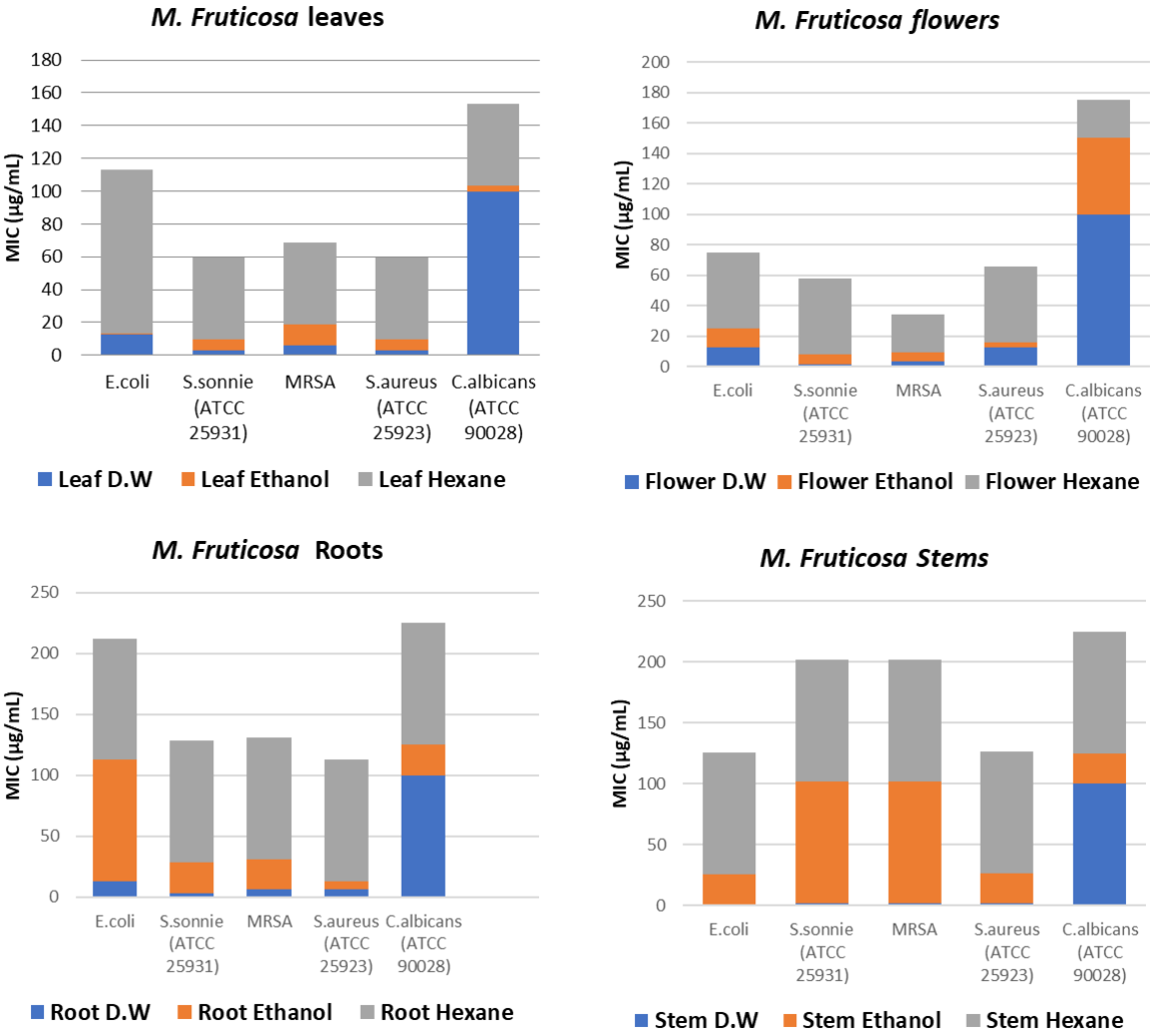

Figure 3. Minimum Inhibitory concentration (MIC) (µg/mL) of botanical parts (leaves, flowers, stems and roots) extracted with water, ethanol and hexane against different pathogens.

