GRIGORE T. POPA UNIVERSITY OF
MEDICINE AND PHARMACY IASI

HABILITATION THESIS

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Compounds of pharmaceutical interest and multifunctional systems: analysis and characterization

- Habilitation thesis -

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CONTENTS

REZUMAT

Societatea modernă necesită un sistem de educație de calitate înaltă. Prin natura ei, profesia didactică presupune o permanentă formare și dezvoltare a cadrului didactic, astfel încât acesta să poată oferi celui care învață o perspectivă comprehensivă asupra domeniului predat. Teza de abilitare prezintă pe scurt cele mai importante cercetări din evoluția cadrului didactic, după acordarea titlului de doctor, fiind un pas important în recunoașterea abilităților științifice, didactice și academice ale candidatului. In același timp, reprezintă un punct de plecare în activitatea de îndrumare a celor care își doresc o aprofundare a activității de cercetare, după finalizarea studiilor universitare, prin realizarea unei teze de doctorat. Astfel, lucrarea de față își propune o sinteză a activităților de cercetare și didactice din perioada postdoctorală, punând accent pe direcţiile din domeniul cercetării ştiinţifice.

Teza de abilitare intitulată "*Compuși de interes farmaceutic şi sisteme multifuncționale: analiză şi caracterizare*" este alcătuită conform recomandărilor Consiliului Național de Atestare a Titlurilor, Diplomelor și Certificatelor Universitare (CNATDCU) şi cuprinde principalele direcții de cercetare, alături de un plan de evoluție şi dezvoltare a carierei academice, fiind structurată în trei secţiuni principale.

Secţiunea I cuprinde o scurtă prezentare a activităţii ştiinţifice din perioada postdoctorală.

Secţiunea II prezintă proiectele viitoare referitoare la activitatea ştiinţifică, profesională şi academică.

Secţiunea III enumeră referinţele bibliografice utilizate în teză.

Secţiunile sunt precedate de o sinteză a activităţii profesionale şi ştiinţifice, de la începutul carierei până în prezent, punând accentul pe perioada postdoctorală. Activitatea didactică urmărește etapele evoluției profesionale, pornind de la preparator până la conferenţiar şi include cursuri şi lucrări practice de *Analiza medicamentului* şi *Legislație şi deontologie farmaceutică* pentru studenții Facultății de Farmacie, seriile în limba română şi engleză, şi a studenților din cadrul secției Asistenți Farmacie. De asemenea, activitatea didactică include ore în cadrul programelor de rezidențiat, specializările *Farmacie clinică* şi *Laborator farmaceutic* şi în cadrul masterului de *Biotehnologii medicale avansate* al Facultății de Bioinginerie Medicală.

Cercetarea din perioada postdoctorală s-a concretizat în peste 50 de lucrări științifice publicate *in extenso*, dintre care 24 sunt în reviste cotate ISI şi 15 sunt indexate BDI, 4 cărţi (1 ca prim-autor), 4 capitole (1 ca prim-autor). Factorul de impact pentru lucrările autor principal este peste 10, iar indicele Hirsch este 7 conform ISI Web of Science, Core Collection, Thomson Reuters. De asemenea, în această perioadă am fost implicată în patru granturi de cercetare, dintre care două pe domeniile tezei de abilitare (1 în calitate de director).

Secţiunea I este centrată pe trei direcţii de cercetare. Prima direcție de cercetare intitulată „*Cercetări privind design-ul şi caracterizarea unor sisteme multifuncţionale pe bază de ciclodextrine*" continuă studiile din cadrul tezei de doctorat, axându-se pe tehnica încapsulării în ciclodextrine în vederea îmbunătățirii calităților biofarmaceutice ale unor substanțe din diferite clase şi de diferite origini (naturală, semisintetică, sintetică), cu o solubilitate scăzută în apă. Deoarece ciclodextrinele sunt recunoscute a îmbunătăți proprietățile farmaceutice ale substanțelor, în special solubilitatea, viteza de dizolvare, stabilitatea şi implicit

biodisponibilitatea, printre ciclodextrinele utilizate se numără: beta-ciclodextrina, dar şi unii derivaţi, cum ar fi hidroxipropil-beta-ciclodextrina, sulfat-beta-ciclodextrina, sulfobutil eter-beta-ciclodextrina, monoclorotriazinil-beta-ciclodextrina. Compușii de incluziune au fost sintetizați prin diferite tehnici şi apoi caracterizați prin aplicarea unor metode moderne de analiză, cum ar fi cele spectroscopice, cromatografice, termice. De asemenea, s-a realizat studiul cineticii de dizolvare a compușilor de incluziune comparativ cu substanţa părinte, *in vitro*, în aceleași condiții testându-se şi activitățile antimicrobiană şi antioxidantă a compușilor de incluziune, rezultatele confirmând eficiența încapsulării, prin îmbunătățirea calităților biofarmaceutice ale substanțelor cercetate.

A doua direcție de cercetare intitulată „*Cercetări privind analiza produselor farmaceutice şi a compuşilor bioactivi naturali*" prezintă pe de o parte unul dintre cele mai importante aspecte ale activității din domeniul farmaceutic şi anume controlul calității produsului farmaceutic şi pe de altă parte, evaluarea calitativă şi cantitativă a unor compuşi din produse naturale. Alegerea metodei de analiză potrivită este o provocare pentru orice farmacist analist, care va trebui să țină cont de cunoștințele sale legate de proprietățile fizico-chimice ale probei analizate, parametrii de calitate corespunzători fiecărei forme farmaceutice, posibilitățile laboratorului în care lucrează, etc. Astfel, în această parte am dezvoltat, validat şi aplicat diferite metode de analiză (UV-Vis, SAA, HPLC), pentru cuantificarea unor substanțe active din produse farmaceutice atât de tip medicament, cât şi supliment alimentar. In cazul probelor naturale s-au utilizat: spectroscopia UV-Vis (analiza totalului polifenolic, a totalului flavonoidic şi a acizilor polifenolcarboxilici totali), HPLC-DAD şi HPLC-DAD-MS (analiza polifenolilor), LC-MS (analiza flavonelor metoxilate, a fitoestrogenilor, a sterolilor). Metodele aplicate s-au dovedit a fi simple, precise şi rezultatele obținute au demonstrat aplicabilitatea lor în analiza substanțelor din diferite matrici.

Ca o continuare a cercetărilor, din dorința de a îmbina domeniul plantelor medicinale cu cel de obținere a unor sisteme nanoparticulate cu un important potențial în dezvoltarea de agenți terapeutici, a treia direcție de cercetare se intitulează "*Cercetări privind design-ul şi caracterizarea diferitelor nanoparticule de argint, obținute prin intermediul plantelor*". Astfel, a fost studiată posibilitatea utilizării unor extracte din plante în vederea sintezei de nanoparticule, biomoleculele prezente în acestea având un rol atât în procesul de reducere şi obţinere a nanoparticulelor, cât şi în cel de stabilizare. De asemenea, confirmarea obținerii nanoparticulelor s-a realizat prin diverse metode de analiză, cum ar fi UV-Vis, FTIR, EDX, DLS şi TEM. Pentru a studia posibilele activități biologice ale nanoparticulelor sintetizate, s-au determinat activitățile antioxidantă şi antimicrobiană, comparativ cu extractele inițiale. Rezultatele au demonstrat că, utilizarea extractelor din plante reprezintă o metodă simplă, ieftină şi eco-friendly, cu un potenţial important în dezvoltarea de agenți terapeutici.

Secţiunea II cuprinde planurile de evoluție şi dezvoltare a carierei științifice şi academice care se vor axa pe continuarea direcțiilor menționate, dar și pe extinderea ariei de interes, în acord cu progresul științei.

Secţiunea III include lista referințelor bibliografice care susțin datele prezentate pe parcursul tezei de abilitare.

ABSTRACT

Modern society requires a high-quality education system. The teaching profession involves a permanent training and improvement of the teacher so that he/she can provide the learner with a comprehensive perspective on the subject being taught. The habilitation thesis summarizes the most important researches in the evolution of the teacher after being awarded the doctoral title. It is an important step in recognizing the candidate's scientific, teaching and academic/organizational abilities. At the same time, it is a starting point for the guidance of those who want to expand their research activity by preparing a PhD thesis, after obtaining a bachelor's degree. Thus, this paper presents a synthesis of the research and didactic activities from the postdoctoral period, which will emphasize the research directions.

