GRIGORE T. POPA UNIVERSITY OF MEDICINE AND PHARMACY IASI

HABILITATION THESIS

"*RESEARCH ON MORPHO-CHEMICAL VARIABILITY OF MEDICINAL PLANTS AND BIOACTIVITY OF NATURAL PRODUCTS*"

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2019

"Logic will get you from A to Z, imagination will get you everywhere."

Albert Einstein (1879-1955)

Acknowledgements

I would like to express my deep gratitude to all the colleagues and collaborators, who contributes with their knowledge and works to this habilitation thesis. I would like to thank each and every one of you with who I share these results.

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THESIS SUMMARY

The habilitation thesis entitled "***Research on morpho-chemical variability of medicinal plants and bioactivity of natural products***" presents the most important scientific achievements obtained during the postdoctoral period (2005-in present). The main professional and academic achievements as well as the future development of all these directions are also discussed. The scientific achievements were initiated and developed starting from the research activity carried out within the doctoral studies completed with the public defence of my PhD thesis entitled "***Studies concerning the influence of pesticides treatment on medicinal plants: morphological and biochemical aspects"***, having as Scientific Supervisor, Professor Ursula Stănescu PhD, University of Medicine and Pharmacy Grigore T. Popa Iasi (Diploma D No.0002889/07.12.2005, Order of the Ministry of Education and Research No. 4802/15.08.2005).

The present thesis is written in compliance with the recommendations of the *National Council for Attestation of University Titles, Diplomas and Certificates* (CNATDCU), being organized in three main sections that include my scientific achievements in the postdoctoral period (**Section I**), the perspectives in my career (**Section II**) and the list of references related to my research accomplisments and the current knowledge (**Section III**).

The three sections are preceded by a synthesis of my entire academic career after the graduation of Faculty of Pharmacy, University of Medicine and Pharmacy Grigore T.Popa Iasi, in 1994, underlining the most important professional (the acquisition of the titles of pharmacist specialist and primary pharmacist in General Pharmacy), academic (the academic positions from junior assistant to associate professor won by competition) achievements and scientific research in the field of *Pharmacognosy* and *Phytochemistry*.

The scientific achievements of the postdoctoral period (**Section I**) are grouped into 2 main research directions, namely:

• The first research direction entitled "*Research achievements in the field of the assessment of the morpho-anatomical and chemical characters variability of some medicinal plants under the influence of abiotic and biotic factors*" continues the theme of the doctoral thesis and it was developed through the scientific activity carried out for the implementation of 4 national research projects and one international project. The biological activity and therapeutic use of medicinal plants or even the presence of toxic effects are influenced by their chemical composition, which in its turn is determined by endogenous and exogenous factors. The morphological and histo-anatomical features are directly correlated with the quality of the plant material, influencing not only the production of biomass, but also the biosynthesis of some active metabolites (volatile terpenes).

- **The second research direction** entitled "*Research achievements in the field of the assessment of natural products biological activities*" comprises two main subdirectoires, as follows:

  - **The first sub-direction** named "*Research achievements in the field of the assessment of antioxidant and cytogenoprotective activities*" brings together the most important contributions regarding on one hand the investigation and highlighting of the antioxidant properties of polyphenolic extracts and essential oils obtained from indigenous and exotic plant species and on on the other hand, the research on the antigenotoxic/genotoxic potential of polyphenolic extracts obtained from plant species and mushrooms, as well as of essential oils. It continues some of the

aspects addressed in the doctoral thesis related to the investigation of the biological properties of medicinal plants, especially extending the spectrum of plant species, their geographical origin and the type of tests that highlight different antioxidant mechanisms. In addition, the research approaches also include the correlation of the results of biological determinations with those regarding the chemical composition, chemical analysis being also an important objective of the studies undertaken. The development of the topic regarding the investigation of antioxidant properties was also possible through the active participation as a member in the team of 8 research projects, gained through international (3) or national (5) competitions.

The research regarding the investigation of the cytogenoprotective activity have developed in the last 6 years starting from the investigation of the antioxidant potential of medicinal and aromatic plants. The results reported in this direction were also possible thanks to funding through the research grant of the University of Medicine and Pharmacy Grigore T. Popa Iasi, the Team-Ideas 2012 Competition (*Investigations on the radioprotective potential of some plant extracts*-contract 1639/01.02.2013) where I participated as project manager at its implementation.

o **The second sub-direction e**ntitled "*Research achievements in the field of the assessment of antimicrobial activity*" refers to some of the most important results obtained in testing the antibacterial and antifungal activity of some essential oils from indigenous and exotic plant species. The research topic continues as in the first direction, aspects addressed in the doctoral thesis, but the subject is developed by diversifying the investigated plant species, the pathogens and investigating the combinatorial effects of essential oils and antibiotics against the pathogens involved in the lower respiratory tract infections.

The section also contains a series of general conclusions regarding the results of the scientific research.

- **Section II** describes the plans for the evolution and development of the scientific, professional and academic activity.

- **Section III** contains the list of bibliographic references related to my own works discussed within the present thesis, as well as titles used to substantiate the studies undertaken and the state of knowledge in the investigated field.

The results of the scientific research from the **postdoctoral period** were published in **197** papers, out of which **41** are articles in **ISI** journals (**cumulative impact factor**: **84.546**; **21 main author** papers), and 156 are articles published in BDI journals and communicated at national and international scientific events. The visibility of the professional activity in the same period is also illustrated by the publication as main author of **4 international book chapters**, as first author of a book and as co-author of 3 national books.

Also, during the postdoctoral period I participated as project manager in the implementation of a national research grant and as a member in the research team, in 13 other projects, of which 3 are international. Currently, I am involved in achieving the scientific objectives of a new grant attributed by competition (internal UMF Iasi-*Investigations grant on antifungal activity of natural products with putative use in onychomycosis*, project 7246/2018), as a member of the research team. I am co-author of an patent.

The Hirsch index is **11** in the Thomson ISI Web of Science Core Collection, **11** in All Database, **11** in Scopus and **16** in the Google Scholar Database.

In the future, **professionally and academically speaking**, my efforts will be oriented towards the development of my teaching competences in order to carry out activities that will lead to an increase in the efficiency of the university teaching and learning process, that is,

developing the student-centered education process as the main objective of the axis: ***teaching-learning-competence-certification-quality***. Also, I will support through my activity the process of professionalizing the students and developing a functional behavior regarding students' guidance in carrying out scientific works, participating in specific events or being involved in different research or social projects. Another important objective will be the development of relational skills by strengthening or developing relationships with the other disciplines within the Faculty of Pharmacy of the UMF "Grigore T. Popa" Iași, within the University or with the disciplines of the Faculties of Pharmacy/Medicine in the country, as well as through partnerships with other educational or research institutions.

From a scientific point of view, I aim to continue the research in the presented directions, with the extension of the studies in order to identify plant agents that offer protection to normal cells during certain antitumor treatments and radiotherapy, as well as to identify optimized formulations of plant polyphenols and essential oils in terms of bioavailability, with therapeutic and non-therapeutic applications. In this respect, accessing funds for research projects focused on these topics will be another major objective pursued.

The development of my future career will also consider a harmonization of the three fields of activity for a good integration in the general effort of our academic community to increase the prestige and visibility of UMF Grigore T. Popa from Iasi, institution in which I have been carrying out my activity for more than 22 years.

In all these directions of future career development I consider that the desire for knowledge, teamwork, communication, respect and honesty are essential values that define you professionally and humanly.

The realization of the present habilitation thesis is not just a personal effort. The research activity, as well as the didactic one, is a team work, with more and more interdisciplinary accents nowadays, and it also implies the accumulation of "gratitude debts", as stated so beautifully by the writer and esthete Andrei Plesu. I express my thanks and consideration to all my colleagues and collaborators with whom I have worked and shared these results.

REZUMATUL TEZEI

Teza de abilitare cu titlul "***Research on morpho-chemical variability of medicinal plants and bioactivity of natural products***" sumarizează cele mai importante realizări ştiinţifice ce au fost obținute în perioada postdoctorală (2005-în prezent). Sunt prezentate de asemenea şi cele mai importante realizări profesionale şi academice, precum şi perspectivele de dezvoltare în toate aceste trei direcții (profesional, academic and ştiințific). Realizarile ştiințifice au fost inițiate şi dezvoltate pornind de la activitatea desfăşurată în cadrul studiilor doctorale finalizate cu susținerea publică a tezei de doctorat intitulată „***Cercetări privind acțiunea unor pesticide aplicate în cultura plantelor medicinale: aspecte morfologice și biochimice***" - Coordonator ştiințific: Prof. univ. dr. Ursula Stănescu, UMF Grigore T.Popa -Iasi) şi conferirea titlului de Doctor în Ştiințe Medicale-Domeniul Farmacie, prin Ordin MEC 4802/15.08.2005.

Teza este redactată conform recomandărilor *National Council for Attestation of University Titles, Diplomas and Certificates* (CNATDCU), fiind structurată în **3 secțiuni** principale ce includ realizările ştiințifice în perioada postdoctorală (Secțiunea I), perspectivele de dezvoltare a activității ştiințifice, profesionale şi academice (Secțiunea II) şi lista referințelor bibliografice utilizate (Secțiunea III).

Cele 3 secțiuni sunt precedate de o sinteză a întregii mele cariere. Sunt prezentate cele mai importante realizări profesionale, academice şi ştiințifice.

Realizările ştiințifice din perioada postdoctorală (**Secțiunea I**) sunt grupate în 2 direcții principale de cercetare, şi anume:

- **Prima direcție de cercetare** cu titlul "*Realizări ştiintifice în domeniul investigării variabilității caracterelor morfo-anatomice şi chimice ale unor specii vegetale medicinale sub influența unor factori abiotici şi biotici"* continuă tema tezei de doctorat şi ea a fost dezvoltată prin activitatea ştiințifică desfăşurată pentru implementarea a 4 proiecte de cercetare naționale şi a unui proiect internațional. Activitatea biologică și utilitatea terapeutică a plantelor medicinale sau chiar prezența unor efecte toxice sunt influențate de compoziția lor chimică, care la rândul său este determinată de factori endogeni şi exogeni. Caracterele morfologice, precum şi cele histo-anatomice se corelează direct cu calitatea materialului vegetal, influențând nu doar producția de biomasă, dar şi biosinteza unor metaboliți activi (terpene volatile).
- **A doua direcție de cercetare** intitulată "*Realizări ştiințifice în domeniul evaluării activității biologice a produselor naturale"* cuprinde 2 subdirecții principale, după cum urmează:
  Prima subdirecție cu titlul "*Realizări ştiintifice în domeniul evaluării activității antioxidante şi citogenoprotectoare a produselor naturale***"** reuneşte cele mai importante contribuții referitoare pe de o parte la investigarea şi evidențierea proprietăților antioxidante ale unor extracte polifenolice şi uleiuri volatile obținute din specii vegetale indigene şi exotice şi pe de altă parte la cercetarea potențialului antigenotoxic/genotoxic al extractelor polifenolice obținute din specii vegetale şi ciuperci, precum şi al uleiurilor volatile din specii vegetale condimentare. Ea continuă unele din aspectele abordate în teza de doctorat referitoare la investigarea proprietăților biologice ale plantelor medicinale, extinzând în special spectrul de specii vegetale, proveniența geografică şi tipul de teste ce evidențiază mecanisme antioxidante diferite. În plus, abordările de cercetare includ şi corelarea rezultatelor determinărilor biologice cu cele privind compoziția chimică, investigațiile

chimice fiind de asemenea obiective importante ale studiilor întreprinse. Dezvoltarea tematicii cu privire la investigarea proprietăților antioxidante a fost posibilă şi prin participarea activă ca membru în colectivul a 8 proiecte de cercetare, din care 3 internaționale şi 5 naționale.

Cercetările privind investigarea activității citogenoprotectoare s-au dezvoltat în ultimii 6 ani pornind de la investigarea potențialului antioxidant al plantelor medicinale şi aromatice. Rezultatele raportate în aceasta direcție au fost posibile şi datorită finanțării prin grantul de cercetare al Universității de Medicină şi Farmacie Grigore T.Popa Iaşi, Competiție Echipe-Idei 2012 (*Investigations on the radioprotective potential of some vegetal extracts*- contract 1639/01.02.2013) la a cărei implementare am participat în calitate de director de proiect.

A doua subdirecție de cercetare denumită "*Realizări ştiințifice în domeniul evaluării activității antimicrobiene a uleiurilor volatile*", se referă la unele dintre cele mai importante rezultate obținute în cadrul testării activității antibacteriene şi antifungice a unor uleiuri volatile provenite din specii vegetale indigene şi exotice. Tema de cercetare continuă ca şi în cazul primei direcții, aspecte abordate în teza de doctorat, dar subiectul este dezvoltat prin diversificarea speciilor vegetale investigate, patogenii urmăriți şi investigarea efectelor combinațiilor de uleiuri volatile/componente volatile şi antibiotice asupra patogenilor implicați în infecțiile de tract respirator inferior.

Secțiunea cuprinde şi o serie de concluzii generale vizând rezultatele cercetării ştiințifice.

- **Secțiunea II** descrie planurile de evoluție şi dezvoltare a activității ştiințifice, profesionale şi academice.
- **Secțiunea III** cuprinde lista referințelor bibliografice aferente lucrărilor proprii discutate în cadrul prezentei teze de abilitare, dar şi titluri utilizate pentru fundamentarea studiilor întreprinse şi a stadiului cunoaşterii în domeniul investigat.

Rezultatele cercetării ştiințifice din **perioada postdoctorală** au fost publicate în **197** lucrări, din care un număr de **41** sunt articole în reviste cotate ISI (**factor cumulat de impact**: **84.546**; **21** lucrări-autor principal), iar **156** sunt articole publicate în reviste BDI/comunicate la manifestări ştiințifice naționale şi internaționale. Vizibilitatea activității profesionale în aceeaşi perioadă este ilustrată şi prin publicarea ca autor principal a **4 capitole de carte internațională**, ca prim-autor a unei cărți şi drept coautor a 3 cărți naționale.

De asemenea, în perioada postdoctorală am participat în calitate de director de proiect la implementarea unui grant național de cercetare şi ca membru în echipa de cercetare, în alte 13 proiecte, din care 3 sunt internaționale. În prezent, particip la realizarea obiectivelor ştiințifice ale unui nou grant câştigat prin competiție (Grant intern UMF Iasi-*Investigations on antifungal activity of natural products with putative use in onychomycosis*, contract 7246/2018), în calitate de membru în echipa de cercetare. Sunt coautor al unui brevet de invenție şi a 3 patente.

Indicele Hirsch este **11** in Thomson ISI Web of Science Core Collectionn, **11** în All Database, **11** in Scopus şi **16** in baza Google Scholar.

În viitor, **pe plan profesional şi academic**, eforturile mele vor fi orientate spre dezvoltarea competențelor didactice în vederea realizării unor activități care să conducă la o sporire a eficacității procesului universitar de predare şi învățare, în sensul dezvoltării învățământului centrat pe student ca obiectiv principal al axei: ***predare-învățare-competență-certificare-calitate***. De asemenea, voi susține prin activitatea mea procesul de profesionalizare a studenților şi de dezvoltare a unui comportament funcțional în ceea ce priveste consilierea şi îndrumarea studenților în realizarea de lucrări ştiințifice, participarea la manifestări specifice sau implicarea

în diferite proiecte de cercetare sau sociale. Un alt obiectiv important îl va constitui şi dezvoltarea unor competenţe relaţionale prin consolidarea sau dezvoltarea relaţiilor cu celelalte discipline din cadrul Facultăţii de Farmacie a UMF ”Grigore T.Popa” Iaşi, din cadrul Universităţii ori cu discipline ale Facultăţilor de Farmacie/Medicină din ţară, precum şi prin realizarea de parteneriate cu alte instituţii de învăţământ sau de cercetare.

Pe plan ştiinţific, îmi propun continuarea cercetărilor în direcţiile prezentate, cu extinderea studiilor în vederea identificării de agenţi de natură vegetală care să ofere protecţie celulelor normale în timpul anumitor tratamente antitumorale şi radioterapiei, precum şi al identificării de formulări optimizate ale polifenolilor vegetali şi uleiurilor volatile în ceea ce priveşte biodisponibilitatea, cu aplicaţii terapeutice şi non-terapeutice. În acest sens, accesarea de fonduri pentru proiecte de cercetare centrate pe aceste teme va fi un alt obiectiv major urmărit.

Dezvoltarea carierei mele viitoare va avea în vedere şi o armonizare a celor 3 domenii de activitate pentru o bună integrare în efortul general al comunităţii noastre academice de creştere a prestigiului şi vizibilităţii UMF Grigore T.Popa din Iaşi, instituţie în care îmi desfăşor activitatea de mai mult de 22 de ani.

În toate aceste direcţii de dezvoltare a carierei viitoare consider că dorinţa de cunoaştere, munca în echipă, comunicarea, respectul şi onestitatea sunt valori esenţiale care te definesc profesional şi uman.

Realizarea prezentei teze de abilitare nu este doar un efort personal. Activitatea de cercetare, ca şi cea didactică este o muncă în echipă, cu tot mai multe accente de interdisciplinaritate în prezent şi ea presupune şi acumularea unor “datorii de recunoştinţă”, după cum afirma în exprimarea sa atât de plastică, scriitorul şi estetul Andrei Pleşu. Îmi exprim mulţumirea şi consideraţia pentru toţi colegii şi colaboratorii mei cu care am lucrat şi împart aceste rezultate.

OVERVIEW OF PERSONAL PROFESSIONAL, ACADEMIC AND SCIENTIFIC ACHIEVEMENTS

Professional and academic achievements

My academic career started in 1996, when I won by competition the position of *Teaching Assistant* at the Discipline of Organic Chemistry and then, starting with 1998, the position of *Teaching Assistant* at the discipline of Pharmacognosy, Faculty of Pharmacy, University of Medicine and Pharmacy Grigore T.Popa Iași. In 2000 I was promoted, by competition, to the position of *Assistant Lecturer* at the same discipline, and in 2005, to the position of *Senior Lecturer*. Since March 2014 I was promoted by competition on the position of *Associate Professor* (position 3), at the Discipline of Pharmacognosy.

In parallel with the didactic activity I obtained the titles of *Specialist Pharmacist* in General Pharmacy (Order MS no. 2707/1996) and of *Primary Pharmacist* in the same specialty (Order MS no.538 / 07.08.2001).

The didactic activity included/includes teaching courses and stages of practical works, as follows:

- *Pharmacognosy* - Course, Pharmacy series, year III (2005-present);
- *Medicinal plants and vegetal products; toxic plants* – Course and practical works, Pharmacy Assistance specialty, II year (2009-2012);
- *Phytotherapy* - Optional course, Pharmacy series, year IV (2008-2017);
- *Phytotherapy and Orthomolecular Medicine* - Course and practical works, Nutrition and Dietetics specialization, Faculty of Medicine, II year (2014);
- *Pharmacognosy* - Practical works, Pharmacy series, III year (1998-present);
- Courses and seminars in the Master's University Program, Faculty of Pharmacy, at the specializations: *The drug of vegetal origin from manufacturing to use* (2006-2007); *Products of plant origin: medicine, food supplement, food* (2007-2011) - modules: *Modern phytotherapeutics and evidence-based phytotherapy*; *Bioequivalence of some phytopreparations, food supplements*;
- Doctoral course for Pharmacy within the Doctoral School (2011-2013) - module: *Advanced experimental techniques in the development of new drugs of natural origin*;
- Applications for Pharmacy PhD students within the Doctoral School (2011-2013).

In order to develop good professional skills, I attended several postgraduate courses, as follows:

- *How to write a successful Horizon 2020 project* (*Transnational support actions for successful participation in EU Framework Programme for Research and Innovation Horizon 2020-actHoriz*) (2016);
- *Cromatografie şi spectrometrie de masă* (2015);
- *Programul de perfecționare a competențelor IT în utilizarea soluției Tablou de Bord Management Resurse* în cadrul Proiectului „Management Educațional și Învățământ de Calitate în Societatea Informațională", POSDRU/86/1.2/S/62594 (2013);
- *Aspecte privind acreditarea laboratoarelor; detecție chiroptică în cromatografia de lichide; cromatografia și extracția cu fluide supercritice; utilizarea spectroscopiei de*

dicroism circular în studiul interacțiunilor moleculare; purificare cu ajutorul flash-cromatografiei (2009);

- *Noi metode analitice pentru detectarea reziduurilor de pesticide prezente în Produse alimentare şi agricole* (2003);
- The Workshop on Pharmaceutical Tablet Technology: *Design, Formulation, Unit Operations, Manufacturing, Quality Assurance and Bioavailability* (2000);
- *Expanding pharmacist's roles in primary health care delivery* (1999-2002).

Teaching was and it is an important part of my professional achievements. The motto that drive my teaching activity is: "education is not the learning of facts, it's rather the training of the mind to think" (*Albert Einstein*).

Communication with students, interactive and interdisciplinary problematization, stimulating their curiosity, imagination and desire to know by asking, identifying methods to stimulate and motivate students regarding the acquisition of knowledge in the field of pharmacognosy, were major objectives of my teaching activity. Academic and curricular quality standards, as well as the educational challenges in the context of modern society were also important reference elements. All this meant a permanent search for the improvement of my teaching skills, to make Pharmacognosy and Phytotherapy courses and Pharmacognosy practical works more attractive for the pharmacist student, but also to support their optimal integration into the modern pharmacist profession.

The presence of numerous and extremely different herbal products on the pharmaceutical market, the attraction of the population towards green medicine represent challenges for the pharmacist. In order to overcome the simplistic and sometimes derisory treatment considerations, medicinal products need a correct and evidence-based understanding of the use in the pharmaceutical and medical context of the plant product and of phytopreparations, as well as what their quality standards mean.

Preparing the pharmacist student in the sense of a correct knowledge and understanding of what the plant product means and its study (botanical investigations, phytochemistry, phytopharmacology, technology of extraction and conditioning of plant extracts, quality analysis of phytopreparations), on the one hand, and on the other the stimulation of imagination and creativity in order to provide efficient and safe herbal products for administration and an adequate counseling of the patients were the benchmarks of my didactic activity.

I introduced new topics for the module of alkaloids within the course and practical work stages of Pharmacognosy, namely: *Camptotheca acuminata and camptotecinoids, Alkaloids from Uncaria sp, Alkaloids from Taxus species, Galanthus nivalis and galantamine,* chemical analysis of purine alkaloids and *Opium* alkaloids. In 2019, I proposed a new optional course for the students of year III from Faculty of Pharmacy, namely: *Aromatherapy and perfumery*. The course was accepted and included in the curriculum for 2019/2020 academic year.

Also, I was involved in the preparation and updating of the course and practical works of Pharmacognosy and the course of Phytotherapy on the e-learning platform.

The publishing activity includes **8 books** (one as first author and 7 as coauthor) and **4 international book chapters**.

My didactic activity was also materialized in the coordination of more than **150 diploma papers** for the students of Faculty of Pharmacy, for the graduates of the Specialization Assistance of Pharmacy (Faculty of Pharmacy) and of the Specialization Nutrition and Dietetics (Faculty of Medicine), University of Medicine and Pharmacy Grigore T.Popa Iași, in the field of Pharmacognosy and Phytotherapy.

At the same time, I coordinated **10 research works** of pharmacy students presented at student scientific events, of which **7 were awarded with prizes**, as follows:

- *The antioxidant activity of some chitosan films with porous structure containing Thymus vulgaris L. essential oil* (author: Platon Ioana-Victoria; co-advisor Senior Researcher II Dr. Maria Valentina Dinu, Institute of Macromolecular Chemistry Petru Poni, Iasi) presented at **the National Congress of Pharmacy Students**, the 17th edition, Cluj-Napoca, April 10-14 2019; **Mention prize**;
- *The bioactivity of adaptogenic phytopreparations in the burnout syndrome* (authors: Ioana-Alexandra Calin, Trofin Marin-Aurel), ***National Congress of Pharmacy Students***, 17th edition, Cluj-Napoca, April 10-14, 2019; **Best poster award**;
- *The chemical composition and biological activity of some essential oils from Thymus species grown in the Republic of Moldova* (authors: Tiron Mădălin, Profiroiu Antonia) presented at ***the National Congress of Pharmacy Students***, the 15th edition, Bucharest, April 5-9, 2017; **Third prize**;
- *Phytotherapeutic opportunities in obesity. WEB application in the prophylaxis and the individualized treatment of obesity* (authors: Mihai Alina Andreea, Taras Raluca, Scutaru Andrei) presented at ***the Pharmacy Students' Gala***, the 6th edition, Timisoara, December 3-6, 2015; **Second prize**;
- *Mentha gattefossei Maire - chemical investigations and evaluation of antioxidant activity in vitro* (author: Ciot Ioana-Alexandra), presented at ***the National Congress of Pharmacy Students***, Cluj-Napoca, April 1-5, 2015;
- *Green or white tea? Total polyphenols, catechin profile and antioxidant activity* (authors: Andriescu Ana-Maria, Luca Vlad Simon), presented at ***the National Congress of Pharmacy Students***, Iasi, April 2-6, 2014; **First prize**;
- *Silymarin - drug versus food supplement* (authors: Pungă Olga, Ştefan Ioana Lavinia; co-advisor – Lecturer Oana Cioancă, PhD), presented at ***the National Congress of Pharmacy Students***, the 10th edition, Iasi, April 6-10, ***2011***.
- Contributions to the characterization of an *Aronia melanocarpa* (Michx) Elliot fruit extract (author: Strugaru Anca-Monica; co-advisor: Professor Anca Miron, PhD), presented at ***the National Congress of Pharmacy Students in Romania***, the 9th edition , Bucharest, April 21-25, 2010; **Third prize**;
- *Pharmacognostic and phytochemical study of some samples of Foeniculi fructus (Foeniculum vulgare Mill, fennel) of different origin* (authors: Bratu Mihaela, Teodoru Andreea), presented at ***the National Congress of Pharmacy Students in Romania***, the 6th edition, Gura-Humorului , April 11-15, 2007; **Mention prize**.

Being awarded the "**Bologna Professor**" distinction within the **Bologna Teacher 2014 Gala** by the *National Alliance of Student Organizations of Romania* (ANOSR) represents an important and meaningful reference for my entire teaching activity, a title that honors and obliges to continue and improve my teaching style in order to meet the study requirements of the students for the purpose of adequate professional training.

In addition to the didactic activities for students, I was also involved in the postgraduate teaching activities, as follows:

- Coordinator of the postgraduate course: *Current knowledge in the phytotherapy of hepatobiliary disorders* (2011-2012);
- Lecturer in the postgraduate courses:
  - *Phytotherapy of gastrointestinal disorders* (2010-2012);

- *Orthomolecular therapy of metabolic diseases* (2011-2012);
- *Current knowledge in the phytotherapy of cardiovascular disorders* (2010-2012);
- *Phytotherapy of CNS disorders* (2006-2007);
- *Qualified person for the release of the drug manufacturing series* (2007) (postgraduate employee training course for SC Antibiotice SA Iaşi).

Over the years I have participated in various activities of the university community, of wich I mention:

- Member of the Senate of University of Medicine and Pharmacy Grigore T.Popa, Iaşi (2015-present);
- Chairman of the Commission of Faculty of Pharmacy for the evaluation of the scores on the faculty for the Admision Competition for self-funded places (foreign currency) in the study program with tuition in Romanian, English or French language (2016-2019);
- Member of Professional Commission for Admission Competition (2017-2019),
- Member of the Evaluation Commission of the files for Admission Competition of Romanians from everywhere (2017, 2018);
- Member of Admission Commission and of Commission for the Bachelor Exam-Faculty of Pharmacy;
- Member of the Commissions for the presentation of dissertation master's theses (2007-2011);
- Member of the Commission for International Relations and Academic Partenerships of Faculty of Pharmacy (2016-present);
- Member of the Commission of Quality Management of Faculty of Pharmacy (2011-2012);
- Member of the Competition Commission for the position of Lecturer at Discipline of Pharmacognosy, position 5 (2012).

Research achievements

The elaboration of this habilitation thesis is a moment of evaluation of my professional activity. The scientific achievements presented here were initiated and developed starting from the activity carried out within the doctoral studies completed with the public presentation of the doctoral thesis entitled „*Studies concerning the influence of pesticides treatment on medicinal plants: morphological and biochemical aspects*" - Scientific Supervisor, Professor Ursula Stănescu PhD, University of Medicine and Pharmacy Grigore T. Popa Iaşi and for which I was conferred the title of Doctor in Medical Sciences-Pharmacy Domain, by 4802/15.08.2005 MEC Order. The subject of the doctoral degree was related to the investigation of the structural, chemical and biological changes induced by antifungal treatments with Topsin M in medicinal and aromatic plants (*Mentha* and *Melissa* species). Subsequent research expanded the spectrum of investigated medicinal and aromatic plants and also, of the abiotic or biotic factors. Also, biological investigations were developed, antioxidant, antimicrobial and cytogenoprotective studies being carried out, as well as the evaluation of the effects of the combinations of essential oils and antibiotics against standard or isolated clinical pathogens.

The results of the scientific research carried out during the **14 years of postdoctoral studies** were published in **197** papers, out of which **41 are articles in ISI** journals (**cumulative impact factor: 84.546**; **21** papers as **main author**), and 156 are articles published in BDI journals and

presented at national and international scientific events. The Hirsch index is **11** in the Thomson ISI Web of Science Core Collection and All Databases, **11** in Scopus and **16** in the Google Scholar Database. The visibility of the scientific activity in the same period is also illustrated by the publication as main author of **4 international book chapters**.

Also, I participated as project manager in the implementation of a national research grant and as a member of the research team, in 13 other projects, of which 3 are international, as follows:

Project Director:

- Internal Grant won in the competition of research projects of University of Medicine and Pharmacy Grigore T.Popa Iasi, Teams-Ideas section, 2012): *Investigations on the radioprotective potential of some vegetal extracts*", contract no.1639/2013, run between 2013-2015 years.

Member in the team of international projects:

- Project REART, Nr. IZ73Z0_152265: *Capitalization of the natural potential of several medicinal and aromatic species in the Artemisia genus with economic and ecological value in Moldov*a. Coordinator: Mediplant - Swiss Research Institute for Aromatic and Medicinal Plants, Conthey, Switzerland, Dr. Xavier Simonnet, run between 2014-2017 years.
- Bilateral cooperation Romania-Republic of Moldova (PN-II-CT-RO-MD-2012–1): *Evaluation and characterization of genetic resources from Lamiaceae species with potential anti-inflammatory properties, their in situ and ex situ conservation* (contract no. 694/2013) run between 2013-2014 years, Project Responsible: Prof. Anca Miron, PhD;
- Bilateral cooperation with Department of Biological Sciences, University of Cyprus: *Identification of new medicinal plants with possible uses in cancer prevention and treatment*, run between 2008-2009 years, Project Responsible: Prof. Anca Miron, PhD;

Member in the team of national projects:

- Project PNCDI II, Programme 4 Partnerships in priority areas, complex projects (PC)/contract no. 62-065/2008: *Complex characterization of cytostatic active extracts of Claviceps purpurea strains obtained by parasexual hybridization biotechnology, to harness the veterinary therapy*, run between 2008-2011 years, Project Responsible: Prof. Monica Hăncianu, PhD;
- Project PNCDI II, Programme 4 Partnerships in priority areas, complex projects (PC)/contract no. 61-39/2007: *Biotechnologies for obtaining plant metabolites used in the prophylaxis and orthomolecular therapy*, run between 2007-2010 years, Project Responsible: Prof. Monica Hăncianu, PhD;
- Project CDI (Innovation)/contract no.33/2007: *Phytomedicines used for metabolic imbalances counteracting*, run between 2007-2010 years, Project Responsible: Prof. Monica Hăncianu, PhD;
- Project PNCDI II, Programme 4 Partnerships in priority areas, complex projects (PC), contract no. 51-060/2007: *Obtaining of phytopreparations with preventive effects in some cardiovascular diseases*, run between 2007-2010 years, Project Responsible: Prof. Anca Miron, PhD;
- Project CEEX/BIOTECH/Module I, project category: research-development (P-CD), contract no. 77/2006: *Biotechnologies to obtain some preparations active over the neuro-immune-cutaneous system*, run between 2006-2008 years, Project Responsible: Prof. Anca Miron, PhD;

- Project CEEX/BIOTECH/ Module I, project category: research-development (P-CD), contract no.21/2005: *Phytopreparations with anti-aging potential obtained by means of biotechnologies*, run between 2005-2008 years, Project Responsible: Prof. Ursula Stănescu, PhD;
- Project CNCSIS type A, contract no. 1146/2007: *Natural polyphenols and modulation of cardiovascular reactivity and endocrin-metabolic dysfunctions in streptozotocin- and stress overload-induced diabetes*, run between 2007-2008 years, Project Responsible Project: Prof. Manuela Ciocoiu, PhD;
- Project CNCSIS type A, contract no. 1225/2004: *The mutual influence between degradable polymers and environment*, run between 2004-2006 years, Project Responsible Project: Prof. Mihaela Pascu, PhD;

Member in the team of internal research grant UMF Grigore T. Popa Iasi:

- *Investigations on antifungal activity of natural products with putative use in onychomycosis* (contract no. 7246/2018), run between 2018-2020 years, Responsible project: Assistant Adriana Trifan, PhD;
- *Characterization of the biological effects of essential oils rich in monoterpene alcohols with relevance in neuroprotection* (contract no. 1642/2013), run between 2013-2014, Responsible project: Lecturer Oana Cioancă, PhD.

The results of the research projects were materialized in obtaining an OSIM patent and other three patents indexed ISI WEB OF SCIENCE:

- OSIM Patent 3/86 from 30.07.2014. *Clavinic antitumour preparation for veterinary use and process for preparing the same* (authors: Roșu CM, Rotinberg P, Olteanu Z, Surdu Ș, Truță E, Mihai CT, Hrițcu L, Gherghel D, Hăncianu M, Miron A, **Aprotosoaie AC**, Cioancă O);
- Patent RO 127813-A0: Procedeu de obținere a unui dermopreparat cu acțiune cicatrizantă din *Allium cepa* L. (authors:Tătărîngă G, Hăncianu M, **Aprotosoaie AC**, Stănescu UH, Mihăilescu RL, Dănilă D, Druțu AC, Miron A, Cioancă O); International Patent Classification: A61K-036/8962; A61P-017/02;
- Patent RO 127808-A0: *Procedeu de obținere a unei fracțiuni macromoleculare din Telekia speciosa (Schreb.) Baumg. cu acțiune imunostimulatoare* (authors: Gille E, Hăncianu M, Stănescu UH, Mihăilescu RL, Dănilă D, Miron A, **Aprotosoaie AC**, Cioancă O, Necula, R, Ghiță LG); International Patent Classification: A61K-036/00; A61K-036/28; A61K-009/14.
- Patent RO 127795-A0: *Procedeu de micropropagare la specia Veronica officinalis L. prin culturi de țesuturi* (authors: Dănilă D, Ștefanache CP, Gille E, Necula, R, Druțu AC, Trotuș E, **Aprotosoaie AC**, Miron A, Cioancă O); International Patent Classification: A01G-031/00; A01H-004/00.

The scientific research activity carried out so far has been rewarded with the **Prize for Excellence in Pharmaceutical Research 2018** by the jury of the Pharmaceutical Gala on the 22nd November 2018, organized by the College of Pharmacists in Romania. At the same time, the results obtained in 3 papers presented at national and international scientific events were awarded as follows:

- **Diploma of Excellence** for the paper presented as poster: *Mentha gattefossei Maire: chemical profile and antioxidant activity of essential oil* (authors: **Aprotosoaie AC**, Trifan A, Ciocârlan N,

Brebu M, Miron A), National Congress of Pharmacy in Romania, 16th edition, Bucharest, September 28-October 1, 2016.

- **TEVA Prize** for the best presentation: *Phenolic profile and in vitro screening of Cyprus cedar bark for antioxidant and antitumor activities* (authors: Miron A, Creţu E, Trifan A, Karonen M, Salminen J-P, Mihai CT, Rotinberg P, **Aprotosoaie AC**), *Phytochemical Society of Europe Meeting, Phytochemicals in Medicine and Pharmacognosy*, Piatra-Neamt, Romania, 27-30 April 2014.
- **Bronze Medal-EUROINVENT 2012**, Iași (*Antineoplastic clavinic alkaloid type product of veterinary use and preparation process thereof*, authors: Rosu CM, Rotinberg P, Olteanu Z, Surdu S, Truță E, Mihai CT, Hrițcu L, Gherghel D, Hăncianu M, Miron A, **Aprotosoaie AC**, Cioancă O).

Also, for 5 papers, we obtained as co-author UEFSCDI Awards: Awarding the Research Results, as follows:

- **UEFSCDI Awards: Awarding the Research 2017**: **Aprotosoaie AC,** Gille E, Trifan A, Luca VS, Miron A. Essential oils of *Lavandula* genus: a systematic review of their chemistry. *Phytochem Rev* 2017; 16 (4): 761-799.
- **UEFSCDI Awards: Awarding the Research 2016**:
  Aprotosoaie AC, Luca, Simon Vlad, Miron Anca. Flavor chemistry of cocoa and cocoa products-an overview. *Compr Rev Food Sci* F 2016; 15 (1): 73-91.
  Aprotosoaie AC, Mihai CT, Vochita G, Rotinberg P, Trifan A, Luca SV, Petreus T, Gille E, Miron A. Antigenotoxic and antioxidant activities of a polyphenolic extract from European *Dracocephalum moldavica* L. *Ind Crops Prod* 2016: 79: 248-257.
- **UEFSCDI Awards: Awarding the Research 2015**: **Aprotosoaie AC**, Trifan A, Gille E, Petreuş T, Bordeianu G, Miron A. Can phytochemicals be a bridge to develop new radioprotective agents? *Phytochem Rev* 2015; 14 (4): 555-566.
- **UEFSCDI Awards: Awarding the Research 2014**: **Aprotosoaie AC**, Hăncianu M, Costache I-I, Miron A: Linalool: a review on a key odorant molecule with valuable biological properties. *Flav Fragr J* 2014; 29 (4): 193-219.

Since 2012 I have participated as a member in the guidance committees for the elaboration of the doctoral theses of the PhD students from the Discipline of Pharmacognosy, the research directions approached by them, being:

- *the pharmacognostic and biological study of some medicinal plants cultivated in the sub-Carpathian area of Moldova: establishing the chemical variability* (pharm. Adriana Trifan; Scientific Supervisor: Prof. Ursula Stănescu, PhD), the defense of PhD thesis on the October 5, 2012;
- *pharmacognostic and biological study of fennel fruits and essential oils extracted from them* (pharm. Mihai Băjan; Scientific Supervisor: Prof. Ursula Stănescu, PhD), the defense of PhD thesis on the February 21, 2012;
- *substances of plant origin - precursors for obtaining by synthesis/semisynthesis of some compounds with potential biological activity* (pharm. Alexandra Rotariu Jităreanu), Scientific Supervisor: Prof. Ursula Stănescu, PhD), the defense of PhD thesis on the October 5, 2012;
- *chemical and biological studies on the Pinus cembra L. species* (pharm. Cristina Lungu Apetrei; Scientific Supervisor: Prof. Anca Miron, PhD); the defense of PhD thesis on the September 19, 2013;

- *study of the chemistry and of the possibilities of therapeutic use of the Pinus brutia Ten and Cedrus brevifolia (Hook.F) A Henry species* (pharm. Elena Crețu; Scientific Supervisor: Prof. Anca Miron, PhD); the defense of PhD thesis on the September 19, 2013;
- *studies on compounds of species of the Paeonia L. genus* (Ana Maria Balan Zbancioc; Scientific Supervisor: Prof. Anca Miron, PhD); the defense of PhD thesis on the March 3, 2014;
- *phytochemical screening of some species of the Asteraceae and Lamiaceae family; evaluation of the antimicrobial activity against pathogens of the lower respiratory tract* (pharm. Adina Catinca Grădinaru; Scientific Supervisor: Prof. Anca Miron, PhD); the defense of PhD thesis on the July 21, 2014;
- *the chemical and biological study of some Crataegus species* (Catrinel Florentina Giurescu Bedreag; Scientific Supervisor: Prof. Anca Miron, PhD); the defense of PhD thesis on the October 29, 2014;
- *chemical and biological studies on some tannins rich plants* (Roxana Laura Amalinei Mihăilescu; Scientific Supervisor: Prof. Anca Miron, PhD); the defense of PhD thesis on the October 29, 2014;
- *phytochemical and biological studies on some plant species of spontaneous African flora* (pharm. Alexandru Vasincu; Scientific Supervisor: Prof. Anca Miron, PhD); the defense of PhD thesis on the September 30, 2014;
- *evaluation of the antioxidant and antidiabetic potential of some edible mushrooms* (Daniela Elena Axinte Zavastin; Scientific Supervisor: Prof. Anca Miron, PhD); the defense of PhD thesis on the April 21, 2016;
- *studies on the isolation and structural and biological characterization of some compounds with anti-inflammatory and cytotoxic activities from Verbascum species* (pharm. Simon Vlad Luca; Scientific Supervisor: Prof. Anca Miron, PhD); the defense of PhD thesis on the May 23, 2019;
- *studies on the chemical and biological characterization of some anthocyanin/ proanthocyanidolic fractions with possible uses in the prophylaxis and the adjuvant treatment of cardiovascular diseases* (pharm. Alexandra Bujor; Scientific Supervisor: Prof. Anca Miron, PhD);
- *studies on the antimicrobial activity of some phytocompounds/plant extracts against pathogens of the skin and the gastrointestinal tract; interactions with antibiotics/ chemotherapeutics* (pharm. Petruța Aelenei; Scientific Supervisor: Prof. Anca Miron, PhD);
- *studies on the isolation, chemical characterization and screening of the chemopreventive and antitumor action of some anthocyanins/anthocyanin fractions* (pharm. Adriana Crăciunescu; Scientific Supervisor: Prof. Anca Miron, PhD);
- *studies on nano-encapsulation of active phytocompounds used in the treatment of non-melanoma skin cancer* (pharm. Irina Macovei; Scientific Supervisor: Prof. Anca Miron, PhD);
- *studies regarding the identification and characterization of some tyrosinase inhibitors of plant origin* (pharm Raluca Taraș Olaru; Scientific Supervisor: Prof. Anca Miron, PhD);
- *studies on the identification and characterization of plant extracts from indigenous or acclimatized species with favorable action in neurodegenerative diseases* (Veronica

Grădinaru; Scientific Supervisor: Prof. Monica Hăncianu, PhD); the defense of PhD thesis on the September 30, 2015;

- *studies on the chemical composition and biological activity of some Pelargonium species* (pharm. Cristina Elena Iancu; Scientific Supervisor: Prof. Monica Hăncianu, PhD); the defence of PhD thesis on the November 24, 2016;
- *studies on secondary plant metabolites with antibacterial potential in urinary infections* (Romeo Dorneanu; Scientific Supervisor: Prof. Monica Hăncianu, PhD);
- *study of plant extracts with potential anti-inflammatory effects* (pharm. Florin-Andrei Păduraru; Scientific Supervisor: Prof. Monica Hăncianu, PhD);
- *assessment of possible interactions between plant extracts/phytocompounds and synthetic drugs* (pharm. Ioana-Andreea Iacob Bucişcanu; Scientific Supervisor: Prof. Monica Hăncianu, PhD).

Also, I was of committees for admission to doctoral studies and I participated as official reviewer in the board of PhD thesis: *Contribuţii la evaluarea calităţii unor produse vegetale de interes farmaceutic*, elaborated by pharm. Elisabeta Ioan Oprea (Scientific Supervisor: Prof. Monica Hăncianu, PhD); the defence of PhD thesis on the 15.12.2015.

My concerns in the field of scientific research also included the activity as reviewer for numerous ISI journals, of which the most important are:

- *Comprehensive Reviews in Food Science and Food Science* (FI^{2018}=8,738);
- *Food Chemistry* (FI^{2017}=4,946);
- *Food Hydrocolloids* (FI^{2017}=4,747);
- *Waste Management* (FI^{2018}=4,723);
- *Journal of Advanced Research* (FI^{2017}=4,327);
- *Scientific Reports* (FI^{2017}= 4,122);
- *Journal of Functional Foods* (FI^{2016}=3,973);
- *Industrial Crops and Products* (FI^{2019}=3,849);
- *Journal of Affective Disorders* (FI^{2017}= 3,786);
- *Food and Chemical Toxicology (*FI^{2017}=3,778);
- *Biomedicine and Pharmacotherapy* (FI^{2017}=3,457);
- *Planta* (FI^{2018}=3,249);
- *Saudi Pharmaceutical Journal* (FI^{2017}=3,11);
- *Phytochemistry Reviews* (FI^{2016}=2,686);
- *Journal of Translational Medicine* (FI^{2017}=3,786);
- *Arabian Journal of Chemistry* (FI^{2015}=2,969);
- *Current Organic Chemistry* (FI^{2017}= 2,193);
- *Pesticide Biochemistry and Physiology* (FI^{2014}=2,009);
- *Journal of Herbal Medicine* (FI^{2018}=1,685);
- *Phytochemistry Letters* (FI^{2019}=1,575);
- *Chemistry&Biodiversity* (FI^{2019}=1,444);
- *Natural Product Research* (FI^{2018}=1,225);
- *Journal of Essential Oil Research* (FI^{2013}=0,815),

as well as an international bool chapter (Tunick M, Nasser J. *The chemistry of chocolate and pleasure*, ACS Books, 2018).

I participated in organizing various national and international scientific events, being a member of the organizing /scientific committees, such as:

- *Conference of Practical Phytotherapy* (2017, 2019);

- *National Congress of Pharmacy in Romania*, 15th edition (2014),
- *Phytochemical Society of Europe Meeting: Phytochemicals in Medicine and Pharmacognos*y (2014),
- *National Symposium: the Drug from Conception to Use*, 2nd edition (2009),
- *National Conference of Phytotherapy* 4th edition (2008),
- *4th Conference on Medicinal and Aromatic Plants of South-East European Countries* (2006).

SECTION I. SCIENTIFIC, PROFESSIONAL AND ACADEMIC ACHIEVEMENTS

The first section presents the scientific achievements of the postdoctoral period (2005-present), which are grouped in 2 main research directions, namely:

1. investigation of morpho-anatomical and chemical variability of medicinal plants under the influence of abiotic and biotic factors;

2. assessment of the biological activities of natural products, as follows:

- evaluation of antioxidant and cytogenoprotective potential of polyphenolic extracts and essential oils;
- investigation of the antimicrobial activity of essential oils, as well as of the combinatorial effects of essential oils/volatiles with antibiotics.

Within each research direction the major contributions are discussed, contributions that have been reported in articles published in specialized journals indexed in international databases, international book chapters, or that have been presented at national and international congresses and conferences.

I.1. Research achievements in the field of the assessment of morpho-anatomical and chemical variability of medicinal plants under the influence of abiotic and biotic factors

The aforementioned research direction continues the theme of the doctoral thesis ("*Studies concerning the influence of pesticides treatment on medicinal plants: morphological and biochemical aspects*") and it was developed through the scientific activity carried out for the implementation of 4 national research projects and one international project, as a member of the research team.

Medicinal plants produce a variety of secondary metabolites with different biological, sensory, or nutritional properties and multiple functionalities. Biosynthesis and accumulation of these metabolites constitutes a response of the plant organism to external stimulation, to ensure adaptation and survival in adverse conditions.Various abiotic factors mainly related to the environmental conditions (ex: temperature, soil composition and nutrients, water availability and drought stress, salt stress, light variations, UV radiations, or altitude, pesticide treatments) and biotic factors (plant interactions with microorganisms, plant ontogeny and phenology) may influence the metabolic profile of plants affecting the quality and quantity of vegetal secondary metabolite pool. Besides, these factors may influence some structural features of plants (density and functionality of stomata, photosynthetic rate, secretory structures as glandular hairs, morphological characters) which could cause the impairment of biomass and secondary metabolites production and of plant material quality. Finally, all these issues can be translated into an impaired plant bioactivity and usage [Aprotosoaie 2005; Ncube et al., 2012].

Identification and understanding the structural and chemical variability of plants under influence of various factors may extend the knowledge of ecologic interactions with their environment and allow alternative approaches to increase productivity of cultivated plants or to ensure quality and safety in phytomedicine.

In the research carried out I focused mainly on the influence of the treatment with fungicides, of the climate, altitude and of the geographical area on the structural characteristics and especially on the production of volatile terpenes and polyphenols and their spectrum in medicinal

and aromatic plants such as: *Foeniculum vulgare* [Băjan et al., 2006; Aprotosoaie et al., 2008a; Aprotosoaie et al., 2009a; Aprotosoaie et al., 2010a; Băjan et al., 2011a; Băjan et al., 2011b; Aprotosoaie et al., 2013a], *Lavandula sp*. [Aprotosoaie et al., 2009b; Robu et al., 2009; Robu et al., 2011; Aprotosoaie et al., 2013b; Aprotosoaie et al., 2014a; Aprotosoaie et al., 2017a], *Origanum* sp. [Aprotosoaie et al., 2009c], *Thymus* sp. [Aprotosoaie et al., 2010b; Dănilă et al., 2010; Necula et al., 2011], *Ocimum sp*. [Hăncianu et al., 2007; Hăncianu et al., 2008], *Hyssopus officinalis* [Aprotosoaie et al., 2010c], *Salvia officinalis* [Dănilă et al., 2008; Şpac et al., 2009; Aprotosoaie et al., 2010d; Aprotosoaie et al., 2013a], *Rosa* sp. [Adumitresei et al., 2009; Delinschi et al., 2009], *Marrubium vulgare* [Ivănescu et al., 2008], *Nepeta nuda* ssp. *nuda*. [Ivănescu et al., 2007).

The results of such research provide useful data on establishing the impact of climate variability on the metabolome, but also on reducing the input of agronomic techniques on the therapeutic and nutritional qualities of plants.

Some of the most important contributions in this direction materialized in the first years of postdoctoral research as follows: 4 articles in extenso and the communication of 4 posters at international (3) and national (1) events, as well as a chapter in an international book.

Aprotosoaie AC, Şpac A, Hăncianu M, Miron A, Tănăsescu VF, Dorneanu V, Stănescu U. The chemical profile of essential oils obtained from fennel fruits (*Foeniculum vulgare* Mill.). *Farmacia* 2010, 58 (1), 46-53 (FI^{2010}=0,850).

Adumitresei L**,** Gostin I, **Aprotosoaie C**, Spac A, Stănescu I, Toma C. Chemical compounds identified in the leaf glands of *Rosa agrestis* Savi and *Rosa rubiginosa* L. *Analele ştiinţifice ale Universităţii "Alexandru Ioan Cuza" din Iaşi (serie nouă), tomul LV, fasc.1, S II a. Biologie vegetală* 2009, 39-48.

Delinschi-Floria V, **Aprotosoaie C**, Stănescu I, Toma C. Morpho-anatomical considerations upon the shoot of some Rosa L. cultivars from the Botanic Garden of Iasi (2nd Note). *Analele ştiinţifice ale Universităţii "Al. I. Cuza" Iaşi, tomul LV, fasc. 2, S. II-a, Biologie vegetală* 2009, 47-54

Aprotosoaie AC, Rugină R, Tănăsescu V, Gacea O, Hăncianu M, Miron A, Stănescu U. Histo-anatomical researches regarding the influence of Topsin M treatments on *Foeniculum vulgare* Mill. (Apiaceae). *Analele ştiinţifice ale Universităţii "Alexandru Ioan Cuza" din Iaşi (serie nouă): S II-a. Biologie vegetală* 2005, tom LI, 39-46.

Aprotosoaie AC, Gille E, Şpac A, Necula R, Druțu C, Stănescu U, Hăncianu M. Influence of planting density on yield and essential oil composition of hyssop (*Hyssopus officinalis* L.). *Phytochemical Society of Europe (PSE), International Symposium on Terpenes-Application, Activity and Analysis*, Istanbul-Turkey, October 26-29, 2010, 55.

Aprotosoaie AC, Gille E, Şpac A, Goncearіuc M, Hăncianu M, Stănescu U. Chemical composition of essential oils from leaves and twigs with leaves of two new varieties of common sage (*Salvia officinalis* L.). *Planta Medica* 2010, 76(12), 1207; 58th International Congress and Annual Meeting of the Society for Medicinal Plant and Natural Product Research, Berlin-Germany, August 29 - September 2, 2010.