Results of the current study showed that aqueous leaf extract had the highest antimicrobial activity against *S. sonnie* (ATCC 25931) and *S. aureus* (ATCC 25923) at the concentration (3.12 µg/mL), while the ethanolic leaf extract had the highest antimicrobial and antifungal activity against *E. coli* (0.78 µg/mL) and *C. albicans* (ATCC 90028) (3.12 µg/mL). Aqueous flower extract had the highest antimicrobial activity against *S. sonnie* (ATCC 25931) (1.56 µg/mL) and MRSA (3.12 µg/mL), while ethanolic flower extract had the highest antimicrobial activity against *S. aureus* (ATCC 25923) (3.12 µg/mL). Hexane flower extract had the most top action against *C. albicans* (ATCC 90028) compared with aqueous and ethanolic flower extracts.

Aqueous stem extract had the highest antimicrobial activity against both Gram-positive: *S. aureus* (ATCC 25923) and MRSA and Gram-negative bacteria: *S. sonnie* (ATCC 25931) and *E. coli*. Hexane stem extract had the highest activity against *C. albicans* (ATCC 90028) compared with aqueous and ethanolic stem extracts. Aqueous root extract had the highest antibacterial activity against both Gram-positive: *S. aureus* (ATCC 25923) and MRSA and Gram-negative bacteria: *S. sonnie* (ATCC 25931). Ethanol root extract had the highest activity against *C. albicans* (ATCC 90028) compared with aqueous and hexane root extracts. Minimum Inhibitory concentration of different plant parts and extracts types against different pathogens (**Figure 3**).

3.5. Anti-tumer activity

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Plant-derived anticancer agents are effective inhibitors of tumor cell lines (Sivaraj et al., 2014)[24], they cause apoptosis and low growth in cancerous cell without causing cytotoxic effect on health cells [25].

Micromeria fruticosa aqueous, ethanol and hexane extract anticancer bioactivity effects was tested on Colon cell line. This effect was investigated by the exposure of Colon cell line to the of the different extracts at their variable concentrations (1000, 500, 250, 125 µg/mL) under study for 24 hrs. Inverted microscopy examination of the cells under treatment revealed a morphological alteration in which cancerous cells became detached in comparison to the normal, which caused a decrease in the cells attached. These observed bio-alterations could be an indication for cell growth inhibition.

Colon cell line viability % after 24 hrs demonstrated that the highest colon anticancer cytotoxic effect was found for the root ethanol extract of *M. fruticosa* as it reduced the cell viability up to 5.9 and 8.5% at 1000 and 500µg/mL, respectively. However, another cytotoxic effective was for the leaf hexane extract, as it reduced the cell viability up to 10.8% at 1000 µg/mL. Nevertheless, at the lowest studied concentration (125 µg/mL), all examined plant extracts displayed a decrease in the colon cell line viability, in which the highest decrease was found for the root ethanol extract (15.5%) and the lowest was for the root aqueous extract (99.1%) (**Table 3** and **Figure 4**).

The recorded data revealed that the different extracts of the studied plant parts have exhibited a cytotoxic effect rather than cytostatic effect on the Colon cell line. For example, the colon cell line viability was reduced up to 5.9% and 48.8 %, respectively for root ethanol extract at 1000 µg/mL. This cytotoxic effect rather than cytostatic effect is recognized in all *M. fruticosa* different extracts under the screened concentrations. Therefore, they have shown a killing effect on the Colon cancer line under study rather than growth arrestting effect. These observations coincide with the morphological inverted microscopy examination. In addition, all used tests went in harmony proving that the different plant extracts effects are in concentration dependent manner.

