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FACULTY OF CHEMISTRY AND CHEMICAL ENGINEERING

Ph.D. THESIS SUMMARY

INVESTIGATIONS REGARDING THE EFFECT OF MICROWAVES ON BIOACTIVE CONSTITUENTS FROM SOME AROMATIC PLANTS BELONGING TO THE APIACEAE FAMILY

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LIST OF ABBREVIATIONS

AA	ascorbic acid
AC	acetic acid
DHAA	dehydroascorbic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
EPR	electron paramagnetic resonance
FID	flame ionization detector
GAE	gallic acid equivalents
GC	gas chromatography
GLV	green leaf volatiles
GSM	mobile telephony (Global System for Mobile Communication)
HPLC	high-performance liquid chromatography
LOD	limit of detection
LOQ	limit of quantification
M	maceration
M1	plants irradiate with microwaves of GSM frequency
M2	plants irradiated with microwaves of WLAN frequency
MAE	microwave-assisted extraction
MM1	dill plants irradiate with microwaves of GSM frequency
MPA	metaphosphoric acid
MS	mass spectrometry
PDA, DAD	photodiode array detector
PM1	parsley plants irradiated with microwaves of GSM frequency
PM2	parsley plants irradiated with microwaves of WLAN frequency
QE	quercetin equivalents
R	reference plants, nonirradiated (or C, control)
RES	electron spin resonance
TLC	thin-layer chromatography
TM1	celery plants irradiated with microwaves of GSM frequency
TM2	celery plants irradiated with microwaves of WLAN frequency

Trolox	6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
WLAN	wireless internet connection (Wireless Local Area Network)
UAE	ultrasound-assisted extraction
UV-VIS	ultraviolet-visibil
VOC	volatile organic compounds
TEM	transmission electron microscopy
TCA	trichloroacetic acid

Keywords: bioactive constituents, Apiaceae, microwaves, extraction, chromatography, UV-VIS spectroscopy, ultrastructural analysis.

INTRODUCTION

Due to their multiple properties, plants represent a particular interest for human nutrition, therefore it is important to know their chemical composition and how it is affected by external factors of climate or pollution generated by human technology.

As consequence, within the last years, some important research activities have been started concerning the influences of environmental stress factors on both crop and natural growing plants. It was concluded that both biological and chemical composition changes may occur in plants [2-6]. Among stress factors acting on plants, with strong impact on the global economy, are temperature (cold, heat), water deficit, ozone, salinity, mineral deficiency and toxicity of soil, electromagnetic radiations etc.

Nowadays, the use of mobile telephony and wireless devices has become more demanding and "mandatory", generating an exponentially increased level of electromagnetic radiations. Thus, a new stress factor occurred, namely the electromagnetic field, especially within the microwaves frequencies range (1 - 100 GHz) [7, 8]. As a result of this type of stress, a series of experiments related to the effects of electromagnetic field on plant growth and development, were performed [7-9, 12].

Consequently, the accumulation of new data and experimental evidence regarding the influence of microwaves on plant metabolic processes and, implicitly, on the nutritional quality of plants, has become an area of real interest and importance for scientific research.

PERSONAL CONTRIBUTIONS

In the Ph.D. thesis entitled: "Investigations regarding the effect of microwaves on bioactive constituents from some aromatic plants belonging to the Apiaceae family", the main objective was to determine the effect of microwaves of mobile telephony frequency (GSM) and wireless frequency (WLAN) on bioactive chemical constituents of three seasoning plants belonging to the Apiaceae family. Selected plants: parsley (*Petroselinum crispum*), dill (*Anethum graveolens*) and celery (*Apium graveolens*), are frequently used do to their multiple medicinal and culinary properties.

The main novely of this thesis consists in: the growth of parsley, dill and celery plants in low microwave power field, in identical anechoic chambers, and the monitoring of variation in the amount of bioactive compounds in irradiated plants compared to control plants (or reference plants, grown in similar chambers, but in the absence of the microwave field).

In order to achieve this objective studies have been conducted on establishing effective methods of extracting the investigated compounds (ascorbic acid, volatile oils, polyphenolic compounds), methods that were subsequently used for the extraction of these compounds from irradiated and non-irradiated plants.

The category of novelty elements of the thesis also includes: the establishing of differences occurred in emissions of volatile organic compounds released from irradiated plants compared to control plants, and the ultrastructural and morphological analyses on leaves of the plants considered for this study.

As result of the investigations performed, was determined an increase in the content of volatile oils, ascorbic acid and polyphenolic compounds in plants irradiated with GSM microwaves, while the effect of WLAN microwaves was variable, depending on the plant analyzed.

Ultrastructural and morphological analyses led to the conclusion that microwaves of WLAN frequency affect more significant the plants compared to GSM microwaves.

The present studies are important because, to date, the literature does not provide sufficient data and clear conclusions regarding the effects of microwaves on living world. Investigating the influence of low power microwaves on biologically active plant chemicals is an important step in this research field.

EXPERIMENTAL PART

CHAPTER 4

SELECTION OF THE EFFICIENT METHOD FOR EXTRACTION OF VOLATILE OILS FROM STUDIED PLANTS

4.1. Extraction methods

Parsley (*Petroselinum crispum*), dill (*Anethum graveolens*) and celery (*Apium graveolens*), aromatic plants belonging to the Apiaceae (Umbelliferae) botanical family, are

among the most popular plants used in Romanian cuisine, due to their rich content in volatile oils and other bioactive compounds.

The essential oils are very complex natural mixtures that consist of molecules produced through different secondary metabolic pathways, characteristically containing terpenoids, benzenoids and sometimes aliphatic compounds [52]. Both the composition and content of essential oils has been shown to strongly depend on plant species and environmental conditions [161, 165, 209].

Extraction of volatile oils from *Petroselinum crispum, Anethum graveolens* and *Apium graveolens*. For extraction of volatile oils from the seasoning plants studied, was used 1 gram of fresh vegetal material (leaves), which was subsequently subjected to extraction by different techniques, using different solvent systems [211], namely: E1 – ethanol - diethil ether (1:1, v/v), E2 – ethanol, E3 – *n*-hexane, E4 – diethyl ether, E5 – diethyl ether - *n*-hexane (1:1, v/v). All extractions were performed in triplicate, and the extraction temperature was 30ºC. The plant extracts were prepared by three extraction methods: maceration, ultrasound-assisted extraction (UAE) and microwave-assisted extraction (MAE).

The establishment of effective extraction conditions (extraction solvent and method) was performed using reference plants, and the most efficient method was further used for analysis of microwave irradiated plants. The analysis of extracts was performed by chromatographic techniques (TLC, GC-FID, GC-MS).

4.2. Determination of volatile oils from studied plants by chromatographic methods

4.2.1. Determintion of volatile oils from studied plants by gas chromatography with flame ionization detector (GC-FID)

GC-FID analyses were carried out on a Shimadzu GC-2010 gas chromatograph with flame-ionization detector equipped with AT-5 type 30 m long capillary column. The injection temperature was set to 250°C and the injection volume was 1 μL. Helium was used as carrier gas with a constant flow of 4 mL/min, starting from 50ºC and maintaining for 2 min, a heating rate of 8°C/min to 250°C and maintaining for 15 min [211].

In order to establish the most effective method for extraction of volatile oils, the registered chromatograms and the peak areas were compared. From these data was concluded that the most efficient extraction technique was maceration, followed by ultrasound-assisted

extraction, while the best extraction solvent system was E5 (diethyl ether: *n*-hexane, 1:1, v/v), followed by E3 (*n*-hexane).

4.2.2. Determination of volatile oils from studied plants by gas chromatography coupled with mass spectrometry (GC-MS)

For this type of chromatographic analysis was employed a system consisting of a gas chromatograph coupled with mass spectrometer, Shimadzu GCMS-QP2010 Plus (Kyoto, Japan), equipped with DB-5-MS Agilent Technologies column type (30 m, inner diameter 0.25 mm, film thickness 0.25 mm). The conditions for volatile oils analysis were as follows: injector temperature was 215°C, initial oven temperature at 40°C was held for 1 min; ramped at 5°C/min up to 200°C, held at this temperature for 1 min; ramped at 10°C/min up to 220°C and held for further 5 min. Helium (purity 99.9999 %, Elmer Messer Gaas AS, Tallinn, Estonia) was employed as carrier gas with a constant flow rate of 1 mL/min. The mass spectrometer was operated in electron-impact mode (EI) at 70 eV, in the scan range *m/z* 30 – 400, the transfer line temperature was set at 240°C and ion-source temperature at 150°C [211].

The volatile compounds were identified based on mass spectra of individual compounds using a spectral library, and respectively, comparing the GC retention data with reference standards (Table 1) [211].

Table 1. Major volatile compounds identified in parsley, dill and celery extracts.