The habilitation thesis entitled *"Compounds of pharmaceutical interest and multifunctional systems: analysis and characterization*" is structured as recommended by CNATDCU and presents the main research directions along with the academic career plan of development, being structured into three main sections.

Section I contains a brief presentation of the postdoctoral scientific achievements.

Section II presents future projects regarding the scientific, professional and academic areas.

Section III includes the list of references used in the thesis.

The sections are preceded by a synthesis of the professional and scientific activities, focusing on the postdoctoral period. The didactic activity follows the steps of professional development, from research assistant to associate professor, and includes courses and practical lessons of *Drug analysis* and *Pharmaceutical Legislation and Deontology* for Pharmacy students in the Romanian and English teaching sections and for Pharmacy Assistance Program students. The didactic activity also included courses and practical lessons in residency programs, *Clinical Pharmacy* and *Pharmaceutical Laboratory* specialties and in the *Advanced Biotechnology Master* of the Faculty of Medical Bioengineering.

Scientific accomplishments of the postdoctoral period include over 50 scientific papers published *in extenso*, of which 24 are found in ISI quoted journals and 15 are BDI indexed, 4 books (1 as the first author), 4 chapters (1 as the first author). The impact factor for first author papers is over 10 and the Hirsch index is 7, according to ISI Web of Science, Core Collection, Thomson Reuters. During this period, I also took part in four research grants, two of them involving the scientific area of the habilitation thesis (1 as a director).

Section I is focused on three research directions. The first research direction entitled *"Research on the design and characterization of some multifunctional systems based on cyclodextrins"* continues the doctoral studies using cyclodextrin encapsulation to improve biopharmaceutical qualities of substances from different classes of compounds and of different origins (natural, semisynthetic, synthetic), all having a low water solubility. Since cyclodextrins are recognized to improve pharmaceutical properties of substances, particularly the solubility, dissolution rate, stability and, implicitly, bioavailability, among the used cyclodextrins were the following: beta-cyclodextrin and some derivatives such as hydroxypropyl beta-cyclodextrin, sulfated beta-cyclodextrin, sulfobutyl ether-beta-cyclodextrin, monoclorotriazinyl-beta-

cyclodextrin. The inclusion compounds were synthesized by various techniques and then characterized by applying modern methods of analysis (spectroscopic, chromatographic, thermal). Moreover, the dissolution kinetics, the antimicrobial and antioxidant tests of the inclusion compounds were carried out *in vitro* in comparison with the parent compounds, the results confirming the efficiency of the encapsulation through the improvement of biopharmaceutical qualities of the investigated substances.

The second research direction, entitled *"Research on the analysis of pharmaceutical products and natural bioactive compounds"* presents, on the one hand, one of the most important aspects of the pharmaceutical activity, namely the quality control of pharmaceutical products and on the other hand, the qualitative and quantitative evaluation of some compounds from natural products. Finding the suitable method of analysis is a challenge for any pharmacist who will need to take into account his knowledge regarding the physico-chemical properties of the sample, the appropriate quality parameters for each pharmaceutical form, the equipment of the laboratory they are working in, etc. Thus, we developed, validated and applied various methods of analysis (UV-Vis, AAS, HPLC) for the quantification of active substances found in different medicines and food supplements. For samples of natural origin, UV-Vis spectroscopy (total polyphenols, total flavonoids and total polyphenol carboxylic acids analyses), HPLC-DAD and HPLC-DAD-MS (analysis of polyphenols), LC-MS (analysis of methoxylated flavones, phytoestrogens, sterols) were used. The applied methods have proved to be simple, inexpensive and accurate and the obtained results have shown that they can be applied for the analysis of substances from different matrices.

As continuation of research, given the desire to combine the medicinal plant area of study with that of nanoparticle systems with important potential in the development of therapeutic agents, the third research direction is entitled *"Research on the design and characterization of different plant-mediated silver nanoparticles".* Therefore, the possibility of using plant extracts for nanoparticles synthesis was studied. Biomolecules found in such extracts participate in the process of reduction and production of nanoparticles, also acting as stabilizers. The successful synthesis of nanoparticles was confirmed by various analytical methods, such as UV-Vis, FTIR, EDX, DLS and TEM. In order to study potential biological activities of the synthesized nanoparticles, the antioxidant and antimicrobial activities were tested in comparison with the initial extracts. Results have shown that the use of plant extracts represents a simple, cheap and eco-friendly synthesis method with important significance for the development of therapeutic agents.

Section II contains the development strategies for the scientific and academic career which will focus on the continuation of the mentioned directions, but also on the extension of the area of interest in agreement with the progress of science.

Section III includes the list of references supporting the data presented in the habilitation thesis.

OVERVIEW OF THE PROFESSIONAL, SCIENTIFIC AND ACADEMIC CAREER

Professional achievements

I graduated the Faculty of Pharmacy, "Grigore T. Popa" University of Medicine and Pharmacy Iasi in 2000, as head of the promotion. In 2001, I worked as a pharmacist during an internship at the "St. Spiridon" County Hospital, Iași. During that time, I passed the national residency exam, being confirmed as a resident (General Pharmacy specialty) and the exam for research assistant in the *Drug analysis* department.

I started my didactic activity in 2002, initially as a research assistant, and then continued as an assistant professor in 2005, lecturer in 2009 and associate professor since 2014. I carried out my activity in the *Drug Analysis* department, where I taught courses and practical lessons of Drug analysis, Basics of Economic Evidence, Pharmaceutical Legislation and Deontology, for pharmacy students, for Pharmacy Assistance Program students between 2005-2013 and, since 2013, for pharmacy students in the English Program.

Between 2015 - 2018, I implemented an optional course entitled "Ensuring and Improving Quality Standards in Pharmaceutical Units," which was addressed to students of the 5^{th} year.

Since 2010, I was co-opted in the Advanced Biotechnology Master's team at the Faculty of Medical Bioengineering. Thus, in addition to supervising bachelor's theses, I also coordinated dissertations.

The didactic activity also included courses and practical lessons in residency programs, Clinical Pharmacy and Pharmaceutical Laboratory specialties. I was also a lecturer, and currently a coordinator of postgraduate courses in the field of drug quality assurance, pharmaceutical quality standards and pharmaceutical legislation.

In 2007 I obtained the Qualified Person Certificate, issued by the Ministry of Public Health - National Medicines Agency.

I completed a master's degree in "Pharmaceutical and cosmetic products" in 2011 at "Gheorghe Asachi" Technical University of Iasi, Faculty of Chemical Engineering and Environmental Protection. I became a primary pharmacist in General Pharmacy specialty in 2009 and finished my second specialty (Clinical Pharmacy) in 2010.

During this period, I have attended and finished training courses regarding drug analysis, chromatography, spectrometry, legislation, management, quality audit.

In order to update the information presented during courses and practical lessons, I participated in writing four specialized books for students, residents, PhD students and all those interested in the field of drug analysis:

- Andreia Corciovă, Constantin Ciobanu, Drugs analysis. Volumetric, chromatographic and UV-Vis spectrophotometric methods. Concepts and applications. "Gr. T. Popa" Publisher, UMF Iasi, 2015
- Doina Lazăr, Andreia Corciovă, Mihai Lazăr, Drug Analysis. Guidelines for laboratory practice, Pim Publisher, Iasi, 2011

- Mihai Lazăr, Doina Lazăr, Andreia Corciovă, Drugs Analysis, vol. 2, Pim Publisher, Iasi, 2008
- Mihai Lazăr, Doina Lazăr, Andreia Corciovă, Drugs Analysis, vol. 1, Pim Publisher, Iasi, 2007

Throughout my entire professional activity, I have emphasized the importance of the taught subjects for the future activities carried out by graduates of the Faculty of Pharmacy.