Aprotosoaie AC, Spac A, Necula R, Stănescu U, Hăncianu M. Chemical variability in *Thymus pulegioides* L. populations growing wild in the northeast of Romania. *Pharmacognosy Magazine*, 2010, vol. 6 (sup. 22), S132; *6th Conference on Aromatic and Medicinal Plants of Southeast European Countries*, Antalya-Turkey, April 18-22, 2010.

Miron A, **Aprotosoaie AC**, Poiată A, Hăncianu M, Tănăsescu V, Stănescu U. Study on influence of phytosanitary treatment with pesticides upon the biological activity of fennel. *Timisoara Medical Journal* 2005, 55, 5/supl, 172-17; Simpozionul *Farmacia astăzi, între promovare şi cercetare*, Timișoara, 26-28 mai, 2005.

Hăncianu M, **Aprotosoaie AC**. The effects of pesticides on plant secondary metabolites. In *Biotechnological production of plant secondary metabolites*, Erdogan Orhan I (ed). Bentham Science Publishers-Bentham eBooks, 2012.

In recent years, the accumulated experience regarding the chemistry and bioactivity of volatiles has materialized in 3 reviews in ISI journals (*Flavour and Fragrance Journal*, *Phytochemistry Reviews* and *Comprehensive Reviews in Food Science and Food Safety*) and 2 international book chapters.

Aprotosoaie AC, Hăncianu M, Costache I-I, Miron A. Linalool: a review on a key odorant molecule with valuable biological properties. *Flavour Fragr J* 2014, 29 (4), 193-219 (FI^{2014}=1,97).
Aprotosoaie AC, Luca SV, Miron A. Flavor chemistry of cocoa and cocoa products-an overview. *Compr Rev Food Sci F* 2016, 15 (1), 73-91 (FI^{2016}=5,974).
Aprotosoaie AC, Gille E, Trifan A, Luca VS, Miron A. Essential oils of *Lavandula* genus: a systematic review of their chemistry. *Phytochem Rev* 2017, 16 (4), 761-799 (FI^{2017}=3,875).

Aprotosoaie AC, Costache I-I, Miron A. Anethole and its role in chronic diseases. In *Drug Discovery from Mother Nature*, Gupta SC**,** Prasad S**,** Aggarwal BB(eds.). Springer International Publishing Switzerland, 2016, pp. 247-267.
Aprotosoaie AC, Costache I-I, Miron A. Sensory qualities and nutraceutical applications of flavors of terpenoid origin. In *Flavors for Nutraceutical and Functional Foods*, Selvamuthukumaran M, Pathak YV (eds.). CRC Press Taylor&Francis Group 2018 (Online), 2019 (Print), pp.167-201.

I.1.1. Research on influence of treatment with pesticides upon the histo-anatomical features, chemical composition and the biological activity of *Foeniculum vulgare* Mill.

Current state of the arte and research objectives

Large-scale cultivation of medicinal and food plants is not possible today without pesticides. Due to the high costs, "organic" (pesticide-free) cultivation is only possible on a small scale. Most research about the influence of pesticides is focused on metabolism and toxicity studies or the development of mathematical models to estimate the maximum residual potential of pesticides in plant material and in the food circuit.

The widespread use of pesticides can have repercussions not only on humans and the environment, but also on the quality and on the nutritive and therapeutic values of medicinal/food plants. Besides the issue of pesticide residues, an equally important role in determining the quality of vegetal products is represented by the structural changes that pesticides may induce on plants. Such changes may lead to an alteration in plant growth or changes in their physiology, affecting the production of biomass or their chemical composition [Hăncianu and Aprotosoaie, 2012]. Most often, pesticide intervention was evidenced in conducting and assimilating tissues [Rugină et al., 1985; Niță et al., 1999; Aprotosoaie, 2005], in the secretory structures [Niță et al., 1999] or in pollen structure [Öztürk, 2008].

Pesticides or even plant growth regulators may interfere to a greater or lesser degree with byproducts of secondary metabolism in plants. Among the secondary metabolites, essential oils in particular are strongly influenced in terms of their chemical composition (quantitative and qualitative), by the type of pesticide, the quantity and the period of application. Herbicides such as nitrofen combined with simazine or metabromuron applied in sage cultures (*Salvia officinalis*) lead to a decrease of essential oil production, as well as of thujone content. The application of the terbacil herbicide in *Mentha × piperita* cultures causes an increase in the production of essential oil, as well as its content in menthol [Aprotosoaie, 2005; Talat et al., 2016].

Anise plans (*Pimpinella anisum*) treated with fungicides as azoxystrobin, difenoconazol or fosetyl showed an increase of essential oil content. Besides, fosetyl treatment leads to the higher levels of *trans*-anethole than in untreated plants [Ullah, 2012].

Commonly called fennel, *Foeniculum vulgare* Mill. (Apiaceae) is a well-known medicinal and aromatic plant.The fennel fruits have expectorant, carminative, spasmolytic, galactagogue and diuretic properties.They are widely used in treatment of gastrointestinal and respiratory disorders as well as in cosmetic, flavor, food and beverages industries. Fennel fruits are an important source of *trans*-anethole for pharmaceutical, flavor and food industries. The biological effects of fennel are ascribed mainly to the essential oil and to the polyphenolic fraction (flavonoids, polyphenolic acids) [Choi et al., 2004; Manzoor et al., 2016; Stănescu et al., 2018].

Topsin M (methyltiophanate) is a common systemic fungicide used to protect crops of food and medicinal plants, including the cultures of fennel [Aprotosoaie, 2005].

Taking into account the above mentioned considerations, the aim of the study was to investigate effect of treatment with Topsin M on structural and chemical features (essential oil and polyphenols) of fennel and subsequently, on the antioxidant and antimicrobial properties of fruits.

Antioxidant activity is one of the most properties of polyphenols while antimicrobial effects are particularly characteristic of essential oils.

Material and methods

Plant material

Vegetal materials (stems, leaves and fruits) of fennel were harvested from experimental lots in "Anastasie Fătu" Botanical Garden, Iaşi (Fig. 1). In this experimental area, parallel cultures of *Foeniculum vulgare* have been made during three consecutive years.Thus, every year, there have been three experimental fields: a field which had no fungicide treatment (control area) (C) and two fields which have been treated with different concentrations of pesticide (0.1% and 0.4%). The land on which the crops were organized has cambic chernozem and illuvial clay soil type.

Fungicide treatments

The antifungal treatment was achieved in vegetative phase by spraying a wettable powder of Topsin M 70 PU (methyl thiophanate) (TM) (Oltchim Rm. Vâlcea-România) as 0.1% (TM 0.1%) and 0.4% (TM 0.4%) aqueous solutions. The 0.1% concentration is the usual concentration used in agriculture, and the 0.4% concentration is recommended for other substances similar to methyl thiophanate. The application of the fungicide was carried out by spraying parcels with a surface of 2 m/2 m, at a pressure of 2 atmospheres, thereby ensuring a uniform distribution of the product. The administration of fungicide Topsin M was done during the full vegetative period, when the foliar system was well developed. Two applications were made at an interval of 10 days between them.

Figure 1. *Foeniculum vulgare* plants ("Anastasie Fătu" Botanical Garden, Iaşi)

Histo-anatomical study

The anatomical features were analysed using superficial and cross sections through the stems, leaves and fruits belonging to the treated and untreated plants. Besides, some data items of the stem and the leaf were established.

The measurements and the section examinations have been performed at Amplival 30-G 048 a (Carl Zeiss Jena, Germany) microscope.

The microphotographes of the sections were done with Olimpus BH-2 (Japan) microscope.

Chemical study

Chemicals

Topsin M 70 PU (TM) was supplied by Oltchim SA Rm. Vâlcea (in collaboration with Sumitomo-Nippon Soda, Japan). Rutin, caffeic acid, anethol were from Merck (Merck Schuchardt, Hohenbrunn, Germany) while quercetin dihydrat was purchased from Roth (Karlsruhe, Germany). Soybean lipoxygenase type 1 (LO), diphenylpicryhydrazyl (DPPH) and linoleic acid were from Sigma (St. Louis, USA). All other chemicals were of reagent grade quality.

Isolation of essential oil

Dried fennel fruits (100 g) were subjected to hydrodistillation for 4 h in a Clevenger-type apparatus. The essential oils were dried over anhydrous sodium sulfate and stored in sealed dark vials at 4 °C. The yield of essential oils was expressed as mL/100 g dried fennel fruits.

Extraction

Crude hydromethanolic extracts were obtained by extracting dried and powdered fennel fruits with aqueous methanol (80%) on water bath at 60 °C followed by removal of solvent in vacuo (Büchi R-114 rotavapor). Both flavonoids and polyphenolic acids were determined in hydromethanolic extracts. For this purpose, 100 mg dried extract were solved in 100 mL

methanol (solution A). In all assays, a Jasco V-550 UV-VIS spectrophotometer (Japan) was used for the absorbances measurement.

Quantification of flavonoids

The flavonoid content was assessed in a solution A according to a spectrophotometric method based on the formation of aluminium-flavonoid complexes. The absorbance was measured at 413 nm [Romanian Pharmacopoeia, 1993]. Total flavonoids were expressed as g of rutin per 100 g extract (w/w). All analyses were carried out in triplicate.

Quantification of polyphenolic acids

The total polyphenolic acids content was measured using the method based on the formation of blue coloured complexes with phosphowolframic acid solution in alkaline medium. The absorbance of the solution was measured at 660 nm [Romanian Pharmacopoeia, 1993]. Total phenolic acids content was expressed as g of caffeic per 100 g extract (w/w). All determinations were carried out in triplicate.

Biological study

In vitro antioxidant activity

The antioxidant activity was assessed by 15-LO inhibitory activity and free radical scavenging assays. For each extract there were made three dilutions in dimethylsulfoxide (DMSO), namely: 10, 5 and 2.5 mg/mL.

Quercetin dihydrat in dimethylsulfoxide (1.69 mg/mL) was used as positive control in antioxidant assays.

The spectrophotometric measurements were performed using a Shimadzu 160A instrument equipped with a Shimadzu CPS240A thermostatted cell changer (Shimadzu, Japan).

15-LO inhibition assay

The inhibition of soybean 15-LO activity was determined using the method described by Lyckander and Malterud (1992). Briefly, 50 μL of each dilution or DMSO alone (as blank) were added to 2.90 mL of 134 μM solution of linoleic acid in borate buffer (0.2 M, pH=9). Then, a solution of 15-LO in borate buffer (10000 UI/mL) was added and the increase of absorbance at 234 nm for 30-90 s was measured.

Percent inhibition of 15-LO was calculated as:

$$100 \times [(\Delta A_1/\Delta t)-(\Delta A_2/\Delta t)/(\Delta A_1/\Delta t),$$

where:

$\Delta A_1/\Delta t$ = value for increase in absorbance at 234 nm for mixture without sample,

$\Delta A_2/\Delta t$ = value for increase in absorbance at 234 nm for mixture with sample.

The assay was conducted in triplicate.

DPPH radical scavenging assay

The assay was carried out as described by Malterud et al. (1993). 50 μL of each dilution was mixed vigorously with 2.95 mL DPPH in methanol (A_{517}=1.0±0.05). The decrease of absorbance at 517 nm was measured over a period of 5 min.

The value of DPPH scavenging activity was calculated as:

$$100 \times (A_{start}-A_{end})/A_{start},$$

where:

A_{start} = absorbance before sample addition

A_{end} = absorbance over a period of 5 min after sample addition.

All measurements were carried out in triplicate.

Antimicrobial assay

Microorganisms

Staphylococcus aureus ATCC 25923 (Gram-positive), *Bacillus subtilis* ATCC 6633 (Gram-positive), *Escherichia coli* ATCC 25922 (Gram-negative) and *Candida albicans* ATCC 10231 (pathogenic yeast) were used as test strains.

Agar diffusion method

Antimicrobial activity of essential oils isolated was investigated by agar-diffusion method using Mueller-Hinton agar (Biolab Zrt., Hungary) inoculated with microbial suspension at a density adjusted to a 0.5 McFarland standard (10^6CFU/mL). Sabouraud medium (Oxoid, UK) was used for *Candida albicans*. Briefly, 10 μL of each sample were applied on sterile paper discs. After incubation at 37°C for 24 h (bacteria) and at 24 °C for 48 h (yeasts), the diameters of microbial growth inhibition were measured [Miron et al., 2005].

Anethole, the main component of fennel essential oils was used as control. 0.1% and 0.4% solutions of Topsin M were also tested in order to exclude possible interferences of pesticide residues and/or pesticide metabolites in evaluating the antimicrobial activity.

Results and discussion

Histo-anatomical study

We analyzed the effects of fungicide treatments on the fennel stem, leaf and fruits tissues.

Stem: The reaction of *Foeniculum vulgare* plants is visible on TM treatment at wood tissue level and it is mainly reflected in stimulatory effects. The analysis of data items of the stem from treated and control plants revealed the differences mainly related to the thickness of cortex, vascular bundles and woody vessels (Table 1).

Table 1. The variation of data items for the stem of *Foeniculum vulgare* (F.v.) under Topsin M (TM) treatment [Aprotosoaie et al., 2005]

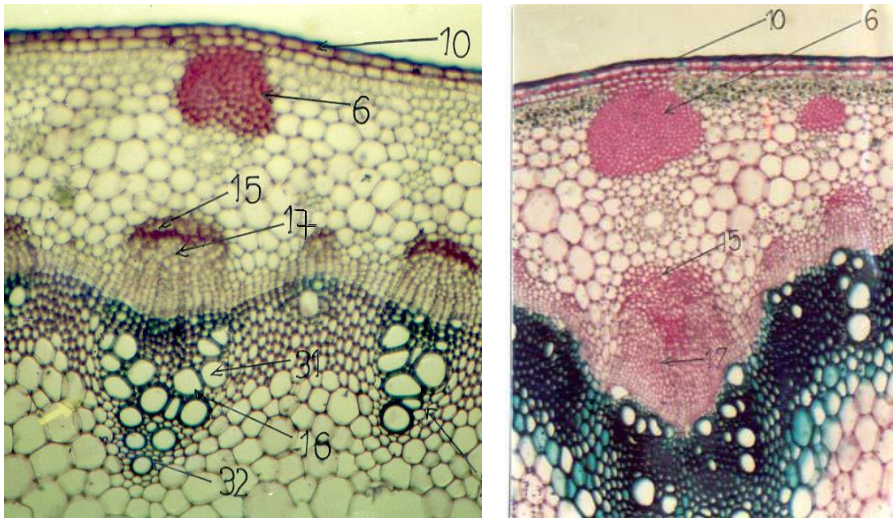
Variant	Diameter (μm)	Number of cortical layers	Thickness of cortex (μm)	Number of libero-ligneous bundles	Number of woody vessels from large	Diameter of woody vessels (μm)
Year I						
F.v. C	8850-10.000	7-9(10)	100-280	80-84	15-20	(20)30-50(60)
F.v. TM 0.1%	9500-10.000	8-9 (10)	200-400	87-90	30-32	(20)50-70(90)
Year II						
F.v. C	4900-5450	10-11(12)	350-400	49-50	32-40 (45)	(20)30-50(60)
F.v. TM 0.4%	9000-9100	10-12 (13)	450-500	93-95	25-39 (40)	(20)50-70(120)
Year III						
F.v. C	4800-5500	10-12	300-450	56-58	40-42	40-50
F.v. TM 0.1%	9500-10.000	6-7	28-40	66-67	35-36	(30) 80-90
F.v. TM 0.4%	9200-10.000	9-10	35-45	100-104	35-50	(30) 80-100

In treated plants, the thickness of cortex is larger comparing to control, because of the increased size of cells, especially of the ones from the internal cortex (Fig. 2). Therefore, the

number of cortical layers was much higher than at control. The stem of TM 0.1% treated plants presented almost twice times more woody vessels than the control; the large fascicles of the stem from treated TM 0.4% plants have, at the internal pole, a more developed mechanical tissue. Therefore, antifungal treatments with Topsin M stimulated the formation of large (F.v.TM 0.1%) or very large (F.v.TM 0.4%) woody vessels (Fig. 3). The stimulation of intrafascicular cambium activity and the formation of new conducting vessels are most often not accompanied by the sclerification and lignification of their walls. Most vessels remain immature.

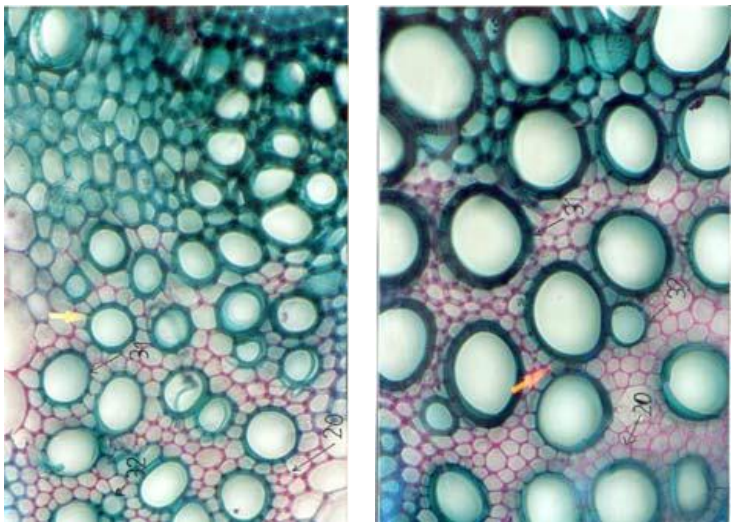
Leaf: The antifungal treatment exerts a stimulating effect, visible by the increase in the thickness of the petiole and the rachis of the treated plants in the direction of the adaxial-abaxial/latero-lateral diameter. At treatments with Topsin M, the epidermal cells are much more numerous than the control, especially for the lower epidermis of the 0.4% TM treated plants (Table 2). 0.4% TM treated leaves show in both epidermis three types of stomata: diacitic, anomocytic and anisocytic, while paracitic type stomata predominate in the leaves of untreated plants.

The mesophyll has a unistratified or bistratified palisade; the palisadic cells are anticline elongated; in the central part of the mesophyll is developed a colourless parenchyma. The conducting tissue is of a fascicular type, the number of the bundles varying between 3-5(7). The thickness of the petiole and the rachis increases at treated plants comparative to control (Table 3).Therefore, in the treated plants, parenchymatic hipertropy leads to the appeareance of twin secretory ducts. Generally, the tickness of the lamina from treated TM 0.1% and 0.4% plants increased by 1-2 times comparative to control plants, due to the almost three times elongation of palisadic cells (Fig. 4).



F.v.C (year III) F.v.TM 0.4% (year III)

Figure 2. Structure of stem in cross section (2,5 × 10) [Aprotosoaie et al., 2005]
6-collenchyma; 10-epiderme; 15-pericycle fibres; 16-xylem; 17-phloem; 31-metaxylem; 32-protoxylem.



F.v.C (year III) F.v.TM0.4% (year III)

Figure 3. Structure of stem in cross section (2,5 × 40) [Aprotosoaie et al., 2005]
20-cellulosic parenchyma; 31-metaxylem; 32-protoxylem.

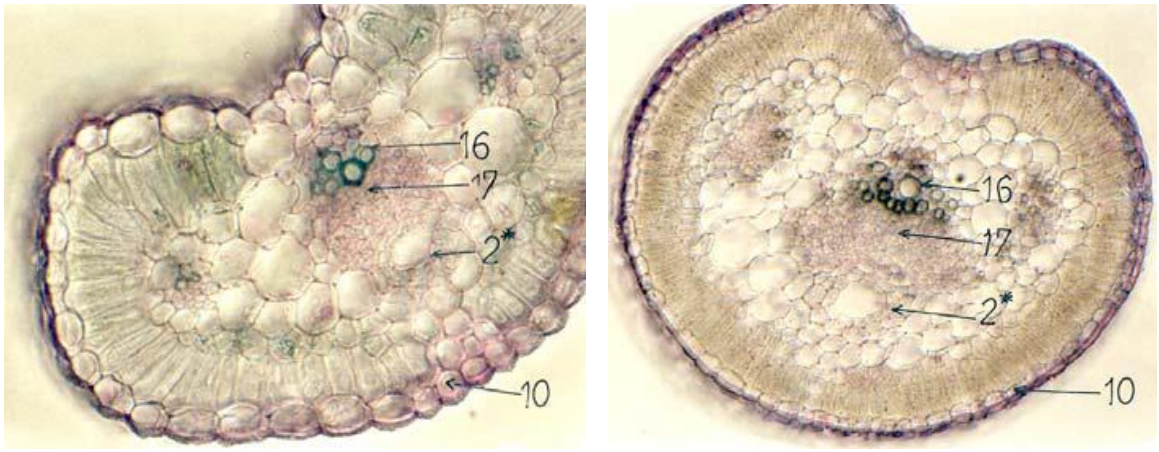
Table 2. Epidermal data items for the leaf of *Foeniculum vulgare* (F.v.) under Topsin M (TM) treatment [Aprotosoaie et al., 2005]

Variant		Upper epidermis		Lower epidermis	
		Number of cells	**Stomata number**	**Number of cells**	**Stomata number**
Year I	F.v. C	101	11	108	14
	F.v. TM 0.1%	109	16	115	19
Year II	F.v. C	104	13	110	10
	F.v. TM 0.4%	109	19	112	18
Year III	F.v.C	99	10	105	12
	F.v. TM 0.1%	106	14	110	17
	F.v. TM 0.4%	109	16	114	20

Fruit: At TM treatment, the fruits are obviously ribbed compared to the control which is only slightly edged. In the mesocarp, cells appear slightly hypertrophied and hyperplasiated near the secretory channels. The secretory channels are noticeably wider than in the control, but usually flattened, due to the pressure of the surrounding hypertrophic and hyperplastic cells. In the TM 0.4% treated plants, in the mesocarp, especially near the conducting fascicles at the flat face of the mericarp, numerous mechanical elements (stereids) are distinguished, strongly sclerified and lignified in the latter case.

Table 3. The variation of data items for the leaf of *Foeniculum vulgare* (F.v.) under Topsin M (TM) treatment [Aprotosoaie et al., 2005]

Variant	**PETIOLE**		**RACHIS**		**LACINIA**	
Year I	**Diameter (μm)**	**Number of bundles**	**Diameter (μm)**	**Number of bundles**	**Thickness (μm)**	**Thickness of palisade (μm)**
F.v. C	1500-1550	6-7	3400-2000	11-12	230-400	50-60
F.v. TM 0.1%	1750-1800	8-9 (10)	4000-3500	13-14 (15)	400-500	50-60
Year II						
F.v. C	700-750	5-8	2300-1900	10-12	200-300	20-30
F.v. TM 0.4%	830 (850)-950 (980)	5-7 (8)	2700-1800	10-12	300-500	70-80
Year III						
F.v. C	1900-2000	10-11	2200-1200	8-9	200-300	40-50
F.v. TM 0.1%	1500-1800	9-10	3100-2100	14-15	400-500	50-60
F.v. TM 0.4%	700-1000	5-6	2600-1500	10-11	260-500	60-70



F.v. C (year III) (2,5×40) F.v.TM 0.1% (year III) (2,5× 20)

Figure 4. Structure of leaf (lacinia) in cross section [Aprotosoaie et al., 2005]
2*-secretory duct; 10-epidermis; 15-pericycle fibres; 16-xylem; 17-phloem.

Chemical study

Polyphenols and essential oil contents

Quantitative analysis of polyphenols and essential oils from samples of fennel fruits (Fig. 5, Fig. 6) showed that:

- 0.1% Topsin M treatd fennel fruits contained a 24.89% lower amount of flavonoids than the control while 0.4% Topsin M treated fennel had a 79.02% higher content of flavonoids than their control;
- 0.1% Topsin M treated fennel fruits contained a 10.81% higher amount of polyphenolic acids than the corresponding control;
- the polyphenolic acids content in 0.4% Topsin M treated fruits was slightly different from polyphenolic acid content in the control;
- at treated plants, content of flavonoids varied more pronounced than the level of polyphenolic acids;
- compared with control, the essential oil content in 0.1% Topsin M treated fruits increased with 15% and decreased with 9% in 0.4% Topsin M treated fennel. The lower amount of essential oil in 0.4% treated fennel might be due either to a high content of very volatile components or to a high content of unstable components which easily react and form non-volatile compounds.
- the flavonoids, polyphenolic acids and essential oil levels of fennel fruits collected from control fields were different from year I to year II, other factors such as weather being also involved.

Different categories of plant metabolites may be affected by phytosanitary treatments, and essential oil is one of the fractions most significantly influenced in this regard. Thus, herbicide treatments can slightly lower the essential oil content in some aromatic plants, such as chamomile, melissa, valerian, sage or the Moldavian dragonhead (*Dracocephalum moldavica*) [Aprotosoaie, 2005].

Furthermore, some herbicides may adversely affect the proportion of the main components of chamomile essential oil (bisabolol oxide B, bisabolol) [Reichling et al., 1979] or the anethole content of fennel essential oil [Pank, 1990]. Treatments with phospho-organic insecticides reduce the essential oil content in *Lavandula angustifolia*, *Mentha × piperita*, *Salvia officinalis* or *Rosa sp*. plants.

Saponosides, alkaloids, phytosterols or carotenoids are other metabolites whose content and spectrum vary in different species under treatment with herbicides, insecticides or fungicides [Talat et al., 2016]. Plant polyphenols being part of the plant's defensive response may be an important target of pesticide action.

Personal research has shown that flavonoid biosynthesis was affected in *Mentha longifolia* and *Melissa officinalis* after Topsin M treatments [Aprotosoaie, 2005]. The results obtained in the case of fennel support this assertion, the flavonoids reacting most visibly in this regard from a quantitative point of view.

For a complete evaluation, the analysis of the flavonoid spectrum is required. Regarding the chemical composition of essential oil this is different depending on the sample: control *vs.* treated plants and control year I *vs.* control year II.

Trans-anethole (69.01-80.86%), fenchone (11.33-18.57%) and estragol (2.91-3.58%) were main compounds of essential oils from all fennel samples.

We noticed an increase of the aromatic fraction content at TM 0.4% treated plants comparative to control (84.72 *vs.* 82.41%) and the decrease of monotepenes content (15.06 *vs.* 17.38%).

Also, we observed a variation of these components in the case of controls from different years:

- year I: monoterpenes -14.90% and aromatic compounds - 81.46%;

- year II: monoterpenes -17.38% and aromatic fraction - 82.41%)(unpublished data).

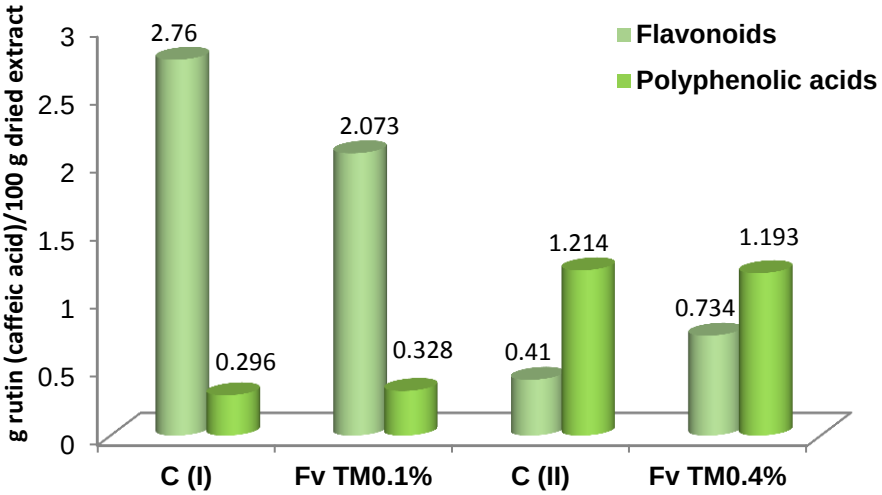


Figure 5. Flavonoids and polyphenolic acids concentrations in extracts from *Foeniculi fructus*
[C (I)-control year I; C(II)-control year II; FvTM 0.1%-treated fennel with 0.1% Topsin M; FvTM 0.1%-treated fennel with 0.4% Topsin M].

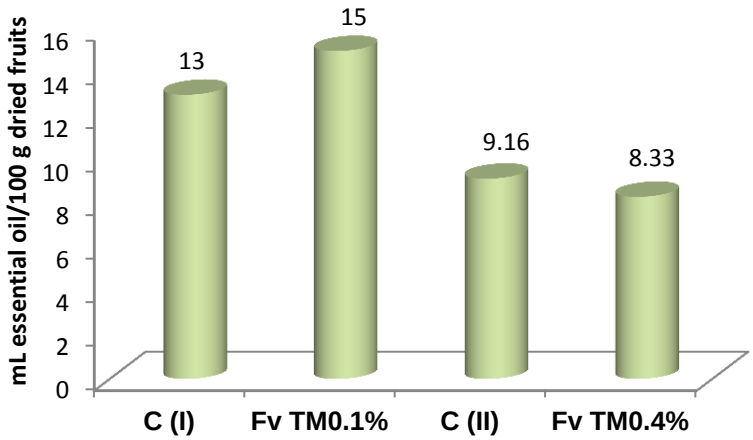


Figure 6. Essential oil content in samples of *Foeniculi fructus*
[C (I)-control year I; C(II)-control year II; FvTM 0.1%-treated fennel with 0.1% Topsin M; FvTM 0.1%-treated fennel with 0.4% Topsin M].

Biological study
Antioxidant activity

Plant polyphenols and flavonoids in particular are very notable from the point of view of their bioactivity, through their antioxidant properties that also explain many of the benefits of the medicinal and aromatic plants. This was the reason why we chose to investigate this type of effects in the case of treated fennel plants.

Investigation of 15-LO inhibitory activity of fennel extracts (Fig. 7) showed that:

- all extracts developed a concentration-dependent inhibitory activity towards 15-LO, an enzyme critically involved in inflamamatory processes, vascular pathology or cancer. At 10 mg/mL, the extracts showed the most potent inhibitory activity (78.1-92.7%); at lower concentrations (5 mg and 2.5 mg/mL), the extracts demonstrated weaker inhibitory activity;
- all dilutions of extract from 0.1% Topsin M treated fennel were more active than the corresponding controls. This fact is associated with an increased content of phenolic acids for the treated samples. A 10.81% higher amount of phenolic acids caused a significant increase in 15-LO inhibitory activity (from 80.5% to 92.7% in case of 10 mg/mL dilution);
- at 10 mg/mL and 5 mg/mL, the extract from 0.4% Topsin M treated fennel was more active than control. In this case even if the treated samples are richer in flavonoids than controls, we noticed only a very slight increase of 15-LO inhibitory activity values (from 78.1% to 81% for dilution of 10 mg/mL).

For fennel fruits it is obvious the fact that polyphenolic acids are more responsible for 15-LO than flavonoids [Miron et al., 2005].

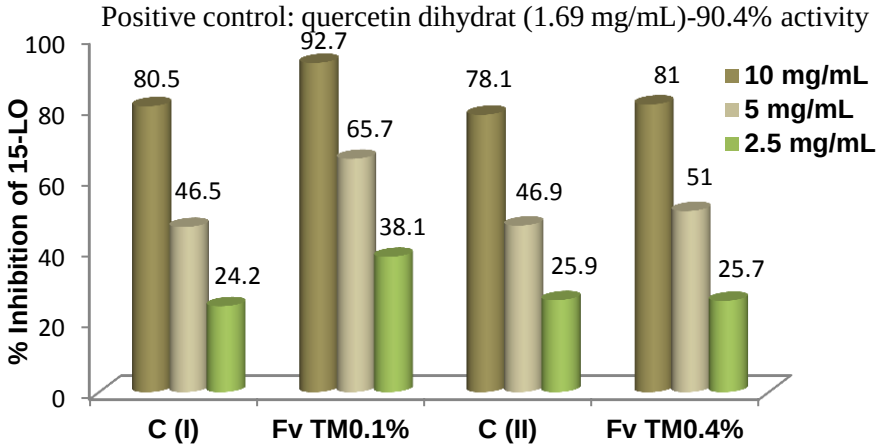


Figure 7. 15-LO inhibitory activity of fennel extracts
[C (I)-control year I; C(II)-control year II; FvTM 0.1%-treated fennel with 0.1% Topsin M; FvTM 0.1%-treated fennel with 0.4% Topsin M].

Investigation of DPPH radical scavenging activity of fennel extracts (Fig. 8) demonstrated that:

- all extracts possessed a weak DPPH radical scavenging activity (less than 50%);
- all tested extracts showed a concentration-dependent DPPH radical scavenging activity. At 10 mg/mL, the extracts showed the most potent activity (29.1-44.7%);
- all dilutions of extracts from treated fruits were more active than the corresponding controls but the differences are modest;
- all dilutions of extracts from control fruits in year I were more active than dilutions of extracts from control fruits in year II. Control fruits in year I contained a 7 times higher amount of flavonoids and a 4 times lower amount of polyphenolic acids than control fruits in year II. It is difficult to correlate the flavonoids and polyphenolic acids levels with DPPH radical scavenging properties and it is possible that besides polyphenols other components are also responsible for antioxidant activity [Miron et al., 2005].

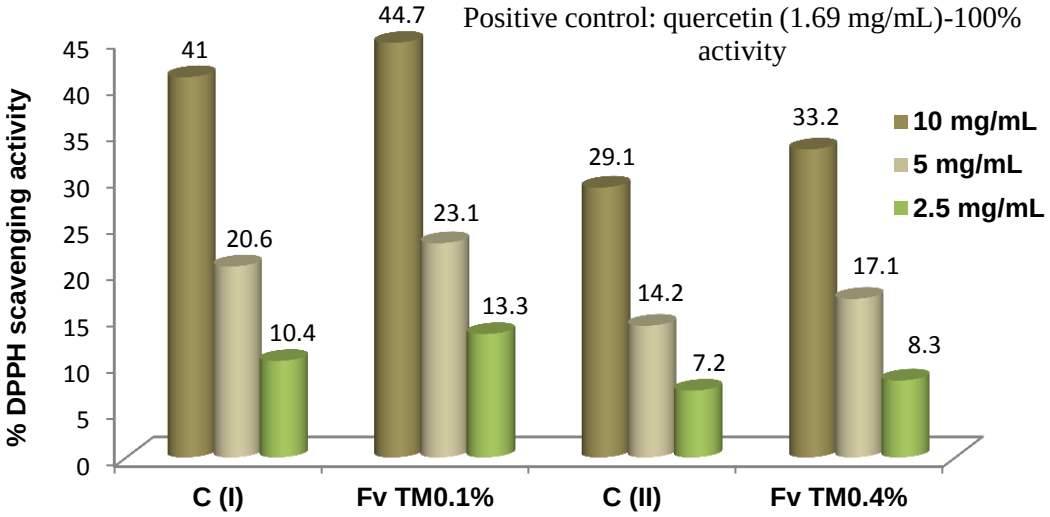


Figure 8. DPPH radical scavenging activity of fennel extracts
[C (I)-control year I; C(II)-control year II; FvTM 0.1%-treated fennel with 0.1% Topsin M; FvTM 0.1%-treated fennel with 0.4% Topsin M].

Antimicrobial activity

Antimicrobial effects of essential oils are one of the most known and well-founded activity of this class of plant metabolites. The chemical composition of the essential oil and the type of compounds are extremely important in defining their antimicrobial properties.

Investigation of antimicrobial properties of fennel essential oils (Table 4) showed that:

- all essential oils were more active against *Bacillus subtilis* and *Candida albicans*;
- only essential oil isolated from untreated fennel in year II (control II) active on *Escherichia coli*;
- only essential oils obtained from untreated fennel (both years I and II) were active on *Staphyloccocus aureus* bacteria;
- Topsin M was not active in the antimicrobial tests.
- anethole showed a poor activity against tested strains; it is obvious the fact that the other components of the fennel essential oils contribute significantly to the antimicrobial effects;
- generally, fennel essential oils were more active as antifungal than antibacterial agents; thus, *Foeniculum vulgare* EOs exhibited potent antifungal activity against *Candida spp*. dermatophyte species (*Tricophyton mentagrophytes*, *T. rubrum*, *T. tonsurans*, *Microsporum gypseum*)[Zeng et al., 2015], but also on food spoilage fungi such as *Aspergillus niger*, *A. japonicus*, *A oryzae*, *Fusarium oxysporum*, *Rhizophus oryzae* and *R. stolonifer* [Mota et al., 2015]. Our results for essential oils obtained from untreated fennel are similar or close to other reported data [Ozcan et al., 2006; Manzoor et al., 2016];
- Topsin M treatment decreased antibacterial effects of fennel essential oil but it did not affect the antifungal activity.

Table 4. Antimicrobial activity of fennel essential oils from untreated and Topsin M-treated plants)

Microorganisms		**Diameter of inhibition zone (mm)**					
		C (I)	**FvTM 0.1%**	**C (II)**	**FvTM 0.4%**	**Anethole**	**Topsin M**
G (+)	*Staphylococcus aureus* ATCC 25923	10	0	10	0	0	0
G (+)	*Bacillus subtilis* ATCC 6633	8	12	30	14	7	0
G (-)	*Escherichia coli* ATCC 25922	0	0	16	0	0	0
yeast	*Candida albicans* ATCC 10231	30	30	35	35	12	0

C (I)-control year I; C(II)-control year II; FvTM 0.1%-treated fennel with 0.1% Topsin M; FvTM 0.1% treated fennel with 0.4% Topsin M.

Conclusions

The antifungal treatments with Topsin M 0.1% and 0.4% applied on the *Foeniculum vulgare* Mill. cultures leads to some structural and chemical changes.

The histo-anatomical changes are mostly quantitative rather than qualitative. They depend on the treatment concentration and the climatic conditions of the culture year, being more pronounced in the case of high concentration of fungicide (0.4%).

The observed changes are included in a general picture of some adaptive response of the plants that signal a stress situation due to the antifungal treatment, especially at the 0.4% concentration of Topsin M.

As regard chemical profile, Topsin M treatments affect the polypenols content (mainly flavonoids) and the production of fennel essential oil. The changes are related to the year of plant culture and they must be interpreted in the context of year weather.

Treatment with Topsin M did not consistently influence antioxidant and antifungal activities but antibacterial properties of fennel essential oil are adversely affected.

The application of different phytosanitary treatments must take into account the monitoring of pesticide residues, but also of the possible influences on the nutritional quality and bioactivity of the plant product.

The monitoring of such changes requires phytochemical studies in several crop years in correlation with the climatic conditions, and also the determination of pesticide residues.

I.1.2. The chemical profile of *Foeniculum vulgare* essential oils dependent on the weather conditions during three years

Current state of the arte and research objectives

Previous personal studies showed the changes of morpho-anatomical and chemical features of fennel plants under phytosanitary treatments with Topsin M fungicide [Aprotosoaie et al., 2005; Miron et al., 2005]. Besides pesticide treatments, the biosynthesis and chemical composition of essential oils are strongly influenced by other factors as the environmental and climate conditions, season of harvesting, age of plants, the stage of fruits ripening, agronomic practices, or genetic data [Dudai, 2005]. Consequently, the chemical variability may cause differences in terms of pharmacological action and/or pharmaceutical quality of the fennel fruits and their essential oil.

There are numerous reports about the variation of chemical composition of essential oils obtained from fennel and other aromatic and medicinal plants. Özcan et al. (2006) showed that essential oils of *Foeniculum vulgare* ssp. *piperitum* exhibited qualitative and quantitative variations of the chemical composition with respect to the different parts of plants and the stage of fruit maturation. The content of some monoterpenes (sabinene, α-phellandrene, β-ocimene and γ-terpinene) decreases with advanced vegetation. Also, the content of sesquiterpene germacrene D decreases after the flowering period.

For *Achillea* species collected from Iran, the high germacrene D content was associated with cold climates whereas high levels of camphor and spathulenol were shown for plants that grown in soils contained higher organic matter in surface horizon [Rahimmalek et al., 2009].

The essential oils obtained from peppermint plants that grown at mild climates have high menthol content and low menthone levels. Also, for *Mentha spicata* (spearmint), the level of monoterpene hydrocarbons (D-limonene, β-phellandrene) is higher in temperate climates while the amount of volatile sesquiterpenes (germacrene D, β-caryophyllene) is higher in warmer locations [Telci et al., 2011].

The present study aimed to evaluate the influence of weather conditions on the essential oil chemical composition of fennel fruits harvesting during three different culture years.

Material and methods

Plant material

The ripe fruits of *Foeniculum vulgare* were harvested in September of each year (Year I, II and III) from cultivated field of "*Anastasie Fătu*" Botanical Garden, Iaşi. In this experimental area, cultures of fennel have been made during three years.

The type of soil was cambic chernozems and clay-illuvial chernozems.

Essential oils isolation

100 g of fennel fresh fruits were crushed and mixed with 1000 mL of bidistilled water and subjected to hydrodistillation for 3 h in a Clevenger-type apparatus.

The oil was separated, dried over anhydrous sodium sulfate and kept in a dark glass bottle at 4 °C until analysis.

The yield of fennel essential oils (v/w %) was calculated on a dry weight basis.

Gas chromatography-mass spectrometry (GC-MS) analysis of essential oils

GC-MS analysis was carried out by an analytical system, consisting of HP 5890 Series II gas chromatograph and HP 5971 mass spectrometer selective detector with electron impact ionization, using a HP-5MS capillary column (30 m × 0.25 mm internal diameter × 0.25 μm film thickness) and He as carrier gas (1 mL/min).

The analysis was performed using the following temperature program: 35 °C held for 5 min, then heated up to 260 °C at a rate of 10 °C per min, and maintained for 3 additional minutes at this temperature.

The temperature of injector was 250 °C and of the MS interface was 280 °C. Injection volume was 0.1 μL essential oil.

Evaluation of the results was performed using ChemStation Software and Wiley mass spectral library.

Identification of components

The compounds of fennel essential oils were identified by matching their recorded mass spectra based with those of analytical standards from Wiley Mass Spectral Library.

Meteorological data

Meteorological data covering March to October intervals of each year (I-III) were obtained from Moldova Regional Weather Station (Iasi, Romania).

The average values for surface soil temperature (° C), atmospheric temperature (° C), sunlight duration (hours), rainfall (mm/m^2), relative humidity (%) are illustrated in the Fig. 9-11.

Results and discussion

The yield of essential oils obtained from fennel fruits harvested during three consecutive years ranged widely from 2% (year III) to 12.60% (year II) (Table 5).

Also, the chemical composition of fennel essential oils showed a great qualitative and quantitative variability (Table 5). Thirteen (year I), six (year II) and fourteen (year III) compounds were identified, accounting for 97.77% (year I), 98.36% (year II) and 99.79% (year III) of the total essential oils.

The major compounds in all oils were *trans*-anethole (60.42-77.61%), fenchone (8.56-13.12%), estragole (methyl chavicol) (2.53-8.28) and limonene (0.34-1.85%).

The aromatic components and oxygenated monoterpenes constitute the bulk of all fennel oils.

We noticed a discret variation in the aromatic fraction content from 80.50% (year III) to 83.81% (year I) and 85.81% (year II). On the other hand a significant increase of the monoterpenes level from 12.55% (year II) and 13.96% (year I) to 19.29% (year III).

Generally, the decrease of the aromatic fraction content was accompanied by an increase in the levels of total monoterpenes.

The amount and composition of the oils seem to correlate with the meteorological data. Colder surface soil and atmospheric temperature (Fig. 9, Fig. 10) and abundant rainfall (Fig. 11) at the beginning of the vegetation period determined lower essential oil yield and higher level of monoterpenes (including oxygenated monoterpenes) for year 3 (2%; 19.29%, 13.61% respectively) compared to year 2 (12.6%; 12.55%, 11.15% respectively) and year 1 (11%; 13.96%, 12.69%, respectively).

Table 5. Main compounds identified in fennel essential oils [Aprotosoaie et al., 2010a]

RT (min)	Compound	(%)		
		Year I	**Year II**	**Year III**
4.30	1,8-cineole	4.00	-	-
5.04	α-pinene	0.34	-	1.88
5.13	β-pinene	0.59	-	0.33
6.34	cymene	1.36	-	-
8.65	**limonene**	**0.34**	**1.04**	**1.85**
9.11	γ-terpinene	-	0.95	-
9.35	camphene	-	-	0.16
10.18	myrcene	-	-	0.72
10.46	α-felandrene	-	-	0.34
10.69	α-terpinene	-	-	0.03
11.08	**fenchone**	**8.56**	**10.56**	**13.12**
11.48	γ-terpinene	-	-	0.36
13.08	camphor	-	-	0.41
13.78	terpinen-4-ol	0.02	-	0.08
14.62	**estragole**	**2.53**	**8.28**	**2.88**
14.93	α-terpineol	0.18	0.59	-
16.82	***trans*-anethole**	**60.42**	**75.81**	**77.61**
19.24	*p*-anisaldehyde	-	-	0.01
19.77	2,3,4,6-tetramethyl-phenol	-	1.72	-
20.22	thymol	**7.41**	-	-
20.59	carvacrol	**12.09**	-	-
Monoterpenes, of which:		13.96	12.55	19.29
Monoterpene hydrocarbons		*1.27*	*1.40*	*5.68*
Oxygenated monoterpenes		*12.69*	*11.15*	*13.61*
Aromatic compounds		83.81	85.81	80.50
Total identified (%)		**97.77**	**98.36**	**99.79**
Yield (v/m%)		**11**	**12.60**	**2**

RT= retention time (min.)

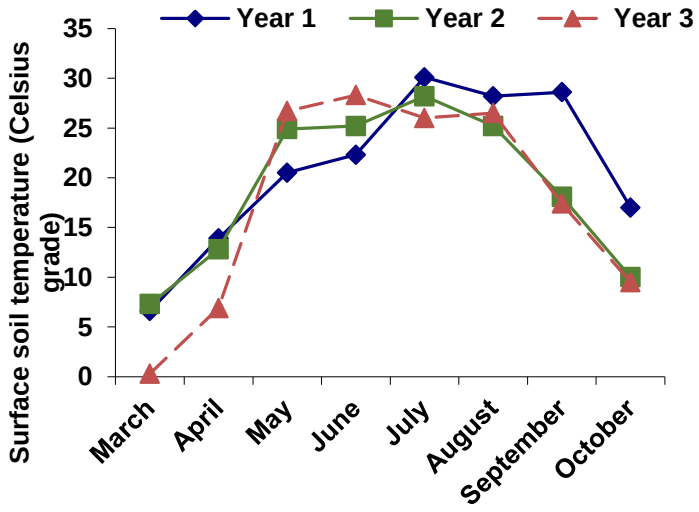


Figure 9. Surface soil temperature (° C) - monthly average data for March-October intervals of culture years [Aprotosoaie et al., 2010a]

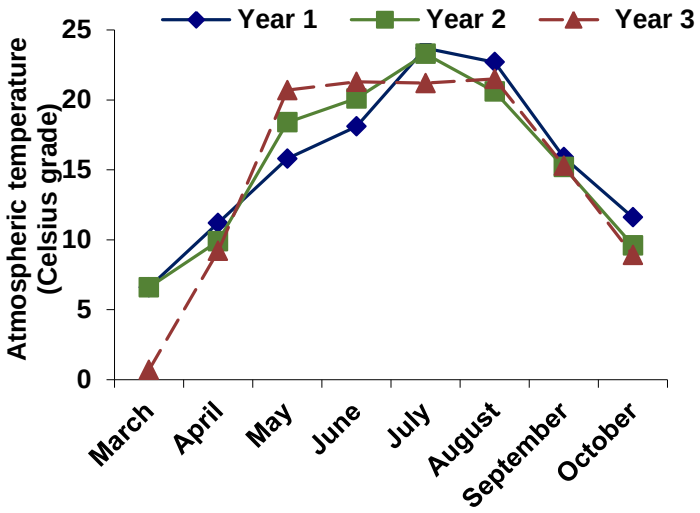


Figure 10. Atmospheric temperature (° C) - monthly average data for March-October intervals of culture years [Aprotosoaie et al., 2010a]

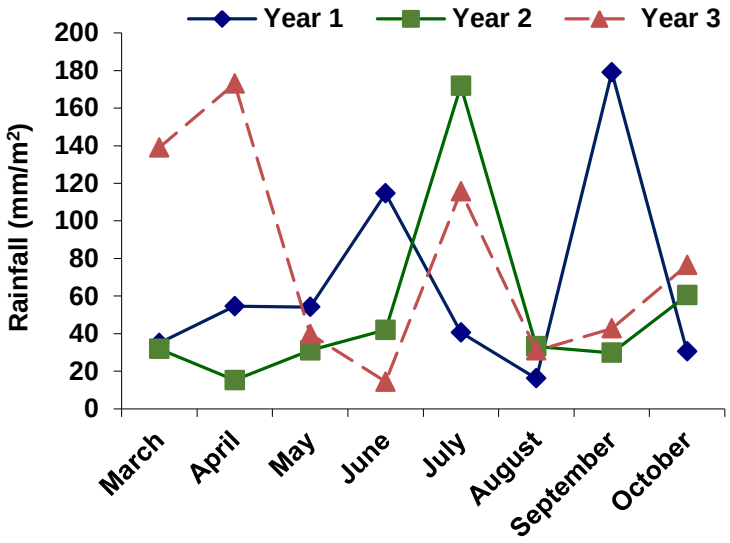


Figure 11. Rainfall (mm/m^2) - monthly average data for March-October intervals of culture years [Aprotosoaie et al., 2010a]

Conclusions

The essential oil of fennel fruits (*Foeniculum vulgare* Mill.) cultivated in Iasi (Romania) showed a characteristic chemical profile from year to year. While *trans*-anethole, estragole, fenchone and limonene were the main components in all oils, qualitative and quantitative differences in composition were observed.

The content and its monoterpenes components were the most susceptible features of fennel essential oil to be affected by weather conditions (temperature and rainfall).

I.1.3. Influence of planting density on yield and essential oil composition of Romanian *Hyssopus officinalis* L.

Current state of the arte and research objectives

Hyssopus officinalis L. (Lamiaceae) (hyssop) is an aromatic, shrubly perennial plant, native to Southern of Europe and the temperate zones of Asia. The aerial part of the plant is used for expectorant, stomahic, antispasmodic and diuretic properties; it is also employed as flavouring agent in the food industry, in liquers, cosmetic field and in perfumery.

The essential oil and various extracts from hyssop have been shown to possess antimicrobial, antiplatelet and α-glucosidase inhibitory activities [Jankovsky and Landa, 2002].

It is well-known that the production of essential oils depends upon the metabolic state and the physiology of the plant, but also the environmental factors and agronomical practices, such as fertilization, water supply or planting density [Ncube et al., 2012].

Therefore, the optimization of planting density can lead to achieve a higher yield in the crop by favorable effect on the plant exposure to the light or moisture and the absorbtion of nutrients.

Khorshidi et al. (2009) showed that fennel essential oil content was affected significantly by different planting densities and the maximum essential oil percentage was obtained with the minimum plant density (30 cm). Similar results have been reported for *Thymus vulgaris* [Naghdibadi et al., 2002; Khazaie et al., 2008] *Ocimum basilicum* [El-Gendy et al., 2001] and *Nigella sativa* essential oils [Ahmed et al., 1986].

Also, our group reported that the coriander essential oil yield, monoterpenols content and fruit productivity per hectare decreased at the greatest row spacing (50 cm)[Trifan et al., 2010].

In the present study we investigated the effect of different densities of planting on yield and essential oil composition of *Hyssopus officinalis* L. cultivated in the Northeast of Romania.

Material and methods

Plant material

A bifactorial experiment was carried out in field of Agricultural Research and Development Centre, Secuieni - Piatra Neamț (România), in 2008 and 2009.

The experiment was performed with three plant densities on the row (0, 20 and 30 cm); the distance between plant rows was 50, 70 and 100 cm, respectively.

Aerial parts were collected from the plants in the second year of vegetation, in July 2009.

The samples and their codification are given in Table 6.

Table 6. The samples of *Hyssopus officinalis* [Aprotosoaie et al., 2010c]

Variant	H1	H2	H3	H4	H5	H6	H7	H8	H9
Plant densities on the row (cm)	0	20	30	0	20	30	0	20	30
Distance between plant rows (cm)	50			70			100		

Isolation of essential oils

100 g of powdered dried aerial parts were subjected to hydrodistillation for 3 h in a Clevenger apparatus.The essential oils were dried over anhydrous sodium sulfate and stored in dark glass tubes at 4°C until analysis.

The yield of essential oils was calculated as L/ha.