Component	R.T. (min)	Area (%)		
		Parsley	Dill	Celery
α-Phellandrene	10.21	0.73	14.68	-
β-Phellandrene	10.94	15.47	-	-
1,3,8-*p*-menthatriene	13.45	12.17	0.21	-
Dill ether	15.62	-	8.39	-
Myristicin	24.52	14.87	2.86	0.08
Elemicin	25.29	-	5.06	-
Apiol	28.22	16.91	0.83	-
Allyl phenoxyacetate	29.27	-	-	53.58
Cyclotetracosane	34.47	8.35	-	-
(*E*)-5-Eicosene	38.61	8.29	-	1.62

The major components identified in parsley extracts were β-phellandrene (15.47%), 1,3,8-*p*-menthatriene (12.17%), myristicin (14.87%) and apiol (16.91%). The major volatile

components identified in dill and celery extracts were α-phellandrene (14.68%) and, respectively, allyl phenoxyacetate also known as pineapple ether (53.58%).

4.2.3. Determination of volatile oils from studied plants by thin layer chromatography (TLC)

Analysis was performed on 10 x 10 cm HPTLC 0.2 mm silica gel 60 plates (Merck, Darmstadt, Germany). The extracts and standard solutions were applied as 1.2 cm bands at 1.5 cm from bottom edge and at 1 cm from lateral edges, using a Camag Linomat V (CAMAG, Switzerland) semiautomatic sample applicator with a 500 μL syringe. The volume of extract applied on each band was 200 μL. Plates were developed on 8 cm distance in a chromatographic presaturated chamber for 10 min. The separation was performed using petroleum ether-dichloromethane (30:70, v/v) as mobile phase. After spraying with vanillin/ sulfuric acid reagent and heating the plates at 120°C for 4 min, in order to record the peak areas of compounds, the plates were analyzed at 600 nm using a photodensitometer (III TLC scanner CAMAG, Switzerland) in order to record the peak areas of compounds. The vanillin/sulfuric acid stained used for visualization was prepared by adding concentrated sulfuric acid (2.5 mL) to a solution obtained by dissolving vanillin (15 g) in ethanol (250 mL).

Table 2. Retention factors of the compounds identified in parsley, dill and celery by TLC.

Component	**R_F**
α-Phellandrene	0.23
β-Pinene	0.42
Myristicin	0.72

Determination of the compounds α-phellandrene and β-pinene from dill extracts (*Anethum graveolens*) prepared with the solvent systems E1-E5 was performed by HPTLC method. The following parameters were determined for the validation of the method: selectivity, linearity, limits of detection and cuantification, precision and accuracy. The amounts of volatile compounds from extracts were calculated based on the calibration curves, obtained by applying a 10 μL volume from the stock solutions of some standard compounds (α-phellandrene and β-pinene) [213].

CHAPTER 5

SELECTION OF THE EFFICIENT METHOD FOR EXTRACTION OF L-ASCORBIC ACID FROM STUDIED PLANTS BELONGING TO THE APIACEAE FAMILY

Vitamin C (L-ascorbic acid, ascorbic acid or ascorbate, AA) is a highly effective antioxidant and an essential compound for human body, being supplemented from fruits and vegetables.

5.1. Extraction methods

In this activity, was performed the extraction of ascorbic acid by three methods: wet grinding followed by centrifugation (1), sonication (2) and microwave field extraction (3), using as extracting agents different aqueous solutions, of concentration 8%, of the following acids: trichloroacetic acid (TCA), metaphosphoric acid (MPA) and acetic acid (AC).

5.2. Determination of L-ascorbic acid by high performance liquid chromatography

HPLC method. The analysis of L-ascorbic acid was carried out on a Shimadzu HPLC model LC-2010 with PDA detector and a Grace Alltima C18 column, 3 µm, 100 x 3 mm. The elution was performed with gradient elution using a mobile phase consisting of 15 mM phosphate buffer at pH 2.7 (A) and methanol (B), and the flow rate was set at 0.4 mL/min. The gradient elution program was as follows: min 0: 10% B, min 5: 20% B, min 10: 10% B. The column was thermostated at 30°C, and the injection volume was 20 µL. The wavelength for detection of L-ascorbic acid was 243 nm, and the retention time was 1.85 min [221]. Based on experimental data, was determined that the most efficient extraction method for ascorbic acid was ultrasound extraction, and 8% acetic acid was the most efficient extracting agent (Figure 26).

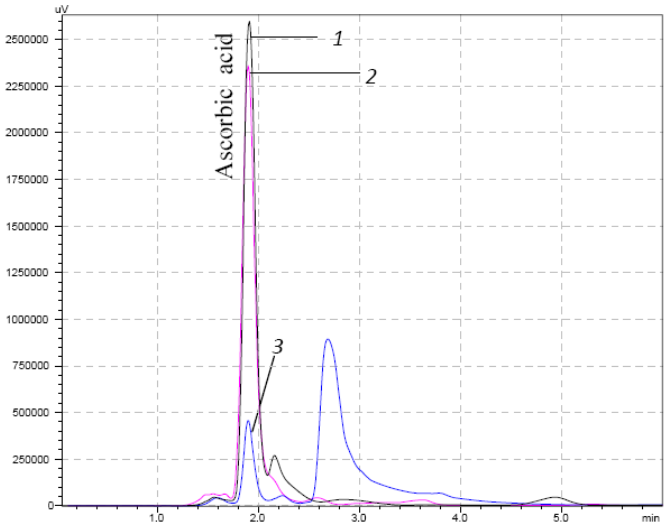


Figure 26. Chromatograms of *Petroselinum crispum* extracts obtained by ultrasonic-assisted extraction with 8% aqueous solutions of different acids: (*1*) AC; (*2*) MPA; (*3*) TCA.

Based on experimental data and from equation of the calibration curve, were determined the amounts of ascorbic acid (Figure 28) from the three seasoning plants studied: parsley (264 mg AA/100 g fresh plant), dill (121 mg AA/100 g fresh plant) and celery (103 mg AA/100 g fresh plant) [221].

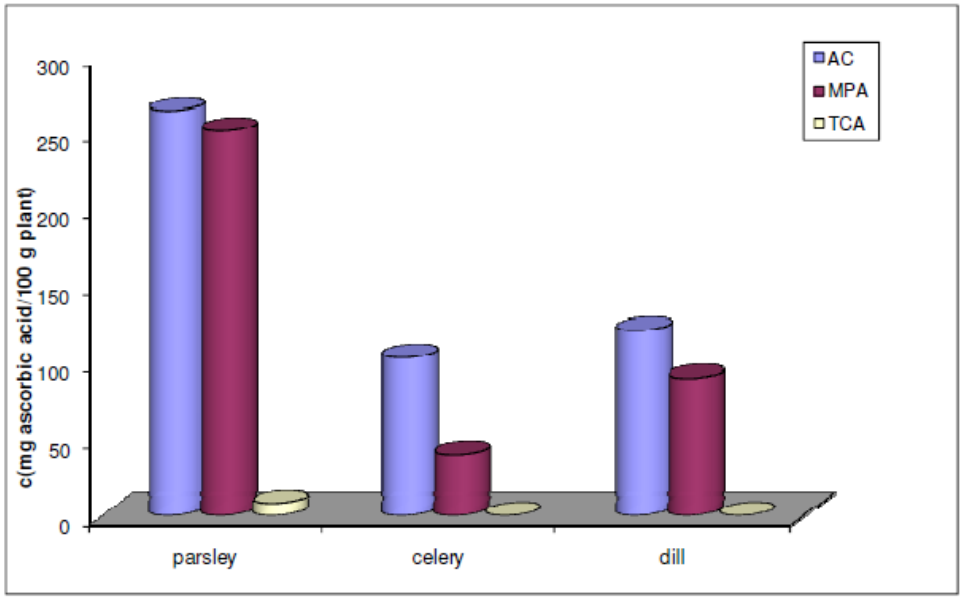


Figure 28. The content of L-ascorbic acid determined from extracts of parsley, celery and dill obtained by UAE extraction method with 8% aqueous solutions of acetic acid (AC), metaphosphoric acid (MPA) and trichloroacetic acid (TCA).

CHAPER 6

SELECTION OF THE EFFICIENT METHOD FOR EXTRACTION OF FLAVONOIDS FROM STUDIED PLANTS

6.1. Extraction and identification of some flavonoids from *Petroselinum crispum* extracts

For the following study vegetal material of commercial origin was used. The parsley extracts were prepared by there extraction procedures: maceration, ultrasound-ssisted extraction and solvent extraction in microwave field. For extraction purpose a systematic variation of different proportions of ethanol-water (v/v) mixtures (100:0, 90:10, 80:20, 70:30, 60:40, 50:50, 40:60) were used. 0.5 g plant (leaves), finely powdered, was extracted with 20 mL solvent mixture. After filtration and washing, the final volume of extracts was adjusted to 25 mL with the

same solvent mixute used for extraction. All extractions were performed at 35°C. The quantitative determination of flavonoids from parsley extracts was performed by colorimetric method using alluminium chloride [122].

According to spectrophotometrical data, the highest quantity of flavonoids was determined for the extracts obtained by maceration with ethanol-water (50:50, v/v) [229], followed by sonication with etanol-apă (60:40, v/v), and extraction in microwave field with ethanol-water (40:60, v/v).