Scientific achievements

The presentation of the scientific achievements starts with 2006, when I completed my doctoral studies at "Grigore T. Popa" University of Medicine and Pharmacy Iasi, *Doctoral Advisor, Professor Mihai Ioan Lazăr*, PhD, with the thesis entitled "*Physico-chemical characterization of cyclodextrin inclusion compounds with synthesis substances*", for which I was conferred the title of *Doctor in Medical Sciences - Pharmacy domain*, by Ord MEC no. 5764 / 28.11.2006 (Diploma series E, No. 0002820). During the doctoral research, given the need to improve the substances' properties, we focused on the characterization of three synthetic compounds (two with isoflavonic structure and a bis-Schiff base) and of rutoside, substances which are not water-soluble. In order to increase the solubility, we combined these substances with beta cyclodextrin, and the obtained inclusion compounds have been characterized in terms of quality, quantity and some of their biological actions have been investigated. The performed analyses demonstrated that the inclusion compounds were obtained, and that physico-chemical and biological properties improved after complexation.

Taking into account the knowledge gained during doctoral studies regarding the synthesis of inclusion compounds and the physico-chemical characterization by different spectroscopic, chromatographic, thermal and biological methods, I wished to improve these competencies. Thus, in the postdoctoral period I continued the research on the development and use of methods for the identification and quantification of some compounds from different matrices and on the optimization of their properties through various formulations.

The results of the postdoctoral scientific research can be quantified according to ISI Web of Science, database as follows: indexed full paper 24; sum of total number of citations - 135; sum of citations, excluding self-citations - 121; H-index - 7.

During the postdoctoral period, I have established different collaborations with department teams from the Faculty of Pharmacy of "Grigore T. Popa" University of Medicine and Pharmacy Iasi and the Faculty of Pharmacy of "Iuliu Haţieganu" UMF Cluj-Napoca and with the research group of the "Center for Advanced Research for Bionanoconjugates and Biopolymers", "Petru Poni" Institute of Macromolecular Chemistry Iasi.

In addition to the previously mentioned scientific works, these collaborations led to my participation in four research grants, two of them following the main research directions. These three research grants include, on the one hand, the synthesis and characterization of cyclodextrins inclusion compounds and on the other hand, the quantitative analysis of some active principles from different plant species:

- "Grigore T. Popa" University of Medicine and Pharmacy Iasi Internal Grant, no. 4872/2013, entitled "Design and characterization of hesperidin-cyclodextrin inclusion

compounds in order to improve biopharmaceutical and pharmacological qualities", Project manager: Andreia Corciovă

- "Grigore T. Popa" University of Medicine and Pharmacy Iasi Internal Grant, no. 31590/2015, entitled "Assessment of antitumor, antioxidant and antimicrobial activity of extracts from *Tanacetum* species", Project manager: Bianca Ivănescu

The concern for the development of separation and analysis methods for various bioactive substances and the study of the legislation in force led to the publication of four chapters in books of prestigious international publishing houses, three of which can fit into the main research directions and include methods of separation, identification and quantification of plant compounds:

- Bianca Ivănescu, Andreia Corciovă, Chapter 12. Artemisinin in cancer therapy, in *"Artemisia annua – Pharmacology and Biotechnology",* editors: Tariq Aftab, Jorge F S Ferreira, M. Masroor A. Khan, M. Naeem, Springer Publisher, 2014, p. 205-229.
- Andreia Corciovă, Daniela Matei, Călin Corciovă, Bianca Ivănescu. Chapter 6. Natural compounds from plants targeting Alzheimer's disease, in „*Frontiers in clinical drug reserch-Alzheimer disorders"- volume 5*, Atta-ur-Rahman editor, Bentham Science Publishers, 2016, p. 3-91.
- Bianca Ivănescu, Andreia Corciovă. Chapter 5. *Artemisia annua* and its bioactive compounds as anti-inflammatory agents, in *"Artemisia annua: Prospects, Applications and Therapeutic Uses"*, CRC Press Taylor & Francis Group, 2018, p. 83-114.

I have been a reviewer for various articles in journals such as: Natural Products Communications, Bioanalysis, Eurasian Journal of Analytical Chemistry, Current Pharmaceutical Analysis, Journal of Molecular Structure, Farmacia, Chemical Science International Journal, Journal of Materials Science Research and Reviews, European Journal of Medicinal Plants.

Moreover, during the postdoctoral period, I was a member of scientific guidance commission of six doctoral thesis.

Academic achievements

During this period, I was involved in various academic activities: coordinator of the Drugs analysis and Pharmaceutical legislation and deontology Departments (since 2011), member of the Teaching council (since 2012) and of the Department council, member of the Scientific commission and Student problems commission of the Faculty of Pharmacy (2012-2016), member of the Curriculum bureau and of the University studies commission of the Faculty of Pharmacy (since 2016), member of the Commission for the recognition and equivalence of studies (2015-2016), member of the Commission for analysis of decommissioning and disposing of expired fixed assets and inventory items and a member of the Commission for the decommissioning and disposal of expired fixed assets and inventory items (since 2012), member of the Professional committee for the Bachelor exam (since 2009), member of the Professional committee for the residency exam (2011, 2014, 2015), member of various committees for the admission exam, specialist / primary pharmacist exams, dissertation, research assistant/assistant professor/lecturer/associate professor exams and member of organizing committees of various national scientific congresses.

SECTION I.

SCIENTIFIC ACHIEVEMENTS FROM THE POSTDOCTORAL PERIOD

CHAPTER 1. RESEARCH ON THE DESIGN AND CHARACTERIZATION OF SOME MULTIFUNCTIONAL SYSTEMS BASED ON CYCLODEXTRINS

1. 1. Background

Continuing the study started in the PhD thesis, the first research direction consisted in obtaining inclusion compounds with natural and synthesis cyclodextrin.

The appearance of new substances requires formulations to a higher standard than was considered satisfactory a few years ago. The search for new formulations is undoubtedly expensive and determines the appearance of new products on the market. Research to improve side effects and optimize medicines has led to new pharmaceutical forms. The solubility of drug substances plays an important role in drug formulations. This property of substances can be improved by complexing, which can be defined as the formation of the reversible interaction between the active substance and host molecules in order to form a new compound by means of intermolecular forces such as: covalent bonds, Van der Waals bonds, ion - dipole forces, dipole - dipole forces, hydrogen bonds [Corciovă and Ivănescu, 2014].

Among the examples of substances that can form inclusion compounds, wherein the guest molecule is partially or totally included in the host molecule, are cyclodextrins.

Cyclodextrins are cyclic oligosaccharides obtained biotechnologically from the enzymatic degradation of starch using a glucosyltransferase most commonly derived from *Bacillus macerans* [Batt and Garala, 2013], usually formed by 6, 7 or 8 glucopyranose monomers (named α-, β-, or γ-cyclodextrins, respectively), covalently bounded by α-1,4 linkages. Due to their hydrophilic outer surface and their hydrophobic cone-like central cavity, they can form host-guest inclusion complexes with various organic/inorganic molecules [Loftsson and Jarvinen, 1999] and increase drug solubility in aqueous solutions, chemical stability and bioavailability.

β-cyclodextrin (β-CD) can be used in oral administration but in the parenteral administration it has the disadvantage of having a relatively low solubility in water. Thus, more suitably would be some derivatives with greater water solubility and low toxicity such as: hydroxypropyl-beta-cyclodextrin (HP-βCD), sulfated-beta-cyclodextrin sodium salt (sulfated-β-CD), sulfobutyl ether-β-cyclodextrin sodium salt (SBE7-β-CD, Captisol), monochlorotriazinyl-β-cyclodextrin (MCT-β CD, Cavasol W7 MCT).

HP-βCD, a hydroxyalkyl derivate, is used as an alternative to β-CD and is obtained through the reaction of propylene oxide and β-CD in alkaline aqueous solutions, have improved water solubility properties [Gould and Scott, 2005], is toxicologically safe both in humans and animals when administered either orally or intravenously [Qiu et al., 2014].

In the case of sulfated-β-CD, the substituent consists of the sodium sulfate salt directly linked to the hydroxylic oxygen of the pyranose unit, without the butyl ether spacer [Uekama et al., 1998]. The literature provides that it also has its own action: protective action against the

nephrotoxicity of gentamicin and activity of the promoter or inhibitor of angiogenesis, depending on the concentration [Strauss et al., 2002].

SBE7-β-CD is a β-CD derivative statistically substituted with an average degree of substitution of approximately seven, at the 2, 3 and 6 positions of the glucopyranose units. The substituent consists of a sodium sulfate salt separated from the hydrophobic cavity by a butyl ether spacer group and thus extending the original hydrophobic cavity which determined an improve complexation [Garcia-Rio et al., 2007]. It has a high solubility in water and is virtually non-toxic [Gopinathan et al., 2013].

