GRIGORE T. POPA UNIVERSITY OF
MEDICINE AND PHARMACY IASI

MEDICINAL PLANTS FROM TRADITIONAL USE TO SCIENTIFIC-BASED THERAPY: OLD REMEDIES AND INNOVATIVE APPROACHES

HABILITATION THESIS

ASSOC. PROF. OANA CIOANCA PHD, MSC, PHARMD

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Abstract

This habilitation thesis summarizes the most relevant part of my research results obtained since my PhD thesis, defended in June 2010. It is a cumulative document providing an overview of various scholarly works studying medicinal plants interdisciplinarity and modern approaches within the last 11 years of research and 18 years of academic activity.

Initially, the thesis includes a brief presentation of my academic and scientific career evolution in section I. This part of the thesis indicates the most significant steps undertaken to evolve in knowledge, pedagogic and technical skills. The type of lectures/practical lessons and teaching programs in which I was involved are presented. Also, there are elements of my implication in student research coordination and academic visibility (as guest editor or reviewer for prestigious ISI journals). Moreover, some elements from my professional experience and jobs are mentioned. Section I represents a comprehensive collection of all my academic and didactic skills, along with my implication in various admission committees, exam committees, research grants, national and international awards conferred to me.

The second section of the habilitation thesis presents the most important results obtained during the last decade of my research development and knowledge acquisition in the vast area of medicinal plants. This section is divided into two main parts, chemical assessment and current scientific evidence of the biomolecular potential of selective herbal extracts.

Chemical profile assessment of herbal extracts is an important element in phytochemistry and phytotherapy and represents the basis for the fundamentalism of future use of vegetal extracts in tests and therapy. Therefore, my thesis indicates two main groups of natural molecules that were of interest for my research: fixed or non-volatile compounds and volatile terpenoids. Each of these two classes was investigated by high-resolution hyphenated techniques correlated to their chemical structure and physicochemical properties. This first chapter of Section I presents briefly the methodology and results obtained for some of the investigated herbal extracts containing either flavonoids, polyphenolic acids, iridoids, alkaloids or essential oils rich in monoterpenes.

Current scientific evidence of the biomolecular potential of selective herbal extracts is the chapter that presents the most significant research in terms of evidencing the biologic potential of previously investigated herbal extracts and natural molecules. Starting from the most known antioxidant potential, investigated by assays that indicate the scavenging or chelating potential of free radicals and enzyme inhibition tests, to the evaluation of the antibacterial and antifungal activity of various selective extracts against standard or clinically isolated strains, all data were obtained through interdisciplinary collaboration with various departments and labs from “Grigore

T. Popa" University of Medicine and Pharmacy from Iasi, Research Institute of the University of Bucharest or "Victor Babes" University of Medicine and Pharmacy from Timisoara.

The collaboration with these departments was extended to the evaluation of *in vitro* cell viability, on normal or melanoma cell lines. The obtained results being published in prestigious national and international ISI journals.

The second subchapter of *Current scientific evidence of the biomolecular potential of selective herbal extracts* indicates the most prolific part of my research interest which was part of my postdoctoral scholarship program and one Young research team grant funded by "Grigore T. Popa" University of Medicine and Pharmacy from Iasi in which I was the coordinator. The aria is related to neuroprotection and the beneficial potential of terpenoids or flavonoid-rich selective herbal extracts in various *in vivo* models of Alzheimer's disease (AD), mild cognitive impairment (MCI) and Parkinson's (PD). The animal models (rat or *Danio rerio*) were obtained by the collaboration with the Department of Animal physiology from the Faculty of Biology, "Al. I. Cuza" University of Iasi. The obtained results starting from 2012 till the present were published in 27 ISI papers in national and international journals, some of which are either Q1 and Q2 rated.

Most of the obtained data suggest that the investigated extracts possess neuroprotective, antidepressant, anxiolytic and antidementia properties by antioxidative and memory-enhancing mechanisms. This underlines the significance of innovative approaches for medicinal plant research. All correlations, challenges and significance of the results are included in the third subchapter of this part.

The third chapter, Th*eory vs. research: current trends and future challenges*, presents briefly two new research directions that are related to medicinal plants extracts. These areas involve nanoparticle and multifunctional systems (based on large particle inclusion – e.q. cyclodextrins) formulation. Such areas will enhance the bioavailability of the natural molecules that often have lipophilic characteristics and lower solubility in usual pharmaceutical preparations.

The second section of the thesis briefly describes the future trends on the academic level with the incorporation of the research results in educational materials and research topics of interest in the proposed field.

Given all the presented facts, defending my habilitation thesis ***Medicinal plants from traditional use to scientific-based therapy: old remedies and innovative approaches*** is a critical next step in my independent research activity, with all my previous experiences, skills, results and publications (in natural compounds research and education, pharmacognosy, cell biology, animal testing, neurosciences) supporting, in every single part, the proposed career plan and all aspects of the presented investigations.

Rezumat

Teza de abilitare intitulată „***Plantele medicinale de la utilizarea tradițională la terapia bazată pe dovezi: remedii vechi și abordări inovatoare*** " reflectă principalele repere ale activității profesionale, academice și științifice începând cu perioada postdoctorală și până în prezent. Totodată sunt incluse perspectivele academice și stiințifice urmărite în vederea dezvoltării profesionale viitoare.

Inițial, în secțiunea I teza include o scurtă prezentare a evoluției carierei mele academice și științifice. Această parte a tezei indică cei mai relevanți pași întreprinși pentru a dobândi cunoștințe, abilități pedagogice și tehnice. Sunt prezentate tipul de cursuri/lecții practice și programe de predare în care am fost implicată. De asemenea, sunt surprinse contribuțiile proprii în coordonarea cercetării studenților și vizibilitatea academică (în calitate de editor invitat sau recenzor pentru reviste ISI de prestigiu). Mai mult, sunt menționate câteva elemente din experiența mea profesională și locurile de muncă. Secțiunea I reprezintă o colecție cuprinzătoare a tuturor abilităților mele academice și didactice, împreună cu implicarea mea în diferite comisii de admitere, comitete de examen, granturi de cercetare, premii naționale și internaționale obținute pe parcursul carierei mele didactice și de cercetare.

Cea de-a doua secțiune a tezei de abilitare prezintă cele mai importante rezultate obținute în ultimul deceniu al cercetării mele și al achiziției de cunoștințe în vastul domeniu al plantelor medicinale. Această secțiune este divizată în două părți principale, evaluarea chimică și dovezile științifice actuale ale potențialului biomolecular al extractelor selective de origine vegetală.

Evaluarea profilului chimic al extractelor vegetale este un element important în fitochimie și fitoterapie și reprezintă fundamentarea utilizării viitoare a extractelor vegetale în variate teste și în terapie. Astfel, teza mea indică două grupuri principale de molecule naturale care au fost de interes pentru cercetarea mea: compuși fixi sau nevolatili și terpene volatile. Fiecare dintre aceste două clase a fost investigată prin tehnici moderne, de înaltă rezoluție, corelate cu structura lor chimică și proprietățile fizico-chimice. Acest prim capitol al secțiunii I prezintă pe scurt metodologia și rezultatele obținute pentru unele dintre extractele de plante investigate care conțin fie flavonoide, acizi polifenolici, iridoide, alcaloizi sau uleiuri volatile bogate în monoterpene.

Dovezile științifice actuale ale potențialului biomolecular al extractelor selective de origine vegetală este capitolul care prezintă cele mai importante rezultate în ceea ce privește evidențierea potențialului biologic al extractelor vegetale și moleculelor naturale cercetate anterior. Toate datele în ce privește potențialul antioxidant (investigat prin teste care indică efectul de scavenger sau chelare al radicalilor liberi și testele de inhibare a unor enzime) și evaluarea activității antibacteriene și antifungice a diferitelor extracte selective față de tulpini standard sau izolate clinic, s-au obținut prin colaborare interdisciplinară cu diverse departamente și laboratoare de la Universitatea de Medicină și Farmacie „Grigore T. Popa” din Iași, Institutul de Cercetare al

Universității din București sau Universitatea de Medicină și Farmacie „Victor Babeș" din Timișoara.

Colaborarea cu aceste departamente a fost extinsă pentru evaluarea viabilității celulare *in vitro*, pe linii celulare normale sau de melanom uman. Rezultatele obținute au fost publicate în reviste ISI naționale și internaționale de prestigiu.

Al doilea subcapitol al dovezilor științifice actuale ale potențialului biomolecular al extractelor selective de origine vegetală, indică cea mai prolifică parte a cercetării proprii, care a făcut parte din programul meu de bursă postdoctorală și un grant de cercetare de Tinere echipe finanțată de Universitatea de Medicină și Farmacie „Grigore T. Popa" din Iași, în care am fost director de proiect. Domeniul de cercetare include neuroprotecția și potențialul benefic al terpenoidelor sau al extractelor selective bogate în flavonoide evaluate pe diferite modele *in vivo* de boală Alzheimer (AD), afectare cognitivă ușoară (MCI) și Parkinson (PD). Modelele animale (șobolan sau *Danio rerio*) au fost obținute prin colaborarea cu Departamentul de Fiziologie animală de la Facultatea de Biologie, Universitatea „Al. I. Cuza" din Iași. Rezultatele obținute începând cu 2012 până în prezent au fost publicate în 27 de lucrări ISI în reviste naționale și internaționale, dintre care unele sunt cotate fie Q1 sau Q2.

Majoritatea datelor obținute sugerează faptul că extractele investigate posedă proprietăți neuroprotectoare, antidepresive, anxiolitice și antidementive prin mecanisme antioxidante și de îmbunătățire a memoriei. Acest lucru subliniază semnificația abordărilor inovatoare pentru cercetarea plantelor medicinale. Toate corelațiile, provocările și semnificația rezultatelor sunt incluse în cel de-al treilea subcapitol al acestei părți.

Al treilea capitol, Teorie vs. cercetare: tendințe actuale și provocări viitoare, prezintă pe scurt două noi direcții de cercetare care pentru extractele din plante medicinale. Aceste domenii implică formulări de nanoparticule și sisteme multifuncționale (bazate pe incluziunea în particule mari - ex. ciclodextrine). Prin astfel de formulări noi se urmărește creșterea biodisponibilității moleculelor naturale care au adesea caracteristici lipofile și solubilitate mai mică în preparatele farmaceutice uzuale.

A doua secțiune a tezei descrie pe scurt tendințele viitoare la nivel academic cu integrarea rezultatelor cercetării în materiale educaționale și subiecte de cercetare de interes în domeniul propus.

Având în vedere toate elementele prezentate, susținerea tezei mele de abilitare „ ***Plantele medicinale de la utilizarea tradițională la terapia bazată pe dovezi: remedii vechi și abordări inovatoare*** " reprezintă un pas critic în activitatea mea independentă de cercetare, cu toate experiențele, abilitățile, rezultatele și publicațiile mele anterioare (în cercetare și educație a compușilor naturali, farmacognozie, biologie celulară, teste *in vitro/in vivo*, neuroștiințe) susținând, fiecare în parte, planul de carieră propus și toate aspectele investigațiilor prezentate.

Teaching and researching career evolution

After graduating from the Faculty of Pharmacy, in 2003, I enrolled in a PhD in Pharmacy - Pharmacognosy, when I analyzed the pharmaceutical quality of commercial samples of chamomile under the coordination of Professor Dr Ursula Stanescu. I continued to deepen my knowledge in this domain during the master "Herbal medicines from origination to use" (2005, "Grigore T. Popa") and the residency in Clinical Pharmacy (2006–2010). I defended my thesis in 2010 and in the same year I obtained my Board Certification in Clinical Pharmacy.

In terms of teaching, my career started with an initial year (October 2003-October 2004) of voluntary tutorship within Pharmacognosy Discipline, Department of 2nd Pharmaceutical Sciences, from the Faculty of Pharmacy, "Grigore T. Popa" University of Medicine and Pharmacy from Iasi, Romania. The next year I applied for a tutor post within the same discipline and after taking the examination I was hired as a full employee. Due to unforeseen circumstances, dependent on the institution, my employment was delayed till the next university year. However, during this time I acted as an honorary tutor for disciplines of Pharmacognosy and Cell biology, attending both laboratories, seminars and assisting during lectures. Later on, I was allowed to teach during seminars and lab work. Also, I was involved in adapting the laboratory lessons to the newer requirements from the pharmaceutical network.

During my teaching years I taught labs, seminars and lectures both in Romanian and English in the following areas:

- Pharmacognosy
- Phytotherapy/Phytochemistry
- Cell and molecular biology
- Toxic plants
- Vegetal product technology
- Bioactive compounds of natural origin
- Food supplements and functional foods
- Orthomolecular medicine.

Such subjects were part of my activity as a tutor, assistant, lecturer and as an associate professor for the Faculty of Pharmacy - Pharmacy and Assistant Pharmacy Bachelor degree programs and for the Faculty of Medicine –Nutrition and dietetics Bachelor degree program, Nutrition and Prophylactic and Curative diet Master's degree program and The Complementary Certificate programme in Practical Phytotherapy and Phytopharmacology for medical doctors, under the supervision of Prof Dr Monica Hancianu as the national coordinator.

Moreover, a part of these subjects (cell biology, pharmacognosy and phytotherapy) were adapted to English and included in the English Bachelor degree program for Faculty of Pharmacy,

which were taught by me to the 1st year, 3rd year and 4th year students respectively. For the 3rd year English students, I put together a coursebook published in 2015 (Cioancă O, Miron A, Trifan A, Aprotosoaie AC, Hăncianu M. *Concepts of pharmacognosy*. Iaşi: Ed. UMF „Gr. T. Popa" Iaşi, 2015, 178 pag, ISBN 978-606-544-341-9).

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

My publishing activity includes 10 books (2 as first author and 8 as coauthor), 9 chapters and one international book chapter.

On the other hand, my didactic activity was also materialized in the coordination of more than 180 diploma and dissertation theses for the students of the Faculty of Pharmacy, for the graduates of Assistance of Pharmacy Program (Faculty of Pharmacy), Nutrition and Dietetics Bachelor degree program, Nutrition and Prophylactic and Curative diet Master's degree program (Faculty of Medicine), University of Medicine and Pharmacy Grigore T. Popa Iasi, in the field of cell biology, Pharmacognosy, Phytotherapy, Food supplements and functional foods. I also coordinated several diploma theses for the students from the English program in the Faculty of Pharmacy.

Starting with 2005, I was involved in the coordination of a student research group from the Discipline of Pharmacognosy, some of the obtained results being presented within national and international Student and young doctors' congresses/meetings. The most significant accomplishment, besides various prizes won by the students (Adriana Trifan, Gabriel Moroșan, Olga Pungă, Ioana – Lavinia Ştefan, Liviu Croitoru, Bogdan Cimpoiașu, Roxana Mihaela Costea, Bogdan Crețu, Ionuț Lungu, Mihaela Magdalena Flutur etc.), is the winning of the scientific performance scholarship by Roxana Mihaela Costea (in 2016), a student I collaborated through all her studying years and for which I supervised the diploma thesis. Also, Adriana Trifan for whom I was the Bachelor diploma supervisor became my youngest colleague in the discipline of Pharmacognosy.

Moreover, I attended various workshops and students' conferences as an invited speaker:

- SSFI National Congress Workshop, Iasi 2018 – 2 workshops,
- Specialized courses for international students from France, May 2019, Iasi,
- Erasmus Plus - „You are my challenge", 10-15 February 2020, Iasi.

As a challenge for my teaching abilities, I applied and was selected for an Erasmus Plus teaching mobility. The mobility took place at the Faculty of Pharmacy, Lithuanian University of Health Sciences, Kaunas, Lithuania, during the first weeks of September 2019. I hold several lectures on Phytotherapy for the English program students within the Department of Pharmacognosy; I met several peers in the same field but I also met various researchers from the biomedical field from other faculties from Kaunas. Through this experience, I established a new collaboration and was later (November 2019) invited as a plenary speaker for pharmacy specialists, doctors, nurses and students at the 10th International Pharmaceutical Conference „Science and

Practice 2019" 15th of November in the Lithuanian University of Health Sciences, Faculty of Pharmacy, Kaunas, Lithuania.

The experience in biomedical research I have gained by participating as a member of numerous research projects (12), two of which were bilateral cooperation (with the University of Cyprus - Department of Biological Sciences and the University of Presov - Slovakia, Department of Ecology). In the four years after the completion of the thesis, I acquired several practical and technical skills, especially in the isolation and fractionation of extracts/plant components such as polyphenols, flavonoids, anthocyanins, and volatile components from both indigenous and exotic plant products.

I evaluated the intra- and inter-species variability of the same genus, in this direction by working with the Centre for Biological Research in Piatra Neamt and "Anastasie Fatu" Botanical Garden of Iasi. We have also assessed the quality of the active principles from biological cultures, thus emphasising the influence and impact of environmental factors on raw materials of plant origin. To this end, I attended training in modern analytical techniques such as spectrophotometry, gas chromatography coupled with mass spectrometry, liquid chromatography high performance (RP-HPLC) and liquid chromatography-ultra fast (reduced time of analysis and pressures above 400 bar). To complete spectrum analysis and especially to assess the biological potential of compounds/extracts of medicinal and aromatic plants, we used *in vitro* analysis of the antioxidant potential (free radical scavenger, iron-chelating ion, lipoxygenase inhibitor, reducing capacity).

I have also studied the influence of pesticides on microorganisms and morphological characteristics and their metabolites synthesis capacity. Based on these studies, we used subsequently *in vitro* tests showing antimicrobial and cytotoxic properties of active principles.

Since 2012, I have initiated a close collaboration with Prof. dr. Lucian Hritcu from the Department of Animal Physiology, Faculty of Biology of the University "Al I Cuza", which was a new area of research, namely, expanding areas phytotherapy Ethnopharmacology and the neurophysiology of behaviour by highlighting the benefits of plant components isolated from the raw material of known and safe origin on animal models (neuroprotective, anxiolytics, antidepressants, antioxidant, stimulation of memory).

Deepening the research areas in the 8 years of close collaboration with Prof. Habil. Lucian Hriţcu has resulted in an internal grant, funded by the "Grigore T. Popa" University of Medicine and Pharmacy Iasi, that I coordinated as the project manager and in 30 ISI articles using animal models of AD induced by scopolamine administration or amyloid-beta (1–42) who were administered oils (lavender, juniper, coriander) and plant extracts rich in polyphenols and alkaloids (piperine) (Hritcu et al., 2012; Hancianu et al., 2013; Cioanca et al., 2014). From 2013 through Mr Prof. dr. Lucian Hritcu, we extended collaboration with Dr Galba Jean Beppe (University of Maroua, Cameroon) and Dr Jaures Noumedem (University of Dschang, Cameroon). The phytochemical and biochemical studies have been conducted on exotic species (*Albizia adianthifolia, Piper nigrum, Markhamia tomentosa, Lactuca capensis*), with results demonstrating

the neuroprotective effects of selected extracts, published in several articles in ISI journals (Beppe et al., 2014; Hritcu et al., 2014; Ionita et al., 2017) with high impact factor.

Recently, the collaboration was extended to Turkey. In the last 2 years, we have published together with the collective of researchers conducted by Prof. Ilkay Erdogan Orhan two papers in Q1 and Q2 ISI indexed journals (Antioxidants and Molecules).

All the results above illustrate effectively scientific achievements in the fields of medicinal plants and herbal medicines as sources of bioactive compounds for pharmaceutical formulations providing a solid foundation for future development of medicines.

Thanks to valuable results obtained in the period 2012–2021, I published 87 articles in ISI (ResearcherID: E-2127-2014), with 682 citations (without self-citation) with a Hirsch index of 16, which shows high visibility, with the fact that most of the ISI papers published are the last 6 years.

(a) ***Information about the degrees and diplomas***

- 1998–2003, Bachelor of Pharmacy, "Grigore T. Popa" University of Medicine and Pharmacy Iasi, Romania
- 2004–2005, Master: The drug of vegetable origin from the obtaining to marketing, University of Medicine and Pharmacy "Grigore T. Popa" Iasi, Romania
- 2003–2010, PhD in Pharmacy - Pharmacognosy, Thesis (Research on the pharmaceutical quality of *Chamomillae flos*, Scientific Supervisor: Prof. Univ. dr. Ursula Stanescu), University of Medicine and Pharmacy "Grigore T. Popa" Iaşi, Romania
- 2006–2010, Board Certified Specialist in Clinical Pharmacy, OM 1535 / 23.12.2010 - 19. Oct.2010 session certified MS S1 series, no. 007 312, Ministry of Health.

(b) ***Professional experience and jobs***

1) 2020-present Associate Professor - Department of Pharmacognosy Phytotherapy, Cell Biology "Grigore T. Popa" University of Medicine and Pharmacy Iasi
2) 2013–2020 Lecturer - Department of Pharmacognosy Phytotherapy, Cell Biology "Grigore T. Popa" University of Medicine and Pharmacy Iasi
3) 2008–2013 Assistant Professor - Department of Pharmacognosy, Phytotherapy, Cell Biology "Grigore T. Popa" University of Medicine and Pharmacy Iasi
4) 2005–2008 Tutor - Department of Pharmacognosy, Phytotherapy, Cell Biology "Grigore T. Popa" University of Medicine and Pharmacy Iasi
5) 2004–2005 Honorary Teaching - Department of Pharmacognosy, Phytotherapy, Cell Biology "Grigore T. Popa" University of Medicine and Pharmacy Iasi
6) 2003–2004 Trainee Pharmacist - Pharmacy Hipocratfarm Iasi, Romania

Skills: Pharmacognosy, Phytochemistry, Clinical Pharmacy, Phytomedicine, Neurobiology and Cell Biology. Researcher ID: E-2127-2014, Address Profile **http://www.researcherid.com/rid/E-2127-2014**

I participated as a project manager in the implementation of an internal research grant and as a member of the research team, in 12 other projects, of which 2 are international, as follows:

1) Internal Grant UMF Grigore T. Popa Iasi: Young Team section - 1642 / 2.1.2013 from the University of Medicine and Pharmacy Iasi Characterization of biological effects of oils rich in monoterpene alcohols with relevance to neuroprotection; grant director; 2013 - 2014, 5000 euro.
2) National Grant PN-III-P1-1.1-TE2019-1894 - Compounds of plant origin with potential use in onychomycosis: strategies based on synergies and nanoformulations; member, 2019-2022.
3) Internal Grant UMF Grigore T. Popa Iasi: Young Team section - contract no. 7246/2018, Investigations on antifungal activity of natural products with putative use in onychomycosis, member, 2018-2020.
4) Internal Grant UMF Grigore T. Popa Iasi: Ideas section, 2012, contract no.1639/2013: Investigations on the radioprotective potential of some vegetal extracts, member, 2013-2015.
5) International Bilateral cooperation with the University of Presov, Slovakia - Monitoring of anthocyanin content in selected plant species and determination of their antioxidant activity (funded UEFISCDI), member; April 2013-December 2014.
6) Internal Grant TE UMF Iaşi 17539/09/2011 Demonstration of antioxidant effect in vitro and in vivo macromycete species; member; 2011 - 2012, 5000 euro.
7) NP II Project, Programme 4 Partnerships in priority areas Complex Projects (CP) - Biotechnology for obtaining plant metabolites useful in the prophylaxis and therapy Orthomolecular (project no. 61–39/2007); member (Partner 3 - UMF "Gr. T. Popa" Iasi); 2007–2010; 350,000 lei,
http://www.umfiasi.ro/Cercetare/Granturi/Pages/parteneriate2007.aspx
8) Project-oriented CDI (INNOVATION) - Phytopreparations counteracting metabolic imbalances (contract no. 33 / 25.09.2007); member (Partner 3 - UMF "Gr. T. Popa" Iasi); 2007–2010; 150,000 lei,
http://www.umfiasi.ro/Cercetare/Granturi/Pages/parteneriate2007.aspx
9) International cooperation with the Department of Biological Sciences, University of Cyprus - Identification of new medicinal plants with potential use in the prophylaxis and treatment of malignancies; member; 2007–2009; 7126 lei,
http://www.umfiasi.ro/Cercetare/Granturi/Pages/Proiecte-Internationale-finalizate.aspx
10) Project PNCDI II 60/2007 - Obtaining Phytopreparations prevention of cardiovascular disease; member (Partner 3-UMF "Gr. T. Popa" Iasi); 2007–2010; 500.000lei,
http://www.umfiasi.ro/Cercetare/Granturi/Pages/parteneriate2007.aspx

11) Grant CNCSIS 817/2006 - Studies on the possibility of recovery of cabbage leaves in therapeutics: Synthesis and characterisation of extractive fractions immunomodulatory and antioxidant properties, member; 2006–2008.
12) CEEX/BIOTECH / Mode I type P-CD 77/2006 - Getting biotechnology of preparations acting on the neuro-immuno-cutaneous (2006–2008), member (partner 3-Pharmacy "Gr. T. Popa »Iaşi) 2006–2008.
13) CEEX/BIOTECH / Mode I type P-CD 77/2006 - Phytomedication potential anti-ageing obtained from biotechnology (2005–2008), member (partner 3 - Pharmacy "Gr. T. Popa" Iasi); 2005–2008.

Specialised training courses

1) "Recognition of pain, suffering and distress and its application in the evaluation of the severity of the procedures (species-specific: mice and rats)" Edition 2, provided by Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise «G. Caporale», from 22 October 2020 to 31 December 2020.
2) Postdoctoral Scholarship - 2014 in biomedical research, under the "Programme of Excellence in doctoral and postdoctoral research in multidisciplinary chronic diseases", contract no. POSDRU/159 / 1.5 / S / 133377.
3) Participation PU course "Applications of GC and HPLC analytical", Iasi, March-May 2011, the University of Medicine and Pharmacy "Gr T Popa" Iasi.
4) Participation in IT skills training programme in the use Campus Management Dashboard Solution within MEDICALIS, HRD/86 / 1.2 / S / 62594.

Awards

1) "Professor Bologna" Distinction, Professor Bologna Gala, ANOSR, Iasi, between May 23-26, 2019.
2) Excellence Award for Pharmaceutical Book - Pharmacists Gala, Bucharest, 08.12.2016, 5th Edition, (Stănescu U, Hăncianu M, **Cioancă O**, Aprotosoaie AC, Miron A. Medicinal plants from A to Z, 2nd ed., Revised and added, Polirom Publishing House, Iasi, 2014, ISBN 978-973- 46-4694-4.)
3) Best Presentation Award from the Phytochemical Society of Europe – MAPPPS/PSE - within the National Symposium with International Participation "MEDICINAL PLANTS - PRESENT AND PERSPECTIVES", Piatra Neamt, Romania, September 6 to 9, 2016, for the oral presentation of the scientific research entitled "New insights regarding the biologic potential of a standardized chamomile extract"
4) "Young Researchers Award " in the Romanian National Pharmacy Congress, 24–27 September 2014, Iasi, Romania
5) Best Presentation Award - SGEM 2013 Albena, Bulgaria, June 16 to 22, 2013 for outstanding accomplishment and Contribution 1314-27-04 ISSN, DOI: 10.5593/ sgem2013

6) Bronze Medal - EUROINVENT 2012 Iasi, 12.05.2012, (Clavinic antineoplastic alkaloid product type and Preparation process of veterinary use thereof, authors: Red CM, Rotinberg P, Z Olteanu, Surdu S, E Truta Mihai CT, Hritcu L, D. Gherghel, Hăncianu M, Miron A, Aprotosoaie AC, **Cioancă O**)
7) Best Lecture Presentation - 1st International Symposium Chamomile, June 2006, Presov, Slovakia

Since 2012, I have been a member of the advisory and guidance committees for several PhD students from the Discipline of Pharmacognosy and one from the Discipline of Nuclear Medicine:

- Cristina Elena Iancu (studies on the chemical composition and biological activity of some *Pelargonium* species, Scientific coordinator Prof dr. Monica Hancianu),
- Gradinariu Veronica (Contributions to the Identification and Characterization of Some Vegetable Extracts from Indigenous or Acclimated Species in Romania with favourable Activity in Neurodegenerative Disorders, Scientific coordinator Prof dr. Monica Hancianu),
- Simion Vlad Luca (Bioactive Metabolites From Verbascum Species, Scientific coordinator Prof dr. Anca Miron),
- Denisa Batir-Marin (The Chemical and biological study of some species of *Pteridophytae* endemic to Moldova county, Scientific coordinator Prof dr. Monica Hancianu),
- Flavia Burlec (Chemical and biological evaluation of extractive fractions from ornamental *Asteraceae* species, Scientific coordinator Prof dr. Monica Hancianu)
- Ioana Humulescu (Contributions to the pharmacognostic study of some species of *Sideritis* and *Teucrium*, Scientific coordinator Prof dr. Monica Hancianu),
- Adam Gigi (Pharmacognostic and biological study of *Perilla frutescens*, Scientific coordinator Prof dr. Monica Hancianu),
- Ioana Buciscanu (Assessment of possible interactions with the concomitant administration of extracts/substances of plant origin with synthetic medicinal products, Scientific coordinator Prof dr. Monica Hancianu),
- Andrei Florin Paduraru (Study of extracts of plant origin with potential neuroprotective effects, Scientific coordinator Prof dr. Monica Hancianu),
- Remo Dorneanu (Secondary Metabolites of Vegetable Origin with Antibacterial Potential in Urinary Tract Infections, Scientific coordinator Prof dr. Monica Hancianu),
- Roxana Iacob Gherasim (Research on early and evolutionary molecular imaging diagnosis in cognitive deficits of various aetiologies and Alzheimer's disease, Scientific coordinator Prof dr. Cipriana Stefanescu),

Lately, I participated as an official member of the Reviewer Board of PhD thesis: *Study of extracts of plant origin with potential neuroprotective effects*, elaborated by pharm. Andrei Florin Paduraru (Scientific Supervisor: Prof. Monica Hăncianu, PhD); the defence of PhD thesis on the 15.04.2021.

Also, I am a member of various committees for admission to doctoral, master and licence (bachelor degree) studies and I am a member of the Ministry of Health Specialized Examination Committee for doctors who want to obtain the certificate of complementary studies in Phytotherapy and Practical Phytopharmacology.

Over the years, I took part in different academic activities as part of the University community, as follows:

- Member of the Senate of University of Medicine and Pharmacy Grigore T. Popa, Iaşi (2020-present);
- Member of the 2nd Pharmaceutical sciences Department Council, Faculty of Pharmacy, University of Medicine and Pharmacy Grigore T. Popa, Iaşi (2016-2020);
- Research Ethics Committee of University of Medicine and Pharmacy Grigore T. Popa, Iaşi (2016-2019);
- Doctoral Admission Commission - Faculty of Pharmacy University of Medicine and Pharmacy Grigore T. Popa, Iaşi (2016-2020);
- Professional Commission for Plant Biology involved in formulating admission tests to the Faculty of Pharmacy (since 2016);
- Member of the Commissions for the presentation of dissertation master's theses (since 2016);
- Member of the Commission for International Relations and Academic Partnerships of Faculty of Pharmacy (2016-present);
- Member of the Teacher's Consulting Group of the University during the World Federation For Medical Education Evaluation, November 2020
- Member of the Job Competition Committee for the position of Associate Professor in Cell Biology, Genetics and Medicinal Plants, at the Ovidius University, Constanta - 5879 / 28.12.2020.

Since 2019 I have participated in promoting the Faculty of Pharmacy and the University of Medicine and Pharmacy Grigore T. Popa, Iaşi, by coordinating special classes in high schools (Falticeni, 2019).

Moreover, in the last five years, I acted as a reviewer for different ISI and IDB journals, of which the most important are:

- ✞ Antioxidants (Q1, IF 6.312)
- ✞ Biomolecules (Q2, IF 4.879)
- ✞ BMC Complementary and Alternative Medicine (Q1, IF 3.659)

- Complementary Therapies in Clinical Practice (Q2, IF 2.446)
- Farmacia (Q4, IF 1.433)
- Fitoterapia (Q3, IF 2.882)
- Food and Chemical Toxicology (Q1, IF 6.023)
- Food Bioscience (Q2, IF 4.240)
- Industrial Crops and Products (Q1, IF 5.645)
- Journal of Pharmacy and Pharmacology (Q2, IF 3.765)
- Molecules (Q2, IF 4.411)
- Pharmaceutical Biology (Q2, IF 3.503)
- Pharmaceutics (Q1, IF 6.321)
- Phytomedicine (Q1, IF 5.340)
- Phytotherapy Research (Q1, IF 5.878)
- Processes (Q3, IF 2.847)
- Current Journal of Applied Science and Technology (IDB)

Also, since October 2020 I am a member of the editorial staff of ISI-listed journals:

- Topic editor - Biomolecules
- Guest editor - BMC Complementary and Alternative Medicine.

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

- Conference of Practical Phytotherapy (2017, 2019, 2021);
- MAPPPS/Phytochemical Society of Europe Meeting: Phytochemicals in Medicine and Pharmacognosy (2016),
- National Congress of Pharmacy in Romania, 15th edition (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 achievements

In the last two decades, the importance of medicinal plants has increased tremendously. Often the discussions on medicinal plants are divided into two main groups, those that favour the traditional medicine approaches and those who rely solely on scientific proofs. Therefore, there are many controversies between practitioners. Although traditional medicine (TM) has used plants and natural compounds for therapy, most of the indications are empirical and come from acquired pieces of information from elders within a certain society. Alternatively, scientific-based therapy represents the proof of an efficient treatment strategy and brings light on the less known natural compounds and their biological implications. Nowadays, there are numerous herbal or food supplements, which can be divided into traditional herbal preparations (THP) and herbal medicinal products (HMP). The last group includes all pharmaceutical formulations with vegetal extracts and should comply with specific regulations (certification of the plant origin and identity – scientific name of the used species, standardization where possible, if not at least the drug extract ratio – DER specification). However, the regulations vary from one country to another and are mainly related to the development and the economic status of the region. Although harmonisation was discussed between European and American provisions, a consensus is yet to be reached.

Interestingly, traditional medicine use is more affordable and to many, the traditional concepts are close to their ideology (natural, non-toxic, no side effects, no age limits). Moreover, TM is adopted in chronic and life-threatening ailments (cancer, various infectious diseases), especially when patients are not compliable with the synthetic compounds. Nevertheless, THP is taken as food supplements or over-the-counter medicines with no consideration to other prescription drugs or indications given by the specialist, medicinal plants have an unexpected outcome. For example, the most common clinical cases indicate that patients do not consult with their doctor before THP use, thus resulting in a series of incompatibilities with the current medication. More frequently antagonistic or synergic effects have been documented, leading to a necessity in medication change or prolonged treatment after toxic events clearance. Moreover, THP products may be misidentified, adulterated, microbiologically contaminated or even containing toxic substances. This is especially true for uncontrolled products, their safety and efficacy are questionable (Ernst, 2005; Ribnicky, 2008). Besides, most of the herbal products available on the market are uncharacterized both chemically and qualitatively. This poses a challenge for the authorities and consumers mainly because there is no certainty that the mixtures included in such preparations will present similar effects and potency as the high-quality plant extracts obtained and evaluated in a controlled environment (for which there is scientific proof). The mare inclusion of such testing for a single plant or several plants within the leaflet or packaging (as most of the food supplements producers do) is not enough to certify a mixture of unknown provenience is effective or safe, as long as the research is not referring to the product itself.

Currently, the HMP market gains more credit due to higher quality products that are controlled according to food supplements or even medicine requirements. In such cases, the producer provides a series of evidence-based indications and documents. Furthermore, the provenience of the raw material and the plant extract is known. Also, the dose amount is specified and within the efficacy limit, which ensures a higher-quality product. There is still a great need for research, standards and reliable fingerprints to promote consistent quality herbal medicines which can be translated to clinical trials and human consumption.

Studies conducted during my doctoral thesis (Studies regarding the pharmaceutical quality of *Chamomillae flos*, coordinated by Prof. Ursula Stanescu PhD, PharmD) led me to understand the importance of vegetable raw material quality for therapeutic use. Given all of the above, postdoctoral research was orientated towards the evaluation of the chemical composition as well as of the biological properties of some extracts or fractions obtained from indigenous and exotic plant species. The most important results of my research collaboration are given below comprehensively.

I. Chemical profile assessment

I.1. General aspects

As I mentioned previously, the quality and origin of the plant material have a great impact on the chemical compounds. Although there is a natural interspecific and intraspecific variance of the component's spectra, various factors can change tremendously the chemical profile of a plant. Extrinsic (climate, soil, nutrients, humidity, sunlight, pollinators etc.) and intrinsic (genetics, age, growth mode, dispersal and habitat preference etc.) factors have a great influence on the biosynthetic capacity of each individual. This is why there is an important difference between THP and HMP, which is made of less or more standardised plant materials. More than that, the dose and effect are different and there is no chance to find common ground between these herbal products.

In pharmacognostic research, the first steps include the macroscopic and the microscopic features of the plant material, which confirm the identity of the dry vegetal product which is used to obtain the extracts/fractions. Since such aspects are considered basics and have long been discussed in my PhD thesis, I have not considered them as necessary for this thesis although for every vegetal product I have considered every aspect of the origin and identity of the investigated plants.

Beginning with the chemical aspects, one can establish reliable parameters for standardization, thus ensuring the existence of a pharmacological/biological effect. This is why my postdoctoral research documented first various methods for the identification and quantification of several types of active compounds. A higher and richer compound spectrum represents a desirable parameter for all extractive fractions that is under investigation.

Qualitative and quantitative methods are often used, such as TLC (Thin Layer Chromatography), spectrophotometry, GC (gas chromatography) and HPLC (high-pressure liquid chromatography). Recently, hyphenated techniques are preferred, GC-MS (gas chromatography coupled with mass spectrometry) or UHPLC-DAD-MS/QTOF (ultra-high-pressure liquid chromatography coupled with various detectors – multiarray diode detector, mass spectrometry or even quadrupole time of flight).

My research used both basic analytical methods and hyphenated techniques. My colleague, Assoc. Prof. Adrian Spac taught me the basics and assisted me in acquiring such techniques. With his help, I learnt to develop methods for various classes of natural compounds such as flavonoids, polyphenolic acids, clavinic alkaloids, anthocyanidins, iridoids, ecdysone, sesquiterpene lactones and volatile terpenes.

Some of the published articles in this area are:

1. Rosu C, Aprotosoaie AC, Rotinberg P, Gherghel D, Mihai C, Olteanu Z, Miron A, Surdu S, **Cioanca O**, Hancianu M. The biochemical investigations of some *Claviceps purpurea* bioproducts and their in vitro cytostatic potential. Farmacia. 2011;59(5):713-720.
2. Hritcu L., **Cioanca O.,** Hancianu M. Effects of lavender oil inhalation on improving scopolamine-induced spatial memory impairment in laboratory rats. Phytomedicine, 2012. 19(6):529-534.
3. Grădinariu V, **Cioancă O**, Gille E, Aprotosoaie AC, Hriţcu L, Hăncianu M. The chemical profile of basil biovarieties and its implication on the biological activity. Farmacia. 2013;61(4):632–9.
4. Salamon I, Grulova D, Hancianu M, **Cioanca O**. Optimization of lyophilization technology for purification and stabilization of anthocyanins from elderberry fruits. InI International Symposium on Elderberry 2013. Acta Horticulturae. 2015, 1061:245–252.
5. Hritcu L, Noumedem JA, **Cioanca O,** Hancianu M, Kuete V, Mihasan M. Methanolic extract of *Piper nigrum* fruits improves memory impairment by decreasing brain oxidative stress in amyloid-beta (1–42) rat model of Alzheimer's disease. Cellular and molecular neurobiology. 2014 Apr 1;34(3):437–49.
6. Mircea C, **Cioancă O,** Draghia L, Hăncianu M. Morphological characteristics and polyphenol variations in *Rudbeckia hirta* L. Romanian Biotechnological Letters. 2015a;20(4):10688.
7. Mircea C, **Cioancă O**, Draghia L, Hăncianu M. Morphological Characteristics, Phenolic and Terpenoid Profiles in Garden *Chrysanthemum* Grown in Different Nutritional Conditions. Notulae Botanicae Horti Agrobotanici Cluj-Napoca. 2015b;43(2):371–379.
8. Mircea (Arsene) CC, **Cioancă O,** Ivănescu B, Draghia L, Hăncianu M. The influence of fertilisation on the ornamental characters and on the alantolactone and triterpenic acids

profiles in *Echinacea purpurea*. Lucrări Ştiinţifice Seria Horticultură, U.S.A.M.V. Iaşi 2015c; 58(2): 261-266.

9. Burlec AF, Arsene C, Gille E, Hăncianu M, **Cioancă O.** Ornamental Asteraceae Species as New Sources of Secondary Metabolites. Indian J Pharm Edu Res. 2017;51(3): S425-428.
10. Iancu C, **Cioanca O**, Hancianu M, Mircea C. Dried extracts and essential oils of some *Pelargonium* species: chemical and biological assessment. Indian J Pharm Edu Res. 2017;51(3): S421-424.
11. Dorneanu R, **Cioancă O,** Chifiriuc O, Albu E, Tuchiluş C, Mircea C, Salamon I, Hăncianu M. Synergic benefits of *Aronia melanocarpa* anthocyanin-rich extracts and antibiotics used for urinary tract infections. Farmacia. 2017;65(5):778–83.
12. **Cioancă O**, Pagonakis A, Trifan A, Hriţcu L, Ioniţă R, Burlec AF, Postu P, Mircea C, Hăncianu M. Pharmacognostic and pharmacologic screening of *Crocus sativus* of Greek origin. Farmacia 2017, 65(3): 401-406.
13. Sadiki FZ, El Idrissi M, Sbiti M, Lemrhari A, Trifan A, **Cioanca O**, Postu PA, Hritcu L. Chemical composition and antibacterial activity of essential oil of *Tetraclinis articulata* (Vahl) Masters branches of eastern Morocco. Chemical and Biological Technologies in Agriculture. 2018;5(1):24.
14. Danciu C, Zupko I, Bor A, Schwiebs A, Radeke H, Hancianu M, **Cioanca O**, Alexa E, Oprean C, Bojin F, Soica C, Paunescu V, Dehelean CA. Botanical Therapeutics: Phytochemical Screening and Biological Assessment of Chamomile, Parsley and Celery Extracts against A375 Human Melanoma and Dendritic Cells. Int J Mol Sci. 2018;19(11):3624.
15. Burlec AF, Pecio Ł, Mircea C, **Cioancă O,** Corciovă A, Nicolescu A, Oleszek W, Hăncianu M. Chemical Profile and Antioxidant Activity of *Zinnia elegans* Jacq. Fractions. Molecules. 2019;24(16):2934.
16. Păduraru AF, **Cioancă O,** Mircea C, Trifan A, Aprotosoaie AC, Miron A, Gille E, Hritcu L, Hăncianu M. Bioactive Extracts from Cultivated *Ajuga genevensis* L. and *A. reptans* L.: In Vitro/In Vivo Pharmacological Effects. Farmacia. 2019;67:603-9.
17. Danciu C, **Cioanca O,** Hancianu M, Racoviceanu R, Muntean D, Zupko I, Oprean C, Tatu C, Paunescu V, Proks M, Diaconeasa Z. Botanical Therapeutics (Part II): Antimicrobial and In Vitro Anticancer Activity against MCF7 Human Breast Cancer Cells of Chamomile, Parsley and Celery Alcoholic Extracts. Anti-Cancer Agents in Medicinal Chemistry (Formerly Current Medicinal Chemistry-Anti-Cancer Agents). 2021;21(2):187-200.
18. **Veterinary patent no. 128512 B1, OSIM - C12N 1/14; C12N 1/38; C12N 15/00**; Clavinic antitumor preparation for veterinary use and process for preparing the same, Patent Number: RO128512-A2; RO128512-A8; authors: Craița Maria Roșu, Pincu Rotinberg, Zenovia Olteanu, Ștefania Surdu, Elena Truță, Cosmin Teodor Mihai, Luminița Hrițcu, Daniela Gherghel, Monica Hancianu, Anca Miron, Ana Clara Aprotosoaie, **Oana Cioancă**

I. 2. Methods

The **chromatographic methods for volatiles** (GC-MS) were based on the following method, which was adjusted to each vegetal product particularity's (debit, flow, temperature, scan time): GC-MS/FID analysis of the volatile oil was performed using an Agilent 6890 GC-MS system, equipped with a split/splitless injector (200°C) and the used parameters were as follows: temperature 280°C, carrier gas (1 mL/min) helium, capillary column DB 5MS (30-m x 0.25 mm; film thickness 0.25 µm; Agilent, Palo Alto, CA, USA), injected volume 0.30 µL, scan time 32 min. The thermal program was 40°C−280°C at a rate of 10°C /min; a split ratio of 100:1. The temperature programme was established to grow with 10 °C/min up to 280 °C (Adams, 2007; Uhlig et al., 2007).

The comparison of the retention indices (RI), retention times (RTs) and mass spectra with those obtained from authentic Wiley libraries (available through Hewlett Packard) and published mass spectra (Adams, 2007) were used to identify the compounds. The GC-FID analysis used an Agilent type 6890 GC connected to an FID detector and all method and analysis parameters were the same as described above.

Kovats retention index (KI) is used to convert RT into non-dependent constants, has no measurement unit and is calculated by interpolation of the peak of a certain compound between two adjacent n-alkanes. To record KI values of n-alkanes (8–20 and 21–40) for further reference, aliquots of the solutions (0.30 µL) were subjected to GC analysis in similar conditions to the samples. The calculation equation is as follows:

$$KI_x = 100\,n + 100\,(t_x - t_n) / (t_{n+1} - t_n)$$

where,

- ✓ n is the number of carbon atoms in the n-alkane eluting before (the smaller alkane)
  - the investigated compound,
- ✓ t_x is the retention time of the chosen compound (X),
- ✓ t_n is the retention time of the reference n-alkane hydrocarbons eluting immediately before (the smaller alkane) the compound,
- ✓ t_{n+1} is the retention time of the reference n-alkane hydrocarbons eluting immediately after the compound.

For each analysis, two replicates of the volatile oil were processed in the same way. Acquisition mass range 40–400 Amu (atomic mass unit, Dalton) (Adams, 2007; Boiangiu et al, 2020; Van den Dool and Kratz, 1963).

Regarding the **liquid chromatography** (UHPLC), the method included an HPLC system (either Agilent 1200 or Thermo UltiMate 3000) that included a quaternary pump, a variable range

injection loop (usually a 25 µL loop), an autosampler (for Thermo UltiMate 3000), a column (selected depending on the investigated samples or correlated to the active compounds that will be identified) and a diode array detector (PDA) used especially for UV and VIS spectral detection. Table I includes several columns used for the analysis and quantification of various classes of compounds.

Table. I. Chromatographic columns used in my research

Column type	LC system	Analyzed compounds
Zorbax Eclipse XDB-C18 column (150 x 4.65 mm, 5 µm)	Agilent 1200 - PDA	flavonoids, polyphenolic acids, anthocyanidins
Micropak Amino (250 x 2.1 mm, 10 µm)	Agilent 1200 - PDA	clavinic alkaloids
Zorbax Eclipse XDB-C18 column (150 x 4.6 mm, 5 µm)	Agilent 1200 - PDA	piperine
Accucore XL C18 column (150 × 4.6 mm, 4 µm).	UHPLC Thermo Ultimate 3000 -PDA	flavonoids, polyphenol carboxylic acids, hydroxycinnamic acids anthocyanidins, crocin and picrocrocin esters, sesquiterpene lactones
Luna PFP (150 x 4.6 mm, 4 µm)	UHPLC Thermo Ultimate 3000 -PDA	iridoids, ecdysones

Each of these classes of compounds can be separated and isolated based on their solubility in a mixture of solvents of variable polarity. Due to quaternary pumps, both systems enable the mixture of the mobile phases in various gradients without the intervention of the analyst. Therefore, concentration mixtures are more precise. However, the choice of solvents and their concentrations must be chosen before analysis as their mixture impacts greatly the separation, identification and quantification of all substances present in the herbal extract. Usually, reverse-phase liquid chromatography uses gradients of polar (water, alcohol, acids) and non-polar solvents (acetonitrile or other lipophilic solvents according to the column characteristics). For phytocomplexes that are a mixture of various compounds from different chemical groups, isocratic phase elution is interposed with the linear gradient of the solvents.

Another variable in LC /HPLC/UHPLC is the flow rate, which is chosen depending on the complexity of the investigated sample, the column and the system capabilities.

As a reliable and quicker technique, I recently adapted the known methods to a newer system - UHPLC (Thermo Ultimate 3000 system). UHPLC means ultrahigh-pressure liquid

chromatography and enables faster analysis at higher pressures (above 400 bar) compared to normal HPLC (max. 400 bar). This method decreases the analysis costs and diminishes the volume of the solvents used during sample elution.