Maximum absorbance for the hydroalcoholic extracts was obtained at wavelength 390 ± 2 nm (Figure 29). In order to reduce the extraction time, the extraction of flavonoids from irradiated plants will be performed by ultrasound extraction (UAE).

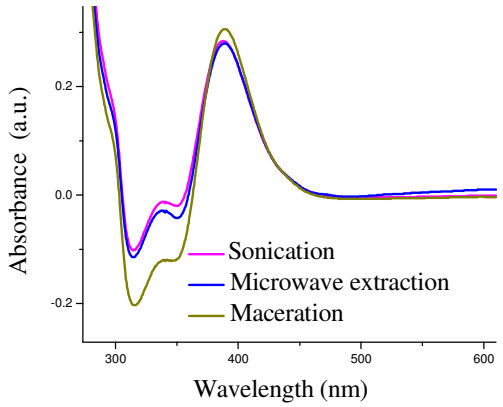


Figure 29. Determination of total flavonoid content from parsley extracts obtained by the three extraction methods.

6.1.2. Analysis by high performance liquid chromatography

Identification of some flavonoid compounds from parsley extracts was performed by HPLC analysis using a HPLC Shimadzu 2010 system equipped with two detectors: mass spectrometer (MS) and photodiode array detector (PDA). The HPLC column utilized was Grace Alltima C18 (100 x 3 mm, 3µm). The mobile phase consisted of acetonitrile (A) and distilled water - formic acid (99.9:0.1, v/v) (B) [126]. The gradient elution program used was as follows: min 0: 5% A, min 30: 42% A, min 35: 5 % A. The flow rate was 0.43 mL/min, and the detection wavelength was 220 nm. By comparing the retention times with those of some flavonoid standards (100 µg/mL), and based on mass spectrometry data, in parsley extracts were identified flavonoid compounds such as flavones (apigenin and luteolin) and flavonols (quercetin and kaempferol) [229].

CHAPTER 7

INFLUENCE OF MICROWAVES ON BIOACTIVE COMPOUNDS FROM THE STUDIED PLANTS BELONGING TO THE APIACEAE FAMILY

7.1. Growth of the plants selected for study in microwave field

The plants considered for this study (parsley, celery and dill), were grown both in classical, reference conditions, and in microwave field. The seeds were purchased from Agrosel company (Câmpia Turzii, Romania): leafy parsley (*Petroselinum crispum*, Plain leaved 2), dill (*Anethum graveolens ssp. hortorum*, Common) and leafy celery (*Apium graveolens*, Pascal Giant). An equal number of seeds have been planted in identical pots of the same volume (150 mL), filled with equal amounts of gardening soil of commercial provenience.

Three weeks after seeding, the vessels with plants were placed in three identical anechoic chambers with 37x37x37cm^3 dimenssions and the walls lined with pyramid structure of 4 cm height, and were maintained under the same conditions of temperature and humidity, and fully-closed [231]. The chambers were maintained in the same conditions: light intensity (300 μmol m^{-2} s^{-1}), temperature (25°C), CO_2 concentration (385 ± 20 ppmv) and humidity (65%), and were characterized by a degree of isolation of 60 dB at radio-frequency range (about 3 kHz to 300 GHz) between the exterior and interior. One chamber was for non-treated control plants, while plants in the other two chambers were subjected to microwave irradiation (Figure 33).

The microwave irradiation was performed with microwaves at bands corresponding to:

1) GSM (mobile devices) using a modified AP5200 generator (D-LINK, China), operating in four bands, 860 – 910 MHz frequency range, Pout 29 dBm (1000 mWatt) (**M1** plants);

2) WLAN (wireless router) using a D-LINK wireless router 802.11g/2.4 GHz (2.412 – 2.48 GHz frequency range, main operating channel at 2.42 GHz, Pout 19 dBm ($10^{1.8}$ mWatt) (**M2** plants).

The power density to the base of chambers was measured with a spectrum analyzer SPECTRAN HF 4060, AARONIA AG (Germany). Inside chambers the plants were grown for three weeks and were periodically watered with equal volums of water (10 mL per pot). After three weeks the plants were removed from the chambers, the leaves were excised, and subsequently were prepared and analyzed the extracts.

Figure 33. Plants of celery introduced in the anechoic chamber for irradiation with GSM frequency microwaves.

7.2. Determination of volatile oils from the seasoning plants subjected to microwave irradiation

7.2.1. Determination of volatile oils by gas-chromatography coupled with mass spectrometry

For both essential oil and volatile organic compounds (VOCs) analysis, a GC-MS system composed of a Shimadzu QP2010 Plus gas chromatograph coupled with quadrupole mass spectrometer (Kyoto, Japan) was used.

For analysis of volatile oils was used the method previously presented in chapter 4 (section ***4.2.2*****).** The volatile oils and volatile organic compounds emitted by plants were identified by comparing the mass spectra of individual compounds with the spectra of GC purity external standards (Sigma Aldrich, St. Louis, MO, USA), using a spectral library, and quantitative determinations were performed based on the calibration curves corresponding to identified compounds.

Our study observed complex composition of essential oils in the seasoning plants studied: 10 compounds were detected in *Petroselinum crispum,* 11 compounds in *Anethum graveolens* and 7 compounds in *Apium graveolens*. In all species, monoterpenes constituted a significant component of the essential oil (Figure 36), in addition, several specific benzenoids were also dominating components: apiol in *Petroselinum crispum*, and myristicin and dillapiol in *Anethum*

graveolens (Figure 36). The main volatile compounds identified in *Apium graveolens* were 3-hexen-1-ol, myrcene, α-ocimene and γ-terpinene (Figure 36).

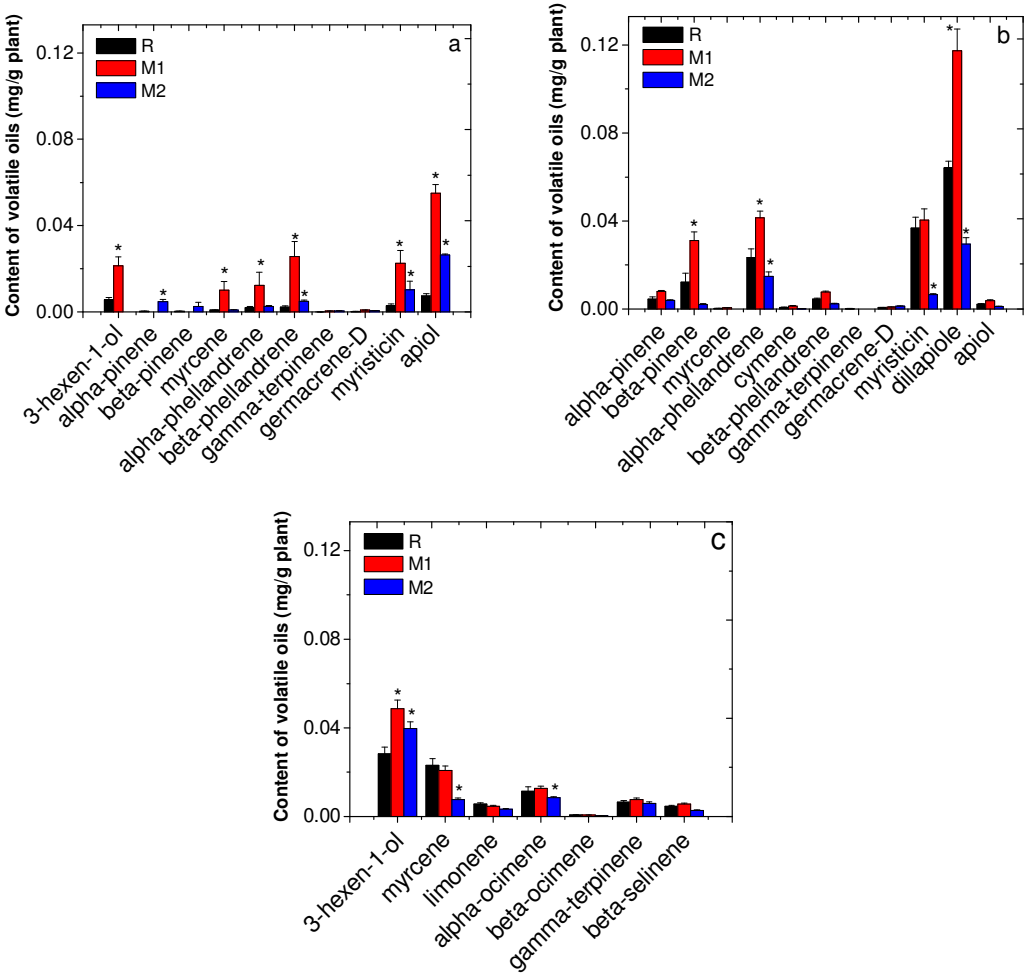


Figure 36. Changes in terpene content (mg/g fresh weight, FW) in *Petroselinum crispum* (a), *Anethum graveolens subsp. hortorum* (b) and *Apium graveolens* (c) foliage in response to microwave irradiations at bands corresponding to wireless router (WLAN) and mobile devices (GSM). Each data point is the mean (± SE, standard error) of three independent replicate experiments with a different plant. *significant statistical differences between irradiated and control plants ($P < 0.05$).