MCT-β–CD is a neutral cyclodextrin having triazinyl substituent, with an average degree of substitution of 0.4 per pyranose unit, non-toxic and with a high solubility in water [Yao et al., 2014].

Cyclodextrins, has the ability to change the physico-chemical properties of the inclusion compound, without changing their original structures. So, these physical properties that can be modified during complexation include: increased solubility and stability of substances, protection against degradation, reduced gastric toxicity and irritancy, masking the unpleasant smell and taste, allowing the formation of homogeneous drug delivery systems, increasing the bioavailability of substances [Davis and Brewster, 2004; Loftsson and Brewster, 2010].

The substances chosen for the synthesis of inclusion compounds belong to the category of flavonic compounds (hesperidine, diosmine), phenolic compounds (protocatehuic acid) and propiconazole derivatives.

This research direction has been materialized by publishing the following articles:

- ✓ Corciovă A, Ciobanu C, Poiată A, Nicolescu A, Drobotă M, Varganici CD, Pinteală T, Fifere A, Marangoci N, Mircea C. Inclusion complexes of hesperidin with hydroxypropyl-β-cyclodextrin. Physico-chemical characterization and biological assessment. *Dig. J. Nanomater. Biostruct.* 2014; 9(4): 1623 – 1637.
- ✓ Corciovă A, Ciobanu C, Poiată A, Mircea C, Nicolescu A, Drobotă M, Varganici CD, Pinteală T, Marangoci N. Antibacterial and antioxidant properties of hesperidin:β-cyclodextrin complexes obtained by different techniques. *J. Incl. Phenom Macrocycl. Chem*. 2015; 81(1-2): 71-84.
- ✓ Corciovă A, Cioroiu B, Mircea C, Tuchiluş C, Ciobanu C, Dimitriu C, Ivănescu B. Influence of hydroxypropyl-cyclodextrin on the physicochemical and biological characteristics of a flavone with important pharmacological properties. *Environ. Eng. Manage. J.* 2015; 14(2): 311-319.
- ✓ Corciovă A, Ivănescu B. Study on the hesperidin – cyclodextrins interactions by thin layer chromatography. *Eur. Chem. Bull*. 2014; 3(6): 548-551.
- ✓ Lungoci AL, Turin-Moleavin IA, Corciovă A, Mircea C, Arvinte A, Fifere A, Marangoci NL, Pinteală M. Multifunctional magnetic cargo-complexes with radical scavenging properties. *Mater. Sci. Eng., C*. 2019; 94: 608–618.

- ✓ Minea B, Marangoci N, Peptanariu D, Roşca I, Nastasa V, Corciovă A, Varganici C, Nicolescu A, Fifere A, Neamţu A, Mareş M, Bărboiu M, Pinteală M. Inclusion complexes of propiconazole nitrate with substituted β-cyclodextrins: the synthesis and *in silico* and *in vitro* assessment of their antifungal properties. *New J. Chem.* 2016; 40: 1765-1776.
- ✓ The research regarding hesperidin was funded by the "Grigore T. Popa" University of Medicine and Pharmacy through its Internal Grants Program, project number 4872/18.03.2013 with title "Design and characterization of hesperidin-cyclodextrin inclusion compounds in order to improve biopharmaceutical and pharmacological qualities ".

1. 2. Design and characterization of inclusion complexes with flavonoids and derivatives

Flavonoids are a large class of natural polyphenolic compounds characterized by a flavan nucleus, which are widely distributed in fruits, vegetables, teas, wines, etc. From this class of compounds we chose to study hesperidin.

Hesperidin (HES) (5, 7, 3'- trihydroxy - 4'- methoxy - flavanone 7 – rhamnoglucoside) is a naturally occurring flavanone glycoside (hesperetin-7-rutinoside) and is the most abundant flavonoid in citrus fruits. HES reduces the permeability and fragility of capillary walls and can be used in chronic venous insufficiency, haemorrhoids, varicose veins, various ulcers and bruising [Garg et al., 2001]. Also, manifests antioxidant activity and radical scavenging properties and a significant antiinflammatory activity. Lately, a considerable amount of research has been carried out on the anticarcinogenic activity of HES against different human cancer cell lines, with remarkable results, having the ability to suppress cell proliferation in oral carcinogenesis [Gil-Izquierdo et al., 2003]. Also, HES significantly decreases the cholesterol levels, total lipids and triglycerides and may be used in the prevention of atherosclerosis and hypertension diseases. Likewise, HES decreases the bone density loss and manifests anti-nociceptive and sedative activity [Corciovă et al., 2014]. HES has found to possess antibacterial, antifungal, antiviral properties, antiallergic and antianaphylactic activities. Further, it has been shown to inhibit platelet and cell aggregation and the activity of some enzymes (hyaluronidase, aldol reductase, aromatase). The aglycone, hesperetin, protects liposomes from UV-irradiation induced peroxidation and might be successfully employed as a topical photo-protective agent [Kuntić et al., 2014].

Diosmin (D) is a HES semisynthetic derivative with important pharmacological properties: vasoprotective [Tong et al., 2013], antioxidant, anti-inflammatory [Sezer et al., 2011], antiproliferative and anti-cancer [Alvarez et al., 2009], antihyperglycemic and hepatoprotective properties [Tahir et al., 2013]. Also, diosmin exhibits a neuroprotective effect and might have potential in the treatment of neurodegenerative diseases [Abdel-Salam et al., 2012].

However, their poor solubility in water, increases the difficulty of formulation and the administration is rather limited, therefore, it is important to develop new methods for enhancing

their solubility. In order to overcome this disadvantage, the complexation method with host substances of cyclodextrins type was used.

Research in this area has focused on:

- investigation of the interaction of HES with β-CD, HP-β-CD, sulfated-β-CD by thin layer chromatography and calculating the inclusion constants and the thermodynamic parameters of the inclusion process
- the solubility studies by determining the phase solubility at different temperatures and different concentrations of cyclodextrins in supersaturated water solutions of HES and diosmin establishing the stability constants and the thermodynamic parameters of the complexation reaction
- the synthesis of inclusion compounds by different techniques
- the physico-chemical characterization of the inclusion compounds by modern methods of analysis
- *in vitro* testing of the obtained inclusion compounds by: dissolution tests, determination of antioxidant and antimicrobial activities

1. 2. 1. Application of thin layer chromatography to investigating host-guest interactions

Thin layer chromatography (TLC), an inexpensive and rapid method, was used to investigating the inclusion complexes and for determination of some thermodynamic parameters. The inclusion and dissociation constants are a measure of changes in the physicochemical properties of a compound as a result of the inclusion process. The thermodynamic parameters, i.e. Gibbs free energy change (ΔG^0_{tr}), free energy change (ΔG^0), enthalpy change (ΔH^0) and entropy change (ΔS^0) can be calculated according to the effect of temperature on the stability constants of the complex.

Materials and methods

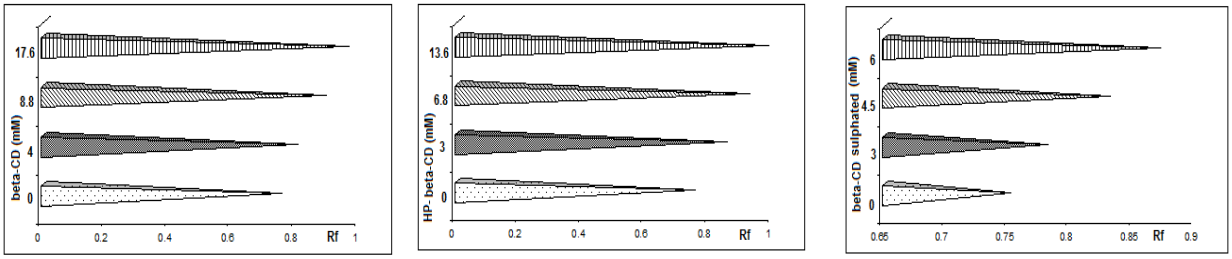
Materials used were HES solution (1 mg/ml) and cyclodextrins such as βCD, HP-βCD and sulfated-β-CD.