For example, the most recent UHPLC method included a Thermo Ultimate 3000 system with a Luna PFP column. The following parameters were used for iridoid identification: detection wavelength - 245 nm, flow rate – 0.8 mL/min, linear-gradient (1% phosphoric acid: acetonitrile) from 38% to 55% in 20 min, and then 55%-100% in 15 min; injection volume – 2.5 µL. Harpagide, catalpol, aucubin and 8-O-acetyl-harpagide (Sigma Chemical Co.) were used as standards. Polyphenols were identified by scanning absorbance from 240 nm to 520 nm with the same system, using a gradient of acetonitrile (A) and 0.1% acetic acid (B) (10% - 23% (A) in 5 min; 23% (A) isocratic for 10 min and then 23% - 35% (A) in 12 min; 35% - 70% (A) for 5 min).

Authentic standards of HPLC grade (Sigma Chemical Co.) were used to obtain the calibration curves. Samples of UV spectra were automatically compared by Chromeleon 7.2.v12 software and the concentration was expressed as a percentage of the standard's aria/concentration.

All chromatographic experiments were performed at 28°C in triplicate and average values were used for calibration curves and quantification of the identified compounds. NIST and Wiley libraries were used for confirmation.

I.3. Results and discussions

Some of the most significant results are presented in the figures below and their relevance for the study is also discussed accordingly.

I.3.1. Analysis, identification and quantification of non-volatile compounds

As a continuation of my PhD studies and for the broadening of my research three sources of apigenin were investigated chemically and biologically. This study represented the beginning of my collaboration with colleagues from the same department in the Faculty of Pharmacy from "Victor Babeş" University of Medicine and Pharmacy, Timisoara, Romania. In this research, we selected three sources, all rich in apigenin and its derivatives that were used for the obtaining of selective extracts to be investigated for the cytotoxic potential The most significant results were published in two papers Danciu C, Zupko I, Bor A, Schwiebs A, Radeke H, Hancianu M, **Cioanca O**, Alexa E, Oprean C, Bojin F, Soica C, Paunescu V, Dehelean CA. Botanical Therapeutics: Phytochemical Screening and Biological Assessment of Chamomile, Parsley and Celery Extracts against A375 Human Melanoma and Dendritic Cells. Int J Mol Sci. 2018;19(11):3624 and Danciu C, **Cioanca O**, Hancianu M, Racoviceanu R, Muntean D, Zupko I, Oprean C, Tatu C, Paunescu V, Proks M, Diaconeasa Z. Botanical Therapeutics (Part II): Antimicrobial and In Vitro Anticancer Activity against MCF7 Human Breast Cancer Cells of Chamomile, Parsley and Celery Alcoholic

Extracts. Anti-Cancer Agents in Medicinal Chemistry (Formerly Current Medicinal Chemistry-Anti-Cancer Agents). 2021;21(2):187-200).

Chamomile, with the scientific name *Matricaria chamomilla* L., also known as German chamomile is an aromatic plant belonging to the Asteraceae family. Asteraceae Bercht. & J.Presl family is also called Compositae due to the composite character of flowers within this family. It is one of the largest families comprising more than 23 000 species included in over 1900 genera (Jeffrey et al., 2007). It was asserted that the biological activity of different types of extracts is due to the flavonoids (apigenin, luteolin, quercetin, patuletin) and essential oil (α bisabolol and its oxides, azulenes) (Srivastava et al., 2009). The main classes of phytochemicals with biological activity that can be found in the flowers of chamomile include volatile oil (which in turn is composed of sesquiterpenes azulenes and non-azulenes as well as monoterpenes), flavonoids, coumarins, terpenoids, and mucilage. The main biological activities include antioxidant, antimicrobial, antiinflammatory, cytotoxic, antispasmodic, antiviral, sedative potential (Park et al., 2017). The antiproliferative potential of chamomile extract was described for various cell lines including human prostate epithelial PZ-HPV-7 cells and human prostate cancer LNCaP, DU145, and PC-3 cells, T-47D breast carcinoma, HeLa -cervical adenocarcinoma, HT1080- fibrosarcoma, RKO-colon carcinoma cells (Srivastava et al., 2007).

Parsley and celery are also aromatic plants belonging to the family Apiaceae. The Apiaceae family is also known under the name Umbelliferae Juss. represents the 16th largest family of flowering plants and comprises approximately 3000–3750 species included in 300–455 genera (Downie et al., 2000). Parsley and celery are two important constituents of this family used both for their culinary and medical benefits. A comprehensive review that presents the ethnopharmacology, phytochemistry and biological activities of parsley, also known under the scientific name of *Petroselinum crispum* (Mill.) Nym. ex A.W. Hill, assigns *in vitro* antioxidant, analgesic, spasmolytic, immunosuppressant, laxative and diuretic properties to the seeds extract (Farzaei et al., 2013). A recent study has shown that extracts obtained from the leaves and stem of English parsley present antioxidant capacity as well as a protective effect against DNA damage induced by H2O2. Moreover, the extract has inhibited the proliferation and migration of the MCF7 breast cancer cell line (Tang et al., 2015). Celery seeds extracts have been described for their antioxidant, antimicrobial, antiarthritic and antiulcer potential (Kooti et al., 2017; Powanda et al., 2015). The research group of Mansi has also found that the extract can induce a hypolipidemic effect in rats (Mansi et al., 2009). Anti-inflammatory, gastro-protective, anti-*Helicobacter pylori* activity and no toxicologically significant sub-chronic effects in experimental models using rats were reported by the group of Powanda et al. (Powanda et al., 2011). The wild celery oil was assigned with antiproliferative potential against HCT116 human colon carcinoma cells (Quassinti et al., 2014).

In a comprehensive review about this medicinal aromatic plant, Srivastava et al., have estimated the presence of approximately 120 secondary metabolites including 28 terpenoids and 36 flavonoids (Srivastava et al., 2010). From the class of flavonoids, apigenin represents one of the most promising compounds from the point of view of bioactivity. Within the vegetal product, it exists predominantly in the glycosidic form whereas the aglycone can be found in small amounts (Svehlikova et al., 2004). It is unanimously accepted that the most usual source of apigenin intake for the human body is represented by chamomile tea (Mojzer et al., 2016).

In the attempt to establish chamomile flowers and parsley or celery seeds as valuable natural sources of apigenin, we analyzed a hydroalcoholic extract based on the idea postulated in the literature that optimum extracts obtained from these vegetal products contain about 50% alcohol (Srivastava et al., 2010). According to the European Pharmacopeia (EP), to have a biological effect, the minimum percentage of apigenin 7-glucoside in the flowers should be 0.25% (Haghi et al., 2014).

Our results confirm that the vegetal product complies with the pharmacopoeia provisions. In a similar approach, Fonseca et al. concluded that the percentage of free and glycosylated apigenin in the methanolic extract is 106 and 903 μg/g whereas in the ethanolic extract the amount is 11 and 247 μg/g (Fonseca et al., 2004). Analyzing the amount of pure and conjugated apigenin in different types of extracts Haghi et al., have observed that the methanolic extract leads to the highest amount of pure apigenin (Haghi et al.,2014). In an aqueous extract, apigenin 7-O-glucoside was found to be the major constituent (Bhaskaran et al., 2010). In similar research, using different solvents for the extraction of active compounds from the aerial parts at the flowering stage of chamomile (e.g.water, methanol, ethanol), Haghi et al., have concluded that the amount of total phenolic compounds and total flavonoids range in the interval 1.77 - 50.75 g GAE/100 g dry plant, respectively 0.82 - 36.75 g quercetin equivalent (QE)/100 g dry plant (Haghi et al., 2014).

Our results from the UHPLC/DAD analysis indicated the presence of several flavonoids and polyphenol carboxylic acids such as chlorogenic acid, apigenin-7-glucoside, rutin, cynaroside, luteolin, apigenin and derivatives of apigenin-7-glucoside (Fig. 1).

The main compounds that were identified in all investigated samples belong to the polyphenolic acids and flavone groups. The most important, quantitatively, are included in Table II and the detected amounts were: chlorogenic acid- 222.54 mg/100 g of dry flowers, caffeic acid - 57.04 mg/100 g of dry flowers, catechin - 35.22 mg/100 g of dry flowers, apigenin-7-glucoside - 927.62 mg/100 g of dry flowers, rutin - 163.54 mg/100 g of dry flowers, cynaroside - 72.32 mg/100 g of dry flowers, luteolin - 139.08 mg/100 g of dry flowers and apigenin - 377.04 mg/100 g of dry flowers (Table II).

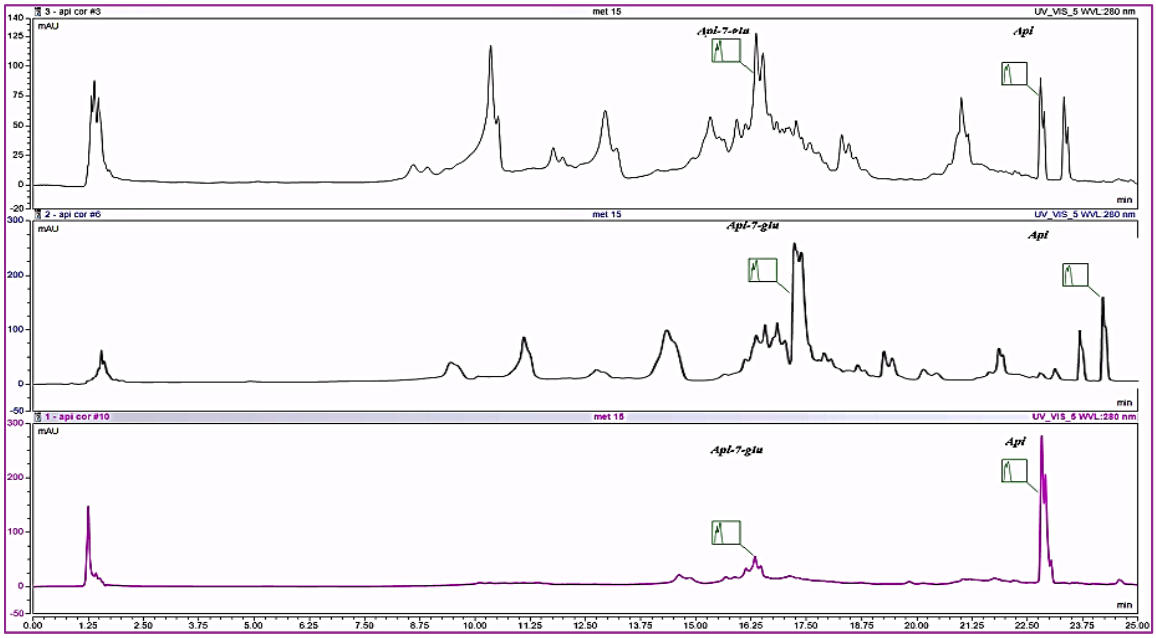

Fig. 1. UHPLC chromatogram of the investigated extracts: MC – chamomile flower extract, C- celery seeds extract, P – parsley seeds extract; Api-7-glu apigenin glucoside, Api – apigenin

Table II. The major polyphenolic compounds of the investigated plant samples by RP-UHPLC (µg/100 µg extract).

	Chlorogenic acid	*Caffeic acid*	*Catechin*	*Apigenin glucoside*	*Rutin*	*Cinaroside*	*Luteolin*	*Apigenin*	*Kaempferol*
MC extract	8.180	1.296	5.179	34.103	6.013	2.659	5.113	1.388	-
P extract	4.572	2.213	-	22.371	-*	0.776	4.078	14.562	2.662
C extract	6.609	1.980	-	18.015	-	6.146	1.539	16.967	8.480

** the extracts from Petroselinum crispum contain also kaempferol derivatives that amount to 2.421 µg/100 µg dry extract*

As indicated in the table, each extract has variations concerning the main compounds, but it is visible that the seeds contain mainly aglycons and fewer glycosides. In regards to parsley and celery extracts, the majority of the available data involved different plant organs usually leaves, stems and culture cells (Leyla Yıldız et al, 2008, Wong et al 2006). Also, the essential and fatty

oils were mostly investigated. In terms of our choice of vegetal product, the seeds are commonly believed to contain the genuine and simple compounds found in the future plant. The chemical analysis of parsley and celery seed alcoholic extracts revealed that the chosen vegetal material contains flavone aglycons and apigenin glucoside (Y. Yao, G. Ren, 2011). Other studies indicated the presence of gallic acid, catechins and their derivatives in ethanolic leaves extract, which were also present in our samples in small quantities. The authors concluded that polyphenols and mainly kaempferol derivatives were responsible for the antioxidant activity (Vranjes M et al, 2016). Similar results were noted for our samples.

A newer tendency in biomedical research is the use of bioreactors and sustainable natural resources such as submerged hybrid strains (Lohmeyer and Sander 1993; Rosu et al., 2010; Uhlig et al., 2007). In this regard, I have participated actively in the investigation and isolation of several ergot alkaloids obtained from extracts of submerged hybrid strains of *Claviceps purpurea*, in a collaboration with the Institute of Biological Research from Iasi, Romania.

The pyrenomycete *Claviceps purpurea* (Fr.) Tul. (Hypocreaceae) is probably the best-known species of the *Claviceps* genus. The fungus produces different ergot alkaloids (ergoline and clavine type) with a wide range of biological activities. The broad spectrum of ergot alkaloids effects is mostly based on their interactions with adrenergic, serotoninergic or dopaminergic receptors, as well as on their interference with some cellular and molecular processes (Mantegani, 1999; Schiff, 2006).

The ergoline alkaloids are used in the treatment of uterine atonia, postpartum bleeding, migraine, orthostatic hypotension, cerebral insufficiency, hyperprolactinemia or Parkinson disease. The antitumoral effect observed in the case of some clavine-type alkaloids reinforced the interest for *Claviceps purpurea* as a possible source of new onchotherapeutical agents (Pažoutová, 2001; Mantegani, 1999; Schiff, 2006; Rosu et al., 2010).

Mycelium aqueous extracts and the concentrated supernatants (the culture liquid) obtained after centrifugation were taken into study. Samples were collected after 6, 8 and 12 days of fungal fermentation and were prepared according to the steps described by Rosu et al., biochemically analyzed and tested for their cytostatic activity (Rosu et al., 2010; Rosu et al., 2011).

The total alkaloid content was measured using van Urk colour reagent (sulfuric acid 70%, p-dimethylaminobenzaldehyde 0.125 g, 1:1 $FeCl_3$ solution, 0.3 mL, per 100 mL of colour reagent). The calibration curve was generated using 0.01% ergotamine tartrate in 1% tartaric acid (Rosu et al., 2011; Pažoutová, 2001; Mantegani, 1999; Schiff, 2006; Rosu et al., 2010). The fungal extracts were characterized by a content of total alkaloid ranging from 1.25 to 11.10 mg/mL for the mycelium extract, and from 6.14 to 16.40 mg/mL for the supernatant (Figure 2).

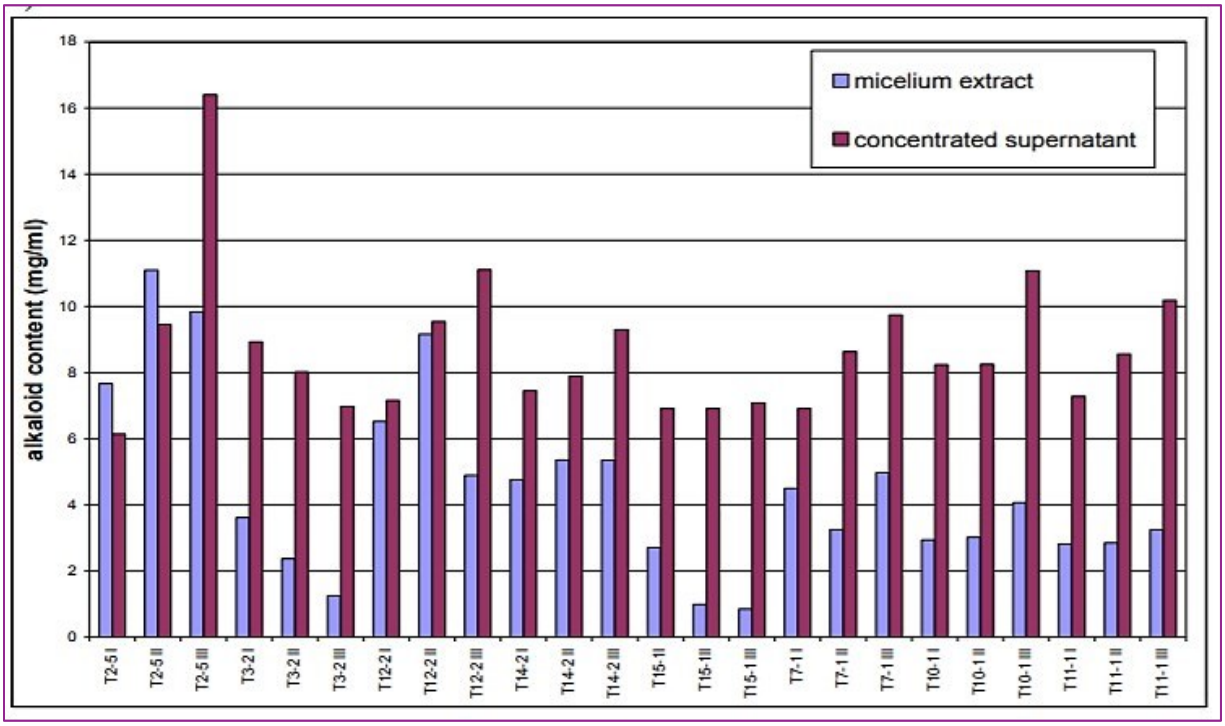

Fig. 2. Total alkaloid content (mg/mL) of fungal aqueous extracts obtained from different strains of *Claviceps purpurea*

The clavinic alkaloids identified in the selective fractions obtained from *Claviceps purpurea* submerged hybrid strains 'extracts (the biological material was obtained by somatic hybridization at the Institute of Biological Research from Iasi, Romania) are presented in Table III.

Table III. RP-HPLC Identified clavinic alkaloids

Alkaloid	RT (min)	System conditions/solvents	Purity factor
Ergocornine	8.910	Temp. 20°C, Flow 0.5 mL/min, Ethanol: Diethyl ether	996.661
Agroclavine	12.076		999.788
Ergocrystine	13.321		996.718
Ergocryptine	13.714		984.081
Agroclavine I	14.693		999.561

The registered peaks for agroclavine in one of the selective fractions are shown in figure 3, and represent the same compound at different wavelengths. The single peak indicates that the compound was isolated correctly, although the purity factor confirms if the component is pure or because of the higher concentration doesn't allow similar compounds to be detected.

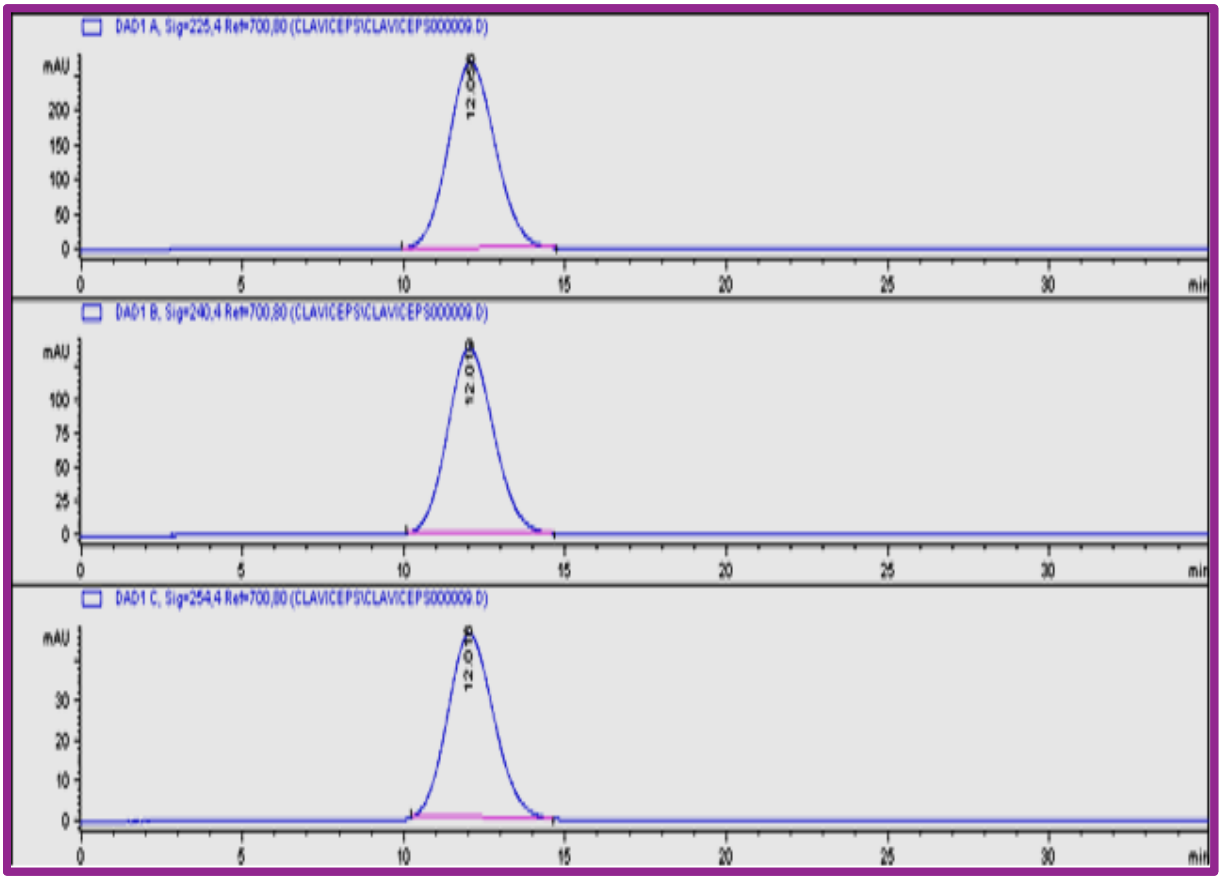

Fig. 3. Agroclavine peaks identified in fraction FIII from *Claviceps purpurea* submerged hybrid strains

It is noted that the peaks have a minimal deviation in the retention time, which is normal for all analyses, without exceeding the accepted limit of 0.05%, according to standard guidelines.

The UV spectra of agroclavine and purity factor analysis are given in figure 4 as registered by the Agilent 1200 HPLC software. In the image, the threshold, noise threshold, the standard deviation for the purity factor evaluation, the total number of spectra used to certify the identity of the peak are also indicated. It is notable that the purity (999.561 of 1000) exceeds the threshold limit. Moreover, the spectra is identical with the available data from spectrum libraries.

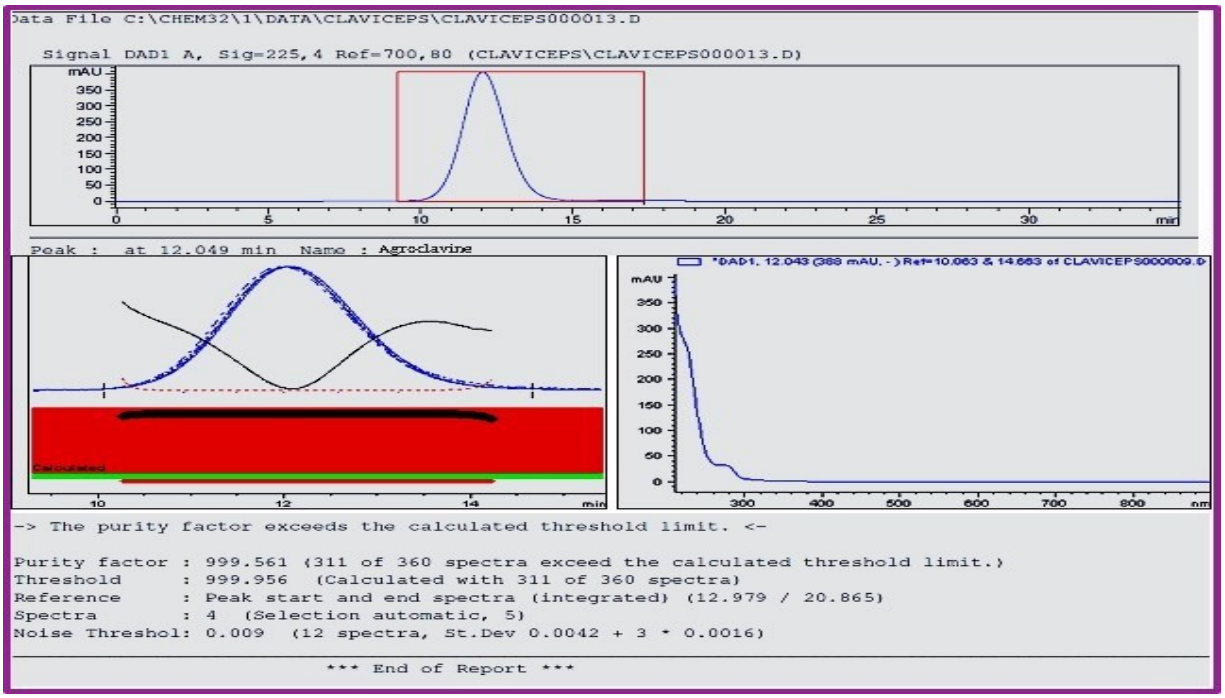

Fig. 4. The main characteristics, UV spectra and purity assessment of agroclavine peak

All these and several more results became part of the preparations used for antitumoral activity in veterinary medicine and are included in the **Veterinary patent no. 128512 B1, OSIM** - C12N 1/14; C12N 1/38; C12N 15/00; Clavinic antitumor preparation for veterinary use and process for preparing the same. The patent is still under protection at this time and this is why I will not include more data.

Another compound that I isolated and analysed, on the same system, was piperine from the methanolic extract of *Piper nigrum* fruits. *Piper nigrum* L. (Piperaceae) is one of the most popular spices in the world and has been used as a flavouring agent, but also in various traditional systems of medicine for treating cholera and dyspepsia, as well as various gastric ailments and arthritic disorders. Piperine is the major alkaloid of black pepper and is responsible for the pungent taste. Recently, pharmacological studies showed that piperine possesses analgesic, anti-inflammatory, anticonvulsant, antioxidant, antidepressant, and cognitive-enhancing effects (Mao et al. 2011). The air-dried and powdered sample (1 kg, fruits harvested in Dschang, West Region of Cameroon in June 2010 and identified by Victor Nana at the National Herbarium Yaounde) was extracted with methanol for 48 h at room temperature. The extract was then filtered and concentrated under reduced pressure and we obtained the crude extract.

The actual isolation of piperine followed the normal procedures of alkaloid separation techniques: 50 mg dry crude extract was macerated with 10 mL glacial acetic acid for 5 minutes at

room temperature. The macerate was then extracted with hexane (3 x 10 mL) in a separation funnel. The pooled extracts were dried and concentrated to obtain yellow crystals of piperine. The UV spectra and RT were superimposable upon the standard available data in online libraries.

By HPLC techniques, the analysis of the initial extract (the sample used also for *in vivo* testing) presented the chromatogram given below (Fig. 5).

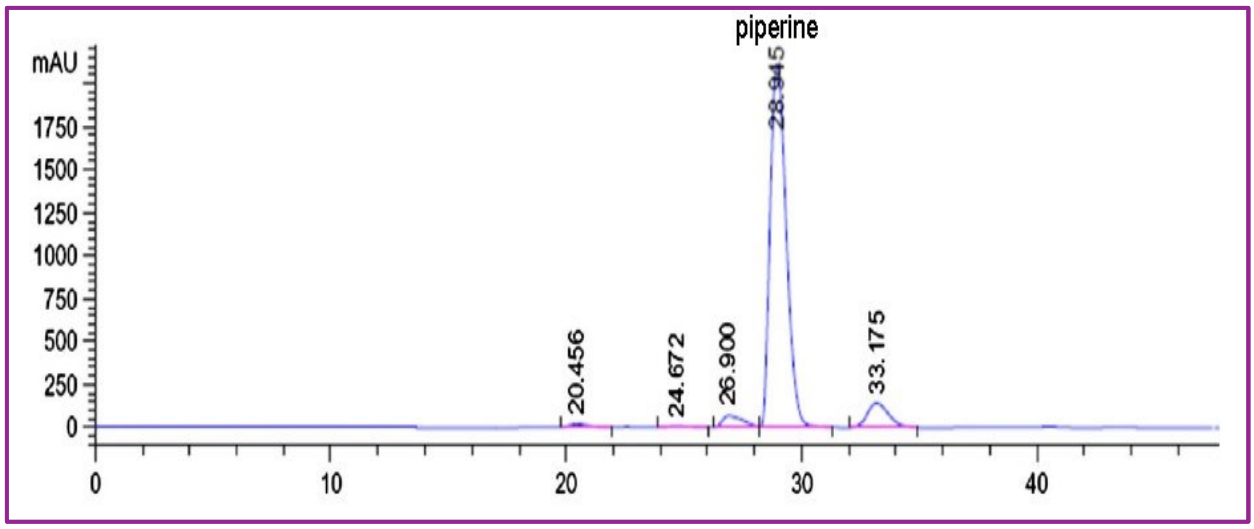

Fig. 5. HPLC chromatogram of the methanolic extract of *Piper nigrum* fruits

The fourth peak (RT 28.945) was represented by piperine, one of the major compounds found in pepper species. The purity factor indicates that the peak contains piperine almost 90%. The quantity in our extract is higher than that of the other similar amines. The amount of piperine in 1 mg methanolic extract was 0.917 mg. The experiment was in triplicate and the calculated amounts represented the average yield (SD ± 0.0001). Our results were consistent with literature data for other *Piper* species (*P. nigrum, P. guineense*, and *P. tuberculatum*) (Scott et al. 2005). The exact detection of piperine was possible by comparison with UV spectra and RT from the isolated piperine and the available data in the NIST Chemistry webbook for standard piperine peak.

The presence of piperine in such concentrations enabled its separation from the extract and its characterisation. Moreover, the biomolecular implications of this compound were studied *in vivo* and the results are described in the corresponding chapter.

Another investigated exotic plant was *Markhamia tomentosa* (Benth.) K. Schum. (Bignoniaceae). This species is known in West African countries, including Cameroun, where various parts of the plant are used as antimalarial, anti-inflammatory, analgesic and antioxidant agents. The research brought the possibility of collaborative work with Professor Galba Jean Beppe from the Department of Biological Sciences, Faculty of Science, University of Maroua, Maroua, Cameroon.

Regarding *M. tomentosa* stem bark, there are no studies available concerning the chemical composition of this vegetal product. Although some researchers have evaluated the biological properties of *M. tomentosa* leaves extracts, no data are confirming the presence of specific compounds, but rather major groups of substances. Thus, from the 14 standards used, we could identify and quantify catechin, epicatechin, rosmarinic acid and several catechin/epicatechin derivatives (without being able to specify which) as indicated in the chromatogram below (Fig. 6).

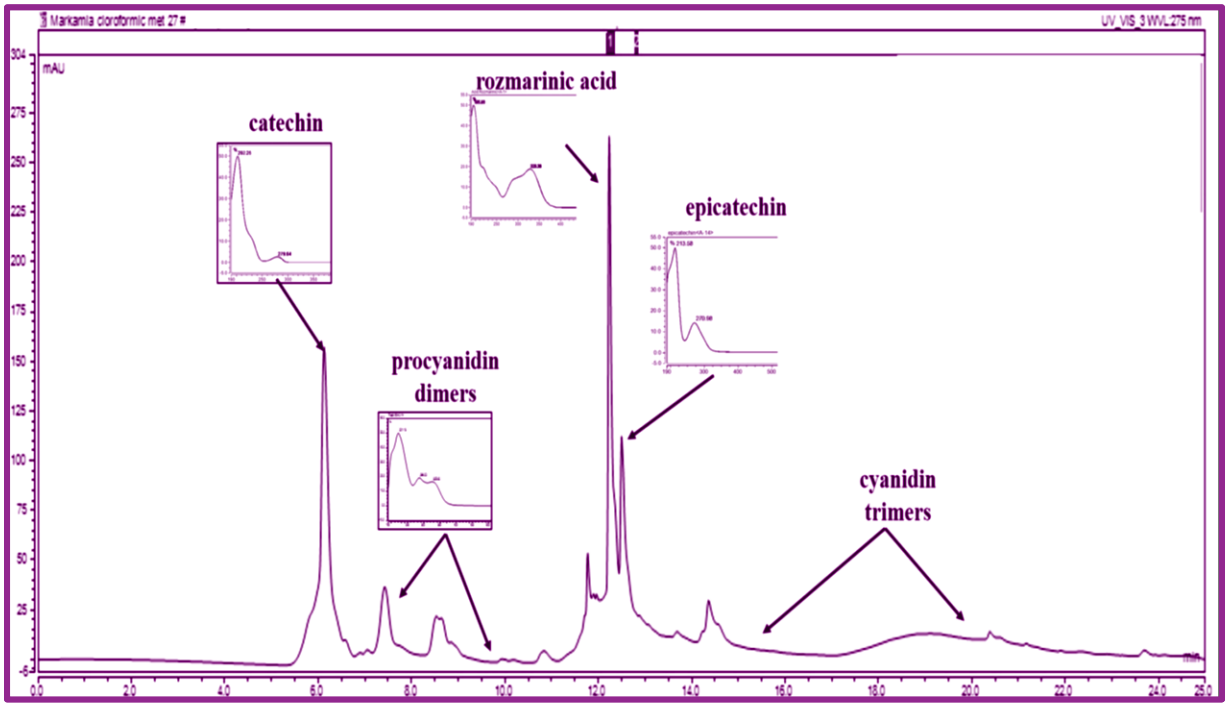

Fig. 6. HPLC chromatogram of the aqueous extract from *Markhamia tomentosa* stem bark

The amounts detected/mg of dry extract were 541.5 µg/mg rosmarinic acid; 11.37 µg/mg (+)-catechin; 15.86 µg/mg procyanidin dimer; 42.47 µg/mg (−)-epicatechin and 14.65 µg/mg cyanidin trimmers. Such chemical composition is related to strong antioxidant and radical chelating activities, as many researchers state the importance of catechin derivatives in protective mechanisms (Kim et al., 2014).

The aqueous extract obtained from the leaves of *Ceratonia siliqua* L. (Leguminosae) was investigated similarly. This species is known as carob and it is widespread in the Mediterranean basin. The analysed specimen was harvested, extracted and brought to us by a Eugen Ionescu PhD student that came from southern Morocco, Laboratoire de Biochimie et Génétique Moléculaire, Faculté des Sciences et Techniques, Université Abdelmalek Essaadi, Tanger.

Different parts of *Ceratonia siliqua* (leaf, flower, fruit, wood, bark, and root) are used in Moroccan pharmacopoeia (Sidina et al., 2009). There have been few studies that have evaluated

the antioxidant activity of leaves from this species (El Hajaji et al., 2011; El Hajaji et al., 2010). For this reason, the aqueous extract from *C. siliqua* (CsAE) leaves was screened concerning its total phenolic, flavonoids and condensed tannin contents. The registered UPLC chromatogram indicated several flavonoids quercetin-3-glucoside, luteolin-7-glucoside, apigenin-7-glucoside, epicatechin and various phenolic acids (gallic acid, chlorogenic and caffeic acids).

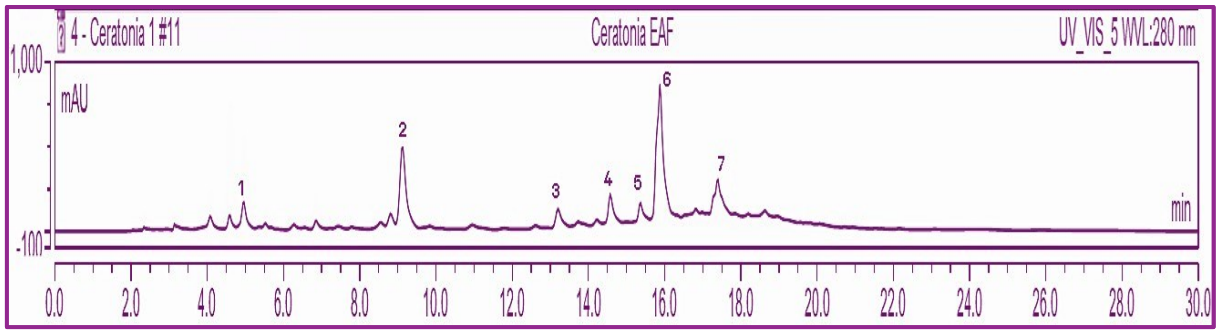

Fig. 7. UHPLC chromatogram of the aqueous extract from the leaves of *C. siliqua* registered at 280 nm

The major compound detected at 280 nm (wavelength specific for flavonoids) was luteolin-7-glucoside (23.78 w/w in the extract) followed by epicatechin (17.79 w/w in the extract), whereas apigenin and quercetin glycosides amounted to equal concentrations. The acidic fraction was less represented and each of the identified phenolic acids measured approx. 4 w/w in the extract (table IV). These results enforce the fact that the chemical composition is closely related to the plant organ that was used and the polarity of the solvent (water) that has a higher affinity for glycosides than for aglycones.

Table IV. The most important compounds identified in the aqueous extract from leaves of *C. siliqua* by UHPLC analysis

Sample	**Compound (mg/mL extract)**						
	1*	2	3	4	5	6	7
CsAE	4.54	17.79	3.59	4.84	7.41	23.78	7.50

* 1 – gallic acid, 2 – epicatechin, 3-chlorogenic acid, 4 – caffeic acid, 5 – quercetin-3-glucoside, 6 – luteolin-7-glucoside, 7 – apigenin-7-glucoside

The results indicate that flavonoids amount up to three times more than the phenolic acids and twice as the catechin. Moreover, such quantities indicate that *Ceratonia* leaves represent a good source of natural bioactive compounds. No aglycons were identified in our sample. Goulas

and Georgiou mentioned the presence of seven well-known phenolic antioxidants (gallic acid, syringic acid, catechin, epicatechin, quercetin, rutin, and myricetin) in the chemical composition of the carob extract (Goulas and Georgiou, 2019). Among them, gallic acid and rutin were noticed in the water extract in significant amounts. Corsi et al. reported the presence of gallic acid, (−) epigallocatechin-3-gallate, and (−) epicatechin-3-gallate in the pod and leaf of *C. siliqua* extracts by HPLC analysis (Corsi et al., 2002). Therefore, our results are comparable to literature data.

Conyza canadensis (CC) belongs to the well-known Asteraceae family and is a species closely related to Moroccan traditional medicine [4]. This annual, biennial, or perennial plant seems to grow well in very damp ground. In-ground submerged in freshwater it rarely shrubs, growing 1–2 m tall. The stems are erect and branched with alternating leaves. It is mostly considered a weed, but the traditional medicine of various countries of origin indicates its utility in treating gastrointestinal problems. CC is reported to have massive biological activities, encompassing antiproliferative activity (Csupor-Löffler et al., 2011), effect on gastrointestinal problems—most commonly diarrhea and dysentery, and as a diuretic agent (Gruenwald et al., 2007). It is also reported to be useful in the treatment of internal hemorrhages, gonorrhea, and bleeding piles (Weiner, 1980). A literature survey revealed that the whole plant is antirheumatic, astringent, balsamic, emmenagogic, styptic, tonic, and vermifuge (Chiej, 1984) and that its leaves are experimentally hypoglycemic (Duke and Ayensu, 1985). CC was reported to exhibit a significant anti-inflammatory effect on rats with carrageenan- and formalin-induced edema (Lenfeld et al., 1986). However, most of the chemical studies were oriented on the essential oil isolated from various organs of this plant.

To date, the scientific literature is rich in data and research regarding the chemical composition of the essential oil extracted from *C. canadensis* roots and aerial parts, but other references to the polyphenolic derivatives existent in this species are scarce. The extract included in this study is an aqueous solution that justifies the primary extraction of catechins, flavone glycosides and polyphenolic acids. The choice of this type of extraction was based on the general use of infusions or brewed teas in the general population. Moreover, data regarding such extracts obtained from CC lacks in international databases. At the time of writing, only four articles refer to the polyphenols from CC (Ding et al., 2010; Liu et al., 2011; Pietta, 2000). Some only quantify the total amount of flavonoids, polyphenols, and tannins and measure the antioxidant and antimicrobial activity of the extracts. However, Liu et al. identified six compounds from CC referred to as eugenyl beta-Psd, scutellarin, luteolin-7-O-beta-D-glucuronide, quercetin, quercetin-3-O-beta-D-glucopyranoside, and luteolin.

Starting from literature data, we investigated the chemical composition and the biological activity of an aqueous extract obtained from the aerial parts of this species. Compared with all published data on *Conyza canadensis*, our research is original due to the type of plant material and extract that was investigated. The chromatographic identification of the active compounds was assessed using a fast liquid chromatography method which lasted up to 30 min. The general aspect

of the HPLC-RP chromatogram is shown in Figure 8, and it comprises the main compounds that were found and quantified in the investigated extract.

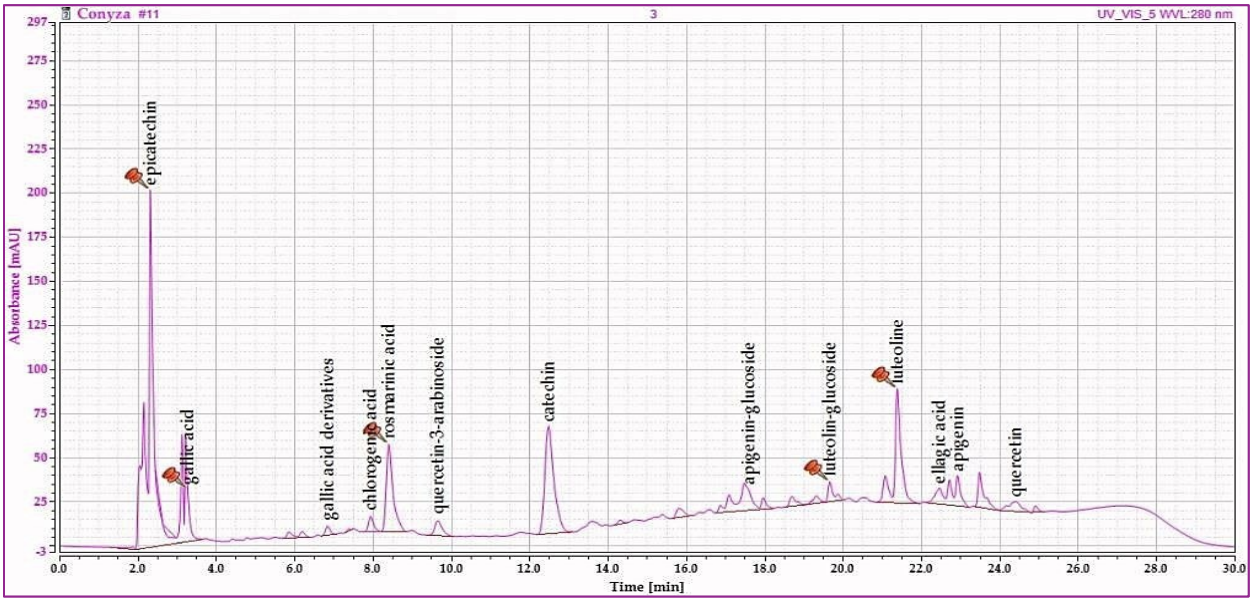

Fig. 8. Chromatogram of the *Conyza canadensis* (CC) aqueous extract.

Our results are similar to some extent, but one should never forget that the pedoclimatic conditions have a significant impact on the biosynthetic capacity of plants. The plant material included in our study was harvested from a dry tropical climate, in contrast to the humid subtropical climate of the area from which other investigated materials were harvested. However, the found components are mainly flavonoids such as catechin and epicatechin, luteolin and its glucoside, apigenin, and apigenin-7-O-glucoside, as well as quercetin and quercetin-3-arabinoside. Some phenolic acids were also identified, but their quantity amounted to less than a third (45.020 µg/mg dry extract) of the flavonoid fraction (Table V).

Table V. Quantification of the major active compounds identified in *Conyza canadensis* aqueous extract

No	**Flavonoids (µg/mg dry extract)**		**Polyphenolic acids (µg/mg dry extract)**	
1	epicatechin	59.343*	gallic acid	13.785
2	catechin	40.818	chlorogenic acid	3.243

No	Flavonoids (µg/mg dry extract)		Polyphenolic acids (µg/mg dry extract)	
3	quercetin-3-arabinoside	4.650	rosmarinic acid	24.557
4	apigenin-7-O-glucoside	6.345	ellagic acid	3.435
5	luteolin-7-O-glucoside	3.440		
6	luteolin	26.308		
7	apigenin	6.170		
8	quercetin	6.468		
	Total identified	**153.542**		**45.020**

** Values included in the table represent the mean of triplicate quantification; limit of detection (LOD): 280 ng/mL; limit of quantification (LOQ): 145 ng/mL.*

The results suggested that the aerial parts of *C. canadensis* are rich in catechins and flavonoids, which sustain the antioxidant potential of the extracts used in therapy.

The ongoing trend in pharmacognostic research is to find new sources of natural compounds. This is mainly represented by a broader search area that includes also food and ornamental plant species. In this regard, my research led to a new collaboration with the Faculty of Horticulture, University of Agricultural Sciences and Veterinary Medicine Ion Ionescu de la Brad Iasi, Romania. The starting point was represented by the fact that the bioactive constituents of ornamentals are insufficiently characterized and many environmental factors, such as pedoclimatic conditions and agro techniques greatly influence the development of the plants. The objectives were to identify and quantify the phenolic compounds in several ornamental Asteraceae species such as *Rudbeckia hirta, Chrysanthemum indicum* and *Tagetes erecta*. Moreover, these species are known for their allergenic potential, therefore the sesquiterpene lactone compounds were also investigated (Mircea (Arsene) et al., 2015 a, 2015 b, 2015 c).

Two *Rudbeckia hirta* cultivars ('Goldilocks' and 'Moreno') were analyzed to measure the total polyphenols in aerial parts of the plants and the macro-morphology of the cultivars related to local conditions in two nutritional statuses: local (V1) and enriched soil conditions (V2). The chosen fertiliser was osmocote-type, ecofriendly with a slow release and 5-6-month availability. The experiment was performed as a plot design based on randomized complete blocks with three replications. Results showed that both cultivars reached ornamental performance in local conditions, despite the higher rainfalls in 2014. The biometric parameter values were similar to the existing data from the literature, with the particular aspect of plant height, which registered smaller values. The quantity of polyphenols in the leaves is positively influenced by fertilization for both

cultivars, yet there was a slight decrease in these compounds in the flowers of the 'Goldilocks' cultivar. This is uncommon and we presume that a different fertilizer is more suitable for the growth of the plant. Notable was the impact of the used treatment on the 'Moreno' cultivar when the total quantity of polyphenols was doubled in flowers. This is expected and indicates a positive influence of the used nutritional substrate on plant growth and biosynthetic metabolism (Mircea (Arsene) et al., 2015 a).

The results indicated that the ornamental Asteraceae species being studied represent rich sources of phenols. Nevertheless, the fertilization lowers the plant biosynthetic capacity for polyphenols for both *Rudbeckia* (1146-mg GAE/100g V1f2 dry extract, compared to 871.9 mg GAE/100g V2f2 dry extract) and *Tagetes* flowers (526.62-mg GAE/100g V1f2 dry extract compared to V2f2, 428.55 mg GAE/100g dry extract). Total phenols are important for plant defence against UV rays and harsh environmental conditions. However, as others have observed the fertilization (V2) with N and P reduced plant chemical defence, although no other changes were made to our crops. Furthermore, the ornamental properties of both species increased significantly with fertilization, both biomasses, the number and diameter of inflorescences were greater in the fertilized samples (V2). Thin layer chromatography analysis indicated qualitative differences between the phenophases (f1 and f2) and less important changes between the fertilized samples (Mircea (Arsene) et al., 2015 a).

Alantolactone, eudesmanolid-type sesquiterpene lactones, have been found to have pharmacological actions including anti-inflammatory, antimicrobial and anticancer properties (Chadwick et al., 2013; Rasul et al., 2013). On the other hand, methylated sesquiterpene lactones are redoubtable allergens of the *Chrysanthemum* genus (Stampf et al., 1982; Warshaw and Zug, 1996) and could induce allergic contact dermatitis (Tanaka et al., 1987; Lowell, 1993; Crosby, 2004; Swierczynska-Machura et al., 2006). The offending allergen may be present in cultivated plants, botanical extract itself or another ingredient such as fragrance (Jack et al., 2013). The allergenic lactones generally are contained in trichomes that cover the stems, leaves or flowers of plants. Many plant species produce glandular trichomes that biosynthesize toxic substances such as terpenes, lactones, dermatotoxic quinones (Burzo and Toma, 2013). In the Asteraceae family, glandular trichomes are found along with other secretory structures, such as secretory channels (NiQa et al., 2001). The evaluation of the allergenic potential of these plants is a problem of occupational and public health.

In our research, terpenoid compounds were also found to be important components of these species, indicating possible implications in allergic reactions. Alantolactone was found in *Rudbeckia* flower buds (0.2358 μg/100g dry flowers), its quantity is decreasing in the unfertilized plants (Mircea (Arsene) et al., 2015 a). Alternatively, alantolactone was found in both phenophases of *Tagetes* flowers, the fertilized fully grown flowers presenting the highest content (0.2459 μg/100 g plant material).