In our study, microwave irradiation by GSM-frequency microwaves generally increased the volatile oil contents (Figure 36), while the effect of WLAN-frequency microwaves was less clear, their content varying for different compounds and species (Figure 36). Among the three plant species tested in these experiments, the strongest effects of microwave irradiation on volatile oils were observed on dill plants, *Anethum graveolens* (Figure 36b). The structure of

Apium graveolens leaves was the least affected by microwave irradiation and the effect on "leaf chemistry" was also the least in this species, celery plants being the most tolerant to microwave action.

7.2.2. Determination of volatile oils from studied plants by thin layer chromatography

In this study, the quantification of linalool and myristicin from parsley, dill and celery extracts of microwave irradiated and non-irradiated plants, based on the equations of calibration curves for the two compounds was performed [232].

7.2.3. Determination of volatile oils by high performance liquid chromatography

The amounts of myristicin and linalool from irradiated and non-irradiated plants were also determined by HPLC analysis. Analyses were performed using a HPLC Shimadzu liquid chromatograph with diode array detector (DAD). Chromatographic separation was performed on LiChrosorb RP-18 colum type (5 µm, 25 x 0,4 cm, Merck, Germany) thermostated at 25°C with gradient elution, with the mobile phase consisting of ultrapure water (A) and acetonitrile (B). The program of gradient elution started from 0 to 1 min with 100% B and then the eluent B decreased in 15 min to 25%. The injection volume was 5 µL, and the flow rate was set to 1 mL/min. The chromatographic peaks corresponding to the studied compounds showed maximum absorptions at 197 nm for linalool and 201 nm for myristicin. Based on the equations of the corresponding calibration curves were calculated the amounts of the two compounds analyzed (Figure 40).

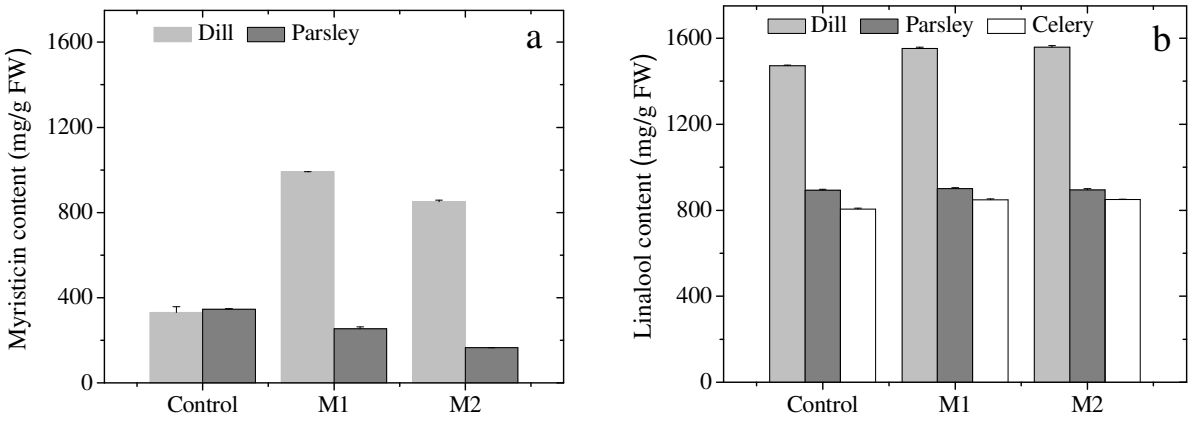


Figura 40. Quantities of myristicin (a) şi linalool (b) (µg/g fresh weight, FW) in aromatic plants (dill – *Anethum graveolens*, parsley – *Petroselinum crispum*, and celery – *Apium graveolens*) after irradiation of GSM frequency microwaves (M1 plants) and WLAN frequency microwaves (M2 plants).

7.3. Analysis of the volatile organic compounds emissions generated by plants under microwave effect

Volatile organic compounds (VOC) sampling was performed using a portable gas exchange system GFS-3000 (Waltz GmbH, Effeltrich, Germany). For each analyzed plant, volatile organic compounds were sampled in a multibed stainless steel cartridge (8.88 × 0.65 cm, Supelco, Bellefonte, PA, USA). Blank air samples were taken from the room before and after the measurements at a flow rate of 200 mL/min for 20 min using a 1003-SKC pump at constant rate (SKC Inc., Houston, TX, USA) at room temperature.

For VOC analysis, an automated cartridge desorber Shimadzu TD20 (Kyoto, Japan) was used connected to a gas chromatograph with MS-Shimadzu QP2010 Plus (Kyoto, Japan). Analysis of volatile organic compounds emitted by analyzed plants was performed following the protocol described by Copolovici et al. [144]. The amounts of volatiles released by control and microwave irradiated plants (parsley, dill and celery), is expressed as Φ (nmol m^{-2} s^{-1}), representing the number of nmoles of volatile substances emitted per 1 m^2 leaf area per second (s).

Our data demonstrate that the emissions observed did reflect a mixture of both storage emission consisting of compounds present in essential oils and "de novo" emissions.

The blend of volatiles was very complex and, in all plant species, the non-stressed plants also emitted monoterpenes and benzenoids present in essential oils, in some cases even compounds not-present in essential oils (Figure 41). The number of compounds detected in the emissions was greater than in the essential oils, and characteristic "de novo" released stress volatiles were observed (Figure 41). 16 compounds were detected in the emissions of *Petroselinum crispum*, 16 compounds in *Anethum graveolens* and 20 compounds in *Apium graveolens.*

In our study, all microwave-irradiated plants emitted the following GLVs: (*E*)-2-hexenal, (*Z*)-3-hexenol, 1-hexanol, while the emissions of GLVs ("green leaf volatiles") were very low, at the level of detection limit of our device in control plants (Figure 41). In general, in all plant species studied, the emissions of GLV were greater for WLAN-frequency microwaves compared to GSM-frequency microwaves (Figure 41, $P < 0.001$ for all).

These results suggest greater stress in the case of WLAN microwave irradiation, and are in agreement with the more significant changes in anatomy of leaves induced by WLAN microwaves (section 7.7., Table 13).

The total amount of volatile compounds emitted by microwave irradiated plants of parsley (*Petroselinum crispum*) and dill (*Anethum graveolens*) was five times higher than for the celery (*Apium graveolens*). As observed for volatile oils from this plant (Figure 36), *Apium graveolens* was less sensitive to microwaves than *Anethum graveolens* and *Petroselinum crispum*.