Thin layer chromatography: Chromatographic analysis was performed by ascendant method, at room temperature. The chromatographic plates were coated with silica gel, 10 x 20 cm and 0.2 mm thickness. The composition of the mobile phase used was butanol: acetic acid: water (4:1:5) containing different concentrations of cyclodextrin. The migration distance was 12 cm from the start line. After the plates were dried, the detection was performed initially by examination in UV light and then by exposure to iodine vapors. The R_f values were calculated by the ratio of the distance traveled by the substance to the distance traveled by the mobile phase which contains increasing concentrations of cyclodextrins [Corciovă and Ivănescu, 2014].

Results

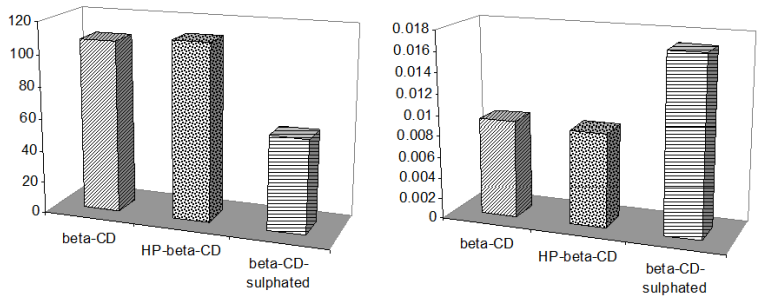
The Rf values obtained were represented graphically based on the cyclodextrins concentrations and the inclusion constants (K) were calculated, at increasing temperatures: 293 K, 303 K and 313 K. The effect of different concentrations of cyclodextrin on the R_f values of

HES is represented in Figures 1. 1. a-c. After K calculations, we represent the inclusion constants and the dissociation constants (1/K) depending on the cyclodextrin used (Figure 1. 2.).



a **b** **c**

Figure 1. 1. The effect of cyclodextrin concentrations on the R_f values of HES
a. β-CD, **b.** HP-β-CD, **c.** sulfated-β-CD



a **b**

Figure 1. 2. a. Inclusion constants; **b.** Dissociation constants of HES depending on the cyclodextrin used

The thermodynamic parameters calculated are presented in Table 1. 1-1.3.

Table 1. 1. ΔG^0_{tr} (kJ mol^{-1}) values depending on CD concentration

CD	**CD concentration, mM**	**ΔG^0_{tr}, kJ mol^{-1}**		
		293 K	**303 K**	**313 K**
β -CD	4	- 0.068	0.239	0.703
	8.8	- 0.178	0.155	0.523
	17.6	- 0.258	0.042	0.453
HP-β-CD	3	- 0.13	0.681	0.546
	6.8	- 0.212	0.506	0.43
	13.6	- 0.280	0.082	0.307
sulfated-β-CD	3	- 0.041	0.314	0.569
	4.5	- 0.106	0.222	0.322
	6	- 0.154	0.120	0.345

Table 1. 2. ΔG^0 (kJ mol^{-1}) values depending on temperature

Temperature	**293 K**	**303 K**	**313 K**
β-CD	- 11.28	- 11.76	- 12.73
HP-β –CD	- 11.35	- 12.08	- 12.93
sulfated-β-CD	- 9.79	- 11.71	- 12.46

Table 1. 3. ΔS^0 (J mol^{-1} K^{-1}) and ΔH^0 (kJ mol^{-1}) values depending on the cyclodextrin used

CD	**ΔS^0**	**ΔH^0**
β-CD	0.042	1.10
HP-β–CD	0.043	1.28
sulfated-β-CD	0.044	3.21

Discussions

By raising the concentration of cyclodextrin in the mobile phase, the migration distance decreases and thus the value of R_f increases. The most obvious change in R_f values was observed for HP-β-CD, followed by β-CD and sulfated-β-CD.

The inclusion capacity decreases in order of HP-β-CD > β-CD > sulfated-β-CD. The HP-β-CD and β-CD compounds are more stable than those with sulfated-β-CD. The values of inclusion constants rise with the increasing temperature, which indicates that the high temperature favors the inclusion process.

Negative values of ΔG^0_{tr} indicate advantageous conditions for migration of HES in the presence of cyclodextrin, especially for HP-β-CD. Values decline with increasing concentrations of CD, which demonstrates that the reaction becomes more favorable with higher CD concentrations. However, at greater temperature the ΔG^0_{tr} values become positive, which indicates that a higher temperature is unfavorable for the migration of HES along the stationary phase. Negative values of ΔG^0 show that the inclusion process takes place spontaneously.

Positive ΔH^0 values indicate an endothermic process and positive ΔS^0 values can be attributed to the transfer of HES into the cyclodextrin cavity and to the formation of hydrophobic bonds [Guo et al., 2004].

Conclusions

The inclusion interaction between HES and βCD, HP-βCD and sulfated-β-CD was studied by TLC method. The results show that, the reaction between HES and cyclodextrins with the formation of inclusion compounds is an endothermic and spontaneous process, and is more effective at increasing temperatures and CD concentrations. The best inclusion and more stable compounds were obtained with HP-β-CD. The method has proved to be useful and easily applied in investigating the HES-cyclodextrin interaction.

1. 2. 2. Application of phase solubility analysis to investigating host-guest interactions

Materials and methods

Materials: Phase solubility analysis was conducted by studying the solubility of HES in βCD and HP-βCD solutions and the solubility of diosmin in βCD, HP-βCD and sulfated-β-CD solutions.

Phase solubility studies were performed according to the Higuchi and Connors method [Higuchi and Connors, 1965]. Briefly, an excess amount of HES was mixed in a series of water solutions of different concentrations of cyclodextrins (β-CD 1.0 - 16.0 mM, HP-β-CD 0.684 - 13.69 mM) [Corciovă et al., 2014; 2015a]. In case of diosmin the cyclodextrin solutions concentrations were: 0.969 - 16.29 x 10^{-3} M for β-CD, 0.684-13.69 x 10^{-3} M for HP-β-CD and 0.305 - 6.10 x 10^{-3} M for sulfated-β-CD [Corciovă et al., 2015b]. In both cases, the mixtures were stirred for 24 hours and the determinations were made at different temperatures in 20-40 ^{0}C range

After the samples were filtered, concentration of substance was determined spectrophotometrically by measuring the absorbance of samples at 286 nm (for HES) and at 257

nm (for diosmin) against a blank containing the same concentration of cyclodextrin. The phase solubility diagrams were drawn by plotting the concentration of HES/diosmin *vs* the concentration of cyclodextrin. The apparent stability constant (K_S) was calculated, according to equation 1 and the dissociation constants (K_d), has been assessed through the equation 2 [Domanska et al., 2011].

$$Ks = \frac{slope}{S_0\,(1-slope)} (1) \quad Kd = \frac{1}{Ks} (2)$$

where: slope – was calculated from the graph; S_0 – was intrinsic solubility of HES/diosmin in the absence of cyclodextrin

The thermodynamic parameters of the inclusion of HES/diosmin into cyclodextrin cavity were calculated using the stability constant's temperature dependence and equations 3-7 [Corciovă et al., 2014; 2015a; 2015b]:

$$\Delta G^0_{tr} = -RT\log\frac{S}{S_0} (3) \quad \Delta G^0 = -2.303\,RT\log K_s (4)$$

$$Slope = \frac{\Delta H^0}{2.303R} (5) \quad \log Ks = -\frac{\Delta H^0}{RT} + \frac{\Delta S^0}{R} (6) \quad \Delta G^0 = \Delta H^0 - T\Delta S^0 (7)$$

where:
ΔG^0_{tr} - Gibbs free energy change (kJmol^{-1}); ΔG^0 - Gibbs free energy (kJmol^{-1}); ΔH^0 - enthalpy change (kJmol^{-1}); ΔS^0 - entropy change (Jmol^{-1}K^{-1}); R - the gas constant (Jmol^{-1}K^{-1}); T - the absolute temperature of the reaction (K); S/S_0 = was the ratio between the solubility of HES/diosmin in cyclodextrin water solution and the solubility of HES/diosmin in water; Slope - slope of log Ks versus 1/T line graph; K_s - the equilibrium constant of the complex formed with 1:1 stoichiometry (M^{-1}).