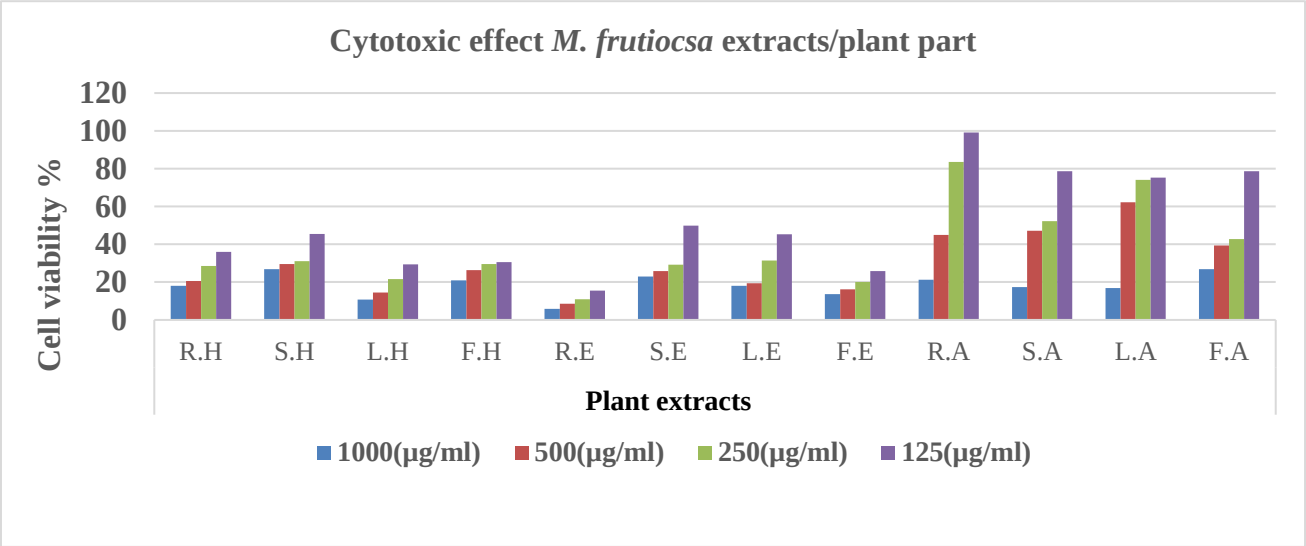

Figure 4. Cytotoxic effect of *Micromeria fruticosa* extract on Colon cell line viability at different concentrations after 24 hours. **R**: Root, **S**: Stem, **L**: Leave, **F**: Flower, **H**: Hexane, **E**: Ethanol and **A**: Aqueous.

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Table 3. Colon cell line viability under hexane, ethanol and aqueous extract of *Micromeria* fruticosa at the studied Concentration after 24 hrs (Cytotoxic effect).

Concentration (µg/mL)	Cytotoxic effect cell viability%											
	R.H	**S.H**	**L.H**	**F.H**	**R.E**	**S.E**	**L.E**	**F.E**	**R.A**	**S.A**	**L.A**	**F.A**
1000	18.0	26.8	10.8	20.9	5.9	22.9	18.0	13.6	21.3	17.4	16.8	26.9
500	20.5	29.6	14.5	26.3	8.5	25.9	19.4	16.2	45.0	47.2	62.3	39.3
250	28.5	31.1	21.6	29.6	11.0	29.2	31.5	20.1	83.6	52.2	74.1	42.7
125	36.0	45.5	29.4	30.6	15.5	49.8	45.3	25.9	99.1	78.6	75.2	78.6

* Cell viability was calculated as percentage of absorbance of treated cells to absorbance of normal control (untreated cells).
** **R**: Root, **S**: Stem, **L**: Leave, **F**: Flower, **H**: Hexane, **E**: Ethanol and **A**: Aqueous.