Chemical analysis of essential oils

The constituents of the essential oils have been characterized by gas chromatography coupled with mass spectrometry (GC-MS). GC-MS analysis was carried out on Agilent 6890N gas chromatograph coupled with a mass spectrometer selective detector (Agilent 5975 inert XL), and using a HP-5MS capillary column (30 m × 0.25 mm internal diameter × 0.25 μm film thickness) and He as carrier gas (1 mL/min).

The oven temperature was programmed as follows: 35 °C held for 5 min, then heated up to 280 °C at a rate of 10 °C per min; the final temperature was held for 5.5 min. The temperature of injector was 250 °C and of the MS interface was 280 °C. Injection volume was 0.1 μL essential oil.

Identification of components

The compounds of hyssop essential oils were identified by matching their recorded mass spectra based with those of analytical standards from Wiley Mass Spectral Library.

Biomass yield and morphological data

Dried biomass yield (kg/ha) was calculated and some morphological parameters of hyssop plants as number of branches/plant and length of inflorescence (cm) were measured.

Results and discussion

The dried hyssop herbage biomass yield varied between 1754.30 Kg/ha to 2536.10 Kg/ha, while the yield essential oil ranged from 31.57 L/ha to 60.86 L/ha (Table 7).

The highest essential oil yield (60.86 L/ha) was obtained with 70 cm row spacing and 20 cm plant space on the row (H5 sample). In fact, we noticed that the essential oil productivity and herbage biomass yield per hectare increased at the medium planting densities (70 cm row spacing).

These findings are correlated with morphological data. Thus, at medium planting space, the plants produced more branches and the length of inflorescence increased (Table 8).

Table 7. Productivity of hyssop herbage biomass and essential oils [Aprotosoaie et al., 2010c]

Variant/ Parameter	**H1**	**H2**	**H3**	**H4**	**H5**	**H6**	**H7**	**H8**	**H9**
Yield of herbage biomass (kg/ha)	1936.80	2054.60	2005.60	**2425.80**	**2536.10**	**2341.50**	1826.30	1808.10	1754.30
Yield of essential oil (L/ha)	32.92	41.09	35.09	**48.51**	**60.86**	**49.17**	42.00	47.01	31.57

Table 8. Some morphological features of hyssop plants [Aprotosoaie et al., 2010c]

Variant/Parameter	**H1**	**H2**	**H3**	**H4**	**H5**	**H6**	**H7**	**H8**	**H9**
Average number of branches/plant	15.4	15.9	16.30	**26.80**	**25.30**	**24.70**	23.20	22	21.90
Average length of inflorescence (cm)	10.22	10.78	10.96	**13.46**	**12.71**	**12.26**	12.33	11.49	11.63

The hyssop essential oils are composed mainly of monoterpenes ans sesquiterpenes. A total of forty (H4) to sixty-one (H2) components were identified in the hyssop essential oil samples, amounting to a total percentage between 92.04% (H1) and 94.15% (H9).

The main components in all hyssop essential oils were: isopinocamphone (*cis*-pinocamphone) (48.65-56.40%), β-pinene (7.45-10.68%), myrtenol (3.57-4.53%) and germacrene D (0.12-3.43%) (Table 9).

Monoterpene fraction predominates in all essential oils (71.18-80.44%) being represented mainly by monoterpene ketones (49.34-57.05%).

The bicyclic monoterpene ketones such as *cis*-isopinocamphone and *trans*-pinocamphone are generally known as the main characteristic components of the essential oils from the hyssop plants [Figueredo et al., 2012].

The investigated oils from Romanian hyssop are rich in *cis*-pinocamphone.This monoterpene ketone was also reported for hyssop essential oils from other countries as Bulgaria [Hristova et al., 2015], Italy [Piccaglia et al., 1999; Manitto et al., 2004] or former Yugoslavia [Chalcat et al., 2001].

The highest concentrations of monoterpenes (80.44%) and monoterpene ketones/isopinocamphone were obtained for medium planting space (70 cm row spacing) (H5, H4 variants).

Table 9. Main components of hyssop essential oils [Aprotosoaie et al., 2010c]

RT (min)	**Compound**	**%**								
		H1	**H2**	**H3**	**H4**	**H5**	**H6**	**H7**	**H8**	**H9**
5.89	sabinene	3.79	1.71	1.61	1.65	1.42	1.96	1.57	1.67	2.07
5.97	**β-pinene**	**8.01**	**7.70**	**7.45**	**9.96**	**7.93**	**8.66**	**9.43**	**10.68**	**9.25**
6.11	β-myrcene	1.25	1.34	1.27	1.84	2.07	1.44	1.63	1.60	1.59
6.98	ocimene	1.52	1.30	1.52	1.27	1.31	1.65	1.17	0.47	2.15
7.80	linalool	2.21	1.55	1.35	1.04	1.10	1.05	0.97	1.03	1.08
8.86	**isopinocamphone**	**48.65**	**49.41**	**53.83**	**56.40**	**53.14**	**52.43**	**56.29**	**50.75**	**52.92**
8.97	L-menthol	-	-	-	-	2.17	-	-	-	0.37
9.18	β-fenchyl alcohol	0.86	0.70	0.59	-	-	0.65	-	0.72	0.60
9.19	α-terpineol	-	-	-	-	1.02	-	-	-	-
9.29	**myrtenol**	**3.57**	**3.60**	**4.35**	**4.07**	**4.24**	**4.53**	**3.93**	**4.03**	**4.45**
12.43	β-caryophyllene	3.08	1.20	0.81	0.73	0.82	0.81	0.72	0.88	0.53
12.95	aromadendrene	0.57	0.84	0.83	1.08	0.96	1.25	1.09	1.51	1.35
13.20	**germacrene D**	**3.43**	**2.99**	**2.58**	**2.37**	**2.31**	**2.47**	**2.34**	**3.11**	**0.12**
13.38	germacrene B	2.16	-	-	-	-	-	-	-	-
13.39	bicyclogermacrene	-	2	1.70	1.53	1.38	1.80	1.70	2.12	1.57
13.98	elemol	0.92	2.31	2.11	1.49	1.19	1.57	1.88	2.08	1.59
14.37	(+)-spathulenol	1.58	1.74	1.68	1.24	1.04	1.50	1.27	1.52	1.40

14.46	caryophyllene oxide	2.48	-	-	-	-	-	-	-	-
14.98	γ-eudesmol	0.39	0.82	0.63	-	0.49	-	0.63	0.66	-
15.08	Δ-cadinene	1.64	0.31	0.23	0.18	0.20	0.21	0.19	0.23	0.22
15.23	α-eudesmol	-	1.61	-	-	-	0.72	-	-	0.50
15.24	γ-gurjunene	1.83	0.54	0.52	0.26	0.38	1.61	0.27	0.50	0.41
15.69	γ-muurolene	0.30	0.90	1	0.72	0.51	0.94	0.78	0.70	0.60
Monoterpenes		71.18	71.78	76.47	80.40	80.44	76.93	79	74.91	79.55
Sesquiterpenes		20.86	21.26	17.41	12.05	12.01	17.04	14.65	17.34	14.60
Total identified (%)		**92.04**	**93.04**	**93.88**	**92.45**	**92.45**	**93.97**	**93.65**	**92.25**	**94.15**

According to the International ISO9841/2013 standard, the amount of β-pinene and isopinocamphone in hyssop essential oil should be 13.6-23% and 34.5-50%, respectively.

The levels of β-pinene recorded in samples of hyssop oils were slightly less than the standard lower limit while the values of isopinocamphone have slightly exceeded the upper limit.

The sesquiterpenes ranged from 12.01% to 21.26%; the main compounds are germacrene D, spathulenol, β-caryophyllene and elemol.The level of sesquiterpenes increased at the narrow spacing (H1-H3 variants).

In addition to the quantitative changes of hyssop volatiles, we also noticed significant quantitative changes.

These refer to the presence of menthol (H5 and H9 samples) and α-terpineol (H5 sample) monoterpenoids as well as of germacrene B (H1 sample), caryophyllene oxide (H1 sample) and α-eudesmol (H2, H6, H9 samples) sesquiterpenes.

Conclusions

In the pedoclimatic conditions of the experimental field, hyssop plants showed an increase of essential oil productivity and herbage biomass yield per hectare and of monoterpene ketones (isopinocamphone) levels at the medium planting density (70 cm between rows).

Acknowledgements

The work was sustained from the PNCDI-2 Program 4: 61-039 and 31-061, financed by National Authority for Scientific Research - Romanian Government.

I.1.4. Chemical composition of essential oils from leaves and twigs of two new varieties of Moldavian *Salvia officinalis* L.

Current state of the arte and research objectives

Salvia officinalis L. (common sage, Lamiaceae) is a small evergreen subshrub, native to mediterranean region, with a long history of medicinal, culinary and ornamental use. Extracts of sage have been shown to exhibit anti-inflammatory, antimicrobial, antioxidant, antihydrotic, spasmolytic and neuroprotective properties [Ghorbani and Esmaeilizadeh, 2017; Sharifi-Rad et al., 2018].The typical products of the plant are two major chemical classes of secondary metabolites: terpenoids and phenolics.

Among the terpenoids, the essential oil produced in the aerial parts of the plant has acquired a special interest for the broad range of applications in aromatherapy and in food industry. As known, the biosynthesis of the essential oils is influenced by various factors, such as: genetic structure and environmental conditions.

The analysis of 48 clones of Greek sage (*Salvia fruticosa* Mill.) as respects the chemical composition of essential oil and genetic profile evidenced by the use of Random Amplified Polymorphic DNA (RAPD) markers demonstrated the existence of a genetic determinism in the contouring of the chemical profile [Skoula et al., 1999]. Böszörményi et al. (2009) showed that there is a strong relationship between the chemical profile and the genetic pools among the Greek sage and ornamental cultivars of *Salvia officinalis* (Purpurascens, Tricolor and Kew Gold). Duarte et al. (2010) have shown that chemovariation in *Eugenia dysenterica* may be genetically determined. Also, Echeverrigaray et al. (2001) identified a perfect correlation between the chemical and the genetic profile in commercial cultivars of *Thymus vulgaris*. The same authors showed that the diversity in the composition of the *Cunila gallioides* Benth. (dittany) essential oil is affected primarily by genetic background rather than environmental factors [Agostini et al., 2008].

In this context, the main goal of this study was to assess the chemical profile of the essential oils obtained from two new varieties of common sage, Miracol and GB Nikita, cultivated in Republic of Moldova.

Miracol variety was created by the Institute of Genetics and Physiology of Plants, Chişinău and it is approved and registered in Republic of Moldova. The plants of Miracol variety are drought and low temperature-resistant with middle period of technical ripening; a single harvest provides an yield of 18 kg essential oil /ha or 8400 kg dry leaves/ha.

GB Nikita was created by the Botanical Garden Nikita, Crimea and it is largely cultivated in the South of Republic Moldova (Cahul district).

Material and methods

Plant material

The leaves and twigs with leaves from two new varieties of *Salvia officinalis* (Miracol and GB Nikita) were harvested during flowering stage (June 2009) from the fields of Institute of Genetics and Physiology of Plants from Chişinău, Republic of Moldova.

Extraction of essential oils

The dried plant material of each sample was submitted to hydrodistillation for 3 h using a Clevenger-type apparatus. The essential oil obtained was dried over anhydrous sodium sulfate and kept in amber vials at 4 °C until analysis.

Gas chromatography-mass spectrometry analysis

An Agilent 6890N gas chromatograph equipped with a mass spectrometer selective detector (Agilent 5975 inert XL and an HP-5MS capillary column (30 m × 0.25 mm internal diameter × 0.25 μm film thickness) was used for the chemical analysis of essential oils. Helium was used as the carrier gas at a flow rate of 1 mL/min, and the split ratio was set to 100:1.

The initial oven temperature was set at 40 °C then increased at 10 °C per min to 280 °C and finally held isothermally for 5.5 min. The temperature of injector was 250 °C and of the MS interface was 280 °C. Injection volume was 0.1 μL of essential oil.

Mass spectra in the electron mode were generated at 70eV.

Identification of components

The compounds of common sage essential oils were identified by matching their recorded mass spectra based with those of analytical standards from Wiley Mass Spectral Library.

The percentage composition of the essential oils was computed from GC peak areas without correction factors.

Results and discussion

The analysis of sage essential oils allowed the identification of thirty-eight compounds accounting for 96.58-97.72% of the total oils composition (Table 10). The major compounds in all oils were: α-thujone (29.60-36.55%), L-camphor (12.17-18.13%), 1,8-cineole (12.85-15.18%), camphene (3.62-5.50%), α-pinene (2.40-5.52%) and β-caryophyllene (2.71-4.10%).

Moldavian sage essential oils are composed mainly of monoterpenes and sesquiterpenes and monoterpenes predominate (86.16-90.33%).

Oxygenated monoterpenes constitute the bulk of the oil in all sage samples (69.99-73.32%), and monoterpene ketones are the most abundant components (49.49% - 53.73% for Miracol variety and 50.73% - 53.03% for GB Nikita variety, respectively) (Table 10).

β-Thujone, a characteristic compound of sage essential oils, was identified only in GB Nikita variety; in this case, the level of β-thujone was 53 times higher in essential oil obtained from twigs and leaves than in the essential oil isolated only from the leaves (11.26% *vs.* 0.21%).

The sage essential oils from Miracol variety contained significant amounts of monoterpen-hydrocarbons (17.32 - 20.34%), the high values being identified for leaf essential oils.

Sesquiterpenes are minor constituents of investigated sage essential oils (6.17-10.04%). Nikita variety contains high levels of sesquiterpenes (8.76-10.04%), particularly the essential oil obtained from twigs and leaves.

Manool, a labdane-type diterpene, was identified only in Nikita sage essential oils. High concentrations of manool (over 6%) have been reported for the sage essential oils (mainly flower essential oils) from Portugal, Serbia, Montenegro and Lithuania [Mockutë et al., 2003].

Three different chemotypes were described for European *Salvia officinalis*, as follows:

a) *α-pinene, camphor, β-thujone*;

b) *α-thujone, camphor, 1,8-cineole* and

c) *β-thujone, camphor* [Mockutë et al., 2003].

Also, depending on thujone content, chemotypes with low (9%), medium (22-28%) and high (39-44%) thujone content in sage essential oil have been characterized.

The analysis of volatile chemical composition sage Moldavian varieties showed that they belong to the same chemotype *thujone/camphor/1,8-cineole*.

Table 10. Compounds identified in sage essential oils [Aprotosoaie et al., 2010d]

RT (min)	**Compound**	**%**			
		Miracol		**GB Nikita**	
		Leaves	**Twigs with leaves**	**Leaves**	**Twigs with leaves**
5.10	tricyclene	0.13	-	0.10	0.09
5.16	α-thujene	0.20	0.23	0.21	0.23
5.28	α-pinene	5.07	5.52	2.60	2.40
5.51	camphene	5.50	4.23	3.80	3.62

5.87	sabinene	0.24	0.26	0.13	0.14
5.93	β-pinene	2.41	2.43	2.42	2.28
6.10	myrcene	1.26	1.34	1.17	1.12
6.34	α-phellandrene	0.13	0.10	0.09	0.07
6.53	α-terpinene	0.24	0.24	0.31	0.30
6.65	*p*-cymene	0.20	0.22	0.33	0.30
6.97	*trans*-β-ocimene	0.38	-	0.37	0.12
6.71	limonene	1.90	1.92	1.63	1.39
6.77	**1,8-cineole**	**12.85**	**13.92**	**15.18**	**13.94**
7.17	γ-terpinene	0.55	0.50	0.61	0.59
7.31	*cis*-sabinene hydrate	0.27	-	0.16	0.19
7.63	terpinolene	2.19	0.55	0.52	1.67
7.76	linalool	-	0.88	1.19	-
7.92	**α-thujone (*cis*)**	**31.36**	**38**	**36.55**	**29.60**
8.32	isothujol	-	-	0.27	-
8.44	sabinol	0.43	-	-	-
8.80	**β-thujone**	**-**	**-**	**0.21**	**11.26**
8.53	**camphor**	**18.13**	**15.73**	**13.97**	**12.17**
8.83	borneol	3.27	2.04	3.14	2.42
8.99	terpineol-4	0.58	0.39	0.52	0.46
9.08	isoborneol	0.25	-	-	-
9.17	α-terpineol	0.31	-	0.23	0.16
9.27	estragol	0.34	-	-	-
10.03	vetiverol	-	0.44	-	-
10.20	allo-ocimene	0.14	-	-	-
10.54	(-)-bornyl acetate	2.54	1.91	1.90	1.94
12.41	β-caryophyllene	2.98	2.71	3.61	4.10
12.53	(Z,Z)-α-farnesene	0.07	-	-	-
12.84	α-humulene	2.39	2.14	3.16	3.49
12.94	alloaromadendrene	0.13	-	-	-
14.45	caryophyllene oxide	0.10	-	0.20	0.25
14.55	γ-gurjunene	0.68	0.88	1.72	2.11
15.06	γ-muurolene	-	-	0.07	0.09
19.33	manool	-	-	0.33	0.39
Total monoterpenes, of which:		90.33	90.19	87.28	86.16
Monoterpene hydrocarbons		20.34	17.32	13.96	14.02
Oxygenated monoterpenes		69.99	72.87	73.32	72.14
Sesquiterpenes		6.35	6.17	8.76	10.04
Aromatic compounds		0.54	0.22	0.33	0.30
Diterpenes		-	-	0.33	0.39
Total identified (%)		**97.22**	**96.58**	**96.70**	**96.89**

Similarly, these compounds have been identified as major constituents for sage oils from Estonia, France, Hungary, Belgium, Russia, Greece, Albania, Montenogro, Ukraine or Scotland,

although their proportions vary [Pop Cuceu et al., 2014; Raal et al., 2007; Craft et al., 2017]. The highest content in thujone was obtained for sage from Albany (59.8%) [Asllani, 2000]. Also, Oniga et al. (2010) identified for Romanian sage, high concentrations of thujone (31.23-52.86%). 1,8-Cineole predominates (45.3%) in Greek sage, and camphor in samples from Albania (37.8%), Scotland (29.8%) and Russia (28.5%) [Pop Cuceu et al., 2014].

Regarding the essential oil in sage varieties from Moldova, one can mention:

- from the point of view of the content in camphor and 1,8-cineole, there are a profile closer to the oils generally obtained from plants which grown in Balkans or Northeast of Europe;
- from the point of view of the content in thujones, a less differentiated profile depending on the geographical area (Table 11).

Table 11. Profile of Moldavian sage essential oils *vs.* profile of different European sages

Compound	Miracol variety (%)	Similar profile		GB Nikita variety (%)
thujones	31.36-38	France, Ukraine, Estonia, Hungary, Egypt		36.76-40.86
camphor	15.73-18.13	Russia, Estonia Belgium, Romania	Hungary, Greece, Ukraine, Romania	12.17-13.97
1,8-cineole	12.85-13.92	France, Belgium, Ukraine, Estonia, Lithuania, Montenegro	Estonia, Russia	13.94-15.18

Only the essential oil obtained from twigs and leaves of the GB Nikita variety can be characterized as falling within the high content thujone chemotype (40.86%). Although thujone, along with camphor and 1,8-cineole, is one of the main compounds responsible for the biological properties of sage oil, it should be kept in mind that it is also responsible for toxic effects as well. A high concentration of thujone (more than 50%) and the levels of monoterpenes-ketones greater than 70% may limit the therapeutic use of the product [Oniga et al., 2010].

Monoterpen-ketones are generally considered to be neurotoxic and epileptogenic. α-Thujone is considered to be more toxic than β-thujone. It is a rapid-acting and readily detoxified modulator of the GABA-gated chloride channel [Höld et al., 2000].

If the sage essential oils chemical profiles are compared with the ISO 9909/1997 criteria [*cis*-thujone: 18-43%; camphor:4.5-24.5%; 1,8-cineole: 5.5-13%, *trans*-thujone: 3-8.5%; α-humulene ≤12%; α-pinene: 1-6.5%; camphene: 1.5-7%, limonene: 0.5-3%, bornyl acetate (≤ 2.5%)], we noticed that they are not respected in terms of 1,8-cineole (twigs with leaves of Miracol variety

and both samples of GB Nikita variety) and *trans*-thujone content (twigs with leaves of GB Nikita variety).

Conclusions

The investigated sage samples showed a characteristic profile of their essential oils, with some qualitative and quantitative differences between varieties, but also between leaves and twigs with leaves from each variety.

The most notable difference is the lack of *trans*-thujone in Miracol sage variety.

For the extraction of essential oil it is indicated to use the leaves of these varieties, the twigs with leaves allowing to obtain large quantities of thujone and to limit the medical use.

I.1.5. Chemical variability of volatiles in some natural populations of *Thymus pulegioides* L. growing in the Northeast of Romania

Current state of the arte and research objectives

Thymus species are well known as aromatic and medicinal plants. In traditional medicine, flowering parts and leaves of *Thymus* plants are widely used as antiseptic, antitussive, stomahic and carminative as well as treating colds. Various studies have showed that the *Thymus* species have strong antibacterial, antifungal, spasmolytic and antioxidant properties. In addition, *Thymus* essential oils are used in pharmaceutical, cosmetic and perfume industry and also for flavouring and preservation of several food products [Stănescu et al., 2018].

The uses of *Thymus* species depend largely on the chemical composition of the essential oil. Also, non-volatile polyphenols are another major category of bioactive compounds. Many *Thymus* species were studied primarily for the composition of essential oils and more chemotypes have been reported.The qualitative composition of essential oils of *Thymus* chemotypes are controlled genetically but the environment and harvesting time can influence the chemical profile of volatiles.

The presence of the major monoterpenes in thyme essential oils is controlled by a dominance and/or epistatic effect of five biosynthetic loci possessing the following sequence: geraniol > α-terpineol > thuyanol > linalool > carvacrol > thymol [Vernet et al., 1986].

Ložienė and Venskutonis (2005) reported that a sudden change of environmental conditions can lead to the occurrence of two types of *Thymus pulegiodes* plants, namely:

- plants that preserve their chemical composition of volatile oil and
- plants that considerably change their chemical profile of volatiles.

It appears that the environmental factors influence the interaction of the genes that determine plant chemotype.The proportion of linalool chemotype plants rises and that of phenol chemotype declines with increasing carbonate content in soil [Mártonfi et al., 1994].

Also, the linalool content in *Thymus vulgaris* plants was higher at low soil water level [Letchamo et al., 1994]. In *Thymus capitatus* plants, the monoterpene hydrocarbons content decreases at the beginning and during the flowering whereas the amount of carvacrol increases drastically [Casiglia et al., 2015].

As a part of our screening programme of the aromatic flora of Northeast of Romania, we here report on the volatile constituents from four wild populations of *Thymus pulegioides* from two counts of this area. *Thymus pulegioides* L. (large thyme or larger wild thyme) is an Euro-Siberian species distributed mostly in northern and middle Europe.This species is resistant to frost and the plants can be harvested easily by mechanical gathering [Ložienė et al., 2003; Ložienė, 2009]. It

is used for similar purposes or as substitute for *Thymus vulgaris* L (common thyme) [Zarzuelo and Crespo, 2002].

Material and methods

Plant material

The aerial parts of *Thymus pulegioides* populations were collected at the flowering stage (june 2009) from Northeast of Romania, namely Suceava and Neamt counties (Table 12). The climate in the two counties is of temperat continental type. Voucher specimens of each population were deposited in the Herbarium of the "Stejarul" Biological Research Center from Piatra Neamț.

Extraction of essential oils

The fresh aerial parts of each sample were submitted to hydrodistillation for 3 h using a Clevenger-type apparatus. The essential oils obtained were dried over anhydrous sodium sulfate and kept in amber vials at 4 °C until analysis.

Table 12. Sites of collection of the populations of *Thymus pulegioides* [Aprotosoaie et al., 2010b]

Plant source	Collection site	Altitude (m)	Population codification
Thymus pulegioides ssp. *montanus* (Bentham) Rouniger	Vama (Suceava County)	533	Tp1
Thymus pulegioides L. ssp. *chamaedrys* (Fries) Gusuleac	Valea Putnei (Suceava County)	835	Tp2
Thymus pulegioides ssp. *pulegioide*s L.	Secuieni (Neamt County)	205.70	Tp3
Thymus pulegioides L. ssp. *chamaedrys* (Fries) Gusuleac	Galu (Neamt County)	800-1000	Tp4

Gas chromatography-mass spectrometry analysis

An Agilent 6890N gas chromatograph equipped with a mass spectrometer selective detector (Agilent 5975 inert XL and an HP-5MS capillary column (30 m × 0.25 mm internal diameter × 0.25 μm film thickness) was used for the chemical analysis of essential oils. Helium was used as the carrier gas at a flow rate of 1 mL/min, and the split ratio was set to 100:1. The initial oven temperature was set at 40 °C then increased at 10 °C per min to 280 °C and finally held isothermally for 5 min. The temperature of injector was 250 °C and of the MS interface was 280 °C. Injection volume was 0.1 μL of essential oil. Mass spectra in the electron mode were generated at 70eV.

Identification of components

The compounds of common sage essential oils were identified by matching their recorded mass spectra based with those of analytical standards from Wiley Mass Spectral Library. The percentage composition of the essential oils was computed from GC peak areas without correction factors.

Results and discussion

The essential oils of *T. pulegioides* yielded from 0.4% to 0.7% (Table 13). The highest level of essential oil was identified for plant population from Neamt County that grow at the lowest altitude (205 m). The chemical constituents identified by GC-MS in the essential oils of *Thymus pulegioides* populations are listed in Table 13.

Table 13. Main compounds identified in *Thymus pulegioides* volatile oils [Aprotosoaie et al., 2010b]

RT (min.)	**Compound**	**(%)**			
		Tp1	**Tp2**	**Tp3**	**Tp4**
6.81	*trans*-β-ocimene	-	2.44	1.30	3.48
7.17	γ-terpinene	-	2.05	-	4.12
7.77	**α-terpinolene**	**12.94**	**24.58**	**3.14**	**5.81**
8.54	camphor	0.81	2.44	-	0.69
8.83	borneol	0.56	0.79	0.58	0.30
8.99	terpinene-4-ol	1.21	0.85	1.33	0.48
9.18	β-fenchyl alcohol	2.09	0.80	0.89	0.57
9.67	nerol	1.46	4.79	1.62	0.67
9.87	Z-citral	5.24	4.21	-	1.58
9.92	carvacrol methyl ether	-	-	2.45	2.98
10.03	β-myrcene	**9.57**	0.73	0.52	2.31
10.04	*trans*-geraniol	-	-	8.51	-
10.28	**E-citral (geranial)**	**6.74**	**5.04**	**9.59**	**2.29**
10.52	lavandulyl acetate	3.01	-	-	-
10.53	thymol	-	-	3.10	1.02
10.68	**carvacrol**	**3.85**	**1.62**	**15.22**	**8.69**
11.51	neryl acetate	0.99	1.14	0.23	0.35
11.95	β-bourbonene	0.80	1.34	0.66	0.66
12.41	**β-caryophyllene**	**2.96**	**3.96**	**4.62**	**5.49**
13.18	**germacrene D**	**6.38**	**12.46**	**3.39**	**9.66**
13.37	farnesene	-	2.08	0.54	-
13.43	β-bisabolene	2.83	1.54	5.23	2.79
14.05	**nerolidol**	**-**	**5.54**	**1.17**	**8.20**
14.06	**farnesol**	**11.24**	-	-	-
14.45	caryophyllene oxide	1.15	-	0.99	-
15.06	Δ-cadinene	4.92	2.52	-	1.18
Monoterpenes, *of which*		46.99	51.19	30.32	28.82
Monoterpene hydrocarbons		*22.51*	*30.53*	*6.21*	*20.69*
Monoterpenoids		*24.48*	*20.66*	*24.11*	*8.13*
Sesquiterpenes		36.56	31.75	18.89	35.02
Aromatic compounds		4.05	1.62	25.60	12.97
Yield (%)		**0.5**	**0.6**	**0.7**	**0.4**

The essential oils of Romanian *Thymus pulegioides* populations were dominated by monoterpenes (28.82-51.19%) and sesquiterpenes (35.02-36.56%) excepting the oil obtained from Secuieni (Neamt County) population where monoterpenes (30.32%) and aromatic fraction (25.60%) are predominant. In the oil obtained from the *Thymus pulegioides* subsp. *montanus* population growing in Suceava County (Tp1), monoterpenes attained 46.99%, and α-terpinolene (12.94%), E-citral (6.74%) and β-myrcene (9.57%) were the major components of this fraction (Fig. 12); sesquiterpenes constituted 36.56% of the oil and the most abundant compounds were germacrene D (6.38%), farnesol (11.24%) and Δ-cadinene (4.92%).

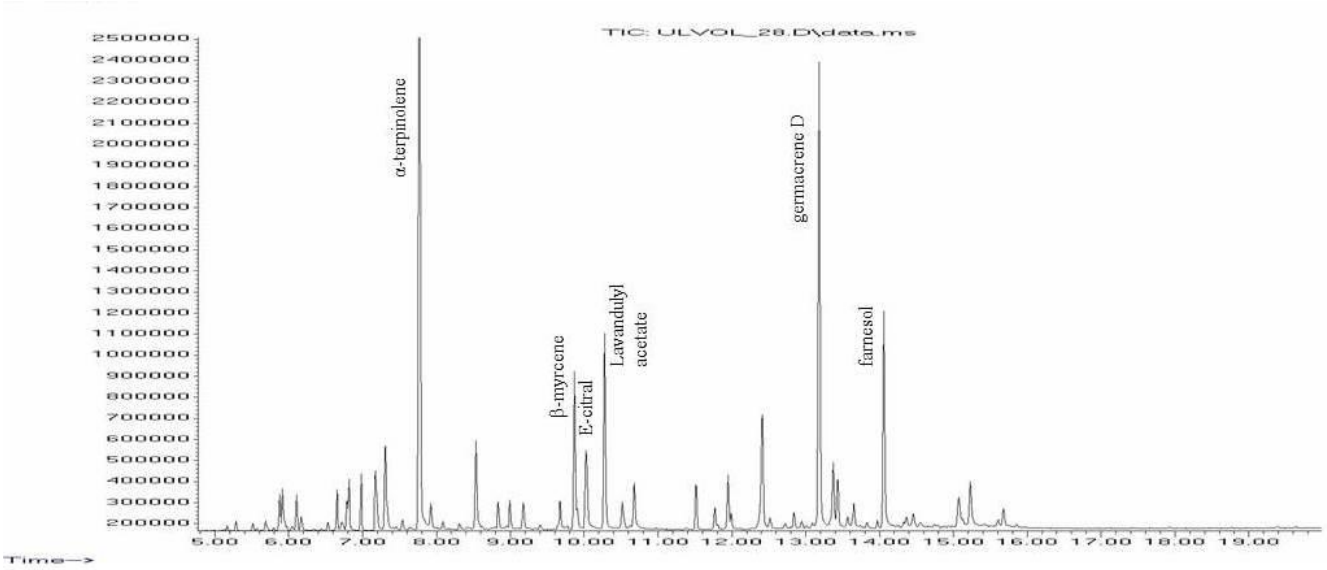


Figure 12. GC-chromatogram of Tp1 essential oil

The composition of essential oils from *Thymus pulegioides* subsp. *chamaedrys* populations (Tp2, Tp4) showed mainly a quantitative variability. The oil of population from Suceava County (Tp2) are richer in monoterpenes than the oil obtained from Neamt County population (Tp4) that grown at higher altitude (51.19% *vs*. 28.82%).

The main monoterpenes in the Tp2 oil are: α-terpinolene (24.58%), E-citral (5.04%) and nerol (4.79%).

In the Tp4 oils, the major monoterpenes are: α-terpinolene (5.81%), γ-terpinene (4.12%) and E-citral (2.29%). The level of sesquiterpenes is similar in the essential oils from Suceava and Neamt populations (31.75% and 35.02%, respectively). The major sesquiterpenes in both essential oils were: germacrene D (12.46% and 9.66%), β-caryophyllene (3.96% and 5.49%) and d-nerolidol (5.54% and 8.20%).

The aromatic compounds attained an high level in the oil of population from Neamt County (Tp4) (12.97%), and carvacrol predominates (8.69%). Also, the population of *Thymus pulegioides* ssp. *pulegioides* from Neamt district (Tp3) that grown at low altitude yielded a rich oil in aromatic fraction (25.60%) and carvacrol was major compound (15.22%) (Fig. 13).

The main monoterpenes of Tp3 were E-citral (9.59%), geraniol (8.51%) and α-terpinolene (3.14%).

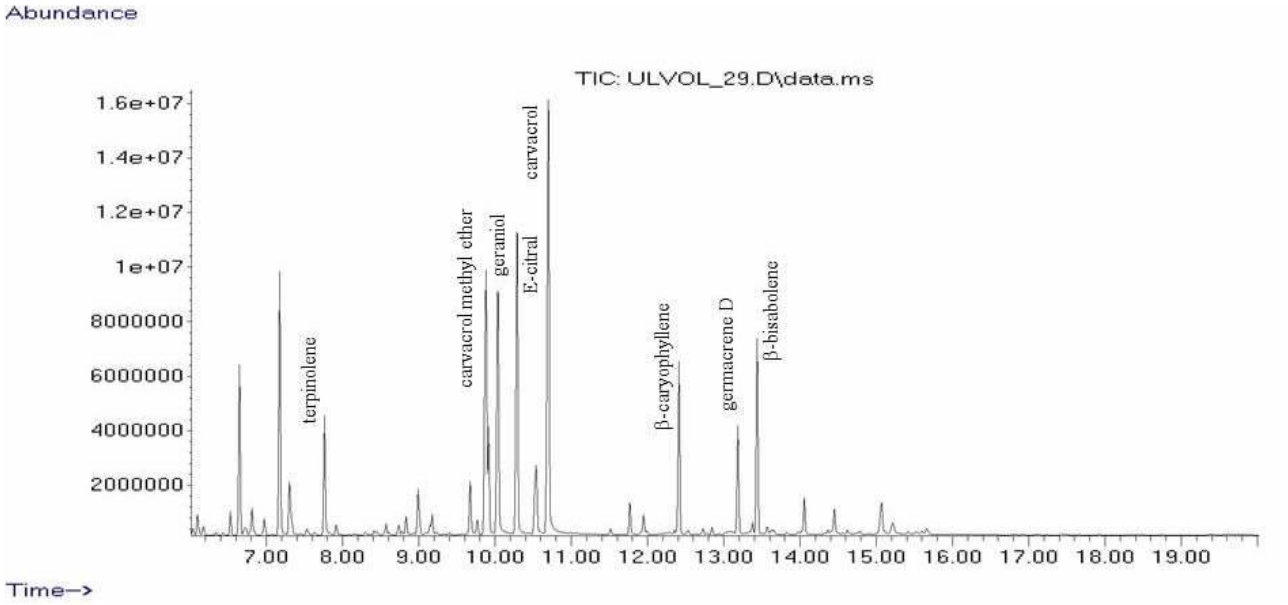


Figure 13. GC-chromatogram of Tp3 essential oil

These findings are in agreement with data reported by Ložiene et al. (2005) for *T. pulegioides* populations from Lithuania. Thus, the phenolic compounds biosynthesis is favoured in warmer and drier climatic areas while non-phenolic compunds accumulate in higher levels in plants from cooler and damper regions.

It is well-known that the genus *Thymus* displays an high chemical polymorphism. A survey of literature showed that the following chemotypes of *Thymus pulegioides* growing wild in Europe were reported, namely: *thymol* chemotype (Southern Europe); *linalool/linalyl acetate*, *carvacrol* and *geranial/geraniol* chemotypes (widespread in most of Europe); *fenchone* chemotype (Slovakia); *cis-sabinene hydrate* chemotype (Denmark), and α-terpenyl acetate [Mártonfi et al., 1992; Mockutë and Bernotienë 2001; Stahl - Biskup and Saéz, 2002].

In our case, the oils from the populations of *Thymus pulegioides* growing wild in Northeast of Romania (Suceava and Neamt counties) may better characterised as following:

- *Thymus pulegioides* ssp. *montanus*: α-terpinolene/farnesol/β-myrcene type;
- *Thymus pulegioides* ssp. *chamaedrys* -Tp2: α-terpinolene/germacrene D type;
- *Thymus pulegioides* ssp. *pulegioides* – Tp3: carvacrol/geranial/geraniol type;
- *Thymus pulegioides* ssp. *chamaedrys*– Tp4: carvacrol/germacrene D/nerolidol type.

Conclusions

Thymus pulegioides plants growing wild in the Northeast of Romania showed a distinct profile for volatile fraction for each type of population. The oils obtained from the plants that grow in Neamț belong to the phenolic carvacrol type, with a more important accumulation of aromatic compounds for the plants that grow at a lower altitude. The populations from Suceava County belong to the non-phenolic type (α-terpinolene), the higher altitude favoring the accumulation of monoterpenes.

I.1.6. Volatiles in the leaf glands of *Rosa agrestis* Savi and *Rosa rubiginosa* L. species dependent on phenological status and solar exposition

Current state of the arte and research objectives

The genus *Rosa* L. (Rosaceae) comprises between 100 and 250 species that are widely distributed in Europe, Asia, the Middle East and North America [de Cock et al., 2008]. Roses are one of the most important ornamental plants with a great value in perfumery, cosmetics, food industries. They are also used for medicinal purposes [Uggla, 2004; Kaur et al., 2007].

In Europe, the most complex and polymorphic section of the genus is the section *Caninae* DC. em. Christ; the species of this section are also known as dog-roses. Previous morphometrical and molecular genetic research on wild dog-rose parents and their interspecific progeny supported the further classification of these species in 3 main subsections (Rubigineae, Villosae and Caninae). The subsection Rubigineae is characterized by the presence of numerous glands on the leaflets that spread a strong fragrance of apples or vines.

Adumitresei et al. (2009) have described the multicellular clavate glands observed in the adaxial face, the edge of the leaf, and on the rachis and stipules of two Rubigineae, namely *Rosa agrestis* Savi and *Rosa rubiginosa* L. species.

Rosa agrestis (fieldbriar) is a perennial shrub mostly distributed in Southern Europe, Asia Minor and the Caucasus. In folk medicine, it has been used in the treatment of allergy, atopic dermatitis and some inflammatory diseases.

Rosa rubiginosa (Rosa mosqueta, sweet briar) grow in natural habitats in Europe but it is also found in South America and Australia. Their leaves and flowers have astringent and refreshing properties. Due to the presence of high content of essential fatty acids, vitamin E, carotenoids, and *trans*-retinoic acid, the seeds are of interest in clinical and cosmetic applications. Also, *Rosa rubiginosa* is used in food industries and beverages, and it is potential source of plant material for breeding new varieties. In addition, they can be used as ornamental plants and rootstock for grafting [Pawłowska and Szewczyk-Taranek, 2014].

Although data on the chemical composition of the volatiles from the flowers of *Rosa* species, especially those valuable in perfumery, are reported in literature, information on the chemical composition of the essential oils produced by the foliar structures of the dog-roses is lacking. These phytochemical data are interesting for identifying new resources of odorant compounds, but also as an additional taxonomic criterion.

In this context and starting from the botanical researches, the chemical composition of the essential oils from the leaves of *R. agrestis* and *R. rubiginosa* growing in Romania was investigated.

We also evaluated the variation of the composition depending on the time of harvest coupled with some phenological events (budding/ blooming) and the exposure of plants (sun/shadow).

Material and methods

Plant material

The leaves of *Rosa agrestis* Savi and *Rosa rubiginosa* L. were collected during two different moments (budding and blooming) from the field of "Anastasie Fătu" Botanical Garden of Iaşi. For *R. rubiginosa*, the harvesting was done both from plants exposed to sun and to shadow.

Isolation of essential oils

The essential oils were obtained from fresh leaves by hydrodistillation in Clevenger apparatus (3 hours). The oil was separated, dried over anhydrous sodium sulfate and kept in a dark glass bottle at 4 °C until the analysis.

Gas chromatograph and mass spectroscopy analysis

An analytical system, consisting of HP 5890 Series II GC and HP 5971 MSD, was used. GC/MS analysis of the essential oils was performed on HP-5MS capillary column (30 m × 0.25 mm diameter × 0.25 μm thickness) coated with cross-linked methyl silicone gum. Carrier gas was helium with 1 mL/min flow rate.

Temperature program was as follows: 35 °C held for 5 min, then heated up to 260 °C at 10 °C per min, and maintained for 3 additional minutes at this temperature. The temperature of injector was 250 °C and of the MS interface was 280 °C. Injection volume was 0.3 μL essential oil in hexane.

The identification of the components was based on comparison of their mass spectra in the apex of each peak with those of analytical standards from Willey Mass Spectral Library.

Results and discussion

Sesquiterpenes are the major compounds of essential oils from *Rosa sp*. leaves (Table 14).

The main compounds in all essential oils of *Rosa rubiginosa* are: R-ar-himachalene (1.58-3.47%), hexatriacontane (0.19-3.11%), α-longipinene (0.32-2.22%), α-farnesene (0.26-2.18%), α-ylangene (0.60-1.85%), junipene (0.87-1.58%), and α-calacorene (0.76-1.02%).

Essential oils obtained from blooming plants have a greater number of components than those isolated from budding plants, regardless of plant exposure (sun/shadow) (42 *vs*. 25 compounds and 31 *vs*. 22/24, respectively). The level of monoterpenes decreases by about 50% in blooming plants exposed to sun compared to the budding phase. Also, the content of sesquiterpenes decreases by about 6% in blooming plants exposed to sun and the level of aliphatic compounds (mainly, fatty acid derivatives which contribute at green aroma) increases about 14 times.

Compared to the budding phase, for the blooming plants exposed to shadow, we noticed:

- the content of monoterpenes decreases by more than 95%,
- the level of aliphatic compounds increases by about 59 times,
- the content of sesquiterpenes increases by about 18%,
- the level of aromatic compounds declines by 42%.

Trans-β-damascenone, a C-13 norisoprenoid characterized as a rose ketone compound, has been identified only in the blooming plants, and its content decreases in plants exposed to shadow. Damascenone is formed biosintetically *via* carotenoids and contributes significantly to the total aroma impression of rose volatile oils [Baldermann et al., 2009]. It imparts a floral green scent with woody, fruity, apple-baked nuances.

Also, other compounds such as: estragole, aristolene, germacrene D, methylhexadecanoate, 9,12-octadecanodienoic (Z,Z)-methyl ester, 9,12,15-octadecatrienoic acid (Z,Z)-methylester, phytol, have been identified only in the blooming, no matter what type of exposure had the plant. Similarly, *trans*-α-bergamotene, isoledene, perillyl alcohol, tetradecanal, have been identified only in the budding.

Interesting is the fact that some valuable rose odour compounds such as geraniol, nerol or linalool have been detected only in the budding plants.

2-phenylethanol, the most proeminent odor component of European rose flowers (*Rosa damascena*) has not been identified in *Rosa rubiginosa* leaves.

The essential oils isolated from *Rosa agrestis* leaves contain sesquiterpenes, aliphatic compounds and monoterpenes. The main compounds of *Rosa agrestis* oils are: R-ar-himachalene (2.76-5.21%), α-amorphene (0.88-4.18%), germacrene D (4.02-4.11%), widdrene (0.66-3.34%), α-calacorene (1.14-2.46%), alloaromadendrene (1.07-1.52%), α-guaiene (0.78-1.25%), α-farnesene (0.26-0.83%), β-farnesene (0.69-0.80%), and ledene (0.67-0.69%).

As in *Rosa rubiginosa*, the oils from the leaves of blooming *Rosa agrestis* plants has an increased number of components (35 towards 28).

Also, in the plants of *Rosa agrestis* in blooming, the levels of mono- and sesquiterpenes decreased with 50% and 5%, respectively, in comparison with the budding phase. In addition, the blooming is associated with the appearance of aromatic compounds and the increase of concentration of aliphatic components (primarily, fatty acid derivatives) of about 13 times.

Trans-β-damascenone rose-ketone as well as sesquiterpenes of muurolene type with herbal woody fragrance are present only in the leaves harvested from blooming plants (Table 14).

Nerol and geraniol appear only in the leaves harvested in the budding phase.

Conclusions

The leaves of both species (*R. rubiginosa* and *R. agrestis*) present volatile oils with different chemical profile depending on the harvest moment and type of plant exposure (sun/shadow).

The leaf monoterpene levels are adversely affected in the blooming phase and plant exposure to shadow for *Rosa rubiginosa* and the blooming phase for *Rosa agrestis*. Contrary, the accumulation of aliphatic compounds is influenced positively in the same conditions for *Rosa rubiginosa* and *Rosa agrestis*.

Trans-β-damascenone, the scented compound marker for the rose volatile oils has been identified only in the leaves harvested from the blooming plants of both dog-roses species.

Table 14. Chemical composition of *Rosa rubiginosa* and *Rosa agrestis* leaf essential oils

RT (min)	Compound	Chemical class*	***Rosa rubiginosa* (%)**				***Rosa agrestis* (%)**	
			Sun		Shadow		Budding	Blooming
			Budding	Blooming	Budding	Blooming		
7.28	6-methyl-hept-5-en-2-ol	Al	-	0.16	-	-	-	-
7.82	*para*-cymene	Ar	-	0.12	-	-	-	0.11
7.89	limonene	Mh	-	0.70	0.13	0.14	-	0.55
9.08	linalool	Mo	1.97	0.57	2.33	-	1.50	-
10.55	linalyl propionate	Mo	-	-	0.74	-	0.36	-
10.72	estragole	Ar	-	0.89	-	0.42	-	-
11.05	nerol	Mo	0.12	-	-	-	0.08	-
11.55	geraniol	Mo	0.42	0.17	0.36	-	0.31	-
12.12	*trans*-anethole	Ar	-	1.92	0.74	0.90	-	1.52
13.02	α-copaene	Sh	1.19	-	0.20	0.48	-	-
13.11	α-cubebene	Sh	0.20	-	-	-	-	-
13.16	α-longipinene	Sh	0.32	1.71	0.33	2.22	0.22	0.32
13.41	α-ylangene	Sh	0.81	1.85	0.85	0.60	1.15	1.58
13.49	(+)-longicyclene	Sh	-	0.63	-	-	-	-
13.56	*trans*-β-damascenone	Rk	-	0.59	-	0.12	-	0.28
13.64	calarene	Sh	-	-	1.32	-	1.15	-
13.82	β-bourbonene	Sh	-	-	-	-	0.24	-
13.88	γ-cadinene	Sh	4.12	0.26	-	-	-	-
14.01	junipene	Sh	1.58	1.18	0.87	1.20	0.25	-
14.11	γ-muurolene	Sh	-	-	-	-	-	0.71
14.12	α-amorphene	Sh	4.07	0.58	-	4.67	4.18	0.88
14.25	α-farnesene	Sh	2.18	0.73	0.26	0.29	0.26	0.83
14.42	β-funebrene	Sh	-	-	0.43	-	0.60	-
14.43	β-farnesene	Sh	-	-	-	-	0.80	0.69
14.59	*trans*-α-bergamotene	Sh	0.44	-	0.37	-	-	-
14.63	R-ar-himachelene	Sh	2.08	3.47	2.70	1.58	5.21	2.76
14.70	germacrene D	Sh	-	3.47	-	1.86	4.11	4.02
14.81	(-)-isoledene	Sh	1.49	-	2.17	-	-	-
14.87	β-himachalene	Sh	-	-	-	0.65	-	-

14.88	cadina-1,4-diene	Sh	-	-	-	-	-	1.12
14.97	widdrene	Sh	-	-	7.52	4.50	3.34	0.66
14.99	α-muurolene	Sh	-	-	-	-	-	2.86
15.02	γ-himachalene	Sh	3.50	-	-	-	-	-
15.10	eremophyllene	Sh	-	-	-	0.47	-	2.53
15.12	β-selinene	Sh	-	1.68	-	-	-	-
15.24	β-bisabolene	Sh	2.23	1.20	-	-	5.28	-
15.47	α-curcumene	Sh	-	-	-	-	0.89	-
15.54	calamenene	Sh	-	0.64	0.65	-	-	-
15.65	6-methoxy-1-acetonaphtone	Ar	-	3.06	1.20	-	-	-
15.82	α-calacorene	Sh	1.02	0.86	0.82	0.76	1.14	2.46
15.93	alloaromadendrene	Sh	2.02	0.91	-	-	1.07	1.52
16.27	Δ-cadinene	Sh	0.71	-	-	-	-	-
16.49	1,2,3,10-tetramethyltricyclo[5.2.1.1]-2-hydroxydibenzotiophene	O	-	-	-	-	0.57	-
16.68	patchoulane	Sh	-	2.09	-	-	-	-
16.99	α-guaiene	Sh	-	-	1	0.47	0.78	1.25
17.16	5-ethylidene-1-methyl-cycloheptene	Mh	-	1.93	-	-	-	-
17.42	ledene	Sh	-	-	-	0.55	0.69	0.67
17.43	sesquisabinene hydrate	So	-	-	-	-	-	0.67
17.46	isocaryophyllene	Sh	-	0.96	-	-	-	-
17.50	1,4-dimethyl-7(1-methylethyl)-azulene	So	-	-	-	0.72	-	-
17.51	cadalene	Sh	-	-	-	-	-	1.03
17.70	bicyclo[4.1.0] heptane, 7-(1-methylethyl)-capnellane-8-one	So	-	-	-	-	-	1.60
17.76	β-caryophyllene	Sh	-	1.74	-	-	-	-
18.36	tetradecanoic acid	Al	-	1.25	-	-	-	1.65
18.46	aristolene	Sh	-	2.67	-	1.92	-	5.76
18.63	sinularene	Sh	-	-	-	0.56	-	-
18.91	1,5-cyclooctadecadiene, 1,6-dimethyl	Mh	-	-	-	-	-	1.25
18.92	1-cyclohexene-1-carboxaldehyde	Al	-	0.97	-	-	-	-
18.95	valerenol	So	-	-	-	-	2.15	-
19.19	6,10,14-trimethyl-2-pentadecanone	Al	-	0.64	-	-	-	-
19.90	perillyl alcohol	Mo	4.12	-	2.87	-	-	0.51

20.02	methylhexanoate	Al	-	0.20	-	1.94	-	0.81
20.26	globulol	So	-	-	0.45	-	-	-
20.44	zingiberene	Sh	0.43	-	-	-	0.30	-
20.52	*trans*-farnesal	So	-	-	-	-	0.58	-
20.62	hexadecanoic acid	Al	0.41	2.19	-	7.15	-	1.84
21.80	9,12-octadecadienoic acid (Z, Z)-methyl ester	Al	-	0.20	-	1.21	-	-
21.88	9,12,15-octadecatrienoic acid (Z,Z)-, methyl ester	Al	-	0.73	-	5.90	-	0.86
22.00	phytol	Dt	-	0.79	-	3.52	-	0.86
22.32	ethyllinoleate	Al	-	-	-	6.24	-	0.58
22.35	9,12,15-octadecatrienal	Al	-	0.62	-	-	-	-
22.75	tetracontane	Al	-	0.11	-	-	-	-
23.84	tricosane	Al	-	0.29	-	-	-	-
24.81	11,14,17-eicosatrienoic acid, methyl ester	Al	-	-	-	0.33	-	-
25.07	eicosane	Al	-	0.32	-	-	-	-
26.55	pentacosane	Al	-	2.46	-	-	-	-
26.56	triacontane	Al					-	1.07
28.13	9,12,15-octadecatrienoic acid	Al	-	-	-	0.37	0.23	-
28.26	1-octadecane	Al	-	-	-	0.17	-	-
28.32	7-hexyldocosane	Al	-	-	-	-	-	0.40
28.34	hexatriacontane	Al	0.19	0.55	0.43	3.11	0.48	-
30.65	9-butyldocosane	Al	-	-	-	-	-	2.23
32.92	1-dotriacontanol	Al	0.15	-	-	-	-	-
34.12	tetradecanal	Al	0.09	-	0.08	-	-	-

*Al, aliphatic compounds; Ar, aromatic compounds; Dt, diterpenes; Mh, monoterpene hydrocarbons; Mo, oxygenated monoterpenes; O, others; Rk, Rose ketones; Sh, sesquiterpene hydrocarbons; So, oxygenated hydrocarbons.

Conclusions about topic 1

Identification and understanding the structural and chemical variability of plants under influence of various factors (pesticide treatments, agronomic practices, climate, harvesting time, phenological events) may extend the knowledge of ecologic interactions with their environment and allow alternative approaches to increase productivity of cultivated plants or to ensure quality and safety in phytomedicine.