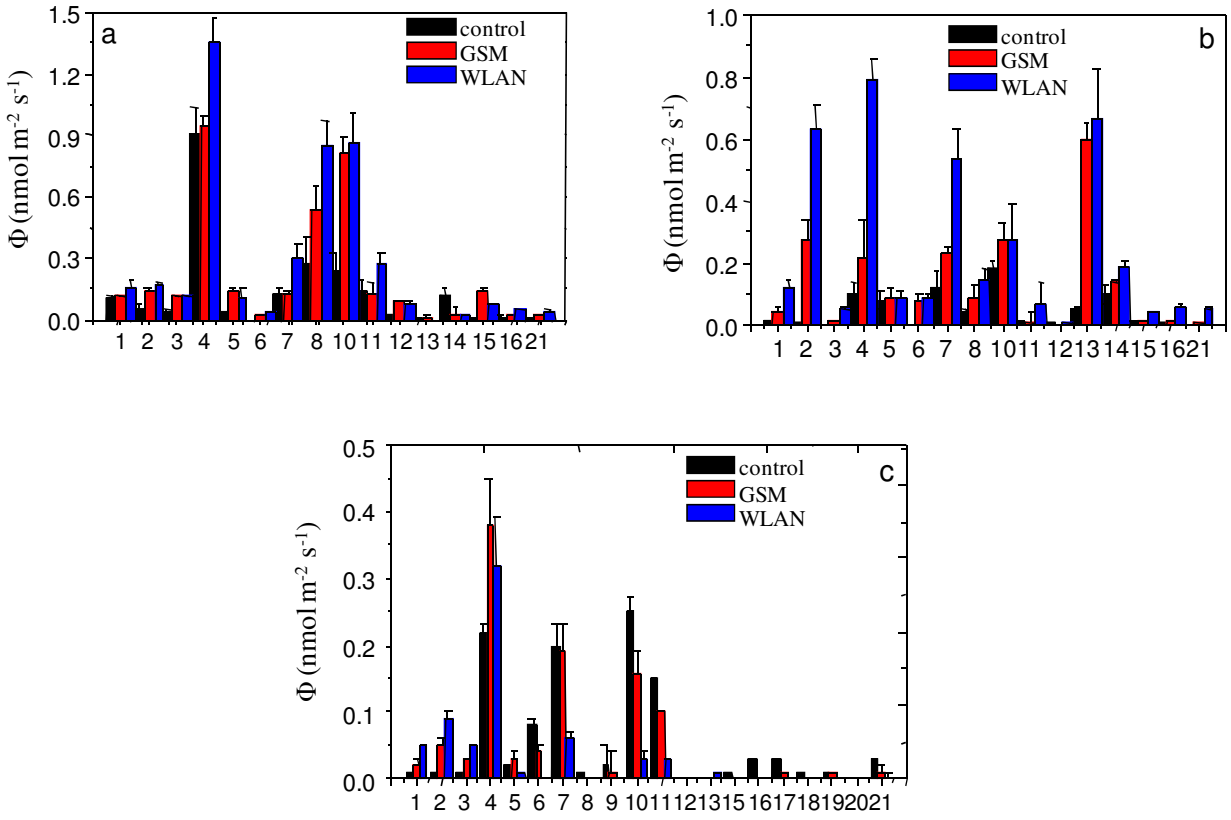


Figure 41. Alteration of the emission of volatile organic compounds (nmol m^{-2} s^{-1}) from foliage of *Petroselinum crispum* (a), *Anethum graveolens subsp. hortorum* (b) and *Apium graveolens* (c) in response to microwave irradiations at bands corresponding to wireless router (WLAN) and mobile devices (GSM). Each number corresponds to a particular volatile compound as follows: **1.** 1-hexanol; **2.** *(Z)*-3-hexen-1-ol; **3.** (*E*)-2-hexenal; **4.** α-pinene; **5.** camphene; **6.** β-myrcene; **7.** β-pinene; **8.** α-phellandrene; **9.** Δ-3-carene; **10.** D-limonene; **11.** *para*-cymene; **12.** β-phellandrene; **13.** (*E*)-β-ocimene; **14.** 1,8-cineol; **15.** iso-bornyl acetate; **16.** longicyclene; **17.** caryophyllene oxide; **18.** α-selinene; **19.** (*Z*)-β-farnesene; **20.** α-caryophyllene; **21.** geranylacetone.

Regarding emissions of monoterpenes detected in the three plants studied (α-pinene, β-pinene, camphene, limonene, Δ-3-carene, *para*-cymene, β-phellandrene, (*E*)-β-ocimen, eucalyptol and bornyl acetate) could be concluded the following:

- In *Petroselinum crispum*, emissions of α-pinene, α-phellandrene and limonene were dominant and enhanced by microwave irradiation, especially in the case of WLAN-frequency microwave treatment (Figure 41a).

- Monoterpene emissions from *Anethum graveolens* were dominated by α-pinene, β-pinene, and limonene, and these emissions were enhanced by microwave irradiation (Figure 41b).

- In *Apium graveolens*, the emissions were almost four times lower than in the other two plants and were dominated by α-pinene, β-pinene and limonene (Figure 41c).

Among the characteristic, stress induced monoterpenes [237, 240], it was observed that in dill the (E)-β-ocimen and 1,8-cineol emissions were strongly enhanced as result of microwave irradiation (Figure 41b). In addition, both parsley and dill plants emitted small amounts of longicyclene (a stress induced sesquiterpene) under WLAN-frequency irradiation.

7.4. Quantitative analysis of L-ascorbic acid

7.4.1. Analysis by high performance liquid chromatography

The ascorbic acid was quantified by external standard method following the validation protocol described described in chapter 5 (section 5.2). According to this previous study, L-ascorbic acid was detected at wavelength 243 nm, and the retention time was 1.85 min [221]. To obtain the calibration curve working ascorbic acid standard solutions in the concentration range 0.3 – 1 µg/mL were prepared by successive dilutions with ultrapure water from a stock solution of ascorbic acid. The limit of detection (0.2 µg/mL) and the limit of quantification (0.22 µg/mL) were calculated. The regression equation was expressed as y = 6E+07x - 9E+06 (where x–concentration, y–area), and the coefficient of correlation (R^2) was 0.9986.

Overlay of HPLC chromatograms of the extracts (the peaks corresponding to L-ascorbic acid) registered for irradiated and non-irradiated plants have shown an increase in the ascorbic acid content of plants grown in microwave fields (Figure 42).

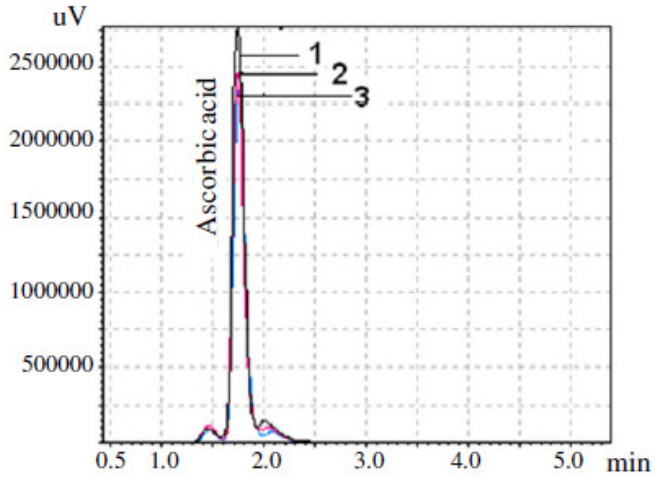


Figure 42. Chromatograms of celery extracts obtained by HPLC analysis method. Plants irradiated with microwaves: GSM (1) and WLAN (2). Non-irradiated, control plants (3).

Quantitative determinations of ascorbic acid from irradiated and non-irradiated plants were performed in order to establish the percentage variations (%) in the ascorbic acid content from these plants [241]. The amount of ascorbic acid per 100 g of fresh plant was determined for both irradiated (M1, M2) and control plants and are presented in Table 10.

Table 10. The amounts of ascorbic acid (AA) and the percentage increase (+%) reported to control plants determined by HPLC method.

Plant lot	Ascorbic acid content (mg AA/100 g plant) ± RSD (%)		
	Parsley	**Dill**	**Celery**
M1[a]	285 ± 0.78 (+8%)	201 ± 0.63 (+66%)	320 ± 0.56 (+211%)
M2[b]	282 ± 0.50 (+6.8%)	180 ± 0.95 (+49%)	290 ± 1.03 (+181%)
R[c]	264 ± 1.32	121 ± 1.41	103 ± 1.51

[a] plants irradiated GSM with GSM frequency microwaves; [b] plants irradiated with WLAN frequency microwaves; [c] control plants.

For non-irradiated (control) plants, the comparative evaluation of experimental data showed the highest amount of ascorbic acid in parsley. In irradiated plants, a greater increase in levels of L-ascorbic acid was determined in plants irradiated with GSM microwaves (Table 10).

The highest quantity of ascorbic acid was found in celery (*Apium graveolens*) plants irradiated with GSM microwaves, for which L-ascorbic acid concentration in celery leaves increased 3.1 times after three weeks of GSM microwave treatment (percentage increase of 211%). The lowest percentage increase in the ascorbic acid content was determined in parsley leaves under the influence of WLAN frequency microwaves (6.8 %).

7.5. Determination of the influence of microwaves on polyphenolic compounds from irradiated plants

The flavonoids and polyphenolic compounds were extracted by ultrasound extraction of the vegetal material (from microwave irradiated plants and control plants) with the solvent system ethanol + water (60:40, v/v), which proved to be the most effective extraction system (section 6.1.1).

7.5.1. Quantitative spectrophotometric analysis of flavonoids

The total content of flavonoids was evaluated by colorimetric method with aluminium chloride [122]. In Figure 46 was illustrated the content of flavonoids, calculated based on the calibration curve, from hydroalcoholic extracts of parsley, dill and celery.

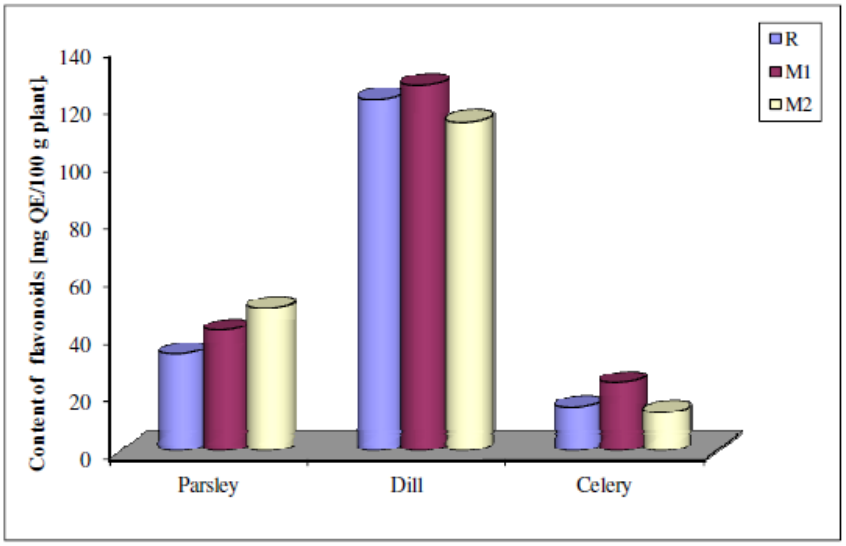


Figure 46. Comparative diagram of the total flavonoid content (expressed as quercetin equivalents, mg QE/100 g fresh plant) from extracts of parsley, dill and celery obtained by sonication with solvent system ethanol:water = 60:40 (v/v). M1-plants irradiated with GSM microwaves; M2-plants irradiated with WLAN microwaves.