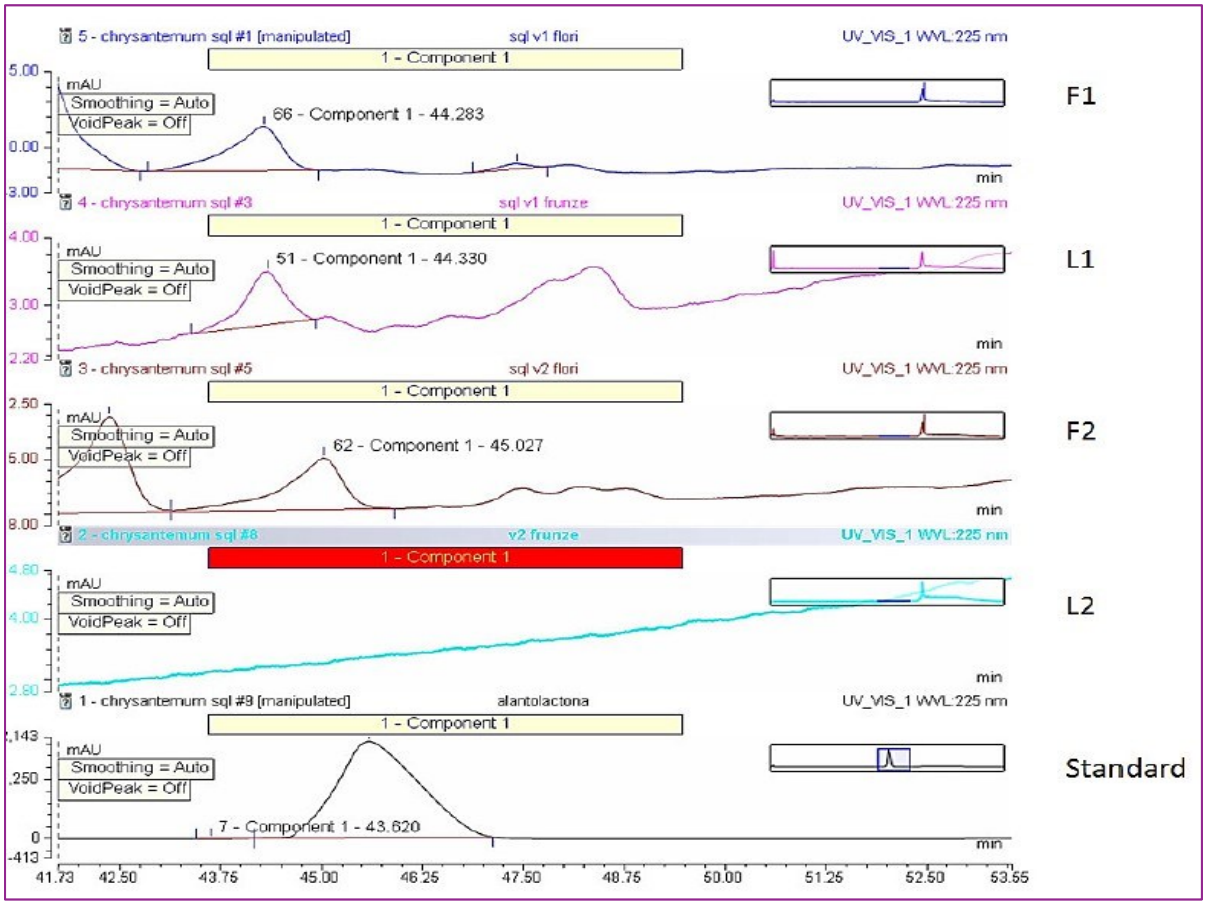

Fig. 8. Alantolactone identification by UHPLC in Avalone red cultivar of *Chrysanthemum indicum*, where the codes represent: F1/L1-inflorescences/leaves from unfertilized plants, F2/L2-inflorescences/leaves from fertilized plants

The morphology, hardiness and easy cultivation, with reduced labour cost, make *Chrysanthemum indicum* cv. 'Avalone Red' an economically advantageous option both for growers and for employing it in the landscape. Fertilization intensifies the ramification of the plant and the growth of its biomass, increases the number of inflorescences but not their diameter. The qualitative chemical analyses confirmed the presence of flavonoids and polyphenolcarboxilic acids, more so in flowers than in leaves. Total phenolic content was higher in leaves than in flowers. The fertilized plants have a higher phenolic content but smaller alantolactone content than unfertilized plants. Ursolic acid was not detected in the studied cultivar. The tested cultivar is a potent source of polyphenols, but alantolactone is present only in small quantities, as confirmed by the HPLC analysis. However, the presence of alantolactone alerts to the allergenic potential of plants and plant extracts, especially the extracts obtained from flowers. In addition to their use as ornamental

plants, chrysanthemum can have a wide array of uses due to their bioactive compounds with antioxidant activity (Mircea (Arsene) et al., 2015 a; 2015 b).

Along with these three ornamentals, we have investigated three medicinal species (*Echinacea purpurea, Carthamus tinctorius* and *Calendula officinalis*) from the same family, that were cultivated and harvested in identical conditions, observing similar parameters for research.

The influence of fertilization on the ornamental characters, the alantolactone and triterpenic acids profiles in *Echinacea purpurea* was evaluated. *Echinacea purpurea* (L.) Moench is a hardy perennial plant that belongs to the Asteraceae family and is one of the most common species of the genus, used as ornamentals and commercially treated as medicinal plants due to their antiviral, antibacterial and immunostimulatory activities to humans (Chen et al., 2008; Draghia, 2004; Hăncianu, 2014; Stanciu, 2008; Mircea (Arsene) et al., 2015 c). The aerial parts of the plant contain flavonoids, polyphenolic acids, essential oils, alcohols, pseudoguaianolide, sesquiterpenes, monoterpenes, sesquiterpene oxides, polyacetylenes, alkylamides and other chemical constituents which have been analyzed by applying various chromatographic techniques (Diraz et al., 2012; Hăncianu, 2014).

Information regarding the effects of genetic diversity, growing climates and cultivation practices on active constituents (secondary metabolites) and biomass production of *Echinacea* are still very limited (Chen et al., 2008). It is known that biosynthesis and concentration of secondary metabolites of plants depend on growing sites, climate conditions, cultural practices, vegetation phases and on cultivar and specific organs of the plant (Mircea (Arsene) et al., 2015c; Chen et al., 2008; DruŃu et al., 2010).

The HPLC analysis confirmed the presence of alantolactone and the semiquantitative quantification showed that alantolactone is present at low levels (some micrograms at 100 gr dried plant). Moreover, the unfertilized plants contain only traces of alantolactone compared to the fertilized samples.

Table VI. Qualitative and semiquantitative identification of alantolactone in *Echinacea purpurea*

Method	**Buds / Open inflorescences**				**Stems and leaves**			
	V1f1	V1f2	V2f1	V2f2	V1-1	V1-2	V2-1	V2-2
TLC	u	–	u	–	–	u	–	+
HPLC	0.0763	0.1010	0.3649	0.0898	0.0135	0.1639	0.0213	0.3065

Note: u = traces; + = present; – = not identifiable.

The fertilization increases the plant height, number of ramifications and number of inflorescences per plant, and diameter of inflorescences. Moreover, alantolactone is positively correlated with biomass, especially with ramification and number of inflorescences. These findings are in accordance with Lerdau et al. (1997) and Ormeño et al. (2008) which have found a positive correlation between the soil and leaves nutrient concentration (especially N) and the concentration of terpenoids from leaves. Gershenzon (1994) stated that nutrient-terpenoid relation has species specificity.

Triterpenic profile confirmed the presence of traces of ursolic acid (Rf 0.40) in open inflorescences while oleanolic acid (Rf 0.58) was confirmed in whole plant.

Overall, more than 100 samples have been analyzed by UHPLC. Synthetic data analysis and its corroboration with the spectrophotometry led to the following conclusion, the profile and the concentration of polyphenols showed variability of species, organ, phenophase and fertilization. Moreover, there is an indirect correlation between the terpenoids concentration, fertilization and polyphenolic content as indicated in the following table.

Table VII. Variability of the bioactive compounds depending on species, organ and fertilization

Species	Alantolactone		Total Polyphenols	
	Flowers V2 vs V1 (f2)*	Leaves and stems V2 vs V1 (f2)	Flowers V2 vs V1 (f2)	Leaves and stems V2 vs V1 (f2)
Rudbeckia hirta	⇗	⇔	⇘	⇗
Chrysanthemum indicum	⇘	-	⇗	⇗
Tagetes erecta	⇗	⇔	⇘	⇘

* V1 – unfertilised plants, V2 – fertilised plants, f2- second phenophase (mature plants)

As indicated in the table, the influence of fertilization on the biosynthetic capacity varies depending on the species, organ and group of metabolites. The most important differences were noted for alantolactone found in flowers that are in higher quantities after fertilization in *Rudbeckia* and *Tagetes*, whereas its quantity decreases in *Chrysanthemum*. The fertilization does not have a positive impact on *Chrysanthemum*, for which the concentration of polyphenols is lower in all aerial parts of the plants compared to the unfertilized lot (Mircea (Arsene) et al., 2015 a, 2015 b).

The concentration of secondary metabolites from the investigated species was found to be influenced by the nutritional status. Moreover, the obtained values indicated that the studied ornamental Asteraceae species represent reliable sources of different polyphenols.

Given that in the pharmaceutical industry the freeze-drying process is a widely used method for obtaining stable preparations that can be easily compressed, facilitating processing into pharmaceutical forms, anthocyanin selective extracts have been obtained by fractionations in different solvents (Salamon et al. 2015a; Salamon et al. 2015b). The parameters of the lyophilisation process were followed comparatively and were carried out by collaboration with a Slovak firm (Medicproduct, Co. Lipany, Slovakia) specialized in the processing of plant extracts at the pilot level. This collaboration was introduced by Prof. Ivan Salamon PhD, during a bilateral research project (International Bilateral cooperation with the University of Presov, Slovakia - Monitoring of anthocyanin content in selected plant species and determination of their antioxidant activity). Some of the results of the preliminary studies have been presented at specialized scientific congresses (Salamon I, Grulova D, Hancianu M, **Cioanca O**. Optimization of lyophilization technology for purification and stabilization of anthocyanins from elderberry fruits. InI International Symposium on Elderberry 2013. Acta Horticulturae. 2015, 1061:245–252).

For such processing, the fruits from *Aronia melanocarpa*, Rosaceae, chokeberry, *Sambucus nigra* L., Caprifoliaceae, elderberry, and *Vaccinium myrtillus*, Ericaceae, blueberry/wild bilberry, were harvested in three consecutive years 2012-2014. Then, the plant material was quickly frozen to preserve the maximum of the active principles. For the lyophilisation, the fruits were subjected to successive extractions (5 times) with ethanol-water mixture and acetone of different concentrations in a double weight. The pooled extracts were then subjected to liquid-liquid separation with chloroform and differentiated fractionation. The obtained fractions were freeze-dried in an automated vial-filling line to a fine, uniform, violet-red and glassy powder. Various parameters were studied and considered for the optimization of this process.

The first of the studied parameters was the dilution ratio of the extracts in the water (A*qua purificata* PhEur) for optimal lyophilization. Starting from one to one up to five to one we established the satisfactory dilution rate that was between 4:1 and 5:1. After segmenting the dilution by 0.1 we obtained the best results at 4.5 and 4.7 for a consistent dryness and yield, first for fine powder and the second for tablet form.

In terms of the lyophilization process, time, temperature and pressure were studied consecutively. The optimal process included special parameters for each step (loading, freezing, evacuation, drying) such as 36 h the lengths of lyophilization, -36ºC temperature for freezing at a pressure ranging from 0.05 to 0.2 mBar.

The initial freezing step is the most critical in the whole freeze-drying process because the product can be spoiled at this stage. In this case, the freezing was done rapidly, to lower the material to below its eutectic point quickly, thus avoiding the formation of ice crystals. Amorphous materials do not have a eutectic point, but they do have a critical point, below which the product must be maintained to prevent melt back or collapse during primary and secondary drying (Deluca and Lachman, 1995). The amount of heat necessary can be calculated using the sublimating

molecules' latent heat of sublimation. In the initial drying phase, about 95% of the water in the material is sublimated. This phase may be slow (sometimes several days in the industry), because, if too much heat is added, the material's structure could be altered.

Pressure is controlled through the application of a partial vacuum. The vacuum speeds up the sublimation, making it useful as a deliberate drying process. Furthermore, a cold condenser chamber and/or condenser plates provide a surface(s) on which the water vapour can re-solidify. This condenser plays no role in keeping the material frozen; rather, it prevents water vapour from reaching the vacuum pump, which could degrade the pump's performance. Condenser temperatures are typically below -50°C (Deluca, 1985; Salamon, 2012). It is important to note that, in this range of pressure, the heat is applied mainly by conduction or radiation; the convection effect is negligible due to the low air density.

Freeze-drying is a relatively expensive process. The equipment is about three times as expensive as the equipment used for other separation processes, and the high energy demands lead to high production costs. Furthermore, freeze-drying also has a long process time, because the addition of too much heat to the material (to speed the process) can cause melting or structural degradation. The low operating temperature of the process leads to minimal damage of these heat-sensitive products – in our case the anthocyanins. Therefore, freeze-drying is often reserved for materials that are heat-sensitive, such as proteins, enzymes, microorganisms, and blood plasma (Timothy, 1990; William, 1984).

Our successful experiments yielded a pure and stable anthocyanin product. Anthocyanins extracted from elderberry stored as a powder are protected against degradation. This product could be used for many different purposes in dietary supplements. These results set the stage for additional research on the use of lyophilization to isolate and stabilize beneficial plant metabolites in elderberry and other plants or fruits.

Other indigenous species known from Romanian Traditional Medicine are *Ajuga reptans* and *Ajuga genevensis* harvested from the experimental field of INCDBSB/CCB Piatra Neamț and the spontaneous flora respectively. These two species were investigated for their chemical composition of polyphenols and iridoids. All significant results were included in one research paper (Păduraru AF, **Cioancă O,** Mircea C, Trifan A, Aprotosoaie AC, Miron A, Gille E, Hritcu L, Hăncianu M. Bioactive Extracts from Cultivated *Ajuga genevensis* L. and *A. reptans* L.: In Vitro/In Vivo Pharmacological Effects. Farmacia. 2019;67:603-9) and a PhD thesis (*Study of extracts of plant origin with potential neuroprotective effects*, elaborated by pharm. Andrei Florin Paduraru, scientific supervisor Prof. Monica Hăncianu, PhD) defended on the 15.04.2021.

After an initial observation of the microscopic features (glandular and surface hairs, epidermis structure, the number of and type of stomata) we confirmed their identification: *Ajuga genevensis* L. (blue bugle) and *Ajuga reptans* L. (common bugle or bugleweed).

The two soft extracts obtained from *A. reptans* and *A. genevensis* according to the above methodology, weighed 0.96 g for bugleweed (which means a 19.2% yield) and 1.00 g respectively for blue bugle, representing a yield of 20%. The investigated extracts represented hydroalcoholic solutions in 70% ethanol obtained from 5 g of vegetal material in 100 mL solvent. The obtained results indicated that in the flowering aerial part of the two species, in addition to polyphenolic derivatives (mainly flavonoids and polyphenolic acids), there are iridoids, some of which are structurally close to 8-O-acetylharpagide and some ecdysteroids.

The quantification of these compounds is given in the table below and it represents the average of three identical determinations for each sample.

TableVIII. Polyphenols identified and quantified in the investigated *Ajuga* samples

Sample	Component (mg/g pv)							
	caffeic acid	**p-coumaric acid**	**ferulic acid**	**rosmarinic acid**	**catechin**	**luteolin-O-glucoside**	**luteolin**	**apigenin**
Ajuga reptans	0.85 ± 0.2	0.646 ± 0.3	2.43 ± 0.3	19,877± 0.1	1,631 ± 0.2	9,796 ± 0.1	0.21 ± 0.2	3,235 ± 0.1
Ajuga genevensis	0.33 ± 0.1	0.333 ± 0.2	4.75 ± 0.2	17,434± 0.1	2,352 ± 0.1	7,697 ± 0.4	3.84 ± 0.3	2,721 ± 0.2

As indicated in Table VIII the proportion between the polyphenolic compounds is different from one sample to another. But there are situations in which *A. genevensis* is richer (ferulic acid, catechin, luteolin) than *A. reptans*. Similar values have been registered for the wild flora samples, but there is no general pattern other than the fact the flavonoid fraction is usually found in higher amounts in *A. genevensis* extracts (Toiu et al., 2016).

In terms of the chemical profile, the polyphenols (flavonoid and polyphenolic acids) were richer in *Ajuga genevensis* (168.3 ± 0.13 mg % and 230.4 ± 0.42 mg % respectively), whereas *Ajuga reptans* contained higher quantities of iridoids (1860 ± 0.21 mg % as compared to 1250 ± 0.03 mg %) as shown in Figure 9.

The analysis of the spectra of the compounds from the investigated samples showed chemical similarities and differences at the same time, thus indicating an interspecific variability between which is present although the plant material has been grown in identical pedoclimatic conditions.

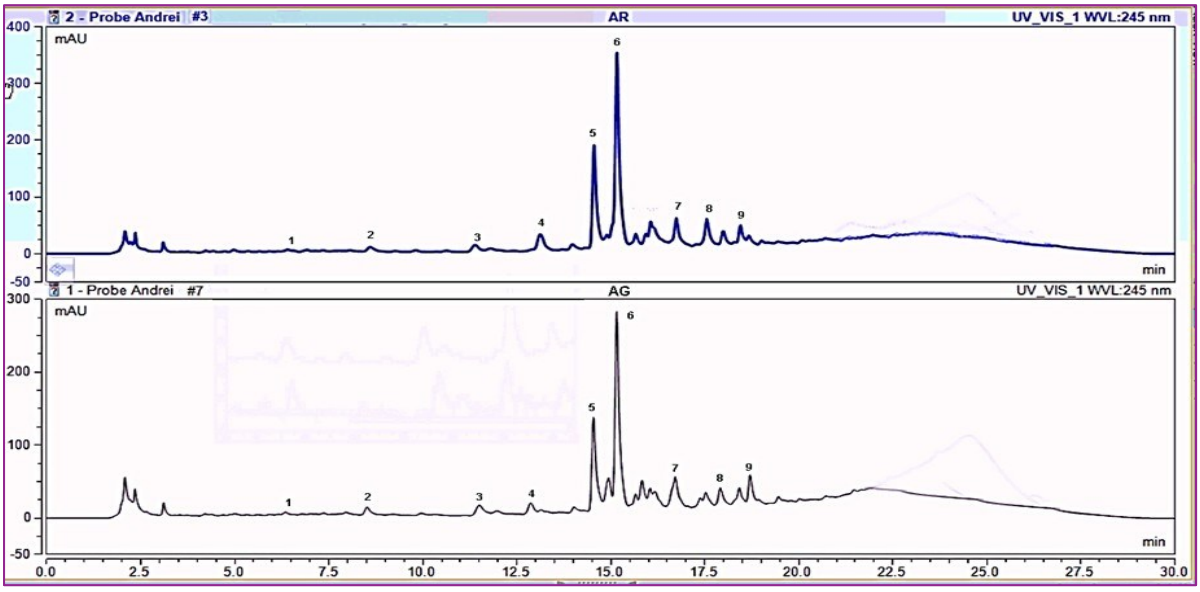

Fig. 9. UHPLC chromatogram of the investigated *Ajuga extracts*, where AR (blue) - *Ajuga reptans*; AG (black) - *Ajuga genevensis*. Legend: 1. chlorogenic acid; 2. p-coumaric acid; 3. catechin; 4. rutoside; 5. luteolin-glucoside; 6. rosmarinic acid; 7. 8-acetyl-harpagoside; 8. harpagoside; 9. apigenin

For a better view regarding the identified iridoids, the zoomed-in fragment of the same chromatograms for the peaks separated from minute 7 to minute 15 is presented in figure 10.

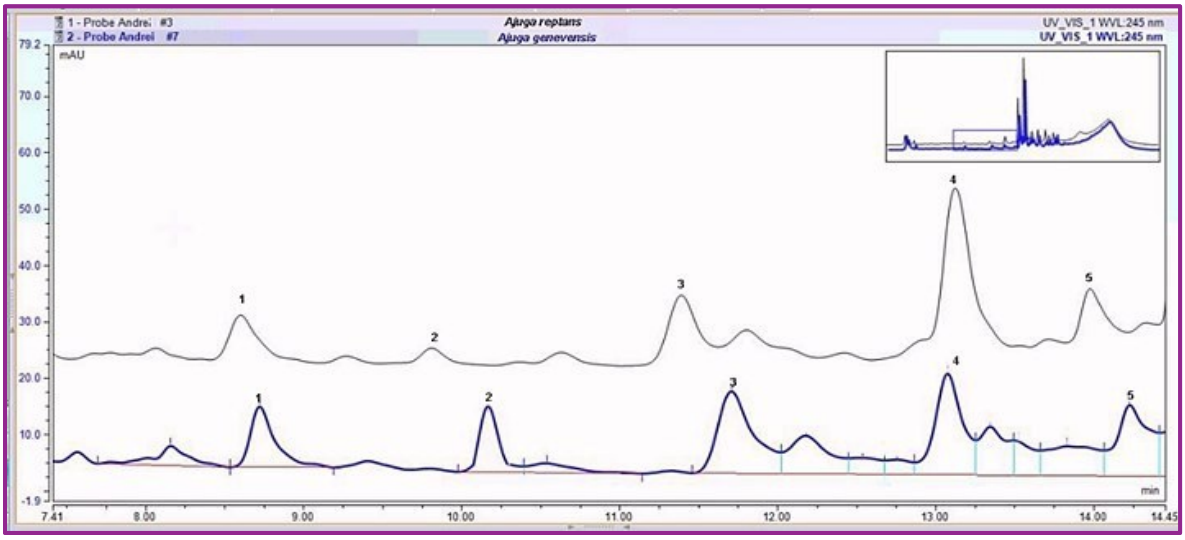

Fig. 10. Detail of UHPLC chromatograms registered in parallel for *Ajuga reptans* (black) and *Ajuga genevensis* (blue), where the numbers represent the following compounds: 1. swertiamarin; 2. catalpol; 3. 20-OH-ecdysone; 4. α-ecdysonone; 5. aucuboside.

Leuzea carthamoides (Willd) Iljin and *Leuzea salina* were investigated for the same compounds similarly and several observations were made regarding the intraspecific variability of the secondary metabolites. However, most of the data regarding these species were included only in the mentioned PhD thesis and are not yet published.

Pelargonium species can be found in the history of modern medicine from the time of British Major Stevens, who, in 1897, launched the Stevens' Consumption Cure drug which treated tuberculosis (Bladt, 1974). The experience he had gained in South Africa was the basis for the introduction of umckaloabo preparations (decoction of the roots of *Pelargonium sidoides*), although the first clinical trial was conducted in 1920 on 800 patients. The promising results obtained by Doctor Adrien Sechehaye were published in 1930 and established the first tuberculostatic treatment before synthetic cytostatic started to be used.

Therefore, we focused in our research on one indigenous species (*Pelargonium zonale*) and three species acclimatized in our country (*Pelargonium hispidum, Pelargonium grandiflorum* and *Pelargonium radens*) of the *Pelargonium* genus, Geraniaceae Family. Various types of extracts were obtained and their chemical composition was assessed by UHPLC means (Iancu et al., 2017a).

The most important compounds identified in the investigated extracts by UHPLC techniques are presented in figure 11 (only for *P. zonale*) and table IX (comparatively for all species).

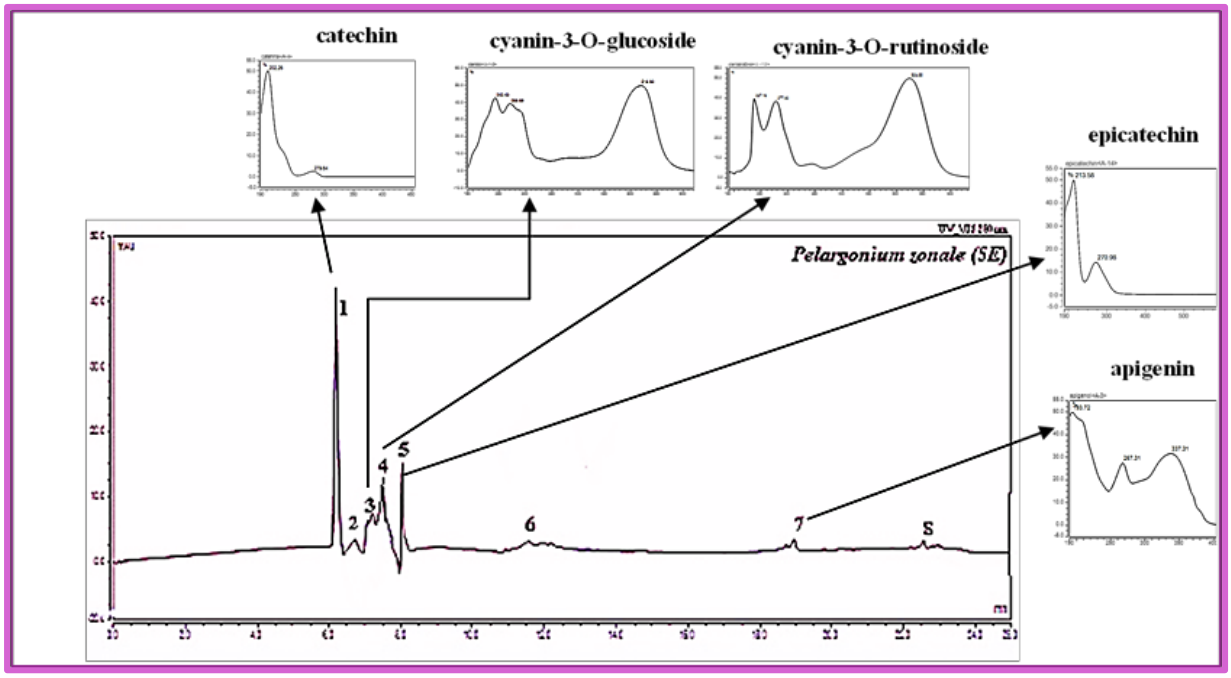

Fig. 11. UHPLC chromatogram for the ethanolic extract obtained from *P. zonale* and the UV spectra of the identified compounds from the Chromeleon 7.2.v12 library

Table IX. Compounds identified and quantified in *Pelargonium sp*. methanolic extracts

Compound (µg/mg dry extract)	Source			
	Pelargonium hispidum	***Pelargonium grandiflorum***	***Pelargonium radens***	***Pelargonium zonale***
catechin	8.717	4.332	1.169	10.930
epicatechin	1.422	0.785	0.568	2.144
cyanidol	-	-	-	2.138
cyanidol derivatives	-	-	-	33.413
delphinidin-3-o-rutinoside	-	-	-	23.398
quercetin-3-arabinoside	3.996	0.516	-	8.001
quercetin	1.007	-	0.154	4.460
luteolin	0.407	0.092	0.918	0.071
kaempferol	0.4199	0.113	0.246	0.133
rutinoside	-	10.760	5.126	-
apigenin	-	0.758	2.246	-
rosmarinic acid	-	2.202	-	-
caffeic acid	-	-	-	0.459
chlorogenic acid	-	0.489	4.451	-
cinnamic acid	0.0519	-	-	-

The identified compounds were catechins, flavones, polyphenolic acids and anthocyanidins, secondary metabolites that are also mentioned by the scientific literature for this Genre. Anthocyanidins and catechins were present especially in *Pelargonium zonale* extract, a fact sustained by the presence of a dark purple-brown horseshoe-shaped mark on the leaves of this species. Moreover, recent studies have proven that the topical administration of leaf extract or juice obtained from this species has a haemostatic effect (Paez and Hernandez, 2003). Most probably, such compounds and tannins would be the active principles that act as protein precipitating agents.

The best plant product proved to be *Pelargonium zonale* which yielded the largest quantity of extract, whereas, the leaves of the *Pelargonium radens* species yielded the lowest quantity of extractible. At the same time, the richest sources were Pelargonium zonale with approx. 5694 mg gallic acid/100 g dry extract and *Pelargonium hispidum* respectively; *P. radens* contained almost the same amount of polyphenols. Comparing the rest of the data for *P. grandiflorum*, one might state that there is no direct correlation between the amount extracted (extractible) from the plant and the total phenolic content.

I.3.2. Qualitative and quantitative analysis of volatile terpenoids

Volatile compounds represent the fragrant substances that belong to terpenoids and are most commonly used in perfumery. In household use, they are referred to as essential oils that can be widely applied for room deodorizing, to repel insects, as antimicrobials or antivirals and also as aromatherapeutic for soothing properties on the mind and body. In the food industry, they are preferred to synthetic antimicrobials and chemical preservatives. Generally, they are secondary metabolites synthesized in various organs and play important roles for the plants (attractant, repellant, antimicrobial, pesticide etc.) (Bakkali et al., 2008; Gershenzon, 1994; Hanif et al., 2019). Chemically speaking, essential oils represent complex mixtures of different compounds usually colourless with aromatic smell, spicy-burning taste and a density lower than water. The common skeleton is composed of a hydrocarbon moiety and a group of oxygen. However, the chemical composition and the existence of oxygenated or aromatic terpenes with smaller (C10) or larger (C15-20) skeletons are closely related to species (genetics, pedo-climatic conditions, weather changes etc.) and the extraction method employed to obtain the essential oil (Bakkali et al., 2008; Hanif et al., 2019). The quality of the plant material is therefore extremely important for high-quality volatile oil. Although the plant material can be either raw or dry, the preference for a certain type is given by the purpose of the use of the essential oil. In general, dry plants are included in pharmaceutical preparations for such material is easier to weigh and less variable in time, whereas, fresh plants are used for aromatherapeutic oils or in perfumery. Also, there is a great difference between the chemical composition of essential oil obtained from fresh or from dry plant material. This is why the pharmaceutical requirements and standards are different from those for aromatherapy (Angioni et al., 2006; Bakkali et al., 2008; Hanif et al., 2019; Masotti et al., 2003).

The choice of the extraction method is according to the final purpose of the essential oil, pharmaceutical use, aromatherapy and human use imposing stricter rules for the chosen methodology. For example, in perfumery, supercritical fluid extraction or nonpolar solvent extraction can be employed. Nevertheless, hydrodistillation is preferred for internal use or biological testing, as recommended by pharmacopoeia. Such a method is less toxic, almost costless but it takes a longer time than the modern techniques (lipophilic solvent extraction, supercritical carbon dioxide extraction, microwaves, low or high-pressure distillation etc.). Each of the mentioned techniques impacts greatly the stereochemistry of the essential oil spectra, the extracted molecules being usually optically active. Often, a change in its stereochemistry induces a variation in the biological activity of a compound (Bakkali et al., 2008; Hanif et al., 2019).

Taking into consideration all of the above, for the chemical and biological evaluation of the aromatic plants, we commonly use dry plant material and hydrodistillation in Neo-Clevenger type apparatus, which ensures a good extractability and quality of the volatile fractions. Although attempts of using variants (microwaves, ultrasounds, solvent extraction) for obtaining the essential oils were made in the past, our lab experience showed that the best quality and composition is attained by water distillation from the dried plants.

Among the more endemic species known to have medicinal value are *Lavandula angustifolia* Mill. (Lamiaceae) and *Lavandula hybrida* Rev. (Lamiaceae). The genus *Lavandula* contains at least 28 different species (Barrett, 1996). Flowers of different species of lavender have been known for their wide therapeutic use for centuries (Woelk and Schläfke, 2010).

Generally recognized, the main constituents of lavender oil for pharmaceutical use are linalool, linalyl acetate, cineole, terpinene-4-ol and camphor (corresponding to GC chromatogram of lavender oil, European Pharmacopoeia (Ph. Eur.) 6th edition, 2008). Ph. Eur. 6th edition describes a capillary gas-chromatographic method and imposes limits for the main terpenoids linalool, linalyl acetate and terpinene-4-ol (values which must be in the range of 20.0–45.0, 25.0–46.0 and 0.1–6.0, respectively). These constituents can vary significantly in different oils. Pure oil is most often used in aromatherapy and massage (Woelk and Schläfke, 2010). Despite its popularity and long tradition of use, only recently scientifically based investigations into the biological activity of the various *Lavandula* species have been undertaken to a greater extent.

As international scientific data sustains, *Lavandula angustifolia* Mill. contains at least 38 different compounds, and *Lavandula hybrida* Rev. contains at least 50 compounds (Pascual Teresa et al., 1991; Reverchon et al., 1995). Linalyl acetate and linalool are the most important chemical constituents in the essential oil of *Lavandula angustifolia* Mill., accounting for up to 90% of the oil by volume (Bissett, 1994; Jager et al., 1992). They also comprise over 70% of the essential oil of *Lavandula hybrida* Rev. (Buckle, 1993; Marotti et al., 1989; Peracino et al., 1994).

Lavandula angustifolia ssp. *angustifolia* Mill. and *Lavandula hybrida* Rev. were harvested from the Botanical Garden Galati (South-East of Romania) in July 2010 and identified. Vouchers specimens are preserved at the Department of Pharmacognosy, Faculty of Pharmacy ("Grigore T. Popa" University of Medicine and Pharmacy, Iasi, Romania), for ready reference. Organic volatile fractions of *Lavandula angustifolia* (LO1) and *Lavandula hybrida* (LO2) were obtained by hydro-distillation of dried flower heads.

The identification of the volatile compounds was based on a comparison of their retention indices (RIs), and mass spectra with those obtained from authentic samples and/or NIST/NBS, Wiley libraries and literature. Our results indicated that the main components in both analyzed samples, LO1 and LO2, were linalool (28.0% and 21.5%, respectively) and linalyl acetate (17% and 22.5%, respectively) followed by terpinene-4-ol (3.3% and 16.7%) and lavandulyl acetate (8.3% and 8.4%, respectively). Interesting is that the presence of camphor and borneol was in trace amounts, which is inconsistent with literature data (Adam, 2007; Lis-Balchin 2002).

The aspect of the GC-MS chromatograms for both samples are included in figure 12 and the main groups of components are indicated in table X, along with the pharmacopeial requirements for lavender essential oil for pharmaceutical use.

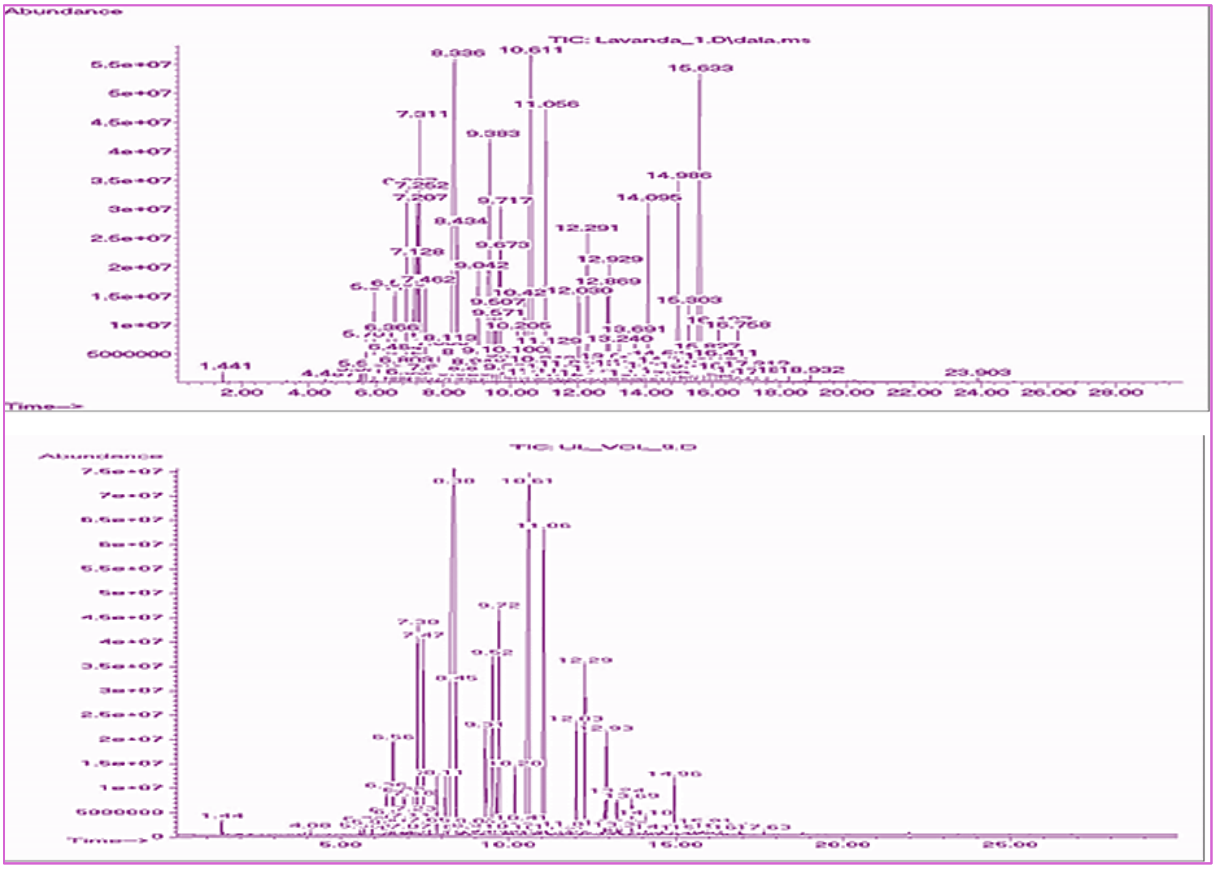

Fig. 12. GC chromatograms for the volatile fractions of *Lavandula angustifolia* (LO1) and *Lavandula hybrida* (LO2)

Table X. Main components identified in *Lavandula angustifolia* (LO1) and *Lavandula hybrida* (LO2) essential oils

Compound	Pharmacopoeial requirements	Samples	
		LO1	LO2
limonene	less than 1 %	2.9	0.8
1,8-cineole	less than 2.5 %	tr	tr
camphor	less than 1.2%	0.5	tr
linalool	20.0 – 45.0 %	28.8	21.5
linalyl acetate	25.0 – 46.0 %	14.7	22.5
terpinen-4-ol	0.1 – 6.0%	4.8	16.7
lavandulyl acetate	more than 0.2 %	7.6	8.4
lavandulol	more than 0.1 %	1.0	tr
α-terpineol	less than 2 %	5.7	7.5

The obtained results confirmed the interspecific variability, but their correlation to the pharmacopoeial provisions is not strict. Depending on the sample, each has parameters that are in agreement but also some components do not meet the requirements. Interesting is that α-terpineol is much higher than the imposed limit. This, however, is related to the environmental conditions for the plants, a higher altitude and dry climate is not favorable for the biosynthesis of linalyl acetate, but increases the terpineol concentration.

The common characteristic is represented by the presence of oxygenated monoterpenes in a concentration of more than 82 % and monoterpenes in an average concentration of at least 10%.

Another species-rich in linalool is coriander. In traditional medicine, *Coriandrum sativum* L. (Apiaceae) has been indicated for several medical problems such as dyspeptic complaints, loss of appetite, convulsion, insomnia and anxiety (Emamghoreishi et al., 2005; Gray and Flatt, 1999). There are two varieties of *C. sativum*: *vulgare* Alef. and *microcarpum* DC. These varieties differ in the fruit size and oil yield: *vulgare* has fruits of 3–5 mm diameter and yields 0.1%–0.35% essential oil, while *microcarpum* fruits are 1.5–3 mm and yield 0.8%–1.8% essential oil (Burdock and Carabin, 2009).

The main constituents of coriander volatile oil are linalool, α-pinene, γ-terpinene, geranyl acetate, camphor and geraniol (corresponding to GC chromatogram of coriander oil, European Pharmacopoeia (Ph. Eur.) 6th edition, 2008). Ph. Eur. 6th edition describes a capillary gas-chromatographic method and imposes limits for linalool (65.0%–78.0%), α-pinene (3.0%–7.0%), γ-terpinene (1.5%– 8.0%), geranyl acetate (0.5%–4.0%), camphor (3.0%–6.0%) and geraniol (0.5%–3.0%), respectively.

The data available on the toxicity of coriander volatile oil are limited. However, coriander and its oil have a long history of dietary use, with no record of harm caused by the consumption of these ingredients. Therefore, the use of coriander volatile oil is considered safe (Burdock and Carabin, 2009).

Mature fruits of coriander were collected from the experimental fields of Agricultural Research and Development Center, Secuieni, Neamt (Eastern Romania) in June 2012 and identified. Air-dried fruits of *C. sativum* var. *microcarpum* sample were subjected to hydro-distillation for 3 h using a Clevenger-type apparatus to obtain the volatile oil. The volatile oil was dried over anhydrous sodium sulfate and kept at −4 °C until analysis.

After calculation of the extraction yield, the chemical composition was analyzed by GC-MS and GC-FID by comparison with standard alkanes (general methodology is given previously in 1.2. Methods).

In the separated volatile fraction, we identified more than 60 components. From the total of 64 separated peaks, monoterpenes were the predominant class of compounds, with linalool (69.358%) being the major component.

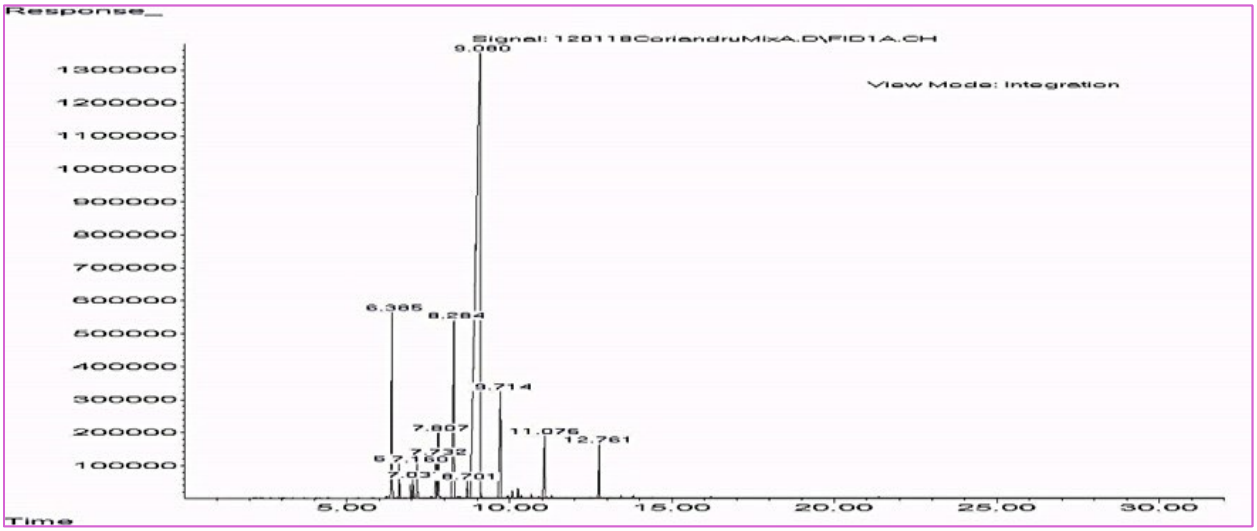

Fig. 13. The GC chromatogram for *C. sativum* var. *microcarpum* essential oil

Other important substances were: γ-terpinene (7.729%), α-pinene (6.509%), pinocarvone (4.388%), carvone (2.314%), β-ocimene (E + Z) (3.105%) and geranyl acetate (1.580%) (Table XI). The results were in accordance with the literature, where monoterpenes have been reported to be the main class in *C. sativum* fruit oils (Teuscher et al., 2006) with linalool being the predominant compound, while camphor and limonene were absent from the oil, but pinocarvone and carvone were present in significant amounts.

Table XI. Chemical composition of volatile oil from *C. sativum* var. *microcarpum* (selection)

RT	KI lit	KI exp	Compound	%
6.385	**939**	**940**	**α-Pinene**	**6.509**
6.607	954	955	CAMPHENE	0.853
7.031	975	974	SABINENE	0.806
7.160	991	991	MYRCENE	0.861
7.732	1032	1032	(*Z*)-β-ocimene	1.175
7.807	1044	1040	(*E*)-β-ocimene	1.930
8.284	**1060**	**1065**	**γ-Terpinene**	**7.729**
9.080	**1097**	**1098**	**Linalool**	**69.358**
9.714	**1165**	**1163**	**Pinocarvone**	**4.388**
11.076	1243	1246	Carvone	2.314
12.761	1381	1379	Geranyl acetate	1.580

RT = retention times; KI lit = reference retention indices; KI exp = experimental retention indices relative to C9–C23 n-alkanes. Significant compounds within the chemical composition of the volatile oil are shown in bold.

The *Ocimum* species are one of the most popular species worldwide, known for more than 2000 years and are one of the most versatile medicinal plants, with a wide spectrum of pharmacological activities like antimicrobial, immunomodulatory, anti-inflammatory, antioxidant, memory-enhancing and anti-diabetic (Beric´ et al. 2008; Kedlaya and Vasudevan 2004; Gradinariu et al. 2013a, b; Oboh 2008). *Ocimum basilicum* L. and *Ocimum sanctum* (known as tulsi) are the most studied *Ocimum* species. These species are also known as good sources of essential oil rich in monoterpenes such as linalool.

The early blooming stage plants (*Ocimum basilicum* L. - Ob, *Ocimum basilicum* var. *purpurascens* – Obr and *Ocimum sanctum* L. - Os), included in bio cultures from the Biological Research Center "Stejarul" Piatra Neamt, Romania, were harvested during August 2011. The plant material was dried in a special room (airflow, humidity and temperature were controlled all the time). The voucher specimens (no.2011/Ob, no.2011/Obr and no.2011/Os, respectively) were deposited at the Department of Pharmacognosy, Faculty of Pharmacy, "Grigore T. Popa" University of Medicine and Pharmacy Iasi, Romania, before the experiments. The basil essential oils were prepared by hydrodistillation of the dried plant material and its chemical composition was analyzed by GC–MS/GC-FID.

The yield of the essential oils varied from one sample to another: 0.91 mL% for Os sample, 1.18mL % for Ob and 2mL/100g dried plant for Obr sample. Monoterpenols (mainly linalool) and sesquiterpenes (mainly β-caryophyllene) were the most important groups of components for the essential oils. The main compounds found in basil and tulsi oil samples were linalool (31 %–Ob, 19 % Os), camphor, b-elemene, α-bergamotene and bornyl-acetate, estragole (15.57 %, respectively 7.59 %), eugenol (2.64 %, respectively 1.39 %) and 1,8-cineole (3.29 % respectively 3.90 %) (Fig. 14).

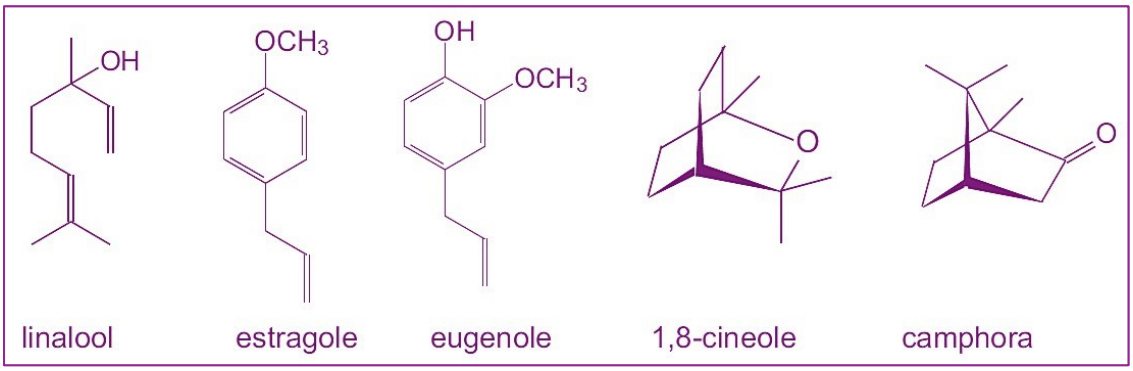

Fig. 14. Chemical structures of the main compounds identified in both samples of basil essential oil

The full chemical analysis results have been previously published (Gradinariu 2013b) and our results are partially consistent with the literature (Carovic´-Stanko et al. 2010). Comparing the composition of the analyzed essential oils, both quantitative and qualitative differences were revealed. The essential oils were dominated by L-linalool, which was found in impressive

quantities in the Obr sample (up to two, respectively, three times higher than in Ob sample, respectively, Os sample).