In the case of phytosanitary treatments, the interpretation of these data could also serve to elaborate models of prognosis that will target some of the most important categories of bioactive compounds, but also the implementation of an ecological management for pesticide use in the cultivation of medicinal plants.

I.2. Research achievements in the field of the assessment of natural products biological activities

I.2.1.Research achievements in the field of the assessment of antioxidant and cytogenoprotective activities

Antioxidant activity of plant extracts and phytochemicals is of particular interest to medicine, food and cosmetic industry or in the development of effective genoprotective/radioprotective agents. Natural plant antioxidants mainly phenolic compounds can protect cell constituents against oxidative damage including that caused by ionizing radiation or other genotoxicants and, therefore, limit the progress of various human pathologies associated with oxidative stress (cancer, chronic inflammation, cardiovascular and neurodegenerative diseases).

Many other beneficial effects of plant-derived products involve antioxidant mechanisms. Understanding these mechanisms, the dual antioxidant/prooxidant behavior of some phytochemicals and plant products contributes to a good use in clinical practice or in non-therapeutic fields (food and beverage industries and cosmetics).

My own research in this direction continue some of the aspects addressed in the doctoral thesis regarding the investigation of the biological properties of medicinal plants, extending the spectrum of plant species, the geographical origin and the type of assays that show different antioxidant mechanisms. In addition, research approaches also include correlating the results of biological determinations with those regarding the chemical composition, chemical investigations being also important objectives of the studies undertaken.

An important role in the development of the theme regarding the evaluation of the antioxidant properties of the herbal products was played by the participation as an active member in the teams of 8 research projects obtained through international (3) or national (5) competition. Some of the important objectives of the respective projects were:

- evaluation of the antioxidant activity of polyphenolic fractions from Moldavian Lamiaceae species (contract 694/2013);
- evaluation of the antioxidant activity of some populations of *Artemisia sp*. from the Republic of Moldova (REART Project, No. IZ73Z0_152265);
- evaluation of the antioxidant activity of some proanthocyanidolic fractions from conifer species in order to establish the chemopreventive potential in cancer (Bilateral Cooperation Romania-Cyprus, 2009);
- chemical characterization and antioxidant properties of some Romanian Lamiaceae species in order to establish the radioprotective potential (contract 1639/2013);

- chemical characterization and antioxidant potential of polyphenolic fractions from *Cynara scolymus* and *Sorbus aucuparia* plants in order to develop phytopreparations for the prophylaxis of cardiovascular diseases (contract 51-060/2007);
- evaluation of the antioxidant activity of some extracts from *Medicago sativa, Vitis vinifera* and *Trigonella foenum-graecum* in order to obtain phytopreparations with effect on metabolic imbalances (contract 33/2007);
- evaluation of the antioxidant activity of the volatile and polyphenolic fractions of *Ajuga sp., Coriandrum sativum* and *Hyssopus officinalis* in order to substantiate some phytopreparations used in prophylaxis and orthomolecular therapy (contract 61-39/2007);
- evaluation of the antioxidant activity of the volatile fractions of *Origanum vulgare, Ocimum basilicum, Satureja hortensis, Thymus vulgaris* and *Lavandula angustifolia*, as well as the characterization of the antioxidant potential of some antiaging formulas (contract 21/2005).

Also, we investigated antioxidant activity of *Lavandula sp*. [Robu et al., 2012], *Melissa officinalis, Thymus vulgaris, Origanum majorana*, [Aprotosoaie et al., 2014b; Aprotosoaie et al. 2014c], *Mentha gattefossei* [Aprotosoaie et al., 2019b] and *Camellis sinensis* plants [Luca et al., 2016].

Since 2012, research regarding the evaluation of the antioxidant potential of medicinal and aromatic plants have been completed and developed in the direction of investigating the cytogenoprotective activity of some polyphenolic extracts (*Melissa officinalis, Dracocephalum moldavica, Verbascum ovalifolium*, and *Ramaria largentii* mushrooms) [Aprotosoaie et al., 2015b; Aprotosoaie et al., 2016; Aprotosoaie et al., 2017b; Luca et al., 2018] and essential oils (*Thymus vulgaris, Thymus calcareus*) [Aprotosoaie et al., 2019a].

The environmental stressors (ionizing radiation, UV light, chemicals, air and food pollutans) or metabolic imbalance cause elevated levels of oxidative stress and redox imbalance which can alter the structure and functions of different biomolecules (DNA, proteins, polyunsaturated fatty acids) and impair genomic stability. The cumulative DNA damage causes mutations, aging, degenerative diseases or cancer [Aziz et al., 2012; Luca et al., 2016].

The identification of genoprotective agents that prevent or offer resistance to genotoxic insults is of increasing interest given the current dramatic exposure of the human body to various genotoxicants (oxidative stress from pathological processes, ultraviolet light, ionizing radiations, air, water and food pollutans, alkylating agents, smoking, mycotoxin-producing fungi [Aprotosoaie et al., 2018a].

Research in this area can also provide support for the development of efficient radioprotective agents, another crucial objective. The exposure to ionizing radiations is both voluntary (radiotherapy) and involuntary (nuclear industry, naturally occurring radiations from the environment or other sources), especially in the context of different threats (nuclear and climate disasters, terorism) the present society faces or should face. Radiotherapy is an important therapeutic alternative in cancer treatment being used worldwide for more than half of all cancer patients. The therapeutic use of ionizing radiations has side effects as a consequence of radiation-induced lesions in normal tissues. The use of radioprotective agents providing a selective protection of normal tissues would allow the administration of higher doses of radiations for a better management of cancer disease and a probable cure [Aprotosoaie et al., 2015a; Saaya et al., 2017]. Also, the problem of prolonged exposure of astronauts to cosmic ionizing radiation is a great challenge that needs to be addressed in order to make deep space expeditions possible. The

evaluation of the antigenotoxic/genotoxic potential of herbal products is also important in terms of their safe use, especially in chronic administration.

The results reported in this direction were also possible thanks to the funding through the research grant of the University of Medicine and Pharmacy Grigore T. Popa Iasi, Team Competition-Ideas 2012 (*Investigations on the radioprotective potential of some vegetal extracts*), where I participated at its implementation as project manager.

The most important contributions materialized by publishing 5 articles in the journals *Industrial Crops and Products* (FI^{2016} = 3,181), *Food and Chemical Toxicology* (FI^{2017} = 3,977), *Flavour and Fragrance Journal* (FI^{2018} = 1,337) and *Farmacia* (FI^{2015} = 1,162; FI^{2018} = 1,527) and 2 review type articles in *Phytochemical Reviews* (FI^{2016} = 3,393; FI^{2015} = 2,686), as well as a chapter in the international book.

Aprotosoaie AC, Mihai CT, Vochița G, Rotinberg P, Trifan A, Luca SV, Petreus T, Gille E, Miron A. Antigenotoxic and antioxidant activities of a polyphenolic extract from European *Dracocephalum moldavica* L. *Ind Crops Prod* 2016, 79, 248-257 (FI^{2016}=3,181**)**.
Aprotosoaie AC, Zavastin DE, Mihai CT, Vochița G, Gherghel D, Silion M, Trifan A, Miron A. Antigenotoxic and antioxidant potential of *Ramaria largentii* Marr &D. E.Stuntz, a wild edible mushroom collected from Northeast Romania. *Food Chem Toxicol* 2017, 108, 429-437 (FI^{2017}=3,977).
Aprotosoaie AC, Miron A, Ciocârlan N, Brebu M, Roşu C, Trifan A, et al. Essential oils of Moldavian *Thymus species*: chemical composition, antioxidant, anti-*Aspergillus* and antigenotoxic activities. *Flavour Fragr J* 2019, 34, 175-186 (FI^{2018}=1,337)
Trifan A, **Aprotosoaie AC** (corresponding author), Brebu M, Cioancă O, Gille E, Hăncianu M, Miron A. Chemical composition and antioxidant activity of essential oil from Romanian *Satureja montana* L. *Farmacia* 2015, 63 (3), 413-416 (FI^{2015}=1,162).
Aprotosoaie AC, Ciocârlan N, Brebu M, Trifan A, Grădinaru AC, Miron A. Chemical composition, antioxidant and antimicrobial activities of *Mentha gattefossei* Maire essential oil. *Farmacia* 2018, 66 (5), 778-782 (FI^{2018}=1,527).

Luca VS, Miron A, **Aprotosoaie AC**.The antigenotoxic potential of dietary flavonoids. *Phytochem Rev* 2016, 15 (4), 591-625 (FI^{2016}=3,393).
Aprotosoaie AC, Trifan A, Gille E, Petreuş T, Bordeianu G, Miron A. Can phytochemicals be a bridge to develop new radioprotective agents? *Phytochem Rev* 2015, 14 (4), 555-566 (FI^{2015}=2,686).
Aprotosoaie AC, Luca VS, Trifan A, Miron A. Antigenotoxic potential of some dietary non-phenolic phytochemicals. In *Studies in Natural Products Chemistry*, Atta-ur-Rahman (ed.), Amsterdam: Elsevier, 2018, 60, pp.223-297.

I.2.1.1. Assessment of antioxidant and cytogenoprotective activities of *Dracocephalum moldavica* against bleomycin-induced genomic damage in human dermal fibroblasts

Current state of the arte and research objectives

Bleomycin (BLM) is a glycopeptide-derived antitumor antibiotic and a well-known radiomimetic drug. It produces genotoxic insults similar to low energy transfer radiation. BLM induces *single strand DNA breaks* (SSBs), *double strand breaks* (DSBs), and even clustered DNA lesions (locally multiple damaged sites, LMDS) [Aziz et al., 2012] *via* a free radical attack on deoxyribose moieties in DNA nucleotides [Povirk, 1996; Regulus et al., 2007]; the process is ferrous ion and oxygen-dependent (Kruszewski et al., 2001).

The oxidative attack of DNA bases (cytosine) is associated with the appearance of electrophilic intermediates that lead to cleavage of DNA bases or the appearance of adducts (Fig. 15).

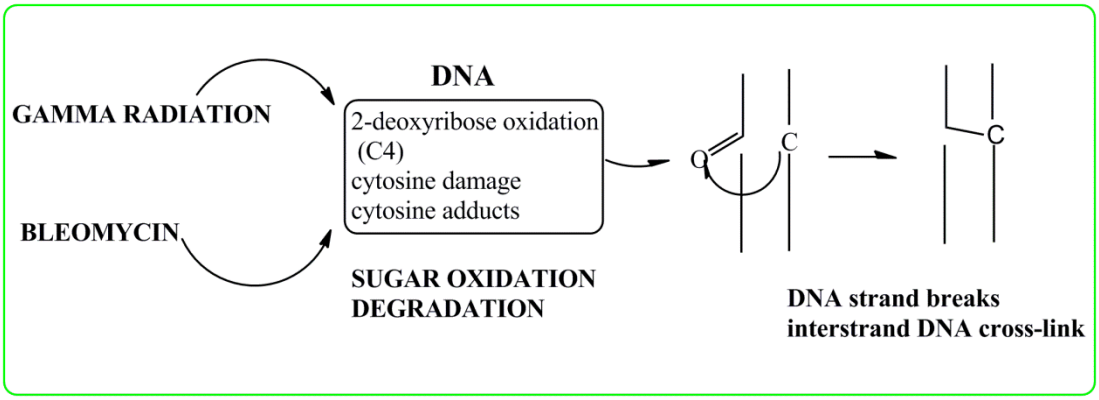


Figure 15. Genomic damage induced by bleomycin [modified after Regulus et al., 2007]

Plant molecules such as polyphenols are powerful antioxidants that act through different mechanisms, being extremely promising compounds in studies on the identification of efficient genoprotective agents.

Dracocephalum moldavica is a perennial plant belonging to Lamiaceae family and it is native to Central Asia and naturalized in Central and Eastern Europe. It is traditionally used in gastric, liver and cardiovascular disorders, headache, but also as food additive [Dastmalchi et al., 2007].

The aerial parts of Moldavian balm are reported to contain various polyphenols, especially hydroxycinnamic acids (rosmarinic and caffeic acids) and flavonoids (apigenin, luteolin and their glycosides, quercetin, kaempherol) [Yang et al., 2014]. Recent pharmacological studies reported significant beneficial effects of D. *moldavica* extracts such as: cardioprotective, antiplatelet [Jiang et al., 2014], neuroprotective [Sun et al., 2014], sedative [Martínez-Vásquez et al., 2012], antiaging and the ability to atenuate the systemic hypoxia and cardiac pathological state in chronic mountain sickness [Maimaitiyiming et al., 2014].

The polyphenols and their antioxidant properties are involved in most of the aforementioned activities. Although some studies have reported a high antioxidant activity of the polar extracts or flavonoid fraction obtained from *D. moldavica* [Krishnaiah et al., 2011], its protective capacity on oxidative DNA-damage has not been investigated.

In this respect, in the present study, a polyphenolic extract obtained from the aerial parts of *Dracocephalum moldavica* (Moldavian balm, Moldavian dragonhead) cultivated in Romania was investigated for its ability to protect against DNA damage induced by BLM in normal human dermal fibroblasts (NHDF).The analysis of polyphenols was carried out using RP-HPLC-DAD. DNA damage was monitored by comet assay and cytokinesis-block micronucleus assay. Amifostine, a well-known cytoprotective agent, was used as positive control. Normal human dermal fibroblasts were used because the skin is one of the most susceptible organs to the BLM toxicity probably due to a poor inactivation of BLM [Sikic, 1986].The antioxidant activity of extract was assessed by six different and complementary tests.

Material and methods

Chemicals

Gallic acid, (+) catechin hydrate, 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), 2,2_-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), (R)-(+)-6-hydroxy-2,5,7,8- tetramethylchroman-2-carboxylic acid (Trolox), thiobarbituric acid, potassium ferricyanide, iron (III) chloride were purchased from Sigma–Aldrich (Steinheim, Germany). Tris(hydroxymethyl) aminomethane (Tris), Folin–Ciocalteu's phenol reagent, hydrochloric acid, 3-(4,5-dimethyl-2-thiazolyl)-2-5-diphenyl-2H-tetrazolium bromide (MTT), Giemsa stain, dimethyl sulfoxide (DMSO) and ferrous chloride were from Merck (Darmstadt, Germany). Ethylenediaminetetraacetic acid (EDTA), 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4',4''-disulfonic acid sodium salt (ferrozine), 2-deoxy-d-ribose, iron (II) sulfate heptahydrate and pyrogallol were supplied by Fluka (Steinheim, Germany). Trichloroacetic acid and potassium persulfate were from Riedel-de-Haën (Seelze, Germany). Ethidium bromide was purchased from Carl Roth (Karlsruhe, Germany).

Normal and low melting point agarose, amifostin, bleomycin, sodium lauroyl sarcosinate, cytochalasin B, rosmarinic acid, apigenin, apigenin 7-glucoside, quercetin, caffeic, ferulic and chlorogenic acids were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Dulbeco's Modified Eagle's Medium (DMEM), 10% foetal bovine serum, phosphate buffered saline (PBS), streptomycin, penicillin were procured from Biochrom AG (Berlin, Germany). Ultrapure water was obtained using a SG Water Ultra Clear TWF water purification system (Siemens Water Technologies Corp., USA).

Plant material

Aerial parts of *Dracocephalum moldavica* (DM) were collected from the experimental field of Stejarul Biological Research Centre (Piatra Neamt, Romania) in July 2012.

The plant material was air dried in shade at room temperature.

Extraction

The powdered plant material of each species (10 g) was extracted under reflux with 100 mL of 80% methanol for 1 h at 60 °C. The extracts were filtered and the residues were re-extracted twice as described. The combined extracts were concentrated in a rotary evaporator under reduced pressure at 40 °C and finally freeze-dried. The *Dracocephalum moldavica* extract (DME, $\eta = 47.74\%$) was stored in a freezer at -18 °C until use.

Quantification of total polyphenols

Total phenolic level was determined using Folin-Ciocalteu reagent [Singleton and Rossi, 1965]. 0.04 mL of extract (5 mg/mL in DMSO:water, 7:3, v/v), 3.16 mL of ultrapure water and 0.2 mL of Folin-Ciocalteu reagent were mixed and allowed to stand for 5 min followed by the addition of 0.6 mL of 20% sodium carbonate. After 2 h incubation at ambient temperature in dark, the absorbance was measured at 765 nm. The calibration curve was plotted using gallic acid (0.2-2 mg/mL) as standard. Total phenolic content was expressed as mg of gallic acid equivalents/g of dry extract (mg of GAE/g of extract).

RP-HPLC analysis of polyphenols

Reversed-phase high-performance liquid chromatography (RP-HPLC) analysis was performed on an Agilent Technologies 1200 Series HPLC system with a diode array detector.

The separation was carried out on a ODS Hypersil column (Thermo Scientific, 250 × 4.6 mm, 5 μ particle size) using the method of Kamden et al. (2013) with minor changes. The mobile phase consisted of (A) acetonitrile and (B) water and acetic acid (99:1, v/v). The elution profile for DME analysis was: 13% A in B (0–10 min, isocratic), 13–20% A in B (10-20 min, linear gradient), 20–27% A in B (20-35 min, linear gradient), 27-40% A in B (35–60 min, linear gradient), 40-70% A in B (60-70 min, linear gradient), 70-100% A in B (70-80 min, linear gradient), 100% A (80-90 min, isocratic), 100-13% A in B (90-100 min, linear gradient).

For qualitative analysis, extracts and the standards (rosmarinic acid, apigenin, apigenin 7-glucoside, quercetin, ferulic, caffeic and chlorogenic acids) were dissolved in methanol and methanol-water mixtures at concentrations of 20 and 0.5 mg/mL, respectively. Volumes of 20 μL were injected. The flow rate was 0.5 mL/min. The detection wavelengths were set at 254, 280, 325 and 365 nm.

The phenolic compounds were identified by comparing their UV spectra and retention times with those of commercial standards. Rosmarinic acid, the major polyphenol in DME, was quantified on the basis of a calibration curve (0.5–1.25 mg/mL); the result was expressed as mg/g of DME.

Antioxidant activity

a) DPPH radical scavenging assay

DPPH radical scavenging activity was assessed according to the method described by Malterud et al. (1993). Various dilutions of DME were prepared in concentrations ranging from 20 to 1.25 mg/mL. 0.05 mL of each dilution were mixed with 2.95 mL of DPPH in methanol (A_{517nm} = 1.00 ± 0.05). The absorbance was measured at 517 nm before adding the extract (A_{start}) and after 5 min reaction time (A_{end}). Gallic acid (0.078-1.25 mg/mL) was used as positive control. The percent of DPPH radical inhibition was calculated as follows:

$$\text{DPPH scavenging activity (\%)} = 100 \times [(A_{start}-A_{end})/A_{start}]$$

b) ABTS radical cation scavenging assay

The assay was carried out following the method described by Re et al. (1999). ABTS radical cation was generated by incubating ABTS stock solution (7 mM) with potassium persulfate (2.45 mM) at room temperature in dark for 16 h followed by dilution with ethanol to an absorbance of 0.70 ± 0.02 at 734 nm. 0.02 mL of different DME dilutions (0.625-5 mg/mL) were mixed with ABTS radical solution in a total volume of 2 mL. The absorbance at 734 nm was measured after 6 min reaction time. Gallic acid (0.0125-0.1 mg/mL) was used as positive control. The ABTS radical cation scavenging activity was calculated using the formula:

$$\text{ABTS radical cation scavenging activity (\%)} = 100 \times [(A_{control}-A_{sample})/A_{control}]$$, where

$A_{control}$ = absorbance of the control

A_{sample}= absorbance in the presence of extracts/gallic acid.

Also, the ABTS scavenging activity was expressed as Trolox equivalent antioxidant capacity (TEAC). In this respect, the scavenging effects of different concentrations of Trolox (0.5-2 mM) against ABTS radical cation were evaluated. TEAC values were calculated by dividing the slope of dose-response curve of DME/gallic acid by the slope of dose-response curve of Trolox.

c) Superoxide anion radical scavenging assay

The assay was performed according to a slightly modified method of Marklund and Marklund based on pyrogallol autoxidation [Wang and Luo, 2007]. Briefly, 0.1 mL of each extract dilutions (10-20 mg/mL), 2.8 mL of 50 mM Tris-HCl buffer (pH=8) containing 1 mM EDTA and 0.2 mL of 6 mM pyrogallol were mixed thoroughly. The absorbance of the mixture

was recorded at 325 nm every 30 s for 4 min. (+)-Catechin hydrate (20-80 mg/mL) was used as positive control. The capacity to scavenge superoxide anion radical was calculated using the following formula:

Superoxide anion radical scavenging activity (%) = 100 × [($slope_{control}$-$slope_{sample}$)/$slope_{control}$], where $slope_{control}$ and $slope_{sample}$ are the slopes of the plots of absorbance *vs*. time for control and DME/gallic acid, respectively.

d) Hydroxyl radical scavenging assay

The hydroxyl radical scavenging ability of the extract was determined using the deoxyribose method [Gutteridge, 1984; Goel et al., 2002] with minor changes. In brief, 63 μL of various DME dilutions (10-120 mg/mL) were mixed with 0.4 mL of 5 mM 2-deoxy-d-ribose and 0.1 M phosphate saline buffer (pH = 7.4) up to a final volume of 1.8 mL. After addition of 0.2 mL of 2 mM ferrous sulfate heptahydrate, the mixture was incubated at 37 °C for 1 h. Then, 1% thiobarbituric acid and 2.8% trichloroacetic acid (1 mL of each) were added followed by heating at 80 °C for 1 h. The mixture was cooled and the absorbance was measured at 532 nm. (+)-Catechin hydrate (0.625-10 mg/mL) served as positive control. The ability to scavenge hydroxyl radical was calculated using the following expression:

Hydroxyl scavenging activity (%) = 100 × [($A_{control}$-A_{sample})/$A_{control}$], where

$A_{control}$ = absorbance of control (containing all reagents except the test compound)

A_{sample} = absorbance of sample (containing all reagents including the test compound).

e) Reducing power assay

The assay was carried out according to a previously described method [Berker et al., 2007] with minor changes. 0.05 mL of extract dilutions (0.89-4.42 mg/mL), 1.2 mL of 0.2 M phosphate buffer (pH = 6.6) and 1.25 mL of 1% potassium ferricyanide were mixed and incubated at 50 °C for 20 min. 1.25 mL of 10% trichloroacetic acid were then added to the mixture and then centrifuged at 3000 rpm for 10 min. To 1.25 mL of the supernatant was added 2.5 mL of ultrapure water and 0.25 mL of 0.1% ferric chloride. After 90 s, the absorbance of the mixture was measured at 700 nm. (+)-Catechin hydrate (0.16-0.82 mg/mL) was used as positive control. A high absorbance of the reaction mixture indicates a high reducing capacity.

f) Ferrous ion chelating assay

The ferrous ion chelating activity was evaluated as described by Dinis et al. (1994) with minor changes. 0.05 mL of each extract dilutions (1.25-10 mg/mL), 0.05 mL of 2 mM ferrous chloride and 2.7 mL of 50 mM Tris-HCl buffer (pH= 7.4) were mixed followed by the addition of 0.2 mL of 5 mM ferrozine solution. Then, the mixture was incubated at room temperature for 10 min followed by measuring the absorbance at 562 nm. Gallic acid (0.0781-2.5 mg/mL) was used as positive control.The percentage inhibition of ferrous ion-ferrozine complex formation was calculated using the equation:

Ferrous ion chelating activity (%) = 100 × [($A_{control}$-A_{sample})/$A_{control}$], where,

$A_{control}$ = absorbance of control (containing all reagents except the test compound) and

A_{sample} = absorbance of samples (containing all reagents including the test compound).

Assessment of antigenotoxic potential of DME

Cell culture

Normal Human Dermal Fibroblasts (PromoCell, Heidelberg, Germany) were cultivated in a DMEM medium supplemented with 10% foetal bovine serum, 100 μg/mL streptomycin and 100 IU/mL penicillin.

Cell viability assay

Cytotoxicity assessment was performed using the MTT assay previously described (Mosmann, 1983). Dermal fibroblasts (1.5×10^4 cells) were seeded in 96-well plates (TPP Techno Plastic Products AG, Trasadingen, Switzerland) and incubated in the absence (control) or presence of different DME concentrations (25, 100 and 200 μg/mL), at 37 °C for 24 h.

Then, 10 μL of MTT (5 mg/mL) were added to each well. The plates were further incubated for 3 h. The blue formazan precipitate was dissolved in 300 μL DMSO/well and the absorbance was measured at 540 nm (PG Instruments T70, UK). The assay was carried out in fiveplicate. The results were expressed as percentages of control viability (100% viability).

Treatment of the cells

Cells were seeded at a density of 2×10^4 cells/well in 24-well plates (TPP Techno Plastic Products AG, Trasadingen, Switzerland) and maintained in a 5% CO_2 atmosphere (Binder CB 150, Tuttlingen, Germany) at 37 °C. When the cells reached the confluence in the monolayer stage, they were incubated with BLM (10 μg/mL) for 6 h, followed by rinsing the cellular film with cold PBS; the rinse was repeated twice. After incubation with BLM, the cells were further exposed to DME (25 and 100 μg/mL) for 18 h. The same experimental protocol was applied in the case of the positive control, amifostine (AMI). It was used in a dose of 100 μg/mL. After 24 h incubation, the cells were detached by trypsinization and the cell suspension was obtained. The groups of treated cells and controls are presented in Table 15.

Table 15. Assessment of DME antigenotoxic potential: treatments and controls

Codification	Group
Sham control	normal dermal fibroblasts
BLM	fibroblasts treated with bleomycin (10 μg/mL)
AMI	fibroblasts treated with amifostine (100 μg/mL)
DME 25	fibroblasts treated with DME 25 μg/mL
DME 100	fibroblasts treated with DME 100 μg/mL
DME 25+BLM	fibroblasts exposed to bleomycin (10 μg/mL) and posttreatment with DME (25 μg/mL)
DME 100+BLM	fibroblasts exposed to bleomycin (10 μg/mL) and posttreatment with DME (100 μg/mL)
AMI+BLM (positive control)	fibroblasts exposed to bleomycin (10 μg/mL) and posttreatment with amifostine (100 μg/mL)

Alkaline single-cell gel electrophoresis assay (Comet assay)

The degree of oxidative DNA damage was estimated by Comet assay [Olive and Banath, 2006]. 200 μL of cell suspension in cold PBS were mixed with 1000 μL of low-gelling-temperature agarose at 40 °C. The agarose cell suspension was mixed and rapidly pipeted onto the agarose-covered surface of a precoated slide, avoiding to produce bubles.The agarose was allowed to gel for aprox. 5 min. After agarose has gelled, the slides were submerged in a covered dish containing lysis solution (1.2 M NaCl, 100 mM Na_2EDTA, 0.1% sodium lauryl sarcosinate,

0.26 M NaOH, pH > 13). The samples were maintained in the lysis solution overnight at 4 °C in the dark. After lysis, slides were submerged in the rinse solution (0.03 M NaOH, 2 mM Na_2EDTA, pH ≈ 12.3, room temperature) for three times. After three rinses, the slides were moved into the electrophoresis chamber filled with migration buffer (0.03 M NaOH, 2 mM Na_2EDTA, pH ≈ 12.3), the level of the migration buffer being 1–2 mm above the slides. The electrophoresis was conducted for 25 min at a voltage of 0.6 V/cm. After electrophoresis, the slides were removed from electrophoresis chamber, rinsed and neutralized with 400 mL of ultrapure water. 1% solution of ethidium bromide was spread on all the surface of slides and incubated for 20 min. The excess of stain was removed by rinsing the slides with 400 mL of ultrapure water.

The images were captured using a Nikon Eclipse 600 epifluorescence microscope (Nikon, Japan). For each experimental variant, 600 cells were analyzed.

Comet analysis was performed using AutoComet software (TriTek Comet Score, 1.5.2.6 version).

Micronucleus assay

Cytokinesis-blocked micronucleus (CBMN) assay was performed for assessing BLM-induced DNA damage in dermal fibroblasts [Fenech and Morley, 1986; Kirsch-Volders et al., 2000]. After cells treatments, 3 μg/mL of cytochalasin B (stock solution: 1 mg cytochalasin B/mL DMSO) were added to the cultures. After 28 h incubation, the cells were treated with mild hypotonic solution of KCl (75 mM) for 3 min and fixed in 3:1 ethanol-acetic acid. After centrifugation (10 min, 4 °C) and removal of the supernatant (process repeated 3 times), the cells were gently dropped on a wet slide and stained with 5% Giemsa for 15 min.

All slides were scored as the number of micronuclei per thousand binucleated cells. The analysis was done using a Nikon Eclipse 600 epifluorescence microscope (Nikon, Japan).

Statistical analysis

Results of chemical and antioxidant assays were expressed as mean±standard deviation (SD) from three determinations. EC_{50} values (μg/mL) were calculated from linear interpolation between values above and below 50% activity. In reducing power assay, EC_{50} values were the concentrations giving an absorbance of 0.5 [Ferreira et al., 2007].

Results of genotoxicity tests were expressed as mean±standard error of mean (SEM). In comet assay, Bonferroni multiple comparison test was used for the statistical evaluation and the results were considered statistically significant for $p < 0.001$.

In MTT and micronucleus assays, the values were statistically analyzed using Student's t test; $p < 0.01$ and $p < 0.05$ were considered statistically significant.

Results and discussion

Total Phenolics

The total phenolic content of DME was 285±2.63 mg GAE/g (Table 16). This value is lower than the one reported for the methanol extract from Iranian *D. moldavica* (488.4±1.8 mg/g) [Dastamalchi et al., 2007].

It is known that the phenolics depend on the environmental conditions, plant ontogenesis and time of harvest, and also method of extraction [Duda et al., 2015]. So, it is not surprising that an important diversity of phenolics composition may occur.

Profile of phenolics

The chlorogenic, caffeic and ferulic acids, apigenin 7-*O*-glucoside, rosmarinic acid and apigenin were identified in DME (Fig. 16). Rosmarinic acid (107.11±0.83 mg/g of DME) was found to be the major polyphenol representing 36.99% of the total phenolic content of extract.

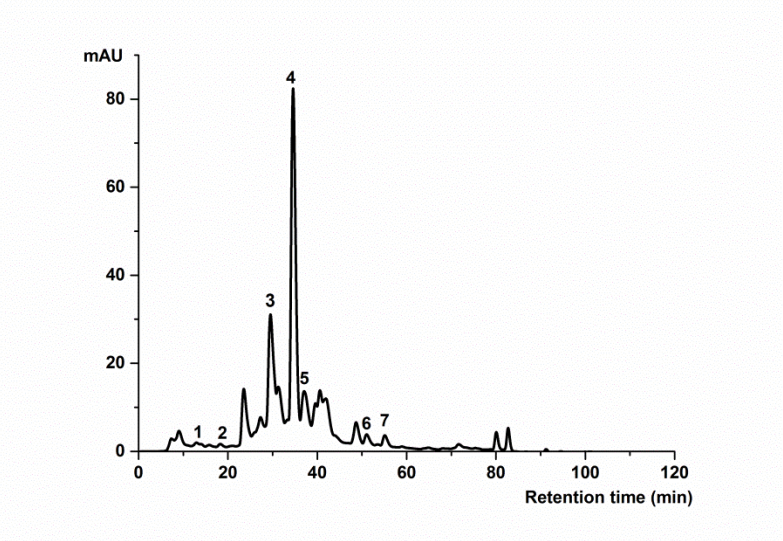


Figure 16. HPLC-DAD chromatogram of DME at 280 nm [Aprotosoaie et al., 2016]
Chlorogenic acid (peak 1; retention time, Rt= 12.94 min); caffeic acid (peak 2; Rt= 18.29 min); ferulic acid (peak 3; Rt= 29.52 min); rosmarinic acid (peak 4; Rt= 34.58 min); apigenin 7-O-glucoside (peak 5; Rt= 37.06 min); quercetin(peak 6; Rt= 51.05 min); apigenin (peak 7; Rt= 55.10 min). Calibration curve for rosmarinic acid: Y = 990.94x + 663.75 (r = 0.9998). HPLC analysis was performed in triplicate.

In vitro antioxidant activity

The genotoxicity of BLM is primarily mediated by free radicals and oxidative damage. In the presence of a redox-active metal such as Fe^{2+}or Cu^{+} and molecular oxygen, BLM is activated and generates superoxide and hydroxyl radicals which attack DNA, oxidize DNA bases, produce protein-damage and promote lipid peroxidation. Antioxidants can protect against oxidative injuries and can act as potential antigenotoxic agents. In this respect we assessed antioxidant properties of DME by six different assays.

The DPPH scavenging activity of DME ranged from 46.27±0.18% at 20.83 μg/mL to 93.74±0.18% at 166.66 μg/mL (Fig. 17A). Dastmalchi et al. (2007) reported that the methanol extract of Iranian *D. moldavica* aerial parts at 33.33 μg/mL (in the reaction mixture) scavenged DPPH radical by 89.5±0.2% after 30 min reaction time. By comparison, a similar effect (91.19±0.22%) was exhibited by DME at 83.33 μg/mL after 5 min of reaction, suggesting that the DME is an efficient free radical scavenger. Taking into account the EC_{50} values (Table 16), DME was less active than gallic acid, a phytochemical with well-known significant antioxidant activity [Villaño et al., 2007]. The high free radical scavenging properties of DME were confirmed by ABTS assay. ABTS scavenging activity of DME increased dose-dependently from 37.70±0.35% at 6.25 μg/mL to 96.63±0.04% at 50 μg/mL after 6 min reaction time (Fig. 17B). According to the EC_{50} and TEAC values (Table 16), DME was less active than gallic acid. Our results are similar with those reported by Dastmalchi et al. (2007) for Iranian *D. moldavica* (TEAC values of 0.56±0.02 and 17.56±0.99 mM Trolox for the methanolic extract and gallic acid, respectively).

Superoxide anion radical has a remarkable high activity and it is a powerful oxidizing agent and an initiator of radical reactions being involved in the production of hydroxyl radicals and the generation of peroxynitrite (ONOO−) by the reaction with endogenous NO. All these radicals lead to DNA impairment and denaturation of -SH-pool [Parihar et al., 2007]. Besides, superoxide anion radical may reactivate BLM for damaging DNA [Yen et al., 2005]. DME efficiently scavenged superoxide anion radical, being almost 4.5 times more active than the positive control, catechin (EC_{50}=445.5±2.3 μg/mL *vs*. 1960.0±13.7 μg/mL) (Fig. 17C, Table 16). At concentrations of 750 μg/mL, DME inactivated superoxide anion radical in a higher proportion than 90%; for the same concentration, catechin scavenged superoxide anion radical by 24%.

Hydroxyl radical is the most reactive oxygen species that attacks directly most biomolecules. It causes deleterious effects that lead to the lethal damage in biological systems [Aprotosoaie et al., 2015a]. At 1890 μg/mL, DME scavenged hydroxyl radicals by 69.94±0.72% (Fig. 17D) and a further increase in concentration did not result in an increasing activity. The extract was less active than the positive control, catechin (Table 16). However, DME (EC_{50}=269.3±11.1 μg/mL) inactivated hydroxyl radicals in 2-deoxy-d-ribose degradation assay more pronounced than the water extract from Iranian *D. moldavica* (EC_{50}=19400±1.5 μg/mL)[Dastmalchi et al., 2007].

Compounds with reducing abilities are electron donors and they can act as primary and secondary antioxidants [Chanda, 2009]. In our study, the reducing power of DME reached maximum value (absorbance 0.75±0.00 at 700 nm) at concentration of 26.74 μg/mL (Fig. 17E). Although DME was 4.3 times less active than catechin (Table 16), it exhibited a good reducing capacity (EC_{50}=17.07±0.21 μg/mL). Additionally, the water extract from Iranian *D. moldavica* had a moderate reducing capacity (0.51±0.06 mmol ascorbic acid/g) being less active than well-known antioxidants such as ascorbic acid (5.58±0.32 mmol ascorbic acid/g), butylated hydroxyanisole (3.14±0.14 mmol ascorbic acid/g) or Pycnogenol (2.14±0.13 mmol ascorbic acid) [Dastmalchi et al., 2007].

Iron catalyzes many oxidative changes of different cellular macromolecules. It is involved in the generation of hydroxyl radicals *via* Fenton reaction and in lipid peroxidation [Perron and Brumaghin, 2009]. In the ferrozine assay, DME chelated ferrous ions dose-dependently. The highest activity (98.36±0.06%) was recorded at concentration of 166.66 μg/mL (Fig. 17F). We found a high ferrous chelating capacity of DME although lower compared to the positive control (Table 16). However, DME was more active than the water extract obtained from Iranian *D. moldavica* (EC_{50}= 35.7±0.4 μg/mL compared to EC_{50}=4 mg/mL) [Dastmalchi et al., 2007].

Cell viability

DME (25–200 mg/mL) did not decrease significantly the viability of normal human dermal fibroblasts up to a concentration of 200 mg/mL after 24 h treatment (Table 17). Thus, 25 and 100 mg/mL were selected as non-toxic concentrations in the subsequent assays.

Effects of DME on BLM-induced DNA damage

Similar to ionizing radiation, activated BLM after the binding to a transition metal (iron), can induce different types of DNA damage: DNA strand breakage (SSBs, DSBs, DNA interstrand cross-link), cytosine damage in cellular DNA, apurinic/apyrimidinic sites, release of free bases or generation of base propenals [Sidik and Smerden, 1990; Regulus et al., 2007]. It is appreciated that the DNA damage pattern in HeLa cells caused by either a treatment with 12 μg/mL BLM for 30 min or exposure to 40 Gy ^{137}Cs are astonishingly similar to each other [Grigaravičius et al., 2009]. In our study, the extent of DNA damage has been assessed by the comet assay. The

migration of DNA fragments out of the cell nucleus is quantified by some parameters such as: *tail moment, olive tail moment, %DNA in head* and *%DNA in tail* [Nair and Salvi, 2008; Liao et al., 2009].

A great percentage of DNA in comet tail indicated a high extent of DNA damage. Amifostine (WR-2721), a phosphorylated aminothiol with radioprotective properties was used as positive control [Nici et al., 1998]; the dose of BLM (10 μg/mL) and time de incubation (6 h) were selected according to previous studies [Jagetia et al., 2007; Westbury et al., 2011]. In a concentration-dependent manner, the posttreatment with DME significantly decreased BLM-induced DNA damage when compared to BLM alone-treated cells.

Exposure of dermal fibroblasts to DME 100 μg/mL after preincubation with BLM produces the most pronounced decrease of comet parameters compared to BLM-alone treated fibroblasts: tail moment decreases from 70.05±3.01 to 12.22±1.19, olive tail moment from 46.84±1.51 to 13.24±0.77 and %DNA in tail from 47.85±1.27 to 22.84±0.93 (Fig. 18, Fig. 19). It is noteworthy that DME 100 showed a more pronounced protective effect than amifostine (100 μg/mL); at the same concentration, DME itself did not induce any DNA damage (Fig. 19).

Surprinsingly, DME 25 alone induced some increases of the comet parameters (%DNA in tail, olive tail moment, tail moment) in comparison to sham control.

Various plant extracts (*Camellia sinensis, Origanum vulgare, Curcuma longa, Podophyllum hexandrum*) or polyphenols (naringin, epigallocatechin gallate, rutin, quercetin, silymarin, curcumin, rosmarinic acid, ferulic acid) showed genoprotective properties against damage induced by bleomycin or other genotoxicants (doxorubicin, H_2O_2, mitomycin, radiation) in different mammalian cells [Aherne and O'Brien, 1999; Jagetia et al., 2007; Silva et al., 2008; Friedmann Angeli et al., 2010]. They act mainly via antioxidant mechanisms that involve free radical scavenging properties, iron chelation, inhibition of lipid peroxidation or stimulation of endogenous antioxidant response.

In our study, it is more likely that the protective effects of DME to be mediated via intervention on physiological mechanisms of DNA repair since the normal fibroblasts were treated with DME after an initial exposure of cells to BLM [Friedmann Angeli et al., 2010]. Rosmarinic acid, the main component of DME, provided protection against DNA-damage induced by tert-butyl hydroperoxide in PC12 cells acting on DNA repair processes.

It increases the activity of OGG1 (8-oxoguanine DNA glycosylase 1), an important enzyme from base excision repair (BER) pathway that is involved mainly in the correction of oxidative genomic damage, including BLM-induced DNA injuries [Silva et al., 2008].

Although, the posttreatment of dermal fibroblasts with DME 100 μg/mL provided lower concentration of rosmarinic acid than the dose used in other studies (29.73 μM *vs.* 50 μM)[Silva et al., 2008], the high activity of DME 100 can be explained by the fact that the other polyphenols in DME potentiated the activity of rosmarinic acid.

Thus, quercetin showed a high capacity to protect against the formation of DNA strand breaks [Silva et al., 2008], ferulic acid enhanced DNA repair in animals exposed to whole body gamma-radiation [Maurya et al., 2005] while chlorogenic acid increased the expression of DNA repair enzymes in HCT-116 cells [Bernstein et al., 2007]. Also, the cells type, genotoxic agent and time of incubation can interfere the final result. The antioxidant properties of DME also contribute to its genoprotective activity. DME has a pronounced superoxide anion radical scavenging activity.

Table 16. *In vitro* antioxidant activity and phenolic content of *Dracocephalum moldavica* extract (DME) [Aprotosoaie et al., 2016]

Extract/ positive control	DPPH radical scavenging assay	ABTS radical cation scavenging assay		Superoxide anion radical scavenging assay	Hydroxyl radical scavenging assay	Reducing power assay	Ferrous ion chelating assay	Total phenolics (mg GAE/g extract)
	EC_{50}*	EC_{50}	TEAC**	EC_{50}	EC_{50}	EC_{50}	EC_{50}	
DME	23.1±0.10	8±0.1	0.79±0.0	445.5±2.3	269.3±11.1	17.07±0.21	35.7±0.04	289.55±2.63
Gallic acid	1.6±0.0	0.5±0.1	18.61±0.19	-	-	-	2.7±0.0	-
(+)-Catechin hydrate	-	-		1960.0±13.7	24.0±0.3	3.8±0.0	-	-

The results are expressed as mean±SD from three determinations. *, μg/mL; **, TEAC-μM Trolox equivalent to 1 μg/mL of extracts/positive control.

Table 17. The effects of DME on NHDF viability [Aprotosoaie et al., 2016]

Group	Cell viability (%)
NHDF	100.0±0.0
NHDF + DME 25	92.3±2.2*
NHDF + DME100	96.1±1.6**
NHDF + DME 200	83.3±6.7**

NHDF, normal human dermal fibroblasts. The values are expressed as mean ± SEM from five replicates.
Significant data at $^{*}p<0.01$ and $^{**}p<0.05$ compared to control (t Test).

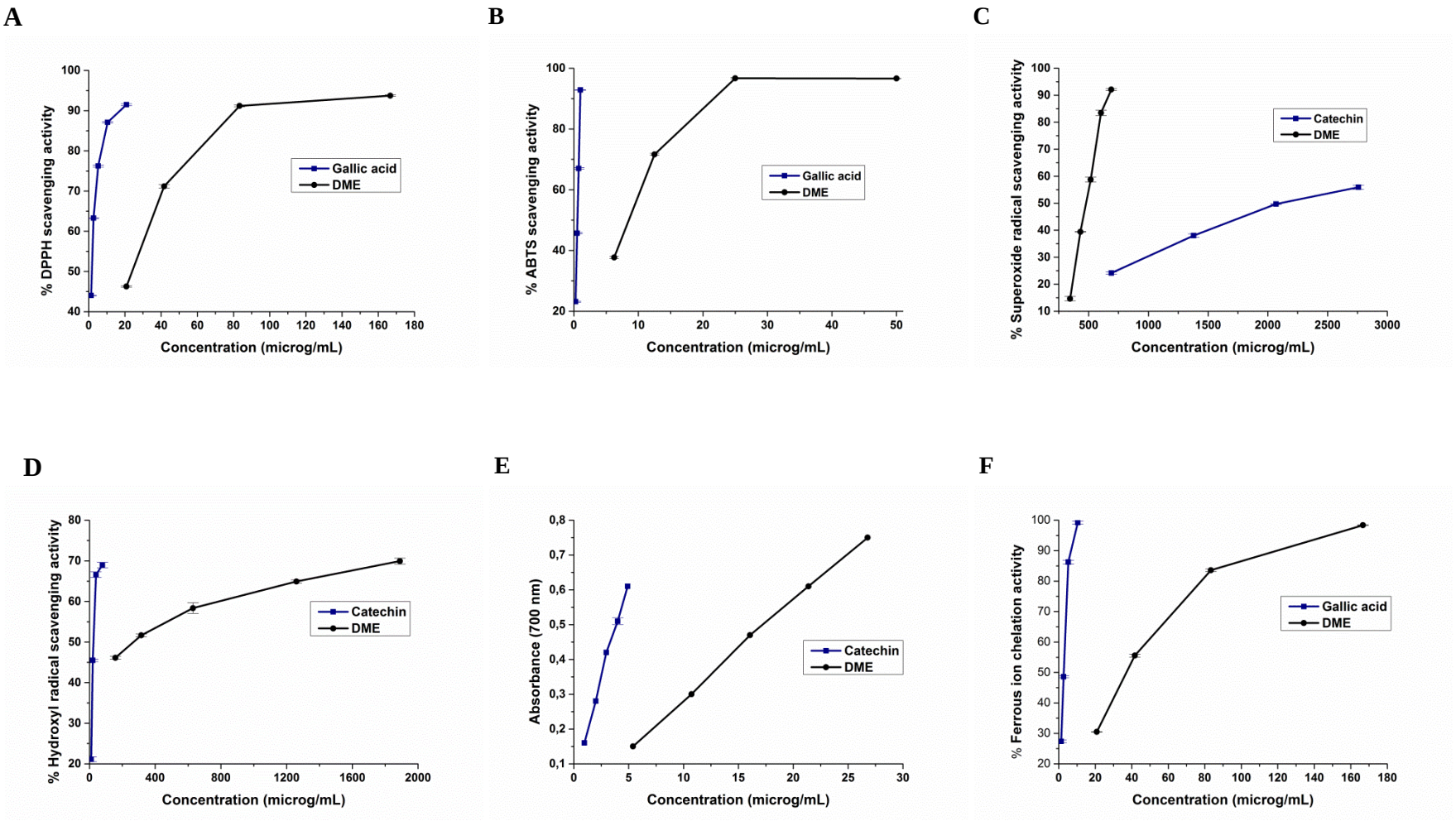


Figure 17. Antioxidant activity of DME [Aprotosoaie et al., 2016]: (A) DPPH radical scavenging activity; (B) ABTS radical cation scavenging activity; (C) superoxide anion radical scavenging activity; (D) hydroxyl radical scavenging activity. (E) reducing capacity; (F) ferrous ion chelating ability. Values are given as mean ± SD of three replicates in each group.

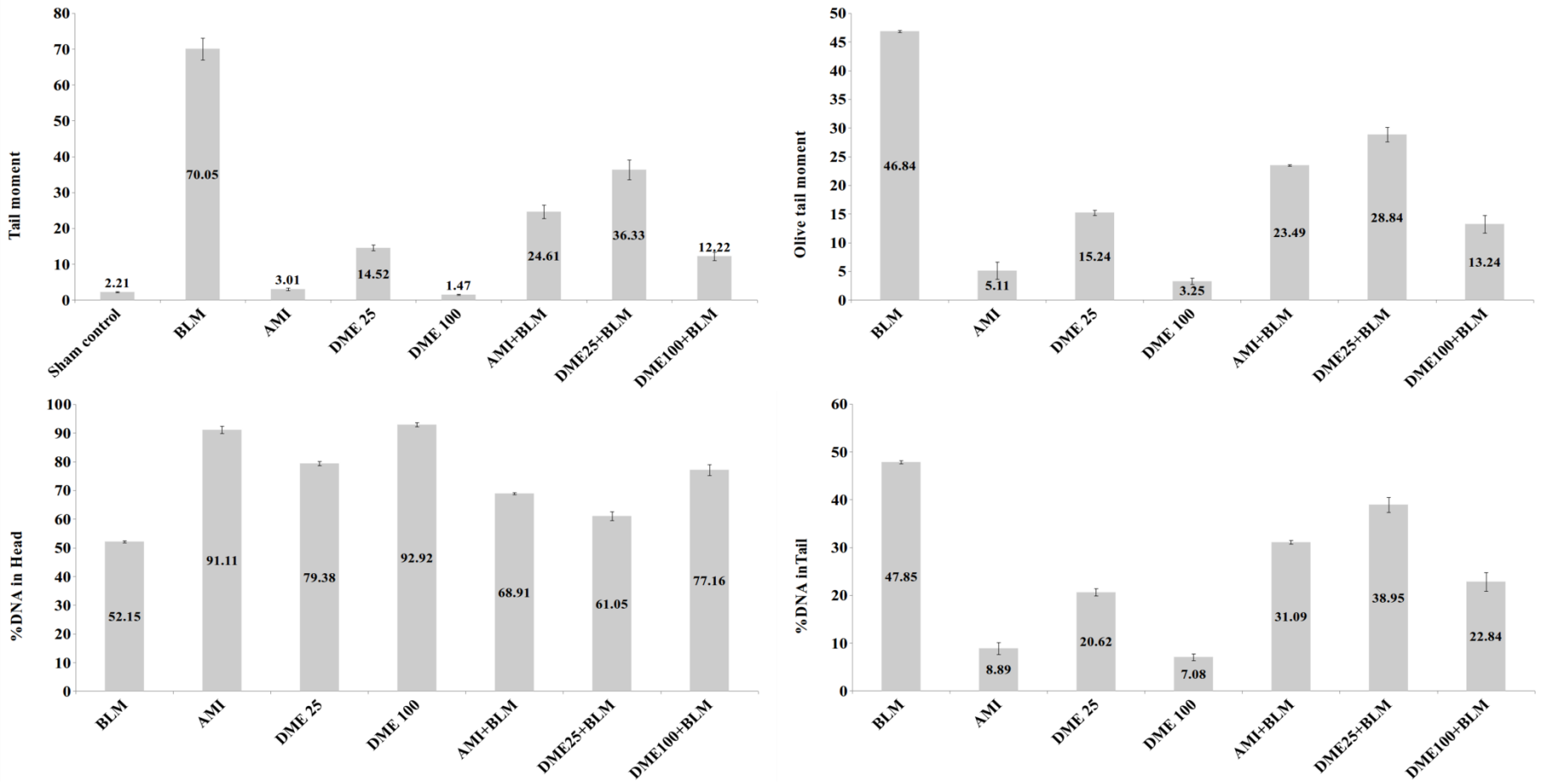


Figure 18. Protective effects of DME and AMI on BLM-induced genotoxicity in normal dermal fibroblasts [Aprotosoaie et al., 2016]. Results are given as mean ± SEM of three experiments in each group; Significant data at $p < 0.001$ compared to sham control (*) or BLM-alone group (**) (Bonferroni's MultipleComparison Test).

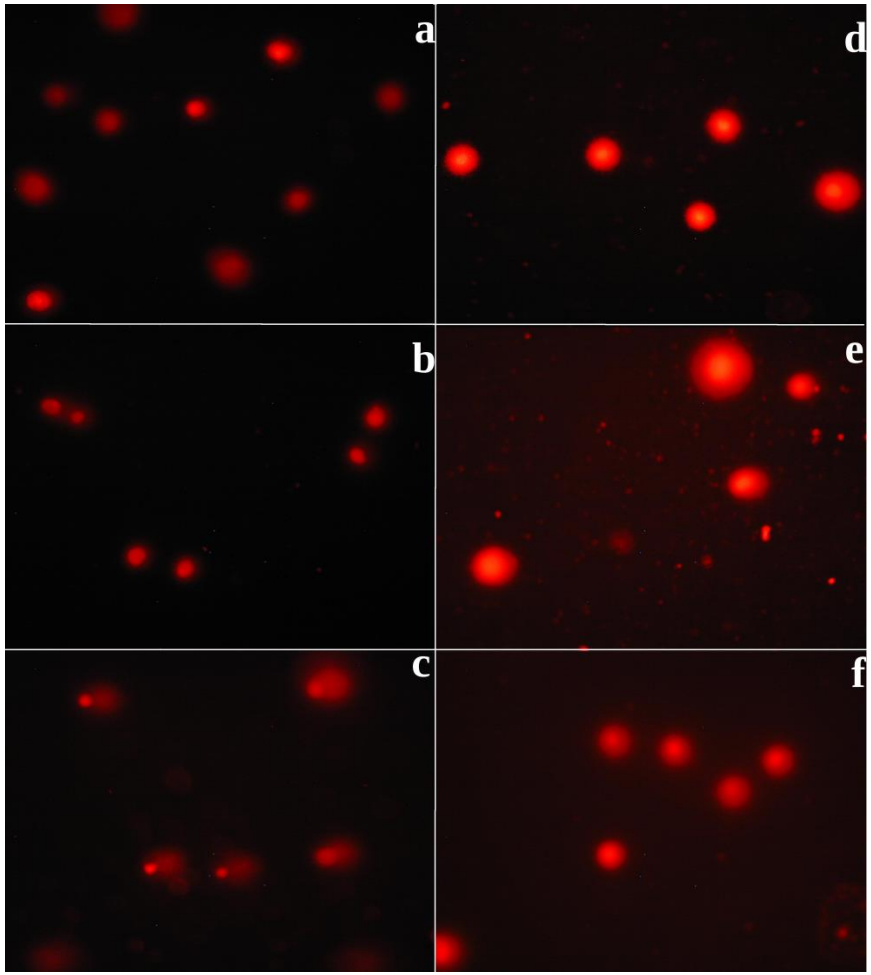


Figure 19. Photomicrographs of comet length in human dermal fibroblasts [Aprotosoaie et al., 2016]: a) normal; b) AMI-treated fibroblasts; c) BLM-treated fibroblasts; d) DME 100-treated fibroblasts; e) (AMI+BLM)-treated fibroblasts; f) (DME100+BLM)-treated fibroblasts.