Results

Figure 1. 3. shows the phase solubility diagrams of HES in water solutions *vs.* β-CD at 20, 25, 37, 40 ± 1 ^{0}C and HP-β-CD concentration at 20, 25, 30, 37 ± 1 ^{0}C and Figure 1. 4. presents the phase solubility diagram in case of diosmin *vs.* β-CD, HP-β-CD and sulfated-β-CD at 20, 25 and 37 ± 1 ^{0}C.

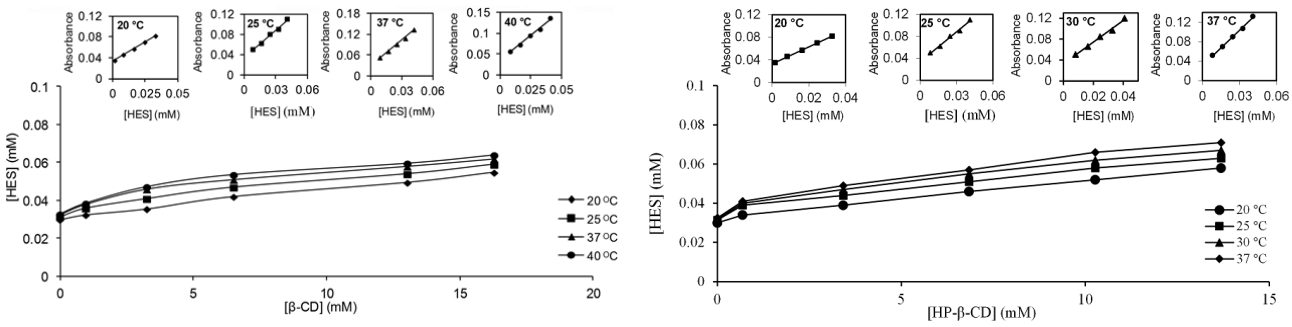


Figure 1. 3. Phase solubility diagrams of HES in water solutions *vs*. cyclodextrin concentrations at different temperatures

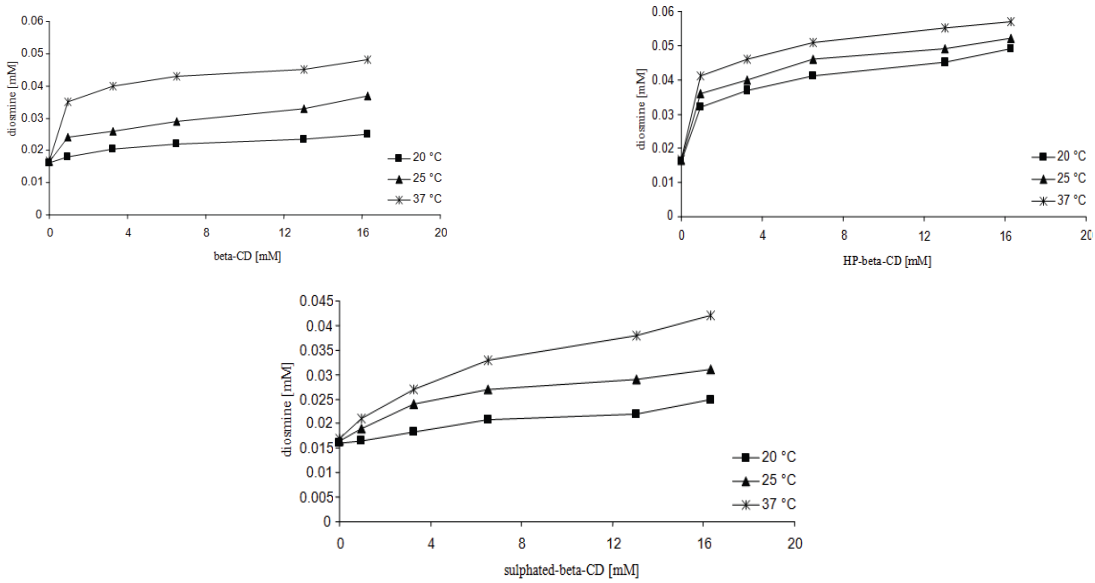


Figure 1. 4. Phase solubility diagrams of diosmin in water solutions *vs.* cyclodextrin concentrations, at different temperatures

From the phase solubility diagrams it can be seen that the solubility of HES and diosmin in water increases linearly with the increment of cyclodextrin concentration and with temperature, also. The stability constants (Ks) in case of HES were presented in Table 1. 4.

Table 1. 4. Ks values of HES-CD complex in water

	Ks (M^{-1})	
Temperature (^{0}C)	**β-CD**	**HP-β-CD**
20	153.87	169.66
25	172.82	198.12
30	-	217.66
37	194.95	242.49
40	198.73	-

Ks values for diosmin were between 112-345 M^{-1} for β-CD, 370-453 M^{-1} for HP-β-CD and 112 – 333 M^{-1} for sulfated-β-CD.

The thermodynamic parameters of the complexation reaction in case of HES are presented in Table 1. 5. [Corciovă et al., 2014; 2015a].

Table 1. 5. Thermodynamic parameters of the inclusion complexes formation between HES and β-CD / HP-βCD

Parameter	**[βCD] (mM)**	**20 ^{0}C (293 K)**	**25 ^{0}C (298 K)**	**37 ^{0}C (310 K)**	**40 ^{0}C (313 K)**
ΔG^0_{tr} (kJmol^{-1})	1.0	- 0.080	- 0.194	- 0.265	- 0.279
	3.0	- 0.182	- 0.335	- 0.471	- 0.504
	6.0	- 0.352	- 0.520	- 0.633	- 0.645
	13.0	- 0.524	- 0.584	- 0.728	- 0.764
	16.0	- 0.632	- 0.722	- 0.801	- 0.845

ΔG^0 (kJmol^{-1})		- 12.146	- 12.638	- 13.585	- 13.635
ΔS^0 (Jmol^{-1})		43.94	44.86	46.18	45.89
ΔH^0 (kJmol^{-1})	0.731				
Parameter	**[HP-βCD] (mM)**	**20 ^{0}C (293 K)**	**25 ^{0}C (298 K)**	**30 ^{0}C (303 K)**	**37 ^{0}C (310 K)**
ΔG^0_{tr} (kJmol^{-1})	0.684	- 0.131	- 0.222	- 0.244	- 0.257
	3.42	- 0.277	- 0.354	- 0.413	- 0.461
	6.84	- 0.449	- 0.517	- 0.587	- 0.626
	10.27	- 0.542	- 0.654	- 0.723	- 0.734
	13.69	- 0.717	- 0.745	- 0.806	- 0.871
ΔG^0 (kJmol^{-1})		- 12.504	- 13.106	- 13.558	-14.150
ΔS^0 (Jmol^{-1})		45.97	47.22	47.93	48.76
ΔH^0 (kJmol^{-1})	0.9669				

Also, in case of diosmin, the ΔG^0_{tr} and ΔG^0 were negative, ΔS^0 was ranged between 52.03 and 53.53 Jmol^{-1}K^{-1} and ΔH^0 was 0.833 kJmol^{-1} [Corciovă et al., 2015b].

Discussions

Both in the case of HES and diosmin, the resulting linear curves can be classified, in general, as an A_L type (linear positive isotherm). The slopes obtained from the phase diagrams were less than 1 indicating that the stoichiometry of each complex was 1:1, which is in agreement with Higuchi and Connors's theory. The values of the stability constant demonstrate that the process is favorable to obtain inclusion compounds, being known that the optimal values for the stability constants are included in the range of 100 -1000 M^{-1} [Hadziabetdic et al., 2012].

In the formation of complexes the thermodynamic interactions between the various components of the system (cyclodextrin, substance, solvent) are critical factors. In order to form a complex, a favorable energy is needed to push the substance into the cyclodextrin cavity, and the change of temperature influences the complexation between the substance and cyclodextrin.

The ΔG^0_{tr} indicates the transfer of HES/diosmin from water in the cyclodextrin microenvironment [Hadziabetdic et al., 2012] and provide data regarding favorable or unfavorable conditions concerning the solubility of HES/diosmin in cyclodextrin solution. Regarding our results the negative values of ΔG^0_{tr} indicate favorable conditions for the solubilization of HES/diosmin in the presence of cyclodextrin. These values decrease with the increasing of the CD concentration and the temperature of the process, which demonstrates that the reaction becomes more favorable with the increasing of the CD concentration and the temperature of the sample.