However, in the cytostatic effect MTT assay, in the cytostatic effect MTT assay for the flower aqueous, after 72 hrs at the low concentrations (250 and 125 µg/mL), an increase in the cell viability % up to around 102.9 and 103.7%, respectively, has been observed, which could be referred to the MTT assay conflicts. Yet, the exact cellular mitochondrial mechanism of MTT reduction into formazan is not well identified, but it likely involves to a reaction with NADH or similar reducing molecules that transfer electrons to MTT. Then again, the pH of the solubilization solution can be adjusted to provide maximum absorbance if sensitivity is deemed as an issue. Therefore, other assay technologies would offer much greater sensitivity than MTT. Additionally, the amount of signal generated may be dependent on several parameters including: the length of the incubation period, the concentration of MTT, the number of viable cells and their metabolic activity. All of which, should be considered when optimizing the assay conditions to generate an adequate amount of product that is detectable and above the background. In addition, the conversion of MTT to formazan by cells in culture was found to be time dependent.

Furthermore, culture conditions that alter the metabolism of the cells might likely affect the rate of MTT reduction into formazan. For example, when adherent cells in culture approach confluence and growth becomes contact inhibited, metabolism may slow down and the amount MTT reduction per cell will be lower. That condition may lead to a loss of linearity between the cell number absorbance and absorbance. Other adverse culture conditions such as altered pH or depletion of essential nutrients such as glucose may lead to a change in the ability of cells to reduce MTT. Otherwise, the MTT assays and related assays (i.e. MTS) are dependant on a mitochondrial reductase to convert the tetrazole to formazan. The assumption is that the conversion is dependent on the number of viable cells. But there is always the possibility that treatment of the cells may result with increased enzymatic activity without actually having an effect on cell number or viability. When it comes to the use of a colorimetric assay (i.e. MTT, SRB, XTT, etc.), they, actually, not fully reflective nor indicative of "cytotoxicity" by only showing "growth inhibitory effects" because a concentration that reduces by 50% the growth of the cell population of interest with the examined compounds of interest does not necessarily mean that 50% of the cells were killed. The possibility remains that indeed 50% of the cells were killed (cytotoxic effects), but also that 50% of the cells faced growth arrest (during the time of the test; cytostatic effects) or that 50% of the cells detached from the bottom of the flask (anti-adhesive and anti-metastatic effects), or a mix of these three biological processes [26].

As a consequence, colorimetric assay should be combined with simple observations with phase-contrast microscopy which can more or less easily provide more clarification about cytotoxic versus cytostatic effects. In addition, performing Trypan blue exclusion assay could be a good idea to complement the attained observations. Together with a more

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direct viability assay (LDH assay) could accomplish much more exact image about the actual antitumor effect of the examined plant species.

On the other hand, the cytostatic effect screening of Micromeria extracts under the examined concentrations indicated that the flower aqueous extract has the highest cytostatic effect on the colon cell line by reducing viability up to (30.4%) at 1000µg/mL followed by the leaf ethanol extract cause of (38.6%) cell viability reduction at the same concentration. In addition it is obvious that at the lowest concentration (125µg/mL) of all examinael plant extract types, the stem ethanol extract has the strongest cytostatic anticancer effect (57.2% cell viability) (**Table 4** and **Figure 5**).

Table 4. Colon cell line viability under hexane, ethanol and aqueous extract of *Micromeria fruticose* at the studied Concentration after 72hrs (Cytostatic effect).

Concentration (µg/mL)	Cytostatic effect cell viability%											
	R.H	S.H	L.H	F.H	R.E	S.E	L.E	F.E	R. A	S.A	L.A	F.A
1000	42.9	49.0	66.2	44.5	48.8	39.7	38.6	51.6	42.3	43.3	53.7	30.4
500	52.2	58.2	70.5	47.3	55.7	49.4	46.6	77.6	47.0	65.4	70.8	102.0
250	69.2	60.2	72.5	48.3	61.7	53.9	58.2	85.0	60.5	65.4	85.4	102.9
125	82.8	79.1	75.3	93.8	81.9	57.2	65.3	88.0	70.4	99.5	94.1	103.7

*Cell viability was calculated as percentage of absorbance of treated cells to absorbance of normal control (untreated cells). ** **R**: Root, **S**: Stem, **L**: Leave, **F**: Flower, **H**: Hexane, **E**: Ethanol and **A**: Aqueous.