7.5.2. Quantitative spectrophotometric analysis of total polyphenolic compounds

The total polyphenolic content was determined by Folin-Ciocalteu method following the experimental protocol described by Ivanova et al. [246]. The content of total polyphenolics (expressed as gallic acid equivalents, mg GAE/100 g fresh plant), calculated based on the calibration curve, from hydroalcohoilic extracts of parsley, dill and celery was presented in Figure 48.

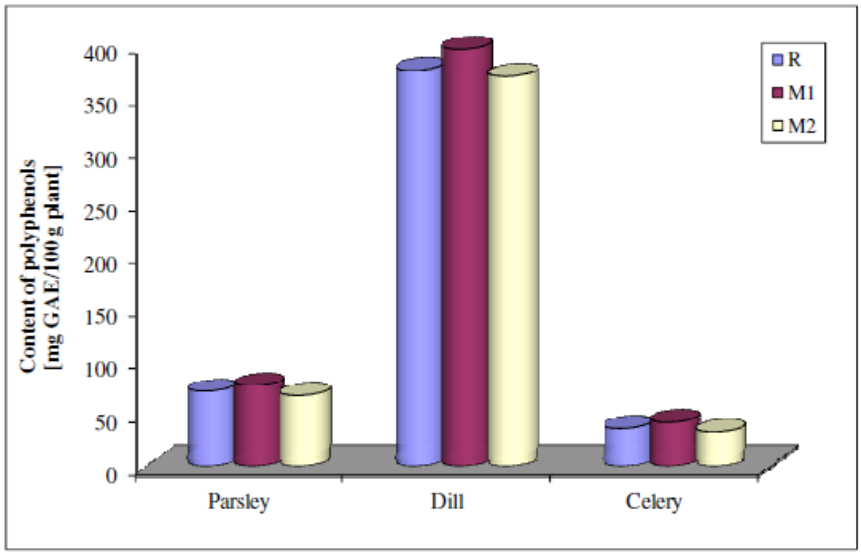


Figure 48. Comparative diagram of the total polyphenolic content (expressed as gallic acid equivalents, mg GAE/100 g fresh plant) from extracts of parsley, dill and celery obtained by sonication with ethanol:water = 60:40 (v/v). M1-plants irradiated with GSM microwaves; M2-plants irradiated with WLAN microwaves.

There were no significant differences between the amounts of total polyphenolic compounds from plants grown under the influence of microwaves compared to control plants.

7.5.3. Determination of antioxidant character of extracts from irradiated and non-irradiated plants by DPPH method

For determination of the antioxidant character of parsley, dill and celery extracts (from microwave irradiated plants and control plants) was employed the experimental method proposed by Garcia et al. [248].

Following the experimental results acquired by the DPPH method was concluded that hydroalcoholic extracts from plants irradiated with microwaves have a more pronounced antioxidant activity than extracts from control plants (Figure 51).

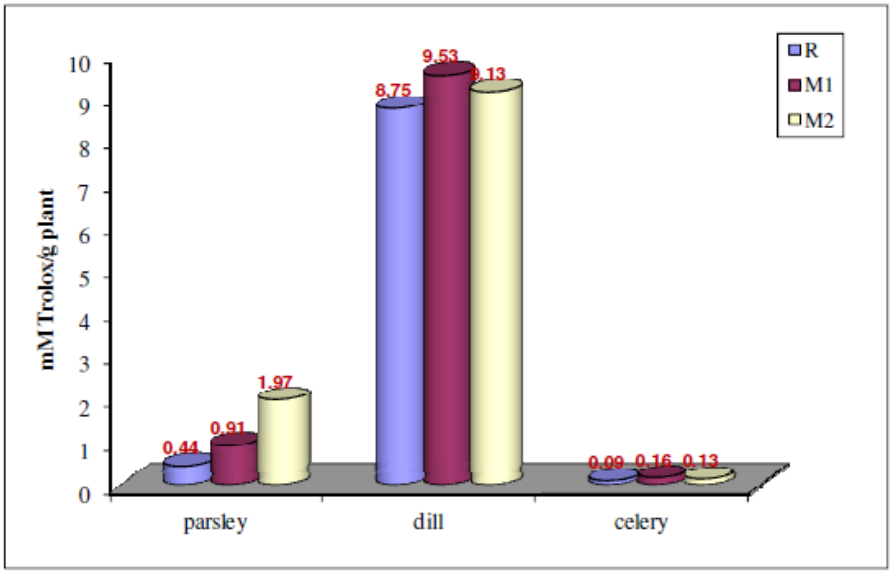


Figure 51. Antioxidant activity of hydroalcoholic extracts of parsley, dill and celery analyzed by DPPH method. R – control plants; M – GSM microwave irradiated plants; M2 – WLAN irradiated plants.

7.7. Ultrastructural and morphological analysis of the seasoning plants studied

The purpose of this study is to analyze the effects of irradiation with GSM and WLAN microwaves in the leaves of parsley, dill and celery plants.

The investigations were performed with a 120 kV TEM Model JEM 1010 (Jeol USA Inc., Peabody, MA, USA) with CCD camera. Samples for transmission electron microscopy (TEM) were processed by fixation in glutaraldehyde and osmic acid, infiltrated and embedded in epoxy resin, Epon 812, and cut (100 nm) in an Ultramicrotome, Leica UC6 with a diamond knife, sections that were contrasted with U and Pb atoms (solutions of uranyl acetate and lead citrate). All procedures were carried out according to conventional techniques and methodologies used in analysis by transmission electron microscopy.

For parsley, experimental groups of plants consist of: R – control plants grown in the reference chamber in the same conditions (PR); M1 – plants irradiate by GSM microwaves (PM1); M2 – plants irradiated by wireless microwaves (PM2). The same experimental model was applied for celery (TR and TM1, TM2) and dill (MR and MM1).

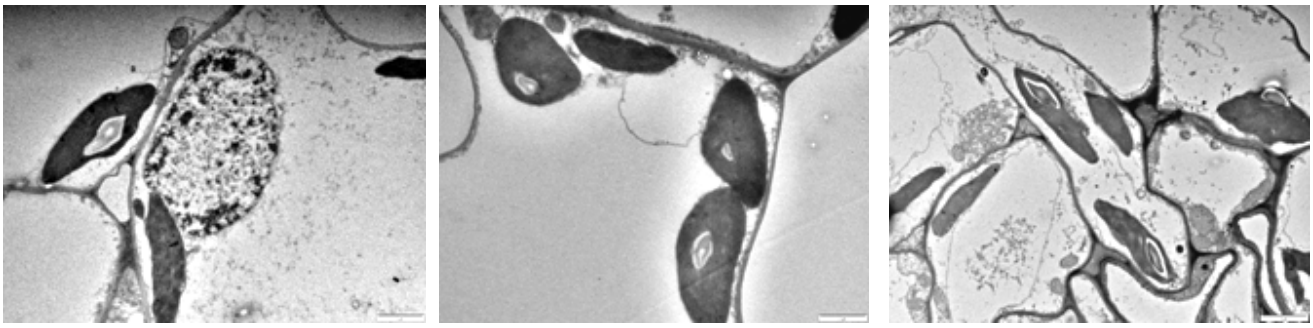
For ultrastructural analysis of leaves collected from all plant experimental groups, were considered the main components of foliar limb, namely: upper epidermis covered by cuticle,

palisade parenchyma having the most chloroplasts within its cells, mesophilic tissues found in the middle of foliar limb, where the bast and wooden vessels are located, lacunar tissues with rarefied arrangement of cells and few chloroplasts, and lower epidermis [256, 258, 259].

Among the cellular components were mainly aimed the photosynthesizing organelles like chloroplasts (the photosynthetic organelles), mitochondria (the energy supplying organelles) and the nucleus as coordinator of cellular metabolism.