The values of ΔG^0 are negative, proving that the inclusion process evolves spontaneously at the chosen temperatures [Lv et al., 2012].

Furthermore, calculating the changes in the ΔS^0 and ΔH^0, informations about the strength of the interaction between HES/diosmin and cyclodextrin can be obtained. The results demonstrate that the inclusion complex formation is an endothermic process given by positive value of ΔH^0 and the transfer of HES from aqueous medium into the cyclodextrin is realized by hydrophobic interactions due to the positive value of ΔS^0 [Guo et al., 2004].

The stability of the complex between HES and HP-β-CD is higher than the complex with β-CD. In case of complexes between diosmin and cyclodextrins, the stability decreases in the following order: HP-β-CD > β-CD > sulfated β-CD.

Conclusions

The phase solubility diagrams of HES/diosmin, in aqueous solutions, showed a linear increase of water solubility with increasing the cyclodextrin concentration and the temperature at which the process takes place, so the reaction becomes more favorable with the increasing of these two parameters. The stability constant was slightly influenced by the temperature of the reaction. In both cases, the best solubility and stability were obtained in the presence of HP-β-CD.

The negative values of ΔG^0_{tr} and ΔG^0 demonstrate that the formation of the HES/diosmin with cyclodextrin inclusion complex is a process that occurs spontaneously at a chosen temperature and the working conditions were favorable for the parent substances solubilization.

Also, the positive values of ΔH^0 and ΔS^0 parameters indicate that the inclusion process is controlled by enthalpy being an endothermic process and it takes place through the establishment of hydrophobic interactions between HES/diosmin and cyclodextrin molecules, when the HES/diosmin is transferred from aqueous medium to the hydrophobic cyclodextrin cavity.

1. 2. 3. Cyclodextrin drug inclusion complexes: synthesis and physico-chemical characterization

Materials and methods

Materials: For HES were obtained inclusion complexes with βCD and HP-βCD and for diosmin was used HP-βCD.

Preparation of inclusion complexes: For the obtaining of the inclusion complexes, in case of HES, three methods were employed: co-evaporation, kneading and lyophilization. For diosmin were used two methods: co-evaporation and co-precipitation. In all cases was used a 1:1 molar ratio [Corciovă et al., 2014; 2015a; 2015b].

*Kneading (*KN) method: at HES and cyclodextrin powders a suitable quantity of water was added and the resulted mass was stirred until the water was evaporated.

Co-evaporation (CV) method: an ethanolic solution of HES/diosmin was added dropwise under stirring, at room temperature, in a clear saturated water solution of cyclodextrin. The mixture was stirred for 24 hours at 30 ^{0}C and after that the temperature was decreased at room temperature, and continuous stirring, until the solvent was evaporated. The paste was dried at 40 ^{0}C until constant weight and the dried complex was grounded to a fine powder.

Co-precipitation (CP): cyclodextrin was dissolved in a given volume of water to the limit of solubility and solid diosmin was added, with vigorous stirring. The mixture was stirred continuously until a precipitate was formed. The precipitate was filtered and dried to constant weight.

Lyophilization (L): HES was added under stirring over a clear cyclodextrin saturated water solution. The resulting suspension was vigorously stirred for 72 hours at room

temperature, and then solution was frozen at - 40 ^{0}C and lyophilized in a VirTis Freeze Mobile 6.6 (Virtis Co., USA) until all the moisture had been sublimated.

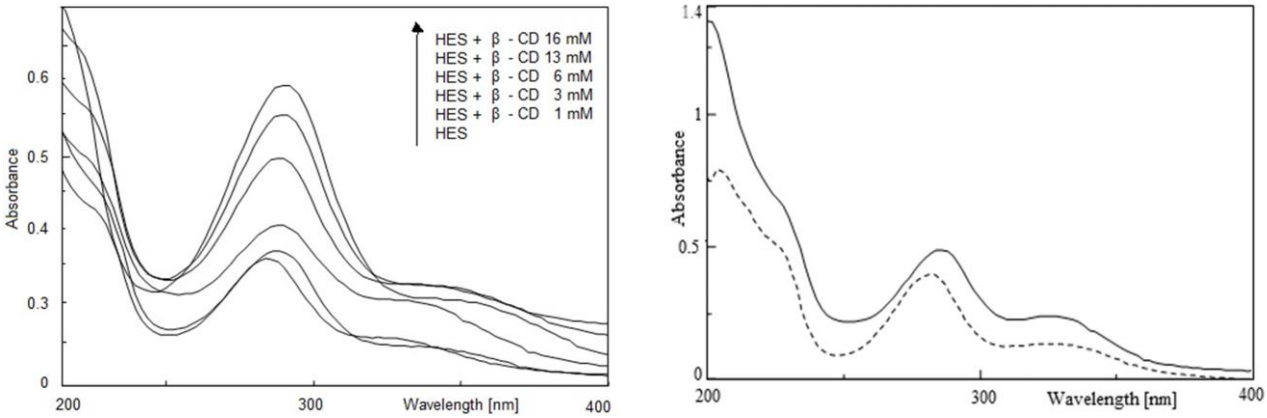
Physico-chemical characterization: The equipment used for physico-chemical characterization was: Jasco UV-Vis 530 spectrophotometer, scan speed 1000 nm min^{-1}, scan range 200-400 nm, 1.0 cm quartz cells; Bruker Vertex 70 device, equipped with ATR device (Golden Gate, Bruker) via Attenuated Total Reflectance (ATR) technique, 4000 - 500 cm^{-1} scan range and 27 Optics FT-IR spectrophotometer from Bruker, Germany, 7500 – 370 cm^{-1} range; DRX 400 Avance Bruker 400 MHz spectrometer; triple quadrupole - TSQ Quantum Access Max mass spectrometer and DSC 200 F3 Maia differential scanning calorimeter (Netzsch, Germany).

Results

For HES, in order to demonstrate the inclusion complexes' formation were used UV-Vis spectroscopy, Fourier Transform Infrared Spectroscopy (FTIR), Nuclear Magnetic Resonance (^{1}H-NMR), Differential scanning calorimetry (DSC) and in case of diosmin were used FTIR and MS analysis.

Characterization of inclusion process by UV-Vis spectroscopy

Figure 1. 5. presents the UV-Vis spectra of free HES and in the presence of βCD and HP-βCD.



a **b**

Figure 1. 5. UV-Vis spectra of free HES and in the presence of **a.** β-CD and **b.** HP-βCD

Free HES exhibits an intense maximum at 284 nm and a shoulder at 328 nm. In the presence of cyclodextrins, a bathochromic displacement of the characteristic peak of HES to 286 nm and 330 nm, respectively were observed.

Characterization of inclusion process by FTIR spectroscopy

In Figure 1. 6. were presented the FTIR spectra for HES free and complexed with β-CD and HP-β-CD, by different techniques, Figure 1. 7. presents FTIR spectra of β-CD, HES, the inclusion compound in 1800–1500 cm^{-1} interval and deconvoluted FTIR spectra for HP-β-CD, HES and inclusion compound in 1400 - 1200 cm^{-1} interval and Figure 1. 8. shows the

deconvoluted FTIR spectra in 3600-3000 cm^{-1} interval for HES, β-CD, HP-βCD and one of the inclusion compounds for each cyclodextrin.