It appears that in BLM-system, superoxide anion radical, not hydroxyl radical, is considered the major DNA-damaging species [Aruoma et al., 1999].

In addition, iron chelating properties and mild reducing abilities of DME can also interfere the redox recycling of BLM.

Effects on bleomycin-induced micronuclei formation

Oxidative DNA lesions induced by BLM may cause micronuclei formation, chromosomal abberations and gene mutation [Cooke et al., 2003; Khouri et al., 2007]. The treatment of fibroblasts with DME in concentrations of 25 and 100 μg/mL resulted in a decrease in micronuclei induction by BLM (Table 18).

Exposure to DME 100 μg/mL showed the highest reduction (69.28%) in the total micronuclei, the effect being significant when compared to BLM-alone group. In addition, DME posttreatment was found to be more effective than amifostine (Table 18) and DME itself had no mutagenic effect. Some polyphenols of DME showed a remarkable protective activity against clastogenic effects of genotoxicants such as certain anticancer drugs or ionizing radiation.

Rosmarinic acid significantly reduced the frequency of micronuclei and DNA damage induced by doxorubicin in V79 cells [Furtado et al., 2010] and gamma irradiation in human lymphocytes [Furtado et al., 2010]. Also, apigenin and ferulic acid protected from micronuclei induction in irradiated human peripheral blood lymphocytes [Prasad et al., 2006; Begum and Prasad, 2012].

It seems that the antioxidant mechanisms are mainly involved in the anticlastogenic potential of plant polyphenols [Furtado et al., 2010].

Table 18. Effect of DME on BLM-induced micronuclei in normal dermal fibroblasts [Aprotosoaie et al. 2016]

Group	Mean total micronuclei ± SEM	Decrease (%)
Sham control	0.89±0.12	
BLM	4.33±0.33	
AMI	3.00±0.58	
DME 25	2.33±0.33*	
DME 100	1.93±0.88*	
AMI +BLM	3.33±0.67	23.10
DME 25+BLM	3.00±0.58	30.72
DME100+BLM	1.33±0.33**	69.28

Values are expressed as mean ± SEM (n=3). Significant data at *p<0.05, **p<0.01 compared to BLM-alone group (t Test).

Conclusions

The polyphenolic extract from European *Dracocephalum moldavica* provided protection against bleomycin-induced genotoxicity in normal human adult dermal fibroblasts *via* the free radical scavenging activity, iron-chelating properties and the possible intervention on DNA repair processes. The extract itself is devoid of genotoxicity.

Further investigations are necessary for a better understanding of mechanisms underlying antigenotoxic effects of *Dracocephalum moldavica*.

The European species are an important source of rosmarinic acid and they are a promising candidate in the studies related on the radioprotection.

Acknowledgements
The study was supported by University of Medicine and Pharmacy "Grigore T.Popa" Iasi, Romania, Internal Research Grant 1639/01.02.2013.

I.2.1.2. Cytogenoprotective activity of *Ramaria largentii* mushrooms against H_2O_2-induced genomic damage in Vero cells

Current state of art and research objectives

Edible mushrooms have been used for their nutritional and medicinal properties for centuries. They are a valuable source of bioactive compounds that exhibit many beneficial effects on human health such as: antioxidant, anti-inflammatory, antitumor, immunomodulatory, antiviral, antihyperglycemic or antihypertensive activities [Roncero-Ramos and Delgado-Andrade, 2017].

Ramaria largentii Marr & D. E. Stuntz (orange coral mushroom) (Gomphaceae) is an edible species usually living in habitats with conifers being largely consumed in those areas. The research conducted by Zavastin (2016) highlighted good free radical scavenging and reducing capacities for a hydromethanolic extract from *Ramaria largentii* (EC_{50} =64.3±0.2 and 61.54±0.46 μg/mL, respectively).

Starting from these findings, this study investigated the antigenotoxic effects of hydromethanolic extract of the fruiting bodies from *Ramaria largentii* against H_2O_2-induced DNA damage in Vero cells.

Material and methods

Mushroom species, extraction and quantification of total phenolic content

The mushrooms were collected in Poiana Stampei (Northeast of Romania). The collection of mushrooms and obtaining hydromethanolic extract were carried out by Zavastin in the context of broader research [Zavastatin, 2016].

Reversed-phase high performance liquid chromatography (RP-HPLC)-*DAD-ESI-MS of phenolic compounds*

The chromatographic analysis of phenolic compounds was performed using an Agilent 1200 Series HPLC system with diode array detector (DAD) coupled to an Agilent 6520 accurate-mass quadrupole time-of-flight (Q-TOF) mass spectrometer equipped with an electrospray ionization (ESI) source. A Hypersil ODS C18 column (250 × 4.6 mm, particle size 5 mm) (Thermo Scientific) was used to separate compounds.

The mobile phase was composed of acetonitrile (A) and water with 0.1% formic acid (B) and a gradient elution varying from 0% to 100% A over 90 min was applied. The injection volume was 20 μL. The flow rate and detection wavelength were set to 0.5 mL/min and 280 nm, respectively.

The mass spectrometric detection was performed in the negative ion mode (capillary voltage -4.0 kV, skimmer voltage -68 V, drying gas flow rate 7 L/min, drying gas temperature 235 °C, nebulizer pressure 25 psig). Masses were scanned from 80 to 2800 amu in steps of 0.3 amu.

Integration and data processing were performed using MassHunter Workstation software. Phenolics were identified by comparing their retention times, UV and ESI-MS spectra with those of authentic standards.

Cell culture

Vero cells (normal African green monkey kidney epithelial) (ATCC CCL 81) were cultivated in DMEM, supplemented with 10% fetal bovine serum, 100 mg/mL streptomycin and 100 IU/mL penicillin. The cells were seeded at a density of 3.5×10^4 cells/well in 24-well plates (TPP Techno Plastic Products AG, Trasadingen, Switzerland) and maintained overnight at 37 °C in a humidified atmosphere containing 5% CO_2 and 95% air (Binder CB 150 Incubator, Tuttlingen, Germany). The cells were used in further experiments when reaching the confluence in the monolayer stage.

Cell viability assay

MTT assay [Mosmann, 1983] was used to evaluate cell viability. This method is based on the reduction by mitochondrial NAD(P)H-dependent dehydrogenases of yellow salt, MTT, into water insoluble purple formazan. After reaching the confluence in the monolayer stage, the growth medium was removed, the cells were washed with PBS, resuspended in fresh medium supplemented with different concentrations of extract (50, 100, 200 and 300 μg/mL) and incubated for 24 and 48 h at 37 °C. The cells treated with ultrapure water (solvent used to dissolve the extract) served as sham control. After incubation, the medium was discarded and the cells were washed with PBS and covered with fresh medium (100 μL). MTT (10 μL, 5 mg/mL) was added to each well followed by a further incubation for 3 h at 37 °C. The formazan dye was solubilized in DMSO (300 μL/well) and quantified at 540 nm. The results were expressed as % viability.

Antigenotoxicity/genotoxicity assay

Alkaline single-cell gel electrophoresis assay (Comet assay) was performed as described earlier [Olive and Banath, 2006; Reis et al., 2016]. The Vero cells were incubated for 24 and 48 h with *R. largentii* extract (100, 200 and 300 μg/mL) or corresponding volume of ultrapure water (sham control). Then, the medium was discarded and Vero cells were washed with PBS and treated with H_2O_2 (50 μM) as genotoxicant for 30 min. Controls treated only with H_2O_2 (50 μM) or extract (100, 200 and 300 μg/mL) were also included in the study. Afterwards, the cells were detached by trypsinization and submitted to Comet assay. For each treatment, the cell suspension (200 mL, aprox. 40,000 cells) was mixed with 1% low melting agarose (1000 mL) at 37 °C and quickly poured onto 1% normal melting agarose precoated slides. After agarose gelation, the slides were immersed in freshly prepared cold lysis solution (1.2 M NaCl, 100 mM Na_2EDTA, 0.1% sodium lauryl sarcosinate, 0.26 M NaOH, pH > 13) and lysed overnight at 4 °C under dark conditions. The slides were further washed with electrophoresis solution (0.03 M NaOH, 2 mM Na_2EDTA, pH~12.3) for three times at room temperature and placed in a horizontal gel electrophoresis tank very close to the anode. The electrophoresis was run at 0.6 V/cm for 25 min. All procedural steps were performed under dimmed light to prevent additional DNA damage. After electrophoresis, the slides were rinsed with ultrapure water. The staining of DNA was accomplished with ethidium bromide (20 μg/mL, 30 s). The slides were examined using a fluorescence microscope (Nikon Eclipse 600, Japan). Comet scoring was performed by OpenComet plugin for ImageJ. The results were expressed as % tail DNA. A higher percentage tail DNA indicated a higher level of DNA damage.

Statistical analysis

Folin-Ciocalteu assay was performed in triplicate and the result was expressed as mean± standard deviation. In case of cell viability and antigenotoxicity assays, five replicates were

performed for each concentration, the results being expressed as mean±standard error of mean. The statistical evaluation was done using the *t*-test. Values of $p < 0.001$, $p < 0.01$ and $p < 0.05$ were considered statistically significant.

Results and discussion

Phenolic profile

The hydromethanolic extract of *Ramaria largentii* is richer in polyphenols than similar extracts from other *Ramaria* species ((*R. botrytis*, *R. flava*, *R. aurea*) (42.33±0.18 mg GAE/g *vs*. 6.66-22.14 mg GAE/g).

The protocatechuic and vanillic acids were detected in *R. largentii* extract by comparing their retention times, MS and UV data with those of commercial standards (protocatechuic acid: retention time 34.3 min, [M-H]- at m/z 153, 260 nm; vanillic acid: retention time 51.1 min, [M-H]- at m/z 153, 260 nm) (Fig. 20). Both phenolic acids are reported for the first time in *R. largentii*.

Phenolic acids were also reported in *R. botrytis* (protocatechuic and *p*-hydroxybenzoic acids) and *R. aurea* (caffeic, cinnamic and gallic acids).

They have important biological activities (antioxidant, antimicrobial, antitumor) and contribute to the beneficial effects of edible mushrooms.

Cytotoxicity of R. largentii extract

In MTT assay, *R. largentii* extract (50-300 μg/mL) did not decrease significantly the viability of Vero cells after 24 and 48 h treatment. High concentrations of extract (200 and 300 μg/mL, respectively) cause only slight reductions in Vero cells viability (8.19 and 9.72%) after 48 h treatment (Table 19) but it does not fall bellow 90%.

Overall, it may be concluded that *R. largentii* extract in concentrations of 50-300 μg/mL is non-toxic to normal Vero cells.

The concentrations of 100, 200 and 300 μg/mL were used in subsequent experiments.

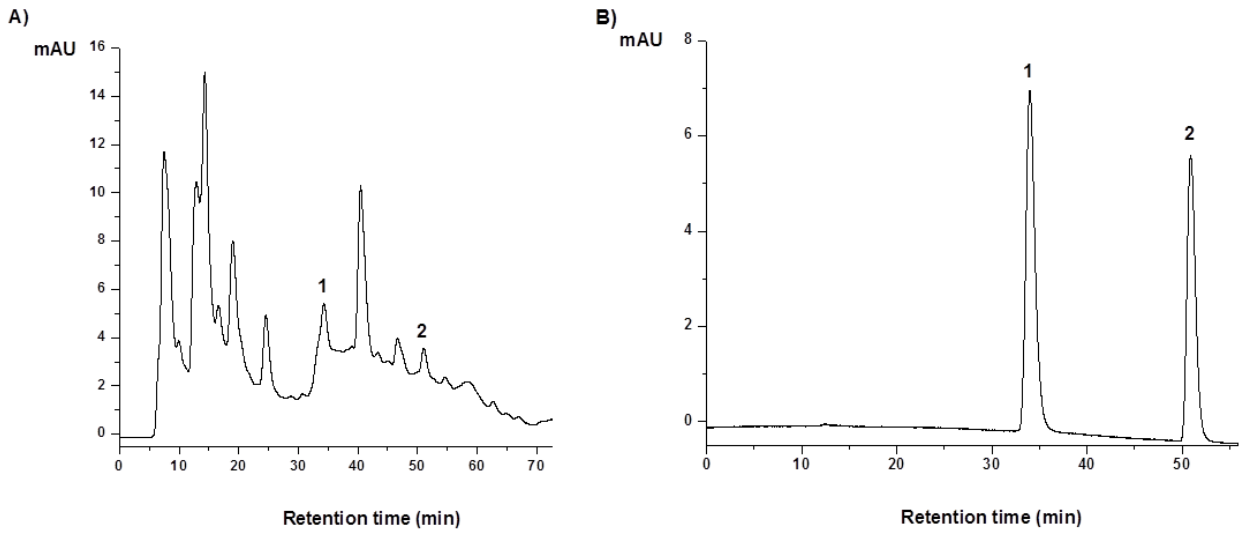


Figure 20. RP-HPLC-UV chromatogram (280 nm) of *Ramaria largentii* extract (A) and standard mixture (B) (1-protocatechuic acid, 2-vanillic acid) [Aprotosoaie et al., 2017b]

Table 19. Viability of Vero cells after 24 and 48 h treatment with *Ramaria largentii* (RL) extract

Group	% Cell viability	
	24 h	48 h
Sham Control	100.0±0.0	100.0±0.0
RL 50	109.47±5.86*	102.46±2.51
RL 100	104.89±3.98	96.87±4.65
RL 200	105.46±6.12	91.81±4.67**
RL 300	100.62±4.00	90.28±2.42***

The values are expressed as mean ± SE from five replicates. Significant data at $^{*}p<0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ when comparing the effects of *Ramaria largentii* extract with sham control (t Test).

Antigenotoxic/genotoxic effects of R. largentii extract

The current constant exposure of the human body to different genotoxic agents requires an adequate supply of safe antioxidants that will contribute to the prevention of genomic damage induced by oxidative stress. In this study, genoprotective potential of *Ramaria largentii* extract was assessed by alkaline single-cell gel electrophoresis assay (Comet assay) using H_2O_2 as genotoxic agent. H_2O_2 is a reactive oxygen intermediate which is generated in various forms of oxidative stress.

At cellular level, H_2O_2 produces highly reactive free hydroxyl radicals *via* Fenton reaction in the presence of transition metals (iron, copper). Hydroxyl free radicals are considered the major mediator of the DNA lesions consisting in strand breaks, oxidized bases and abasic sites. Apart from DNA damage, hydroxyl radicals are also involved in other processes that impair cell viability such as membrane lipid peroxidation and protein carbonylation. The alkaline comet assay allows to detect single/double-strand DNA breaks, alkali labile sites (apurinic/ apyrimidinic sites), DNA cross-links, base/base-pair damages [Nandhakumar et al., 2011].

Exposure of Vero cells to H_2O_2 (50 mM, 30 min) caused a significant increase in DNA damage in comparison with sham control (76.9 *vs*. 5.41% tail DNA) (Fig. 21, Fig. 22). At all tested concentrations, *R. largentii* extract afforded a remarkable protection against H_2O_2-induced DNA damage (2.09-7.91% tail DNA) (Fig. 21, Fig. 22). It is worth mentioning that the antigenotoxic activity was not dependent on the incubation time (24 or 48 h).

For each extract concentration (100, 200 and 300 μg/mL), the genoprotective effects of *Ramaria largentii* after 24 h pretreatment were comparabile with those detected after 48 h pretreatment (Fig. 21, Fig. 22).

The genoprotective activity is due to the phenolic compounds, but also to other components of the extract, which are likely to act synergistically in this regard. The claim is based on the fact that, however, the content of phenolic derivatives has a lower value compared to other phenolic extracts that develop similar effects.

In antioxidant tests, the *Ramaria largentii* extract showed weak iron ions chelating abilities (EC_{50}=2497±6 μg/mL) [Zavastin, 2016], so the extract is more likely to act by free radical scavenging properties and reducing capacities as they were highlighted by Zavastin (EC_{50} = 64.3±0.2 μg/mL in ABTS assay and 61.54±0.46 μg/mL in reducing power assay)[Zavastin, 2016].

The indirect mechanisms such as *up*-regulation of cellular enzymatic (catalases, glutathione peroxidases) and nonenzymatic systems (reduced glutathione) that detoxify H_2O_2 might also be involved in genoprotection.

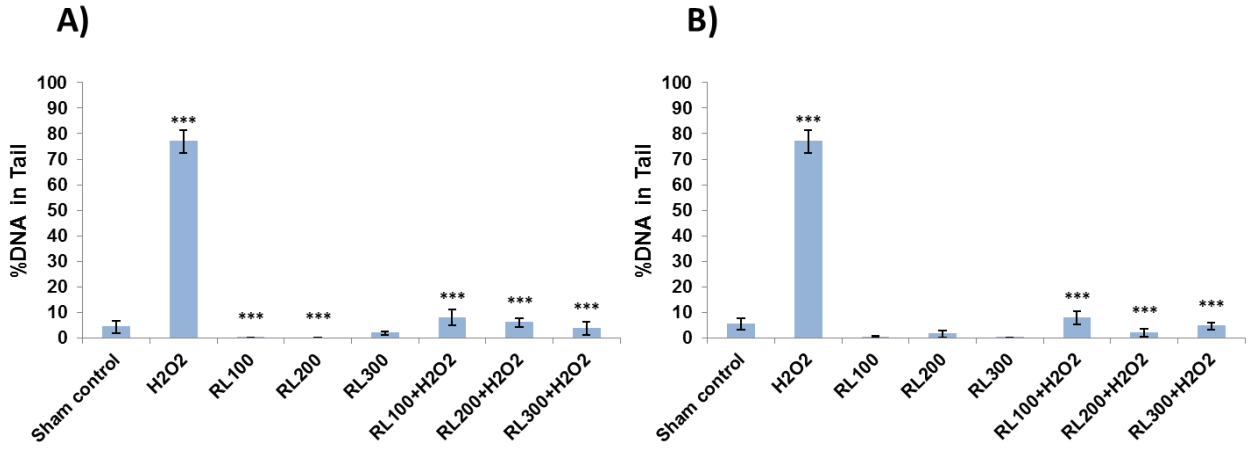


Figure 21. % Tail DNA in Vero cells after 24 (A) and 48 h (B) pretreatment with *Ramaria largentii* (RL) extract (100, 200 and 300 μg/mL) [Aprotosoaie et al., 2017b]. Results represent the mean±SE of five independent experiments. ***p < 0.001 when comparing the effects of *Ramaria largentii* extract with H_2O_2-treated cells and H_2O_2-treated cells with sham control (t-test).

A literature survey revealed cell-based studies on the genoprotective potential of other edible mushroom species but not *Ramaria* species.

Aqueous extracts of *Agaricus bisporus* and *Ganoderma lucidum* fruiting bodies (0.5 mg/mL) respectively, provided almost complete protection against H_2O_2-induced DNA damage in Raji cells (Burkitt's lymphoma, ATCC CCL-86) (Shi et al., 2002b).

Also, DNA damage induced by methyl methanesulfonate in human laryngeal epidermoid carcinoma HepG-2 cells was reduced by the aqueous extracts of *Lentinula edodes* (Shiitake)(1 and 0.5 mg/mL) [Miyaji et al., 2004].

The methanolic extract of *Lactarius vellereus* (Fleecy Milkcap)(500 μg/mL) protected against genotoxicity induced by 2-amino-3-methylimidazo(4,5-f)quinolone (carcinogen heterocyclic amine generated during cooking meat) in human hepatoma HepG2 cells [Mlinaric et al., 2004].

Genoprotective mechanisms of edible mushrooms are not fully understood; only a few mechanisms were highlighted, such as:

- enhancement of the cellular antioxidant defense (*Agaricus bisporus*),
- inhibition of benzo(a)pyrene activation or its penetration into the cell (*Agaricus blazei* β-glucan), or
- stimulation of DNA repair mechanisms (*Agaricus blazei*) [Angelli et al., 2006, Angelli et al., 2009].

Although edible mushrooms are considered to be devoid of toxicity and safe for human consumption, there are studies that shown the genotoxic potential of some mushrooms species.

Thus, the n-butanolic extract from *Agaricus blazei* proved to be genotoxic in HTC rat hepatoma cells. Its genotoxicity was probably due to the generation of toxic metabolites since HTC hepatoma cells are drug-metabolizing cells.

In our study, *R.largentii* extract itself (100, 200 and 300 μg/mL) had no genotoxic effects in Vero cells after 24 and 48 h treatment, the percentage of tail DNA being comparable with that of sham control (0.01-1.48% vs. 5.49%) (Fig. 23).

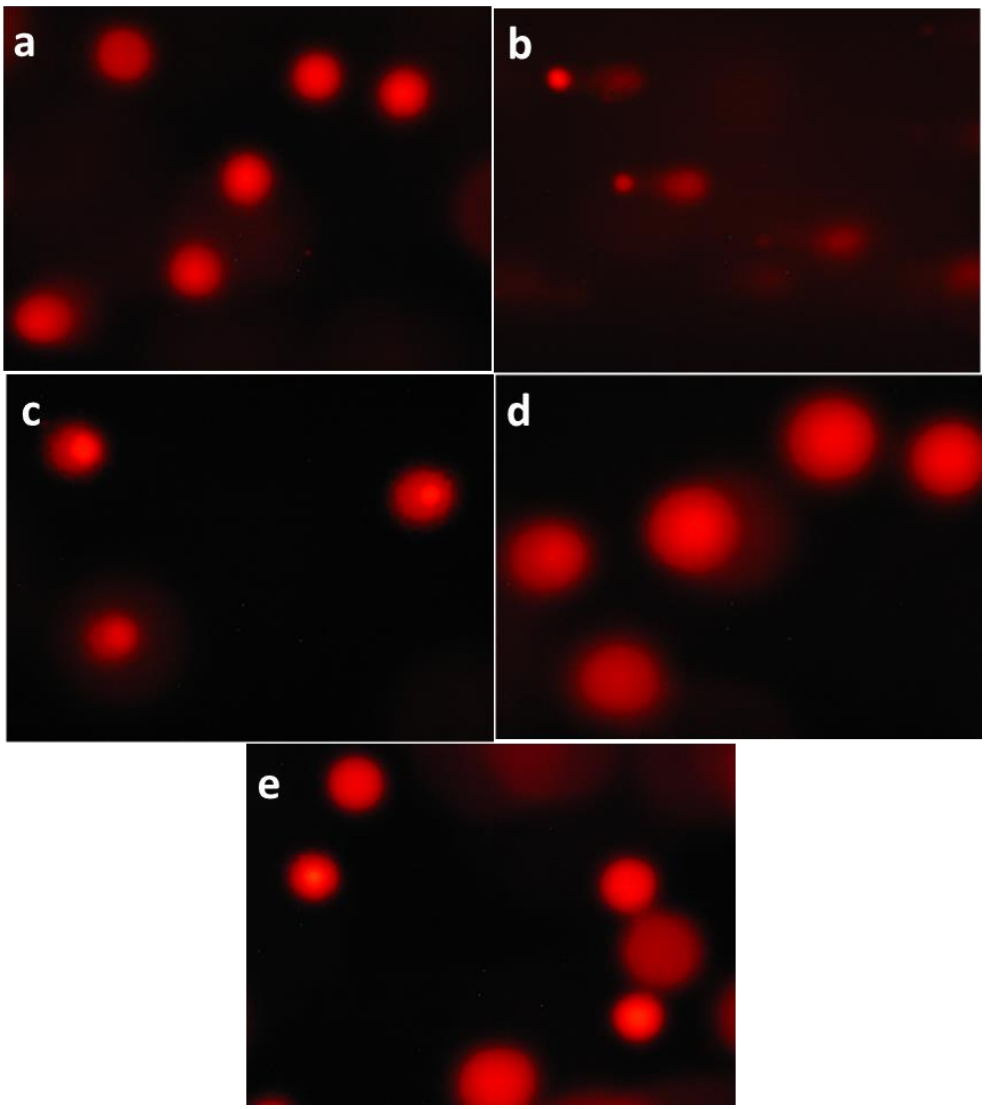


Figure 22. Photomicrographs of comet length in Vero cells: (a) normal, (b) exposed to H_2O_2 (50 mM, 30 min), exposed to H_2O_2 (50 mM, 30 min) after 48 h pretreatment with *Ramaria largentii* extract: (c) 100 μg/mL, (d) 200 μg/mL, (e) 300 μg/mL [Aprotosoaie et al., 2017b].

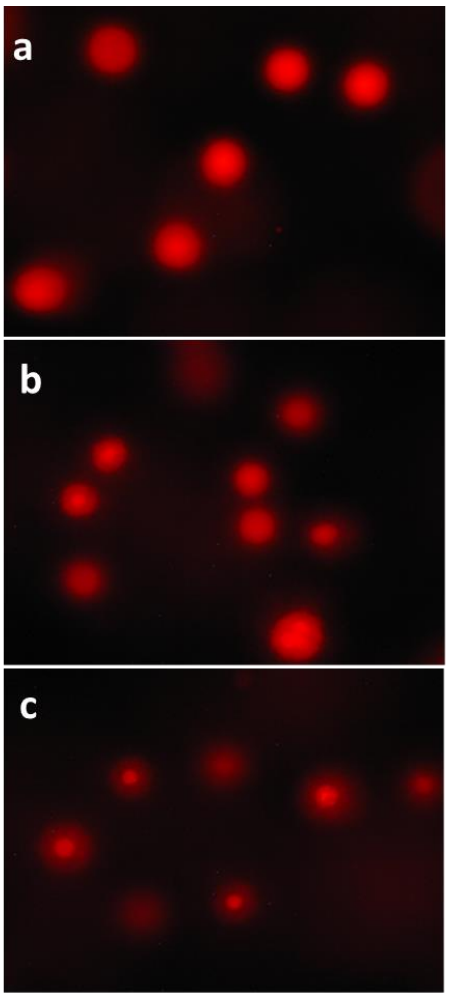


Figure 23. Photomicrographs of comet length in Vero cells: (a) normal, after 48 h treatment with *Ramaria largentii* extract: (b) 200 μg/mL, (c) 300 μg/mL [Aprotosoaie et al., 2017b].

Conclusions

This research is the first report on the chemical composition and bioactivity of *Ramaria largentii* mushroom collected from Northeast Romania.The results of our study show that *Ramaria largentii* extract is devoid of genotoxicity and has a remarkable DNA protective activity against H_2O_2-induced damage. The antioxidant activity of extract contributes to the expression of genoprotective properties but considering their magnitude other mechanisms are also possible. In addition, it appears that not only phenolic compounds are responsible for antigenotoxic effects. All these findings encourage further studies on identification of antigenotoxic constituents and elucidation of the mechanisms underlying their effects as well as research of *in vivo* bioactivities of *Ramaria largentii* mushrooms.

I.2.1.3. Protective effects of Moldavian *Thymus sp*. essential oils against H_2O_2-induced genomic damage in V79 cells

Current state of art and research objectives

The *Thymus* L. is one of the most important and polymorphic genus of the Lamiaceae family comprising about 250 species of perennial, aromatic and medicinal plants and subshrubs native to Europe, North Africa and Asia [Stahl-Biskup, 2004].

The thyme species are extensively used as culinary herbs, flavouring and food preservative agents as well as therapeutical products in traditional medicine. Besides, they have valuable applications in the food, cosmetic, and pharmaceutical industries, and also in perfumery.

Significant bioactivities have been reported for thyme-derived products, such as: antioxidant, antimicrobial, anti-inflammatory, antinociceptive, spasmolytic, expectorant, or antitumor [Kuete, 2017]. These properties and non-therapeutic uses of *Thymus* species have been largely attributed to the presence of essential oils (EOs).

In fact, due to its valuable uses, *Th. vulgaris* EO is among the world's top ten EOs, and demand for thyme EOs is growing [Ballester-Costa et al., 2013] So, there is a strong interest concerning the chemistry, biological effects and safe use of *Thymus* EOs.

Although, an extensive research in this area has been performed, to the best of our knowledge, no data has been reported for the EOs of *Thymus* species from Republic of Moldova.

In this respect, the main goal of the present study was to evaluate the chemical composition, antioxidant and antigenotoxic properties of EOs from three *Thymus* species (*T. vulgaris* L., *T. citriodorus* (Pers.) Schreb and *T. calcareus* Klock. et Bess.-Schost) and two cultivars (*T. vulgaris* 'Faustini' and *T. citriodorus* 'Aureus') from Republic of Moldova.

The data on chemistry and biological properties of *T. calcareus* and *T. citriodorus* 'Aureus' EOs, regardless of geographical area, are reported for the first time.

Material and methods

Chemicals and media

Alkane standard solution C_8-C_{20}, 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), butylated hydroxyanisole (BHA), potassium ferricyanide, ferric chloride, dimethyl sulfoxide (DMSO), sodium lauryl sarcosinate, normal and low melting point agarose were purchased from Sigma-Aldrich (Steinheim, Germany). H_2O_2 was from Fluka (Steinheim, Germany). 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was supplied by Merck (Darmstadt, Germany).

Ethidium bromide was obtained from Carl Roth (Karlsruhe, Germany). Trichloroacetic acid and potassium persulfate were from Riedel-de Haën (Seelze, Germany).

Dulbecco's Modified Eagle's medium (DMEM), phosphate buffered saline (PBS), fetale bovine serum, streptomycin and penicillin were from Biochrom AG (Berlin, Germany).

Ultrapure water was obtained using an Ultra Pure Water System type Ultra Clear TWF UV (SG Water, Barsbüttel, Germany). Ringer powder was purchased from Scharlau (Barcelona, Spain). RPMI 1640 medium was from Biochrom GmbH (Berlin, Germany).

Plant material

Aerial parts of *T. vulgaris*, *T. citriodorus*, and two cultivars, *T. vulgaris* 'Faustini' and *T. citriodorus* 'Aureus' (Fig. 24), were collected at the full flowering stage from specimens grown in the Botanical Garden of Chişinău (*Academy of Sciences of Moldova*, Republic of Moldova) in July 2015.

The plants were cultivated in ecological conditions.

T. calcareus (Fig. 25) was collected at the full flowering stage from spontaneous flora, in the same year.

The aerial parts were dried separately at room temperature (20-23 ºC) in dark.

T. vulgaris

T. citriodorus

T. vulgaris 'Faustini'

T. citriodorus 'Aureus'

Figure 24. *Thymus* species and cultivars from the fields of Botanical Garden of Chişinău

Essential oils isolation

Plant material was powdered (100 g) and subjected to hydrodistillation for 3 h in a modified Clevenger-type apparatus. Two extractions were performed for each plant material. The EOs were dried over anhydrous sodium sulfate and stored in dark glass vials at 4 °C until use. The yield of extraction was expressed as volume (mL) of EO per 100 g of plant dry matter (v/w).

Gas chromatography analysis

Gas chromatography analysis of EOs was performed on an Agilent 6890N gas chromatograph equipped with a flame ionization detector (FID) and 5975 inert XL mass selective detector. A capillary column (30 m × 0.25 mm i.d.) coated with a 0.25 μm film of cross-linked 5% phenyl methyl silicone (HP-5MS, Agilent) was used with helium as carrier gas at a flow rate of 1 mL/min. The injection volume of each EO was 0.2 μL. The split ratio was 1:20 in GC-FID analysis and 1:50 in GC-MS analysis. The oven temperature was programmed from 60 to 250 °C at 4 °C/min and then from 250 to 300 °C at 10 °C/min; the final temperature was maintained for 7.5 min. The front inlet was set at 250 °C. Mass spectra

were acquired in the scan mode (mass range of 15-450 *m/z*) and were recorded under electron electron ionization energy of 70eV.

Figure 25. *T. calcareus* from spontaneous flora (Măscăuți, Criuleni County, Republic of Moldova)

The retention indices were obtained by analyzing a standard solution of *n*-alkanes (C_8-C_{20}) under the same chromatographic conditions. The compounds were identified by comparison of their recorded mass spectral data with those stored in the NIST 14 MSD database as well as by comparison of the calculated retention indices with those reported in literature [NIST 2018]. The relative percentages of the compounds were obtained from the FID peak areas without applying the correction factors. The analysis was carried out two independent times, and average values were reported.

In vitro antioxidant activity

DPPH radical scavenging assay

DPPH radical scavenging activity was assessed as previously described by Apetri et al. (2013). 1 mL of EO dilutions in methanol (concentration range from 80 to 0.156 mg/mL) was mixed with 1 mL of DPPH methanolic solution (0.004%, w/v) and further incubated for 30 min in dark at room temperature. Then, the absorbance was measured at 517 nm. BHA was used as the positive control. The DPPH radical scavenging activity (%) was calculated using the formula: $100\times(A_C - A_S)/A_C$, where A_C and A_S are the absorbances of DPPH radical in the control and in the presence of EOs/BHA, respectively. The results were expressed as EC_{50} values (concentrations giving half-maximal response) calculated by linear interpolation between values above and below 50% activity.

ABTS radical cation scavenging assay

The ability of essential oils to scavenge ABTS radical cation was evaluated as described by Re et al. (1999) with minor changes [Apetri et al., 2013]. ABTS radical cation solution was prepared by the reaction of equal volumes of ABTS (7 mM) and potassium persulfate (2.45 mM) for 12-16 h in the dark at room temperature. Then, the ABTS radical cation

solution was diluted with methanol to an absorbance of 0.70±0.02 at 734 nm (equilibration at 23±2 °C). An aliquot (0.02 mL) of each dilution of EOs (0.156-80 mg/mL) was mixed with ABTS radical cation solution (1.98 mL) and the absorbance was read at 734 nm after 6 min reaction time. BHA was used as reference substance for comparison (positive control). ABTS radical cation scavenging activity (%) and EC_{50} values were calculated as described in DPPH assay.

Reducing power assay

The reducing capacity of EOs was determined based on the method of Ferreira et al. (2007) with minor modifications. The EOs were diluted in methanol in concentrations varying from 600 to 2.5 mg/mL. An aliquot of each dilution of EOs (50 µL) was mixed with 1% potassium ferricyanide (1.25 mL) solution and 0.2 M phosphate buffer, pH 6.6 (1.2 mL). The mixture was incubated in a water bath at 50 °C for 20 min. To this reaction mixture, 1.25 mL trichloroacetic acid (10%) were added and the reaction mixture was further centrifuged at 3,000 rpm for 10 min. The upper layer (1.25 mL) was mixed with ultrapure water (1.25 mL) and 0.1% ferric chloride solution (0.25 mL). The absorbance at 700 nm was measured after 10 min. BHA was the positive control. EOs concentration giving an absorbance of 0.5 was considered as effective inhibitory concentration (EC_{50}).

Cell culture

Chinese hamster lung fibroblast V79 cells ATCC® CCL-93 were grown in DMEM containing 10% fetal bovine serum and antibiotics (100 µg/mL streptomycin, 100 IU/mL penicillin). The cells were plated in 96-well tissue-culture plates (8×10^3 cells/well) and allowed to attach and grow for 24 h at 37 °C in a humidified air incubator containing 5% CO_2. When the cells have reached the confluence in the monolayer stage, they were subjected to viability and genotoxicity/antigenotoxicity assays.

Cell viability assay

After 24 h incubation with EOs in different doses (25-300 µg/mL), the cell viability was assessed by MTT test, with minor modifications [Mosmann, 1983]. In brief, the cells were washed with PBS, the medium was replaced by fresh growth medium (100 µL/well) and then MTT (10 µL/well) was added. After 3 h incubation at 37 °C, the formazan dye, generated by the reduction of MTT in living cells, was dissolved in DMSO (100 µL/well). The absorbance was further read using a microplate Reader at 540 nm. The cell viability (%) was calculated as $100\times(A_S/A_C)$, where A_S and A_C are the absorbances of the formazan dye in the cells incubated with EO dilutions (samples) and sham control, respectively.

Genotoxicity/antigenotoxicity assay

In the genotoxicity assay, V79 cells were exposed to thyme EOs (25 µg/mL) for 60 min. Genoprotective potential of thyme EOs was assessed using H_2O_2 as genotoxicant and two different protocols (pre- and post-treatments). In the first, V79 cells were pre-treated with EOs (25 µg/mL) for 60 min. Then, the medium was removed, the cells were washed with PBS and treated with H_2O_2 (50 µM) for 30 min. In the post-treatment protocol, V79 cells were first exposed to H_2O_2 (50 µM, 30 min) and then to EOs (25 µg/mL, 60 min). The controls exposed only to H_2O_2 (50 µM, 30 min) or EOs (25 µg/mL, 60 min) were also included in both experimental variants. At the end of the treatment, the cells were detached and resuspended in fresh medium for the Comet assay [Kozics et al., 2013; Aprotosoaie et al., 2017b].

Alkaline single-cell gel electrophoresis assay (Comet assay)

In brief, a freshly prepared cell suspension (200 μL, aprox. 40,000 cells) was mixed with 1000 μL of 1% low melting agarose and spread onto the slides precoated with 1% normal melting agarose. After solidification of the gel, the slides were immersed in freshly prepared cold lysis solution (1.2 M NaCl, 100 mM Na_2EDTA, 0.1% sodium lauryl sarcosinate, 0.26 M NaOH, pH>13), lysed overnight (4 °C in dark) followed by three washes with electrophoresis solution (0.03 M NaOH, 2 mM Na_2EDTA, pH≈12.3) and transfer to a horizontal gel electrophoresis tank. The electrophoresis was carried out at 0.6 V/cm for 25 min. After washing with water, the slides were stained with ethidium bromide (20 μg/mL, 30 s). All the above steps were conducted under dimmed light to prevent additional DNA damage. The slides were examined with a Nikon Eclipse 600 fluorescence microscope. Image capture and analysis were performed using ImageJ OpenComet plugin. The percentage of DNA in the comet tail (% tail DNA) was used to measure DNA damage [Olive and Banath, 2006; Reis et al., 2016; Aprotosoaie et al., 2017b].

Statistical analysis

The antioxidant assays were performed in triplicate and the results were expressed as mean±standard deviation. In the cell viability and genotoxicity/antigenotoxicity assays, five replicates were performed for each concentration; the results were expressed as mean±standard error of mean. Student's *t*-test was performed for the statistical comparison between treatments and control. The differences were considered statistically significant at $p < 0.001$, $p < 0.01$ and $p < 0.05$.

Results and discussion

Essential oils yield and chemical profile

The yields of thyme EOs ranged from 1.31 to 3.15% (Table 20). The chemical composition of thyme EOs is summarized in Table 20. 63 compounds were identified in the essential oil of *T. vulgaris* representing 91.78% of total oil composition.Thymol (55.44%), *m*-cymene (11.88%), γ-terpinene (5.74%) and *o*-cymen-5-ol (5.14%) were the main compounds (Table 20, fig. 26). The aromatic terpene fraction predominates (72.58%), being mainly represented by monoterpene phenols (60.58%) (Table 20). The chemical composition of *T. vulgaris* EOs has been intensively investigated.Thymol chemotype has been the most frequently reported chemotype for *T. vulgaris* from different geographic areas: Balkan Peninsula (17.4-80.4%)[Ćavar and Maksimović, 2015], Egypt (32.33%)[Viuda-Martos et al., 2011], Spain (57.7%)[Rota et al., 2008] or Iran (40.97%)[Pirbalouti et al., 2013]. These findings are consistent with our study reporting the thymol chemotype for Moldavian *T. vulgaris*. With respect to *T. vulgaris* 'Faustini' EO, 53 compounds were identified accounting for 93.92% of total EO. Geraniol (31.45%), nerolidol (10.05%), geranial (9.14%), neral (7.05%), germacrene D (5.61%), linalool (4.92%) and nerol (3.75%) were the major components of *T. vulgaris* 'Faustini' EO. The oxygenated monoterpenes predominated in Moldavian *T. vulgaris* 'Faustini' EO (69.07%) whereas the oxygenated sesquiterpenes were the second most abundant compounds (10.79%) (Table 20). Data on the chemical composition of *T. vulgaris* 'Faustini' EO are scarce. Only one study which investigated the chemical composition of *T. vulgaris* 'Faustini' EO from Italy reported thymol as its main component (about 50%) [Bertoli et al., 2010]. The Moldavian 'Faustini' cultivar contained low concentrations of thymol (0.22%); instead, it was a rich source of geraniol, a monoterpene alcohol that, together with monoterpene aldehydes geranial and neral, contributes to the pleasant floral, lemon-like scent of volatile oil.

The analysis of *T. citriodorus* EO enabled the identification of 54 compounds (94.99% of total EO composition). Lavandulol (54.27%), geranial (12.25%), neral (9.00%) and nerol

(3.88%) were the main constituents. Oxygenated monoterpenes constituted the bulk of Moldavian *T. citriodorus* EO (83.87%) being primarily responsible for the green, rosy and lemon-like fragrance of EO (Table 20). In general, *T. citriodorus* EOs are commonly characterized by the presence of geraniol (up to 60%), geranial (8.2%), neral (5.5%) and nerol (2.8%) as the most important constituents [Stahl-Biskup and Holthuijzen, 1995; Omidbaigi et al., 2005]. On the contrary, Wu et al. (2013) have identified borneol (28.82%) and thymol (14.43%) as the most abundant compounds in *T. citriodorus* from China. The compositional diversity of *Thymus* EOs is well-known, the genus *Thymus* being characterized by a high chemical polymorphism. Both environmental and genetic factors cause this variability. The presence of monoterpenol lavandulol as the major component in *T. citriodorus* EO is reported there for the first time. The Moldavian *T. citriodorus* (lemon thyme) can be considered an important source of this valuable aroma compound. On the other hand, geranial and neral, lemon-scented components, are also found in Moldavian thyme in similar levels with those reported in literature. In total, 33 components were identified in *T. citriodorus* 'Aureus' EO representing 98.72% of oil composition and geraniol (60.31%), geranial (9.26%), neral (7.15%) and thymol (3.45%) being the most abundant. Oxygenated monoterpenes (81.59%) were the most prevalent fraction of the EO, their value being comparable with that found in *T. citriodorus* EO (83.87%). On the contrary, the aromatic fraction was higher in *T. citriodorus* 'Aureus' EO than in *T. citriodorus* EO (7.53% *vs.*1.05%) (Table 20). To the best of our knowledge, there are no literature data on the chemical composition of *T. citriodorus* 'Aureus'.

In *T. calcareus* EO there have been detected 44 components (96.16% of total oil). Thymol (55.45%), γ-terpinene (13.38%), *o*-cymene (8.32%) and linalool (4.86%) were dominating compounds (Table 20, Fig. 26). Similar to *T. vulgaris* EO, the aromatic terpene fraction predominated in *T. calcareus* EO (66.73%). The monoterpene hydrocarbons were the second most abundant subclass in *T. calcareus* EO, their level being higher than in *T. vulgaris* EO (17.69 *vs.* 9.24%). Also, *T. calcareus* EO was richer in oxygenated monoterpenes than *T. vulgaris* EO (8.41 *vs*. 4.95%) (Table 20).

Antioxidant activity

In the present study, the antioxidant potential of thyme EOs was screened by free radical scavenging and reducing capacity assays and compared to BHA as reference substance. There are significant differences in the antioxidant activity of EOs in all three assays. *T. vulgaris* EO was the most potent in scavenging DPPH and ABTS free radicals and reducing ferric ion (EC_{50} = 0.147, 0.003 and 0.041 mg/mL, respectively), followed by *T. calcareus* EO (EC_{50} = 0.369, 0.003 and 0.051 mg/mL, respectively) (Table 21). Thymol, a phenolic monoterpene, the major constituent of both EOs, is largely responsible for their remarkable antioxidant activity. It was found to be a potent antioxidant in numerous *in vitro* assays including free radical scavenging, reducing power and anti-lipid peroxidation assays [Ündeğer et al., 2009; Ali et al., 2013]. Similar to other phenolic antioxidants, thymol quenches free radicals by hydrogen donation thus forming phenoxyl radicals. Several structural features underlie the strong antioxidant properties of thymol, namely the phenolic hydroxyl and alkyl groups (in *o*- and *m*-positions) (Fig. 27). The latter have inductive effects and therefore, increase the electron density of the phenoxyl radicals stabilizing them and enhancing the antiradicalar potency [Ali et al., 2013]. Although *T. vulgaris* and *T. calcareus* EOs had similar thymol contents (55.44 and 55.45%, respectively) (Table 20), they scavenged free radicals and reduced ferric ion with different potencies. According to the EC_{50} values, *T. vulgaris* EO was more potent than *T. calcareus* EO in all antioxidant assays (Table 21).

Table 20. Chemical composition of *Thymus* species EOs [Aprotosoaie et al., 2019a]

Compound	**KI[a]**	**KI[b]**	**%**				
			***T. vulgaris* EO**	***T. vulgaris* 'Faustini' EO**	***T. citriodorus* EO**	***T. citriodorus* 'Aureus' EO**	***T. calcareus EO***
α-Thujene	931	931	0.30	0.24	0.03	-	0.44
α-Pinene	939	940	0.41	0.32	0.09	0.20	0.34
α-Fenchene	950	951	0.02	-	-	-	-
Camphene	953	952	0.07	0.16	0.16	-	0.07
β-Thujene	968	968	0.87	-	-	-	1.17
1-Octen-3-ol	975	976	0.75	0.38	0.58	0.57	-
β-Pinene	980	979	0.16	0.13	0.07	0.18	0.11
3-Octanone	985	984	0.03	-	1.32	1.16	0.05
β-Myrcene	992	993	-	0.21	0.03	-	-
3-Octanol	995	995	0.02	0.21	0.80	0.71	-
2-Carene	1001	1001	1.04	0.10	0.01	-	1.42
α-Phellandrene	1003	1003	0.14	-	-	-	0.18
3-Carene	1007	1007	0.05	-	0.01	-	0.06
(E,E)-2,4-heptadienal	1009	1010	-	-	0.02	-	-
4-Carene	1012	1011	-	-	0.01	-	0.15
o-Cymene	1021	1020	-	-	-	-	8.32
m-Cymene	1022	1023	11.88	-	-	-	-
p-Cymene	1026	1026	-	0.12	0.10	2.69	-
Isosylvestrene	1027	1027	-	-	-	-	0.25
Limonene	1030	1030	0.31	0.20	0.03	0.21	-
Eucalyptol	1031	1031	0.61	1.82	0.29	0.37	0.50
(*Z*)-Ocimene	1038	1037	0.04	1.66	0.02	-	0.06
(*E*)-Ocimene	1048	1047	-	0.54	-	-	-
γ-Terpinene	1061	1062	5.74	0.04	0.09	1.20	13.38
2-Ethylidene-6-methyl-3,5-heptadienal	1066	1066	-	-	0.03	-	-

cis-Sabinene hydrate	1070	1071	0.43	-	-	-	0.57
α-Terpinolene	1087	1088	0.16	-	-	-	-
Linalool	1098	1097	0.88	4.92	0.66	-	4.86
cis-Rose oxide	1108	1108	-	-	0.01	-	-
cis-Menth-2-en-1-ol	1119	1123	0.02	-	-	-	-
Chrysanthenone	1125	1125	-	0.02	-	-	-
3-Cyclohexen-1-carboxaldehyde, 3,4-dimethyl	1130	1130	-	-	0.04	-	-
Camphor	1145	1145	0.58	1.12	-	0.08	-
cis-β-Terpineol	1147	1147	0.03	1.08	-	-	-
Nerol oxide	1151	1150	-	-	0.08	0.07	-
Borneol	1165	1166	0.32	1.08	1.23	1.22	0.39
Lavandulol	1170	1170	-	0.89	54.27	-	-
Terpinen-4-ol	1176	1177	1.04	0.74	0.10	0.31	0.78
α-Terpineol	1190	1190	0.22	2.05	0.12	0.16	0.18
cis-Piperitol	1195	1196	0.03	-	-	-	0.06
trans-Dihydrocarvone	1200	1202	0.04	-	0.03	-	0.04
Nerol	1229	1229	0.03	3.75	3.88	1.65	-
Thymol-methyl-ether	1235	1233	0.07	-	0.29	0.43	0.02
Neral	1237	1237	0.06	7.05	9.00	7.15	0.05
Carvone	1241	1241	0.05	-	-	-	0.02
Isothymol-methyl-ether	1244	1244	-	-	0.07	0.65	-
Geraniol	1252	1252	0.33	31.45	-	60.31	0.65
Methyl nerolate	1265	1265	-	-	0.07	-	-
Geranial	1269	1270	-	9.14	12.25	9.26	-
Bornyl acetate	1285	1285	-	0.02	0.01	-	-
Thymol	1297	1297	55.44	0.22	-	3.45	55.45
Geranyl formate	1305	1304	-	0.21	0.20	-	-
o-Cymen-5-ol	1336	1336	5.14	-	0.59	0.31	2.93
α-Cubebene	1348	1348	0.06	-	-	-	0.03
Thymyl acetate	1357	1357	0.05	-	-	-	0.01
Neryl acetate	1365	1364	0.16	0.81	0.11	-	0.26

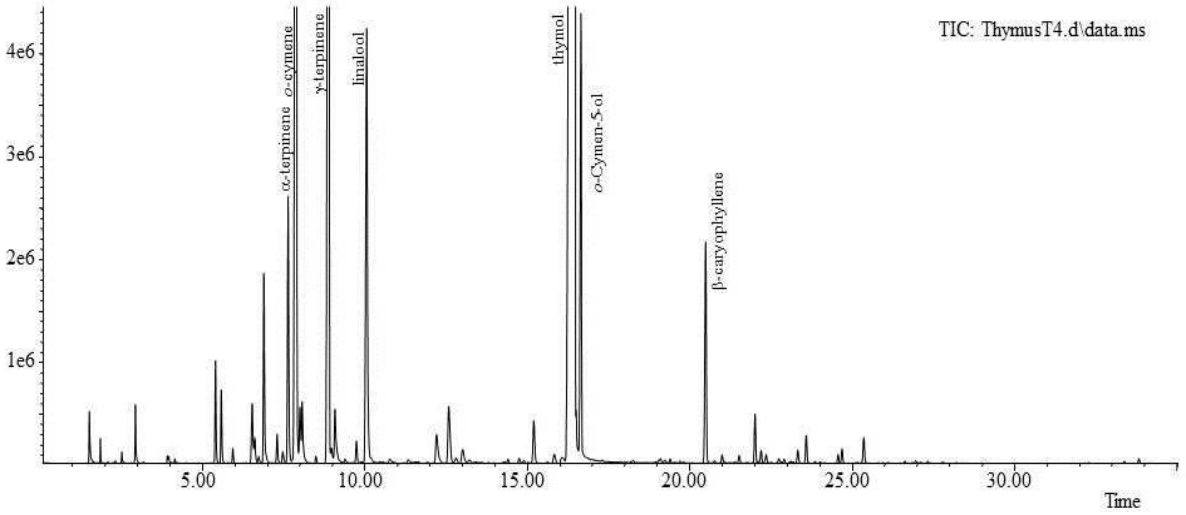
Ylangene	1368	1368	0.02	-	-	-	-
Geranyl acetate	1370	1370	-	2.60	0.67	0.67	-
α-Bourbonene	1374	1376	-	0.04	-	-	-
Copaene	1380	1382	-	0.06	-	-	0.02
β-Bourbonene	1384	1385	0.04	0.96	0.35	0.16	0.03
β-Elemene	1387	1387	-	0.15	-	-	-
β-Cubebene	1390	1389	0.02	0.08	-	-	0.01
cis-Jasmone	1393	1394	0.01	-	-	-	-
α-Gurgujene	1401	1401	-	-	0.02	-	-
β-Caryophyllene	1418	1420	1.53	1.64	2.49	2.53	2.20
Neryl propionate	1430	1430	0.02	0.03	-	-	-
α-Bergamotene	1432	1433	-	0.04	0.02	-	-
Elixene	1437	1437	-	-	0.01	-	-
Aromadendrene	1440	1440	-	-	-	0.08	-
α-Humulene	1452	1452	0.18	0.06	0.21	0.04	0.07
Geranyl propionate	1447	1448	-	-	-	0.13	-
Eudesma-4(14), 11-diene (β-Selinene)	1465	1464	0.01	-	-	-	-
δ-Muurolene	1468	1468	0.14	-	-	-	-
β-Cadinene	1475	1474	0.04	-	-	-	0.12
γ-Muurolene	1477	1477	0.30	0.06	-	-	-
α-Muurolene	1480	1480	0.07	-	-	-	-
Germacrene D	1487	1487	-	5.61	0.55	0.14	0.05
β-Ionone	1490	1489	0.02	-	-	-	-
Linalyl isovalerate	1494	1494	-	0.26	0.16	-	-
α-Amorphene	1495	1495	0.44	0.05	-	-	-
β-Bisabolene	1505	1505	-	-	1.99	2.07	-
α-Farnesene	1507	1507	0.05	0.36	-	-	-
Bicyclo [4.4.0] dec-1-ene, 2-isopropyl-5-methyl-9-methylene	1510	1510	-	-	0.03	-	-
Cadina-1(2), 4-diene	1512	1513	0.04	-	-	-	0.01

Geranyl isobutyrate	1517	1517	-	0.03	-	-	-
Δ-Cadinene	1520	1520	0.54	0.21	0.13	0.07	0.34
γ-Cadinene	1524	1524	0.21	0.20	0.08	-	-
Nerolidol	1531	1531	0.01	10.05	-	-	0.12
α-Cadinene	1536	1537	0.09	-	-	-	0.05
Caryophyllene oxide	1561	1561	0.31	0.13	0.74	0.28	0.19
Geranyl butyrate	1570	1570	0.09	-	0.81	0.21	0.05
Spathulenol	1582	1582	0.01	0.49	0.08	-	-
γ-Eudesmol	1630	1631	0.14	-	-	-	-
α-Cadinol	1653	1653	-	0.12	0.04	-	-
Cadalene	1674	1674	0.01	-	-	-	-
2-Pentadecanone-6,10,14-trimethyl	1848	1848	-	0.01	-	-	-
Monoterpene hydrocarbons			9.24	3.60	0.57	1.79	17.69
Oxygenated monoterpenes			4.95	69.07	83.87	81.59	8.41
Sesquiterpene hydrocarbons			3.72	9.52	5.88	5.09	2.97
Oxygenated sesquiterpenes			0.47	10.79	0.86	0.28	0.31
Aromatic compounds			72.58	0.34	1.05	7.53	66.73
Non-terpenoid aliphates			0.85	0.60	2.76	2.44	0.05
Others			0.02	-	-	-	-
Total identified (%)			**91.78**	**93.92**	**94.99**	**98.72**	**96.16**
Yield (%)			**2.50**	**3.10**	**1.61**	**1.31**	**3.15**

Values are the mean of two different experiments and
SD values were ignored to diminish the complexity of table.