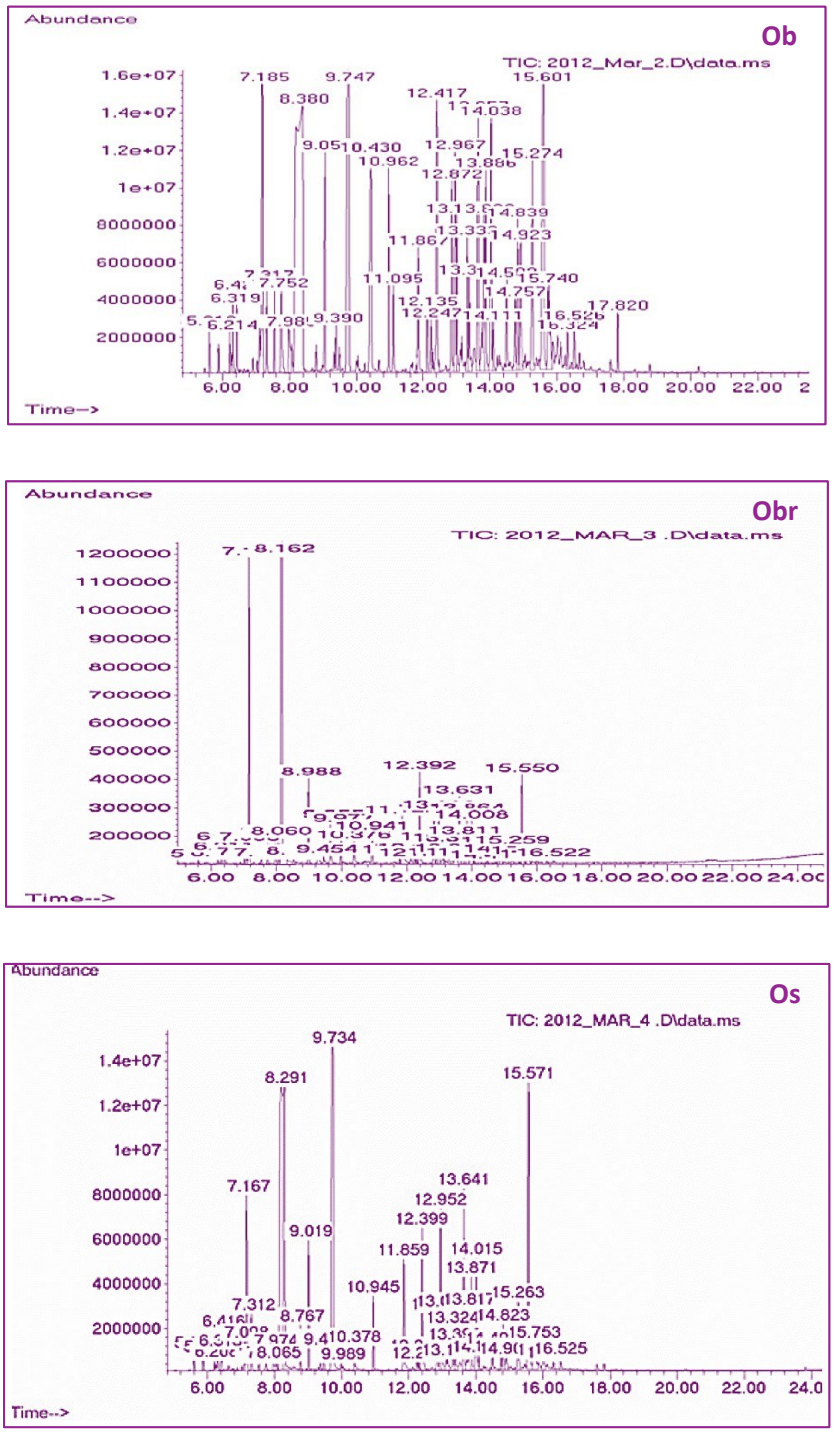

Fig. 15. The gas-chromatograms for the *Ocimum* essential oil samples

From the total amount of compounds (116 for Ob sample, 69 for Os sample, and 55 for Obr sample), we have selected only 39, quantitatively important (Table XII).

Table XII. Chemical composition of *Ocimum* essential oil samples (selection)

No.	RI	RT	Compound	Ob	Obr	Os
1	927	5.474	alpha-Thujene	-	-	0.04
2	935	5.610	alpha-Pinene	0.31	-	0.34
3	938	5.882	Camphene	0.26	-	0.21
4	974	6.213	Sabinene	0.16	0.08	0.3
5	980	6.320	beta-Pinene	0.38	0.21	0.59
6	991	6.427	l-Myrcene	0.74	0.4	0.59
7	1000	6.534	3-Octanol	-	-	0.04
8	1007	6.738	1-Phellandrene	-	-	0.03
9	1010	6.777	l-3-Carene	-	-	0.03
10	1015	6.903	alpha-Terpinene	0.06	-	0.13
11	1020	7.030	m-Cymene	-	0.11	0.11
12	1031	7.107	l-Limonene	0.53	0.39	0.43
13	1034	7.185	1,8-Cineole	3.29	-	3.9
14	1041	7.321	trans-beta-Ocimene	0.97	-	0.67
15	1065	7.545	gama-Terpinene	0.12	-	0.61
16	1075	7.749	l-Linalool oxide	-	0.5	1.21
17	1098	8.196	l-Linalool	31.08	66.72	19.25
18	1150	9.062	Camphor	2.26	1.7	2.31
19	1197	9.655	alpha-Terpinolene	-	1.17	-
20	1200	9.742	Estragole	15.57	-	7.59
21	1241	10.005	l-Fenchyl acetate	0.1	0.74	0.1
22	1254	10.433	l-trans-Geraniol	-	-	3.69

No.	RI	RT	Compound	Ob	Obr	Os
23	1291	10.958	n-Bornyl acetate	1.29	0.69	1.78
24	1354	11.094	Carvacrol	-	-	0.79
25	1357	11.872	Eugenol	2.64	-	1.39
26	1375	12.416	l-beta-Elemene	3.24	2.32	3.71
27	1421	12.873	beta-Caryophyllene	1.12	1.03	1.93
28	1439	12.97	l-alpha-Bergamotene	2.95	0.42	2.53
29	1440	13.019	alpha-Guaiene	1.22	1.03	1.46
30	1456	13.33	l-alpha-Humulene	0.87	0.47	1.24
31	1482	13.398	epi-Bicyclosesquiphellandrene	8.3	1.99	1.39
32	1489	13.661	l-Germacrene	3.82	1.58	3.25
33	1491	13.758	l-beta-Selinene	0.36	-	0.62
34	1504	13.826	l-Bicyclogermacrene	1.44	0.71	1.88
35	1506	13.884	l-delta-Guaiene	1.85	1.15	2.09
36	1566	14.04	l-alpha-Amorphene	2.34	1.07	3.3
37	1580	14.837	l-Spathulenol	0.96	-	1.49
38	1585	14.925	l-Caryophyllene oxide	0.47	-	1.62
39	1515	15.605	l-delta-Cadinene	0.1	-	6.8

The three studied species differ, both quantitatively and qualitatively. The essential oil of *O. basilicum* species is the richest in volatile components, whereas the red variety is the poorest. A few compounds were common to all the essential oils: linalool, camphor, β-elemene, epi-biciclosesquiphellandrene, β-caryophyllene, and 1-α-bergamotene. The highest quantities were recorded for linalool (31.08% for Ob sample, 66.72% for Obr sample, and 19.25% for Os sample). Still, important compounds, such as 1, 8-cineole, estragole, eugenol and trans-beta-ocimene, were missing in the Obr sample.

Estragole, another major component, was present only in Ob sample (15.57%) and Os sample (7.59%) and was missing in Obr sample, a fact that can make Obr suitable for safe administration to children (recent studies reveal that herbal infusions obtained from plants with

high estragole content can induce carcinogenic effects and hormonal imbalance if its concentration exceeds 10%) (Speijers et al., 2010).

A special chemotype (CT) of basil is used in aromatherapy, *O. basilicum* CT linalool. Our sample of *O. basilicum* essential oil can be included in this chemotype, but the estragole quantity is half compared with the aromatherapeutic oil (30 %). However, such results indicate lower toxicity (carcinogenic) that has been established for this compound (Grădinariu V, **Cioancă O**, Gille E, Aprotosoaie AC, Hriţcu L, Hăncianu M. The chemical profile of basil bio-varieties and its implication on the biological activity. Farmacia 2013; 61(4): 632-639; Grădinariu V, **Cioancă O**, Hriţcu L, Hăncianu M. Basil bio-varieties cultivated in Romania and the chemical profile of the volatile oil. Advances in Biotechnology, 13th SGEM GeoConference on Nano, Bio And Green - Technologies For A Sustainable Future, SGEM2013 Conference Proceedings, 2013: 113-120).

Continuing with the evaluation of the volatile components, during the postdoctoral scholarship in biomedical research won by competition within the "Program of excellence in multidisciplinary doctoral and postdoctoral research in chronic diseases" POSDRU/159/1.5 /s/133377 – "Grigore T. Popa" University of Medicine and Pharmacy Iasi, I investigated several aromatic and medicinal plants with the purpose to find the best terpenoids with putative neuroprotective effects. Later on, this themed research was continued on other species in the Young Research Teams Internal Grant (no. 1642/2013) won by competition with the title "*Characterization of the biological effects of volatile oils rich in monoterpenic alcohols with relevance in neuroprotection*", as grant director during 2013-2014.

For this purpose, I selected three species *Salvia officinalis*, *Juniperus communis* and *Foeniculum vulgare*. For each of these species, samples of plant products were harvested and dried, taking into account the strict rules of storage and disposal of plant products intended for use in therapy. The dry samples were crushed (in the electric grinder) to obtain homogeneous powders, which were subjected to entrainment with water vapour in the Neo-Clevenger type installation for obtaining volatile oils.

Plants from the *Juniperus* genus have an extensive extensively history of use in global folk medicine for various disorders, such as common colds, urinary and kidney infections and dermatological disorders (Allen and Hatfield 2004). It has been reported that *Juniperus* sp. exhibited various proprieties as follow: anti-inflammatory (Lesjak et al., 2011), antioxidant and antimicrobial (Miceli et al., 2009), hypotensive, abortifacient, antinociceptive (Akkol et al., 2009), anticholinesterase (Öztürk et al., 2011) analgesic properties (Moreno et al., 1998) and memory-enhancing effects (Cioanca et al., 2014).

Juniperus communis berries were purchased from the Romanian pharmaceutical market in 2012 and identified in the Department of Pharmacognosy, Faculty of Pharmacy, University of Medicine and Pharmacy "Gr T. Popa", Iasi, Romania where a voucher specimen was registered

and deposited for ready reference. The juniper berry volatile oil was produced by hydro-distillation for 2 h using a Clevenger type apparatus (70 g of vegetal material in 600 mL of water).

The main compounds identified were: α-thujene (3.78 %), α-pinene (41.13 %), sabinene (11.73 %), myrcene (10.16 %), limonene (8.63 %), γ-terpinene (1.57 %), terpinene-4-ol (3.42 %), followed by lower quantities of β-elemene (1.1 %), β-caryophyllene (1.03 %), germacrene D (1.51 %), eremophylene (1.17 %) as indicated in Table XIII.

Table XIII. Chemical composition of the volatile oil from *Juniperus communis* berries

RILIT.	RIEXP.	COMPOUNDS	CONCENTRATION (%)
Monoterpenes hydrocarbons			
930	927	α-thujene	3.78
939	934	α-pinene	41.13
953	950	camphene	0.69
976	975	sabinene	11.73
980	981	β-pinene	1.92
991	989	β-myrcene	10.16
1005	1002	α-phellandrene	0.13
1018	1014	α-terpinene	1.20
1026	1027	p-cymene	2.24
1031	1035	limonene	8.63
1062	1059	γ-terpinene	1.57
1088	1087	α-terpinolene	0.84
Oxygenated monoterpenes			
1165	1163	borneol	0.46
1177	1178	terpinen-4-ol	3.42
1194	1194	myrtenol	0.29
1217	1214	*trans*-(+)-carveol	0.24
1285	1286	l-bornyl acetate	0.37
Sesquiterpenes hydrocarbons			
1351	1349	α-cubebene	0.51
1376	1377	α-copaene	0.22
1375	1380	β-elemene	1.1
1418	1416	*trans*-β-caryophyllene	1.03
1430	1432	γ-elemene	0.47
1440	1441	humulene	0.60
1458	1456	β-farnesene	0.81
1461	1457	alloaromadendrene	0.61
1477	1477	α-elemene	0.38
1480	1479	germacrene D	1.51
1494	1493	α-selinene	0.80
1503	1503	eremophilene	1.17
1524	1522	δ-cadinene	0.44
1560	1561	germacrene B	0.93
TOTAL (%)			95.66

RILIT. = literature retention indices; RIEXP. = experimental retention indices relative to C8-C22 n-alkanes on the DB-5MS column.

The chemical composition of the juniper volatile was analyzed by GC–MS/FID (figure 16).

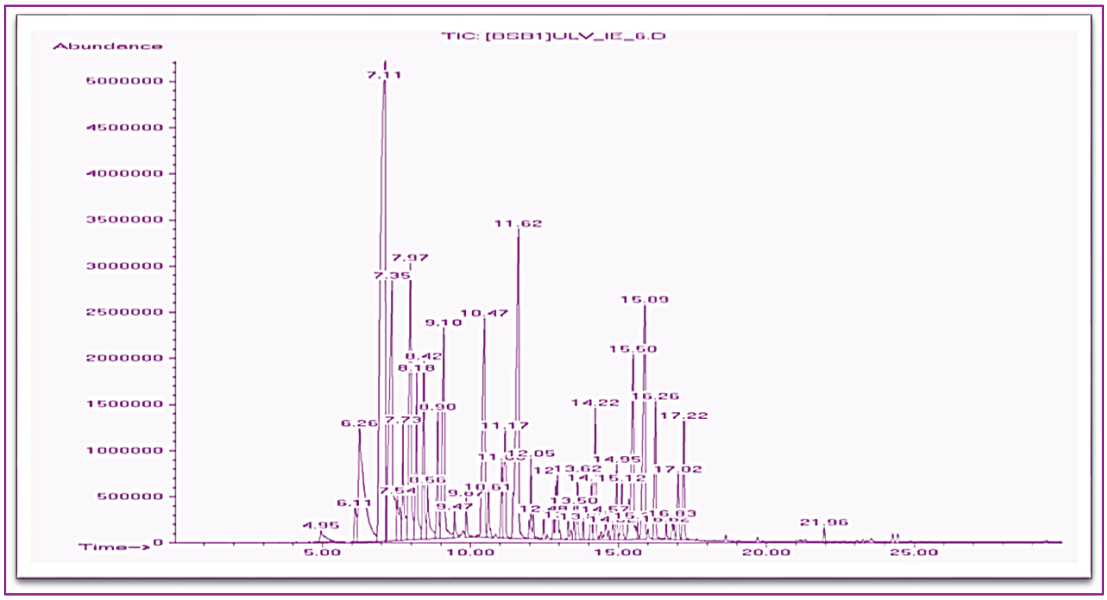

Fig. 16. GC-profile of the investigated juniper essential oil

Among them, α-pinene (41.13%) was identified in high amounts and it has been reported to present anxiolytic effects (Satou et al., 2010). Here we reported the extraction yield concentration of the oil components without any standardization procedure. We noted that α-pinene (41.13%), sabinene (11.73%), myrcene (10.16%) and limonene (8.63%), all monoterpenes hydrocarbons, account for 70% of the chemical composition of the juniper volatile oil, and therefore we might presume that the biological effects of the essential oil would be induced by them. Most of the compounds are within the limits imposed by the European Pharmacopeia, 6th ed., 2007, and therefore we consider that the volatile oil isolated from the samples included in this study could represent an important source for pharmaceutical preparations (Hriţcu L, Hăncianu M, Mihăşan M, **Cioancă O**. Effects of inhaled juniper volatile oil in amyloid beta (1-42)-induced anxiety and depression in laboratory rats. Flavour and Fragrance Journal 2016; 31(2): 149-157; **Cioancă O**, Mircea C, Trifan A, Aprotosoaie AC, Hriţcu L, Hăncianu M. Improvement of amyloid- β-induced memory deficits by *Juniperus communis* L. volatile oil in a rat model of Alzheimer's disease. Farmacia 2014; 62 (3): 514-520).

Considerable attention has been devoted to genus *Salvia*, for example, hydro-alcoholic leaf extracts from *S. officinalis, S. sclarea, S. triloba* and *S. lavandulifolia* were investigated as radical scavengers against 1,1-diphenyl-2-picrylhydrazyl (DPPH), among which *S. officinalis* extracts were the most active (IC50 41 μg/mL) (Lamaison et al., 1990). Nevertheless, sage is mostly known for its essential oil that confers soothing, carminative, tonic and antimicrobial effects, being used

as a medicinal remedy and as a food and cosmetic preservative. In aromatherapy, the essential oil of sage is known for its antiviral, antibacterial, antifungal, secretolytic, febrifuge, carminative, dermal healing and regenerating and estrogen-like qualities. The psychological level induces relaxation, memory and concentration stimulation. Therefore, *Salvia* essential oil is recommended in aromatherapy as a remedy for cough and bronchitis, thrush wounds, herpes and zoster herpes, hyperhidrosis, climacteric syndrome, impaired concentration. Due to its known high content in thujone and camphor the maximum level of inclusion of *Salviae aetheroleum* in foods (Regulatory Status on Thujone-Scientific Committee on Food) and perfumes is 0.013% and 0.8% respectively. However, there is no published data on *in vitro in vivo* comparison of antioxidant potential of the essential oil extracted from *Salviae folium*. Therefore, the goal was to evaluate both *in vitro* and *in vivo* antioxidant effects of sage essential oil isolated from dried leaves of *Salvia officinalis* of certified origin, cultivated in ecological environment conditions.

The plant material was acquired from known sources (Biological Research Centre from Piatra Neamt) to ensure a certified origin and a proper identification of the investigated species: *Salvia officinalis* leaves. The dry plant material was sorted and weighed, then extracted by distillation with water vapours according to the method specified in the European Pharmacopoeia 7th ed. The extraction was carried out in Neo-Clevenger type apparatus for 3 hours, after which the essential oil volume was measured and compared to the 100 g dry plant product. The extraction yield was 2.9 mL to 100g dry plant (Miguel et al., 2011).

The obtained results, GC-MS chromatogram and the selective presentation of the most important terpenoids identified and quantified in the *Salviae aetheroleum* sample are indicated in figure 17 and table XIV.

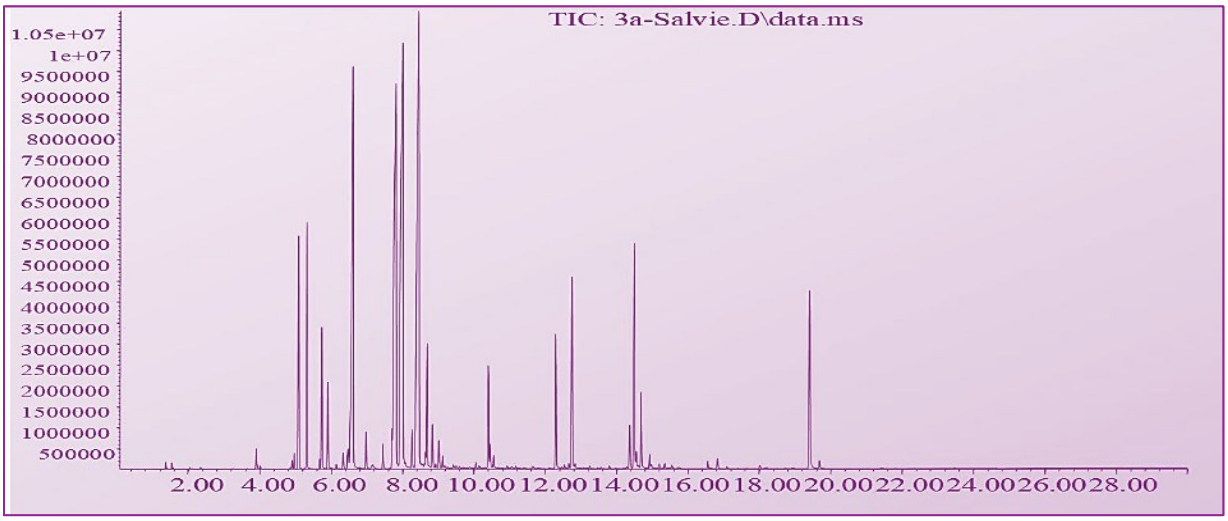

Fig. 17. GC chromatogram for *Salviae aetheroleum* sample

Table XIV. Compounds identified in the volatile oil obtained from *Salvia officinalis*

Compounds	***%***
α-thujene	0.23
α-pinene	2.36
camphene	3.50
sabinene	0.14
β-pinene	2.28
myrcene	1.12
limonene	1.39
1,8-cineole	13.94
terpinolene	1.67
α-thujone	22.60
β- thujone	8.26
L-camphor	12.17
L-borneole	2.42
4-terpineol	0.46
bornyl acetate	1.94
β-caryophyllene	4.10
α-humulene	3.49
γ-gurjunene	2.11

During the GC-MS/FID analysis we identified 45 peaks out of which the majority (84.48%) represented monoterpenes (α- and β-pinene 4.64%, camphene 3.50%, sabinene, myrcene, limonene 1.39%, 1,8-cineole 13.94%, thujone, camphor 12.17%, 4-terpineole, borneol 2.42%, bornyl acetate

1.94%) and only a small amount were sesquiterpenes (3.10% caryophyllene, 3.49% humulene, 2.11 % gurjumene).

This chemical composition is somewhat similar to *Salviae aetheroleum* for aromatherapeutic use that should contain: 30-60% monoterpene ketones (thujone), 8-15% oxides (1,8-cineole), 5-15% monoterpenes (pinene, camphene), 5-15% sesquiterpenes, 5-10% monoterpenols (borneol), 1-4% sesquiterpenols and traces of diterpenes such as salviol (Abdolrasoul et al., 2010; Ben Farhat et al., 2009). Since the total amount of thujone was 30.86%, which indicates low neurotoxicity considering that there are samples of *Salvia officinalis* with amounts above 60%, we concluded that we could use the isolated essential oil in animal testing (Cioancă O, Mircea C, Hriţcu L, Trifan A, Mihăşan M, Aprotosoaie AC, Robu S, Gille E, Hăncianu M. *In vitro-in vivo* correlation of the antioxidant capacity of *Salviae aetheroleum*. Farmacia 2015; 63(1): 34-39).

Used as a spice and to improve the palatability of different meat and vegetable dishes, common fennel, *Foeniculum vulgare* Mill. (Apiaceae), was a traditional remedy for the relief of spasms and colic due to gas accumulation, to stimulate gastrointestinal motility, to alleviate productive coughs as well as for the induction of menstruation and lactation.

Foeniculum vulgare Mill. (Apiaceae) commonly known as fennel is an aromatic plant widely cultivated in temperate and tropical regions (Aprotosoaie, et al., 2010). It has been reported that *F. vulgare* exhibited medicinal effects as evidenced by a different animal and clinical studies include, antibacterial and antifungal (Özcan et al., 2006), antioxidant (Barros et al., 2009), anti-inflammatory (Chainy et al., 2000), anti-atherosclerotic (Oulmouden et al., 2011), gastroprotective (Birdane FM, 2007), hepatoprotective (Özbek et al., 2003) and diuretic (Wright et al., 2007). Moreover, it has been reported that the crude extract of *F. vulgare* has an anxiolytic profile in the mice model (Kishore et al., 2012).

Mature fennel fruits were harvested during August 2011 from biocultures of the Biological Research Center ‘‘Stejarul’’ Piatra Neamt, Romania. The plant material was dried in an oven, controlling permanently the airflow, humidity and temperature (35^{o}C). The voucher specimens (no.2011/Fv) were deposited at the Department of Pharmacognosy, Faculty of Pharmacy, University of Medicine and Pharmacy’’ Gr. T. Popa’’ Iasi, Romania, before the experiments.

The essential oil was obtained by steam distillation in a Clevenger type apparatus for 3 hours. Then, the isolated samples were dried over anhydrous sulphate and stored at -4^{o}C until testing.

The GC-MS/FID analysis of *Foeniculum vulgare* volatile oil indicated trans-anethole (58.135%), a phenylpropanoid, followed by camphor (21.297%), a terpene ketone, as the main

components of this volatile oil. In total, 71 components were identified, but among them only 11 representing 96.8% of the total amount (Table XV, Fig. 18).

Table XV. Chemical composition of the essential oil from *Foeniculum vulgare* fruits

No.	Compound	RT (min)	RI[a]	%
1	α-thujene	6.1	930	tr
2	β-pinene	6.4	939	2.6
3	camphene	6.5	954	tr
4	sabinene	6.6	975	0.2
5	myrcene	6.8	991	0.3
6	*p*-cymene	7.0	1025	0.1
7	L-limonene	7.2	1029	0.9
8	γ-terpinene	7.4	1060	0.2
9	*trans*-sabinene hydrate	7.8	1071	3.1
10	α-fenchone	8.2	1094	0.5
11	α-thujone	8.5	1116	0.1
12	camphor	8.9	1146	21.3
13	estragole	9.6	1195	0.6
14	(+)-carvone	10.4	1243	7.8
15	*p*-anisaldehyde	11.3	1251	1.5
16	*trans*-anethole	11.8	1289	58.1
17	germacrene D	12.7	1561	0.3
Total identified				97.6
Other compounds[b]				2.4

[a]RI provided for HP-5MS column.
[b]this category includes monoterpenes and oxidized terpenes with amounts less than 0.1% that are not included in the table.

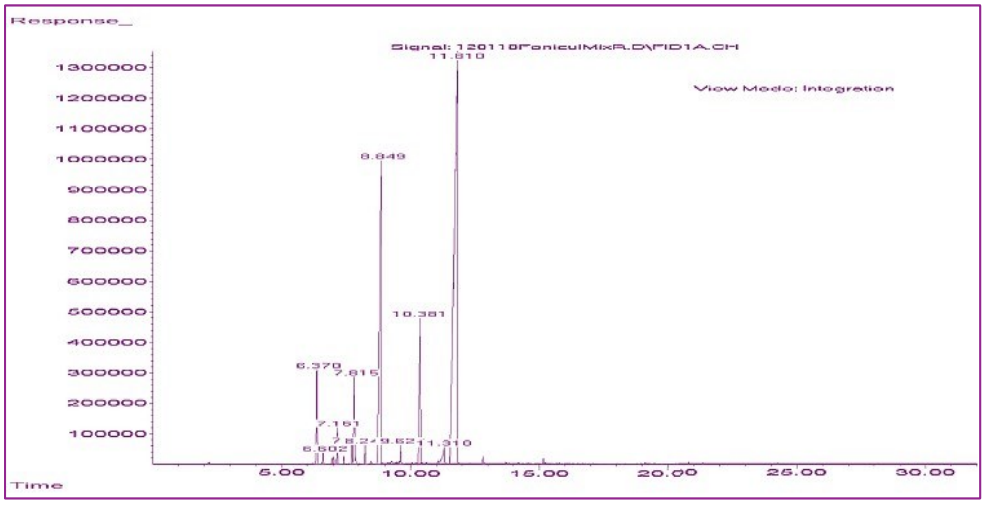

Fig. 18. The GC profile of the *Foeniculum vulgare* essential oil.

Camphor (21.3%), a terpene ketone, was the second major compound detected in fennel essential oil, followed by carvone (7.8%), trans-sabinene hydrate (3.1%), β-pinene (2.6%), and others were found to be the minor components in the essential oil of fennel seeds. According to this profile of the components, our fennel essential oil has anxiolytic activity and works mainly as a tonic agent for the central nervous system.

The major component of the essential oil, trans-anethole, has been reported to display a potent anxiolytic activity in mice (Miyagawa, et al., 2014). All the results were included in the following paper **Cioancă O**, Hăncianu M, Mircea C, Trifan A, Hriţcu L. Essential oils from Apiaceae as valuable resources in neurological disorders: *Foeniculi vulgare aetheroleum*. Industrial Crops and Products 2016, 88: 51-57.

All of the above data suggest that aromatic and medicinal plants represent rich sources of natural molecules that vary depending on the species, cultivation conditions, extraction and processing methods. Nevertheless, this emphasizes the importance of the quality of the plant material and represents the basis for further research with the intent to enlarge the material ground for therapeutics and medicine development.

II. Current scientific evidence of the biomolecular potential of selective herbal extracts

Considering all available data and my own experience in terms of medicinal plants and natural biomolecules, I continued to research possible biological effects for plant components and selective herbal extracts. Starting from the scientific evidence, the research for putative activities of herbal extracts was undertaken in two distinctive directions - *in vitro* and i*n vivo.*

For this purpose, several interdisciplinary collaborations were established with the Department of Pharmaceutical and Clinical Biochemistry, Microbiology Discipline and Drug control Discipline from "Grigore T. Popa" University of Medicine and Pharmacy from Iasi, Animal physiology - Faculty of Biology from "Al. I. Cuza" University of Iasi, Department of Pharmacognosy and Toxicology from "Victor Babes," University of Medicine and Pharmacy from Timisoara, Laboratoire de Biochimie et Génétique Moléculaire, Faculté des Sciences et Techniques, Université Abdelmalek Essaadi, Tanger, Morocco and Department of Pharmacognosy, Faculty of Pharmacy, Gazi University, Ankara, Turkey.

Briefly, *in vitro* evaluation consisted in the assessment of the antioxidant potential, enzyme inhibitory and antimicrobial effects, as well as the evaluation of cell viability on various cell lines and cytotoxicity.

In vivo assessment was represented by animal models, mainly rat and zebrafish models of neurodegenerative dementia (mild cognitive impairment – MCI, Alzheimer's disease – AD, Parkinsons – PD). For each model the impact of the herbal selective fractions was assessed through behavioral tests, memory tests, oxidative parameters and histochemical methods for DNA fragmentation.

The most significant publications in this domain, both as main author as well as a coauthor, are indicated as follows:

1. El-Akhal J, Humulescu I, Ionita R, Postu PA, Ungureanu E, Hancianu M, Bencheikh R, Robu S, **Cioanca O**, Hritcu L. Anxiolytic and Antidepressant-Like Effects of Conyza canadensis Aqueous Extract in the Scopolamine Rat Model. Plants. 2021;10(4):645.
2. Brinza I, Boiangiu RS, Hancianu M, **Cioanca O,** Erdogan Orhan I, Hritcu L. Bay Leaf (Laurus Nobilis L.) Incense Improved Scopolamine-Induced Amnesic Rats by Restoring Cholinergic Dysfunction and Brain Antioxidant Status. Antioxidants. 2021;10(2):259.
3. Batir-Marin D, Boev M, **Cioanca O**, Mircea C, Burlec AF, Beppe GJ, Spac A, Corciova A, Hritcu L, Hancianu M. Neuroprotective and Antioxidant Enhancing Properties of Selective Equisetum Extracts. Molecules. 2021;26(9):2565.
4. Burlec AF, Pecio Ł, Kozachok S, Mircea C, Corciovă A, Vereștiuc L, **Cioancă O**, Oleszek W, Hăncianu M. Phytochemical Profile, Antioxidant Activity, and Cytotoxicity Assessment of Tagetes erecta L. Flowers. Molecules. 2021;26(5):1201.
5. Danciu C, **Cioanca O,** Hancianu M, Racoviceanu R, Muntean D, Zupko I, Oprean C, Tatu C, Paunescu V, Proks M, Diaconeasa Z. Botanical Therapeutics (Part II): Antimicrobial and In Vitro Anticancer Activity against MCF7 Human Breast Cancer Cells of Chamomile, Parsley and Celery Alcoholic Extracts. Anti-Cancer Agents in Medicinal Chemistry (Formerly Current Medicinal Chemistry-Anti-Cancer Agents). 2021;21(2):187-200.
6. Postu PA, Gorgan DL, **Cioanca O,** Russ M, Mikkat S, Glocker MO, Hritcu L. Memory-Enhancing effects of Origanum majorana essential oil in an alzheimer's amyloid beta1-42 rat model: a molecular and behavioral study. Antioxidants. 2020;9(10):919.
7. Abidar S, Boiangiu RS, Dumitru G, Todirascu-Ciornea E, Amakran A, Cioanca O, Hritcu L, Nhiri M. The Aqueous Extract from Ceratonia siliqua Leaves Protects against 6-Hydroxydopamine in Zebrafish: Understanding the Underlying Mechanism. Antioxidants. 2020;9(4):304.
8. Boiangiu RS, Brinza I, Hancianu M, Erdogan Orhan I, Eren G, Gündüz E, Ertas H, Hritcu L, **Cioanca O.** Cognitive Facilitation and Antioxidant Effects of an Essential Oil Mix on Scopolamine-Induced Amnesia in Rats: Molecular Modeling of In Vitro and In Vivo Approaches. Molecules. 2020;25(7):1519.
9. Iancu C, Mircea C, Petrariu F, **Cioanca O**, Stan C, Corciova A, Murarasu A, Filip N, Hancianu M. The Evaluation Of Normo-Glycemic and Cyto-Regenerative Effects of Pelargonium Species Extracts. Farmacia. 2020;68(1):135-41.

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Although the list is this long, for a better comprehension, only several of the obtained results are included in the present thesis.

II.1. In vitro evaluation of biological effects

Oxygen is the essential element for maintaining cellular activities, but also for the body in general (Burton and Jauniaux, 2011). Penetrated in the form of gas, through the respiratory tract, molecular oxygen is mainly transformed into the water in the biochemical processes in the respiratory chain and thus allows the production of the energy required for cellular processes, in the form of ATP (Burton and Jauniaux, 2011). Under physiological conditions, some of this oxygen is transformed into reactive oxygen species (ROS), for which neutralization systems in the form of antioxidant substances or enzymes are developed at the cellular level. Long-term or repeated exposure of the body to oxidative stress causes the development of pathological phenomena,

including cancer (Dröge, 2002; Milovanović et al., 2007; Glasauer and Chandel, 2013). In these situations, it is necessary to supplement the antioxidant defense capacity at the cellular level by administering antioxidant vitamins (vitamins C and E) or plant extracts that contain antioxidants (Lipinski, 2011; Halliwell and Gutteridge, 2015).

Numerous tests to evaluate the antioxidant action *in vitro* have been developed over time, as a preliminary step to testing the antioxidant effects *in vivo* (Čanadanović-Brunet et al., 2009). From these tests, there were selected those based on antioxidant mechanisms that can also occur in the biological environment, such as chelation of ferrous ion, lipoxygenase inhibition, the scavenger capacity of the hydroxyl radical, and the superoxide radical anion (Neha et al., 2019).

The antioxidant capacity of many medicinal plants has been the main goal of different studies. Such properties play an important role in preventing mutagenesis, tumorgenesis and degenerative disorders, induced by oxidative stress (Cioanca et al., 2015; Iova et al., 2014; Linck et al., 2010). Moreover, various aromatic and medicinal plants are indicated for immune system boosting and photoprotection against radiations prior and after exposure to sun or X-rays.

Both free and oxygen radicals have been involved as mediators to cell and tissue injury, inflammation, ischemia, neurodegeneration and dementia. Therefore, a great number of evidence-based studies tried to explain the mechanisms and structure relation activity. Most of these studies are related to polyphenols, renowned compounds for their powerful antioxidant properties (Čanadanović-Brunet et al., 2009; Cioanca et al., 2015; Viskelis et al., 2009).

Phenolic compounds are metabolites derived from L-phenylalanine (Petti et al., 2009), including a large group of substances such as phenolic acids, hydroxycinnamic acids, lignans, tannins, and flavonoids (Almanasrah et al., 2015; Abidar et al., 2020).

Phenolic compounds improve color, flavor, and food quality (El Hajaji et al., 2010). They have shown beneficial effects on human health (Roseiro et al., 2013), suggesting their use in pharmaceutical industries (Kasrati et al., 2014). Indeed, they possess antioxidant activity (Oniszczuk and Podgórski 2015), inhibiting, thus, ROS (Kukula-Koch et al., 2013), such as superoxide anion (O2-), hydroxyl radical (OH-), and hydrogen peroxide (H2O2) (Park and Jhon, 2010) contributing to the prevention of several diseases including cancer, cardiovascular illnesses (El Hajaji et al., 2010) and neurodegenerative diseases (Chonpathompikunlert et al., 2010). These natural compounds will constitute an alternative to the use of synthetic antioxidants (tertiary butyl hydroxy quinone (TBHQ), butylated hydroxyanisole (BHA), and propyl gallate (PG), which have already shown carcinogenic effects (Custódio et al., 2011; Abidar et al., 2020).

There is much literature to support the high antioxidant activity of hydroxycinnamic acid derivates (notably rosmarinic acid), caffeic acid, as well as phenolic diterpenes (carnosic acid, carnosol and derivatives) (Lu and Foo, 2001; Orhan et al., 2013; Pizzale et al., 2002). Also, in 2007 Grzegorczyk et al. proved that extracts of sage roots from *in vitro* cultures exhibited strong

antioxidant activity, dependent on the type of the solvent used for extraction, when low polarity extracts in acetone (containing mainly carnosic acid and carnosol) showed strong activity in lipid peroxidation assay, whereas methanolic extract (characterized by the presence of both rosmarinic acid and diterpenes derivatives) had better radical scavenging properties against DPPH (Grzegorczyk et al., 2007).

Actually, the type of the test and the mechanism are selected depending on the herbal extract chemical composition, although the presence/absence of lypo-/hydrophylic molecules is not directly correlated to the putative antioxidant activity of the investigated extracts.

II.1.1. Free Radical Scavenging/Chelating Activity

Free radicals remain a very challenging problem in today's therapy. They are considered promoters and aggressor at the same time, their balance representing the basis of a healthy organism. Therefore, overproduction of such molecules leads to disruptive effects on cells and tissues. Antioxidants, on the other hand, are compounds that can maintain the balance of redox processes. This is why, adopting antioxidants as preventive therapies is very common nowadays.

The most common methods used in our research to identify the scavenging/chelating activity of herbal extracts/compounds are given briefly below.

- **Diphenylpicrylhydrazyl (DPPH) radical scavenging assay**

DPPH radical is a synthetic compound which is not found in the living world, but which is frequently used for the screening of the antioxidant potential due to its increased reactivity to natural compounds (Iancu et al., 2017b). Free radical scavenger activity was assessed by measuring the ability to neutralize 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical and the transformation of the reduced form by the analyzed plant extracts (Orhan et al., 2013). Due to unshared pair of electrons of the two nitrogen atoms DPPH is a stable radical that strongly absorbs at 517 nm. By reaction with a scavenger of free radicals the DPPH solution changes color from purple to yellow due to the formation diphenyl-picrylhydrazyne (DPPH-H), a compound of low absorbance at 517 nm.

Stock solution (60 mg/mL) of the investigated samples was diluted in methanol to obtain concentrations ranging from 10 µL/mL to 40 µL/mL. Diluted solutions (1 mL each) were mixed with 1 mL of a freshly prepared 4% DPPH radical methanol solution and allowed incubate for 30 min in the dark at room temperature. The absorbance was recorded on an ultraviolet- visible (UV–Vis) spectrometer (ABL&E JASCO) at 517 nm using a blank containing the same concentration of oil without DPPH solution. Inhibitions (%) of DPPH radical were calculated with the following formula: **I% = 100 x scavenger activity (Acontrol- Asample) / Acontrol,** where: **Acontrol** = absorbance of DPPH solution before adding the test solution; **Asample**= absorbance of DPPH solution after 5 minutes after addition of the test solution. In parallel, for each sample **IC_{50}** value

(sample concentration providing 50% inhibition) was calculated, expressed in µL/mL final solution (Cioanca et al., 2015).

- **ABTS assay**

The procedure based on the inhibition of the production of the ABTS radical cation did not involve a substrate. ABTS with an absorption maximum at 342 nm has high water solubility and chemical stability. It is a peroxidase substrate, which, when oxidized in the presence of H_2O_2 generates a metastable radical cation with a characteristic absorption spectrum and high molar absorptivity at 414 nm (Re et al., 1999).

- **Fe2+ chelation activity assay**

The ferrous ion is present in serum and intracellular fluid in extremely small amounts, but when its concentration increases and its protein binding capacity is low, it may be involved in pathological phenomena (Venditti et al., 2010). At the cellular level, it participates in the Fenton and Haber-Weiss reactions of hydroxyl radical generation, one of the most aggressive free radicals involved in the occurrence of oxidative stress (Eid et al., 2017). The chelating capacity of ferrous ions is an important indicator for the evaluation of the antioxidant activity (Batir-Marin et al., 2021).

Freshly prepared iron chloride (2 mM) was added to a reaction mixture containing 400 µL DMSO extract solution, in four concentrations, and 80 µL ferrozine, 5 mM. A first measurement of the absorbance was performed at 562 nm in a Jasco V- 550 UV/VIS spectrophotometer. After 10 min of reaction at RT, the absorbance was measured, for the second time, at the same wavelength. Caffeic acid was used as standard. Sample concentration providing 50% inhibition (IC_{50}) was obtained plotting the inhibition percentage against sample concentration.

- **FRAP assay**

The ferric reducing antioxidant power (FRAP) method is based on the reduction of a ferrin analogue, the Fe^{3+} complex of tripyridyltriazine $Fe(TPTZ)^{3+}$, to the intensely blue coloured Fe^{2+} complex $Fe(TPTZ)^{2+}$ by antioxidants in an acidic medium. Results are obtained as absorbance increases at 593 nm and can be expressed as micromolar Fe^{2+} equivalents or relative to an antioxidant standard.

- **Hydroxyl radical assay**

The hydroxyl radical causes the initiation of oxidation reactions of unsaturated fatty acids that are present in the structure of membrane phospholipids, and their oxidation under conditions of oxidative stress will affect the stability of the cell membrane with the risk of uncontrolled loss of cell contents or reduced cell viability. Last but not least, this hydroxyl radical induces the

oxidation of proteins with the modification of their spatial structure and affecting their biological functions. *In vitro* and *in vivo* studies have shown the negative effect of plasma hydroxyl radical on the stability of fibrinogen which will be more easily converted to fibrin, which ultimately leads to increased blood coagulation [10,15].

II.1.2. Enzymatic Inhibition Potential

Enzymatic systems represent important molecular targets for antioxidants in the attempt to counteract the redox imbalance and overproduction of reactive oxygen species. Each enzyme has its own area of influence in the live organisms and presents specific characteristics. Natural compounds such as the vegetal secondary metabolites can influence and interact with enzymatic systems to inhibit toxicity and degenerative processes progression.

This set of tests were a further step in my research and implicated new collaboration with the colleagues from the

In our research, for i*n vitro* testing, the most relevant enzyme assays are presented briefly as follows:

- **15-Lipoxygenase inhibitory assay**

Lipoxygenase (EC.1.13.11.33) is an enzyme of the oxidoreductase class that catalyzes the oxidation of unsaturated fatty acids with the formation of peroxides [26]. They are involved in oxidative phenomena at the cellular level increasing the oxidation rate of lipids with the appearance of pathological phenomena. The enzyme is also involved in the synthesis of inflammation mediators ().

Natural molecules found in selective herbal extracts have the ability to block the activity of lipoxygenase which catalyzes the oxidation of linoleic acid, thus reducing the absorbance at 234 nm. An aliquot of 15-lipoxygenase in a borate buffer is mixed with the sample solution in DMSO (various concentrations); 10 min later, 2 mL of 0.16 mM linoleic acid borate buffer is added and the absorbances are registered at 234 nm for 90 seconds (Cioanca et al., 2015; Mircea et al.,). Inhibition of 15-lipoxygenase is established with the formula: per cent inhibition = (AEFI - AECI) x 100/AEFI; AEFI is the difference in enzyme absorbance without inhibitor at 90 and 30 seconds, while AECI represents the same difference in the enzyme-inhibitor mixture.

- **Acetylcholinesterase inhibition**

The ability of the investigated extracts to inhibit acetylcholinesterase was assessed by Ellman's method by measuring the absorbance variations at 412 for 5 min. The activity value is

calculated as a percentage of the difference between the absorbance values (enzyme solution with inhibitor after 5 min and the absorbance of the same solution at the initial time).

- **Butyrylcholinesterase inhibition**.

For the inhibitory potential against butyrylcholinesterase, we used acetylthiocholine iodide (0.2 M) as a substrate, but the rest of the assay is conducted similarly on the method described above for acetylcholinesterase evaluation. The measuring time is also 5 min and the temperature for determination was 25°C. Galantamine will be used as a positive inhibitor. The values recorded in the test represent the mean and standard deviation of triplicate measurements.

II.1.3. Antimicrobial evaluation of plant extracts

Currently, the highest threat in the world is the continuous growth of microbial resistance to antibiotics. For example, urinary tract infections (UTI) annually affect over 150 million people worldwide, with immense medical costs and causing significant recurring and chronicling morbidity (Lu et al., 2016; Olson and Hunstad, 2016; Schappert and Rechtsteiner, 2007). The penetration and multiplication of bacteria in the urinary tract is manifested by symptomatic or asymptomatic bacteriuria. Therefore, the need for an effective treatment implies that antimicrobial drugs should be used to stop the multiplication of pathogens (Dorneanu et al., 2017).

Although, with the discovery of penicillin such therapeutic approaches were possible, soon enough (as early as 1944) resistant strains were discovered. Moreover, depending on the type of resistance there are natural and acquired resistant strains. Especially the last type is very problematic due to its properties that allow the bacteria to adapt and become resistant to a drug to which it had been sensitive before (Dorneanu et al., 2017).

Also, in the last decade multi resistant strains have spread all over the world due to irrational use of antibiotics (Horváth et al., 2016; Iancu et al., 2016; Olson and Hunstad, 2016; Lu et al., 2016; Schappert and Rechtsteiner, 2007). Recently, clinical researchers have found that approximately 24% of *Escherichia coli* isolates and 22% of *Klebsiella pneumoniae* isolates were resistant to fluoroquinolones, whereas third-generation cephalosporin resistant pathogens were significantly more common in cases of patient fatality (Horváth et al., 2016; Lu et al., 2016).

On the other hand, fungal infections are a cause of high morbidity and mortality in immunocompromised persons. Increasingly, strains such as *Candida, Cryptococcus neoformans* and *Aspergillus fumigatus* represent the most common cause of these infections. Moreover, multidrug resistance in fungi is increasing, some *Candida* strains are becoming resistant to first-line and second-line antifungal agents such as azoles and echinocandins. Above all, fluconazole-

resistant *Candida* species have been highlighted as a growing problem (Morita et al., 1998; Srinivasan et al, 2014; Xie at al., 2014).

On the attempt to decrease the numbers of resistant bacteria researchers have looked in nature to find compounds that possess antimicrobial properties and could be used as antibacterial agents on their own or in combination with commonly used chemotherapeutics (Horváth et al., 2016; Ioannou et al., 2007). Polyphenols such as flavonoids and anthocyanin derivatives have been proven to possess antioxidant, good antiseptic and cancer preventive activities (Cisowska et al., 2011; Rugină et al., 2012; Viskelis et al, 2009; Shen at al., 2014). Among the richest sources of anthocyanins are the intensely coloured berries (red, blue, black) usually used as foods (Dorneanu et al., 2017) that are safe for human use. Therefore, one important part of our research was finding new herbal sources of natural compounds with antibacterial and antifungal potential. Various methods were employed, however the most common for our publications are presented briefly below.

- ***Disk diffusion method.***

The antimicrobial activity of the selected compounds was evaluated, according to other studies, by the disk diffusion method (Oprean et al, 2016). A Petri dish containing the Mueller-Hinton medium (Sanimed, Bucharest, Romania) was inoculated with 0.1 mL of a physiological saline solution containing 108 CFU/mL of the microorganism under study (CFU-colony forming units). Ten microliters from each sample (10 mg/mL in DMSO) was added to a 6 mm diameter sterile blank filter disk, placed on top of the culture media. Plates inoculated with the bacterial suspensions were incubated at 37°C for 24h. The inhibition zone diameters were measured in millimetres, with a ruler. For all bacterial strains, we performed duplicate disk-diffusion tests and was taken the average reading. For the positive control we used gentamycin or fluconazole disk (BioMaxima, Lublin, Poland). As a negative control, a disk impregnated with DMSO was used (Danciu et al., 2021).

- ***Dilution method***.

The MIC (minimum inhibitory concentration) values were evaluated by the binary microdilution method, in the range of 1.56–50 μg/mL (Ledeti et al., 2015). From stock solutions in DMSO of the tested extracts, serial dilutions of the compounds were prepared and brought, to a final volume of 200 μL with Mueller Hinton broth (Sanimed, Bucharest, Romania). In all tubes 50 μL of bacterial suspensions were added, with a density equal to a 0.5 McFarland. All these tubes were incubated at 37∘C for 24 h. The MIC was recorded as the lowest concentration of the compound which inhibited the visible growth of the tested bacteria. For a negative control 50 μL of DMSO was introduced in a tube with 50 μL of bacterial suspension and 100 μL of Mueller Hinton broth (Danciu et al., 2021).

All the test tubes with no visible growth were inoculated on Columbia agar supplemented with 5% blood in order to determine the MBC (the minimum concentration which killed 99.9% of the bacteria) (Danciu et al., 2021).

- ***Minimum inhibitory concentration quantification***.