Analysis of images obtained for the leaves of plants irradiated with GSM frequency and power, compared to images of reference plants, exhibit the following ultrastructural features:

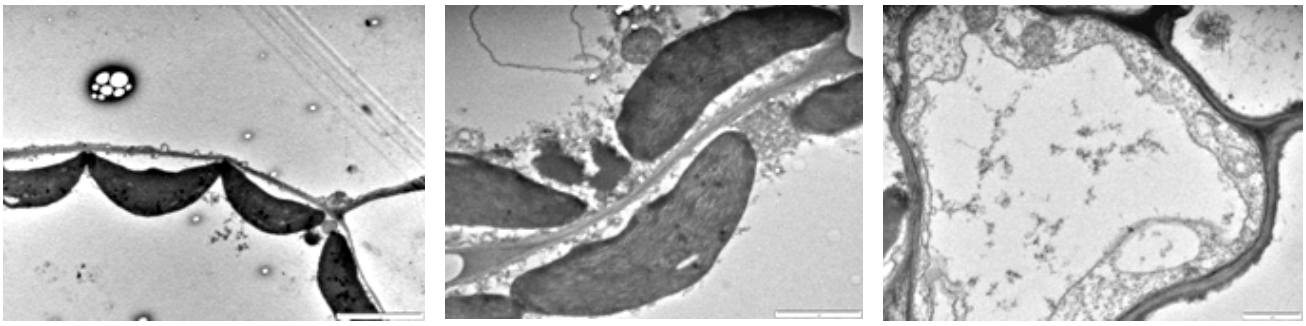
- Chloroplasts retained their ultrastructure and normal arrangement of cells (Figure 55a, 55b), but the presence of starch grains is more evident, suggesting a slightly increase in its synthesis.



a b c

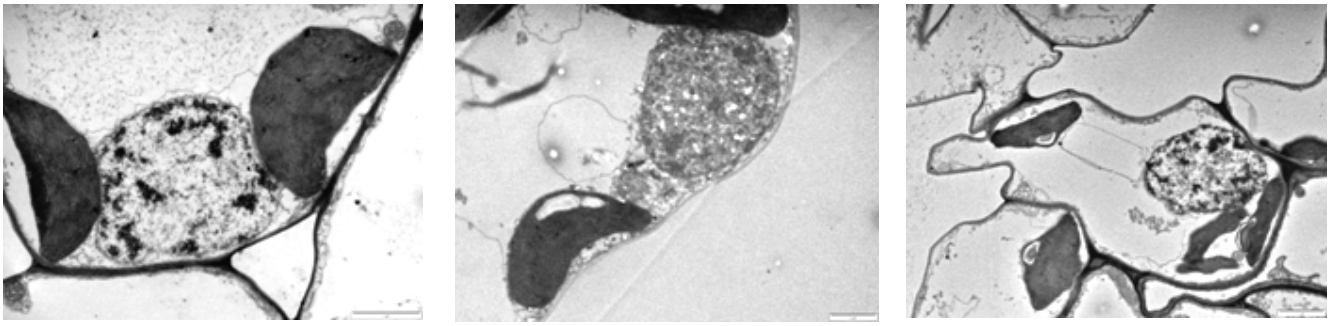
Figure 55. TEM images of chloroplasts for: a) PR; b) PM1; c) PM2.

- Mitochondria have electrondensified matrix and mitochondrial cristae slightly rarefied (Figure 56a, 56b), suggesting a slight decrease in their metabolic activity.



a b c

Figure 56. TEM images of mitochondria for: a) PR; b) PM1; c) PM2.



a b c

Figure 57. TEM images of cell nucleus for: a) PR; b) PM1; c) PM2.

In conclusion, GSM-intensity microwaves used in our experiments induce relatively small changes in the ultrastructure of parsley leaf, evidenced at the cellular walls level, in mitochondria, chloroplasts and especially, in nuclei of some cells, without significantly affecting the overall metabolic activities of the leaf.

Analysis of images obtained for irradiated plants with microwaves of WLAN intensity and power, emphasizes that incipient adverse effects presented for M1 groups of plants have enhanced displaying obvious alterations as follows:

- Chloroplasts are fewer, most do not have the classical horn shape, but all have one starch grain (Figure 55c), suggesting accumulation of energy reserves for protection against the stress generated by wireless high frequency microwaves.

- Mitochondria have rarefied matrix and cristae, showing alteration signs (Figure 56c).

- Nuclei show apparent normal structure, with more than the normal amount of heterochromatin, and slightly irregular shape. The structure in some cells nuclei is altered, without distinct evidence of euchromatin and heterochromatin in caryolympha (Figure 57c).

In conclusion, comparing the effects of the two types of microwaves on leaf cell ultrastructure of the studied plants, it could be concluded that wireless microwaves (WLAN) used in the experimet induce negative changes significantly greater than those observed in the case of GSM microwaves. *Apium graveolens* (celery) has proven to be the anatomically most resistant (tolerant) species to microwaves, while *Anethum graveolens* (dill) was the most affected in this regard (Table13).

Table 13. Ultrastructural analysis of the leaves of studied plants.

Treatment	Cell wall thickness (µm)	Chloroplast length (µm)	Chloroplast area (μm^2)	Mitochondrion length (µm)	Ratio of starch grain area to chloroplast area (%)
Petroselinum crispum					
Control	0,300 ± 0,07	6,78 ± 0,12	13,31 ± 0,22	1,00 ± 0,27	8,93 ± 0,13
GSM	0,250 ± 0,06	6,76 ± 0,28	8,491 ± 0,06	0,90 ± 0,13	9,99 ± 0,12
WLAN	0,200 ± 0,05	6,50 ± 0,16	7,807 ± 0,11	0,70 ± 0,05	6,01 ± 0,08
Apium graveolens					
Control	0,187 ± 0,01	5,90 ± 0,13	8,43 ± 0,23	1,68 ± 0,13	5,21 ± 0,13
GSM	0,175 ± 0,01	5,20 ± 0,17	8,08 ± 0,29	1,00 ± 0,25	8,13 ± 0,08
WLAN	0,175 ± 0,01	4,85 ± 0,31	7,04 ± 0,22	0,80 ± 0,10	0
Anethum graveolens					
Control	0,160 ± 0,01	5,80 ± 0,20	7,68 ± 0,14	1,57 ± 0,08	5,34 ± 0,28
GSM	0,156 ± 0,01	4,33 ± 0,26	7,06 ± 0,35	0,55 ± 0,13	0
WLAN	0,136 ± 0,01	3,33 ± 0,26	6,68 ± 0,14	0,25 ± 0,13	0

± SE for six independent measurements.

CONCLUSIONS

The main objective of the present Ph.D. thesis was to determine the effect of microwave frequency bands GSM (mobile telephony) and WLAN (wireless), respectively, on bioactive compounds from composition of three aromatic plants belonging to the Apiaceae family: *Petroselinum crispum* (parsley), *Anethum graveolens* (dill) and *Apium graveolens* (celery).

Following the studies it was found that:

• Depending on the solvent and the extraction technique used were extracted various classes of bioactive compounds, in various amounts.

• The most effective method for the extraction of volatile oils from parsley, dill and celry was found to be ultrasound extraction with *n*-hexane – diethyl ether (1:1, v/v) solvent system.

• The effective extraction of L-ascorbic acid was achieved by ultrasound extraction with 8% aqueous solution of acetic acid.

• In the case of plants considered for the present study, ultrasound extraction with solvent system ethyl alcohol – water (60:40, v/v) was used for extraction of polyphenolic compounds, as the most effective.

• It was established that changing conditions of plant growth by introducing them in the microwave field with different intensities (GSM and wireless) influenced both plant development and content of biologically active compounds investigated.

• Based on HPLC analyses, it was concluded that the highest increase in the *L-ascorbic acid* content was registered for celery irradiated by GSM microwaves (211%), while the lowest increase was determined for parsley irradiated by WLAN microwaves (6.8%).

• Analysis of *volatile oils*, performed by GC-MS, showed that their content increased in the plants subjected to GSM microwaves, while the effect of WLAN microwaves was variable, depending on the analyzed plant. The strongest effect of microwaves was observed for volatile oils from dill.

• The total amount of *volatile organic compounds* (captured from plant respiration) released by parsley and dill as result of microwave influence, were considerably higher than in the case of celery, because as observed for volatile oils in this plant, celery was less sensitive to microwaves than parsley and dill.

All three plant species irradiated by microwaves emitted the volatile compounds (*E*)-2-hexenal, (*Z*)-3-hexenol, 1-hexanol, while in the case of control plants the emissions of volatile organic compounds were very low. It was observed that in dill the (E)-β-ocimen and 1,8-cineol emissions were strongly enhanced as result of microwaves; both parsley and dill plants emitted small amounts of longicyclene (a stress induced sesquiterpene) under WLAN-frequency irradiation.

• The amount of *total polyphenolic compounds*, determined spectrophotometrically, showed no significant variation in irradiated plants compared to non-irradiated plants.

• After analysis by DPPH method, it was determined that hydroalcoholic extracts have a higher antioxidant activity than the extracts of control plants.

• The EPR studies showed a decrease in the free radical content in GSM and WLAN irradiated plants, respectively, in comparison to control plants, three weeks following the irradiation.