[a]Retention indices relative to C8–C20 n-alkanes calculated on HP-5MS capillary column; [b]Retention indices reported in literature.

T. calcareus EO



T. vulgaris EO

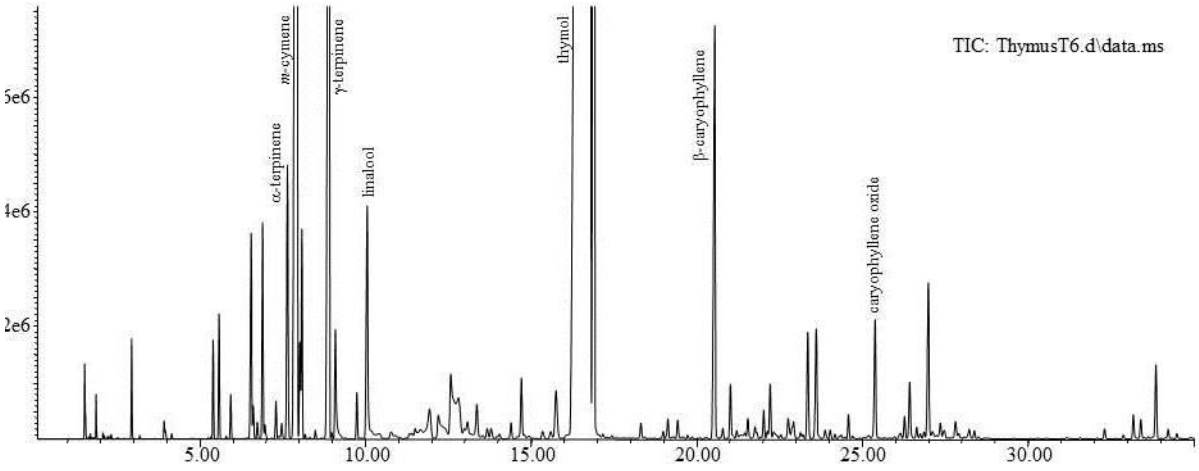


Figure 26. Gas-chromatograms of *Thymus* EOs

These differences are obviously due to other EOs constituents, most probably to cymene derivatives. Cymenes are aromatic compounds with well-known antioxidant effects [Youdim et al., 2002]. They were detected in higher levels in *T. vulgaris* EO (17.02% *vs.* 11.25% in *T. calcareus* EO) (Table 20). Among them, alike thymol (also known as *p*-cymen-3-ol), *o*-cymen-5-ol (5.14% in *T. vulgaris* EO *vs.* 2.93% in *T. calcareus* EO) (Table 20) has a phenolic hydroxyl group and two alkyl (methyl and isopropyl) groups but in *m*- and *p*-positions (Fig. 27) and these structural features enhance the antiradical activity [Ali et al., 2013].

The antioxidant activity of *T. vulgaris* EOs has been previously reported in literature [Viuda-Martos et al., 2011; Teixeira et al., 2013; Mancini et al., 2015] but due to the different experimental protocols, a comparison of our results with those reported in the aforementioned studies is not feasible.

T. citriodorus EO, containing 54.27% lavandulol, showed the weakest antioxidant effects (EC_{50}=34.28 and 0.52 mg/mL in DPPH and ABTS scavenging assays, respectively). Its ferric ion reduction capacity could not be assessed due to low activity (Table 21). Higher antioxidant activity was determined for the EOs isolated from the two cultivars, *T. vulgaris* 'Faustini' and *T. citriodorus* 'Aureus' containing geraniol as the predominant component (31.45% and 60.31%, respectively) (Table 20). The monoterpene alcohols geraniol and lavandulol are weaker antioxidants than thymol, geraniol being more potent than lavandulol [Ruberto and Baratta, 2000]. The higher antioxidant potency of geraniol compared to lavandulol is due to fact that geraniol may lose allylic hydrogens thus neutralizing free radicals (Fig. 27) [Wojtunik et al., 2014]. The stronger antioxidant effects exhibited by *T. citriodorus* 'Aureus' EO may be attributed, in large part, to its higher contents of geraniol (60.31% *vs.* 31.45% in *T. vulgaris* 'Faustini' EO) and aromatic compounds (7.54% *vs.* 0.35% in *T. vulgaris* 'Faustini' EO) (Table 20). Geraniol, an acyclic monoterpene alcohol, showed strong DPPH scavenging activity; in addition, it reduced the lipid peroxidation and generation of reactive oxygen species and increased the glutathione level and superoxide dismutase activity [Chen and Viljoen, 2010].

Cytotoxic activity

Since *T. vulgaris* and *T. calcareus* EOs exhibited the most potent antioxidant effects, they were selected for further cell-based studies (cytotoxicity and antigenotoxicity/genotoxicity assays). V79 cells represent a well-established cell model for the study of DNA damage and it is appropriate for the assessment of EOs effects due to the that the inhalation is one of the main routes of their administration in humans. *T. vulgaris* EO (25-200 μg/mL) did not alter the viability of V79 cells. An important reduction in cell viability (over 20%) has been recorded for the concentration of 300 μg/mL.

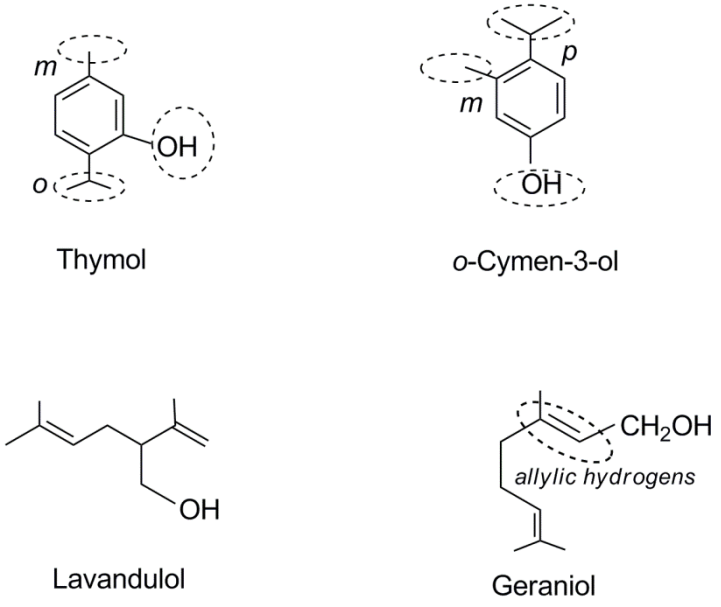


Figure 27. Major components of *Thymus* species EOs

Table 21. *In vitro* antioxidant activities of *Thymus* species EOs [Aprotosoaie et al., 2019a]

EO/Positive control	**EC_{50} (mg/mL)**		
	DPPH radical scavenging assay	**ABTS radial cation scavenging assay**	**Reducing power assay**
T. vulgaris	0.147 ± 0.004	0.003 ± 0.000	0.041 ± 0.000
T. vulgaris 'Faustini'	18.607 ± 0.536	0.371 ± 0.007	nd
T. citriodorus	34.281 ± 0.653	0.522 ± 0,011	nd
T. citriodorus 'Aureus'	1.565 ± 0.045	0.014 ± 0.001	3.057 ± 0.151
T. calcareus	0.369 ± 0.007	0.003 ± 0.000	0.051 ± 0.000
BHA	6.126 ± 0.014*	1.524 ± 0.008*	3.991 ± 0.032*

nd, not determined due to low activity; *, μg/mL; the results are expressed as mean ± SD from three determinations. EC_{50} values (mg/mL or μg/mL) were obtained by linear interpolation between values above and below 50% activity. In reducing power assay, EC_{50} values were the concentrations that lead to an absorbance of 0.5.

Cytotoxic activity

The treatments with medium and high concentrations of *T. calcareus* EO (50-300 μg/mL) produced a decline in the survival of V79 cells (16.69-27.86%). Only low concentration of *T. calcareus* EO (25 μg/mL) did not significantly alter the viability of V79 cells (9.05% reduction in cell viability) (Table 22).

Antigenotoxic/genotoxic activity

The capacity of *T. vulgaris* and *T. calcareus* EOs to provide protection against genomic instability induced by H_2O_2 was investigated by comet assay. Deleterious effects of H_2O_2 against genomic stability primarily result from the generation of highly reactive hydroxyl radicals *via* the iron-mediated Fenton reactions. Hydroxyl radicals can react directly with DNA leading to oxidative damage that involve DNA breaks, sugar base modification and DNA-protein cross linking [Aprotosoaie et al., 2015a].

The genoprotective effects of thyme EOs were examined at preventive (pretreatment 60 min with EOs prior to the exposition to H_2O_2) and interventional (posttreatment 60 min with EOs after exposition to genotoxicant) levels. Based on the results from MTT assay, we used both essential oils in concentration of 25 μg/mL.

Table 22. Viability of V79 cells after 24 h incubation with *Thymus* EOs [Aprotosoaie et al., 2019a]

Group	Cell viability (%)
Untreated V79 cells	100±0.00
TVEO 25	124.21±21.22
TVEO 50	113.41±13.47
TVEO 100	104.29±7.50
TVEO 200	106.42±16.85
TVEO 300	86.03±8.12
TCaEO 25	90.95±6.27
TCaEO 50	83.31±3.80**
TCaEO 100	79.25±7.19*
TCaEO 200	79.22±5.83*
TCaEO 300	72.14±5.93**

TVEO, *Thymus vulgaris* essential oil; TCaEO, *Thymus calcareus* essential oil; The results are expressed as mean ± standard error of mean of five independent experiments; significant data at *$p<0.05$ and **$p<0.01$ compared to untreated V79 cells (t-test).

As can be seen from the Fig. 28, exposition of V79 cells to H_2O_2 (50 μM, 30 min) induced a greater extent of migration of DNA into the comet tail in comparison with sham control (69.80±2.69 *vs*.14.49±1.43%). Pre- and post-treatments with *T. vulgaris* and *T. calcareus* EOs (25 μg/mL) protected against H_2O_2-induced genomic oxidative damage in V79 cells, *T. vulgaris* EO showed similar pronounced protective effects in both types of treatment (93.79±1.29% and 94.48±0.88% DNA in head; 6.21±1.29% and 5.52±0.88% DNA in tail) while post-treatment with *T. calcareus* EO induced a higher protection than the pre-treatment (92.74±4.32% DNA in head and 7.26±0.95% DNA in tail *vs.* 74.87±2.56% DNA in head and 25.13±2.53% DNA in tail) (Fig. 28, Fig. 29). Both EOs are devoid of genotoxicity at tested concentration.

Only few studies have investigated the antigenotoxic/genotoxic activities of *Thymus* species EOs.Thus, *T. vulgaris* EO had no mutagenic or DNA-damaging effects in the Ames test or *Bacillus subtilis* rec-Assay [Zani et al., 1991] while *T. kotschyanus* EOs containing carvacrol (5.3-54.2%) and thymol (8.1-28.1%) as main components, protected human normal lymphocytes against DNA-oxidative damage induced by H_2O_2 (100 μM, 30 min) [Afshari et al., 2016].

Thymol, the major component of *T. vulgaris* and *T. calcareus* EOs, provided protection in a dose-dependent manner against toxicity induced by different genotoxicants, such as: H_2O_2 [Horváthová et al., 2014], mitomycin C and imidazolquinoline [Aydin et al., 2005], gamma-radiation [Archana et al., 2011], or UVA and UVB radiations [Calò et al., 2015] in various mammalian cells.

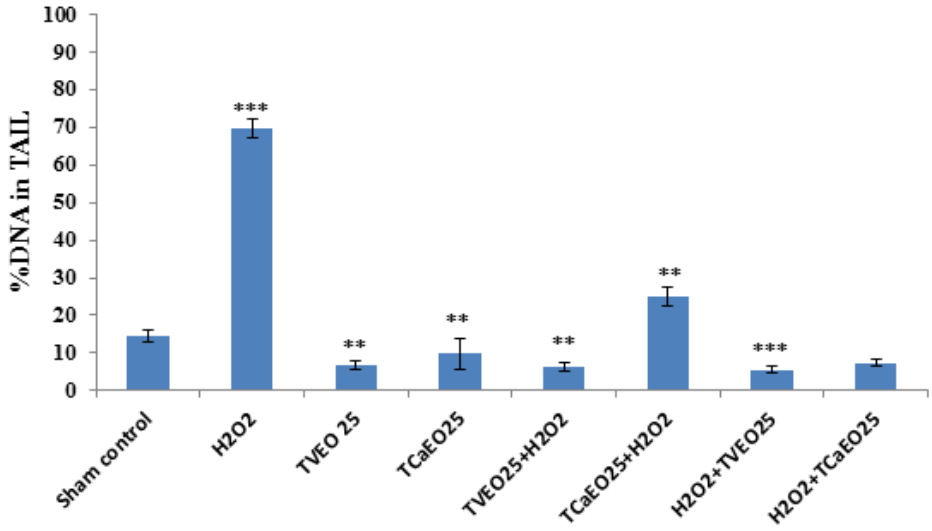


Figure 28. Tail DNA (%) in V79 cells after pre- and post-treatment with thyme EOs [Aprotosoaie et al., 2019a]

TVEO, *Thymus vulgaris* essential oil; TCaEO, *Thymus calcareus* essential oil. The results were expressed as mean±standard error of mean of five replicates. ***p<0.001 and **p<0.01 when comparing the effects of *Thymus* species EOs with H_2O_2-treated cells and H_2O_2-treated cells with sham control (t-test).

Aydin et al. (2005) reported that the thymol acts genoprotective at concentrations below 100 μM while the values above these levels produced DNA damaging effects in human normal lymphocytes. In addition, Ündeğer et al. (2009) showed the lack of clastogenic activity for concentrations up to 5 μM of thymol, whilst 25 μM of thymol induced DNA damage in V79 Chinese hamster lung fibroblast cells.

Similar effects have also been reported for other volatiles of thyme EOs such as carvacrol and γ-terpinene. Thus, they did not induce genotoxicity at concentrations lower than 50-100 μM in human normal lymphocytes [Aydin et al., 2005]. This dual behavior might be explained by the pro-oxidant effects of the volatiles, mainly phenolic ones, at high concentrations.

In our study, we recorded genoprotective activity but concentrations of thymol and γ-terpinene used to treat cells are lower than 1 μM. In the pre-treatment protocols, the beneficial effects of of *Thymus* EOs can be attributed to the antioxidant properties. As we already showed, *Thymus* EOs analyzed in our study have strong free radical scavenging abilities, mainly *T. vulgaris* EO. In the post-treatment protocols, the antigenotoxic activity of *Thymus* EOs can be ascribed to the stimulation of DNA repair process.

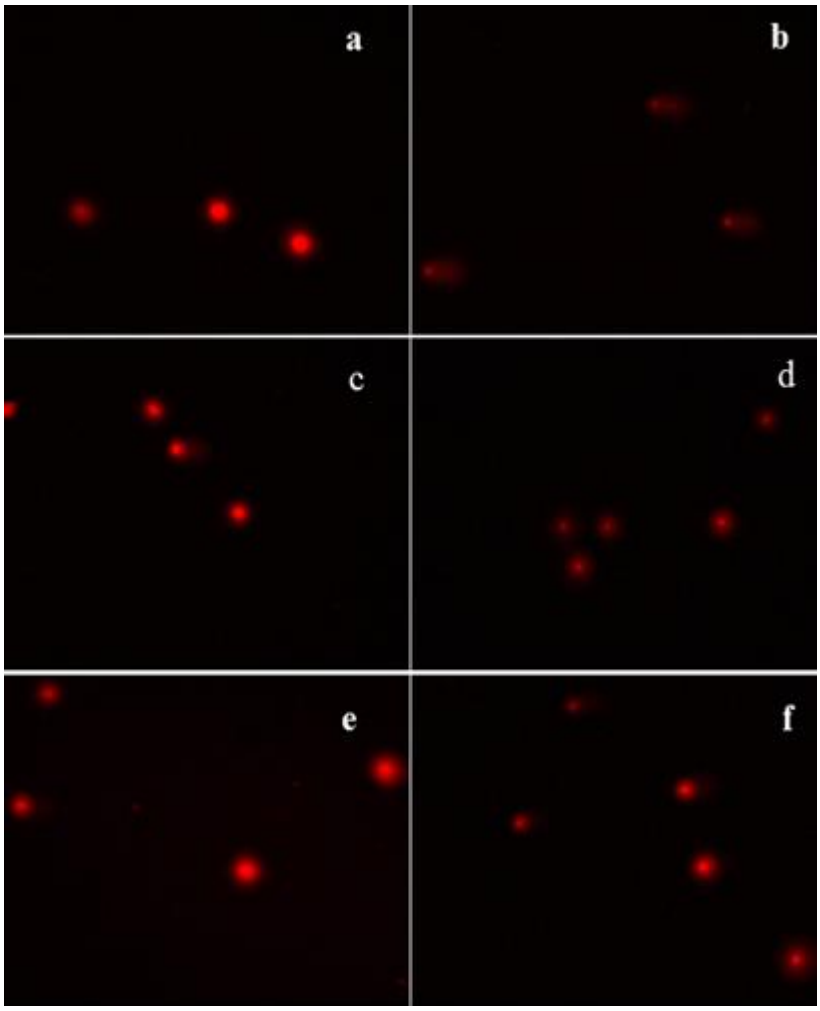


Figure 29. Photomicrographs of comet length in normal and treated V79 cells: a) normal; b) exposed to H_2O_2 (50 μM, 30 min); exposed to H_2O_2 (50 μM, 30 min) after 60 min pre-treatment with thyme EOs (25 μg/mL): c) *T. vulgaris* EO, d) *T. calcareus* EO; exposed to H_2O_2 (50 μM, 30 min) and 60 min subsequent treatment with thyme EOs (25 μg/mL): e) *T. vulgaris* EO, f) *T. calcareus* EO [Aprotosoaie et al., 2019a].

It has been reported that some monoterpenes such as camphor and eucalyptol which are common in thyme EOs, act as bioantimutagens, enhancing DNA repair mechanisms [Nikolić et al., 2011]. Besides, it has been suggested that thymol could decrease DNA damage induced by UVB irradiation by enhancement of nucleotide excision repair genes expression [Calò et al., 2015].

Conclusions

Moldavian thyme species are good sources of bioactive EOs with possible applications in the food and pharmaceutical industries (*Th. vulgaris*, *Th. calcareus*) and perfumery (*Th. citriodorus*, *Th. vulgaris* 'Faustini').

The chemical composition of *T. vulgaris* EO and *T. calcareus* EO is dominated by aromatic components; monoterpenoids are abundant in *T. citriodorus* EO, *T. citriodorus* 'Aureus' and *T.*

vulgaris 'Faustini' EO. Thymol predominates in *T. vulgaris* and *T. calcareus* EOs, lavandulol in *T. citriodorus* EO, while geraniol is the major compound in *T. citriodorus* 'Aureus' and *T. vulgaris* 'Faustini' EOs.

At doses that provided micromolar concentrations of thymol, both *T. vulgaris* and *T. calcareus* EOs were genoprotective at preventive and interventional levels against oxidative genomic damage in V79 cells involving antioxidant mechanisms but also possible stimulation of DNA repair processes.

Further research on *Thymus* EOs is needed for a better understanding of their antigenotoxic mechanisms and effects on genomic stability at chronic exposure.

I.2.1.4. HPLC-DAD-ESI-Q-TOF-MS/MS polyphenolic profile and antioxidant activity of *Mentha gattefossei* Maire

Current state of art and research objectives

Mint species are one of the most useful aromatic and medicinal plants with a significant economic value for food, pharmaceutical, flavor industries, cosmetics, perfumery, confectionery and alcoholic beverages. In therapeutics, mint plants are used mainly for their spasmolytic, carminative, cholagogue, expectorant, antiemetic and antidiarrheal properties [Stănescu et al., 2018].

Mentha gattefossei Maire (Menthe de Perse) is endemic to Morocco and it is used for medicinal purposes and as a food source and also for the extraction of essential oil [Fennane and Tattou, 2005]. This species is listed among the rarest plants in the world and it is mentioned in the IUCN (*World Conservation Union*) Red List of Threatened Plants as Near Threatened being a plant of global conservation interest [Bunsawat et al., 2004; Ciocârlan, 2014].

Despite the fact that the bioactivity of plants from genus *Mentha* is due to both essential oil and polyphenols, data on polyphenols or antioxidant potential of *M. gattefossei* species are lacking.

This study assessed for the first time the polyphenolic profile and antioxidant activity of an alcoholic extract obtained from aerial parts of *M. gattefossei* species cultivated in Republic of Moldova within *ex situ* conservation programmes at international level.

Material and methods

Plant material

The aerial parts of *Mentha gattefossei* were harvested during flowering stage in July 2017 from the experimental field of Botanical Garden, Academy of Sciences of Moldova, Chişinău, Republic of Moldova (Fig. 30).

Mentha gattefossei plants were obtained from seeds received by the international exchange with the Botanical Garden from Coimbra, Portugal.

Extraction

The powdered aerial parts were extracted with ethanol for 18 h and *Mentha gattefossei* ethanolic extract (MGE) was stored in a freezer at -18 °C until use.

Figure 30. *Mentha gattefossei* (Botanical Garden of Chişinău)

Determination of total phenolic content

The total phenolic content was determined with Folin-Ciocalteu reagent [Singleton and Rossi, 1965]. The results were expressed as gallic acid equivalents (mg gallic acid per gram of dried extract).

Qualitative HPLC-ESI-Q-TOF-MS/MS analysis of polyphenols

Qualitative HPLC-ESI-Q-TOF-MS/MS experiments were conducted on an Agilent 1200 HPLC system (Agilent Technologies, Santa Clara, CA, USA) equipped with an auto-sampler (G1329B), degasser (G1379B), binary pump (G1312C), column oven (G1316A), diode array detector (DAD, G1315D) and electrospray ionization-quantum-time of fligt-mass spectrometer (ESI-Q-TOF-MS, G6530B). A Phenomenex Gemini C18 (100 × 2 mm, 3 µm) column was used. The mobile phase consisted of 0.1% formic acid in water (A), 0.1% formic acid in acetonitrile (B). The elution profile started with 0% B and then it was linearly increased to 40% B in 30 min, with a flow-rate of 0.2 mL/min. ESI-Q-TOF-MS analysis was performed in positive ionization mode, using the following parameters: mass range 100-1700 m/z; gas temperature 300 °C; nitrogen flow 12 L/min; nebulizer pressure 40 psig; skimmer 65 V; capillary voltage 4000 V; fragmentor 140 V, fixed collision energy 10 V. Data were processed with Agilent MassHunter Qualitative Analysis Navigator B.08.00.

Quantitative HPLC-DAD analysis of polyphenols

Quantitative HPLC-DAD analysis was carried out on a Shimadzu HPLC system equipped with an automatic degasser (DGU-20A 3R), a quaternary pump (LC-20AD), an auto-sampler (SIL-20A HT) and a photodiode array detector (SPD-M20A). Separation was carried out on a Agilent Eclipse XDB-C18 (250 mm × 4.6 mm, 5 µm) column. The mobile phase consisted of 0.1% formic acid in water (A) and acetonitrile (B). The elution profile was: 10% B (0 min), 25% B (5 min), 40% B (10 min), 70% B (20 min) and 100% B (25–35 min). The flow rate was 1 mL/min, the column temperature was 25 ºC and the injection volume was 10 µL. The detection wavelength was set to 330 nm. Data were processed using a LabSolutions 5.51 software (Shimadzu). The concentrations of the identified compounds were determined from the peak

area, using the linear regression equations of the calibration curves obtained from six concentrations of rosmarinic acid (standard). The results were expressed as mg/g extract.

In vitro antioxidant assays

DPPH radical scavenging assay

DPPH radical scavenging activity was determined by the method described by Malterud et al. (1993). Gallic acid was used as positive control.

ABTS radical cation scavenging assay

The ability of MGE to scavenge ABTS radical cation was evaluated as described by Berker et al. (2007). (+)-Catechin was used as positive control.

Reducing power assay

The assay was carried out following the method described by Re et al. (1999). (+)-Catechin was used as positive control.

Assessment of ferrous ion chelating activity

The iron-chelating capacity of MGE was estimated by the method of Dinis et al. (1994). Gallic acid was used as positive control.

EC_{50} values (μg/mL) were calculated from linear interpolation between values above and below 50% activity. In reducing power assay, EC_{50} values were the concentrations giving an absorbance of 0.5 (Ferreira et al., 2007).

Results and discussion

The total phenol content was 72.38±0.61 mg of GAE/g of dry extract.

The base peak chromatogram (Fig. 31) of the HPLC-ESI-Q-TOF-MS/MS metabolite profiling of MGE revealed the presence of 12 known constituents, such as organic and phenolic acids (citric acid, caffeic acid hexoside, hydroxybenzoic acid hexoside, hydroxybenzoic acid, chlorogenic acid, rosmarinic acid, danshensu, salvianolic acid C) and flavonoids (diosmin, isoquercitrin, acacetin rutinoside) (Table 23). Rosmarinic acid (RA) was by far the major phenolic compound (Fig. 32), its content reaching 12.42±0.23 mg/g extract.

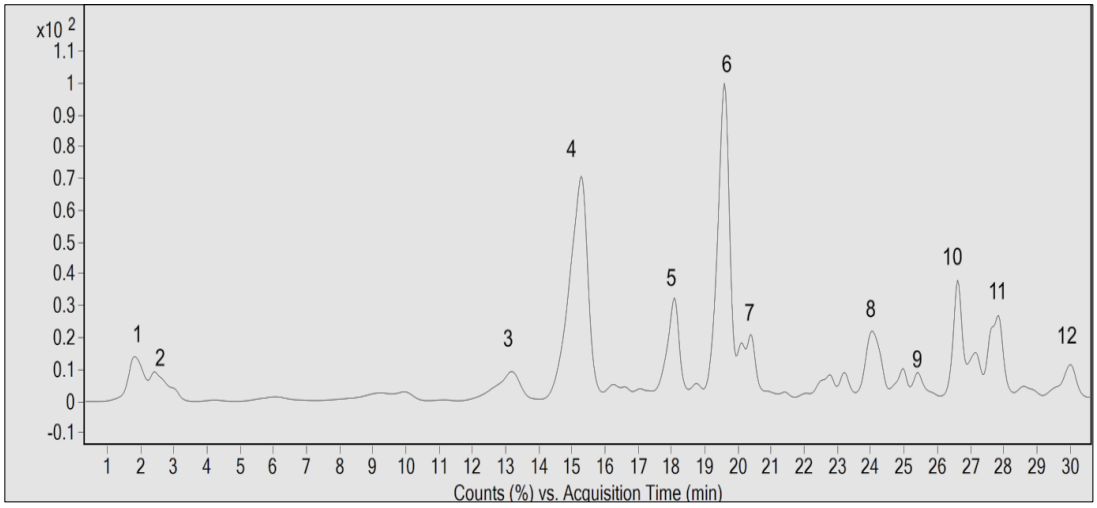


Figure 31. Base peak chromatogram of MGE

Table 23. HPLC-ESI-Q-TOF-MS/MS analysis of *Mentha gattefossei* extract

No.	TR (min)	Identity	MF	[M-H]⁻ (*m/z*)	ESI-MS/MS (*m/z*)
1	1.8	Citric acid	$C_6H_7O_7$	191	111
2	2.5	Caffeic acid hexoside	$C_{15}H_{18}O_9$	341	179, 161, 135
3	13.2	Hydroxybenzoic acid hexoside	$C_{13}H_{16}O_8$	299	179, 137
4	15.3	Hydroxybenzoic acid	$C_7H_6O_3$	137	119
5	18.1	Medioresinol	$C_{21}H_{24}O_7$	387	369, 207, 163, 119
6	19.6	Danshensu	$C_9H_{10}O_5$	197	179, 153, 135
7	20.4	Chlorogenic acid	$C_{16}H_{18}O_9$	353	179, 173, 161, 135
8	24.1	Isoquercitrin	$C_{21}H_{19}O_{12}$	463	301, 271, 245, 151
9	25.3	Diosmin	$C_{28}H_{32}O_{15}$	607	299, 284
10	26.6	Rosmarinic acid*	$C_{18}H_{16}O_8$	359	223, 197, 179, 161, 135
11	27.9	Acacetin rutinoside	$C_{28}H_{32}O_{14}$	591	445, 283
12	30.0	Salvianolic acid C	$C_{26}H_{20}O_{10}$	491	311, 135

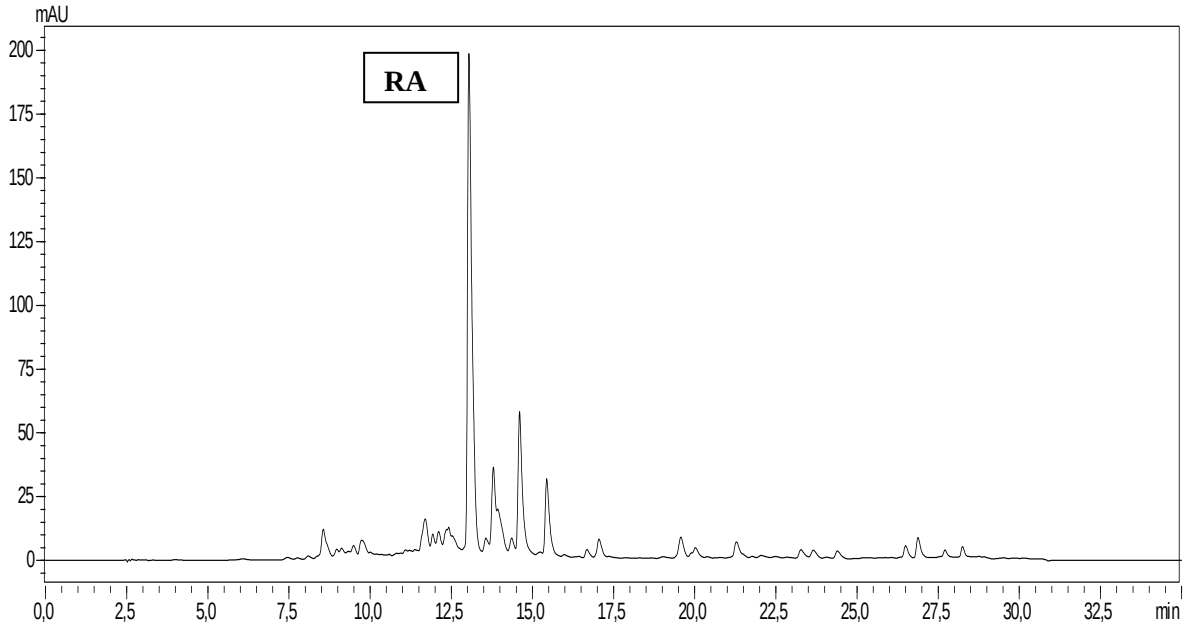


Figure 32. HPLC-DAD chromatogram of MGE (330 nm)

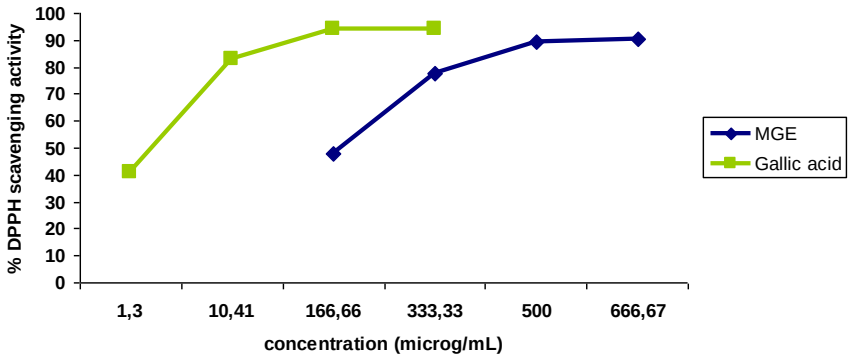
The MGE extract showed a concentration-dependent antioxidant activity. The DPPH scavenging activity of MGE increased dose-dependently from 47.74±0.20% at 166.66 µg/mL to 90.48±0.33% at 666.67 µg/mL (Fig. 33 A).

ABTS scavenging activity of MGE ranged from 48.49±0.33% at 50 µg/mL to 93.77±0.07% at 200 µg/mL after 6 min reaction time (Fig. 33 B). At 833.33 µg/mL, the extract chelated ferrous ions by 76.23±0.28% (Fig. 33 C).

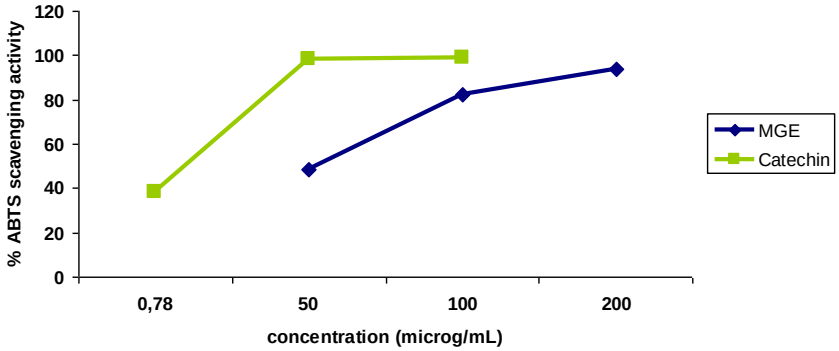
The reducing power of MGE reached maximum value (absorbance 1.40±0.00 at 700 nm) at concentration of 66.84 µg/mL (Table 24). It was found to be more active as reducing agent (EC_{50}=19.56±0.00 µg/mL) and scavenger of ABTS (EC_{50}=51.56±0.35 µg/mL) and DPPH radicals (EC_{50}=105.4±0.5 µg/mL) than ferrous ion chelating agent (EC_{50}=217.96±3.6 µg/mL) (Table 24).

According to the EC_{50} values (Table 24), MGE was less active than controls (gallic acid or catechin).

A.



B.



C.

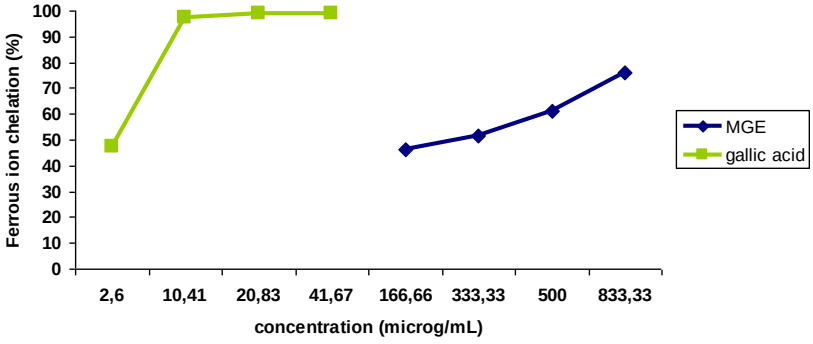


Figure 33. Antioxidant activity of MGE: (A) DPPH radical scavenging activity; (B) ABTS radical cation scavenging activity; (C) ferrous ion chelating capacity

Table 24. The antioxidant activity of MGE and controls

Antioxidant assay	**EC_{50} (µg/mL)**	
	MGE	**Control (gallic acid[a] /catechin[b])**
DPPH radical scavenging activity	105.4±0.50	1.90±0.00[a]
ABTS radical scavenging activity	51.56±0.35	1.80±0.00[b]
Ferrous ion chelating activity	217.96±3.6	2.80±0.00[a]
Ferric reducing power	19.56±0.00	3.61±0.00[b]

Conclusions

This study provided an insight on polyphenols from *Mentha gattefossei* and their bioactivity. *Mentha gattefossei* is a potential source of rosmarinic acid and other antioxidant polyphenols which act mainly as reducing agents and free radical scavengers.

I.2.1.5. Assessment of antioxidant activity of some essential oils of Lamiaceae species

Current state of art and research objectives

The essential oils (EOs) are vegetal metabolites with a wide range of biological properties and uses. They are gaining increasing interest in medicine, food and cosmetic industry. There is an ongoing effort to screen medicinal plants as sources of EOs with significant antioxidant and antimicrobial properties.

Many health benefits (antiaging, chemopreventive, anticancer, neuroprotective, anti-inflammatory) of essential oils are based on the antioxidant effects. Besides, the replacement of synthetic compounds with natural, non-harmful antioxidants is of great importance in the development of new food preservatives. There are many reports about the antioxidant activity of EOs. They are able to scavenge free radicals and to inhibit lipid peroxidation.

Antioxidant properties of EOs is significantly related to their chemical composition. In this respect, terpenoid phenolics (thymol, carvacrol, eugenol) and oxygenated monoterpenoids are the most active antioxidant volatiles. In addition, antioxidant properties of EOs are greatly influenced by species, harvesting time and method extraction [Tongnuanchan and Benjakul, 2014].

The species belonging to Lamiaceae family are important sources of valuable essential oils from both a sensory and biological point of view.

As a part of three research projects (61-39/2007; 21/2005; 1639/2013) or independent research, we investigated antioxidant properties of essential oils from some Lamiaceae species, such as: *Origanum vulgare*, *Origanum majorana*, *Ocimum basilicum*, *Satureja hortensis*, *Satureja montana*, *Thymus vulgaris*, *Lavandula angustifolia*, *Melissa officinalis*, *Dracocephalum moldavica*, *Hyssopus officinalis and Mentha gattefossei*, related to the development of antiaging, radioprotective phytopreparations or products with beneficial effects in orthomolecular therapy [Trifan et al., 2014; Trifan et al., 2015; Aprotosoaie et al., 2018b]

The results of studies on *Satureja montana* and *Mentha gattefossei* EOs are presented below.

Satureja montana L. commonly known as mountain savory or winter savory, is an aromatic, perennial semishrub native to the Mediterranean area. Winter savory is used as medicinal plant, spice, and is one of the best honey plants [Vidic et al., 2009].

The whole plant has antiseptic, stomachic, carminative and expectorant properties [Wesolowska et al., 2014]. The bioactivity and the aromatic profile of *S. montana* are significantly influenced by the chemical composition of its essential oil (EO).

Depending mostly on geographic area and its stages of development, the occurrence of various *S. montana* EO chemotypes was reported [Miloš et al., 2001; Chizzola, 2003]. The prevailing carvacrol/thymol chemotypes determine a strong spicy flavor of the plants. They exhibit a significant antioxidant potential while the linalool or geraniol chemotypes have a lower activity [Ćavar et al., 2008].

Despite the fact that *S. montana* is an important medicinal and aromatic plant in Romania, there is little information on chemical composition and antioxidant activity of EO from the Romanian species.

Many of the biological effects as well as mint specific sensory qualities are chiefly bounded to the composition of essential oil. There is reported for the first time the chemical composition and the antioxidant activities of the essential oil isolated from *Mentha gattefossei* species cultivated in Republic of Moldova.

Material and methods

Chemicals

All chemicals were of analytical grade and were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany) and Merck (Darmstadt, Germany).

Plant material

The aerial parts of *S. montana* were harvested during flowering stage in July 2013 from the cultivated field of Stejarul Biological Research Center, Piatra Neamț, a location in Northeastern Romania.

Data on the *Mentha gattefossei* plant material are described in the section I.2.1.4.

Essential oil isolation

100 g of air-dried aerial parts of plants were ground and mixed with 1500 mL of doubly distilled water and subjected to hydrodistillation for 3 h in a modified Clevenger-type apparatus. The obtained essential oils were dried over anhydrous sodium sulphate and stored in a sealed dark glass at -4 °C until it was analyzed. The extraction yield of EOs (v/w %) was calculated on a dry weight basis.

GC and GC-MS analysis

The GC-FID analysis was performed using an Agilent 6890 gas chromatograph equipped with a flame ionization detector and a HP-5MS capillary column (30 m × 0.25 mm internal diameter, 0.25 μm film thickness). A volume of 0.2 μL of EOs was injected in the split mode (split ratio 1:50). Helium was used as carrier gas at a flow rate of 1 mL/min for *Satureja montana* EO (SmEO) and 1.8 mL/min for *Mentha gattefossei* EO (MgEO), respectively. The analysis was performed using the following temperature programs:

- for SmEO: 4 °C/min from 40 °C to 250 °C, 10 °C/min from 250 °C to 300 °C; the final temperature was held for 7.5 min;

- for MgEO: 4 °C/min from 60 °C to 220 °C, 20 °C/min from 220 °C to 320 °C.

The GC-MS analysis of the oils was carried out on an Agilent type 7890A gas cromatograph, equipped with an Agilent 5975C mass spectrometer selective detector with electron impact ionization. The column and GC-MS conditions were the same as the ones described above. Only for the MgEO, the helium flow rate was 0.5 mL/min and the split ratio was 20:1. Mass spectra were acquired in the scan mode (mass range 15–450 *m*/*z*) [Aprotosoaie et al., 2010a].

Volatile compounds identifications

The retention indices of components from SmEO were determined by analysing a standard solution of *n*-alkanes (C_8–C_{20}) under the same chromatographic conditions. The compounds were identified by comparison of their recorded mass spectral data with those stored in the Wiley 275 mass spectral library from GC-MS database and their retention indices relative to *n*-alkanes with those mentioned in literature. The components of MgEO were identified by comparison of their Kovats Index (KI) relative to standard solution of C8-C23 n-alkanes under the same chromatographic conditions, and by matching their mass spectral data with those *National Institute of Standards and Technology* (NIST) library. The relative percentages of the essential oil constituents were obtained from the FID peak areas without using correction factors [Michaelakis et al., 2007; Ćavar et al., 2008].

In vitro antioxidant assays

Butylhydroxianisole (BHA) was used as positive control. All spectrophotometric measurements were performed on ABLE-JASCO V 550 UV-VIS spectrophotometer (Jasco, Tokyo, Japan).

DPPH radical scavenging assay

DPPH radical scavenging activity was assessed according to the method described by Mighri et al. (2010). A volume of 1 mL of each EOs dilution in methanol was mixed with 1 mL of DPPH methanolic solution (0.04%, w/v). The mixtures were vortexed and kept for 30 min at room temperature, in the dark. Then, the absorbance was measured at 517 nm. The methanolic dilutions of SmEO with concentrations ranging from 290 μg/mL to 4640 μg/mL were used. Also, MgEO was used as dilutions with concentrations ranging from 5 μg/mL to 80 μg/mL. The percentage of DPPH radical scavenging was calculated using the equation: $100\times[(A_0-A_t)/A_0]$, where A_0 is the absorbance of the blank and A_t is the absorbance in the presence of EOs or positive control.

ABTS radical cation scavenging assay

The assay was performed as previously described by Re et al. (1999). ABTS radical cation ($ABTS^{\cdot+}$) was generated by mixing together equal volumes of ABTS aqueous solution (7 mM) with potassium persulfate (2.45 mM). The mixture was left in the dark at room temperature for 12-16 h before use. Prior to testing, $ABTS^{\cdot+}$ solution was diluted with methanol to an absorbance of 0.7±0.02 at 734 nm. 0.02 ml of each methanolic dilution of EOs (concentration ranging from 29 mg/mL to 464 mg/mL for SmEO and from 5 mg/mL to 80 mg/mL for MgEO, respectively) were mixed with 1.98 mL $ABTS^{\cdot+}$ solution. The absorbance at 734 nm was measured after 6 min of reaction. $ABTS^{\cdot+}$ scavenging activity (%) was calculated using the formula: $100\times[(A_{control}-A_{sample})/A_{control}]$, where $A_{control}$ is the absorbance of the control and A_{sample} is the absorbance in the presence of EOs/positive control.

Reducing Power Assay

The assay was carried out using Oyaizu method [Ferreira et al., 2007]. 500 μL of each EOs methanolic dilution (0.52-4.16 mg/mL for SmEO and 0.6336-10.1384 μg/mL for MgEO, respectively) were mixed with 0.2 M sodium phosphate buffer (1.2 mL; pH 6.6) and 1% potassium ferricyanide (1.5 mL). The mixture was incubated at 50 °C for 20 min. After cooling, 10% trichloroacetic acid (1.25 mL) was added to the mixture which was then centrifuged for 10 min at 3000 rpm. The upper layer of the solution (1.25 mL) was mixed with ultrapure water and with 0.1% (w/v) ferric chloride (0.25 mL). After 10 min, the absorbance was measured at 700 nm. An increased absorbance of the mixture indicates a good reducing power.

Statistical analysis

All antioxidants assays were performed in triplicate and the results are expressed as means±SD.

Results and discussion

A. Chemical composition and antioxidant activity of SmEO

The hydrodistillation of *Satureja montana* aerial parts gave a dark yellow oil with warm, peppery flavor. The yield of SmEO was 1.98% (19.8 mL/kg dried aerial parts). Twenty one compounds accounting for 90.79% of the total oil composition were identified (Table 25). The main components were carvacrol (63.40%), *para*-cymene (10.97%) and γ-terpinene (3.70%).

Aromatic compounds were predominant (76.55%) followed by monoterpenes (hydrocarbons and oxygenated derivatives) (8.75%).

A literature survey reveals that the essential oil content in *Satureja montana* plants varies depending on the geographic region. Winter savory plants from Albania contain from 0.22 to 1.60% essential oil while those from Montenegro contain between 1.1 and 1.9%. For the Croatian varieties, the essential oil content ranged from 1.2 to 2.2%. Also, for Polish *Satureja montana* there has been reported a content of 2.27%. French winter savory contains 1.56% essential oil while the plants harvested in Italy contain only 0.59% [Wesolowska et al., 2014]. In our study, the content of *Satureja montana* EO was higher compared to most cited data.

The chemical composition of EOs from *Satureja montana* plants shows an important variability according to various factors (climate, age of plants, vegetative cycle stage, location) [Ćavar et al., 2008]. Depending on the prevalence of phenols or monoterpene alcohols two main chemotypes can be described, namely A and B, each of them including several subtypes such as A (thymol), A (carvacrol), B (linalool), B (geraniol) [Prieto et al., 2007]. Due to high levels of phenols (mainly carvacrol), Romanian *Satureja montana* EO belongs to chemotype A (carvacrol) as well as EOs from former Yugoslavia, Italy [Fraternale et al., 2007; Hassanein et al., 2014] or Portugal [Serrano et al., 2011].

The antioxidant activity of *Satureja montana* EO was assessed by free radicals (DPPH and $ABTS^{\cdot+}$) scavenging assays and also by reducing power assay.

Satureja montana EO exhibited a concentration-dependent antioxidant activity. It possessed a high reducing capacity, and it was more effective as DPPH free radical scavenger than as $ABTS^{\cdot+}$ inhibitor (Table 25). At the concentration of 2320 μg/mL, *Satureja montana* EO caused a 90% inhibition of the DPPH radical. A similar inhibition was noticed in $ABTS^{\cdot+}$ assay for the concentration of 4640 μg/mL (Fig. 34).

The maximum reduction of ferric complex (absorbance 0.89±0.00 μg/mL at 700 nm) was obtained for a concentration of 225.26 μg/mL.

Table 25. Chemical composition of SmEO [Trifan et al., 2015]

RI[a]	**RI[b]**	**Compound**	**%**
923	923	α-thujene	0.23
930	932	α-pinene	0.67
987	990	β-myrcene	0.67
1024	1024	***para*-cymene**	**10.97**
1027	1027	limonene	0.12
1056	1058	**γ-terpinene**	**3.70**
1097	1099	linalool	0.82
1175	1177	terpinen-4-ol	1.03
1232	1235	thymol methylether	1.57
1242	1243	carvone	0.66
1257	1258	geraniol	0.85
1290	1286	thymol	0.61
1307	1306	**carvacrol**	**63.40**
1383	1384	β-bourbonene	1.43
1438	1441	aromadendrene	1.43
1450	1450	α-humulene	0.30
1478	1478	germacrene D	0.35
1506	1508	β-bisabolene	0.48
1511	1513	γ-cadinene	1.01
1520	1524	δ-cadinene	0.23
1580	1581	caryophyllene oxide	0.26
Group of constituents (%)			
Monoterpene hydrocarbons		5.39	
Oxygenated monoterpenes		3.36	
Aromatic compounds		76.55	
Sesquiterpenes		5.49	
Total identified (%)		90.79	

[a]Retention indices relative to C8–C20 n-alkanes calculated on HP-5MS capillary column; [b]Retention indices reported in literature.

Table 26. Antioxidant activity of SmEO [Trifan et al., 2015]

Essential oil/positive control	EC_{50} (μg/mL)		
	DPPH assay	**$ABTS^{\cdot+}$ assay**	**Reducing power assay**
SmEO	243.80±4.93	560.86±23.15	64.35±0.00
BHA	3.6±0.07	1.81±0.05	4.32±0.04

Although, SmEO was less active than the positive control (BHA), it showed better radical scavenging activity as compared to the data reported in literature.

For the essential oils from Bosnia and Herzegovina, Ćavar et al. (2008) have reported the EC_{50} values of 5490±260 μg/mL and 18900±190 μg/mL, respectively in the DPPH assay.

In the same assay, Serrano et al. (2011) found an EC_{50} of 508.45±5.8 μg/mL for the Portuguese EO while Miladi et al. (2013) reported an EC_{50} of 410.5±4.27 μg/mL for French winter savory EO.