Quantitative analysis was performed by the binary liquid serial microdilution method (single bacterial broth and Sabouraud for fungi) in 96-well plates using negative control of sterility and positive microbial growth control. The binary test series tested were: 5, 2.5, 1.25, 0.625, 0.3125, 0.15625, 0.078125, 0.039063, 0.019531, 0.009766. Dilutions were made in 200 μL broth and then each well was seeded with 50 μL of microbial inoculum. The plates were incubated for 24 hours at 37°C and the minimum inhibitory concentration (MIC) values were established as corresponding to the lowest concentration of the test extract which inhibited the growth of microbial cultures compared to the positive control (Imre et al., 2016; Dorneanu et al., 2017).

- ***Determination of minimum biofilm eradication concentration.***

The microtiter method was used to evaluate the influence of the tested extracts on the ability of microbial strains to form biofilms on the inert substrate. The microplates used for MIC testing were emptied and washed three times with saline phosphate buffer. The biofilm formed on the plastic well wall was fixed for 5 minutes with cold methanol, stained for 15 minutes with a purple crystal solution and resuspended with a 33% glacial acetic acid solution. The minimal biofilm eradication concentration (MBEC) was the lowest concentration of the test compound that inhibited the development of biofilm in plaque wells (Balaure et al., 2012; Dorneanu et al., 2017).

- ***Synergy between the tested extracts and antibiotics against uropathogenic microbial strains.***

The principle of the method consists in sowing the microbial standardized inoculum liquid on the surface of an agarized medium. Then, at equal distances there are placed the standardized antibiotic disks, standard antibiotic disks along with 5 μL of stock solution from the investigated extracts, and the sterile filter paper disks only with 5 μL of extract stock solution.

The active substances will diffuse in the medium, achieving a concentration gradient inversely proportional to the diameter of the diffusion zone, so with the distance from the disk. Standardized antibiotic disks were selected according to the International clinical laboratory standards recommendations for each microbial strain.

Depending on the selected samples, their antimicrobial activity was screened against standard strains. The most typical bacterial strains used in our research are listed in the table below.

Table XVI. Reference strains (Danciu et al., 2021)

Bacterial species	*ATCC*	*Producer*
Salmonella enterica serotype typhimurium	14028	ThermoScientific
Shigella flexneri serotype 2b	12022	ThermoScientific
Enterococcus faecalis	51299	ThermoScientific
Escherichia coli	25922	ThermoScientific
Klebsiella pneumoniae	700603	ThermoScientific
Pseudomonas aeruginosa	27853	ThermoScientific
Staphylococcus aureus	25923	ThermoScientific
Candida albicans	10231	ThermoScientific
Candida parapsilosis	22019	ThermoScientific

To assess the antimicrobial profile against uropathogens, four standard pathogens, *E. coli ATCC 13202, Pseudomonas aeruginosa ATCC 27853, Enterococcus faecalis ATCC 29212, Staphylococcus aureus ATCC 29212, and 18 clinical isolates (E. coli 2041, E. coli 1851, E. coli 1992, Pseudomonas aeruginosa 1908, P. aeruginosa 1128, Klebsiella pneumoniae 2110, K. pneumoniae 1074, K. pneumoniae 831, Morganella morganii 2520, Acinetobacter baumanii 1908, A. baumanii 2329, Enterobacter cloacae 2951, E. faecalis 2823, E. faecium 2862, E. faecium 2980, E. faecium 2027, S. aureus 14, S. aureus 17*) from patients with urinary tract infections were used. All colonies were positively identified by MALDI-TOF (Matrix-Assisted Laser Desorption/Ionization Time -of- Flight Mass Spectrometry). This method ascertains the unique molecular fingerprint of each microorganism by mass spectrometry. Then, the protein profile of each microorganism is compared to that existing in an extensive database (Dorneanu et al., 2017).

II.1.4. In Vitro Cell Viability Assays

- **Protection of the erythrocyte membrane in a hypotonic environment**

Inflammation is a pathological process whose evolution involves the presence of enzymes, proinflammatory chemical mediators, extravasation of fluids, cell migration, tissue damage, but also tissue recovery processes (Iancu et al., 2017).

The spectrophotometric method is based on the in vitro evaluation in a hypotonic medium of the erythrocyte membrane stability in the presence of substances with protective effects. The hypotonic medium aggression causes the lysis of erythrocytes with haemoglobin release (Iancu et al., 2017).

Heparinized blood was centrifuged at 2500 rpm for 10 min. The RBCs were separated from plasma and buffy coat and washed three times with 0.9% NaCl. The samples underwent the same preparation steps as described by Dzeletovic et al. and diclofenac was used as a standard (Dzeletovic et al., 1995; Iancu et al., 2017).

- **Cytotoxicity evaluation/ Cell viability evaluation via MTT assay**

Recent studies have described that the global incidence of cutaneous melanoma continues to grow year after year and directly correlated to this the mortality caused by unresectable or metastatic melanoma represents unfortunately a parameter in evolution (Robert et al., 2015). Also, in the field of oncology, but not only, but molecules derived from natural sources also represent a huge contribution to drug discovery today. Worth mentioning that a screening of the number of chemotherapeutic agents concerning their source concludes that over 60% of approved drugs are derived from natural molecules. Vincristine, vinblastine, paclitaxel, docetaxel, topotecan, irinotecan are only some examples of important plant-derived anticancer agents (Da Rocha et al., 2001). Among the phytochemicals with anti-cancer properties flavonoids represent one of the most studied classes of natural compounds (Chahar et al., 2011). A flavonoid that arouses interest to an increased number of research groups due to the plethora of biological activities including also anti-cancer potential is the 4′,5,7-trihydroxyflavone commonly known under the name of apigenin. Research in the field have assigned apigenin both in vitro and in vivo antiproliferative, pro-apoptotic and anti-metastatic effect in experimental models of melanoma (Caltagirone et al., 2000; Hasnat et al., 2015; Zhao et al., 2017).

In order to test the cell viability of MCF7 cells treated with test extracts, the consecrated MTT (3-(4,5-dimethylthiazol-2- yl)-2,5-diphenyltetrazolium bromide) assay was employed. A number of 5000 cells/well was seeded in 96-well plates and left overnight in order to attach to the bottom of the plate. Starting from the second day cells were incubated with different concentrations of extracts (10, 30 and 60 µg/mL) for 72 h. After this step cells were incubated for 4 h with a 5 mg/mL MTT solution. Dimethyl sulfoxide (DMSO) was added and the absorbance of the precipitated formazan crystals solution was measured by the help of a microplate reader at 545 nm.

- **Proliferative and migratory potential by the means of a wound healing technique.**

In order to evaluate the inhibitory activity of the chamomile, parsley and celery extracts on migration and proliferation of human breast adenocarcinoma - MCF7 cells, the scratch assay test was performed. This is a facile and practical technique widely used to express cell to cell interactions and consists in drawing a scratch following the diameter of each well, by using a sterile

tip. 2x105 cells/well in 1500 µL medium were cultured in a 12-well plate and when the suitable confluence was reached (90%) a scratch was made in the middle of each well. After this step, the cells were stimulated with medium containing the test extracts at a concentration of 60µg/mL. For quantification of the antiproliferative and anti-migratory effect, the cell growth was observed by taking pictures initially and 24h respectively, followed by measuring the wound widths. Pictures at a magnification of 10X were taken using an inverted microscope - Olympus IX73, provided with DP74 camera. The scratch area was determined with CellSense Dimension software and the migration percentage was calculated according to the formula described by Felice *et al*. 2015 (Danciu et al., 2021).

- **Cytotoxicity evaluation via lactate dehydrogenase (LDH) release**

Pierce LDH Cytotoxicity assay kit was acquired from ThermoScientific (No 88954). The toxicity of the extracts was assessed by LDH assay, sensitive and well-known technique which detects the extracellular LDH released in the medium when the cellular membrane damage occurs [22]. The quantification of LDH leakage can be measured by a coupled enzymatic reaction, based on the ability of LDH to reduce NAD+ to NADH by conversion of lactate to pyruvate and followed by the reduction of the tetrazolium salt to red formazan *via* diaphorase which uses NADH formed in the first step of the reaction. 5000 cells/well in the 200µL medium were seeded in a 96-well plate and incubated overnight. After that, the medium was removed by gentle aspiration and the cells were stimulated with 100µL media containing 60µg/mL concentration of extracts, for 72h. On the day of the assay, the cytotoxicity reagents were prepared according to the manufacturer's protocol. 50µL of all samples were transferred to a new 96 well plate and 50µL of the reaction mixture was added to each well and incubated at room temperature for 30 minutes, followed by addition of a stop solution. After this step, the concentration of formazan (which is directly proportional with the amount of LDH leakage) was measured at 490nm and 680nm wavelengths, *via* spectrophotometry with a microplate reader (xMarkTMMicroplate, Biorad) (Danciu et al., 2021)..

- **Cell cycle analysis**

Flow cytometry analysis was performed in order to study the percentage of cells in different cell cycle phases. MCF7 human breast cancer cells were seeded onto 9 cm2 6-well plates (3x105cells/well) and were treated with the selected extracts using the concentrations 30 and 60 µM. After 72 h of exposure, cells were collected, fixed with 70% ethanol and stored for 30 min at 4°C followed by centrifugation at 2000 rpm. In order to wash the cells, cold Phosphate Buffer Saline was used. To obtain DNA staining, 50 µL of Propidium Iodide (50µM) (Carl Roth, Karlsruhe, Germany) was added to the cells and the cells were incubated for 10 min in the dark at room temperature. Next, in order to analyze the DNA content, a FACSCalibur flow cytometer (Becton-Dickinson, Franklin Laked, NJ, USA) was used. The percentage of cells in all cell cycle phases was determined using Flowing Software Version 2.5.1 (Danciu et al., 2021).

- **Annexin V-PI Assay**

In order to evaluate the apoptotic potential of selected extracts, Annexin V-PI assay was employed as previously discussed. A number of 5x105cells/well was seeded into 6 well plates (Greiner Bio-one) and left overnight in order to attach to the bottom of the plate. Starting from the second day cells were incubated with the tested extracts at a concentration of 60 µg/mL for 72 h. Annexin V-FITC -PI kit (Invitrogen, ThermoFisher, Vienna, Austria) was employed for the staining following the manufacturer's protocol. Briefly, 2-5x105cells were washed twice in 1 × Annexin V Binding Buffer and afterwards centrifuged for 5 min at 1500 RPM. Fallowing this step cells were resuspended in the binding buffer and incubated for 15 min in the dark with 5 µL of Annexin V-FITC. After another step of washing and centrifugation the pellet was resuspended in 190 µL binding buffer, and 10 µL of PI solution was added immediately prior to analysis by flow cytometry (Danciu et al., 2021).

II.2. In vivo designed models for selected extracts activity evaluation

Neurodegenerative diseases have become increasingly intensively studied, and their early detection is the point of interest in understanding their etiology. Most inducing processes of neurodegeneration cause the initiation of a chemical cascade that results in apoptosis or necrosis of neurons. This manifests itself in the form of loss of certain neurological and cognitive functions [19, 20]. Most often, these degenerative manifestations are accompanied by regenerative or neuroprotective compensatory mechanisms such as the presence of antioxidant enzymes, neurotrophic growth factors, or peptides with a regulatory role [19]. By discovering the most common processes of neurodegeneration, but also neuroprotection, intervention strategies can be drawn in the progress of various neurodegenerative diseases (Ames 1993).

Reactive oxygen species (ROS) and oxidative stress represent key factors for multiple health problems such as metabolic syndrome, neurodegeneration and cancer. The negative impact is expressed as lipid peroxidation, protein misfolding, DNA damage and mitochondrial dysfunction that leads to cell death. Neuroprotection is a major strategy for brain health and may include natural molecules with antioxidant potential. Often, neuronal alteration, subtle cognitive and behavioural changes occur long before the clinical stage of dementia symptoms. Newer trends in neuroscience imply that depression and dementia might have a common cause or they might be linked. It is thought that earlier-onset depressive symptoms are an indicator of a higher risk of developing dementia later in life.

II.2.1. Neuroprotection

Neuroprotection is a sum of strategies to protect the nervous system against neuronal damage or degeneration caused by events such as neurodegenerative diseases, cerebral ischemia, or various traumas that may occur. The goal of neuroprotection is to limit the spread of neural apoptosis and minimize neural dysfunction through mechanisms to maintain the integrity of cellular interactions (Stadtman, 1992).

The most well-known functions of the hippocampus are learning and memory, and awareness of space. The hippocampus also modulates the sensitivity of the hypothalamus-pituitary-adrenocortical (HPA) axis, thereby regulating the stress response. Hippocampal dysfunction and dysregulation of the HPA axis are implicated in the pathogenesis of mood and anxiety disorders.

Smaller hippocampal volumes were observed in stress-induced memory deficits and depressed adolescent patients, and it may also act as an early risk factor for the eventual development of cognitive impairments seen in stress-related psychiatric disorders. The hippocampus also regulates the secretion of glucocorticoids and contains high levels of oxytocin receptors (Abidar et al., 2020).

Reactive oxygen species (ROS) are molecules generated during the normal cellular metabolism in aerobic life (Gulcin et al., 2010) and containing one or more than an unpaired electron in one atomic orbital, giving them a high reactivity degree (Bhat et al., 2015). The excess production of ROS, accompanied by the insufficient antioxidative defense, is described as oxidative stress (Limón-Pacheco et al., 2009). Numerous studies have shown that oxidative stress is correlated with the pathophysiology of PD (Kamdem et al., 2013). Indeed, the brain is more vulnerable to attacks from free radicals leading to neurodegeneration: on the one hand, the brain consumes more oxygen under physiological conditions, but then, it contains fewer amounts of endogenous antioxidant compounds (Roberts et al., 2010). Many lines of evidence prove this hypothesis: the high amounts of ROS detected in particular brain areas (Chong et al., 2013), the increased levels of free iron accumulated in the brain during PD (Gaki et al., 2014) and the presence of lipid peroxidation markers such as the 4-hydroxynonenal and malondialdehyde (MDA) in the substantia nigra of PD patients (Bhat et al, 2015). Furthermore, the superoxide dismutase (SOD), catalase (CAT), peroxidase, and glutathione (GSH)—representing the endogenous antioxidative system—are found at lower levels in the substantia nigra of PD patients (Giordano et al., 2013; Abidar et al., 2020).

II.2.2. MCI/AD models

MCI represents cognitive dysfunction that surpasses age-related memory impairment but is not as severe as senile dementia. There is data that suggests that more than 50% of the patients

diagnosed with MCI will later on progress to AD. Therefore, preventive behaviours should be taken into account as early as possible to maintain the cognitive status and memory.

Neurochemical analyses of the brain samples from Alzheimer's disease (AD) patients indicated a significant loss of the cortical cholinergic innervation, and also cholinergic deficits in the cortex and hippocampus (Savonenko et al., 2012). Acetylcholinesterase (AChE) is a target for AD therapy by inhibiting its activity helps to maintain the acetylcholine (ACh) levels in the neuronal synapses with positive effects in AD patients. Evidence suggests that AChE inhibitors decrease the extrasynaptic metabolism of ACh, being available at high levels of ACh at the synaptic cleft and enhances postsynaptic stimulation. Recently, Haider et al. (2016) proposed that cholinergic dysfunction-induced memory impairment is correlated with increased oxidative stress following the administration of scopolamine. The cognitive impairment induced by scopolamine is temporary, thus mimicking MCI and allows testing of substances/extracts that possess anti amnesic properties along with neuroprotective effects.

Alzheimer's disease (AD) is an irreversible brain disorder mainly characterized by cholinergic deficits, amyloid/tau toxicity and oxidative stress/mitochondrial dysfunctions (Mohamed et al., 2016). Various peptide fragments amyloid beta 1-42 (Aβ1-42) resulted from the abnormal cleavage of amyloid precursor protein (APP) generate deposits in the specific area of the brain leading to losing of memory and cognitive impairment in patients with AD (Castillo et al., 2016). In addition, the peripheral anionic site (PAS), located on the acetylcholinesterase (AChE) enzyme, was previously reported as a facilitator factor that induced aggregation of the Aβ-monomers (Dickerson et al., 2005; Postu et al., 2018).

On the other hand, intracerebroventricular injection of Aβ(1–42) interferes with memory function and subsequently causes impairment of spatial memory within the Y-maze and radial arm-maze tasks, following previous investigation using rats (Cioanca et al. 2013). Treatment with natural compounds (volatile or fixed molecules/extracts) that may act as inhibitors against Aβ(1–42) peptide aggregation leads to fewer memory errors, better cognitive abilities and neuroprotection. For this model, different numbers of animals were used for each tested extract, the number of groups being established dependent on the used concentration and the number of vegetal species investigated.

Generally, male Wistar rats (200 g, housed in a temperature and light-controlled room, fed and allowed to drink water *ad libitum*) were divided into various groups (5 - 10 animals per group): (1) control group with saline treatment (0.9% NaCl); (2) Aβ(1-42) alone-treated group (by intracerebroventricular injection of 400 pmol of beta-amyloid peptide 1-42, Rat, Sigma-Aldrich, Germany), 20 days before testing; (3) Aβ(1-42)-treated group received by inhalation *Salvia oficinalis* essential oil 1% (SO1%+ Aβ(1-42)); and (4) Aβ(1-42)-treated group received by inhalation *Salvia oficinalis* essential oil 3% (SO3%+ Aβ(1-42)). Control and Aβ(1-42) alone-treated groups were caged in similar conditions but without *Salviae aetheroleum*. Tested animals were treated in accordance with the guidelines of animal bioethics in compliance with the European

Council Directive of 24 November 1986 (86/609/EEC) with the approval of the local Ethics Committee.

II.2.3. PD models

Parkinson's disease (PD) represents the other common neurodegenerative disease after Alzheimer's disease (AD) (Lee et al., 2013). PD is characterized by tremor, rigidity, bradykinesia (Daviaud et al., 2013) and postural instability (Gaki et al., 2014). These symptoms are mainly attributed to the dopaminergic neurons' depletion in the substantia nigra pars compacta (Pradhan et al., 2016), leading to a subsequent loss of dopamine in the striatum (Muñoz-Manchado et al., 2013). PD is accompanied by a presence of eosinophilic and intracytoplasmic inclusions called Lewy bodies (Prediger et al., 2012).

There is now considerable evidence showing that the degeneration also affects the cholinergic (Kehagia et al., 2010), noradrenergic (Lee et al., 2012), adrenergic, and serotoninergic systems (Prediger et al., 2012) resulting in non-motor symptoms namely sleep disorders (Erro et al., 2012), cognitive problems ranging from memory impairment to dementia (Kehagia et al., 2010), depression, anxiety, psychosis and apathy, all affecting patients' life quality (Gallagher et al., 2012).

With the high prevalence and the absence of treatment to curate PD (Sekeroglu et al., 2012), several toxin models are provided to understand the pathogenesis and to develop new strategies against PD (Khan et al., 2010). Among the most recognized PD models, the 6-hydroxydopamine (6-OHDA), a neurotoxin inducing depletion of dopaminergic neurons in the substantia nigra pars compacta (Wang et al., 2011). It has been experimented with in cell cultures (Zhang et al., 2017) in rodents, in which the injection is localized in the medial forebrain bundle or other parts of the dopaminergic pathway (Panula et al., 2010) and zebrafish (Zhang et al., 2017).

Rat Models

For an easier comprehension of the procedures and administration of treatment I have included two graphical abstracts, included in the published manuscripts for two variants of rat models:

- **scopolamine-induced AD/MCI**

Scopolamine, a muscarinic acetylcholine receptor (MAChR) antagonist, can block the cholinergic function of the central nervous system by targeting M1AChR and M2AChR. It has been reported that scopolamine can induce anterograde memory impairment, particularly short- term memory and learning acquisition (Navarro et al, 2017). Moreover, scopolamine can significantly increase the activity of AChE and malondialdehyde (MDA) levels in the cortex and hippocampus, and oxidative stress in the brain (Haider et al., 2016; Tao et al., 2014).

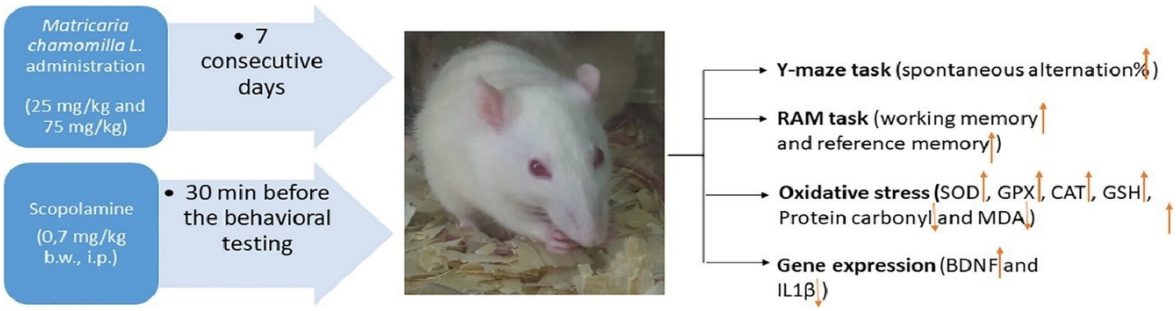

Fig. 19. Graphic indication of scopolamine-induced animal model for testing the efficacy of *Matricaria chamomilla* L. extract on memory loss and oxidative stress at the brain level

- **beta-amyloid-induced AD**

All surgical procedures were conducted under aseptic conditions, under sodium pentobarbital (50 mg/kg b.w., i.p., Sigma-Aldrich, Germany) anesthesia. Rats were mounted in the stereotaxic apparatus with the nose oriented 11° below horizontal zero plane. Animal model of AD was established by intracerebroventricular (i.c.v.) injection of 400 pmol Aβ(1–42) (Sigma-Aldrich, Germany), 20 days prior to exposure to the juniper volatile oil according to the procedure established by Laursen and Belknap in 1986. Aβ(1–42) was dissolved in 0.9 % saline and incubated at 37°C for 3 days to allow the peptide to aggregate. Aβ(1–42) was administered right-unilaterally through Hamilton syringe over 4 min, and the syringe was left in place for 5 min after injection before being slowly removed. The injection volume (4 µL) was delivered gradually (1 µL/min) using the following coordinates: 1.5 mm lateral to the midline; 7.4 mm ventral to the surface of the cortex (Paxinos and Watson, 2005). The sham-operated rats were injected with saline (Cioanca et al., 2015).

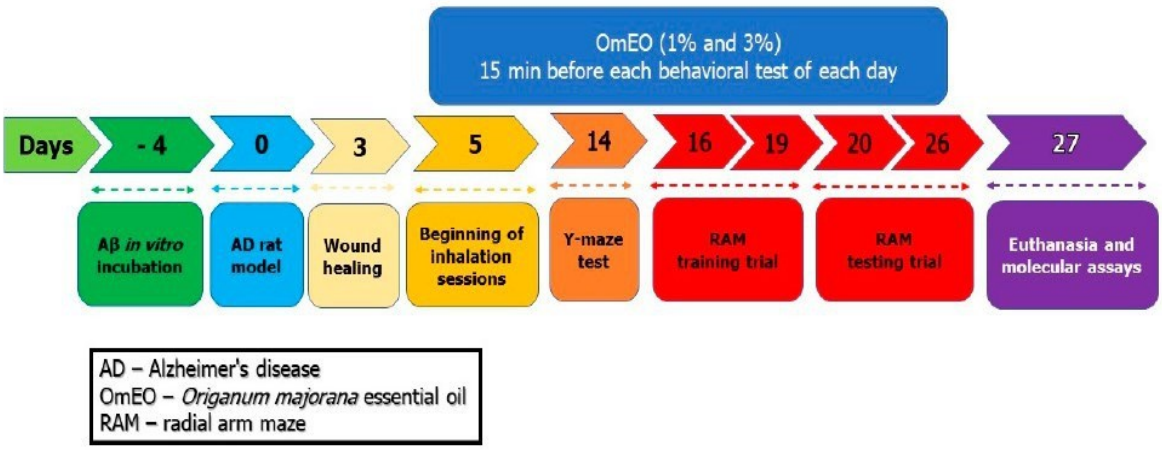

Fig. 20. The experimental design of Aβ-induced AD model, drug treatment and behavioral measurements

Zebrafish Models

Zebrafish (*Danio rerio*) is a model frequently used for the study of neurodegenerative diseases. Its genome has a homology of 70% with human genes, and 84% of the genes that are associated with human diseases can also be found in zebrafish (Kalueff et al., 2014). These things support the translational value of zebrafish models. The brain structure of zebrafish shows a high degree of conservation compared to the mammalian brain. Both zebrafish and mammalian brains have the same parts (Santana et al., 2012). Also, in zebrafish, the dali-lateral pallium is homologous to the mammalian hippocampus, an area affected by Alzheimer's disease. Moreover, in zebrafish, the blood-brain barrier is similar in structure and function to that of mammals, allowing the testing of substances with neurotropic action. Zebrafish have numerous genes involved in Alzheimer's disease such as MAPT, APOE, APP, PSEN1 and PSEN2. In the brain of zebrafish, the major systems of neurotransmitters such as dopamine, serotonin, acetylcholine, glutamate, glycine and GABA have been identified (Santana et al., 2012). There are transgenic zebrafish models that mimic many pathologies characteristic of Alzheimer's disease, but memory impairment is rarely seen in these models.

A pharmacological model of zebrafish induced by administering okadaic acid is currently being used (Koehler et al., 2018a). Okadaic acid is an inhibitor of protein phosphatase 1 (PP1) and PP2. By exposing zebrafish to okadaic acid, memory deficits, Aβ fragment deposits, induction of senile plaques, hyperphosphorylated tau proteins and neuronal apoptosis appear (Koehler et al., 2018b).

Used as a model organism of vertebrate development (Newman et al., 2014), nowadays, zebrafish presents an encouraging model of neurodegenerative disorders such as AD, PD, Huntington disease, and schizophrenia (Richetti et al., 2011) due to its neuroanatomical and neurochemical pathways similitudes with the human (Santana et al., 2012), and its high fecundity and short generation time allowing the evaluation of large drug varieties (Zhang et al., 2017).

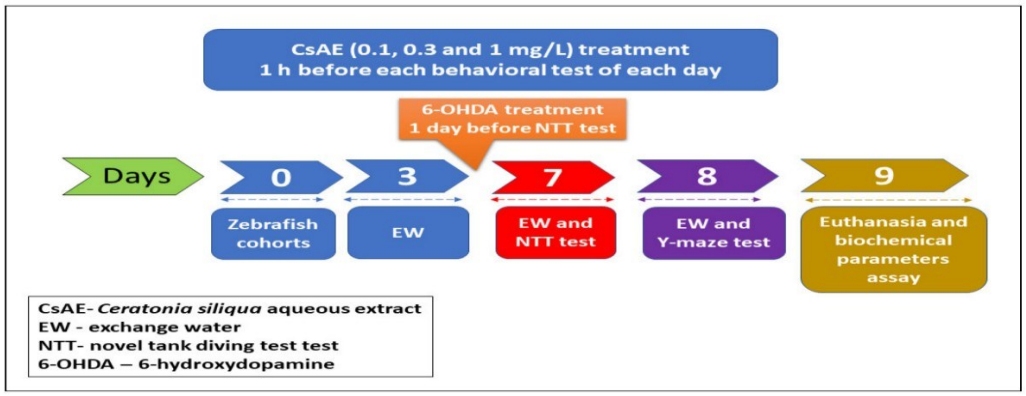

Fig. 21. Schematic representation of the experimental protocol

Experimental protocol was approved by the local board of ethics for animal experimentation (No. 15309/2019) and fully complied with the Directive 2010/63/EU of the European Parliament and of the Council of 22 September, 2010 on the protection of animals. Efforts were made to reduce animal suffering and the number of animals utilized.

Assessment of pharmacological parameters

For memory assessment in rat models, we used two well-characterized hippocampus-dependent spatial memory tasks: Y-maze and radial arm-maze.

The Y-maze task is a specific and sensitive test of spatial recognition memory in rodents. The test relies on an innate tendency of rats to explore a novel environment. The Y-maze used in this study involves no aversive stimuli and was considered suitable for evaluating memory. The specific part of the brain involved in the performance of this task includes the hippocampus (Bagheri et al. 2011).

In behavioural neuroscience trials, the radial arm-maze task is widely used (Cioanca et al. 2013). The radial arm-maze task is useful in evaluating the effect of drugs, stress, and various other environmental factors on learning and memory (Titus et al. 2007). Working memory and reference memory are the two variables that report the physiological status of the brain (Titus et al. 2007).

The elevated plus-maze (designed by Coulbourne Instruments, Allentown, PA, USA) was used to assess anxiety levels. This black Plexiglas apparatus had two open arms opposite each other, two closed arms (49 x 10 cm) opposite each other, and a central sheath raised 50 cm above the surface. Each animal was gently positioned in the center of the device, facing the open arm, and allowed to explore for 5 min in a silent chamber. Rat behavior was filmed using a Logitech HDWebcam C922 Pro Stream camera and the videos were analyzed using ANY-maze software (designed by Stoelting Co., Wood Dale, IL, USA). The following variables were recorded: (1) the time spent in the open arms and the enclosed arms, and (2) the number of entries to any of the four arms. The entry of all four feet of the animal into one arm was described as an arm entry. The test lasted 5 min, and after the rat was removed and the apparatus was thoroughly washed with cotton and a 10% ethanol solution. The reference drug in this study was diazepam, an anxiolytic agent (Ionita et al., 2017; El-Akhal et al., 2021).

The forced swimming test (FST) is the most used animal model for depression. The model's theoretical rationale is that uncontrolled stress causes behaviors that stimulate anhedonia, a common human depression syndrome. Rats were placed in a transparent cylindrical glass tank (height = 59 cm, internal diameter = 30 cm) containing water to a level of 25 cm (26 _ 1 _C). Water was changed for each rat. Rats were given a 15-min pretest swim session, followed by a 6-min test swim session the next day. Both swimming sessions took place from 12.00 to 18.00 h. The rats

were taken out of the water, dried with towels, and put in a warm enclosure for 20 min before being returned to their home cages. The behavior of the rat was filmed using a Logitech HD Webcam C922 Pro Stream camera during a single exposure to forced swimming (6 min), and the videos were analyzed using ANY-maze software (from Stoelting Co.,Wood Dale, IL, USA). Two forms of behavior were tested: (1) immobility (when the animal was immobile and floated in a straight position with only minor movements to hold its head above the water's surface) and swimming (time spent with active swimming movements). Tramadol, an antidepressant and analgesic agent, was used as the reference drug in this study (Ionita et al., 2019; El-Akhal et al., 2021).

For *Danio rerio* models, Novel Tank Diving Test (NTT) and Y-maze tank (YT) were used to assess the animal behavior (Abidar et al., 2020).

The NTT is a specific test used for assessing anxiety in zebrafish, as described by Cachat et al. The trapezoidal tank (1.5 L) used measured 15.2 height x 27.9 top x 22.5 bottom x 7.1 width cm, equally divided into two horizontal sections (top and bottom). After 1 h of sample treatment, the animals were placed individually within the test tank without acclimatization, and swimming behavior was recorded for 6 min. The animals were recorded with a Logitech HD Webcam C922 Pro Stream camera (Logitech, Lausanne, Switzerland) placed 30 cm away from the tank, and the videos were analyzed using ANY-Maze® software (Stoelting CO, Wood Dale, IL, USA). The following parameters were registered: total distance in the tank (m) (to assess the locomotor activity), for the anxiety-like behavior: time spent in the top zone of the tank (s), and time spent in the bottom zone of the tank (s) were recorded. Representative tracking images of zebrafish locomotor activity from each group were obtained at the end of the analysis with ANY-Maze® software. Imipramine (IMP, 20 mg/L, Sigma-Aldrich, Darmstadt, Germany) was used as the positive control in the NTT test (Abidar et al., 2020).

Spatial memory in zebrafish was assessed using the Y-maze task (Dumitru et al., 2019). The apparatus consisted of a Y-maze glass aquarium with three arms (25 cm long, 8 cm wide and 15 cm high), filled with 3 L of the same water used in the home aquarium. After 1 h of CsAE treatment, each fish was tested individually during 5 min session. Donepezil (DP, 10 mg/L, Sigma-Aldrich, Darmstadt, Germany) was used as the positive control in the Y-maze test. The behavioral activity was analyzed using ANY-Maze® software (Stoelting CO, Wood Dale, IL, USA) and with a Logitech HD Webcam C922 Pro Stream camera (Logitech, Lausanne, Switzerland) placed above the Y-maze tank. The following measures were recorded: the percent spontaneous alternation (to assess short-term spatial memory), and for the locomotory activity, the number of arm entries was recorded. Representative tracking images of zebrafish locomotor activity from each group were obtained at the end of the analysis with ANY-Maze® software (Abidar et al., 2020).

Biochemical Parameters Assay

Our group previously showed a strong correlation between memory dysfunctions and the oxidative stress in the rat hippocampus and scopolamine using a rat model of cognitive impairment (Aydin et al., 2016). Among different hallmarks of the AD, oxidative stress was reported (Xu et al., 2017). Moreover, decreasing of the antioxidant enzymes activity, such as superoxide dismutase (SOD), glutathione peroxidase (GPX) and catalase (CAT) have been reported in the early stages of the AD (Boonruamkaew et al., 2017). The hippocampal neurogenesis is regulated by the normal cholinergic system activity through modulating neurogenic mechanisms such as those involving the brain-derived neurotrophic factor (BDNF) and cAMP response element-binding protein (CREB) (Bruel-Jungerman et al., 2011). Evidence suggested that a reduction in the BDNF levels in the entorhinal cortex and hippocampus of patients with the AD (Wu et al., 2016), resulting in decreasing of the patient's score on the mini-mental state examination. In addition, the alterations in BDNF, and phosphorylated CREB occurred following scopolamine treatment were evidenced (Park et al., 2016). Supporting information suggested that the involving of the neuroinflammation in AD pathogenesis which contributes to disease progression and severity (Heneka et al., 2015). Data suggested that IL-1β stimulate the progression of neurodegenerative diseases by inducing nitric oxide production and cholinergic function decline via increased AChE activity (Xian et al., 2015).

After the recording of the behavioral data, rats were euthanized, and their brains were isolated for a biochemical parameters assay. The hippocampi were precisely excised from the rat's brains and gently homogenized in ice and 0.1 M potassium phosphate buffer (pH 7.4) with 1.15% KCl with Potter Homogenizer (Cole-Parmer, Vernon Hills, IL, USA). The homogenates were centrifuged at 960 x g for 15 min at 4ºC. The supernatant was used for the estimation of AChE, superoxide dismutase (SOD), and catalase (CAT) specific activities, and the total content of reduced glutathione (GSH) and protein carbonyl and malondialdehyde (MDA) levels following the methods described in detail by Cioanca et al. 2015 and Postu et al. 2020. Estimation of protein content was done through a bicinchoninic acid (BCA) protein assay kit (Sigma-Aldrich, Darmstadt, Germany).

- **Determination of Hippocampal AChE Activity**

Activity of acetylcholinesterase (AChE) in the rat hippocampus was determined according to the method of Ellman [24] using acetylthiocholine (ATC) as artificial substrate [25]. The reaction mixture (600 µL final volume) contained 0.26 M phosphate buffer with pH 7.4, 1 mM 5,50-dithio-bis- 2-nitrobenzoic acid (DTNB) and 5 mM ATC chloride. The assay was started by adding supernatant and fallowing the developing of the yellow color at 412 nm for 10 min at room temperature. Suitable controls were performed for the nonenzymatic hydrolysis of ATC. The

enzyme activity is expressed as nmol of ACT/min per/mg of protein (Cioanca et al., 2015; Postu et al., 2020).

- **SOD assay**

The activity of superoxide dismutase (SOD) is evaluated by monitoring its ability to inhibit the photochemical reduction of nitro blue tetrazolium (NBT). Monitoring the increase in absorbance at 560 nm following the production of blue formazan. One unit of SOD is defined as the quantity required to inhibit the rate of NBT reduction by 50% as described by Winterbourn et al. (1975). The enzyme activity is expressed as units/mg protein.

Data analysis

Data were entered into the GraphPad Prism version 7.00 (GraphPad Software, La Jolla California USA). One-way ANOVA followed by Tukey's post hoc test was used to analyze the data. The data were expressed as mean ± standard error of the mean (S.E.M.) and $p < 0.05$ was considered a significance level.

II.3. Correlations, challenges and their significance

Taking into account all the published research I have selected only several that will be indicated at the corresponding theme in a manner that will allow easier comprehension of the achieved results in terms of biologic activity of the selected herbal extracts. Correlation of the observed effects with the dose, concentration and chemical composition is also given.

Worth mentioning is that most of these results are correlated to the chemical results obtained in our investigations. The chemical aspect and the proper selection of the extract/compound represent the basis for high-quality research in herbal medicines, as stated at the beginning of Section I. Therefore, all the observed effects contain references to all important aspects that impact the significance for further development of these studies and act as justification for preventive and therapeutical use of the investigated extracts.

However, some results were not significant enough or the observed effects were less intense than standards. Fewer results were contradictory, presenting lower activity at higher doses. In such cases, there is much to be learned, for they represent another step into research and allow the development of the investigation into comprehending the mechanisms and relationship between concentration/dose and activity intensity. Moreover, some aspects could be connected to toxicity and justified based on published scientific data.

II.3.1. Antioxidant activity results

As a winner of a postdoctoral scholarship in bio clinical research through "Program of Excellence in doctoral and postdoctoral research in multidisciplinary chronic diseases", contract no. POSDRU /159/1.5/S/133377, beneficiary "Grigore T. Popa" Iasi, financed from the European Social Fund through Sectorial Operational Program Human Resources Development 2007-2013, I investigated the biologic activity of the essential oil isolated from dry leaves of *Salvia officinalis* L., cultivated in the ecological environment (Cioanca et al, 2015).

For the *in vitro* testing, we chose linalool as a control, but in most of the assays its activity was lower than that of the diluted essential oil, therefore we believe that the actual biologic activity belongs to the phytocomplex maybe by synergistic effects and is less due to a single compound. In regards to the DPPH assay, we noted that the intensity of the scavenger activity is directly proportional to and depends on the concentration of the sample. The IC_{50} value for sage oil was 10.5±0.2 µL/ml. This value, by comparison with other essential oils tested similarly, is higher which signifies an average scavenger activity (Joshi et al., 2014, Kelen and Tepe, 2008; Kolak et al., 2009; Cioanca et al., 2015).

15-lipoxygenase is an enzyme from the oxide-reductases class that catalyzes the oxidation reaction of unsaturated fatty acids to form lipid peroxides (Joshi et al., 2014). Due to its wide distribution in the brain, it has been demonstrated that overexpression could cause Alzheimer's disease and neuropsychiatric anxiety disorders. Thus, the inhibition of this enzyme in the brain leads to reduced oxidation of protein post-ischemia and impaired blood-brain barrier (Maltreud and Rydland, 2000; Perry et al., 2000; Schneider and Bucar, 2005). The sage essential oil tested in this research has a protective action against lipoxygenase because concentrations of 25 µL/mL inhibit the enzyme by over 75% (Table XVII). In addition, low IC_{50} values (0.064 ± 0.03) are an encouragement to use the essential oil in therapy, such low doses being safe and non-toxic (Cioanca et al., 2015).

Table XVII. 15-lipoxigenase inhibition capacity

Sample	**Concentration / %Activity**						**IC_{50}**
	25 µL/mL	12.5 µL/mL	6.25 µL/mL	3.125 µL/mL	1.5625 µL/mL	0.78125 µL/mL	
Sage oil	91.31 ± 0.70	84.73 ± 0.63	56.72 ± 0.71	47.01 ± 0.81	29.18 ± 0.73	15.83 ± 0.68	0.064 ± 0.03
Linalool	76.03 ± 0.14	68.57 ± 0.71	52.15 ± 0.25	28.21 ± 0.37	18.14 ± 0.34	13.56 ± 0.25	0.097 ± 0.0005

The therapy with cholinesterase inhibitors in Alzheimer's disease has been shown to induce beneficial effects of clinical significance in patients with early forms of the disease. Compounds of this class may lead to an improvement of a patient's behaviour and daily activities or of cognitive

functions. Therefore, our research investigated also the capacity of AChE inhibition for sage essential oil (table XVIII). Due to certain limitations imposed by the enzymatic environment, the maximum concentration of essential oil diluted in DMSO was 12.5 µL/mL (Cioanca et al., 2015).

Table XVIII. AChE inhibition capacity for *Salviae aetheroleum*

Sample	Sample concentration / % Activity					IC_{50}
	12.5 µL/mL	6.25 µL/mL	3.125 µL/mL	1.5625 µL/mL	0.78125 µL/mL	
Sage oil	57.00 ± 2.37	34.61 ± 1.75	16.10 ± 0.68	14.21 ± 0.91	7.92 ± 0.20	0.478 ± 0.032
Linalool	13.87 ± 0.78	11.10 ± 0.21	9.46 ± 0.51	6.73 ± 0.35	5.11 ± 0.08	-

There are few scientific publications on the essential oils capacity to inhibit AChE, most studies were conducted on the alcoholic or hydro-alcoholic plant extracts with high content of polyphenolcarboxylic acids (Kolak et al., 2009; Orhan et al., 2007; Orhan et al., 2013; Perry et al., 2000; Pizzale et al., 2002). Our results prove that the essential oil has an AChE inhibitory potential, the IC_{50} value being the proof. Even though there are certain differences between our data and the literature, these are most probably influenced by the environmental conditions from where the plants have been harvested. Also, the extraction process is of great impact on the chemical composition of the essential oil.

The *in vivo* tests confirm the antioxidant potential of the essential oil included in our study, even though there is not a perfect correlation between the concentration of the sample and the intensity of the activity (fig. 22).

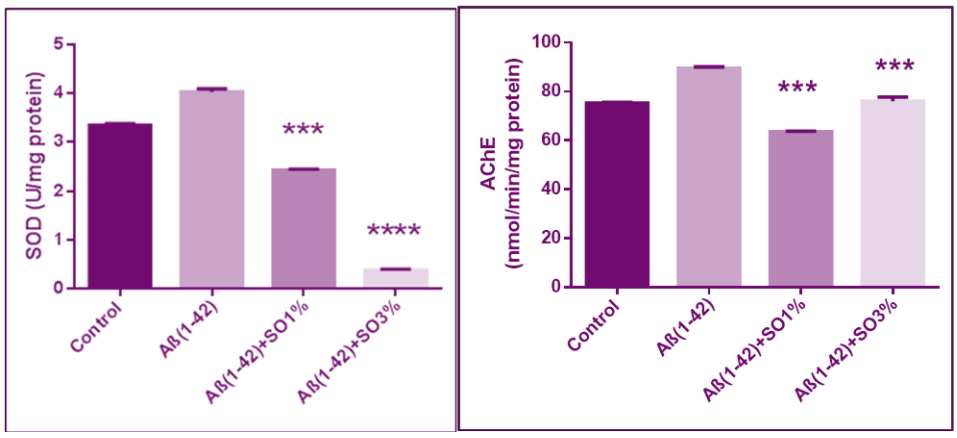

Fig. 22. Diagrams of the *in vivo* assays: SOD activity and AChE inhibition

Among other endogenous enzymes, SOD is part of the first line of defence against free radicals. Therefore, its activity is extremely important for the health of the body. In our research we noted that the intensity of activity is directly correlated to the concentration of the administered essential oil, SO3% induces a strong activity of the fore mentioned enzyme. Still, the lower concentration of sage essential oil (1%) stimulates also SOD activity (Cioanca et al., 2015).

In the AChE assay, SO 1% showed the highest activity, whereas SO 3% has a lower inhibitory potential but is still statistically significant. Such a discrepancy could signify either that there is a competition for the same receptors or it may be due to toxicity since the concentration is three times higher than the maximum admitted for aromatherapy. Thus, we confirmed once more that a concentration of 1% essential oil is sufficient to induce pharmacological effects both *in vitro* and *in vivo* (Cioanca et al., 2015).

Aronia melanocarpa (Maloideae subfamily, Rosaceae family), also known as black chokeberry, was originally thought to have little medicinal importance, but lately has been proven to present antioxidant, hypoglycaemic, immunomodulatory and enzyme inhibitory properties (Bräunlich et al, 2013; Ho et al., 2014; Trifan et al., 2016; Shen et al., 2014; Varela et al., 2016; Viskelis et al., 2009). It is known that the chemical composition of a vegetal material is highly influenced by the environmental conditions and the processing methods. Therefore, in our study we used ripen berries from a controlled environment that were extracted by two different solvents actone and ethanol (Dorneanu et al., 2017).

Our results for the antioxidant assessment indicated that both extracts have a good antioxidant activity that depends on their concentration. The IC50 values and the Trolox equivalent antioxidant capacity (TEAC) values were the highest for *Aronia melanocarpa* ethanolic extract, while 9 times less active proved to be the acetone sample, as indicated in the table below.

Table XIX. Systematic results for the antioxidant assays of the investigated samples

Assays		**Samples**		**Standard**	
		1 (acetone)	**2** (ethanol)	**gallic acid**	**ascorbic acid**
ABTS radical scavenging	IC_{50}*	18.2 ± 0.1	1.7 ± 0.1	0.6 ± 0.02	-
	TEAC**	0.55 ± 0.02	4.22 ± 0.1	20.48 ± 0.14	-
LOX inhibition	IC_{50}	20.83 ± 0.51	12.28 ± 0.1	-	29,91 ± 0,62

* IC_{50} values **expressed** in µg/mL**;** ** TEAC values calculated as µM Trolox equivalent to 1 µg/ mL extract

The LOX inhibition assay indicated that both investigated samples have an average potential comparable to ascorbic acid used as positive control. These activities were in close relation to anthocyanidin concentration of each sample.

Previous research on black chokeberry revealed that *Aronia melanocarpa* juice can reduce different oxidative stress markers (Shen et al., 2014; Varela et al., 2016). Our results come to complete such data, the antioxidant tests we used involved other mechanisms, thus leading to the conclusion that *Aronia* is a rich source of antioxidants not only as food or in its raw juice, but also as a great vegetal material to obtain selective extracts for therapeutic use (Dorneanu et al., 2017).

Three species of basil were investigated in order to establish their antioxidant potential. The analyses were performed on the hidroalcoholic (50%) extracts from *Ocimum basilicum L.* (Ob), *Ocimum basilicum var. purpurascens* (Obr), and *Ocimum sanctum L.* (Os), included in bio-cultures from Romania. The phenolic content was quantified by the Folin-Ciocalteu method. A high performance liquid chromatography method (HPLC) was used to identify the main compounds of the hidroalcoholic extracts. In addition, the antioxidant activity was assessed by the diphenylpycrylhydrazyl (DPPH) radical method and metal chelating method (Gradinariu et al., 2013).

The hydro-alcoholic extracts (ethanol 50%) were obtained from 2.5 g of dried material, still the amount of dry extract that was obtained varied from one sample to the other. Thus, the drug extract ratio (DER) was as follows: Ob sample DER=2.5:1.21, Os sample DER=2.5:1.20 and Obr sample DER=2.5:0.89. Therefore, we might state that the lowest extractability corresponds to red basil variety sample, maybe due to some compounds that partially inhibit the extraction.

As expected, in terms of capacity for scavenging DPPH radicals, Obr extract showed the best activity, since it had the lowest IC50 values (0.8 mg/mL). In contrast, Os had the lowest ability for scavenging this radical, having the highest IC50 value (2.0 mg/mL). The analyses of the extracts revealed that an inverse correlation between the content of the total amount of polyphenols and IC50 values were observed in the case of Os extract. It is notable that at concentrations of 15.67 mg/mL and 5 mg/mL, the values of the scavenging activity resemble for all samples, compared to the reference antioxidant, quercetin (Fig. 23). When lowering the concentration at 1mg/mL, the scavenging activity lowers as well, significantly. Still, even at this dilution, Obr sample has the highest value of all extracts (Gradinariu et al., 2013).

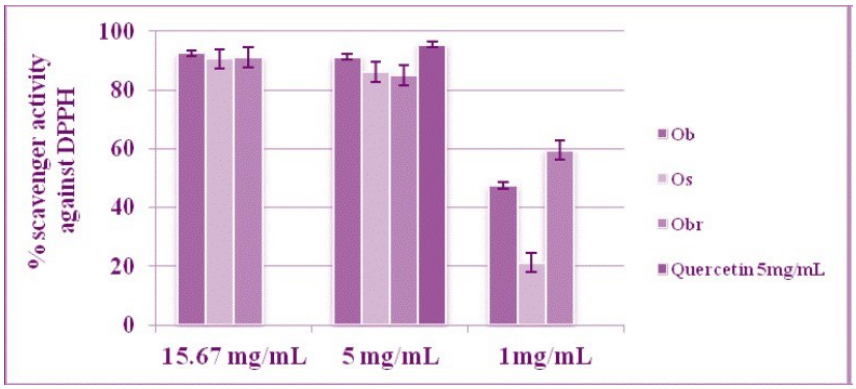

Fig. 23. Free radical scavenging of basil extracts activity (%) for three dilutions

Although some authors reported that the phenol compounds are responsible for the antioxidant activities of plants, more recent data shows that this relationship may vary, depending especially on the method used for evaluating the antioxidant ability (Albano et al., 2012; Barros et al., 2009; Oboh 2008; Gulcin et al., 2007). In this study, an inverse correlation between the high polyphenols content was found for Os sample and its scavenging capacity, which was low. Such results suggest that in this extract there are some phenolic constituents contributing less effectively for the scavenging activity, by comparison to the other two extracts that are more active (Gradinariu et al., 2013).