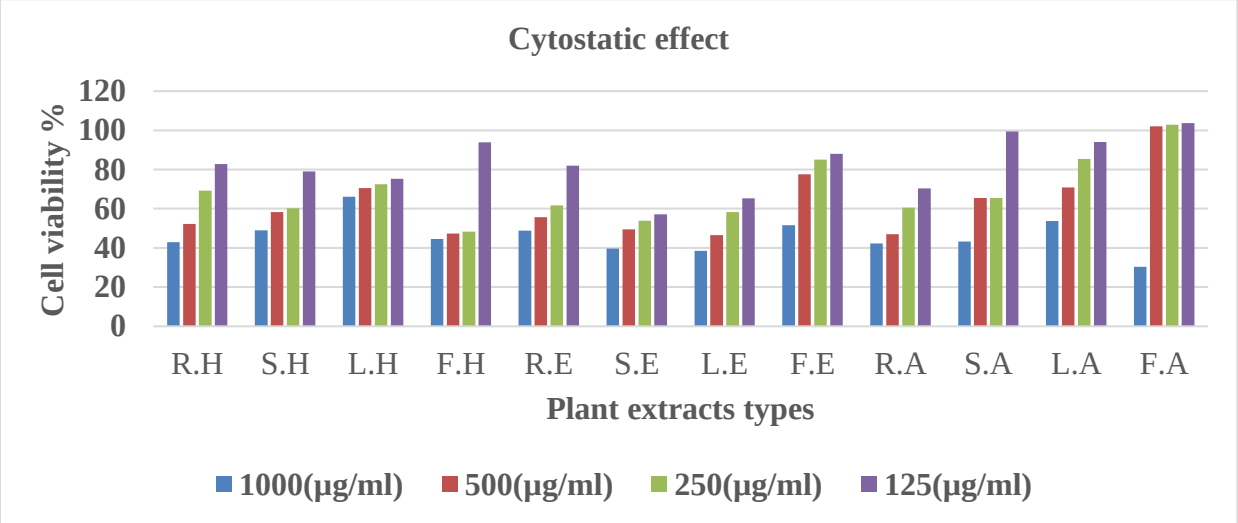

Figure 5. Cytostatic effect of *Micromeria fruticosa*extract effect on Colon cell line viability at different studied concentrations after 72 hours. **R**: Root, **S**: Stem, **L**: Leave, **F**: Flower, **H**: Hexane, **E**: Ethanol and **A**: Aqueous.

4. Conclusion

In this work, a GC-MS based characterization of the chemical composition of the different crude extracts from *Micromeria fruticosa* plant different botanical parts (leaves, stems, roots, and flowers), along with testing the antioxidant, antimicrobial and antitumor activities of the plant different parts and extracts was carried out.

A total of 27 phytochemicals were identified and quantified in the different parts' and extracts of *M. fruticosa* plant under study. *M. fruticosa* extracts attained with solvents of different polarities, showed variable anti-oxidant, anti-tumer and antimicrobial activities depending on the phytochemical composition of each extract. Interestingly, Oleamide,

Pulegone and Menthol were the main the functional ingredients detected with higher percentages in almost all of the samples analysed.

The aqueous extract of *M. fruticose* showed a morphological alteration and detachment in reference to the normal cells. Besides, MTT cell viability decreased at most examined aqueous plant species in a dose-dependent manner. Otherwise, the water extract of leaves showed a maximum percent of inhibition (90%) at concentration of 100 µg/mL. *Micromeria fruticosa* different extracts under study have both cytotoxic and cytostatic effects on Colon cell line in which cell growth is inhibited at all examined concentrations in a dose-dependant manner. One of the key steps in natural product processing is the selection of extraction solvent as was proved from the results of the biological activities variation among the different investigated extracs or/and botanical part. Further *in vivo* studies are needed to confirm the potential biological activities and to evaluate the toxicity and safety of plant extract. As well, additional studies are required to isolate the basic components responsible for potential pharmacological activities.

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