• *Ultrastructural analysis* (TEM) of the leaves from the plants belonging to the experimental groups, was focussed on the investigation of the main components of lamina (upper epidermis, palisade parenchyma, mesophilic tissue, lacunar tissue, lower epidermis) and, among celular components, mainly, the photosynthetic organelles (chloroplasts), the energy providing organelles (mitochondria) and the coordinator of cell metabolism (the nucleus). Comparing the effects of the two types of microwaves acting on cellular ultrastructure of the leaves of studied plants, it was concluded that WLAN microwaves induce negative changes significantly higher than those found in the case of GSM microwaves. Celery has proven to be the anatomically most resistant species to microwaves, while dill was the most affected species in this regard.

• Analyses for the determination of carbon isotopic composition ($\delta^{13}C$) showed the increase in the isotopic composition in celery irradiated by GSM microwaves, while in parsley have not been determined significant changes in the isotopic composition compared to control plants.

• As result of the studies performed, the greatest effect on plant development had the wireless electromagnetic radiation.

• The content of L-ascorbic acid and volatile oils, respectively, was the most affected by the microwaves.

• The experimental data presented in this work collectively suggest that microwave irradiation constitute a stress to the plants, resulting in enhanced emissions of volatile organic compounds, modification in bioactive compounds content and foliage anatomy.

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LIST OF SCIENTIFIC PAPERS

A. Papers published on the thesis topic

1. Extraction and HPLC determination of the ascorbic acid content of three indigenous spice plants
   M. Stan, M.L. Soran, C. Măruțoiu
   Journal of Analytical Chemistry, issue: "Analysis of pharmaceutical", 69(10), **2014** (accepted for publication).
2. Influence of microwave field on the ascorbic acid content in leaves of some common aromatic plants in Romania
   M. Stan, M.L. Soran, C. Varodi, I. Lung
   Studia Universitatis "Babes-Bolyai" Chemia, LIX, 1, **2014**, 125-133.
3. HPTLC quantification of some essential oils from *Anethum graveolens* extracts
   M. Stan, I. Lung, O. Opriş, M.L. Soran
   Journal of Planar Chromatography - Modern TLC, 27(1), **2014**, 33-37.
4. HPTLC quantification of myristicin and linalool from leaf extracts of microwave-irradiated parsley, dill and celery
   M. Stan, O. Opriş, I. Lung, M.L. Soran
   Journal of Planar Chromatography - Modern TLC, 27(2), **2014**, 97-101.
5. Extraction and identification of flavonoids from parsley extracts by HPLC analysis
   M. Stan, M.L. Soran, C. Varodi, I. Lung
   AIP Conference Proceedings, 1425, **2012**, 50-52.
6. Extraction and GC determination of volatile aroma compounds from extracts of three plant species of the Apiaceae family
   M. Stan, M.L. Soran, C. Varodi, I. Lung, L. Copolovici, C. Măruțoiu
   AIP Conference Proceedings, 1565, **2013**, 75-78.
7. Quantification of myristicin and linalool in some microwave-stressed aromatic plants using high performance liquid chromatography – diode array detection
   I. Lung, **M. Stan**, O. Opriş, M.L. Soran
   Analytical Letters (sent for publication).

8. Influence of microwave frequency electromagnetic radiation on terpene emission and content in aromatic plants
   M.L. Soran, **M. Stan**, Ü. Niinemets, L. Copolovici
   Journal of Plant Physiology (accepted for publication).

B. Papers published as communications at international conferences and symposia on the thesis topic

1. Determination of dill essential oil composition using modern extraction techniques
   M. Stan, M.L. Soran, C. Varodi, I. Lung
   5-th Conference on Chemistry and Life, 14 - 16 September, **2011**, Brno, Czech Republic.

2. Identification of flavonoids from parsley extracts by HPLC analysis
   M. Stan, M.L. Soran, C. Varodi, I. Lung
   8-th International Conference Processes in Isotopes and Molecules PIM-2011, 29 September - 01 October, **2011**, Cluj-Napoca, Romania.

3. Determination of vitamin C in parsley grown in the microwave field
   M.L. Soran, **M. Stan**, C. Varodi, I. Lung, C. Tudoran, M.R.C. Truşcă
   1st International Congress of Environmental Science and Technology
   1st National Congress of the Argentinean Society of Environmental Sciences and Technologies, 28 May - 01 June, **2012**, Mar del Plata, Argentina.

4. Chromatographic identification and determination of essential oils from some indigenous spice plants
   M. Stan, I. Lung, M.L. Soran, C. Varodi, C. Măruţoiu
   2nd International Conference on Analytical and Nanoanalytical Methods for Biomedical and Environmental Sciences, "IC-ANMBES 2012", 24 - 27 May, **2012**, Braşov, Romania.

5. Vitamin C from indigenous plants - extraction and electrochemical detection
   C. Varodi, **M. Stan**, M.L. Soran, L.M. Mureşan
   2nd International Conference on Analytical and Nanoanalytical Methods for Biomedical and Environmental Sciences, "IC-ANMBES 2012", 24 - 27 May, **2012**, Braşov, Romania.

6. GC-FID determination of essential oils from parsley extracts obtained by different extraction techniques

I. Lung, M.L. Soran, **M. Stan**, C. Varodi

2nd International Conference on Analytical and Nanoanalytical Methods for Biomedical and Environmental Sciences, "IC-ANMBES 2012", 24 - 27 May, **2012**, Braşov, Romania.

7. HPLC determination of vitamin C from indigenous parsley extracts obtained by different extraction techniques

   M.L. Soran, **M. Stan**, I. Lung, M.R.C. Truşcă

   7-th Conference on Sustainable Development of Energy, Water and Environment Systems", 1 - 7 July, **2012**, Ohrid, Macedonia.

8. Determination of essential oils from *Anethum graveolens* L. by thin layer chromatography

   M. Stan, I. Lung, M.L. Soran

   The First Mediterranean Symposium on Medicinal and Aromatic Plants, MESMAP-2013, 17 - 20 April, **2013**, Gazimagosa (Famagusta), Turkish Republic of Northern Cyprus.

9. Determination of essential oils from three Romanian aromatic herbs using thin layer chromatography

   M.L. Soran, **M. Stan**, I. Lung

   The First Mediterranean Symposium on Medicinal and Aromatic Plants, MESMAP-2013, 17 - 20 April, **2013**, Gazimagosa (Famagusta), Turkish Republic of Northern Cyprus.

10. Extraction and determination of essential oils from celery using modern techniques

    I. Lung, **M. Stan**, M.L. Soran

    The First Mediterranean Symposium on Medicinal and Aromatic Plants, MESMAP-2013, 17 - 20 April, **2013**, Gazimagosa (Famagusta), Turkish Republic of Northern Cyprus.

11. The influence of microwave field on vitamin C from indigenous seasoning plants

    M.L. Soran, **M. Stan**, I. Lung, C. Varodi

    International Congress on Energy Efficiency and Energy Related Materials, 9 - 12 October, **2013**, Kemer, Turkey.

12. Extraction and gas chromatographic determination of essential oils from three plant species of the Apiaceae family

    M. Stan, M.L. Soran, C. Varodi, I. Lung, L. Copolovici, C. Măruţoiu

    9-th International Conference "Processes in Isotopes and Molecules" PIM-2013, 25 - 27 September, **2013**, Cluj-Napoca, Romania.

13. Quantification of ascorbic acid in parsley, dill and celery from Romania

    M.L. Soran, **M. Stan**, I. Lung, O. Opriş

    9-th International Conference "Processes in Isotopes and Molecules" PIM-2013, 25 - 27 September, **2013**, Cluj-Napoca, Romania.

14. Separation and identification of volatile compounds from *Petroselinum crispum* L. by thin layer chromatography

    I. Lung, **M. Stan**, M.L. Soran, O. Opriş

    9-th International Conference "Processes in Isotopes and Molecules" PIM-2013, 25 - 27 September, **2013**, Cluj-Napoca, Romania.

15. Myristicin and linalool content variation in microwave stressed aromatic plants

    I. Lung, M.L. Soran, **M. Stan**, O. Opriş

    XVI YUCORR, Meeting Point of the Science and Practice in the Fields of Corrosion, Materials and Environmental Protection, 23 - 26 June, **2014**, Tara Mountain, Serbia.

16. Effect of microwave field on total polyphenol and antioxidant activity of dill

    I. Lung, M.L. Soran, **M. Stan**

    XVI YUCORR, Meeting Point of the Science and Practice in the Fields of Corrosion, Materials and Environmental Protection, 23 - 26 June, **2014**, Tara Mountain, Serbia.

17. Microwave fields effect on the essential oils from some aromatic plants

    M.L. Soran, **M. Stan**, I. Lung, L. Copolovici, M.R.C. Truşcă

    XVI YUCORR, Meeting Point of the Science and Practice in the Fields of Corrosion, Materials and Environmental Protection, 23 - 26 June, **2014**, Tara Mountain, Serbia.

18. The influence of microwave fields on volatile organic compounds emissions of some common aromatic plants in Romania

    M. Stan, M.L. Soran, I. Lung, L. Copolovici

    XVI YUCORR, Meeting Point of the Science and Practice in the Fields of Corrosion, Materials and Environmental Protection, 23 - 26 June, **2014**, Tara Mountain, Serbia.