Iron can catalyse reactions (e.g., Fenton reaction) that generate ROS. Also, iron decomposes lipid peroxides, favoring lipid peroxidation of biological membranes, thus generating cytotoxic aldehydes (e.g., malondialdehyde [MDA]). These can inhibit protein synthesis, inactivate enzymes, cross-link proteins, generate thrombin, and eventually leading to membrane rupture and to release cell and organelle contents (Ferrari 2000; Geetha et al., 2004; Oboh 2008; Gradinariu et al., 2013).

Experiments revealed that at 40 mg/mL concentration the chelating activities were found to be 73.78% for Ob, 89.09% for Os and 81.16% for Obr. The results (fig. 24) showed that the metal chelating activity lowers proportionally with the concentration. According to their IC50 values, extracts Ob (4.9 mg/mL) and Os (4.3 mg/mL) generally showed a weaker capacity for metal chelating. Obr (3.9 mg/mL) was, again, the most effective as Fe^{2+} chelating agent. As reported for DPPH radical-scavenging activity, in this assay there was also a correlation between flavonoids content and the ability for chelating metals. The analysis of the extracts of each plant revealed a positive correlation between flavonoids content and IC50.

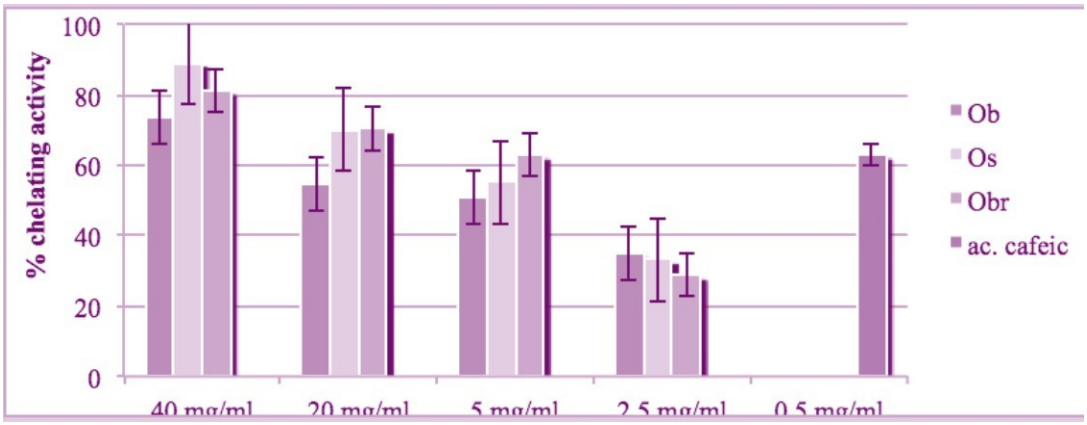

Fig. 24. Metal chelating activity (%) for four dilutions for *Ocimum* samples

The hydroalcoholic extract of Ob had the lowest polyphenolic content in contrast to those of Os and Obr, respectively. On the other hand, the extract of Obr had significantly higher flavonoids content, comparing to the other. One must state that even with the lowest extractability

rate (DER=2.5:0.89) red basil extract has to be considered the richest in antioxidant compounds, both *in vitro* tests indicating its scavenger and chelating potential. Nevertheless, further studies are needed for understanding/discovering the chemical structures of the components in the extracts responsible for the antioxidant activities.

The antioxidant and of some major botanical sources of apigenin—chamomile (MC), parsley (P), and celery (C) methanolic extracts was also investigated and the obtained results were published by Danciu et al. in 2018.

Radical scavenger capacity was assessed by DPPH and ABTS binding capacity. The color changes noted during testing are included in Table XX. The results are expressed as EC50, which represents the efficient concentration to chelate 50% of the free radicals present in the assay environment. These results indicate the extract quantity necessary to be used in order to obtain at least 50% inhibition against free radicals.

Table XX. Radical scavenger capacity of the investigated extracts

Extract/Standard	EC_{50} (μg/mL)	
	DPPH Assay	ABTS Assay
MC extract	47.8 ± 1.4	21.4 ± 0.2
C extract	157.9 ± 2.1	163.7 ± 1.3
P extract	165.4 ± 0.2	164.1 ± 1.5
Caffeic Acid	3.6 ± 0.0	1.6 ± 0.0

The bivalent iron is indirectly involved in the occurrence of oxidative stress because it participates in the Fenton reaction by which hydroxyl radicals are generated. The latter exhibit particular chemical reactivity, and may initiate oxidation reactions, in particular, of unsaturated compounds, with damage to the cell membrane structure or other biologically relevant compounds. Therefore, the chelation of ferrous ion greatly decreases the availability of ions for the Fenton reaction, which might explain the antioxidant and protective activity of the investigated extract. Results can be seen in Table XXI.

Table XXI. Iron chelation potential of the selected extracts

Sample	0.078125 mg/mL	0.15625 mg/mL	0.3125 mg/mL	0.625 mg/mL	1.25 mg/mL	2.5 mg/mL	5 mg/mL	10 mg/mL	EC_{50} (mg/mL Solution Final Tube)
MC extract	3.12 ± 0.05	4.77 ± 0.05	10.25 ± 0.12	13.28 ± 0.10	25.41 ± 0.07	50.59 ± 0.12	69.89 ± 0.13	73.91 ± 0.02	491.94 ± 1.61
C extract	2.25 ± 0.03	3.00 ± 0.04	7.73 ± 0.04	19.91 ± 0.03	51.44 ± 0.01	88.15 ± 0.03	91.65 ± 0.04	92.96 ± 0.01	242.21 ± 0.06
P extract	6.84 ± 0.09	10.75 ± 0.04	12.39 ± 0.07	16.61 ± 0.13	24.21 ± 0.06	74.10 ± 0.02	93.55 ± 0.01	96.91 ± 0.03	357.72 ± 0.19
Caffeic Acid	97.03 ± 0.09	97.09 ± 0.14	98.36 ± 0.21	98.61 ± 0.32	99.27 ± 0.22	100 ± 0.00	100 ± 0.00	-	2.47 ± 0.25

"-" not detected

Determination of lipoxygenase inhibition activity was conducted using the modified Maltreud method, based on the principle that the polyphenolic compounds present in the extract have the ability to block the action of lipoxygenase that catalyzes oxidation of linoleic acid with a decrease in absorbance at 234 nm. There are two possible mechanisms by which the compounds in the tested extracts can block the enzyme activity, as follows: either by blocking the redox reversible transformation process of Fe2+ from Fe3+ and, thus, blocking the oxidation of the substrate; or by modifying the spatial structure of the active site or of the enzyme. The lipoxygenase inhibition activity is linear with the concentration. Results can be seen in Table 5. Such results sustain the anti-inflammatory activity of the tested extracts. The EC50 value indicates that, for a good inhibitory effect, only 166.32 ± 2.03 mg of MC extract, 86.15 ± 4.82 mg of C extract, and 69.46 ± 1.70 mg of P extract need to be used. There is still a great difference between the EC50 calculated for the standard (caffeic acid) and the investigated extracts.

This indicates that the standard should be more active than our samples. Nevertheless, the calculated value of EC50 proves that all three extracts are active against LOX, and their activity decreases in the following order: P > C > MC. Usually, amounts of ca. 70–170 mg of plant extracts are quite low, taking into account that this is the quantity necessary to inhibit 50% of the lipoxygenase activity.

Table XXII. Lipoxygenase inhibition activity of selected extracts

Sample	0.078125 mg/mL	0.15625 mg/mL	0.3125 mg/mL	0.625 mg/mL	1.25 mg/mL	2.5 mg/mL	5 mg/mL	10 mg/mL	EC_{50} (mg/mL Solution Final Tube)
MC extract	4.71 ± 0.03	7.13 ± 0.54	12.30 ± 0.30	23.85 ± 0.37	35.40 ± 0.48	43.03 ± 0.31	59.95 ± 0.28	73.38 ± 0.20	166.32 ± 2.03
C extract	7.88 ± 0.28	12.84 ± 0.17	21.30 ± 0.89	30.06 ± 0.78	44.66 ± 0.97	56.22 ± 0.89	77.01 ± 0.66	100 ± 0.00	86.15 ± 4.82
P extract	7.44 ± 0.48	10.44 ± 0.34	16.73 ± 0.64	28.08 ± 0.30	46.08 ± 0.91	71.84 ± 0.93	80.87 ± 0.72	100 ± 0.00	69.46 ± 1.70
Caffeic Acid	95.13 ± 0.79	96.15 ± 0.48	97.53 ± 0.21	98.22 ± 0.32	99.27 ± 0.17	99.43 ± 0.93	100 ± 0.00	100 ± 0.00	1.24 ± 0.05

The study analyzed a hydroalcoholic extract based on the idea postulated in the literature that optimum extracts obtained from this vegetal product contain about 50% alcohol. According to the European Pharmacopeia (EP), in order to have a biological effect, the minimum percentage of apigenin-7-glucoside in the flowers should be 0.25%. Our results confirm that the vegetal product complies with the pharmacopoeia provisions. In a similar approach, Fonseca et al. concluded that the percentage of free and glycosylated apigenin in the methanol extract is 106 and 903 µg/g whereas, in the ethanol extract, the amount is 11 and 247 µg/g. Analyzing the amount of pure and conjugated apigenin in different types of extracts, Haghi et al. have observed that the methanol extract leads to the highest amount of pure apigenin. In an aqueous extract, apigenin 7-O-glucoside was found to be the major constituent (Bhaskaran et al., 2007; Fonseca et al., 2004; Danciu et al., 2018).

In a similar approach using different solvents for the extraction of active compounds from the aerial parts in the flowering stage of chamomile (e.g., water, methanol, ethanol), Haghi et al. have concluded that the amount of total phenolic compounds and total flavonoids range in the interval (1.77–50.75 g GAE/100 g in dry material) and (0.82–36.75 g quercetin equivalent (QE)/100 g in dry material), respectively (Haghi et al., 2014).

As revealed in the results, it is obvious that the MC extract demonstrates free radical-scavenging potential. The practical approach is that intake of different food supplements or tea based on chamomile flower extract can lead to prevention of an increased number of pathologies related to oxidative stress and, also, prevent cell mutation. In a recent study, Cvetanovic et al. have assessed the antioxidant potential of different types of extracts obtained from chamomile flowers, namely Soxhlet, microwave-assisted, ultrasound-assisted, and subcritical water extraction. They concluded that the best free radical-scavenging ability was generated by subcritical water extraction (Cvetanović et al., 2015). The stamp that confirms the antioxidant potential of water and alcohol extracts of this vegetal product was put down also by Al-Ismail et al., who used a linoleic acid and liposome model system, as well as the well-known DPPH free radical-scavenging assay (Al-Ismail and Aburjai, 2004). The phytochemicals that might be involved in this type of effect are the flavonoids, due to their polyphenolic structure; and the terpenoids, due to their double bond system. A recent study showed the protective role of chamomile decoction extract, using a rat model of alcohol-induced injury of gastric mucosa. This potential was assigned to its capacity to reverse the depletion of antioxidant enzymes activity, such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) induced by ethanol administration (Jabri et al., 2017). Moreover, our results confirm that MC extract has a better activity against LOX than against free radicals, suggesting that different mechanisms are involved for such properties. The LOX-inhibiting activity can be correlated with an anti-inflammatory potential, which may decrease the membrane permeability (Danciu et al., 2018).

In regard to parsley and celery extracts, the majority of research has involved different plant organs, usually leaves, stems, and culture cells (Yıldız et al., 2008; Wong and Kitts, 2006). In terms of our choice of vegetal product, the seeds are commonly believed to contain the genuine and simple compounds found in the future plant. The chemical analysis of parsley and celery seed alcoholic extracts revealed that the chosen vegetal material contains flavone aglycons and apigenin glucoside (Yao et al., 2011). Other studies indicated the presence of gallic acid, catechins, and its derivatives, in ethanolic leaf extract, which were also present in our samples in small quantities. The authors concluded, then, that polyphenols, and mainly kaempferol derivatives, were responsible for the antioxidant activity (Vranješ et al., 2016).

At the same time, the calculated EC50 value for all the antioxidant tests allowed us to observe differences in regard to their activity. Taken together, all investigated extracts showed a better scavenger activity against free radicals, a good inhibitory activity against lipoxygenase, but a lower sensitivity against iron. These confirm that the type of bioactive compound is highly

important for the activity of the extract. Such claims have been previously discussed by Csepregi et al. in terms of structure–activity relationships, when the presence of certain free hydroxyl groups on the phenolic or flavonoidic skeleton can enhance the antioxidant/scavenger/chelation potential, depending on the assay (Csepregi et al., 2016).

However, the total phenolic quantification is not in direct correlation with the extract antioxidant capacity. There is still a correlation between the flavonoid content, the presence of aglycons, and the antioxidant potential for both parsley and celery samples. Every extract reacts differently depending on the assay, and the ratio and distribution of each component; for example, parsley and celery are more potent as enzyme inhibitors, whereas the chamomile extract acts more strongly as a radical scavenger. Moreover, all extracts show a weaker iron-binding activity. Our results sustain the observations made previously on the antioxidant capacity of parsley and celery extracts (Wong and Kitts, 2006; Tanasawet et al., 2017). Some studies also suggest that the type of solvent used for extraction and the temperature used during processing has a great impact on the chemical composition and the biological implication of the final extract. The current study suggests that the chosen extraction method and solvent ensure a good chemical composition of the investigated extracts.

II.3.2. Antimicrobial activity results

As previously mentioned, uropathogens represent a permanent problem in modern life therapeutics. Therefore, finding new antimicrobial agents is an ongoing purpose for researchers. In our study, we investigated the antimicrobial profile of two different type of extracts from *Aronia* berries grown in Romania and aimed to show putative synergy between the investigated samples and some antibiotics (Dorneanu et al., 2017).

Regarding the antimicrobial activity, both extracts showed a variable potential dependent on the used concentration and the tested strains. All registered values are included in table XXIII.

Table XXIII. MIC values for antimicrobial test for *Aronia* extracts

Microbial strain	**Tested extract**	
	1	**2**
Enterobacteria	**MIC (mg/mL)**	
E. coli ATCC	10	10
E. coli 2041	10	10
E. coli 1851	10	10

Microbial strain	Tested extract	
	1	**2**
E. coli 1992	10	5
E. cloacae 2951	10	10
K. pneumoniae 2110	10	10
K. pneumoniae 1074	10	10
K. pneumoniae 831	10	10
Morganella morganii 2520	10	5
non-fermenting Gram-negative bacteria		
Pseudomonas aeruginosa ATCC	10	2.5
A. baumanii 1908	10	10
A. baumanii 2329	10	10
P. aerug. 1908	10	2.5
P. aerug.1128	10	2.5
Gram-positive catalase - negative cocci		
E. fecalis ATCC	10	5
E. fecalis 2823	10	10
E. faecium2862	10	5
E. faecium 2980	10	10
E. faecium 2027	10	5
Gram-positive catalase - positive cocci		
S. aureus ATCC	10	2.5
S. aureus 14	10	2.5
S. aureus 17	10	5

The results indicate a good antibacterial activity for the ethanolic extract (sample 2) obtained from *Aronia* berries, whereas an average potential was noted for the acetone extract. The lowest MIC values corresponding to the most intense antimicrobial activity were obtained for sample 2, which proved to be most active against one strain of *E. coli* and one of *Morganella morganii* (5 mg / mL MIC), three strains of *P. aeruginosa* (2.5 mg/ mL MIC), one *E. faecalis* and

two *E. faecium* (5 mg / mL MIC) strains, and also against three strains of *S. aureus* (2.5-1.5 mg / mL MIC). Good activity against non-fermenting Gram-negative bacteria is extremely important for further studies considering that such microbial strains are ubiquitous in the environment, and can adapt to acquire multiple resistance to antibiotics.

Determination of the antimicrobial activity of the extracts was performed by determining the minimum biofilm eradication concentration (MBEC). Also in this case, similar to the previous test, the most active extract was sample 2, which inhibited the development of biofilm at lower CMEB values in the case of an *E. coli* and a *Morganella morganii* strain (CMEB of 5 mg / ml), three strains of *P. aeruginosa* (2.5 mg / mL CMEB), one *E. faecalis* and two *E. faecium* (5 mg/mL CMEB) strains. Moreover, the same sample was active at concentrations range between 3.5 and 5 mg/mL against three strains of *S. aureus*.

The synergistic effect for both extracts was modest, but an increase in the growth inhibition zone diameter for the antibiotic disks supplemented with the investigated extracts (fig. 25) was noted when amikacin, tetracycline, nitrofurantoin, imipenem and norfloxacin were used against *P. aeruginosa*, *E. coli* and *M. morganii*.

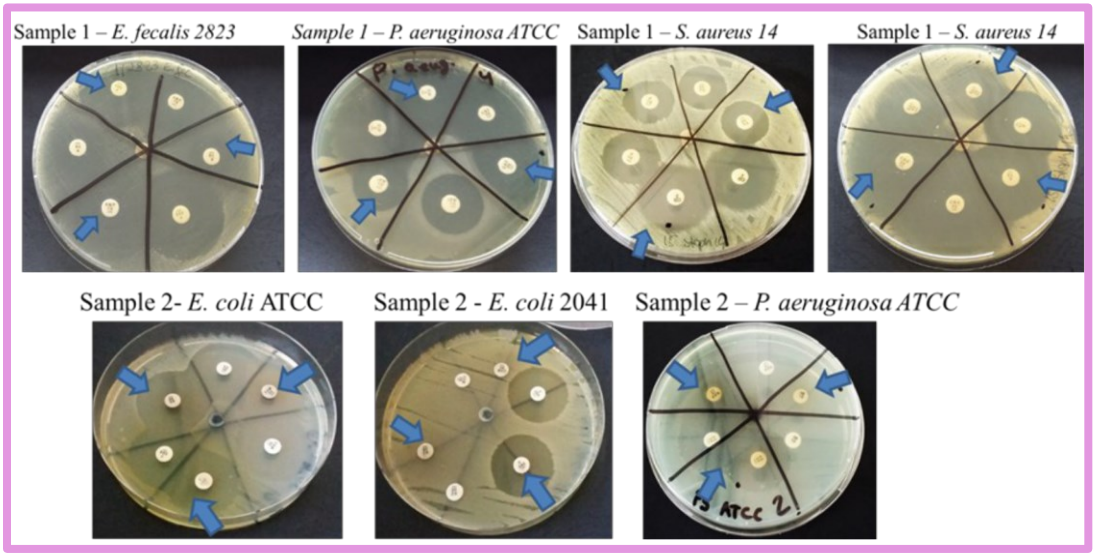

Fig. 25. Synergism testing of the acetone extract - Sample 1 and ethanolic extract – Sample 2 (the arrow indicates the presence of the extract on the disk)

Given all data, we can state that the antimicrobial activity observed in the initial tests is relevant when extracts are used on their own although, a modest to moderate synergy is observed when antibiotics are used. Yet, this study is only the starting point for investigating the correlation between the chemical composition and the mechanism in bacterial antibiotic susceptibility in the presence of *Aronia* extracts. Further studies are necessary to draw a concluding concept regarding synergism-based antibiotic treatment of anthocyanidin-rich extracts and its importance for therapy.

In summary, the present study indicated that ethanol favors the extractability of anthocyanins from chokeberry fruits and the lyophilization is a good method to preserve the biologic activity of these compounds. The ethanolic extracts proved to be active against 10 out of the 18 tested strains at doses that amounted 2.5 – 5 mg/mL (MIC). The same sample, at doses of 2.5 – 5 mg/mL showed significant inhibition of monoculture biofilm formation of 11 out of all tested strains.

Therefore, well obtained and standardized extracts should be taken into consideration for expanding the preventing measures and treatment in case of drug resistant microbes. *In vivo* testing should be the next step towards the use of such berry extracts in patients with urinary tract infections.

In an attempt to evaluate the broader bioactivity of the flavonoid derivatives identified in chamomile flowers (CA), parsley (P) and celery (C) seeds extracts, we first tested the antimicrobial activity of these samples.

Although the establishment of the antibacterial activity of the investigated extracts is difficult to interpret in the absence of standardized values, we considered that the chamomile extract was active only against tested Gram-positive cocci, while the parsley extract inhibited only the *S. aureus* strain. The celery extract did not show antibacterial activity. However, all tested preparations showed antifungal activity (Table XXIII and Table XXIV) (Danciu et al., 2021).

Table XXIII Antibacterial and antifungal activity of selected extracts expressed as inhibition zone (mm) (Danciu et al., 2021)

Sample	*K. pneumoniae*	*S. flexneri*	*S. enterica*	*E. coli*	*P. aeruginosa*	*S. aureus*	*E. faecalis*	*C. albicans*	*C. parapsilosis*
CA	7	7	7	7	7	10	10	10	10
P	7	7	7	7	7	10	9	10	10
C	7	7	7	7	7	7	7	10	10

Table XXIV. MIC and MBC values for each extract on bacteria and fungi (Danciu et al., 2021)

Bacterial species	Extracts	MIC (µg/ml)	MBC (µg/ml)
K. pneumoniae	CA	-	-
	P	-	-
	C	-	-
S. flexneri	CA	-	-
	P	-	-
	C	-	-
S. enterica	CA	-	-
	P	-	-
	C	-	-
E. coli	CA	-	-
	P	-	-
	C	-	-
P. aeruginosa	CA	-	-
	P	-	-
	C	-	-
S. aureus	CA	12.5	12.5
	P	12.5	12.5
	C	-	-
E. faecalis	CA	25	50
	P	-	-
	C	-	-
C. albicans	CA	12.5	25
	P	12.5	25
	C	25	25
C. parapsilosis	CA	12.5	25
	P	12.5	25
	C	25	25

It can be said that the extracts are more active on fungi than on bacteria. A similar approach reported about the antibacterial effect of different type of extracts (using as solvent methanol, ethanol, diethyl ether and hexane), respectively essential oil obtained by hydrodistillation, from the flowers of Egyptian *Matricaria chamomilla* L. The essential oil led to the smallest values for the minimum inhibitory concentration, and the sensitive strains were the fungus *C. albicans* and the gram-positive strains *B. cereus* and *S. aureus* (Roby et al., 2013). It is well known that plant-derived

volatile oils are powerful antibacterial and antifungal agents (Dorman and Deans, 2000). Both extract and essential oil obtained from *Anthemis nobilis* L. were described to possess antimicrobial effect against *P. gingivalis*, a bacteria present in the case of periodontitis (Saderi et al., 2004). The antibacterial potential against *B. subtilis* and *E. coli* of freeze-dried and irradiated parsley leaves and stems were assessed on methanol and water extracts by determining bacterial cell damage and bacterial growth inhibition. Parsley leaf methanol extract was able to induce cell damage against both tested strains (Wong and Kitts, 2006). The group of Wahba *et al*., showed that among other aromatic plants parsley presents antibacterial potential against *S. aureus* as well as natural microflora, yeast and moulds in Kareish cheese (Wahba et al., 2010). In a recent study Linde *et al*., analyzing the antifungal and antibacterial potential of the volatile oil obtained from parsley have concluded that it is a powerful bacteriostatic agent against *S. aureus*, *L. monocytogenes* and *S. enterica*, has powerful bactericidal activity against *S. aureus*. and strong fungistatic activity against *P. ochrochloron* and *T. viride* (Linde et al., 2016). The effect of volatile oil of celery was tested against 21 pathogenic strains. Results have shown bactericidal potential with the gram-positive strains presenting increased sensitivity towards gram-negative strains (Gupta et al., 2004).

II.3.3. *In vitro* cell viability results

Regarding antiproliferative potential, results show (Figure 26) that in the range of tested concentrations the selected extracts present an overall weak antiproliferative activity against MCF7 human breast cancer cell line. Among the screened samples, the antiproliferative potential was in the range: parsley > celery > chamomile.

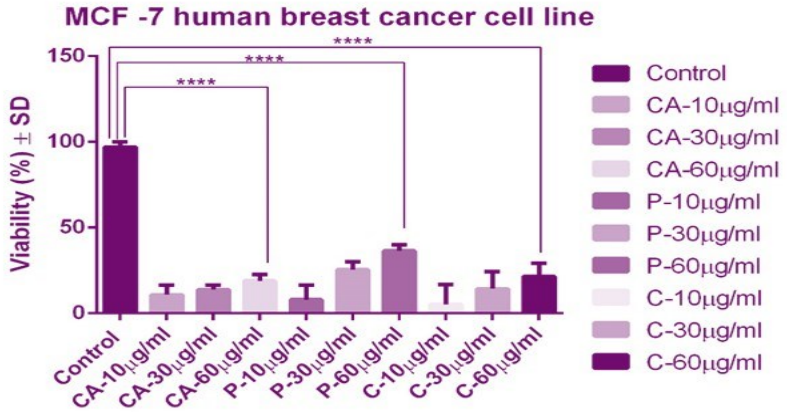

Fig. 26. Antiproliferative potential of tested extracts against MCF7 human breast cancer cell line (One-way ANOVA with Newman-Keuls posttest; *, **, ***, **** indicates $p<0.05$, $p<0.01$, $p<0.001$ and $p<0.0001$ respectively, compared to the control group)

The migratory rate of MCF7 - human breast adenocarcinoma cell line was below 40% after treatment with all of the extracts (Figure 27). However, celery extract manifested the most potent anti-migratory effect, displaying scratch widths of 430 µm initially and 390 µm after 24 h, with a migratory rate of only 9.30%.

Chamomile extract manifested a migratory percentage of 14.93% and parsley extract exhibited a rate of 39.64%, all of the test extracts being statistically significant when compared to control (no stimulated cells).

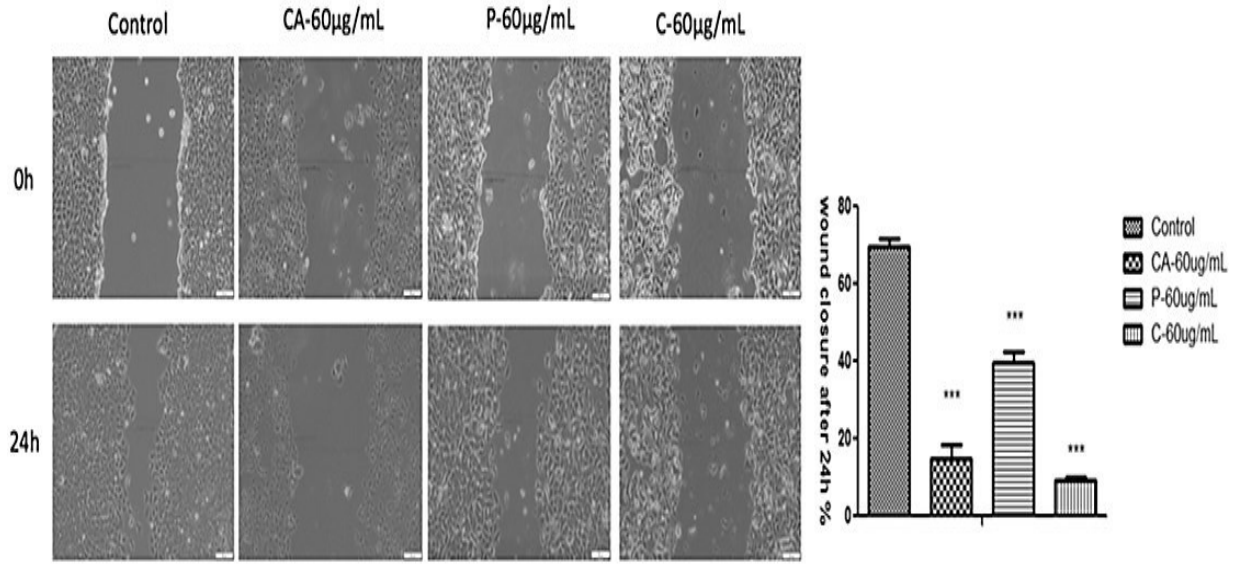

Fig. 27. Migratory ability of human breast adenocarcinoma - MCF7 cell line, after treatment with test extracts at a concentration of 60µg/mL. Wound closure was imaged by light microscopy initially and 24h, respectively. Scale bars represent 100µm. The bar graphs are expressed as the percentage of wound healing after 24h compared to the initial area.

Exposure of MCF7 - human breast adenocarcinoma cells to chamomile, parsley and celery extracts at a concentration of 60µg/mL caused a significant LDH release only in the case of parsley extract, displaying a cytotoxicity percentage of 10.22% (Fig. 28). However, the celery extract manifested a cytotoxicity rate of almost 3%, whereas the chamomile extract showed no cytotoxic effect at all, moreover it displayed a slight proliferative activity. The proliferative effect of the chamomile extract might be explained by causing an intracellular alteration of MCF7 cells' function, which could not be quantified by the LDH assay (Weyermann et al., 2005), the MTT test being more relevant in this case.

Fig. 28. Cytotoxicity percentage of chamomile, parsley and celery extracts at a concentration of 60µg/mL, after 72h stimulation (One-way ANOVA with Newman-Keuls posttest; *, **, ***, **** indicates $p<0.05$, $p<0.01$, $p<0.001$ and $p<0.0001$ respectively, compared to the control group)

Flow cytometric analysis was conducted in order to study the effect on the distribution of the phases of the cell cycle of MCF7 cells after 72 h of incubation with tested extracts (Table XXV and Fig. 29). Results have shown that in the case of CA extract, in both concentrations a slight G0/G1 accumulation can be noticed. Also, P extract elicited the same type of behavior, but in this case, the accumulation of cells in G0/G1 phase was in a dose-dependent manner. Among screened samples, this extract at the highest tested concentration had the most significant effect on the G0/G1 cell cycle arrest. Cells incubated with C extract at the concentration of 30µM led to a G2/M accumulation whereas in case of 60µM a slight G0/G1 accumulation could be detected.

Table XXV. Cell cycle distribution of MCF7 human breast cancer cells after 72 h treatment with selected extracts (mean values of 3 experiments and standard deviation)

Treatment	Cell cycle distribution (%) MCF7		
	G0/G1	S	G2/M
0(CTRL)	61.12 ± 1.38%	21.96 ± 0.25%	16.74 ± 1.66%
CA 30 µg/mL	69.41 ± 4.02%	14.83 ± 2.08%	15.12 ± 1.42%
CA 60 µg/mL	69.33 ± 1.00%	13.87 ± 2.63%	16.71 ± 1.63%

Treatment	Cell cycle distribution (%) MCF7		
	G0/G1	S	G2/M
P 30 µg/mL	69.00 ± 0.53%	11.24 ± 0.25%	19.15 ± 0.90%
P 60 µg/mL	75.82 ± 0.58%	11.66 ± 0.79%	12.21 ± 0.27%
C 30 µg/mL	57.99 ± 0.41%	21.75 ± 0.11%	19.83 ± 0.49%
C 60 µg/mL	66.11 ± 2.47%	16.27 ± 1.60%	17.36 ± 0.99%

where: CA – chamomile extract; P – parsley; C - celery

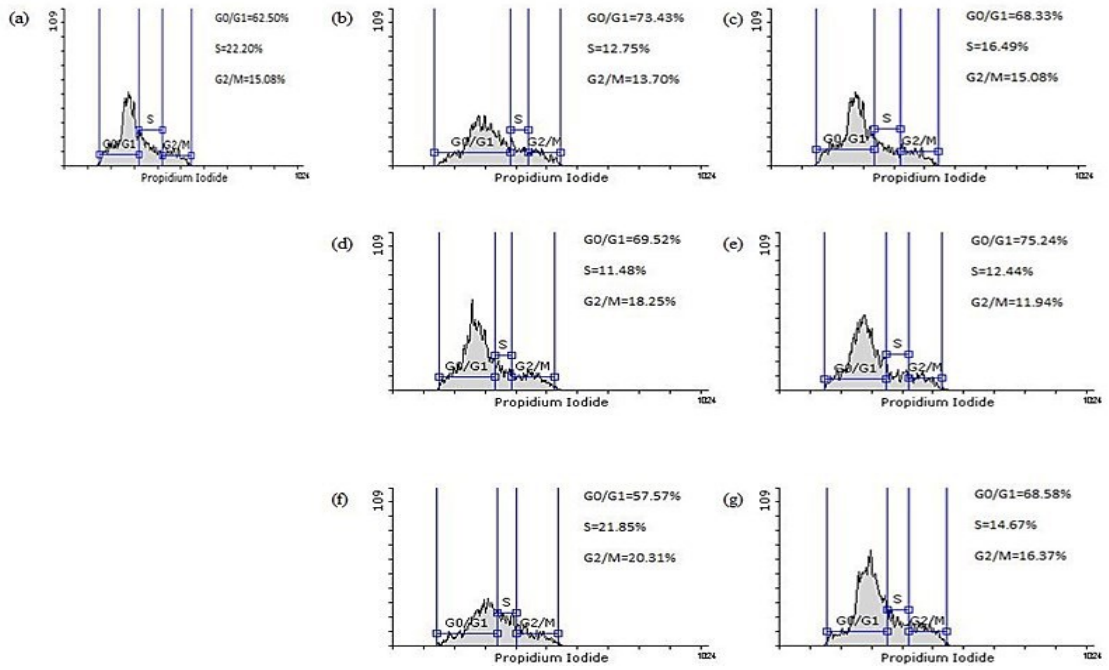

Fig. 29. Representatives histograms of cell cycle analysis for MCF7 cells treated with: (**a**) control; (**b**) 30 µg/mL CA; (**c**) 60 µg/mL CA; (**d**) 30 µg/mL P; (**e**) 60 µg/mL P; (**f**) 30 µg/mL C; (**g**) 60 µg/mL C

In order to check the pro-apoptotic potential of selected extracts including phenomena of early apoptosis, late apoptosis and necrosis the Annexin-PI double staining was employed. Among the three extracts, CA caused the highest percentage of necrosis, namely 17.65 % ± 0.20 In terms

of total apoptotic events C was the most potent extract, inducing a percentage of 8.99 % ±4.67 cells. Results are presented in Table XXVI and representative dot-plots are displayed in Figure 30.

Table XXVI. Viability (mean values) of MCF7 human breast cancer cell line using Annexin V-PI analysis

	Viable	Early apoptosis	Late apoptosis	Necrosis
Control	87.90 ± 1.43	1.53 ± 0.38	0.11 ± 0.05	10.46 ± 0.62
CA	75.26 ± 6.63	5.66 ± 2.74	1.44 ± 1.35	17.65 ± 0.20
P	80.39 ± 6.58	5.70 ± 2.79	0.93 ± 0.86	12.99 ± 0.13
C	82.11 ± 7.50	7.53 ± 3.41	1.46 ± 1.26	8.89 ± 0.57

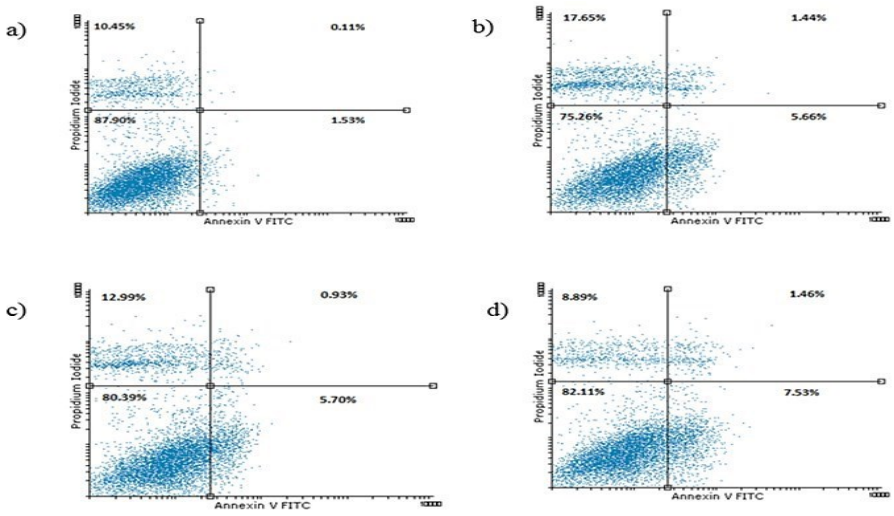

Fig. 30. Representative dot-plots of MCF7 human breast cancer cell line for: (**a**) Control; (**b**) incubated with CA; (**c**) incubated with P; (**d**) incubated with C

As described in the comprehensive review of Srivastava *et al*., an increasing number of papers about the anti-cancer activity of chamomile extract involve studies towards the flavone apigenin, one of the most bioactive constituents. *In vitro* and *in vivo* studies have shown beneficial effects by eliciting growth inhibitory potential in case of skin, prostate, ovarian and breast cancer models (Srivastava et al., 2010). The same author has evaluated the antiproliferative and pro-apoptotic potential of aqueous and methanolic extracts against different human cancer cell lines and underlined the positive effect on cancerous cells associated with minimal growth inhibitory effect for normal cells (Srivastava and Gupta, 2007). In a complex approach about the *in vitro* cytotoxic (PC-3- human prostate, A-549 - human lung carcinoma and MCF7 - human breast cancer cells) and antibacterial potential (*Propionibacterium acnes*) of ten volatile oils, chamomile oil was

also included. The essential oil at a concentration of 0.200% (v/v) exhibited a strong cytotoxic effect against PC-3 - human prostate cancer cells. Also the viability of MCF7 - human breast cancer cells was reduced to 6.93% (Zu et al., 2010). Kandelous *et al*., have shown that extracts obtained from the aerial part of ′Roman chamomile′, known under the scientific name of *Chamaemelum nobile* (L.) All. in the range of concentrations of (0.001- 0.25 mg/mL) elicit antiproliferative effect against MCF7 cells. Also when parsley and celery are mentioned, an increased number of studies related to the chemopreventive potential for *in vitro* and *in vivo* models of breast cancer of apigenin (Navabi et al., 2015; Mocanu et al., 2015). In a similar approach Farshori *et al*., have screened the anticancer activity of alcoholic extracts and oil of parsley seeds against MCF7 - human breast cancer cells. The study concluded that both the alcoholic extract as well as the oil have significantly reduced cell viability in a dose-dependent manner. Moreover, doses over 50 µg/mL for the extract and 100 µg/mL for the oil were found to be cytotoxic for this breast cancer cell line (Danciu et al., 2021).

On the other hand, the same three extracts show an overall weak antiproliferative activity in the range of tested concentrations, C extract being more active, starting from the concentration of 10 µg/mL. At the highest tested concentration, namely 60 µg/mL, and after a period of 72 h incubation, the C extract led to a cell growth inhibition of 28.1 ± 2.0%, the P extract led to a cell growth inhibition of 24.9 ± 2.9%, while the MC extract led to a cell growth inhibition of 5.17 ± 3.4%. Results can be seen in Figure 31 (Danciu et al., 2018).

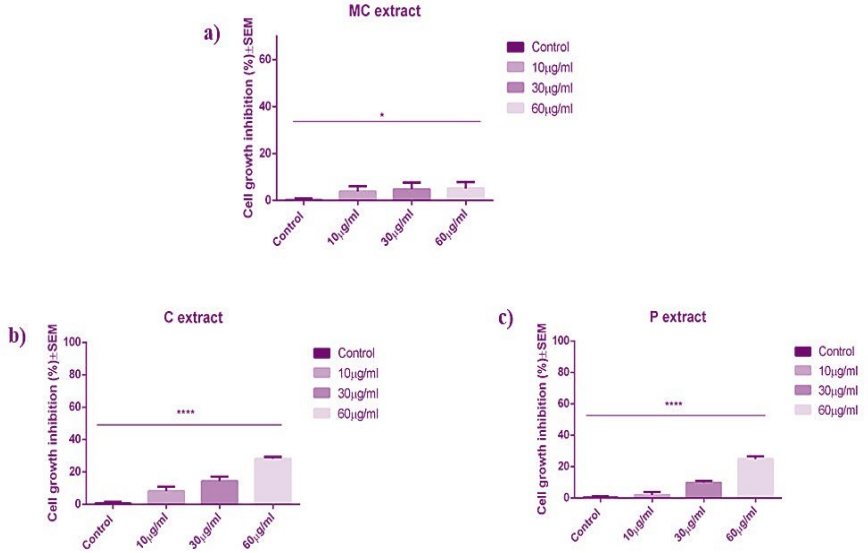

Fig. 31. Cell growth inhibition (%) ± SEM against A375 human melanoma cells after 72 h of incubation with (**a**) MC extract; (**b**) C extract; or (**c**) P extract.

Statistical significance was assessed by one-way ANOVA with Newman–Keuls post-test for comparison of multiple groups *, **, ***, and **** indicate $p < 0.05$, $p < 0.01$, $p < 0.001$, and $p < 0.0001$ respectively, compared to the control group.

The activity of protein caspase 3 as an effector caspase of apoptosis was analyzed for the three extracts using the same two concentrations, namely 30 and 60 µg/mL. However, at 30 µg/mL, no effect was detected for the MC extract while, at the highest tested concentration of 60 µg/mL, a significant increase of caspase 3 activity was induced as compared with control. Results have shown that, in case of C extract, caspase 3 is not activated in the tested dose range. On the other hand, the P extract in the concentration of 30 µg/ml significantly increased the amount of this protein, which shows the pro-apoptotic potential of P extract at this dose. Interestingly, when 60 µg/mL P extract was used, the amount of caspase 3 decreased, probably as a consequence of necrosis. Results can be seen in Figure 32 (Danciu et al., 2018).

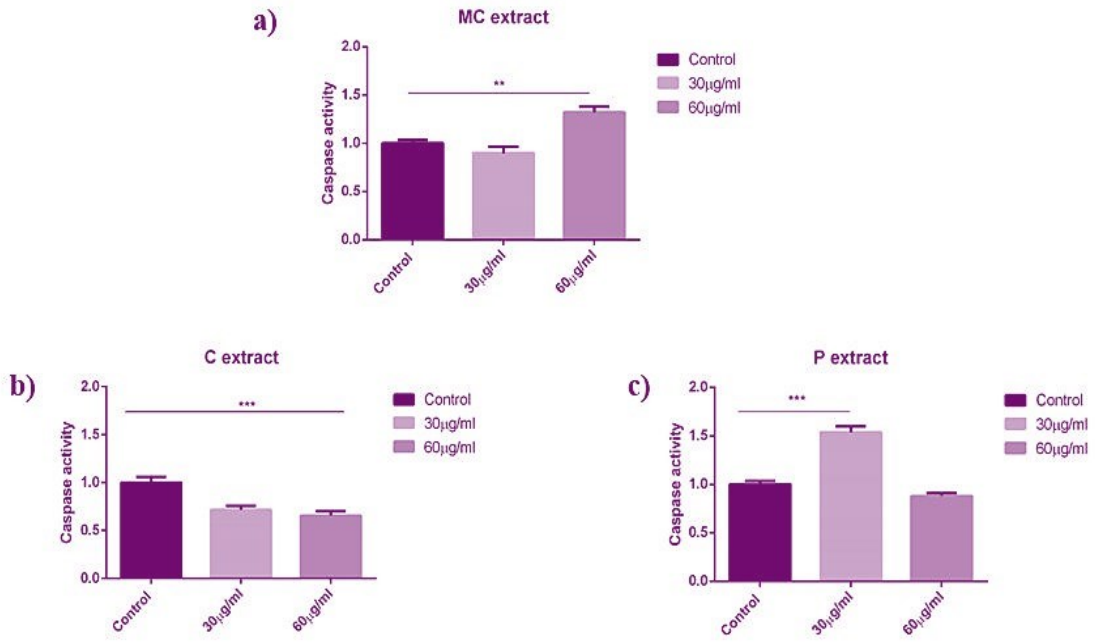

Fig. 32. Caspase 3 activity of A375 human melanoma cells after 72 h of incubation with (a) MC extract; (b) C extract; (c) P extract.

In order to analyze, more thoroughly, the apoptotic events, annexin V-PI staining was used, in order to have data about early apoptosis, late apoptosis, and necrotic events. Correlated to the abovementioned findings, the study showed that P extract at the concentration of 60 µg/mL can induce phenomena of early and late apoptosis, as well as necrosis, compared to control cells. Results can be seen in Table XXVII (Danciu et al., 2018).

Table XXVII. Apoptotic events for A375 human melanoma cell line after incubation with selected extracts

	Viable cells	Early apoptotic cells	Late apoptotic cells	Necrotic cells
Control	95.40 ± 1.89	3.10 ± 1.62	0.67 ± 0.21	0.83 ± 0.05
MC extract (60 µg/mL)	95.76 ± 0.16	1.50 ± 0.08	0.64 ± 0.08	2.1 ± 0.16
C extract (60 µg/mL)	95.19 ± 0.95	2.38 ± 0.60	0.82 ± 0.37	1.70 ± 1.17
P extract (60 µg/mL)	89.00 ± 0.09	7.30 ± 0.65	2.36 ± 0.24	1.34 ± 1.25

Immunocytochemistry was performed in order to detect the expression of caspase 2 as a possible inductor of caspase 3. This technique also allowed for observation of the number of cells along with their morphology. Regarding the marker expression, a similar relative ratio, in control and cells incubated with 60 µg/mL MC and C extract, could be detected. Increased caspase 2 expression, in the case of cells treated with 60 µg/mL P extract, could be observed. A reduced number of cells compared to control, in the case of cells treated with selected extracts, was noticed. The morphology of A375 human melanoma cells is fibroblast-like. Incubation with 60 µg/mL C extract did not generate changes in the morphology of cells. A cytoplasm reduction and elongated cells were present upon incubation with 60 µg/mL MC extract. Incubation with 60 µg/mL P extract led to the reduction of the volume of the cytoplasm, as well as elongated and thinned cell shape. The expression of cellular tumor antigen p53 in A375 human melanoma cells was also checked after incubation with the three extracts in the concentration of 60 µg/mL. A similar expression of p53 marker could be noticed in all samples, thus, this tumor suppressor marker is not activated following incubation with the selected extracts. Results can be seen in Figure 33 (Danciu et al., 2018).

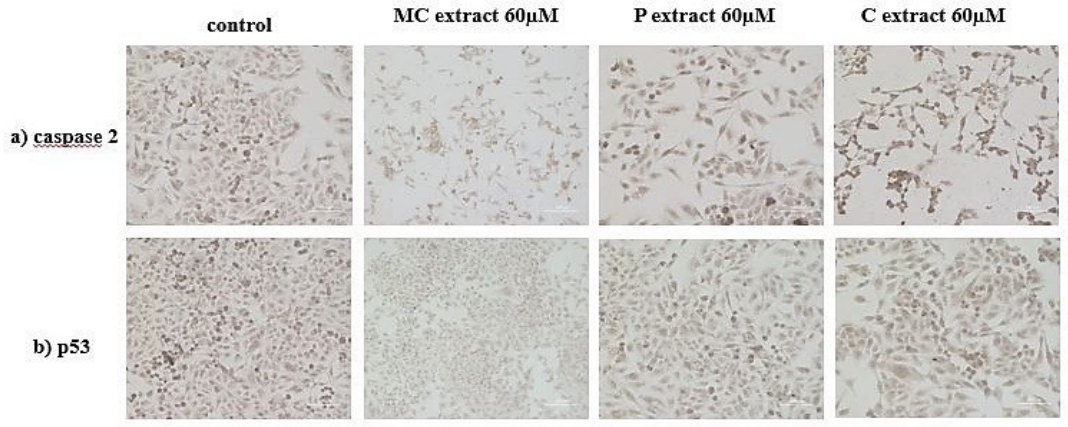

Fig. 33. Immunocytochemical evaluation of A375 human melanoma cells after incubation with selected extracts at a concentration of 60 µM; (a) caspase 2 evaluation; (b) p53 evaluation.

In a recent study Tang *et al*., have analyzed the effect of five types of extracts obtained from leaf and stem of (Mill.) Fuss towards normal 3T3-L1 fibroblasts and MCF-7, MDA-MB-231 (breast) and HT-29 (colorectal) cancer cell lines. Results have shown a weak cytotoxic activity, dichloromethane being the best type of extract, conducting at the highest tested concentration, namely 500 µg/mL to a percentage of inhibition of 48.4% ± 1.8% for MCF7 cells, 25.5% ± 3.0% for MDA-MB-231 cells and 49.9% ± 1.0% for HT-29 cells. Furthermore, at the concentration of 300 µg/mL, the extract inhibited H_2O_2-induced MCF7 cell migration thus assigning the extract with protective effects against metastasis. Schroder *et al*., discussed the dose-dependent effect of root extract for the proliferation of MCF 7 cells and concluded that in the range of concentrations of [0.01 µg/mL-100 µg/mL] the extract does not elicit a cytotoxic effect, however at 500 µg/mL the extract was cytotoxic for more than 70% of cells. Regarding the celery extract, literature is poor in information about the effect against breast cancer cells, however antiproliferative and proapoptotic activity against other cancer cell lines was previously reported (Köken et al., 2016; Momtazi et al., 2017; Danciu et al., 2021).