The antioxidant potential of Romanian winter savory essential oil is due to the high content of phenolic constituents (76.55%) and also to the low levels of sesquiterpenes, compounds considered to be weak antioxidants [Ćavar et al., 2008].

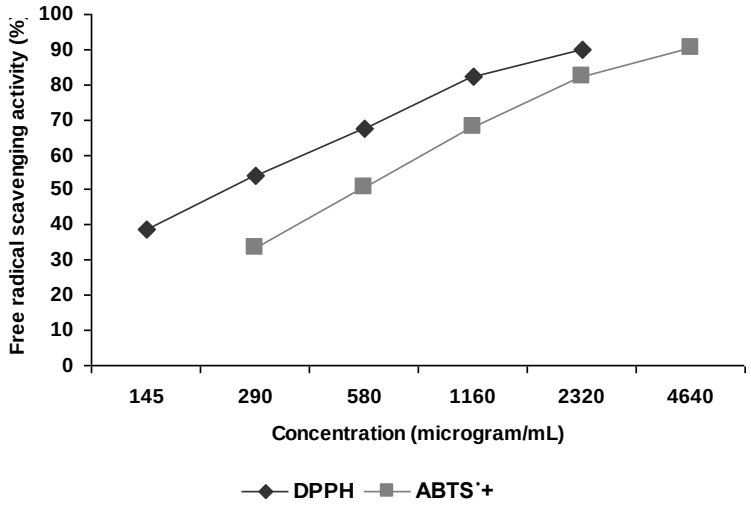


Figure 34. Free radical scavenging activity of SmEO [Trifan et al., 2015]

B. Chemical composition and antioxidant activity of MgEO

The hydrodistillation of dried aerial parts of *Mentha gattefossei* gave a green oil with strong minty and herbaceous flavor (extraction yield of MgEO was 2.5 %).

Twenty-three compounds were identified representing about 90.87% of total oil composition (Table 27).

The major components were pulegone (57.36%), neomenthone (28.74%) and D-limonene (1.20%). Oxygenated monoterpenes constitute the dominant fraction of MgEO (87.03%) followed by monoterpene hydrocarbons (2.73%).

The monoterpene-ketones prevail in the composition of oxygenated monoterpenes (86.53% from 87.03%).

To the best of our knowledge, only one study reported data about the chemical composition of *Mentha gattefossei* essential oil identifying pulegone (56.9%), menthone (30%) and piperitenone (3.4%) as the main compounds [Fujita and Moriyoshi, 2001].

Our results are in agreement with those previously reported. Also, pulegone was detected as major component in the essential oils of *Mentha pulegium* (76-78%), *M. canadensis* (1.5-81.5%), *M. cervina* (31.7-60.8%), or *M. arvensis* (54.6%) while menthone is one of the main components of *M.piperita* essential oil (8.1-31.6%) [Lawrence, 2010; Kapp, 2015].

Among *Mentha* species, *Mentha gattefossei* is closely related to *M. cervina* as suggests the evidence of chloroplast DNA sequences for assessing the phylogenetic relationships in *Mentha* genus [Ciocârlan, 2014].

DPPH scavenging activity of MgEO increased dose-dependently from 36.88±0.81% at 2.5 μg/mL to 81.51±0.63 at 40 μg/mL (Fig. 35A). Also, ABTS scavenging activity of MgEO ranged from 13.40±0.33% at 50 μg/mL to 84.56±0.11% at 800 μg/mL after 6 min reaction time (Fig. 35B).

The positive control, BHA almost completely scavenged the DPPH radical (94.96±0.35%) at 10 μg/mL and it exhibited 100% $ABTS^{\cdot+}$ scavenging effects at 200 μg/mL (Fig. 35).

MgEO was more effective as DPPH free radical scavenger than as $ABTS^{\cdot+}$ inhibitor (Table 28). Also, MgEO was similarly effective as BHA in terms of DPPH scavenging activity (EC_{50}=3.64±0.07 *vs*. EC_{50}=3.30±0.15) (Table 28).

Regarding the reducing power assay, MgEO showed a weak reducing capacity.

Thus, EC_{50} in this assay could not be calculated because MgEO showed an absorbance value of 0.2 at a concentration of 0.4848 μg/mL, and the increase of tested concentration was not possible due to low solubility of the oil in the reaction medium.

Table 27. Chemical composition of MgEO [Aprotosoaie et al., 2018b]

Compound	**RI[a]**	**%**
α-Pinene	939	0.52
Camphene	952	0.03
β-Pinene	980	0.85
β-Myrcene	990	0.08
Octanal	1002	0.05
Pseudolimonene	1018	0.01
p-Cymene	1024	0.08
D-Limonene	1027	**1.20**
β-Phellandrene	1030	0.03

Eucalyptol	1033	0.33
γ-Terpinene	1062	0.01
α-Campholenal	1127	0.02
Hexenyl isobutanoate	1142	0.41
Pinocarveol	1146	0.11
Neomenthone	1158	**28.74**
Myrtenol	1184	0.04
Pulegone	1236	**57.36**
Piperitone	1251	0.43
o-Cymen-5-ol	1334	0.03
Phenylbutyrate	1245	0.05
Mint furanone	1291	0.01
Hydroxyacetophenone	1447	0.06
Caryophyllene oxide	1578	0.42
Group of constituents (%)		
Monoterpene hydrocarbons	2.73	
Oxygenated monoterpenes	87.03	
Sesquiterpenes	0.42	
Aromatic compounds	0.22	
Non-terpenoid aliphates	0.47	
Total identified (%)	**90.87**	

[a]Retention indices relative to C8–C20 n-alkanes calculated on HP-5MS capillary column

The antioxidant activity of mint essential oils was largely investigated but this is the first study on the antioxidant properties of MgEO.

Although a direct comparison can not be achieved, we must say, however, that the MgEO showed more efficient free radical scavenging properties than other *Mentha* essential oils with high concentrations of monoterpene ketones such as pulegone and menthone.

Thus, Kamkar et al. (2010) reported for the essential oil from Iranian *Mentha pulegium* (with 40.5% pulegone and 35.4% menthone) an EC_{50} value of 14736 μg/mL in DPPH assay.

In our study, EC_{50} of MgEO in DPPH assay was 3.64 μg/mL.

Table 28. Antioxidant activity of MgEO [Aprotosoaie et al., 2018b]

Essential oil/ positive control	EC_{50} (µg/mL)		
	DPPH assay	$ABTS^{\cdot+}$ assay	Reducing power assay
MgEO	3.64±0.07	254.12±8.40	-
BHA	3.30±0.15	1.9±0.10	4.26±0.10

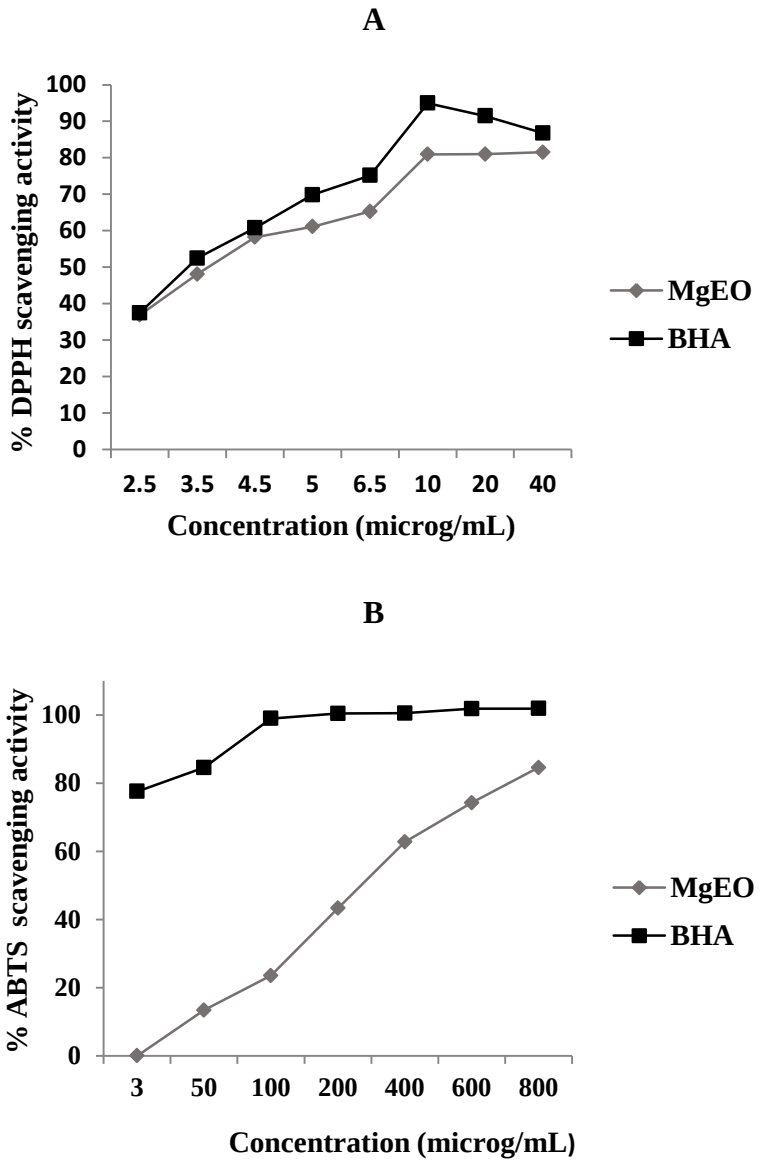


Figure 35. Free radial scavenging activity of MgEO: A) DPPH radical scavenging activity; B) ABTS radical cation scavenging activity; BHA, butylhydroxyanisole as positive control [Aprotosoaie et al., 2018b]

Conclusions

These findings suggest that the essential oil obtained from Romanian *Satureja montana* belongs to the phenolic chemotype (carvacrol). It exhibits a remarkable antioxidant activity with potential applicability in the protection of susceptible matrices from free radical-mediated oxidative stress, including ionizing radiation-induced oxidative damage as well as in food industry.

The essential oil of *Mentha gattefossei* plants cultivated in Republic of Moldova is a rich source of monoterpene-ketones as pulegone and neomenthone. These components recommend the essential oil for further research regarding its potential insecticidal and repellent properties. Further, it showed good free radical scavenging properties.

Conclusions about topic 2

Herbal extracts from Lamiaceae species or mushrooms species and their constituents, non-volatile or volatile molecules, especially of phenolic type, are important sources of antioxidant agents that support various therapeutic and non-therapeutic applications, especially in the food and cosmetics industries. Their superior antioxidant activity also explains the genoprotective potential highlighted in various experimental contexts regarding the bleomycin- or H_2O_2-induced oxidative DNA impairment. Natural products can act at a preventive or interventional level, also intervening on the mechanisms of DNA repair. The genoprotective action is dose dependent, low doses in the case of phenolic compounds being protective.

Future studies should focus on understanding the antigenotoxic mechanisms, testing against other genotoxic agents, including gamma radiation, and monitoring the action on chronic exposure.

I.2.2. Research achievements in the field of the assessment of antimicrobial activity

Aromatic plants have been used since ancient times for their preservative and medicinal properties, and to impart aroma and flavor to foods. These properties of aromatic plants are partially attributed to the essential oils. Essential oils (EOs) are secondary vegetal metabolites and complex blends of small volatile molecules (mainly terpenes) with a broad spectrum of valuable biological properties and multiple recognized uses in various areas (medicine, pharmaceutical, food, beverage, cosmetic and textile industries, perfumery and toiletries, crop protectans and veterinary medicine).

Their antimicrobial activities have generated impressive reports in the medical literature [Hammer and Carson, 2011]. EOs are active on a wide variety of microorganisms, including food-borne pathogens.They may act by multiple and different mechanisms at microbial cell level (alteration on cell selective permeability, destabilization of membranes, inhibition of cellular metabolism, alteration of cell walls, reduction of mitochondrial enzymes activity) [Murbach Teles Andrade et al., 2016; Chouhan et al., 2017]. Also, due to their multicomponent nature, EOs have a low potential for the development of microbial resistance.The emergence of multidrug resistance in common pathogens is a great challenge faced by clinicians and there is an urgent need to identify new antimicrobial agents and therapeutical strategies. In this respect, EOs can be valuable tools in the fight against microbial resistance.

Combinatorial strategies using EOs and antibiotics are one of the most promising alternatives in this direction. Food, pharmaceutical and cosmetic industries have shown a great interest in the antimicrobial properties of EOs. Due to toxicity of some synthetic food additives and the current restrictions imposed by competent authorities, the use of safe naturally occurring

antimicrobial agents has received importance as a trend in the replacement of synthetic preservatives.

Based on these considerations, a third research direction focused on the evaluation of the antimicrobial properties of essential oils obtained from indigenous and exotic aromatic plants from the Apiaceae and Lamiaceae families.

The research topic continues some aspects addressed in the doctoral thesis, but the subject is developed by diversifying the investigated plant species, the pathogens and investigating the effects of the combinations of essential oils/volatiles and antibiotics on the pathogens involved in lower respiratory tract infections. In addition, the antimicrobial study was also correlated with the analysis of chemical composition of the essential oils.

It is known that the antimicrobial activity of essential oils, as well as their biological and sensory qualities, are a result of chemical composition. But the chemical profile of essential oils varies widely depending on various factors, and this can also lead to changes in antimicrobial activity, in one way or another. Even modest changes in the concentrations of volatiles can lead to important variations of antimicrobial effects.

The knowledge of the chemical profile of the essential oils allows their better and more specific application. Beyond the economic impact, we can talk about a strategic capitalization of the local flora.

An important role in the development of this theme was played by the participation as an active member in the teams of three research projects obtained through national competition. Some of the important objectives of the respective projects were:

- assessment of antibacterial effects of essential oils isolated from leaves, flowers and roots of *Telekia speciosa* (Schreb.) Baumg. (project 61-39/2007);
- evaluation of antimicrobial potential of essential oils obtained from *Ocimum basilicum*, *O. citriodora*, *O. sanctum*, *Lavandula angustifolia* and *Origanum vulgare* plants (project 21/2005).

The most important contributions in this research direction materialized in 4 ISI papers (*Flavour and Fragrance Journal*, *Letters in Applied Microbiology*, *Farmacia*) and a BDI paper, as follows:

Aprotosoaie AC, Miron A, Ciocârlan N, Brebu M, Roşu C, Trifan A, et al. Essential oils of Moldavian *Thymus species*: chemical composition, antioxidant, anti-*Aspergillus* and antigenotoxic activities. *Flavour Fragr J* 2019, 34, 175-186 (FI^{2018}=1,337).

Grădinaru AC, Trifan A, Şpac A, Brebu M, Miron A, **Aprotosoaie AC.** Antibacterial activity of traditional spices against lower respiratory tract pathogens: combinatorial effects of *Trachyspermum ammi* essential oil with conventional antibiotics. *Lett Appl Microbiol* 2018, 67, 449-457 (FI^{2018}=1,805).

Aprotosoaie AC, Ciocârlan N, Brebu M, Trifan A, Grădinaru AC, Miron A. Chemical composition, antioxidant and antimicrobial activities of *Mentha gattefossei* Maire essential oil. *Farmacia* 2018, 66 (5), 778-782 (FI^{2018}=1,527).

Grădinaru AC, **Aprotosoaie AC**, Trifan A, Şpac A, Brebu M, Miron A. Interactions between Cardamom essential oil and conventional antibiotics against *Staphylococcus aureus* clinical isolates. *Farmacia* 2014, 62 (6), 1214-1222 (FI^{2014}=1,005).

Aprotosoaie AC, Hăncianu M, Poiată A, Tuchiluş C, Şpac A, Cioancă O, Gille E, Stănescu U. *In vitro* antimicrobial activity and chemical composition of the essential oil of *Foeniculum vulgare* Mill. *Rev. Med. Chir. Soc. Med. Nat. Iaşi* 2008, 112 (3), 832-836.

The research on this topic was done through close collaboration with colleagues from Discipline of Microbiology, University of Medicine and Pharmacy Grigore T.Popa Iasi, Microbiology Laboratory of the Lung Hospital, Iasi and Institute of Biology Iasi.

1.2.2.1. Antimicrobial activity of essential oils from some Apiaceae and Lamiaceae species

Current state of art and research objectives

Apiaceae and Lamiaceae species are aromatic plants containing valuable essential oils. They are commonly used as food, nutraceuticals, cosmeceuticals and for flavoring and medical purposes. Besides, many Apiaceae and Lamiaceae plants are good sources for potent antimicrobial phytochemicals such as thymol, carvacrol, limonene, carvone, cuminaldehyde, linalool or estragole. Previous investigations showed that the essential oils of *Thymus*, *Origanum*, *Satureja* or *Mentha* plants from Lamiaceae family or coriander, cumin, ajowan and caraway plants from Apiaceae family exhibited significant antimicrobial activities [Nikolić et al., 2014; Khalil et al., 2018].

As part of our continuous search for improving the knowledge about the chemical profile of aromatic plants (mostly from Romanian flora) and their biological effects, we investigated the antimicrobial activity of fennel [Aprotosoaie et al., 2008b], lemonbalm [Poiata et al., 2007; Hăncianu et al., 2008], lavender [Dănilă et al., 2008], oregano, thyme or mint plants [Poiată et al., 2006; Poiată et al., 2008]. There we presented the study on the fennel plants (*Foeniculum vulgare* subsp. *vulgare*) harvested from submontaneous areas in Eastern of Romania, antimicrobial activity of *Mentha gattefossei* essential oil obtained from plants growning in Republica of Moldova and anti-*Aspergillus* activity of Moldavian *Thymus sp.* essential oils.

General data on *Foeniculum vulgare*, *Mentha gattefossei* and Moldavian *Thymus sp*. plants are presented in sections I.1.1., I.2.1.4, and I.2.1.3 respectively.

Material and methods

Plant material

The ripe fennel fruits were harvested from submontaneous areas of Piatra Neamț County, România, in September 2004 (F1 and F2 samples from plants in their second vegetative year) and September 2005 (F3 and F4 samples from plants in the third vegetative year), respectively.

Data on plant material of *Mentha gattefossei* and *Thymus sp.* are described in sections I.2.1.4 and I.2.1.3, respectively.

Essential oils isolation

The fennel essential oils were obtained by hydrodistillation for 4 hours in a Clevenger-type apparatus. Then, they were separated, dried over anhydrous sodium sulfate and kept in a dark glass bottle at 4 °C until the analysis.

The isolation of *Mentha gattefossei* essential oil (MgEO) is presented in section I.2.1.4. Experimental data about the isolation of *Thymus* sp. EOs are presented in section I.2.1.3.

Chemical analysis of essential oils

The chemical composition of fennel essential oils was investigated by GC-MS means. An analytical system, consisting of HP 5890 Series II GC and HP 5971 MSD, was used. GC-MS analysis of the essential oil was performed on HP-5MS capillary column (25 m×0.25 mm×0.25 μm) coated with cross-linked methyl silicone gum. Carrier gas was helium with 1 mL/min flow

rate. Temperature program was: 40 °C held for 5 min, then heated up to 260 °C at 10 °C per min, and for 5 additional minutes at this temperature. The temperature of injector was 250 °C and of MS interface was 280 °C. Identification of each individual compound was made by comparison of their retention times with those of the authentic samples, in the same operating conditions, and by computer searching, matching mass spectral data with those held in the computer library. For these purposes Wiley 275 L library, operating with collection of 135.720 mass spectra, was used [Aprotosoaie et al., 2008].

Data on chemical analysis of MgEO and *Thymus sp.* EOs are presented in sections I.2.1.4 and I.2.1.3, respectively.

Microbial strains

The activity of fennel and mint essential oils was investigated using the microbial strains mentioned in Table 29:

Table 29. Microbial strains used in the evaluation of antimicrobial activity of fennel and mint EOs

Organisms		***Foeniculum vulgare* EO**	***Mentha gattefossei* EO**
G (+)	*Staphylococcus aureus* ATCC 25923	+	+
G (+)	*Sarcina lutea* ATCC 9341	+	-
G (+)	*Bacillus cereus* ATCC 14579	+	-
G (-)	*Pseudomonas aeruginosa* ATCC 27853	+	+
G (-)	*Escherichia coli* ATCC 25922	+	+
G (+)	*Streptococcus pneumoniae* ATCC 49619	-	+
Fungus	*Candida albicans* ATCC 10231	+	-
Source		Collection of Microbiology Laboratory from Faculty of Pharmacy, University of Medicine and Pharmacy Grigore T. Popa Iaşi	Collection of Microbiology Laboratory from Lung Hospital, Iaşi

The antifungal activities of thyme EOs and thymol was investigated using *Aspergillus flavus* MUCL 19006 standard strain (MUCL 19006 - BCCM).

Evaluation of antimicrobial activity

Disc diffusion method

In vitro antimicrobial activity of MgEO was determined by using the agar-diffusion assay [CLSI, 2009]. The test was performed on sterile Petri plates (90 mm diameter) using Mueller Hinton agar inoculated with microbial suspension at a density adjusted to a 0.5 McFarland standard (10^6 CFU/mL). The inoculum was spread on the plates using sterile swabs. The wells with 50 mm diameter have been made in agar and every well was completely filled with 50 µL MgEO. Then, the plates were aerobically incubated for 18-24 h, at 35 °C. As positive controls, there were used antibiotic discs of amoxicillin (25 µg/disc) and ciprofloxacin (5 µg/disc) placed on the medium surface. After incubation, the growth inhibition zones were measured and recorded in mm.

Determination of minimum inhibitory concentration

The antimicrobial activity of fennel essential oils was quantitative determined by the broth dilution method with an inoculum of 10^6 CFU/mL. Double dilutions of each oil sample were tested. The tubes with diluted antimicrobial agent solution are inoculated with an equal volume of the bacterial suspension. The last tube resulting in complete inhibition of visible growth after 20 h incubation at 37 °C, represents the minimum inhibitory concentration (MIC). The minimum bactericidal concentration (MBC) was determined by transferring 0.1 mL from each of the tubes showing no growth on the MIC on the surface of agar plate. The subcultures are incubated 20 h and the MBC read as the least concentration which produced ≥ 99.9% killing of the bacteria [Waterworth et al., 1978; Aprotosoaie et al., 2008b].

Anti-Aspergillus flavus assay

A. flavus was cultured on MYA2 (2% malt yeast agar) medium slant at 4 °C. For conidia production, the fungus was cultivated on PDA (potato dextrose agar) medium at 28 ºC for 7 days.Minimum inhibitory and minimum fungicidal concentrations (MICs and MFCs, respectively; µL/mL) of thyme EOs against *A. flavus* were determined using a broth microdilution method [CLSI, 2006]. Initially, the EOs were diluted in a sterile solution of Tween 80 (0.001%). Serial doubling dilutions of EOs (0.03-2.00 µL/mL) in RPMI 1640 medium (with L-glutamine and phenol red but without bicarbonate, supplemented with glucose up to 2%) were prepared in 96-well microtiter trays. The stock inoculum was prepared by recovering the conidia with sterile Ringer solution (containing 0.1% Tween 80, v/v) from the 7 day culture grown on PDA medium; its turbidity was adjusted to 0.09-0.11 yielding $0.4\text{-}5\times10^6$ conidia/mL. An aliquot (100 µL) of the stock inoculum diluted 1:50 in RPMI was added to 0.1 mL of RPMI 1640 medium containing EO dilutions, followed by incubation for 48 h at 28 ºC in a humid atmosphere. Both sterility (uninoculated medium) and growth (inoculated medium) controls were included. Thymol, a natural compound with well-known antifungal properties, was used as positive control and was tested using the same experimental protocol described for EOs.

The MIC values were recorded as the lowest concentrations of EOs/thymol inhibiting the visible growth of *A. flavus*. The MFCs were determined by the spot inoculation of 10 µL from the wells showing no visible fungal growth on PDA medium, followed by incubation for 72 h at 28 °C.

The lowest concentrations of EOs/thymol inhibiting the fungal growth were defined as MFCs [Tullio et al., 2007].

Results and discussion

Antimicrobial activity of fennel essential oils

Major compounds found in all essential oils were *trans*-anethole (31-74%), estragole (7.21-22.25%), fenchone (4.70-17.54%), limonene (1.09-3.88%), and α-pinene (9.64-3.34%), but the concentration of these constituents varied greatly among the oils (Table 30). The fruits harvested from plants 3 years aged (F3, F4) showed large variations in the content of *trans*-anethole. Also, the levels of monoterpene hydrocarbons and fenchone are low in aged plants (Table 30).

Table 30. Main compounds identified in fennel essential oils [Aprotosoaie et al., 2008b]

RT (min.)	Compound	%			
		F1	**F2**	**F3**	**F4**
6.58	α-pinene	3.12	3.34	0.64	0.80
6.80	camphene	0.51	0.53	-	-
7.20	sabinene	0.25	0.21	-	0.07
7.28	β-pinene	0.37	0.34	-	-
7.47	myrcene	0.99	0.92	0.15	-
7.75	α-phellandrene	0.52	0.43	0.94	-
8.25	limonene	3.88	3.51	1.67	1.09
8.71	γ-terpinene	1.28	1.15	0.19	0.33
9.01	β-phellandrene	-	-	1.10	-
9.08	*trans*-sabinene-hydrate	0.17	0.10	-	-
9.45	fenchone	17.54	17.21	4.70	8.91
10.20	camphor	0.30	0.50	1.10	-
10.72	estragole	22.25	20.40	7.21	13.33
11.08	carveol	-	-	5.56	-
13.00	*trans*-anethole	46.95	44.47	31.00	74.00
13.08	hydrocarveolacetate	-	-	7.75	-
13.58	trans-carveolacetate	-	-	2.53	-
14.00	anisyl-acetone	1.48	3.44	-	-
14.05	β-bourbonene	-	-	1.36	-
15.35	germacrene D	0.25	0.76	-	-
15.90	α-cubebene	-	-	0.18	-
Monoterpenes		28.93	28.24	26.33	10.87
Aromatic compounds		70.68	68.31	38.21	87.33
Sesquiterpenes		0.25	0.76	1.54	-

All fennel oils have a good activity against *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 bacteria (Table 31). *E.coli* bacteria were the most sensitive to fennel oils (MIC=0.7-1.4 mg/mL). Both *E. coli* and *S. aureus* are major pathogens that cause a wide range of infections. In addition, they are leading cause of food borne diseases around the globe. Due to its superior activity against these pathogens, the antimicrobial effects of fennel essential oil against *E. coli* and *S. aureus* strains with high pathogenicity could be investigated

Fennel essential oils were less active against *B. cereus* and *P. aeruginosa* strains. The poor activity against *Pseudomonas aeruginosa* could be related to extremely low sensitivity of this

pathogen to essential oils as various studies have already reported [Burt, 2004]. The resistance of *Pseudomonas aeruginosa* seems to be due to its outer membrane structure particularly impermeable to oils [Longbottom et al., 2004].

Antifungal activity was remarkable and similar with F2, F3 and F4 oil samples (MIC=0.36 mg/mL) and higher than F1 sample (MIC=2.8 mg/mL)(Table 31).The oil samples were generally bactericidal at a concentration up to twofold or fourfold higher than the MIC value (Table 32). According to MIC values, no difference was observed among antibacterial activity with F1, F2 and F3, F4 oil samples. Although, *trans*-anethole is major component of fennel essential oils samples, the variations of its content are not reflected in an appropriate variation of antimicrobial activity, especially in the case of F3 sample. The hypothesis of an antimicrobial effect exerted by the contribution of all components seems to be more plausible in the case of fennel essential oil.

Table 31. MIC values (mg/mL) of fennel essential oils [Aprotosoaie et al., 2008b]

Microorganism		**MIC (mg/mL)**			
		F1	**F2**	**F3**	**F4**
G(+)	*S. aureus* ATCC 25923	2.8	1.4	1.4	2.8
G (+)	*Bacillus cereus* ATCC 14579	22	22	22	22
G (-)	*Escherichia coli* ATCC 25922	0.7	1.4	0.7	0.7
G (-)	*Pseudomonas aeruginosa* ATCC 27853	44	44	44	44
Yeast	*Candida albicans* ATCC 10231	1.4	0.18	0.18	0.18

Table 32. MBC values (mg/mL) of fennel essential oils [Aprotosoaie et al., 2008b]

Microorganism		**MBC (mg/mL)**			
		F1	**F2**	**F3**	**F4**
G(+)	*S. aureus* ATCC 25923	5.6	2.8	2.8	5.6
G (+)	*Bacillus cereus* ATCC 14579	44	44	44	44
G (-)	*Escherichia coli* ATCC 25922	1.4	5.6	1.4	1.4
G (-)	*Pseudomonas aeruginosa* ATCC 27853	87	87	87	87
Yeast	*Candida albicans* ATCC 10231	2.8	0.36	0.36	0.36

Antibacterial activity of Mentha gattefossei essential oil

The agar-diffusion assay showed a strong antibacterial activity (inhibition zone ≥ 20 mm) of MgEO against *Streptococcus pneumoniae* ATCC 49619 bacteria but lower than antibiotics used as positive controls (Table 33). Also, MgEO showed moderate activity on *Staphylococcus aureus* ATCC 25923.The standard strains Gram-negative bacteria of *Pseudomonas aeruginosa* and *Escherichia coli* were non-sensitive to MgEO (Table 33).

Although pulegone, the major component of MgEO, has a potent biocide activity on both Gram-positive and Gram-negative bacteria, however the essential oil behaves differently. The antimicrobial efficacy of the MgEO itself is determined by its entire chemical composition. In this respect, other important components of MgEO such as neomenthone and limonene have proven to be less effective against the tested microorganisms [Oumzil et al., 2002].

The higher antimicrobial effects of MgEO against Gram-positive than Gram-negative bacteria could be explained by the structure of cell envelope. Thus, the structure and the composition of cell wall and outer membrane of Gram-negative bacteria are more complex and contribute significantly to the occurrence of certain resistance to the passage of antimicrobial agents such as essential oils [Nazzaro et al., 2013].

Table 33. The antibacterial activity of *Mentha gattefossei* essential oil (MgEO) and conventional antibiotics against tested bacteria [Aprotosoaie et al., 2018b]

Bacterial strains		Diameter of inhibition zone (mm)		
		MgEO	Standard drugs	
			Amoxicillin	Ciprofloxacin
G-	*Pseudomonas aeruginosa* ATCC 27853	0.00±0.00	0.00±0.00	26.33±0.57
G-	*Escherichia coli* ATCC 25922	0.00±0.00	25.00±0.57	40.00±0.57
G+	*Staphylococcus aureus* ATCC 25923	10.00±0.00	25.00±0.00	30.00±0.57
G+	*Streptococcus pneumoniae* ATCC 49619	20.66±1.15	40.00±0.00	30.00±0.00

Anti-Aspergillus activity of Thymus sp. essential oils

Aspergillus flavus is responsible for the contamination of foods and feedstuffs but also for the human invasive aspergillosis and superficial skin infections. *A. flavus* produces many toxic compounds among which aflatoxin B1 is highly carcinogenic, mutagenic and teratogenic to humans and animals [Hedayati et al., 2007].

Among natural products, essential oils are very most promising antifungal and anti-aflatoxigenic agents. They have great advantages such as effectiveness, low resistance and eco-friendly properties.

Starting from the high antimicrobial potential of *Thymus* EOs, we investigated the anti-*Aspergillus* activity of the essential oils obtained from *Thymus sp*. growing in Republic of Moldova.

MIC and MFC values of thyme EOs and thymol are shown in Table 34. *T. vulgaris* and *T. calcareus* EOs, rich in thymol, were the most active against the fungal strain.

According to the MIC and MFC values (0.25 and 0.50 μL/mL, respectively), both EOs exhibited similar strong antifungal effects. In addition, *T. citriodorus* 'Aureus', containing 7.53% aromatic compounds alongside a high level of geraniol (60.31%), displayed an important antifungal activity (MIC=0.50 μL/mL, MFC=1.00 μL/mL).

T. vulgaris and *T. calcareus* EOs, rich in thymol, were the most active against the fungal strain. According to the MIC and MFC values (0.25 and 0.50 μL/mL, respectively), both EOs exhibited similar strong antifungal effects. In addition, *T. citriodorus* 'Aureus', containing 7.53%

aromatic compounds alongside a high level of geraniol (60.31%), displayed an important antifungal activity (MIC=0.50 μL/mL, MFC=1.00 μL/mL).

Thymol, assayed as single compound, proved to be more active than all EOs (MIC=0.125 *vs.* 0.25-1.00 μL/mL, MFC=0.25 *vs*. 0.50-1.00 μL/mL).

Table 34. Anti-*Aspergillus* activity of thyme EOs [Aprotosoaie et al., 2019a]

EO/Positive control	MIC* (μL/mL)	MFC** (μL/mL)
T. vulgaris	0.25	0.50
T. vulgaris 'Faustini'	1.00	1.00
T. citriodorus	1.00	1.00
T. citriodorus 'Aureus'	0.50	1.00
T. calcareus	0.25	0.50
Thymol	0.125	0.25

*MIC, minimum inhibitory concentration; **MFC, minimum fungicidal concentration.

Many studies have highlighted strong anti-*Aspergillus* properties and inhibitory effects on aflatoxin production of EOs from different *Thymus* species. However, there are no data on the antifungal effects of *T. citriodorus*, *T. calcareus* and *T. vulgaris* 'Faustini' EOs and our study firstly reported on this issue. Klaric et al. (2007) showed that *T. vulgaris* EO containing 33% thymol exerted a strong fungicidal activity on *A. flavus* isolated from damp dwellings (MIC=9.35 μg/mL). In addition, Kohiyama et al. (2015) reported that *T. vulgaris* EO (40.6% borneol and 19.9% α-terpineol) inhibited growth of *A. flavus* (MIC=250 μg/mL) and aflatoxin production. With respect to *T. vulgaris* EO (density=0.92 g/mL, at 20 °C), we found a MIC value close to that reported by Kohiyama et al. (2015)(230 μg/mL *vs.* 250 μg/mL). The differences that occur between the reported values can be explained by the sensitivity of the fungal strain, methodology and chemical composition of *Thymus* EOs.

The strong antifungal profile of thyme EOs is mainly related to their content in monoterpene phenols such as thymol. Among the volatile constituents, the phenols are considered to be the most potent antifungal agents. These compounds alter the microbial cell membrane integrity causing the loss of essential cytoplasmic constituents, impair the fungal enzyme systems and interfere with the cell wall synthesis [Moghaddam et al., 2016].

Conclusions

Fennel essential oils showed a good antibacterial activity against *Escherichia coli* and *Staphylococcus aureus* strains. Also, they exhibited a remarkable antifungal activity on *Candida albicans*.

No significant antimicrobial activity variations were observed for *Foeniculum vulgare* essential oil samples obtained from plants after two or three years cultivation period. The antimicrobial activity can not be correlated with the content of *trans*-anethole, the main compound of fennel essential oil.

The results are encouranging for future investigations on the action of fennel essential oil on food-borne pathogens and fungal pathogens.

The essential oil of *Mentha gattefossei* plants cultivated in Republic of Moldova showed promising antimicrobial activity only on *Streptococcus pneumoniae* bacteria.

Among thyme essential oils, *T. vulgaris* and *T. calcareus* EOs showed the most potent antifungal activities. Alongside *T. vulgaris* EO, Moldavian *T. calcareus* EO might have applications in the food and pharmaceutical industries as antioxidant and antifungal agents.

I.2.2.2. Antibacterial activity and combinatorial effects of essential oils with antibiotics against lower respiratory tract pathogens

Current state of art and objectives research

The irrational and inappropriate use of antibiotics has led to the emergence of multi-drug resistant (MDR) bacteria, phenomenon that has acquired outstanding accents during the past decades. These bacterial strains are a major cause of nosocomial infections. *Staphylococcus aureus*, *Streptococcus pneumoniae* and *Pseudomonas aeruginosa* bacteria are among the ones most commonly associated with antibiotic resistance and they can even serve as markers for this phenomenon. To ensure therapeutic control of MDR-bacteria it is imposed to find new antimicrobial agents or to increase the antibiotics'activity by overcoming the resistance. Plants are a rich reservoir of antimicrobial agents and essential oils are among the most promising vegetal metabolites. Numerous studies have highlighted the remarkable antimicrobial potential of essential oils from various plants. Furthermore, the antimicrobial effectiveness of antibiotics can be positively influenced when they are combined with essential oils.

The antimicrobial agents such as essential oils can restore sensitivity to antibiotics, they may decrease the effective dose of the antibiotics on resistant bacteria minimizing side effects, they may extend the antimicrobial spectrum and reduce the cost of anti-infective therapy [Fadli et al., 2012]. The hydrophobic and multi-componential nature of essential oils explain their ability to cross the bacterial membrane and to act on different and multiple targets.

In this respect, the combinatorial effects of ajowan essential oil and conventional antibiotics (amoxicillin, ciprofloxacin) against some clinical isolates of multiresistant bacteria from *Pseudomonas*, *Staphylococcus* and *Streptococcus* genera were investigated. Also, the effects of the combinations of cardamom essential oil and antibiotics against some methicillin-resistant strains of *Staphylococcus aureus* were evaluated.

Ajowan, *Trachyspermum ammi* (L.) Sprague ex Turrill syn. *Carum copticum* Benth & Hook (Apiaceae) is an annual herbaceous and aromatic plant, originating in India and Egypt. It has a long history of use as a cooking spice and herbal remedy, especially in Asian regions [Zachariah, 2008].The fruits are ajowan's most used part. They have a bitter, pungent taste and they develop trough crushing a strong aromatic fragrance, resembling that of thyme. Their characteristic aroma is due to the essential oil (2.5-5%) containing thymol as its major constituent (35-60%), followed by p-cymene, γ-terpinene and β-pinene [Kaur and Arora Singh, 2010].

Ajowan dried fruits, whole or powdered, are used for flavoring foods, snacks, sauces and vegetable preparations or as preservative in the food industry or in alcoholic beverages. Besides their extensive use as a spice, the fruits of ajowan have also been used in traditional Indian herbal medicine as a remedy for a wide array of unrelated ailments including indigestion and colic, dyspepsia, flatulence, diarrhea, arthritis and rheumatism, coughs, bronchial pneumonia or asthma [Malhotra and Vijay, 2004; Zachariah, 2008].

Apart from the traditional use, in the last decade, extensive pharmacological studies have been undertaken in order to assess the biological effects of *Trachyspermum ammi* fruits and their derivated extracts. Several studies have reported a marked antimicrobial activity of ajowan essential oil (AjEO) or its main compound, thymol, against a broad spectrum of Gram-positive and Gram-negative bacteria, including food borne and spoilage bacteria [Paul, 2011] or water borne enteropathogenic bacteria [Kumar et al., 2011].

Elletaria cardamomum (L.) Maton (Zingiberaceae), known as cardamom, is a perennial aromatic plant, native to the evergreen forests of southern India [Korikanthimath and Rao, 2002]. Its dried fruits have great economical importance, being one of the most highly priced spices [Korikanthimath, 2004].

Cardamom fruits have stomachic, spasmolytic, anti-foaming, expectorant and anti-inflammatory properties. Besides medicinal purposes, cardamom fruits are used as a condiment and flavouring agent in food products. Cardamom essential oil (CEO) is used in medicine as antimicrobial, anti-inflammatory and analgesic agent, but also in cosmetic industry and perfumery [Vijayan et al., 2002; Sengottuvelu, 2011].

However, there is a lack of data on the combination effects of these essential oils and antibiotics. How is the antibiotic activity influenced? May such combinations be useful to reduce the emergence of resistant bacteria?

Starting from this background we investigated possible synergistic effects between AjEO or CEO and conventional antibiotics (amoxicillin, ciprofloxacin) against some clinical isolates and standard strains of *Pseudomonas, Staphylococcus* and *Streptococcus* genera, bacteria that are mainly involved in the occurence of lower respiratory tract infections.

Material and methods

Chemicals

An alkane standard solution C8-C20, thymol and dimethylsulphoxide (DMSO) were purchased from Sigma-Aldrich (Steinhem, Germany). 1,8-cineole (purity 99%) was purchased from Sigma-Aldrich (Milwaukee, USA). Mueller Hinton agar and broth were obtained from Biolab Zrt. (Budapest, Hungary). The microcomprimates of amoxicillin and ciprofloxacin were aquired from Bioanalyse Tibbi Malzemeler (Ankara, Turkey).

The horse lysed blood was procured from Oxoid (Basingstoke, UK). All other solvents were of analytical grade.

Plant material

Dried ajowan and cardamom fruits were purchased from a local supermarket and their botanical identity was confirmed in the Department of Pharmacognosy, Faculty of Pharmacy, University of Medicine and Pharmacy "Grigore T. Popa", Iasi, Romania.

The voucher specimens (AJ no. 12013 and EC no. 12012, respectively) were deposited in the same Department.

Essential oil isolation

The volatile fractions were isolated by hydrodistillation of powdered dried fruits (100 g) in a Clevenger type apparatus (3 h for ajowan fruits and 5 h in the case of cardamom fruits, respectively).

The ajowan and cardamom essential oils were dried over anhydrous sodium sulfate and stored in sealed glass tubes at 4 °C until analysis.

GC-MS analysis

GC-MS analysis was carried out on an Agilent type 7890A gas cromatograph, equipped with an Agilent 5975C mass spectrometer and a DB-5MS capillary column (30 m x 0.25 mm internal diameter, 0.25 μm film thickness). The injection volume was of 0.3 μL of pure essential oil. The oil samples were injected in split mode (1/30), using pressured controlled He as carrier gas with a flow rate of 1.5 mL/min. The injector and detector temperatures were maintained at 250 °C. The analyses were performed using the following temperature program: the oven temperature was raised at a rate of 3 °C min^{-1} from 40 °C to 250 °C (isothermal for 4 min), then raised to 280 °C at 10 °C min^{-1} and the final temperature was held for 2 min [Aprotosoaie et al., 2010].

GC-FID analysis

The GC-FID analysis was performed using an Agilent 6890 gas chromatograph equipped with a flame ionization detector and a DB-5MS capillary column (30 m × 0.25 mm internal diameter, 0.25 μm film thickness). The GC-FID conditions were the same as the ones described above. The compounds were identified by comparing their recorded mass spectra with those stored in the Wiley mass spectral library and their retention indices relative to *n*-alkanes with those mentioned in literature [Adams, 2007; Goodner, 2008].

Microbial test strains

The panel of test microorganisms includes standard and clinical isolated bacterial strains (Table 35). The standard strains were provided by Liofilchem (Abruzzi, Italy) and MediMark Europe (Grenoble, France), respectively.

The clinical isolates were obtained from pathological products (sputum, tracheobronchial aspirate) of patients with lower respiratory tract infections, and were provided by the Microbiology Laboratory of the Lung Hospital, Iasi, Romania.

Table 35. Bacterial strains used in the study

Strains	Gram	Standard	Clinical Isolate
Staphylococcus aureus	Positive	ATCC 25923	-
Staphylococcus aureus methicillin resistant	Positive	-	MRSA 37
Staphylococcus aureus methicillin resistant	Positive	-	MRSA 4185
Streptococcus pneumoniae	Positive	ATCC 49619	-
Streptococcus pneumoniae penicillin resistant	Positive	-	PRSP 4423
Streptococcus pneumoniae penicillin resistant	Positive	-	PRSP 4546
Streptococcus pneumoniae penicillin resistant	Positive	-	PRSP 4566
Pseudomonas aeruginosa	Negative	ATCC 27853	-
Pseudomonas aeruginosa amoxicillin-resistant	Negative	-	ARPA 2351

Antimicrobial susceptibility testing

The antimicrobial susceptibility of clinical isolates was determined using disk diffusion method on Mueller Hinton agar (Oxoid, UK) and a panel of 9 antibiotics: amoxicillin (Amx; 25 μg/disk), cefpirome (Cef; 30 μg/disk), doxycycline (Dox; 30 μg/disk), erythromycin (Ery; 15 μg/disk), levofloxacin (Lev; 5 μg/disk), methicillin (Met; 5 μg/disk), penicillin (P; 10 U/disk), streptomycin (St; 10 μg/disk), vancomycin (Van; 30 μg/disk) (Oxoid, UK). After the incubation for 16-18 h or 24 h (*Streptococcus pneumoniae*) at 35 °C, the growth inhibition zones were recorded and the results were interpreted according to *Clinical and Laboratory Standards Institute Guidelines* (CLSI, 2009).

Determination of the minimum inhibitory concentration (MIC)

The MIC values were assessed by the broth microdilution method (CLSI, 2009). Serial double dilutions of AjEO and thymol (Sigma-Aldrich, Germany), ranging from 64 to 0.0015 mg mL^{-1}, were prepared into Mueller Hinton broth, followed by inoculation (10^5 CFU/well). CEO and 1,8-cineole serial dilutions, ranging from 25 mg/mL to 0.0015 mg/mL and 5 mg/mL to 0.015 mg/mL respectively, were also prepared into Mueller Hinton broth.

The solubility of AjEO was enhanced by adding 5% (v/v) DMSO to the 128 mg/mL stock solution of essential oil in Mueller Hinton broth. In each well, was added the inoculum adjusted to 0.5 McFarland standard turbidity. For *Streptococcus pneumoniae* isolates, the Mueller-Hinton broth was supplemented with 5% (v/v) lysed horse blood (Oxoid, UK). Amoxicillin and ciprofloxacin were used as positive controls in a dilution range from 64 to 0.007 μg mL^{-1} for AjEO analysis. In the case of CEO, the same antibiotics were used in a dilution range of 16 μg/mL to 0.007 μg/mL and 8 μg/mL to 0.003 μg/mL, respectively. The solvents used to dilute the antibiotics were phosphate buffer (pH=6) for amoxicillin and sterile double distilled water for ciprofloxacin. Negative controls were also included in the test, namely: bacteria growth control and sterility control, as well as solvents used to dilute samples and antibiotics. The final volume in each well was of 1 mL. The incubation was performed at 35 °C, for 24 h. The amount of bacterial growth was monitored by visual assessment of turbidity.The MIC was defined as the lowest concentration of sample that inhibited the growth of the tested bacteria [Mighri et al., 2010].

Checkerboard assay

This assay was performed to evaluate potential interactions between volatiles and antibiotics [Lorian, 2005; Grădinaru et al., 2014). Serial double dilutions of AjEO, CEO, thymol, 1,8-cineole and antibiotics were prepared as described previously for determination of MIC values. AjEO/thymol and antibiotics were dispensed on the 96-well plates in a checkerboard manner. Thus, AjEO/thymol was added to a Mueller Hinton medium to give two-fold dilutions along the X-axis, whilst the antibiotic was diluted two-fold along the Y axis. The same technique was applied in the case of CEO and 1,8-cineole. The range of dilutions of volatiles and antibiotics comprises for each them the characteristic MIC value. 50 μL of each dilution are found in each well, the final volume being of 100 μL. The inoculum adjusted to 0.5 McFarland standard of bacterial concentration was added in each well of the plate. The plates were incubated at 37 °C for 24 h. The interactions between volatiles and antibiotics were interpreted according to the fractional inhibitory concentration indexes (FICIs) values.The fractional inhibitory concentration (FIC) derived from the lowest concentration of agents combination that does not allow a visible growth of the test organisms on the plates.

The FICI values were calculated as follows:

$FICI = FIC_{\text{essential oil/ volatile compound}} + FIC_{\text{antibiotic}}$,

where $FIC_{essential\ oil/volatile\ compound}$=$MIC_{essential\ oil/volatile\ compound\ in\ combination}$/$MIC_{essential\ oil/volatile\ compound\ alone}$ and $FIC_{antibiotic}$=$MIC_{antibiotic\ in\ combination}$/$MIC_{antibiotic\ alone}$.

A combination of two substances operates synergistically if FICI≤0.5, additively if 0.5>FICI≤1, indifferently if 1>FICI≤ 4 and antagonistically if FICI>4 [Schelz et al., 2006]. The synergistic and additive interactions were illustrated using isobolograms. Points falling below or on 0.5:0.5 line correspond to synergistic interactions, those situated between 0.5:0.5 and 1:1 lines (including the ones on 1:1 line) to additive interactions.

Results and discussion

Antibacterial activity and combinatorial effects of AjEO

Chemical composition of the AjEO

The hydrodistillation of the ajowan fruits gave a yellowish essential oil with yield of 7.4% (v/w). GC-MS analysis of AjEO revealed the presence of sixteen compounds, which accounted for 98.93% of the total oil. The major constituents of the AjEO were thymol (50.75%), γ-terpinene (25.94%), p-cymene (18.31%) and β-pinene (2.27%), while myrcene (0.49%), α-terpinene (0.28%) and α-pinene (0.21%) were minor components (Table 36).

Aromatic compounds (phenols and hydrocarbons) were the most abundant fraction of the AjEO (69.06%). Also, in some previous studies on the chemical composition of ajowan fruits essential oil, the main components were reported as thymol (39.1%-49%), γ-terpinene (23.2-30.8%) and *p*-cymene (15.7-30.8%) (19, 20).

On the contrary, other reports showed that carvacrol (45.2%) and *p*-cymene (41.98%) are major compounds of the oil [Srivastava and Saxena Baby, 1999]. Mohagheghzadeh et al. (2007) suggested the existence of two chemotypes, thymol and carvacrol, for *Trachyspermum ammi* fruits.

It is well-known that the essential oil chemical composition depends upon many factors such as: the environmental and climate conditions, the extraction method, the harvesting time, the plants age or the genetic data. Further, qualitative and quantitative differences in chemical composition could have a significant impact on olfactive and biological properties of essential oil. According to the data obtained in our study, AjEO belongs to thymol chemotype.

Antimicrobial susceptibility assessment

The clinical isolates showed a characteristic antimicrobial resistance profile. Mainly the *Staphylococcus aureus* and *Pseudomonas aeruginosa* isolates were MDR strains, exhibiting resistance to two or three antibiotics from different classes (Table 37).Taking into account the common resistance characteristics of the isolates from each bacterial species and to simplify the exposure, we generally appreciated these pathogens as follows: amoxicillin-resistant *Pseudomonas aeruginosa*, methicillin resistant *Staphylococcus aureus*, and penicillin resistant *Streptococcus pneumoniae* bacteria.

Antibacterial activity of AjEO

Among tested bacteria, both standard and clinical strains of *Streptococcus pneumoniae* were most sensitive to AjEO (MIC=0.125-0.50 mg mL^{-1}). Thymol was mostly active against *Pseudomonas aeruginosa* ATCC 27853 (MIC=0.07 mg mL^{-1}) and *Staphylococcus aureus* ATCC 25923 (MIC=0.003 mg mL^{-1}) bacteria (Table 38). The antibacterial activity of thymol is mainly due to its membrane-permeabilizing effect: being lipophilic, thymol intercalates into the phospholipid bilayer thus causing an increase in membrane permeability, inorganic ions leakage

and cell death [Kon and Rai, 2012]. Althunibat et al. (2016) have found that *Pseudomonas aeruginosa* multi-drug resistant bacteria are more sensitive to thymol as pure compound than to essential oil of *Thymus capitatus*. Compared to other constituents of essential oils, thymol has a high capacity to disturb the outer membrane of Gram-negative bacteria such as *Pseudomonas aeruginosa* [Kon and Rai, 2012]. It is mainly related to the hydrophobicity of the aromatic nucleus and to the position of hydroxyl group on the ring [Althunibat et al., 2016]. Also, the higher susceptibility of *Staphylococcus aureus* standard strain and clinical isolates to thymol than to AjEO (0.003 mg/mL *vs*. 4 mg/mL, and 0.125 mg/mL *vs*. 8 mg/mL, respectively) might be due to the fact that γ-terpinene and *para*-cymene, constituents of AjEO that usually potentiate the antibacterial effects of thymol [Ultee et al., 2002; Souren 2011], have a considerable lower toxicity against *S. aureus* in comparison with thymol [Cristani et al., 2007].

Table 36. The chemical composition of AjEO [Grădinaru et al., 2018]

Compound	RI[a]	RI[b]	% ± SD
Monoterpene hydrocarbons			
α-thujene	922	924	0.41±0.01
α-pinene	928	933	0.21±0.02
camphene	943	946	0.01±0.00
β-pinene	974	974	2.27±0.04
myrcene	987	988	0.49±0.03
Δ-3-carene	1004	1008	0.02±0.00
α-terpinene	1013	1014	0.28±0.02
γ-terpinene	1067	1064	25.94±0.61
terpinolene	1082	1084	0.06±0.01
Monoterpene alcohols			
terpinen-4-ol	1174	1174	0.10±0.01
Monoterpene ketones			
piperitenone	1339	1340	0.01±0.00
Aromatic hydrocarbons			
p-cymene	1029	1030	18.31±0.58
Phenols			
thymol	1296	1295	50.75±0.25
Phenol methyl ethers			
(*E*)-anethole	1280	1282	0.03±0.00
eugenol	1351	1356	0.03±0.00
Sesquiterpenes			
(*E*)-caryophyllene	1409	1411	0.01±0.00
Total (%)			**98.93**

[a]Retention indices relative to *n*-alkanes (C_8–C_{20}) calculated on DB-5MS capillary column; [b]Retention indices reported in literature.