The phytochemical analysis showed that chamomile, parsley, and celery methanol extracts contain natural compounds that belong to the polyphenolic acids and flavone groups. Apigenin in the form of aglycone, as well as heteroside, could be detected in all extracts, but it is clearly visible that the seeds contain mainly aglycons and less glycosides. The tested samples showed radical scavenger capacity, iron chelation potential, as well as lipoxygenase inhibition capacity. The three extracts showed an overall weak antiproliferative activity against A375 human melanoma cell line in the range of tested concentrations. Among the screened extracts, parsley was the most active in terms of pro-apoptotic properties, inducing both caspase 3 and caspase 2, as well as phenomena of early apoptotic, late apoptotic, and necrotic cells. Parsley and chamomile extracts affected the fibroblast-like morphology of A375 human melanoma cells. Regarding the activity on human dendritic cells, chamomile and celery extracts abrogated the expansion of LPS-activated dendritic cells. On the other hand, the metabolic activity of active human DCs was attenuated by stimulation with celery extract, while chamomile and parsley extracts had no effect on the metabolic activity. Extract incubation with naïve dendritic cells did not trigger cytokine secretion (TNF-alpha, IL-6, IL-10), indicating that the extracts themselves have no immune reactivity in the given settings, only the levels of anti-inflammatory cytokine IL-10 being significantly reduced upon celery extract stimulation.

In our study, at the highest tested concentration, namely, 60 µg/mL parsley was the most active extract in terms of reducing the viability of MCF7 human breast adenocarcinoma cell line and inducing the release of lactate dehydrogenase. In the set experimental conditions, chamomile and celery extracts present relevant and moderate antiproliferative and cytotoxic potential. On the other hand, celery extract manifested the most potent anti-migratory effect and was the most active extract in terms of total apoptotic events (both early and late). This preliminary study conducted

for the assessment of the various biological effects of screened samples shows that further steps need to be undertaken for a clear understanding of the complex mechanisms of this bioactive phytocomplexes (Danciu C, **Cioanca O**, Hancianu M, Racoviceanu R, Muntean D, Zupko I, Oprean C, Tatu C, Paunescu V, Proks M, Diaconeasa Z. Botanical Therapeutics (Part II): Antimicrobial and In Vitro Anticancer Activity against MCF7 Human Breast Cancer Cells of Chamomile, Parsley and Celery Alcoholic Extracts. Anti-Cancer Agents in Medicinal Chemistry (Formerly Current Medicinal Chemistry-Anti-Cancer Agents). 2021;21(2):187-200).

II.3.4. *In vivo* bioactivity results

AD is an age-associated neurodegenerative disorder characterized by progressive loss of memory and cognition (Severini et al., 2016). Pathologically, the disease is characterized by the presence of extracellular and intracellular plaques of β-amyloid peptide (Aβ), and intracellular tangles hyperphosphorylated *tau* protein. Basically, brain cells wither away and die, causing disorientation, dementia and severe changes in personality and social interactions (Adams et al., 2007). Memory function is likely to be formed by numerous discrete neural networks than can be disrupted by the pathophysiological processes in mild cognitive impairment (MCI) and AD. The hippocampus, a well-known brain region involved in memory consolidation is especially susceptible to AD. Early degenerative symptoms including significant deficits in the performance of hippocampal-dependent cognitive abilities such as spatial learning and memory have been reported that can be related to MCI rather than AD. Moreover, 80% of the patients with MCI will most certainly develop AD within 5 to 10 years from first starting to show MCI symptoms. Furthermore, anxiety and depression are common in AD, and is often related with duration of dementia, greater severity of dementia and lower education level (Hritcu et al., 2016). Patients also frequently have non-cognitive symptoms, such as depression, apathy and psychosis that impair daily living (Zhang et al., 2004). Currently, there is no cure for most forms of AD dementia type. Pharmacotherapy is focused on symptomatic benefit and slowing disease progression but a number of possible diseases modifying and preventive strategies based on current understanding of AD pathophysiology are under investigation (Anand et al., 2014). The advantage of plant extracts in multifactorial diseases such as AD is the diversity of compounds present in these extracts, each treating another target (as a multi-targeted approach).

As a pharmacological tool in producing a model of dementia in our laboratory, we successfully used scopolamine and amyloid beta (1-42) peptide or amyloid beta (25-35) peptide to induce an Alzheimer's disease rat model. In this direction, we reported that multiple exposure of rats to various essential oils could effectively reverse spatial memory deficits induced by dysfunction of the cholinergic system in the rat brain induced by scopolamine and amyloid beta (1-42) and might provide an opportunity for management neurological abnormalities in dementia conditions. We also reported that these essential oils increased anxiolytic-antidepressant-like behavior and possess neuroprotective effects via anti-oxidative activities. It has been clearly

established that oxidative stress is among the major causative factors in the induction of many chronic and neurodegenerative diseases (Rege et al., 2014). Thus the use of scavenging peroxide molecules may be useful to attenuate oxidative neuronal damage associated with various neurodegenerative diseases. Monoterpenes were identified as the major constituents of our essential oils (40-50%) with different biological activities which might explain our results and the beneficial effect of these oils to AD afflicted rats. Furthermore, in silico studies carried out by employing molecular docking experiments pointed out to existence of strong interactions of monoterpenes from our essential oils with anxiolytic and antidepressant effects with GABAA receptor (Bagci et al., 2016). Moreover, many signaling cascades involved in the induction of long- term potentiation (LTP), including Ca2+/calmodulin-dependent protein kinase II (CaMKII) or protein kinase C (PKC), we assumed that our selected extracts would play an essentia role in learning and memory by enhancing the synaptic plasticity, especially in the hippocampus (Han et al., 2015). Through the PKC/ (cAMP response element-binding protein) CREB signaling pathway, PKC regulates the excitability of nerve cells and affects the synthesis and release of monoamine neurotransmitters, with a relatively high content in the hippocampus.

To sustain the aforementioned elements, I included below the most interesting results obtained in AD or PD models following treatment with volatile fractions or flavonoid rich extracts. Moreover, I included also some biochemical parameter results that emphasizes the benefic impact of herbal extract treatment for neuroprotection.

The most recent study (El-Akhal et al., 2021) on *Conyza canadensis* (CC) aqueous extract on scopolamine (Sco) rat model suggests that the CC extract acts as anxiolytic and antidepressant agent by regulating the cholinergic system.

As illustrated in Figure 34A, Sco exposure significantly decreased the time spent in the open arm ($p < 0.001$), as compared to the control group. Treatment of rats given Sco with CC extract at 100 and 200 mg/kg significantly increased the time spent in the open arm ($p < 0.0001$), as compared to Sco-alone treated group. The number of entries in the open arm (Figure 34B) was significantly ($p < 0.001$) reduced by Sco, whereas administration of the CC extract significantly increased the entries in the open arms, especially at the dose of 200 mg/kg, as compared to Sco- alone treated group.

As noticed in Figure 34C, Sco injection-induced hypolocomotion, as evidenced by a decreased number of crossings ($p < 0.001$), as compared to the control group. Treatment with the CC extract significantly increased locomotion ($p < 0.0001$), as compared to Sco-alone treated group, suggesting an anxiolytic profile. According to a literature survey, several phytochemical studies reported that CC contains terpenes, acetylene derivatives, flavonoids, benzoic acid derivatives, alkaloids, essential oils, sphingolipids, fatty acids, and sterols (Csupor-Löffler et al., 2011; Ding et al., 2010). Among them, it has been reported that quercetin protects against stress-induced anxiety- and depression-like behavior and improves memory in male mice (Samad et al., 2018). Also, Crupi et al. demonstrated that for the first time the luteolin compound exerts a

significant antidepressant effect at a low dose and may be considered as a novel therapeutic strategy in depression.

Our findings showed that the CC extract eliminated Sco's anxiogenic effects, acting as an anxiolytic agent with close-by values related to diazepam (DIAZ), a typical anxiolytic agent. The obtained results are in direct correlation with the chemical composition and the *in vitro* antioxidant activity. Moreover, CC extract administration does not have a sedative effect, nor the side effects usually given by the DIAZ.

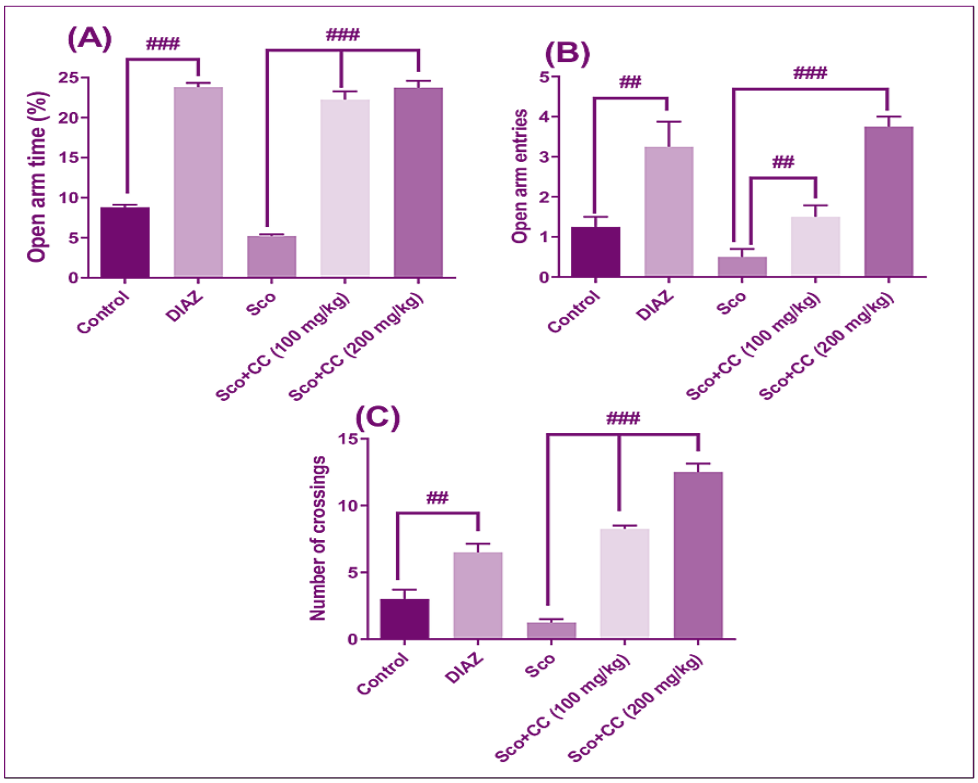

Fig. 34. Effects of the *Conyza canadensis* (CC) aqueous extract (100 and 200 mg/kg b.w.) in the elevated plus-maze test on the percentage of the time spent in the open arms (A), on the number of open-arm entries (B), and on the number of crossing (C) in the scopolamine (Sco, 0.7mg/kg b.w.)-treated rats. Values are means ± S.E.M. (n = 6 animals per group). For Tukey's *post hoc* analyses: ## $p < 0.001$ and ### $p < 0.0001$

The increased number of crossings proves that the animals are not only less anxious but also more active than the positive control group. Translated to further studies this may relate to an increased life standard for mentally impaired persons. However, this needs to be investigated in future research.

The results from the FST are illustrated in Figure 35 A and B. According to this diagram, scopolamine exposure induced a depressive-like behavior as evidenced by decreased swimming

time ($p < 0.01$) (A) and increased immobility time ($p < 0.01$) (B), as compared to the control group. Treatment with CC extract (100 and 200 mg/kg) in rats given Sco, caused a significant increase of the swimming time ($p < 0.0001$) (A) that parallel with a significant decrease of the immobility time ($p < 0.0001$) (B), suggesting antidepressant profile.

The antidepressant profile of the CC extract is supported by the chemical constituents identified ((epi)/catechin, luteolin, rosmarinic acid, gallic acid, and quercetin) in its chemical composition as previously stated. Anjaneyulu et al. indicated that quercetin has the potential to be employed as a therapy for depression in streptozotocin-induced diabetic mice. De La Pena et al. reported that luteolin mediates the antidepressant-like effects in mice, possibly through modulation of the GABAA receptor.

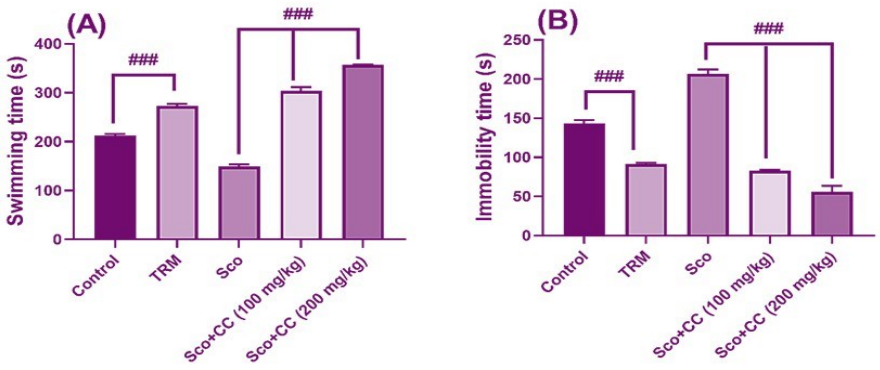

Fig. 35. Effects of the *Conyza canadensis* (CC) aqueous extract (100 and 200 mg/kg b.w.) in the forced swimming test on the swimming time (A), and the immobility time (B) in the scopolamine (Sco, 0.7mg/kg b.w.)-treated rats. Values are means ± S.E.M. (n = 6 animals per group). For Tukey's *post hoc* analyses: ### $p < 0.0001$

Increased swimming time denotes a survival tendency that usually lacks in depressive individuals. The same attitude is characteristic for all living beings, not just for animals. Therefore, our extract effects are better than the impact of the positive control (TRM) and even better than the behavior of the healthy animals. Therefore, we may postulate that an enriched flavonoid diet may or can lower depressive behavior and increase life expectancy.

In another example, we started from Iranian folk medicine where bay leaf (*Laurus nobilis* L.) has been shown to possesses various biological activities such as wound healing activity, antioxidant activity, antibacterial activity, antiviral activity, immunostimulant activity, anticholinergic activity, antifungal activity, insect repellent activity, anticonvulsant activity, antimutagenic activity, including the use used to treat epilepsy, neuralgia, and parkinsonism (Caputo et al., 2017). Despite extensive studies about the biological activities of *L. nobilis* extract, there is currently no study that addressed the benefits of bay leaf incense (BL) in diseases involving brain disfunction, where the incense of the plant is sold with a memory-enhancing claim in

Anatolia. Thus, the study was organized to elucidate the possible potential effects of BL on memory function and brain antioxidant status of rats exposed to Sco by means of scientific approach. For this purpose, we begun a successful collaboration with the Department of Pharmacognosy, Faculty of Pharmacy, Gazi University, Ankara, Turkey, which provided the plant material.

Therefore, we investigated whether the bay leaf incense (BL) elicits the memory formation via the action on the cholinergic system using a scopolamine (Sco)-induced rat model. Thus, rats were exposed to BL over 5 min in a smoking chamber apparatus once daily for 22 days, whereas memory impairment was induced by Sco (0.7 mg/kg), a muscarinic receptor antagonist, delivered 30 min before each behavioral test. The phytochemical composition of BL was achieved by gas chromatograph–mass spectrometry (GC/MS). Behavioral effects in rats were assessed by Y-maze, radial arm maze (RAM), and novel object recognition (NOR) paradigms. Additionally, the acetylcholinesterase (AChE) activity and the oxidative stress markers in the rat hippocampus were also evaluated.

Essential oils are complex mixtures of various small molecules substances and their chemical composition is highly influenced by environmental factors and processing technology. On the other hand, even if it uses the same raw material as an essential oil the incenses or smoke obtained by direct burning of the vegetal product comprises a different spectrum of compounds. This is mainly because the burning induces direct pyrolysis of the plant material and it takes a shorter amount of time than usual hydrodistillation. In our study we investigated the incense obtained during the first 5 min of burning, considering that this process leads to artifacts and metabolites that can suffocate the animals if inhaled for longer.

Today, little is known of the direct implications of the volatiles on the brain, especially the synergistic and multitarget approach of such compounds. Most of the research was directed on monoterpenes containing hydroxyl groups (such as linalool, eugenol, terpineol, terpinene-4-ol, etc.) and their synergistic effects with antibiotics enhancing the antimicrobial activity. However, the activity is correlated with the chirality, the size, the shape, and the physicochemical properties of the molecule and its interaction and integration in the cell membranes influencing the integrity/permeability, receptors, ionic channels, enzymatic systems, etc. [41–43]. Interestingly, the isomers of the volatile compounds can modify the odour, the taste, and the effects on the organisms.

Previous data states that linalool and its derivatives have a great impact on the central nervous system (CNS) by interacting either with Ca^{2+} channels or by interfering with the muscarinic receptors, thus inducing anxiolytic and antidepressant effects. Similar effects and mechanisms have been postulated also for limonene, carvone, and O-methyl jasmonate. The lipophilic character of the compounds allows an easy passage through the blood-brain barrier. Such direct effects on the limbic systems have been proven for the first time for 1,8-cineole [44]. Our data agrees with many other authors working on *L. nobilis* essential oil. Dammak et al. reported that 1,8-cineole was the most abundant compound of *L. nobilis* essential oil (43.2 ± 1.7%).

To explore whether BL exposure attenuated Sco-induced cognitive impairment in rats, the Y-maze, radial arm-maze (RAM), and novel object recognition (NOR) tests were conducted. For the Y-maze test, the Sco administered group demonstrated an increase of locomotor activity, as evidenced by a significant increase ($p < 0.0001$) of the number of arm entries compared to the control group (Figure 36A). When Sco was injected, a significant decrease of short-term memory performance was observed, as evidenced by decreased spontaneous alternation percentage ($p < 0.001$) compared with the control group (Figure 36B).

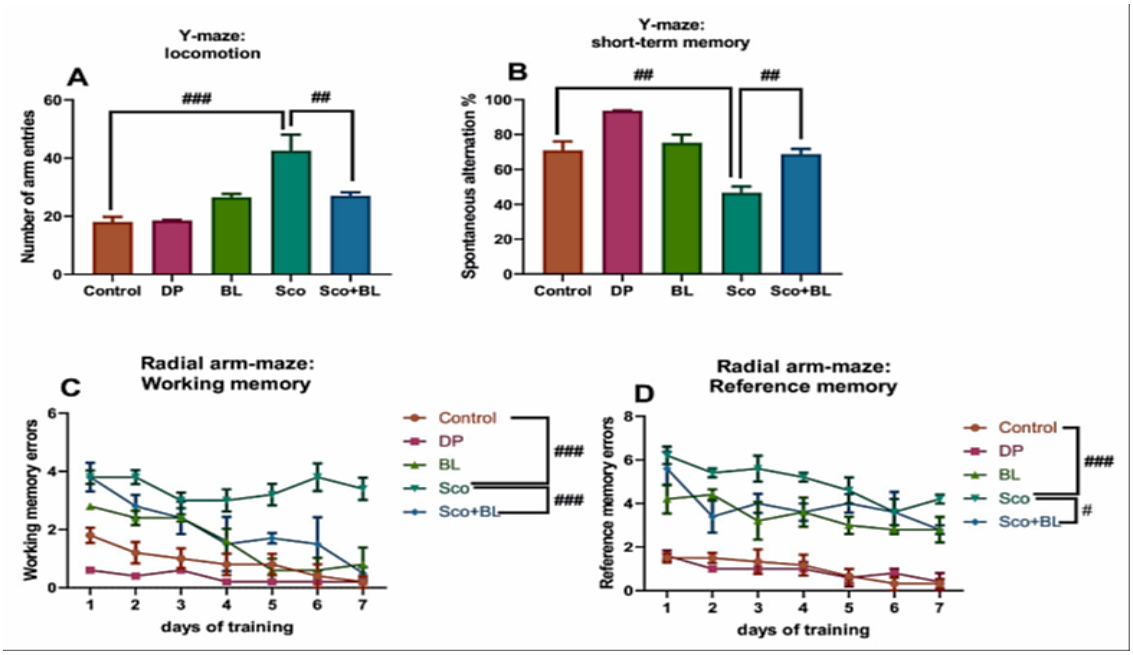

Fig. 36. Behavioral analysis for the Y-maze and the radial-arm maze (RAM) tests. (**A**) represents the graph for the number of entries in the Y-maze test in different groups; (**B**) represents the graph for spontaneous alternation percentage in the Y-maze test in different groups; (**C**) represents the graph for the working memory errors in RAM in different groups; (**D**) represents the graph for the reference memory errors in RAM in different groups.

Data are expressed as means ± SEM ($n = 5$ and statistical analysis by one-way ANOVA followed by Tukey's *post hoc* analyses: (**A**) Control vs. Sco: ### $p < 0.0001$, and Sco vs. Sco + BL: ## $p < 0.001$; (**B**) Control vs. Sco: ## $p < 0.001$, and Sco vs. Sco + BL: ## $p < 0.001$; (**C**) Control vs. Sco: ### $p < 0.0001$, and Sco vs. Sco + BL: ### $p < 0.0001$; (**D**) Control vs. Sco: ### $p < 0.0001$, and Sco vs. Sco + BL: # $p < 0.01$.

Moreover, significant improvement of the spontaneous alternation percentage ($p < 0.001$) was observed in Sco-injected rat following BL exposure when compared to Sco-induced rats, indicating that the memory impairment induced by Sco was reversed. Compared with the control group, in the RAM, rats treated with Sco showed increased number of working memory errors (Figure 36C) ($p < 0.0001$), and number of reference memory errors (Figure 36D) ($p < 0.0001$).

However, rats treated with Sco and BL showed significantly reduced number of working memory errors (Figure 36C) ($p < 0.0001$), and the number of reference memory errors (Figure 36D) ($p < 0.01$), when compared to the Sco-treated group. When BL and DP, a standard drug for AD, were administered, significant effects on memory performance in the Y-maze and RAM tests were noticed.

Based on the outcomes, the Sco groups showed decreased discrimination index of the N (Figure 37A) ($p < 0.01$) as compared to the control group, while exhibited the same preference to explore both F and N, indicating an impaired response to recognizing the N. The Sco-treated rats subjected to BL, displayed significant increase ($p < 0.001$) in both discrimination index (Figure 37A) and the time to explore the N (Figure 37B), suggesting a positive response to novelty. Both BL and DP significantly increased performance in the NOR, suggesting positive effects on recognition memory.

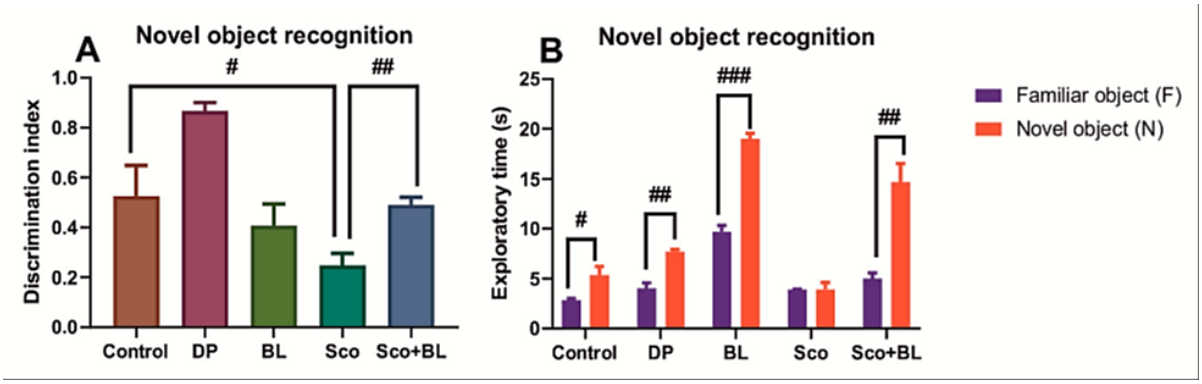

Fig. 37. Behavioral analyses for the novel object recognition (NOR) test. (**A**) represents the graph for discrimination index in different groups; (**B**) represents the graph for the exploratory time (s) in different groups.

Data are means ± S.E.M.(n = 5) and statistical analysis by one-way ANOVA followed by Tukey's post hoc analyses: (**A**) Control vs. Sco: # $p < 0.01$; Sco vs. Sco + BL: ## $p < 0.001$; (**B**) # $p < 0.01$; ## $p < 0.001$ and ### $p < 0.0001$.

Few studies are describing the potential effects of *L. nobilis* against AD-relevant insults, consistent with our data obtained in the current study. People around the world have also used bay leaves in traditional and complementary medicine practices for hundreds of years. It has been demonstrated that bay leaf burning offers a range of health benefits. Anxiety relief is touted as a major benefit of bay leaf burning. This is probably because bay leaf smoke contains linalool, a compound found in several other plants.

Pacifico et al. demonstrated that *L. nobilis* leaf extracts have neuroprotective potential and anti-amyloidogenic efficacy. It has been reported that the chloroform fraction of *L. nobilis* was able to protect against cerebral ischemia neuronal damage (Cho et al., 2010). Correspondingly, the apolar *L. nobilis* leaf extracts exhibited neuroprotective action toward three nervous system cell lines (Pacifico et al., 2013; 2014). Moreover, some of the major compounds identified in the

chemical composition of our *L. nobilis* used samples, supported its cognitive-enhancing profile. Lee et al. demonstrated the neuroprotective potentials of α-pinene against Sco-induced learning and memory impairment in C57BL/6 mice. Moreover, the authors reported that α-pinene significantly increased the spontaneous alternation percentage in the Y-maze test, enhanced spatial recognition in the Morris water-maze test by reducing the escape latency, and increased step- through-latency in the passive avoidance test in the Sco-induced model. Recently, our group demonstrated that an essential oil mix containing β-pinene (1.76%), α-pinene (1.01%), linalool (0.55%), and cymene (0.53%) produced an improving effect on the consolidation of NOR memory (Boiangiu et al., 2020). Goto et al. demonstrated a significant improvement of the cognitive function of elderly people following 1,8-cineole exposure. 1,8-cineole was previously proven to be a good anti-inflammatory and antinociceptive agent in mice models, whereas its isomer 1,4-cineole demonstrated good anxiolytic effects (Gomes et al., 2010). Interestingly, the main oxygenated compound identified in our sample, methyl dihydrojasmonate (syn. hedione) promotes neuronal health as indicated by Pavan et al. Moreover, this compound proved to be efficient in neurodegenerative diseases associated with pigmentation problems due to its binding to the vomeronasal type-1 receptor 1 (VN1R1) found in the amygdala and hippocampus. Furthermore, olivetol is recognized as a diphenol cannabinoid compound with antioxidant and anticholinergic properties (Taslimi and Gulçin, 2018). In line with these results, our *in vivo* findings confirm the ability of the BL to usefully modulate and enhance cholinergic neuronal transmission and cognitive performance under dementia-related conditions.

For the memory and the cognitive functions, the cholinergic transmission plays a significant role. The enzyme AChE is responsible for the degradation of ACh into acetate and choline and decreases neurotransmitter levels in the brain, as can be noticed in the cholinergic dysfunction of AD (Dunant et al., 2017). Acetylcholinesterase inhibitors (AChEIs) increase the amount of ACh, improving memory functions. People with AD are commonly treated with precognitive medicines. However, the hepatotoxicity and the side effects arising from the activation of the cholinergic system limit the use of AChEIs (Kowalczyk et al., 2020; Deng et al., 2019). The effect of BL on the AChE activity in the rat hippocampus caused by Sco is shown in Figure 38A. The activity of AChE significantly increased ($p < 0.001$) in the Sco-treated group as compared to the control group. However, the activity of AChE in the administration of the BL significantly decreased ($p < 0.0001$) compared to the Sco-treated group. DP and BL exhibited anti-AChE activities. Our results demonstrated the high anti-AChE activity of the BL. These outcomes are in line with few past investigations which reported the anti-AChE activity of the *Laurus* extract. Gazwi et al. demonstrated that *Laurus* leaf extract significantly restored AChE of the brain in lead-treated rats, proposing that *Laurus* extract could preserve living organisms against neurotoxicity by reversing the AChE imbalance caused by lead. This anti-AChE activity of the extract could be attributed to its phenolic and flavonoids contents and its antioxidant activity. Ferreira et al. showed that *L. nobilis* extract exhibited high AChE inhibitory activity due to the presence of flavonoids. Our data

suggest that the memory enhancement effects of BL in Sco-induced amnesic rats could be attributed to the inhibition of the AChE activity and restored of the cholinergic system activity.

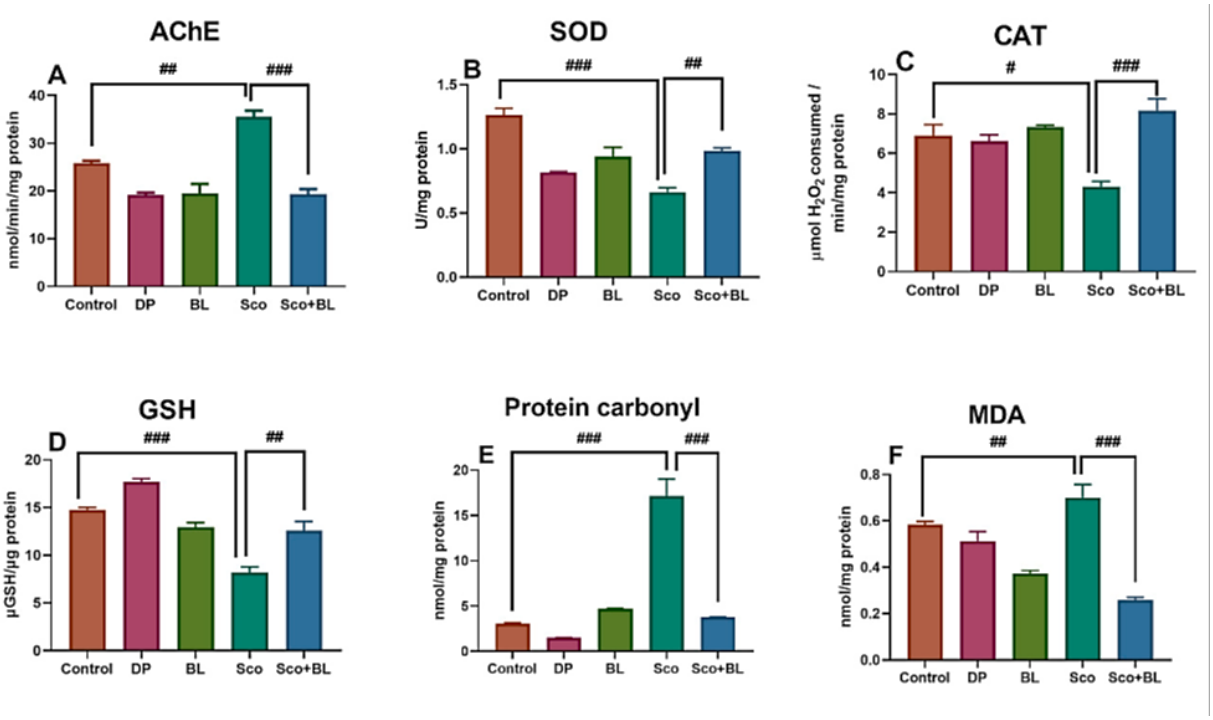

Fig. 38. Effects of the bay leaf incense (BL) on (A) AChE; (B) superoxide dismutase (SOD); (C) glutathione peroxidase (GPX) specific activities; (D) reduced glutathione (GSH); (E) protein carbonyl and (F) malondialdehyde (MDA) level.

Values represent means ± S.E.M. (n = 5) followed by Tukey's post hoc analyses: (A) Control vs. Sco: ## $p < 0.001$, Sco vs. Sco + BL: ### $p < 0.0001$; (B) Control vs. Sco: ### $p < 0.0001$, Sco vs. Sco + BL: ## $p < 0.001$; (C) Control vs. Sco: # $p < 0.01$, Sco vs. Sco + BL: ### $p < 0.0001$; (D) Control vs. Sco: ### $p < 0.0001$, Sco vs. Sco + BL: ## $p < 0.001$; (E) Control vs. Sco: ### $p < 0.0001$, Sco vs. Sco + BL: ### $p < 0.0001$; (F) Control vs. Sco: ## $p < 0.001$, Sco vs. Sco + BL: ### $p < 0.0001$.

Sco exhibited pro-oxidant activity, as evidenced by suppressed activity of SOD ($p < 0.001$) (Figure 38B), CAT ($p < 0.01$) (Figure 38C), the total content of reduced GSH ($p < 0.0001$) (Figure 38D), along with increased levels of protein carbonyl ($p < 0.0001$) (Figure 38E), and MDA ($p < 0.001$) (Figure 38F). As an antioxidant agent, BL exposure restored the antioxidant enzyme activity and decreased the levels of protein carbonyl and lipid peroxidation as compared to Sco-treated animals. Oxidative stress is one of the pathways responsible for Sco-induced amnesia. The pro-oxidative effects of Sco have been documented as it decreases the activity of antioxidant enzymes such as SOD, CAT, and GPX (Uma et al., 2014; Capatina et al., 2020) and increased the concentration of malondialdehyde (MDA), which is the main marker of lipids peroxidation (Capatina et al., 2020; Abd-El-Fattah et al., 2014). Also, numerous studies have shown the pro-

cognitive impact of antioxidant compounds on Sco-induced memory damage, possibly by attenuating oxidative stress markers (Boiangiu et al., 2020; Kowalczyk et al., 2020). As demonstrated by a substantial increase in SOD and CAT specific activities and the total content of reduced GSH, along with a decrease in protein carbonyl and MDA levels, BL significantly restored the antioxidant status in the brain of rats. The current results are supported by different studies that demonstrated the antioxidant effects of *Laurus extract*.

To determine the potential association between memory, antioxidant enzymes and lipid peroxidation, the Pearson correlation analysis was used (Figure 39). In this way, a significant negative correlation between the spontaneous alternation% vs. MDA ($n = 5$, $r = -0.703$, $p < 0.01$) (Figure 39A) and CAT vs. MDA ($n = 5$, $r = -0.776$, $p < 0.0001$) (Figure 39C) was noticed, suggesting that memory improvement in the Y-maze test is well corelated with a low level of MDA. Also, a decreased activity of AChE is positively correlated with a low level of MDA ($n = 5$, $r = 0.772$, $p < 0.0001$) (Figure 39B). A correlation between the AChE inhibitory action of the ethanolic extract of *Laurus* leaf and its antioxidants ability in rats was shown by Gazwi et al. By using Pearson's test, the improvement of spatial memory in behavioral approaches is significantly corelated with the decrease of AChE activity and lipid peroxidation level following BL exposure.

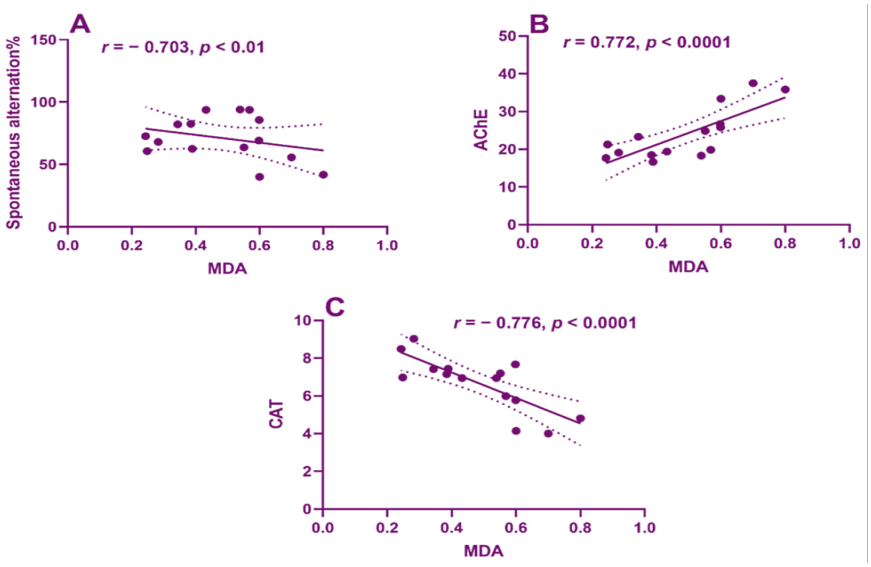

Fig. 39. Statistical Pearson's correlation analyses of behavioral scores and oxidative stress markers (n = 5).

Values expressed are spontaneous alternation%, AChE (nmol/min/mg protein), CAT (U/mg protein), and MDA (nmol/mg protein). (**A**) Spontaneous alternation% vs. MDA ($n = 5$, $r = -0.703$, $p < 0.01$); (**B**) AChE vs. MDA ($n = 5$, $r = 0.772$, $p < 0.0001$); (**C**) CAT vs. MDA ($n = 5$, $r = -0.776$, $p < 0.0001$).

Overall, the exposure to BL may improve memory formation. At the same time, it can effectively diminish the cognitive deficits induced by Sco in the rat brain. The mechanism of the

observed effects can be explained by decreasing in the AChE activity followed by increased level of antioxidant enzymes changed as a consequence of Sco administration. These new findings provide pharmacological and biochemical support for the development of the potential of BL in cognitive deficits.

In a similar manner, exposure to fennel and coriander volatile fraction improves memory and cognition, acting also as anxiolytic and antidepressive. The anxiolytic-and antidepressant-like effects of the fennel essential oil were studied by means of *in vivo* (elevated plus-maze and forced swimming tests) approaches. The beta-amyloid (1-42)-treated rats exhibited the following: decrease of the exploratory activity, the percentage of the time spent and the number of entries in the open arm within elevated plus-maze test and decrease of swimming time and increase of immobility time within forced swimming test.

Inhalation of the fennel essential oil significantly exhibited anxiolytic- and antidepressant-like effects. In the elevated plus-maze task (Fig. 40a) ANOVA revealed a significant overall difference between all groups ($F(4,45) = 11.01$, $p < 0.0001$) on the percentage of the time spent in the open arms. Both doses of the fennel essential oil (1% and 3%), but especially 3%, significantly increased the percentage of the time spent in the open arms in Aβ(1-42)-treated groups as compared to Aβ(1-42) alone-treated group.

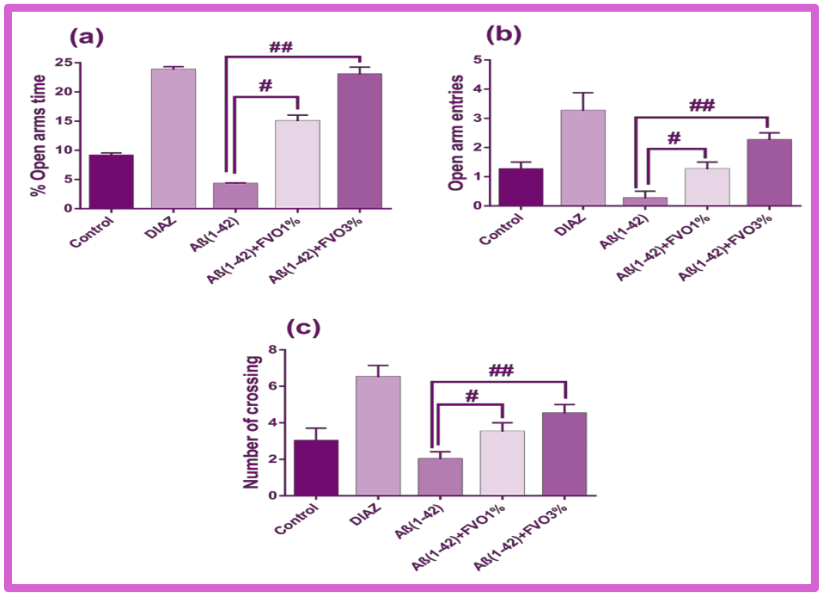

Fig. 40. Effects of inhaled *Foeniculum vulgare* volatile oil (1% and 3%) in the elevated plus-maze test on the percentage of the time spent in the open arms (a), the number of open-arm entries (b) and the number of crossing (c) in the Aβ (1-42)-treated rats.

Values are means ± S.E.M. (n = 10 animals per group). For Turkey's *post hoc* analysis - $^{\#}$Aβ (1-42) vs. Aβ (1-42)+FOV1%: $p<0.0001$ and $^{\#\#}$Aβ (1-42) vs. Aβ (1-42)+ FOV3%: $p<0.0001$ (a), $^{\#}$Aβ (1-42) vs. Aβ (1-42)+ FOV1%: $p<0.001$ and $^{\#\#}$Aβ (1-42) vs. Aβ (1-42)+ FOV3%: $p<0.0001$ (b) and $^{\#}$Aβ (1-42) vs. Aβ (1-42)+ FOV1%: $p<0.001$ and $^{\#\#}$Aβ (1-42) vs. Aβ (1-42)+ FOV3%: $p<0.0001$ (c).

In Fig. 40b ANOVA revealed a significant overall difference between all groups ($F(4,45) = 10.06$, $p < 0.0001$) on the number of open-arm entries. The inhalation of the fennel essential oil (1% and 3%), but especially 3%, significantly increased on the number of open-arm entries of Aβ(1-42)-treated groups as compared to Aβ(1-42) alone-treated group. Significant overall differences between all groups ($F(4,45) = 9.23$, $p < 0.0001$) on the number of crossing (exploratory activity) are shown in Fig. 40c. The inhalation of the fennel essential oil (1% and 3%), but especially 3%, significantly increased the exploratory activity of Aβ(1-42)-treated groups as compared to Aβ(1-42) alone-treated group.

The elevated plus-maze is recognized as a valuable model able to predict anxiolytic- or anxiogenic-like effects of drugs in rodents (Blainski et al., 2010). The percentage of the time spent in the open arms and the number of open-arm entries of Aβ(1-42)-treated rats was significantly decreased (Fig. 40 a and b). This indicates that the Aβ(1-42)-treated rats experienced high levels of anxiety and were suitable for evaluating the presumed anxiolytic substances as our essential oil (Hayashi et al., 2012). Furthermore, after the Aβ(1-42)-treated rats being exposed to *F. vulgare* essential oil, the per-centage of time spent in the open arms significantly increased in a dose-dependent manner as compared to Aβ(1-42)-alone treated rats. Additionally, the number of open arms entries and number of crossing (exploratory activity) (Fig. 2c) increased in the Aβ(1-42)-treated rats exposed to *F. vulgare* essential oil (3%). As expected, diazepam (DZP) as a benzodiazepine drug used as positive control produced significant increase in the percent-age of time spent in the open arms, the number of open-arm entries and the number of crossings as compared to Aβ(1-42)-alone treated rats. These data are consistent with the results of numerous previous studies, which have shown that DZP and other benzodiazepines produce significant anxiolytic effects in a variety of anxiolytic screening procedures, including elevated plus-maze test procedures (Adebesin et al., 2015; Leggio et al., 2015). The pharmacological action of diazepam enhances the effect of the neurotransmitter GABA by binding to the benzodiazepine site on the GABA-A receptor (via the constituent chlorine atom) leading to central nervous system (CNS) depression (Riss et al., 2008). The anxiety indicators in the elevated plus-maze (the percentage of the time spent in the open arms and the number of open-arm entries) showed up being sensitive to the agents which were thought to act via the GABA-A receptor complex (Emamghoreishi et al., 2005). Moreover, it has been reported that trans-anethole display a potent anxiolytic activity in mice (Miyagawa et al., 2014). In light with these reports, our high trans-anethole (58.135%) containing *F. vulgare* essential oil has increased anxiolytic-like behavior and anti-depressive-like response in Aβ(1-42)-treated rats (Cioanca et al., 2016).

The forced swimming test has been validated as a suitable tool for predicting the antidepressant properties of drugs (Cioanca et al., 2014). When rodents are forced to swim in a confined space, after an initial period of struggling, they would become immobile, resembling a state of despair and mental depression. This inescapable stressful situation can be evaluated by assessing different behavioral strategies (Porsolt et al., 1977). As shown in Fig. 41a and b, the

swimming time decreased and the immobility time increased in Aβ(1-42) alone-treated rats as compared to control rats. This indicates that the Aβ(1-42) alone-treated rats exhibited depression.

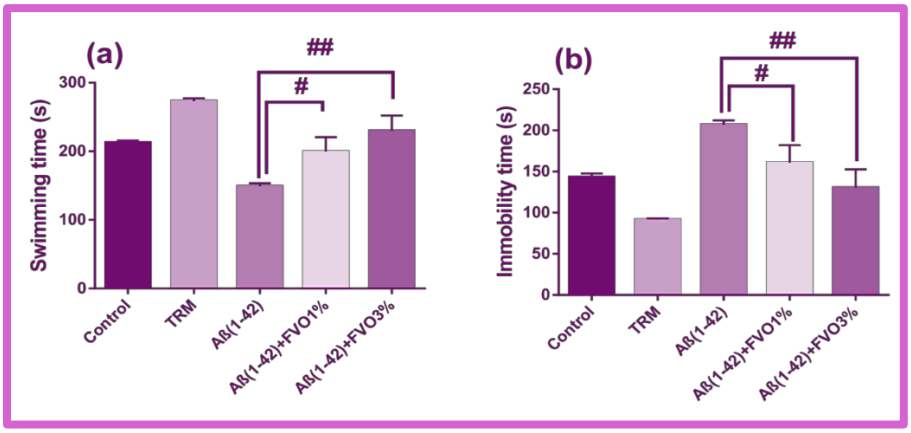

Fig. 41. Effects of inhaled *Foeniculum vulgare* volatile oil (1% and 3%) on swimming time (a) and immobility time (b) in the Aβ (1-42)-treated rats during the 6 min period in the forced swimming test.

Values are means ± S.E.M. (n = 10 animals per group). For Turkey's *post hoc* analysis - $^{\#}$Aβ (1-42) vs. Aβ (1-42)+FOV1%: $p<0.001$ and $^{\#\#}$Aβ (1-42) vs. Aβ (1-42)+ FOV3%: $p<0.001$ (a) and $^{\#}$Aβ (1-42) vs. Aβ (1-42)+FOV1%: $p<0.001$ and $^{\#\#}$Aβ (1-42) vs. Aβ (1-42)+ FOV3%: $p<0.0001$ (b)

After being exposed to both doses of *F. vulgare* essential oil (1% and 3%), the swimming time significantly increased in a dose-dependent manner. Moreover, the decrease of the immobility time in a dose-dependent manner was also observed. These results suggested that *F. vulgare* essential oil possesses a strong antidepressant-like response to an inescapable stress. In our study, tramadol (TRM), as positive control, produced significant increases in the swimming time and decreases the immobility time as compared to Aβ(1-42)-alone treated rats. Tramadol is a unique drug with multiple modes of action. It is a weak agonist of the μ-opioid receptor but it also inhibits the reuptake of serotonin as well as norepinephrine. It is an analgesic and it is also considered as an antidepressant (Caspani et al., 2014; Cioanca et al., 2016).

Our results suggest that the fennel essential oil inhalation ameliorates beta-amyloid (1-42)-induced anxiety and depression in laboratory rats. Thus, the results of the present study indicate that the fennel essential oil may have potential clinical applications in the management of anxiety and depression related to AD conditions (Cioanca et al., 2016).

Returning to one of the most known medicinal plants, chamomile or *Matricaria chamomilla*, we obtained a standardized extract for which we investigated the safety and the impact on a scopolamine-induced memory impairment in rats. Our findings showed no sign of toxicity. The results evidenced that treatment with scopolamine impaired memory processes in laboratory rats. Various behavioral and molecular experiments were conducted and revealed decreased

memory performance, a changed in oxidant/antioxidant balance along with an altered BDNF and IL-1β expression in the rat hippocampus.

Results from the Y-maze and radial arm maze tasks showed that scopolamine-treated rats displayed decreased scores during training sessions (Fig. 42). However, co-treatment with the extract in both doses prevented scopolamine-induced memory deficits as evidenced by increased spontaneous behavior in Y-maze test along with decreased of working memory errors and reference memory errors by performing radial arm maze task.