Table 37.The antimicrobial resistance profile of lower respiratory tract clinical isolates [Grădinaru et al., 2018]

Clinical isolate	Antimicrobial resistance/ inhibition zone of bacterial growth (mm)
ARPA 2351	Amx/5 (R); Lev/10 (R); Cef/9 (R)
MRSA 37	Met/7 (R); Dox/10 (R); Ery/23 (S); Van/24 (S)
MRSA 4185	Met/6 (R); Dox/10 (R); Ery/11 (R); Van/23 (S)
PRSP 4423	P/6 (R); St/9 (R); Ery/10 (R); Van/21 (S)
PRSP 4546	P/7 (R); St/20 (S); Ery/20 (S); Van/21 (S)
PRSP 4566	P/7 (R); St/22 (S); Ery/23 (S); Van/23 (S)

Amx, amoxicillin; Cef, cefpirome; Dox, doxycycline; Ery, erythromycin; Lev, levofloxacin; Met, methicillin; P, penicillin; St, streptomycin; Van, vancomycin; R, resistance; S, susceptible.

Table 38. Minimum inhibitory concentrations (MICs) of AjEO/thymol and antibiotics (mg mL^{-1}) [Grădinaru et al., 2018]

Standard strains/Clinical isolates	MIC			
	AjEO	Thymol	Antibiotics	
			Amoxicillin	Ciprofloxacin
Gram-negative bacteria				
Pseudomonas aeruginosa ATCC 27853	16	0.07	4×10^{-3}	1×10^{-3}
ARPA 2351	16	8	32×10^{-3}	2×10^{-3}
Gram-positive bacteria				
Staphylococcus aureus ATCC 25923	4	0.003	2×10^{-3}	0.5×10^{-3}
MRSA 37	8	0.125	2×10^{-3}	4×10^{-3}
MRSA 4185	8	0.125	4×10^{-3}	4×10^{-3}
Streptococcus pneumoniae ATCC 49619	0.25	4	0.07×10^{-3}	0.5×10^{-3}
PRSP 4423	0.125	0.125	4×10^{-3}	2×10^{-3}
PRSP 4546	0.50	0.25	0.03×10^{-3}	0.5×10^{-3}
PRSP 4566	0.25	0.25	2×10^{-3}	0.5×10^{-3}

In general, AjEO had a better activity against Gram-positive bacteria. Multiple factors influence the antimicrobial activity of essential oils: their multicomponential nature, the ratios between compounds, lipofilia, the composition of bacterial membranes and their interaction with volatile components. Due to an additional outer membrane and overexpression of efflux pumps involved in the antimicrobial resistance, Gram-negative bacteria are less susceptible to the action of antibacterial agents and essential oils [Aelenei et al., 2016].

Interactions between AjEO/thymol and antibiotics

Synergistic interactions were found for some AjEO-amoxicillin combinations against *Staphylococcus aureus* ATCC 25923 and MRSA 37 (FICI=0.37-0.50) (Fig. 36A), resulting an eight-fold reduction in MIC of amoxicillin (0.25×10^{-3} mg mL^{-1} *vs.* 2×10^{-3} mg mL^{-1}) in the case of the best combination (Table 39).

Thymol behaves similarly against MRSA 37 (FICI=0.36-0.49) (Fig. 36B), the best combination leading also to an eight-fold reduction in MIC of amoxicillin (0.25×10^{-3} mg mL^{-1} *vs.* 2×10^{-3} mg mL^{-1}) (Table 39).

Also, additive effects were observed for AjEO-amoxicillin and thymol-amoxicillin combinations against *S. aureus* ATCC 25923, as well as against MRSA 4185 (Fig. 36A, Fig. 36B) and PRSP 4423 clinical isolates (Table 39).

Table 39. Effects of combinations between ajowan essential oil (AjEO)/thymol (TM) and amoxicillin (Amx) against tested bacteria

Strains	**Combination**	**MIC_c**	**FIC^1/ FIC^2**	**FICI**	**Outcome**
Pseudomonas aeruginosa ATCC 27853	$AjEO^1+Amx^2$	$16/1\times10^{-3}$	1/0.25	1.25	I
	TM^1+Amx^2	$0.07/1\times10^{-3}$	1/0.25	1.25	I
ARPA 2351	$AjEO^1+Amx^2$	$2/32\times10^{-3}$	0.125/1	1.12	I
		$16/32\times10^{-3}$	1/1	2	I
		$16/0.5\times10^{-3}$	1/0.01	1.01	I
	TM^1+Amx^2	$8/0.5\times10^{-3}$	1/0.01	1.01	I
Staphylococcus aureus ATCC 25923	$AjEO^1+Amx^2$	$0.5/1\times10^{-3}$	0.125/0.5	0.62	Ad
		$0.5/0.5\times10^{-3}$	0.125/0.25	0.37	S
		$1/0.5\times10^{-3}$	0.25/0.25	0.50	S
		$2/0.5\times10^{-3}$	0.5/0.25	0.75	Ad
		$8/0.25\times10^{-3}$	2/0.12	2.12	I
	TM^1+Amx^2	$0.0015/1\times10^{-3}$	0.5/0.5	1	Ad
		$0.0015/0.5\times10^{-3}$	0.5/0.25	0.75	Ad
		$0.003/0.25\times10^{-3}$	1/0.12	1.12	I
MRSA 37	$AjEO^1+Amx^2$	$1/2\times10^{-3}$	0.12/1	1.12	I
		$1/0.5\times10^{-3}$	0.12/0.25	0.37	S
		$2/0.5\times10^{-3}$	0.25/0.25	0.50	S
		$2/0.25\times10^{-3}$	0.25/0.12	0.37	S
		$8/0.25\times10^{-3}$	1/0.12	1.12	I
	TM^1+Amx^2	$0.015/2\times10^{-3}$	0.12/1	1.12	I
		$0.015/0.5\times10^{-3}$	0.12/0.25	0.37	S
		$0.03/0.5\times10^{-3}$	0.24/0.25	0.49	S
		$0.03/0.25\times10^{-3}$	0.24/0.12	0.36	S

		$0.125/0.25\times10^{-3}$	1/0.12	1.12	I
		$0.125/0.06\times10^{-3}$	1/0.03	1.03	I
MRSA 4185	AjEO[1]+Amx[2]	$1/4\times10^{-3}$	0.125/1	1.12	I
		$1/2\times10^{-3}$	0.125/0.5	0.62	Ad
		$4/2\times10^{-3}$	0.5/0.5	1	Ad
		$4/0.25\times10^{-3}$	0.5/0.06	0.56	Ad
		$8/0.25\times10^{-3}$	1/0.06	1.06	I
	TM[1]+Amx[2]	$0.015/4\times10^{-3}$	0.12/1	1.12	I
		$0.015/2\times10^{-3}$	0.12/0.5	0.62	Ad
		$0.06/2\times10^{-3}$	0.48/0.5	0.98	Ad
		$0.06/0.25\times10^{-3}$	0.48/0.06	0.54	Ad
		$0.125/0.25\times10^{-3}$	1/0.06	1.06	I
		$0.125/0.125\times10^{-3}$	1/0.03	1.03	I
Streptococcus pneumoniae ATCC 49619	AjEO[1]+Amx[2]	$0.25/0.003\times10^{-3}$	1/0.04	1.04	I
		$0.25/0.015\times10^{-3}$	1/0.21	1.21	I
	TM[1]+Amx[2]	$4/0.003\times10^{-3}$	1/0.04	1.04	I
		$4/0.015\times10^{-3}$	1/0.21	1.21	I
PRSP 4423	AjEO[1]+Amx[2]	$0.03/4\times10^{-3}$	0.24/1	1.24	I
		$0.06/4\times10^{-3}$	0.48/1	1.48	I
		$0.06/2\times10^{-3}$	0.48/0.5	0.98	Ad
		$0.125/2\times10^{-3}$	1/0.5	1.5	I
		$0.125/0.125\times10^{-3}$	1/0.03	1.03	I
	TM[1]+Amx[2]	$0.03/4\times10^{-3}$	0.24/1	1.24	I
		$0.03/2\times10^{-3}$	0.24/0.5	0.74	Ad
		$0.06/2\times10^{-3}$	0.48/0.5	0.98	Ad
PRSP 4546	AjEO[1]+Amx[2]	$0.125/0.03\times10^{-3}$	0.25/1	1.25	I
		$0.5/0.03\times10^{-3}$	1/1	2	I
		$0.5/0.003\times10^{-3}$	1/0.1	1.01	I
	TM[1]+Amx[2]	$0.125/0.03\times10^{-3}$	0.5/1	1.5	I
PRSP 4566	AjEO[1]+Amx[2]	$0.06/2\times10^{-3}$	0.24/1	1.2	I
		$0.25/2\times10^{-3}$	1/1	2	I
		$0.25/0.125\times10^{-3}$	1/0.06	1.06	I
	TM[1]+Amx[2]	$0.125/2\times10^{-3}$	0.5/1	1.5	I

MICc, MIC of essential oil/antibiotic combination; FICI, the fractional inhibitory concentration index; I, Indifferent; Ad, Additive; S, Synergistic; ARPA, amoxicillin-resistant *Pseudomonas aeruginosa;* MRSA, methicillin-resistant *Staphylococcus aureus;* PRSP, penicillin-resistant *Streptococcus pneumoniae*.

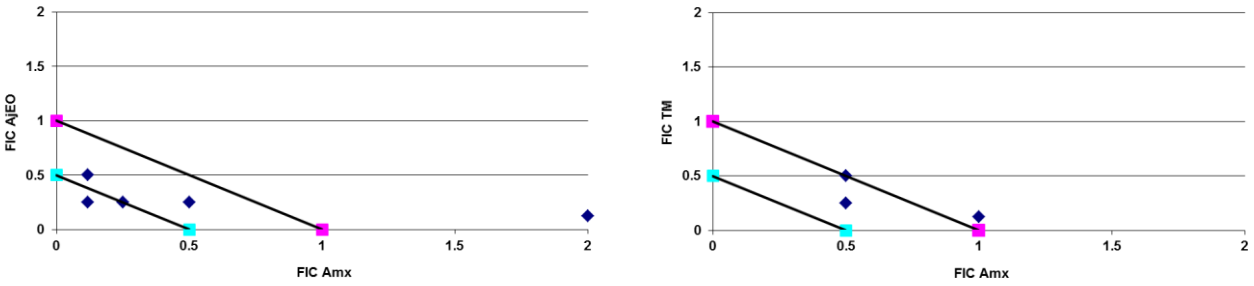
A synergistic interaction was noted for two AjEO-ciprofloxacin combinations against *Staphylococcus aureus* ATCC 25923 (FICI=0.37-0.50) (Fig. 37A) and one AjEO-ciprofloxacin combination against PRSP 4423 (FICI=0.49), causing a four-fold reduction in MIC of ciprofloxacin (0.125×10^{-3} mg mL^{-1} *vs.* 0.5×10^{-3} mg mL-1 and 0.5×10^{-3} mg mL^{-1} *vs*. 2×10^{-3} mg mL^{-1}, respectively) (Table 40). Also, one thymol-ciprofloxacin combination produced a synergistic effect against PRSP 4423 (FICI=0.49); in this case, thymol reduced MIC of ciprofloxacin by four-fold (0.5×10^{-3} mg mL^{-1} *vs*. 2×10^{-3} mg mL^{-1}) (Table 40).

Additive interactions against methicillin-resistant *Staphylococcus aureus* clinical isolates (MRSA 37, MRSA 4185) and *Streptococcus pneumoniae* pathogens (*S. pneumoniae* ATCC 49619, PRSP 4546, PRSP 4566) were also detected for AjEO-ciprofloxacin combinations (Table 40). Besides, thymol-ciprofloxacin combinations caused additive interactions against *Streptococcus pneumoniae* and *Staphylococcus aureus* pathogens (Table 40; Fig. 37B).

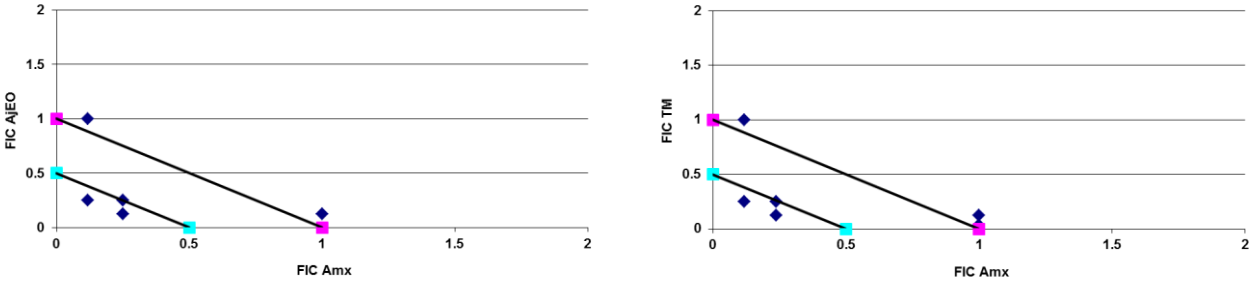
Only two AjEO-ciprofloxacin combinations and a combination of thymol and ciprofloxacin demonstrated synergy against *Pseudomonas aeruginosa* ATCC 27853 (FICI=0.37; FICI=0.46), reducing MIC of ciprofloxacin by four to eight-fold (0.125×10^{-3} mg mL^{-1}, 0.25×10^{-3} mg mL-1 *vs.* 1×10^{-3} mg mL^{-1}) (Table 40).

Overall, no antagonistic effects were observed with AjEO/thymol and ciprofloxacin or amoxicillin combinations.

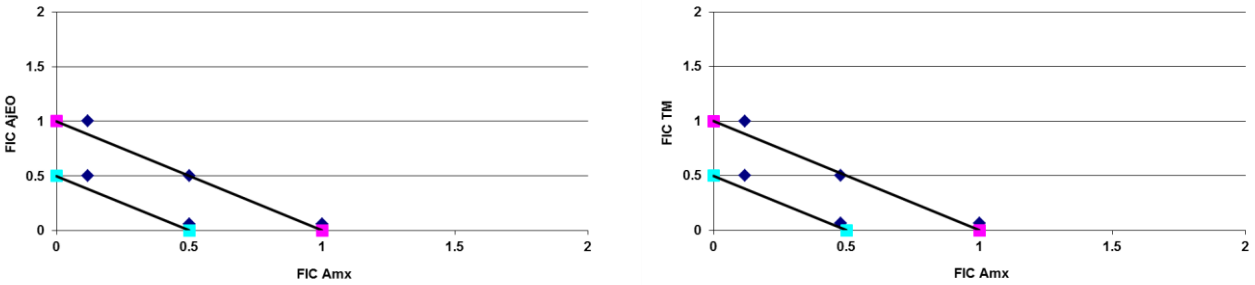
***Staphylococcus aureus* ATCC 25923**



MRSA 37



MRSA 4185



A) **B)**

Figure 36. Isobolographic plot of A) AjEO-Amx, B) TM-Amx combinations against *Staphylococcus aureus* strains [Grădinaru et al., 2018]

Table 40. Effects of combinations between ajowan essential oil (AjEO)/thymol (TM) and ciprofloxacin (Cip) against tested bacteria

Strains	**Combination**	**MIC_c (mg/mL)**	**FIC^1/ FIC^2**	**FICI**	**Outcome**
Pseudomonas aeruginosa ATCC 27853	$AjEO^1+Cip^2$	$2/0.25\times10^{-3}$	0.125/0.25	0.37	S
		$4/0.125\times10^{-3}$	0.25/0.125	0.37	S
		$8/0.125\times10^{-3}$	0.5/0.125	0.62	Ad
	TM^1+Cip^2	$0.015/0.25\times10^{-3}$	0.21/0.25	0.46	S
		$0.03/0.25\times10^{-3}$	0.42/0.25	0.67	Ad
		$0.07/0.125\times10^{-3}$	1/0.125	1.12	I
ARPA 2351	$AjEO^1+Cip^2$	$2/2\times10^{-3}$	0.12/1	1.12	I
		$8/2\times10^{-3}$	0.5/1	1.5	I
		$16/0.25\times10^{-3}$	1/0.12	1.12	I
	TM^1+Cip^2	$16/2\times10^{-3}$	2/1	3	I
		$16/0.25\times10^{-3}$	2/0.12	2.12	I
Staphylococcus aureus ATCC 25923	$AjEO^1+Cip^2$	$0.5/0.25\times10^{-3}$	0.125/0.5	0.62	Ad
		$0.5/0.125\times10^{-3}$	0.125/0.25	0.37	S
		$1/0.125\times10^{-3}$	0.25/0.25	0.50	S
		$2/0.125\times10^{-3}$	0.5/0.25	0.75	Ad
		$4/0.07\times10^{-3}$	1/0.14	1.14	I
	TM^1+Cip^2	$0.0015/0.25\times10^{-3}$	0.5/0.5	1	Ad
		$0.0015/0.125\times10^{-3}$	0.5/0.25	0.75	Ad
		$0.003/0.007\times10^{-3}$	1/0.014	1.014	I
MRSA 37	$AjEO^1+Cip^2$	$2/4\times10^{-3}$	0.25/1	1.25	I
		$2/2\times10^{-3}$	0.25/0.5	0.75	Ad

		$8/2\times10^{-3}$	1/0.5	1.50	I
	TM^1+Cip^2	$0.03/2\times10^{-3}$	0.24/0.5	0.74	Ad
		$0.125/2\times10^{-3}$	1/0.5	1.5	I
MRSA 4185	$AjEO^1+Cip^2$	$1/4\times10^{-3}$	0.125/1	1.125	I
		$1/2\times10^{-3}$	0.125/0.5	0.625	Ad
		$4/2\times10^{-3}$	0.5/0.5	1	Ad
		$4/1\times10^{-3}$	0.5/0.25	0.75	Ad
		$8/1\times10^{-3}$	1/0.25	1.25	I
		$8/0.5\times10^{-3}$	1/0.125	1.125	I
	TM^1+Cip^2	$0.03/4\times10^{-3}$	0.24/1	1.24	I
		$0.03/2\times10^{-3}$	0.24/0.5	0.74	Ad
		$0.06/2\times10^{-3}$	0.48/0.50	0.98	Ad
		$0.06/1\times10^{-3}$	0.48/0.25	0.73	Ad
		$0.125/1\times10^{-3}$	1/0.25	1.25	I
		$0.125/0.5\times10^{-3}$	1/0.125	1.125	I
Streptococcus pneumoniae ATCC 49619	$AjEO^1+Cip^2$	$0.06/0.25\times10^{-3}$	0.24/0.5	0.74	Ad
		$0.125/0.25\times10^{-3}$	0.5/0.5	1	Ad
	TM^1+Cip^2	$1/0.25\times10^{-3}$	0.25/0.5	0.75	Ad
		$2/0.25\times10^{-3}$	0.5/0.5	1	Ad
		$4/0.125\times10^{-3}$	1/0.25	1.25	I
		$4/0.07\times10^{-3}$	1/0.14	1.14	I
PRSP 4423	$AjEO^1+Cip^2$	$0.03/2\times10^{-3}$	0.24/1	1.24	I
		$0.03/0.5\times10^{-3}$	0.24/0.25	0.49	**S**
		$0.125/0.5\times10^{-3}$	1/0.25	1.25	I
		$0.125/0.25\times10^{-3}$	1/0.12	1.12	I
		$0.25/0.125\times10^{-3}$	2/0.06	2.06	I
	TM^1+Cip^2	$0.03/2\times10^{-3}$	0.24/1	1.24	I
		$0.03/0.5\times10^{-3}$	0.24/0.25	0.49	**S**
		$0.125/0.5\times10^{-3}$	1/0.25	1.25	I
		$0.125/0.125\times10^{-3}$	1/0.06	1.06	I
PRSP 4546	$AjEO^1+Cip^2$	$0.125/0.5\times10^{-3}$	0.25/1	1.25	I
		$0.125/0.25\times10^{-3}$	0.25/0.5	0.75	Ad
		$0.25/0.25\times10^{-3}$	0.5/0.5	1	Ad
		$0.25/0.125\times10^{-3}$	0.5/0.25	0.75	Ad
		$0.5/0.125\times10^{-3}$	1/0.25	1.25	I
		$0.5/0.06\times10^{-3}$	1/0.12	1.12	I

	TM[1]+Cip[2]	$0.125/0.5\times10^{-3}$	0.5/1	1.5	I
		$0.125/0.25\times10^{-3}$	0.5/0.5	1	Ad
		$0.25/0.25\times10^{-3}$	0.5/0.5	1	Ad
		$0.25/0.125\times10^{-3}$	1/0.25	1.25	I
		$0.5/0.125\times10^{-3}$	2/0.25	2.25	I
		$0.5/0.06\times10^{-3}$	2/0.12	2.12	I
PRSP 4566	AjEO[1]+Cip[2]	$0.06/0.5\times10^{-3}$	0.24/1	1.24	I
		$0.06/0.25\times10^{-3}$	0.24/0.5	0.74	Ad
		$0.125/0.25\times10^{-3}$	0.5/0.5	1	Ad
		$0.125/0.125\times10^{-3}$	0.5/0.25	0.75	Ad
		$0.25/0.125\times10^{-3}$	1/0.25	1.25	I
		$0.25/0.06\times10^{-3}$	1/0.12	1.12	I
	TM[1]+Cip[2]	$0.06/0.5\times10^{-3}$	0.24/1	1.24	I
		$0.06/0.25\times10^{-3}$	0.24/0.5	0.74	Ad
		$0.125/0.25\times10^{-3}$	0.5/0.5	1	Ad
		$0.125/0.125\times10^{-3}$	0.5/0.25	0.75	Ad
		$0.25/0.125\times10^{-3}$	1/0.25	1.25	I
		$0.25/0.06\times10^{-3}$	1/0.12	1.12	I

MICc, MIC of essential oil/antibiotic combination; FICI, the fractional inhibitory concentration index; I, Indifferent; Ad, Additive; S, Synergistic; ARPA, amoxicillin-resistant *Pseudomonas aeruginosa;* MRSA, methicillin-resistant *Staphylococcus aureus;* PRSP, penicillin-resistant *Streptococcus pneumoniae*.

Streptococcus pneumoniae, *Staphylococcus aureus* and *Pseudomonas aeruginosa* are among the most common etiological agents of the community-acquired lower respiratory tract infections. Although *S. aureus* has been mainly involved in respiratory tract infections occuring in intensive care units, over the last decade, MRSA has caused severe community-acquired pulmonary infections. Clindamycin, linezolid and vancomycin are the most commonly used antibacterial agents but they have limitations related to their severe adverse effects [Granowitz and Brown, 2008].

Combination therapy in which antibiotics are associated with bacterial membrane permeabilizers, which enhance the antibiotics' penetration into the bacterial cell, seems to be very promising for the management of multi-drug resistant bacterial infections [Hu et al., 2015]. In this study, synergistic combinations of AjEO or thymol and amoxicilin against Gram-positive bacteria such as: *Staphylococcus aureus* ATCC 25923 and MRSA 37, have been identified.

A literature survey revealed few studies on the interactions between plant extracts and amoxicillin against MRSA. Lemon grass essential oil, ethanolic extracts of *Emblica officinalis* seeds and *Nympha odorata* stamens showed synergistic activity with amoxicillin against MRSA [Mandal et al., 2010, Grădinaru et al., 2018].

The synergistic effects might be due to the fact that main constituents of AjEO and amoxicillin target the bacterial cell wall: thymol, γ-terpinene and *para*-cymene damage the bacterial membrane by different mechanisms as previously described [Oeyedemi et al., 2009;

Hyldgaard et al., 2012] while amoxicillin inhibits the bacterial cell wall synthesis [Clark et al., 2011].

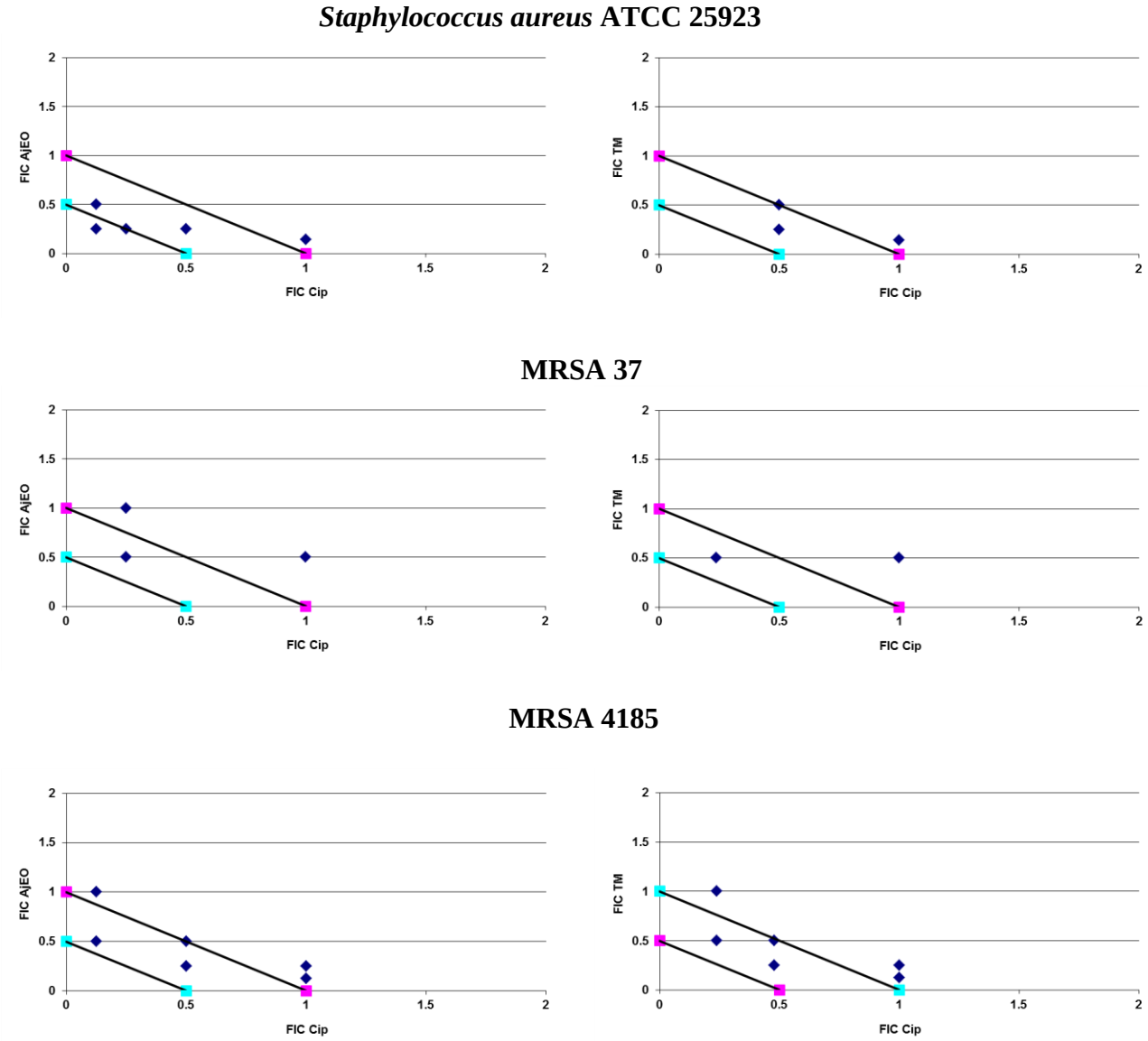


Figure 37. Isobolographic plot of C) AjEO-Cip and D) TM-Cip combinations against *Staphylococcus aureus* strains [Grădinaru et al., 2018]

As our study revealed, the combination of AjEO with ciprofloxacin produces synergistic interactions against both Gram-negative (*Ps. aeruginosa* ATCC 27853) and Gram-positive bacteria (*S. aureus* ATCC 25923, *Streptococcus pneumoniae* clinical isolate). It is worthy to note the synergistic interaction against *Ps. aeruginosa*, a common agent for a broad spectrum of severe opportunistic infections, known for its resistance to many antibiotics.

Similarly, synergistic effects have been reported for some carvacrol-rich essential oils (*Thymus maroccanus, T. broussonetii*) in combination with ciprofloxacin against *Ps. aeruginosa* clinical isolates [Fadli et al., 2012]. As regard to *S. aureus*, few studies have described synergistic interactions for essential oils of *Lavandula angustifolia*, *Pelargonium graveolens*, *Thymus vulgaris*, and *Mentha piperita* in combination with ciprofloxacin, dependent of combination ratios [van Vuuren et al., 2009; Malik et al. 2011; de Rapper et al., 2013]. Also, research conducted by Grădinaru et al. (2014, 2015) showed the activity of phenolic extracts from Romanian *Helichrysum arenarium* subsp. *arenarium* against lower respiratory tract pathogens as well as their synergistic interactions with ciprofloxacin [Grădinaru et al., 2014; Miron et al., 2015].

Phenolic compounds like thymol are able to alter not only the membrane and the cell walls; they also interfere with other bacterial cellular targets: protein and nucleic acid synthesis, enzyme activity, nutrient uptake and energy-generating processes [Souren et al., 2011].

It is likely that synergistic antimicrobial interactions between AjEO and ciprofloxacin are caused not only by the membranotropic properties of the oil, but also by other mechanisms such as capacity to interfere with the bacterial metabolic pathways and enzymes or to enable a better diffusion of antibiotics through the bacterial membrane to reach specific cellular targets [Fadli et al., 2012]. Although thymol is the main compound of AjEO and it is primarily responsible for its effects, the minor constituents can also significantly modulate the antibacterial activity.

The profile of essential oil-antibiotic combination plays also a crucial role in the design of interaction type [van Vuuren et al., 2009].

Antibacterial and combinatorial effects of CEO

Chemical composition of CEO

The hydrodistillation of cardamom fruits gave a yellowish and pleasant-scented essential oil with a yield of 9.25% (v/w). Twenty-three compounds were identified representing 94.34% of the total oil (Table 41). α-terpinyl acetate (39.59%) and 1,8-cineole (31.27%) were the major constituents in CEO. Oxygenated monoterpenes were dominant (84.54%) followed by monoterpenes (8.27%).

The higher level of α-terpinyl acetate compared to 1,8-cineole gives a superior odour quality of CEO. In addition to α-terpinyl acetate, other terpenoids such as α-terpineol, linalool, linalyl acetate and geraniol impart a sweet flavour which counterbalances the camphorated-sharp touch of 1,8-cineole. Results of present study were consistent with some earlier findings that reported α-terpinyl acetate and 1,8-cineole as main compounds in the cardamom essential oils from Guatemala, India [Thomas et al., 2006], Italy and Turkey [Kuyumcu Savan et al. 2013].

Table 41. Chemical composition of the essential oil isolated from cardamom fruits (Grădinaru et al., 2014]

Compound	RI[a]	%	Identification
Monoterpene hydrocarbons			
α-thujene	921	0.17	GC-MS
α-pinene	929	1.35	GC-MS
camphene	943	0.03	GC-MS
sabinene	970	**3.53**	GC-MS
myrcene	987	1.86	GC-MS

Δ-3-carene	1004	0.02	GC-MS
α-terpinene	1015	0.38	GC-MS
trans-β-ocimene	1045	0.14	GC-MS
γ-terpinene	1067	0.62	GC-MS
α-terpinolene	1081	0.17	GC-MS
Monoterpene alcohols			
linalool	1104	**4.73**	GC-MS
terpinen-4-ol	1179	1.75	GC-MS
α-terpineol	1198	**3.43**	GC-MS
carveol	1215	0.08	GC-MS
geraniol	1250	0.25	GC-MS
trans-anethole	1279	0.32	GC-MS
Monoterpene esters			
linalyl acetate	1256	**3.21**	GC-MS
bornyl acetate	1277	0.91	GC-MS
α-terpinyl acetate	1360	**39.59**	GC-MS
Monoterpene ketones			
carvone	1238	0.18	GC-MS
Monoterpene ethers			
1,8-cineole	1038	**31.27**	GC-MS
Sesquiterpene hydrocarbons			
β-selinene	1490	0.32	GC-MS
Sesquiterpene alcohols			
nerolidol	1539	0.03	GC-MS
Total (%)		**94.34**	

[a]Retention indices on a DB-5MS column

Antimicrobial activity of CEO

Cardamom essential oil inhibited the growth of all *Staphylococcus aureus* strains at a concentration of 6.25 mg/mL (Table 42). 1,8-cineole was more active than the CEO (MIC = 1.25-2.5 mg/mL). The methicillin-resistant clinical isolates of *Staphylococcus aureus* were most susceptible to 1,8-cineole (MIC=1.25 mg/mL).The CEO and 1,8-cineole showed an antistaphylococcal activity much lower than that of amoxicillin or ciprofloxacin.

Table 42. MIC values of cardamom essential oil (CEO), 1,8-cineole and conventional antibiotics (mg/mL) (Grădinaru et al., 2014]

Microorganisms		**CEO**	**1,8-cineole**	**Standard drugs**	
		MIC	**MIC**	**MIC amoxicillin**	**MIC ciprofloxacin**
G+	*Staphylococcus aureus* ATCC 25923	6.25	2.5	2×10^{-3}	0.5×10^{-3}
G+	MRSA 37	6.25	1.25	2×10^{-3}	4×10^{-3}
G+	MRSA 4185	6.25	1.25	4×10^{-3}	4×10^{-3}

The combinations of cardamom essential oil and amoxicillin determined additive effects (FICI=0.56-1) on MRSA 37 and MRSA 4185 isolates and indifferent effects (FICI=1.06-2) on the reference strain (Table 43, Fig. 38).

An interaction of additive type was generated with the combination of cardamom essential oil with ciprofloxacin on MRSA 4185 (FICI=1) and *Staphylococcus aureus* reference strain (FICI=0.62) (Fig. 39). One dose pair combination of 1,8-cineole and amoxicillin elicits additive effects on *Staphylococcus aureus* reference strain and MRSA 4185 (FICI=0.56 and 0.74, respectively) (Table 44, Fig. 40). Moreover, an additive interaction was demonstrated at one dose pair combination of 1,8-cineole and ciprofloxacin on *Staphylococcus aureus* reference strain (FICI=0.62).

It has been shown that cardamom essential oil exhibited strong antibacterial activity against *Listeria monocytogenes*, *Staphylococcus aureus, Escherichia coli, Yersinia enterocolitica,* and *Pseudomonas aeruginosa.* Also, cardamom essential oil is reported to inhibit the growth of fungus and food-borne microorganisms [Anwari et al., 2016].

1,8-Cineole is one of the most important antimicrobial compounds of cardamom essential oil. It acts by affecting the structural stability of the cytoplasmic membrane and increasing membrane permeability [Hendry et al., 2009].

Table 43. Combination effects of cardamom essential oil and antibiotics against *Staphylococcus aureus* strains (Grădinaru et al., 2014]

Strains	**CEO[1] + amoxicillin (A)[2]/ ciprofloxacin (C)[2]**	**MIC_c (mg/mL)**	**FIC[1]/ FIC[2]**	**FICI**	**Outcome**
Staphylococcus ATCC 25923	CEO + A	6.25/2 $x10^{-3}$	1/1	2	I[a]
		6.25/0.125$x10^{-3}$	1/0.06	1.06	I
	CEO + C	3.125/0.06$x10^{-3}$	0.5/0.12	0.62	**A**[b]
		6.25/0.06$x10^{-3}$	1/0.12	1.12	I
MRSA 37	CEO + A	0.7/2 $x10^{-3}$	0.11/1	1.11	I
		0.7/1 $x10^{-3}$	0.11/0.5	0.61	**A**
		3.125/1 $x10^{-3}$	0.5/0.5	1	**A**
		3.125/0.125$x10^{-3}$	0.5/0.06	0.56	**A**
		6.25/0.125$x10^{-3}$	1/0.06	1.06	I
	CEO + C	6.25/4 $x10^{-3}$	1/1	2	I
		6.25/0.06$x10^{-3}$	1/0.01	1.01	I
MRSA 4185	CEO + A	0.7/4 $x10^{-3}$	0.11/1	1.11	I
		0.7/2 $x10^{-3}$	0.11/0.5	0.61	**A**
		6.25/2 $x10^{-3}$	1/0.5	1.5	I
		6.25/0.125 $x10^{-3}$	1/0.03	1.03	I
	CEO + C	3.125/4 $x10^{-3}$	0.5/1	1.5	I
		3.125/2 $x10^{-3}$	0.5/0.5	1	**A**
		6.25/2 $x10^{-3}$	1/0.5	1.5	I
		6.25/0.06 $x10^{-3}$	1/0.01	1.01	I

FIC, fractional inhibitory concentration; FICI, FIC indexes; MIC_c, MIC of essential oil/antibiotic combination; [a]Indifferent; [b]Additive.

Table 44. Combination effects of cardamom essential oil and 1,8-cineole against *Staphylococcus aureus* strains (Grădinaru et al., 2014]

Strains	1,8-cineole (Ci)[1] + amoxicillin (A)[2]/ ciprofloxacin (C)[2]	MIC_c (mg/mL)	FIC[1]/ FIC[2]	FICI	Outcome
Staphylococcus aureus ATCC 25923	Ci + A	$1.25/0.125x10^{-3}$	0.5/0.06	0.56	**A**
	Ci + C	$1.25/0.06x10^{-3}$	0.5/0.12	0.62	**A**
MRSA 37	Ci + A	$1.25/2\ x10^{-3}$	1/1	2	I
		$1.25/0.125x10^{-3}$	1/0.06	1.06	I
	Ci + C	$1.25/4\ x10^{-3}$	1/1	2	I
		$1.25/0.06x10^{-3}$	1/0.01	1.01	I
MRSA 4185	Ci + A	$0.3/4\ x10^{-3}$	0.24/1	1.24	I
		$0.3/2\ x10^{-3}$	0.24/0.5	0.74	**A**
		$1.25/2\ x10^{-3}$	1/0.5	1.5	I
		$1.25/0.125\ x10^{-3}$	1/0.03	1.03	I
	Ci + C	$1.25/4\ x10^{-3}$	1/1	2	I
		$1.25/0.06x10^{-3}$	1/0.01	1.01	I

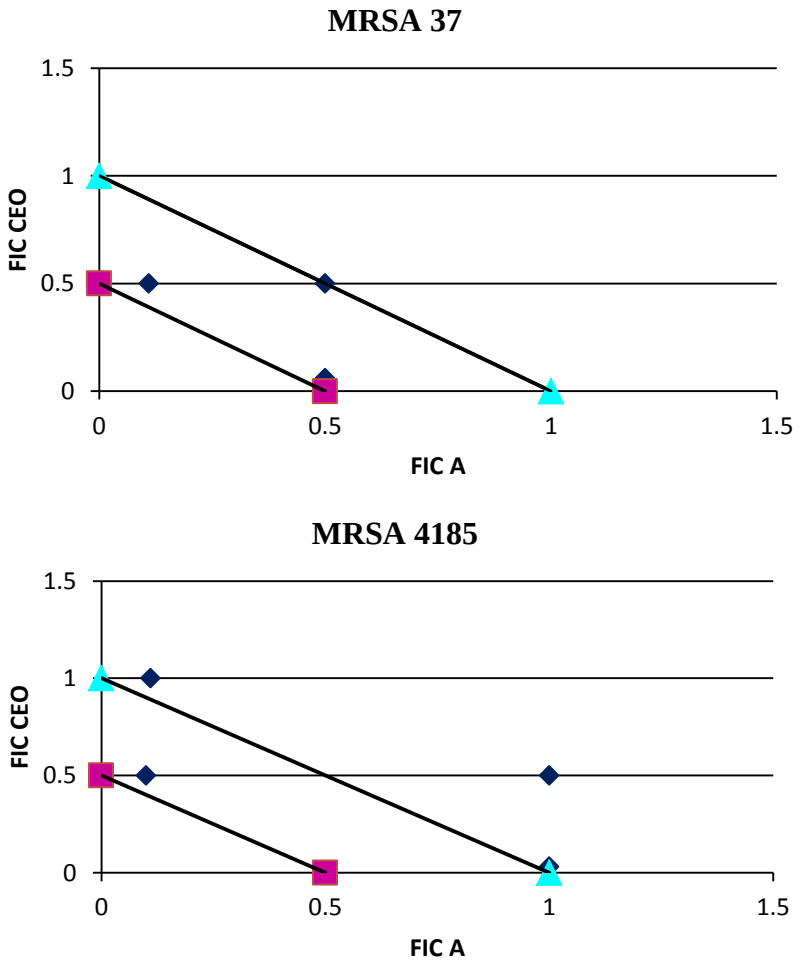


Figure 38. Isobolographic plot of CEO-A combinations against MRSA strains

Our findings are in agreement with data from previous studies. Although antistaphylococcal activity of 1,8-cineole was better compared to that of essential oil, the interaction potential of essential oil with antibiotics was higher.

Thus, we noticed a greater number of combinations of cardamom essential oils with amoxicillin or ciprofloxacin that function additive on *Staphylococcus aureus* bacteria, mainly on MRSA strains.

No antagonistic interactions were identified, a fact which supports the combined use of cardamom essential oil with conventional antibiotics to treat infectious diseases caused by *Staphylococcus aureus*, especially MRSA.

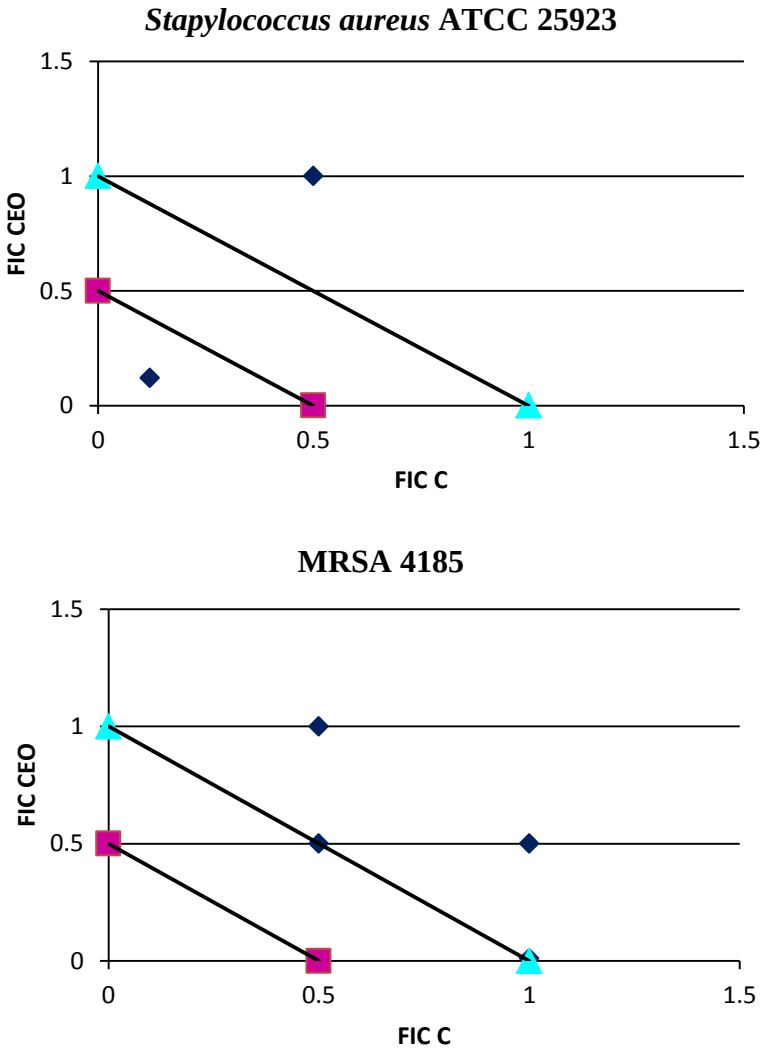


Figure 39. Isobolographic plot of CEO-C combinations against *Staphylococcus aureus* strains

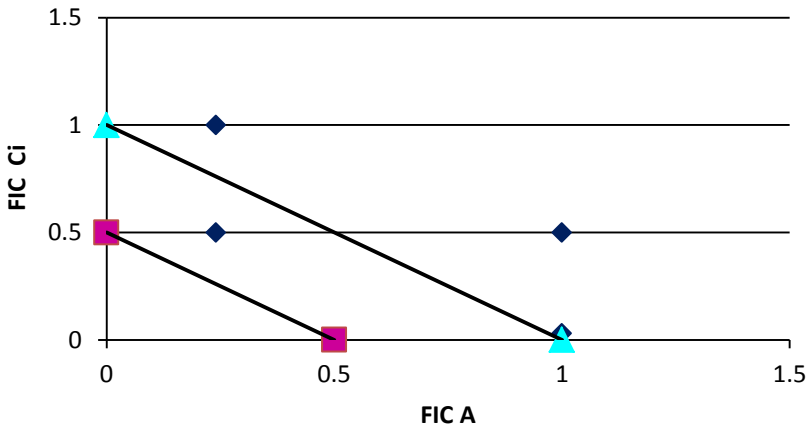


Figure 40. Isobolographic plot of Ci-A combinations against MRSA 4185 pathogens

Conclusions

Our study reveals for the first time the antibacterial effects of combinations based on ajowan and cardamom essential oils and their major volatiles and conventional antibiotics against multi-drug resistant respiratory pathogens. The results are encouraging because of the following findings:

- ajowan essential oil and thymol are able to improve the effectiveness of ciprofloxacin in the treatment of infections with *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and penicillin-resistant *Streptococcus pneumoniae* pathogens;
- ajowan essential oil and thymol can improve efficiency of amoxicillin in the therapy of infections with *Staphylococcus aureus* and methicillin-resistant *S. aureus* bacteria;
- combinations of cardamom essential oil or 1,8-cineole with amoxicillin or ciprofloxacin exerted additive effects on methicillin-resistant clinical isolates;
- the concomitant administration of cardamom essential oil or 1,8-cineole with amoxicillin or ciprofloxacin does not adversely affect the activity of these antibiotics in infections with *Staphyloccocus aureus* strains.

The assessment of the therapeutic application of essential oils-antibiotics synergistic combinations potential requires further investigation to explore their functionality *in vivo* and to understand the synergy mechanism.

Conclusions about topic 3

There has been a growing interest in developing antimicrobial agents to combat the infections resulting from drug (multidrug)-resistant human pathogens. Among various natural agents, essential oils and their major volatiles possess important bioactivities including a promising antimicrobial potential, mainly the essential oils from Lamiaceae plants. The use of essential oils combined with antibiotics is an alternative strategies that enhance the efficacy of antibiotics and it can overcome drug resistance.

Future studies should explore the mechanism of interactions between volatiles and antibiotics on various microbial strains. The initiation of a systematic research including a screening about the dynamic interactions between antimicrobials and pathogens and the study on clinical efficacy of these combinations are also needed.

I.3. General conclusions on research achievements

To summarize, my postodoctoral research in Pharmacognosy domain was mainly focused on:

1) the studies regarding the influence of abiotic (pesticide treatments, climate conditions, agronomic tehniques) and biotic factors (plant phenological events) on morpho-anatomical features, chemical composition and biological activity of medicinal plants, and
2) the assessment of biological properties (antioxidant, cytogenoprotective, antimicrobial) of polyphenolic extracts and essential oils in relation to their chemical composition.

The results were communicated in various national and international scientific events and also published in various journals (**over 100 papers**, of which **41 ISI** articles with **cumulative impact factor 84.546**) and 4 chapters in international books.

These data could be used to determine the impact of climate changes on metabolome and of agronomic techniques on therapeutic and nutritional qualities of medicinal plants.

Also, results of biological studies support the research in order to identify new antimicrobials and cytogenoprotective agents useful in radioprotection.

SECTION II. SCIENTIFIC, PROFESSIONAL AND ACADEMIC FUTURE DEVELOPMENT PLANS

II.1. Perspectives in scientific activity

From a scientific point of view, I will continue my research in the presented directions but I intend to expand and diversify the studies on essential oils and natural polyphenols, as follows:

- assessment of chemistry and biological properties (antioxidant, antimicrobial) of essential oils related to the development of approaches that to enhance the stability, biodistribution and promotes bioavailability and efficacy of these vegetal metabolites.
  In this respect, one of the topics under investigation concerns the encapsulation of monoterpene phenole-rich essential oils (thyme, oregano, savory essential oils) in chitosan-based hydrogels with tailored architectures and responsiveness as drug delivery systems in antimicrobial topical therapy but also with possible food and cosmetic applicability.
- investigation of the antigenotoxic and anticlastogenic potential of polyphenol-rich fractions that then could support the development of products for the protection of people whose jobs involve a chronic exposure to radiations (occupational exposure) or patients undergoing radiotherapy.
  In this respect, the research will focus on the effect of plant polyphenolic fractions (pre-, simultaneous and post-treatments) against genotoxic and cytotoxic damage and clonogenic death of normal and tumor cell lines induced by X-radiation, using tests such as:
  - cell viability assay,
  - analysis of micronucleus induction,
  - quantification of DNA damage using comet assay,
  - enzymatic comet assay (in order to quantify DNA repair),
  - clonogenic survival, free radical scavenging potential,
  - evaluation of endogenous antioxidants activity (measurement of SOD, CAT, GPx activities, and GSH and lipid peroxidation levels),
  - apoptosis quantification (Annexin V – FITC assay) and
  - cell cycle analysis (propidium iodide – RNAse assay).
- assessment of chemistry and biological properties of plant polyphenols (antioxidant, antimicrobial, antitumor, radioprotective) related to the development of approaches that to enhance their stability and bioavailability or to optimize their pharmaceutical formulation (*site-specific carrier targeting*).

Continuing and strengthening the collaboration with my colleagues from the faculty and other research institutions, but also extending the collaboration, is another major objective to achieve these goals.

Teamwork and interdisciplinarity are the important features of current research and I strongly believe that the valuable scientific outcomes can only obtained in a research team.

In order to acces funds for research and to be able to achieve important results I aim to apply in future calls for research projects.

I will use my knowledge for the formation of curious, creative and dedicated future researchers.

II.2. Perspectives in professional and academic activity

In the changing and extremely fluid modern society, with an intense and dynamic information flow and with various challenges in the pharmaceutical and medical fields, there is a continuous need for adaptation to the new requirements. These are also valid issues in educational terms. In this respect, my future efforts will be oriented towards carrying out activities that will lead to an increase in the efficiency of teaching and learning process and to develop a student-centered education.

For the development of didactic activity I will consider the following main objectives:

- continuous improvement and updating of the courses and stages of practic works as well as a good documentations of the lectures,
- coordination of well systematized practical works,
- elaboration of new materials for the course and practical works of Pharmacognosy,
- improving the teaching style and the interactive approach,
- stimulating the interest of students for independent work and the specific thinking skills related to the pharmaceutical and medical fields;
- use of alternative methods of evaluation in compliance with deontological requirements;
- providing permanent positive feedback to students in relation to their results and the capitalization of these findings to optimize teaching activity;
- participation in postgraduate courses or other training programs to improve my professional and practical skills.

In addition, I will support through my activity the process of students professionalization by:

- active involvement of students in the scientific activities within the Student Scientific Circle of Pharmacognosy as well as in research projects,
- coordinating scientific works of students,
- coordinating graduation theses,
- stimulating students to participate to scientific events or to involve in different research or social projects.

Another important objective of my professional activity will be to strenghten or develop relationships with other disciplines within Faculty of Pharmacy, within the University of Medicine and Pharmacy Grigore T.Popa Iasi, or with other Faculties of Pharmacy or Medicine in the country, as well as partnerships with other academic or research institutions.

I will also strengthen my personal development by participating to training courses in the academic or scientific fields within the University of Medicine and Pharmacy Grigore T.Popa Iasi or in other authorized institutions.

I intend to be actively involved in the academic life of university by participating in professional and administrative activities and also in the development of inter-institutional relationships to support high visibility of the institution where I work for over 22 years.

The development of my future career will also follow a harmonization of the mentioned directions, and in all these I consider that the desire for knowledge, teamwork, communication, respect and honesty are essential values that define you professionally and humanly.

SECTION III.

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