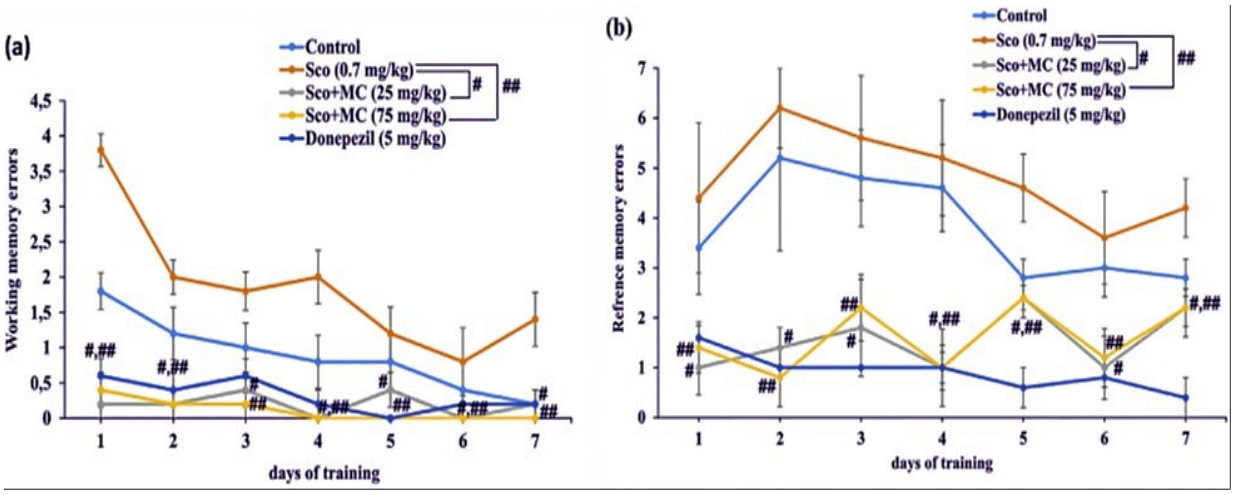

Fig. 42. Effects of the hydroalcoholic extract from *Matricaria chamomilla* aerial parts (25 and 75 mg/kg) administration on the working memory errors (a) and the reference memory errors (b) during 7 days training in the radial arm maze in the scopolamine-treated rats. Values are means ± S.E.M. (n=5 animals per group).

For Tukey's post hoc analyses - #Sco vs. Sco+MC (25 mg/kg): $p<0.001$ and ##Sco vs. Sco+MC (75 mg/kg): $p<0.001$ (a) and #Sco vs. Sco+MC (25 mg/kg): $p<0.001$ and ##Sco vs. Sco+MC (75 mg/kg): $p<0.001$ (b).

In the radial arm maze task, the scopolamine-treated rats exhibited a significant increase of both the working memory errors ($p<0.01$, Fig. 42a) and the reference memory errors ($p<0.01$, Fig. 42b) as compared to control group, but this increase was significantly reversed by the administration of the extract. Additionally, repeated-measures ANOVA revealed a significant time difference ($F(6,112)=2.39$, $p<0.01$) and a significant group difference ($F(3,56)=10.89$, $p<0.001$) and a significant time-group interaction ($F(3,112)=32.23$, $p<0.001$) for the working memory errors evaluation (Fig. 42a). Moreover, repeated-measures ANOVA revealed a significant group difference ($F(3,112)=32.02$, $p<0.001$) for the reference memory errors evaluation. Donepezil has effects similar to those of the extract treatment.

These findings suggest that the extract has anti-amnesic effects in the scopolamine-induced model. Furthermore, this evidence is supported by the decrease of the AChE activity in pretreated scopolamine rats with the hydroalcoholic extract, suggesting that the extract could prevent scopolamine-induced cholinergic dysfunction in the hippocampus by modulating the cholinergic activity. The previous study has demonstrated that decreased in hippocampal ACh level due to over-activity of AChE could disrupt the brain cholinergic system activity, resulting in cognitive impairment (Rogers and Kesner, 2004).

HPLC analyses data of the extract identified the presence of eight active compounds such as chlorogenic acid, cafeic acid, catechin, apigenin-7-glucoside, rutin, cynaroside, luteolin and apigenin. We believe that the anti-AChE activity and cognitive-enhancing effects of the extract are induced by the synergic action of the active phenol compounds.

Accumulating evidence suggested that scopolamine-induced memory impairment by an attenuation of the cholinergic neurotransmission that parallels to decrease the anti-oxidative enzyme activity and increase the level of radical (Zhou et al., 2016). The elevation of brain oxidative status after scopolamine administration further substantiates the value of scopolamine-induced amnesia as an animal model to test for drugs with potential therapeutic benefits in dementia (El-Sherbiny et al., 2003).

In our study, scopolamine injection significantly depleted antioxidant capacity of SOD, GPX, CAT and the total content of reduced GSH in the rat hippocampus, whereas these abnormalities were restored significantly by the treatment with the extract. As expected, scopolamine injection induced oxidative stress in the hippocampus, as evidenced by increased levels of the protein carbonyl and MDA. These alterations were significantly attenuated by the extract pretreatment. Increased LDH activity-induced neuronal apoptosis (neurotoxicity) was evidenced in AD associated with aging (Ho et al., 2007). Scopolamine administration induced neurotoxicity in the hippocampus, as observed by enhancing the LDH activity, while treatment with the extract lowered the LDH activity close to normal values. Our results demonstrated that the extract exhibits an antioxidant and neuroprotective potential by contributing to improving the memory performance in the scopolamine rat model.

Supporting evidence suggested that dysfunction of BDNF is a possible contributor to the pathology and symptoms of the AD (Qin et al., 2017). It has been suggested that scopolamine-induced suppression of the expression of the BDNF, nerve growth factor (NGF) and their receptors in the hippocampus (Lee et al., 2016), resulted in alteration of memory function in mice. In our study, scopolamine suppressed BDNF mRNA which is similar to the results of the previous studies. As expected, treatment of scopolamine rats with the extract reversed the BDNF mRNA copy number close to normal conditions (Fig. 43).

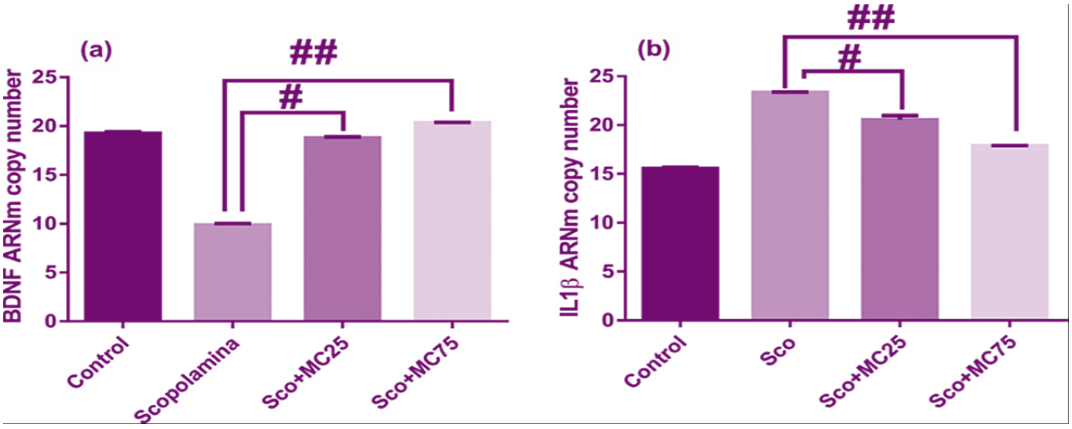

Fig. 43. BDNF mRNA copy number (a) and IL1β mRNA copy number (b) in the scopolamine groups pretreated with the hydroalcoholic extract from *Matricaria chamomilla* aerial parts (25 and 75 mg/kg). Values are means ± S.E.M. (n=5 animals per group).

For Tukey's post hoc analyses - #Sco vs. Sco+MC (25 mg/kg): $p<0.0001$ and ##Sco vs. Sco+MC (75 mg/kg): $p<0.0001$ (a) and #Sco vs. Sco+MC (25 mg/kg): $p<0.001$ and ##Sco vs. Sco+MC (75 mg/kg): $p<0.0001$ (b).

These results indicate that the cognitive-enhancing effects of the extract may be associated with the activation of BDNF gene. Inflammation, as well as cholinergic neuron degeneration, may play a critical role in the pathogenesis of the degenerative changes and cognitive impairments of the AD. Previous data have evidenced that scopolamine-induced a strong inflammatory response such as the hyperexpression of proinflammatory cytokines IL-1β and IL-6 and astrocyte activation in the mice hippocampus (Xu et al., 2016). In our study, hippocampal IL-1β mRNA copy number was increased by scopolamine injection, which was decreased by treatment with the extract, suggesting powerful antineuroinflammatory potential.

Results from Pearson correlation indicated that enhancing of memory in specific behavioral tasks are related to decreasing of oxidative stress damage and cholinesterase activity in the hippocampal tissue of treated scopolamine rats with the extract. All the obtained data supports that the chamomile extract improved the memory deficits induced by scopolamine through modulation of AChE activity, and increasing of BDNF along with decreasing of IL1β expression in the rat hippocampus. Therefore, our extract may be a promising natural therapeutic drug for the prevention of amnesia and aging-/neurodegenerative disease-related cognitive impairment.

Taking into account the benefic effects of antioxidants and the increased ROS in the brain during aging and dementia, we investigated the restorative potential of *Piper nigrum* extract treatment on an Aβ(1–42)-induced Alzheimer rat model. The protective activity was assessed in the amygdala using superoxide dismutase, glutathione peroxidase and catalase specific activities, the total content of the reduced glutathione, protein carbonyl and malondialdehyde levels. Statistical analyses were performed using one-way analysis of variance (ANOVA). Significant

differences were determined by Tukey's post hoc test. F values for which $p < 0.05$ were regarded as statistically significant. Pearson's correlation coefficient and regression analysis were used in order to evaluate the connection between behavioral measures, the antioxidant defence and lipid peroxidation (Hritcu et al., 2015).

As shown in Fig. 44b, the low dose of the methanolic extract (50 mg/kg) in Aβ(1–42)-treated rats significantly improved spatial working memory, as evidenced by the increase in spontaneous alternation percentage compared to Aβ(1–42)-treated rats. Additionally, significant differences were observed between both doses of the methanolic extract on spatial working memory in the Y-maze task. The improvement in spatial working memory within a Y-maze task cannot be attributed to the locomotor activity, because no changes in the number of entries of the groups treated with the methanolic extract were observed (Fig. 44a).

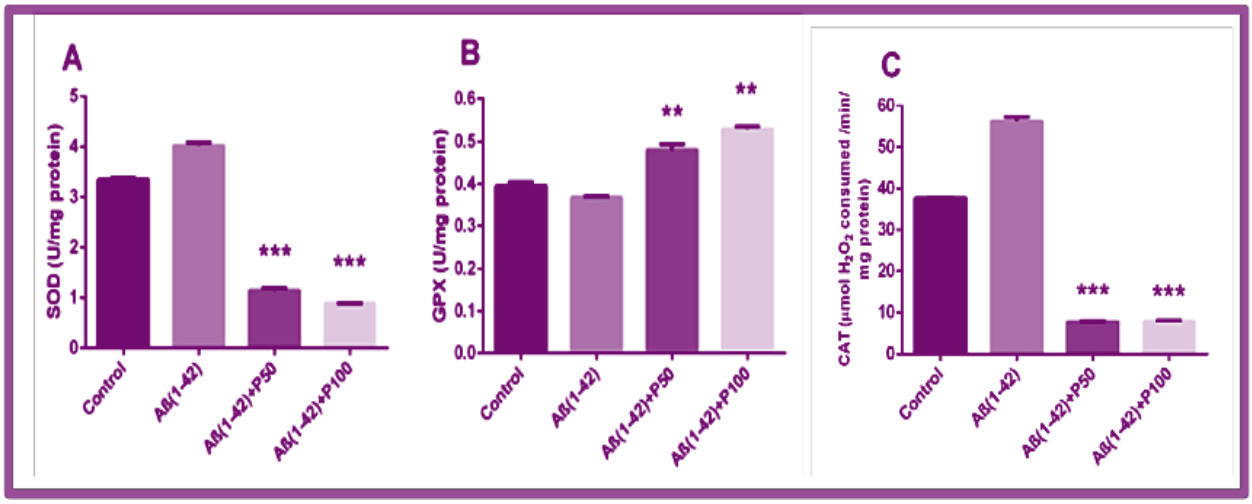

Fig. 44. The activity of the antioxidant enzymes (a – SOD; b – GPX; c – CAT) in the brain is restored by the administration of *Piper nigrum* extracts

Both doses of the methanolic extract (50 and 100 mg/kg), but especially the high dose (100 mg/kg), significantly improved long-term memory of Aβ(1–42)-treated rats, explored by reference memory (Fig. 44d) in radial arm-maze task. These findings could suggest that the methanolic extract plays an important role in spatial memory formation, especially on working and reference memory.

Evidence suggested that oxidative stresses are involved in the mechanism of Aβ-induced neurotoxicity and AD pathogenesis (Jhoo et al. 2004). In addition, exposure to Aβ increased lipid peroxidation, protein oxidation, and the formation of hydrogen peroxide in cultured cells (Behlet al. 1994). Similarly, increases in lipid peroxidation, protein carbonyl, and oxidation of mitochondrial DNA have been observed in the brains of patients with AD (Lyraset al. 1997).

In our study, Aβ(1–42)-treated rats exhibited an increase in SOD- and CAT-specific activities, elevated protein carbonyl and MDA levels, and a decrease of the total content of reduced GSH- and GPX-specific activity in the hippocampal homogenates. The increases in the SOD- and CAT-specific activities appeared to parallel increases in protein carbonyl and MDA levels in the hippocampal homogenates suggesting that these events are needed to scavenge superoxide radicals induced by Aβ(1–42). Protein oxidation is an important factor in ageing and age-related neurodegenerative disorders (Stadtman, 1992).

Protein oxidation is most often indexed by the presence of protein carbonyls (Stadtman 1992), which arise from a direct free radical attack on vulnerable amino acids side chains or the products of glycation, glycoxidation, and lipid peroxidation reactions with protein. The increased protein carbonyl in the AD brain, with the altered activities of antioxidant enzymes (in some cases due to oxidation) coupled with studies showing that Aβ(1–42)- induced neuronal protein oxidation can be inhibited by antioxidants (Yatin et al. 1999), suggests that Aβ-induced protein oxidation accounts, in part, for neurodegeneration in the AD brain. MDA is the most abundant individual aldehyde resulting from lipid peroxidation and can be considered a marker of lipid peroxidation. Consistently, both doses of methanolic extract (50 and 100 mg/kg), but especially 100 mg/kg, restored the activities of SOD (Fig. 44a) and CAT (Fig. 44c) and increased GPX (Fig. 44b) activity in the hippocampal homogenates of Aβ(1–42)-treated rats.

As expected for the antioxidant agents, both the doses of the methanolic extract (50 and 100 mg/kg) decreased the protein carbonyl (Fig. 44e) and MDA (Fig. 44f) levels along with the increase in the total content of reduced GSH (Fig. 40d) in the hippocampal homogenates. Moreover, we found a significant correlation between spontaneous alternation percentage versus MDA, working memory errors versus MDA, reference memory errors versus MDA, SOD versus MDA, GPX versus MDA, CAT versus MDA, and protein carbonyl versus MDA when linear regression was determined.

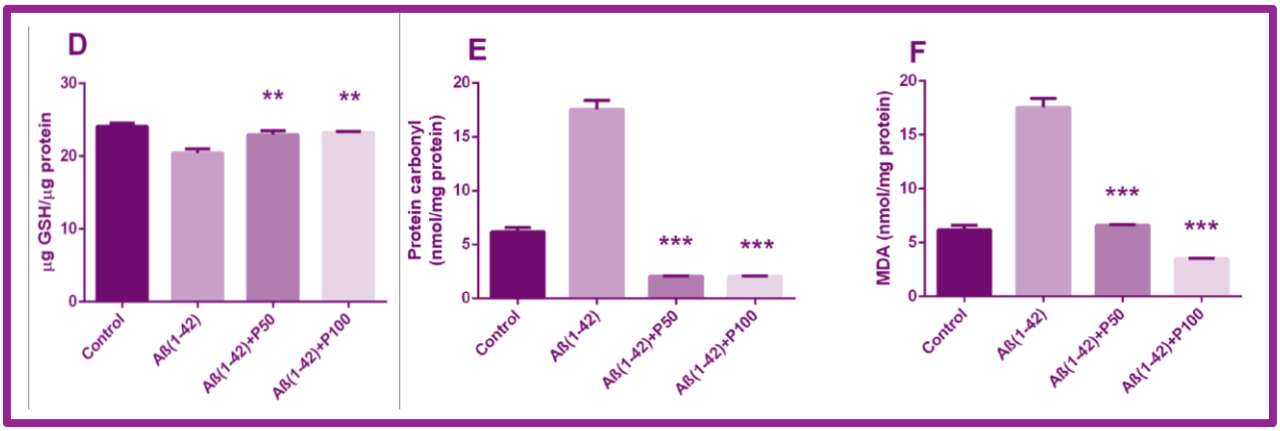

Fig. 44. Effect of *Piper nigrum* extract on the total content of reduced GSH (d), Protein carbonyl (e), and MDA levels (f)

These results suggest that an increase in behavioural parameters in Y-maze and radial arm-maze tasks and the antioxidant defence along with the decrease of lipid peroxidation and protein oxidation is correlated with the involvement of the methanolic extract in neuroprotection against Aβ (1–42)-induced oxidative stress generation in the rat hippocampus. Also, we reported the absence of DNA cleavage patterns in the Aβ (1–42)-treated rats treated with the methanolic extract, suggesting that the methanolic extract possesses neuroprotective and anti-apoptotic activities. In our study, DNA cleavage patterns were absent in the methanolic extract groups (Fig. 45), suggesting that the methanolic extract of *P. nigrum* fruits protects against neurotoxicity and this effect could be related to its antioxidant activity.

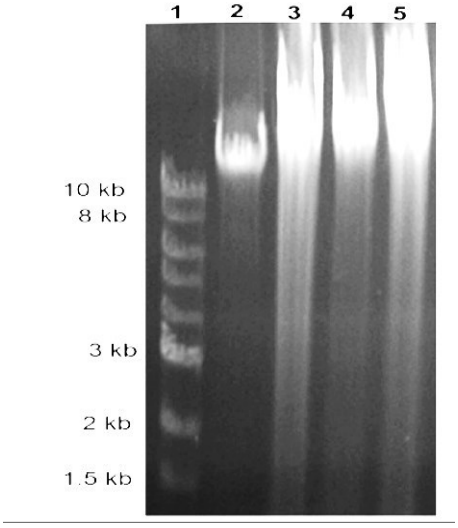

Fig. 45. Effects of the methanolic extract of *Piper nigrum* fruits (50 and 100 mg/kg) on DNA fragmentation by agarose (1.5%) gel electrophoresis

Lane 1 DNA ladder; the lane 2 control group; lane 3 Aβ (1–42)-treated group; lane 4 Aβ(1–42)+P50 group; and lane 5Aβ(1–42)+P100 group

All in all, the treatment with *Piper nigrum* extract protected the DNA structure from alteration at both administered doses. This result along will all other data proves that *Piper nigrum* extract could represent an important source of neuroprotective compounds.

As I mentioned previously, along with the AD model in rats, our research group used also PD models induced by 6-hydroxydopamine (6-OHDA) administration. Also, *Danior rerio* or zebrafish was another species that is commonly used today for *in vivo* testing. In such a model, a zebrafish 6-OHDA-induced PD model, we recently investigated the aqueous extract from *Ceratonia siliqua* (CsAE) leaves. The vegetal product is an exotic species that was collected in Morocco. Therefore, we extended our collaboration through Eugen Ionescu scholarship program to Laboratoire de Biochimie et Génétique Moléculaire, Faculté des Sciences et Techniques, Université Abdelmalek Essaadi, Tanger, Morocco.

In the testing, CsAE (0.1, 0.3, and 1 mg/L) was administered by immersion to zebrafish for eight consecutive days and one hour before each behavioral test of each day, while 6-OHDA (250 µM) treatment was supplied one day before the novel tank diving test (NTT). The memory performance was evaluated through the NTT and Y-maze tests. Additionally, the *in vitro* and *in vivo* antioxidant status and acetylcholinesterase (AChE) activity was also assessed.

Figure 46 shows the results of 6-OHDA (250 µM) and CsAE (0.1, 0.3, and 1 mg/L) treatment of anxiety-like behavior within the NTT test. Representative locomotion tracking pattern (Figure 46A) highlighted the discrepancies between the top and bottom areas in swimming traces. It revealed that 6-OHDA-treated zebrafish exhibited a preference for the bottom zone, indicating an anxiogenic profile.

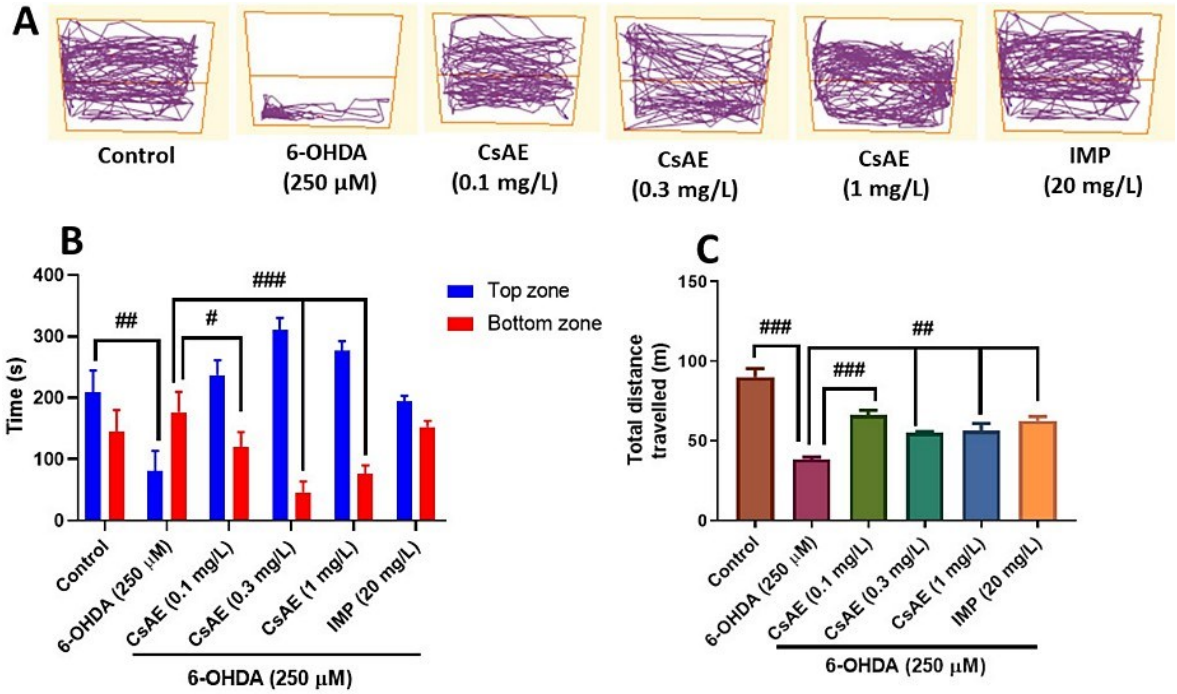

Fig. 46. Ceratonia siliqua aqueous extract (CsAE, 0.1, 0.3, and 1 mg/L) ameliorated locomotion pattern and reduced anxiety in the NTT test. (**A**) Representative locomotion tracking pattern of the control, 6-hydroxydopamine (6-OHDA) (250 µM), CsAE (0.1, 0.3, and 1 mg/L), and imipramine (IMP) (20 mg/L) treated groups. (**B**) Represent the time spent in the top/bottom zone by zebrafish in the tank in different groups. (**C**) Represent the total distance travelled by zebrafish in the tank in different groups.

Values are means _ S.E.M. (n = 10). For Tukey's post hoc analyses: (**B**) Control vs. 6-OHDA (250 µM): ##p < 0.001, 6-OHDA (250 µM) vs. CsAE (0.1 mg/L): # p < 0.01, 6-OHDA (250 µM) vs. CsAE (0.3 mg/L): ### p < 0.0001 and 6-OHDA (250 µM) vs. CsAE (1 mg/L): ### p < 0.0001; (**C**) Control vs. 6-OHDA (250 µM): ### p < 0.0001, 6-OHDA (250 µM) vs. CsAE (0.1 mg/L): ###p < 0.0001, 6-OHDA (250 µM) vs. CsAE (0.3 mg/L): ## p < 0.001 and 6-OHDA (250 µM) vs. CsAE (1 mg/L): ## p < 0.001.

Moreover, the 6-OHDA treatment increased the time spent in the bottom zone ($p < 0.01$), as well as decreased the time spent in the top zone ($p < 0.001$) as compared to the control group (Figure 46B). Reducing the time spent in the top zone of the tank suggests the anxiogenic-like profile of 6-OHDA. By decreasing the total distance traveled in the tank, 6-OHDA treatment created a hypolocomotor effect (Figure 46C) compared to control. By comparison, the time spent in the top zone of the tank (Figure 46B) indicates the anxiolytic-like result of CsAE, particularly at the dose of 0.3 mg/L. Besides, CsAE treatment avoids 6-OHDA anxiogenic effect as shown by decreasing of the time spent in the bottom zone of the tank ($p < 0.01$ for 0.1 mg/L and $p < 0.0001$ for 0.3 and 1 mg/L) (Figure 46B) and by increasing of the total distance travelled ($p < 0.0001$ for 0.1 mg/L and $p < 0.001$ for 0.3 and 1 mg/L) (Figure 46C) relative to 6-OHDA alone-treated zebrafish. Moreover, 6-OHDA alone-treated zebrafish exhibited a decreased total distance travelled in the tank as compared to the control group ($p < 0.0001$), suggesting anxiogenic effects) (Figure 46C). IMP (20 mg/L) used as the positive reference drug exhibited an anxiolytic profile, as evidenced by decreasing the time spent in the bottom zone of the tank and by increasing the total distance travelled in the 6-OHDA-treated zebrafish.

On the other hand, figure 5 illustrates the e_ects of 6-OHDA (250µM) and CsAE (0.1, 0.3, and 1 mg/L) treatment on the Y-maze spatial memory. Representative locomotion tracking pattern (Figure 47A) illustrates the differences between the Y-maze arms in swimming traces and shows that 6-OHDA treated group traveled less distance in the Y-maze, suggesting hypolocomotion. Moreover, the administration of 6-OHDA induced memory deficits as evidenced by decreased the percentage of spontaneous alternation ($p < 0.0001$) (Figure 47B) as compared to the control group. Administration of CsAE significantly counters the 6-OHDA action induced-memory impairment, as evidenced by increased the percentage of spontaneous alternation in a dose-dependent manner. Reducing the percentage of spontaneous alternation suggests the memory impairment effect of 6-OHDA.

Furthermore, 6-OHDA administration affects locomotion, as illustrated by decreased number of arm entries ($p<0.001$) (Figure 47C) and a reduced of the total distance ($p<0.0001$) (Figure 47D) as compared to the control group. By contrast, the administration of CsAE in the 6-OHDA fish significantly improved locomotion, and increased total distance travelled in the Y-maze test. Our findings suggested that CsAE displayed anxiolytic and cognitive-enhancing effects, which could be attributed to the presence of the bioactive compounds belonging predominantly to flavonoids. Our results are in line with those reported by Alzoubi et al., who demonstrated that the methanolic extract from *C. siliqua* prevented short-term memory deficit induced by chronic stress in rats, probably as a result of avoiding reduction in the brain-derived neurotrophic factor (BDNF) levelsin the hippocampus. Moreover, the CsAE exhibited an anxiolytic-like effect and prevented emotional behavior impairment and metabolic disorders induced by estrogen deficiency in rats (Azoubi et al., 2018; Ammari et al., 2020).

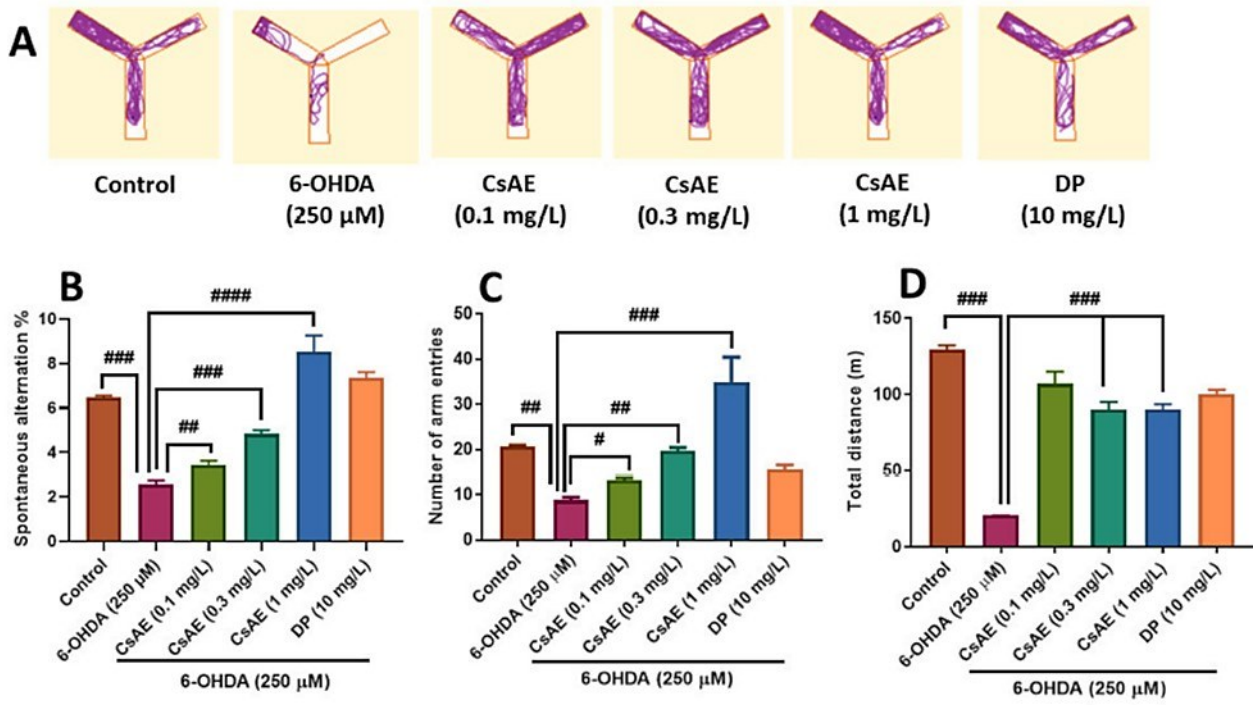

Fig. 47. *Ceratonia siliqua* aqueous extract (CsAE, 0.1, 0.3, and 1 mg/L) improved locomotion pattern and memory in the Y-maze test. (**A**) Representative locomotion tracking of the control, 6-OHDA (250 µM) CsAE (0.1, 0.3, and 1 mg/L), and donepezil (DP) (10 mg/L) treated groups. (**B**) Represent the percentage of spontaneous alternation in the Y-maze in different groups. (**C**) Represent the number of arm entries in the Y-maze in different groups. (**D**) Represent the total distance travelled by zebrafish in the Y-maze in different groups.

Values are means ±S.E.M. (n = 10). For Tukey's post hoc analyses: (**B**) Control vs. 6-OHDA (250 µM): ###p < 0.0001, 6-OHDA (250 µM) vs. CsAE (0.1 mg/L): ## p < 0.001, 6-OHDA (250 µM) vs. CsAE (0.3 mg/L): ### p < 0.0001 and 6-OHDA (250 µM) vs. CsAE (1 mg/L): #### p < 0.00001; (**C**) Control vs. 6-OHDA (250 µM): ## p < 0.001, 6-OHDA (250 µM) vs. CsAE (0.1 mg/L): # p < 0.01, 6-OHDA (250 µM) vs. CsAE (0.3 mg/L): ## p < 0.001 and 6-OHDA (250 µM) vs. CsAE (1 mg/L): ### p < 0.0001; (**D**) Control vs. 6-OHDA (250 µM): ### p < 0.0001, 6-OHDA (250 µM) vs. CsAE (0.3 mg/L): ### p < 0.0001 and 6-OHDA (250 µM) vs. CsAE (1 mg/L): ### p < 0.0001.

In our study, the 6-OHDA-treated zebrafish showed significantly increased AChE activity in the brain of zebrafish as compared to the control ($p < 0.001$). By contrast, CsAE administration (0.1, 0.3, and 1 mg/L) significantly inhibited AChE in the brain by 6.49 ± 0.63 ($p < 0.001$), 6.18 ± 0.51 ($p < 0.001$), and 4.57 ± 0.49 ($p < 0.0001$) nmol/min/mg protein compared with the 6-OHDA-treated group (Figure 48A).

AChE is an enzyme localized in the nervous system and muscles of vertebrates and humans (Scherer et al., 2010). The significant role of this enzyme is the termination of transmission at the cholinergic synapses (Pegan et al., 2010) by hydrolyzing acetylcholine to choline and acetate (Lopez et al., 2010). The inhibition of AChE could be used to treat AD (Ingkaninan et al., 2003). Moreover, the depletion of acetylcholine is also detected in PD, participating in dementia as a non-motor symptom of this pathology (Bohnen et al., 2010). Interestingly, alterations in the cortical cholinergic pathways can affect cognitive capacities and lead to dementia in PD patients (Chou et al., 2014). Several authors have shown that *C. siliqua*—mainly the leaves—exhibited an excellent ability in vitro to inhibit AChE (custodio et al., 2015). However, no studies were found about inhibition in vivo. In contrast, Uysal et al. showed that CsAE inhibited butyrylcholinesterase (BChE) but not inhibited AChE.

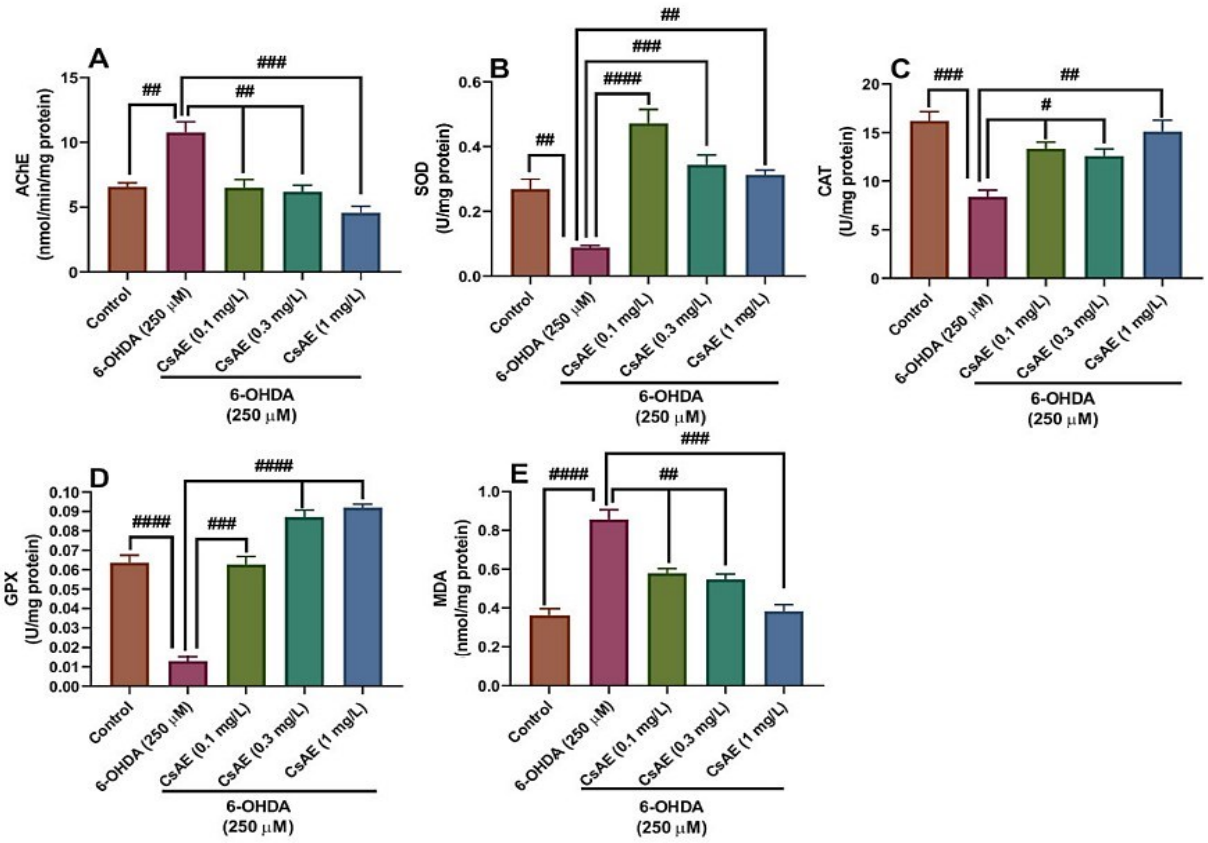

Fig. 48. Ceratonia siliqua aqueous extract (CsAE, 0.1, 0.3, and 1 mg/L) exhibited an anti-AChE effect and improved brain antioxidant status. The enzyme's specific activities: (**A**) Acetylcholinesterase (AChE), (**B**) Superoxide dismutase (SOD), (**C**) Catalase (CAT), and (**D**) Glutathione peroxidase (GPX) and (**E**) Malondialdehyde (MDA) level. Values are means ± S.E.M. (n = 10).

At the same time, the 6-OHDA injection significantly decreased the SOD specific activity ($p < 0.001$) (Figure 48B) in the zebrafish brain as compared to the control group, suggesting facilitation of the oxidative stress. The administration of CsAE in three doses (0.1; 0.3, and 1 mg/L) significantly prevented the decreased SOD activity in the 6-OHDA-treated zebrafish, but 0.1 mg/L remains the best ameliorative dose of SOD ($p < 0.00001$) as compared to 6-OHDA alone-treated zebrafish. Our results also showed a significant decrease in CAT activity following the injection of 6-OHDA as compared to control ($p < 0.0001$) (Figure 48C). The administration of the three doses of CsAE (0.1; 0.3, and 1 mg/L) in the 6-OHDA zebrafish significantly prevented the decrease of the same antioxidant enzyme, especially the dosage of 1 mg/L; however, the levels were inferior to those of control. We also observed that the 6-OHDA injection produced marked decreases in the activity of GPX ($p < 00001$) (Figure 48D) and the three doses of CsAE significantly exhibited a high power to enhance the activity of GPX, especially the doses of 0.3 and 1 mg/L ($p < 0.00001$) in zebrafish brain compared with 6-OHDA-treated zebrafish.

The treatment with the 6-OHDA increases the levels of MDA in the zebrafish brain as compared to control ($p < 0.00001$), and the administration of CsAE inhibits the MDA, especially with 1 mg/L (Figure 48E). The brain is sensitive to oxidative stress, which activates the production of anion superoxide and hydrogen peroxide (Syed et al., 2018). ROS generated leads to neurons loss and, consequently, cognitive impairment observed in PD (Kamden et al., 2013). The antioxidant enzymes such as SOD, CAT, and GPX play a vital role in the human body's defense against oxidative stress (Navabi et al., 2012).

Pearson correlation coefficient (r) was used to test the linear association between memory scores, antioxidant enzymes, and lipid peroxidation. Significant negative correlations between the spontaneous alternation percentage vs. MDA ($n = 10$, $r = -0.730$, $p < 0.0001$) and between the total distance travelled in the tank vs. MDA ($n = 10$, $r = -0.621$, $p < 0.0001$) were observed. The negative value of r indicates that the improvement of memory scores in specific tests such as Y-maze and NTT is well correlated with a decreased level of MDA, a marker of lipid peroxidation. Moreover, strong positive correlation was noticed by linear regression between AChE vs. MDA ($n = 10$, $r = 0.901$, $p < 0.0001$). However, significant negative correlations between CAT vs. MDA ($n = 10$, $r = -0.940$, $p < 0.0001$) and between GPX vs. MDA ($n = 10$, $r = -0.873$, $p < 0.0001$) were reported when linear regression was calculated. In this case, the positive and negative values of the r indicate that decreasing of AChE specific activity as well as increasing of CAT and GPX specific activities is well correlated with a low MDA level (Abidar et al., 2020).

Overall, our findings demonstrated that CsAE presented positive antioxidant and anti-AChE activities, which contributed to the improvement of cognitive function in the 6-OHDA zebrafish PD model.

Conclusions

Our data provide evidence for the link of enriched herbal extracts exposure with antioxidant, anxiolytic, antidepressant and memory enhancing effects in an AD/PD rat/zebrafish model. The observed biologic activities are dose dependent and should be always correlated to the chemical composition of the vegetal extract. Moreover, literature indicates a direct connection between the cognitive decline and anxiety in patients with dementia. Often, depressionand anxiety increase the severity of cognitive impairment for thesepatients (Wuwongse et al., 2010). Therefore, exposure to volatile oils /administration of vegetal extracts might offer a useful alternative or complementary choice in either the prevention or the treatment of psychiatric condition close related to AD/PD conditions. A very important aspect is related to the source of the vegetal material. Our choice was based on our previous experience regarding the chemical and microbial quality of a vegetal product that is to be used for therapy. The organic crops that are the best controlled environment represent the best choices in such cases, the repeatability being assured. For pharmaceutical industry high qualitative crops ensure a good productivity and lower costs for herbal extracts production. Furthermore, the use of phytopharmaceuticals as complementary or alternative medicine, either to prevent or to ameliorate many diseases, still remains a trend for the future generations to come. On the other hand, there are no potentially adverse impacts on the environment and human health.

III. Theory vs. research: current trends and future challenges

The first new research direction is related to the previous obtained results during *in vivo* studies, that allowed us to discover new trends and challenges. The most significant is the link between the observed pharmacological effects and the chemical composition of the administered extract. There is little data available at the moment and the positive results encourage pharmaceutical formulations for human use. However, there is a high necessity to demonstrate the dose-concentration effect and the main effector of the observed results. Therefore, my first investigation trend is the **identification and pharmacokinetics of the natural compounds within biological samples obtained from animal models**, along with the correlation between the administered extract and the identified compounds in the biological samples.

The correct identification will allow better understanding of the metabolism of natural compounds (related to the route of administration), the mechanisms and will give us the justification for a translational study. Metabolomic fingerprinting represent a challenge as well as

necessity for medicine development. For such complex studies I intend to deepen my knowledge and technical skills in mass spectrometry and high-resolution analysis.

Taking into account all presented data and the available scientific literature, there is still much to be investigated in the field of herbal secondary metabolites and their putative effects for prevention and treatment.

Some of the challenges of pharmaceutical formulations of herbal extracts and natural compounds are related to their structural changes, lack of complete solubility in water systems thus influencing the bioavailability and the target reach of these molecules. Targeted treatment is a desired objective of modern medicine since it offers precise therapy and lowered negative impact without affecting the surrounding tissues.

Similarly, I believe that **herbal based nano formulation and multifunctional system formulation with cyclodextrins** is and interesting subject for further consideration and it represents one of my future directions of investigation.

Extracts in nanoparticles

Nanoparticles represent one of the great interest areas in the last decade, with applications in medicine, biology, electronics, etc. One of the methods used for obtaining nanoparticles is represented by the use of plant extracts, which is a cost-effective, non-toxic and eco-friendly method (Corciovă et al., 2018).

Such technologies are usually utilized for an increased pharmacological activity, a better dissolution time or an easier reach of the target cell. Besides, silver, gold or even iron, nanoparticles can be of organic composition (liposomes, micelles, dendrimers, hydrogels) and their use extends from precise diagnosis to packing and delivery of various molecules with biologic activity and effective release to its target with minimal side effects (Falagan-Lotsch et al., 2017).

For plant extracts, the formulation as silver nanoparticles (AgNPs) represents a future trend for better inclusion and bioavailability of partially soluble components. First attempt, on my end, as part of the collaboration with the Department of Drug Analysis, Faculty of Pharmacy, "Grigore T. Popa" University of Medicine and Pharmacy was represented by the obtaining of silver nanoparticles from *Phyllanthus amarus* (Corciovă et al., 2018).

The synthesis of AgNPs was realized by mixing 1 mL plant extract with 10 mL of 5 mM silver nitrate (AgNO3) at 600 rpm for approximately 1 hour at room temperature. AgNPs were separated by centrifugation at 10,000 rpm for 15 minutes. For purification, AgNPs were redispersed in water, followed by centrifugation, and the operation was repeated three times. The obtained AgNPs were dried in the oven and used for characterization. After the first 10 minutes of mixing the extract with silver nitrate, a colour change from yellow to brown was observed which turned

dark-brown within 20 minutes. The mixture colour change is a general feature of the AgNPs synthesis (Ali et al., 2016). The reduction of silver ion was firstly visually monitored and then by UV-Vis spectrophotometry in the 350 - 600 nm range, using a Jasco V 530 double beam UV-Vis spectrophotometer (Corciovă et al., 2018).

The synthesis of AgNPs was monitored by recording the UV-Vis spectra of the extract, the AgNO3 solution, the initial mixture and the colloidal solution after 10, 20, 30 and 60 minutes. On the extract, for AgNPs and initial mixture spectra no peak is observed in the 300 - 500 nm range. The peak can be observed on the colloidal solution spectra of the surface plasmon resonance band at 424 nm, characteristic of AgNPs synthesis after 10 minutes, which demonstrates the start of the reduction process of Ag+. When the reaction time increases, a rise in absorbance can be observed. After 60' the peak remained unchanged, showing that the process was complete. For the determination of hydrodynamic diameter and zeta potential a Delsa Nano Submicron Particle Size Analyzer (Beckman Coulter, USA) was used. The morphology and size of AgNPs were determined using Transmission Electron Microscopy (TEM) with a Hitachi High-Tech HT7700 Transmission Electron Microscope (Japan). Energy dispersive X-ray analysis (EDX) was used to determine the most important elements constituting the chemical composition of AgNPs using an EDX system equipped with a Quanta 200 Environmental Scanning Electron Microscope (ESEM). FTIR analysis was performed with a Bruker Vertex 70 instrument (USA), in the range 4000 cm^{-1} to 310 cm^{-1} (Corciovă et al., 2018).

Cyclodextrins

To achieve improvement of solubility, long-term stability etc, which means an improvement of bioavailability and specificity, we intend to obtain some multifunctional systems able to enclose and to control the release of herbal bioactive substances based on natural and modified cyclodextrins.

Cyclodextrins by their excellent biocompatibility and unique encapsulation ability are particularly attractive for new functional materials engineering and biomedical applications. They have been successfully used to build supramolecular systems and to design new functional materials, with benefits resulting from host-guest interactions between the units of cyclodextrin and bioactive molecules: increased solubility and stability of bioactive substances, better absorption, masking the unpleasant odour and taste, controlling the release profile of the active substance, decreasing local and systemic toxicity of bioactive substances, enhancing the permeability of the substance across biological barriers (Zhang, 2013; Zhou, 2010).

One of the objectives of this area of research is to design, synthesise, characterise and test new therapeutic systems based on cyclodextrins, to obtain supramolecular assemblies with improved biological properties. Such aims can be achieved through synthesis through appropriate, unconventional and clean methods (co-precipitation, lyophilization) of inclusion compounds with some cyclodextrins. Another important step is represented by the study of phase solubility and

dissociation rate by determining the stability constant of the complex and the host/guest ratio in complexation, whereas the development of structure-property relationship by investigation of structural properties of prepared systems may be assessed by different analysis methods such as UV-Vis, FTIR or thermal analysis.

Nevertheless, once obtained, new structure systems need further investigation to confirm improved capabilities of release and dissolution kinetics, as well as the biologic potential by comparison to the parent bioactive substances.

Section II. Academic and professional future development

I intend to continue to develop the personal, teaching and research skills. Therefore, the main coordinates of each direction are given briefly in this section.

The didactic activity will be concretized by:

- teaching courses and conducting practical work sessions/seminars in Pharmacognosy, Cell and molecular biology, Toxic plants, Vegetal product technology, Food supplements and functional foods;
- teaching course modules in postgraduate courses for pharmacists and doctors;
- coordination of diploma papers / dissertations for students in Bchelor and Master degree program in Pharmacy, Cosmetics and Nutrition and dietetics
- teaching within The Complementary Certificate programme in Practical Phytotherapy and Phytopharmacology for medical doctors
- participating as a member in the examination commissions of the undergraduate students,
- participating as a member in commissions for the evaluation of some papers presented during the doctoral internships.

In term of **methodology**, I am going to continually adapt the content of the courses and practical works to the current requirements in the pharmaceutical field and I am going to upgrade my technical skills in accordance to the modern teaching techniques in accordance with the new trends manifested in university pedagogy (use of multimedia equipment, dialogue with the audience). Also, I will try to diversify the topics of practical works and modernize the laboratory activities.

Scientifically, my directions will aim at:

- the continuation of the research directions developed within the postdoctoral studies,

- the increasing of the number of specialized papers published in prestigious ISI and IDB classified journals, specialized in the field of Pharmacy,
- the initiation of new pharmacognosy/cell biology/phytochemistry studies at the level of the Pharmacognosy Laboratory of the Faculty of Pharmacy, within the "Grigore T. Popa" University of Medicine and Pharmacy from Iasi, which should represent the starting point for the submission of project proposals in the competitions opened at national level,
- leading small teams for prospective research;
- maintaining of the relationship with the present collaborators, encouraging partnerships that allow young researchers and PhD students to exchange knowhow and learn new skills;
- the enlargement of the collaboration team for an increased success for project proposals,
- participation in scientific events in the field, for a proper dissemination and an increased national/international visibility for the University.

All in all, I will seek to improve performance and capabilities in all three mentioned areas for the benefit of professional future development and to achieve the status of a research leader and well-known professor.

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