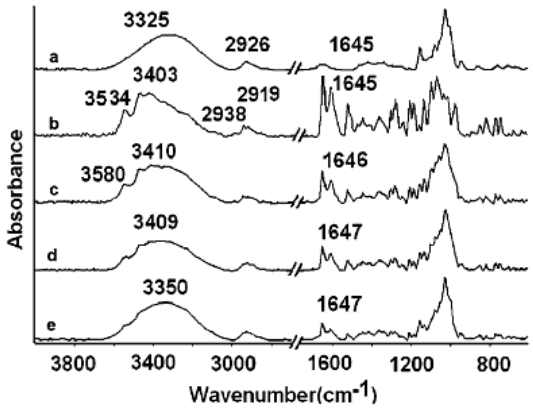


Figure 1. 6. a. Comparative FTIR spectra of βCD (a); HES (b); and the inclusion compounds CV (c); KN (d), L (e)

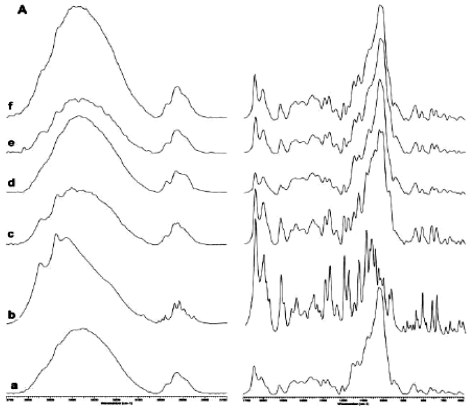
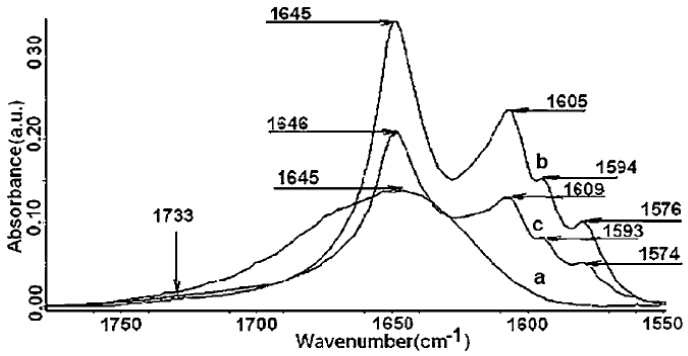


Figure 1. 6. b. Comparative FTIR spectra of HP-βCD (a); HES (b); physical mixture (c); and the inclusion compounds L (d), KN (e); CV (f)



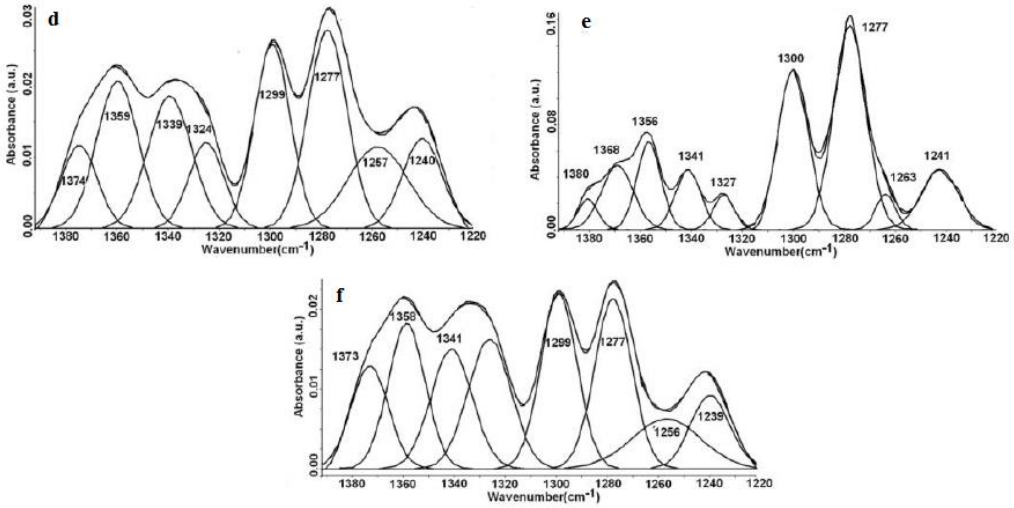


Figure 1. 7.

FTIR spectra of
a. β-CD,
b. HES,
c. inclusion compound in 1800–1500 cm^{-1} interval;

deconvoluted FTIR spectra for
d. HP-β-CD,
e. HES
f. inclusion compound in 1400 - 1200 cm^{-1} interval

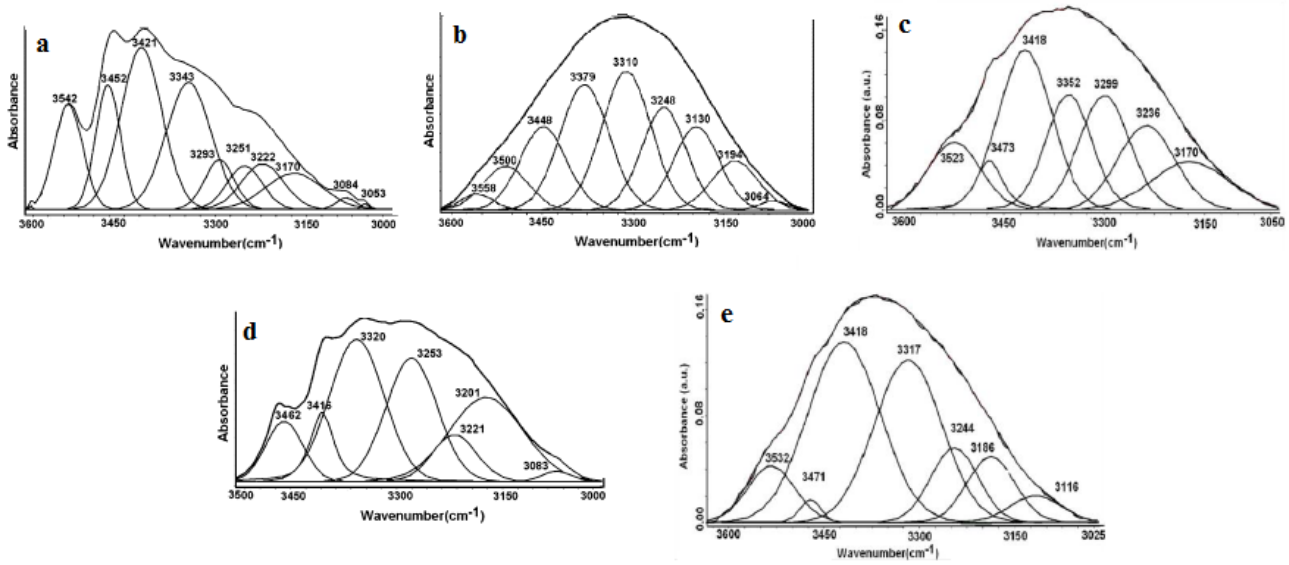


Figure. 1. 8. Deconvoluted FTIR spectra in 3600-3000 cm^{-1} interval for **a.** HES, **b.** β-CD, **c.** HP-βCD, **d.** inclusion complex with β-CD and **e.** inclusion complex with HP-β-CD

In the FTIR spectrum of HES, the characteristic absorption bands were present at 3403 and 3534 cm^{-1} (OH stretching vibrations), 2919 cm^{-1} (CH and CH_2 aliphatic), 1645 and 1607 cm^{-1} (C=O in ketones) and at 1605, 1594, 1576 cm^{-1} (C=C valence vibrations in the benzene rings). The absorption bands in 950-700 cm^{-1} interval are specific for the pulsation vibrations in glucopyranosyl unit and deformation vibrations of C-H bonds [Lin et al., 2011]. The deconvolution of the FTIR spectrum of HES presents 2 deconvoluted bands (3293, 3251 cm^{-1}) of the hydroxyls connected to phenyl rings, 2 bands (3542 and 3452 cm^{-1}) attributed to the intermolecular bonds of primary hydroxyls and 6 bands of intramolecular secondary hydroxyls of the glucopyranosyl units.

The characteristic spectrum of diosmin is defined by the following absorption maxima – 3409, 1661, 1610 1501, 1448, 1319, 1261, 1182, 1069, 854 and 821 cm^{-1}. The bands specific to free OH groups are wide bands, in the 3200 - 3600 cm^{-1} domain. The band at 3409 cm^{-1} indicates the presence of these groups in the diosmin molecule. The bands characteristic of C-H bonds deflection appear in the domain 1275 - 1000 cm^{-1}, confirmed by the band at 1069 cm^{-1} (in the case of plane deformation) and at 900-690 cm^{-1} (in the case of out-of-plane deformation), respectively, bands in the range 854- 821 cm^{-1} of carbonyl group and it is associated with the stretching vibration of the group.

In the β-CD FTIR spectrum, the wide absorption band with maximum at 3325 cm^{-1} (O-H stretching vibrations) was present. Also, there were other characteristic bands at 2926 cm^{-1} (CH and CH_2 aliphatic), 1645 cm^{-1} (OH groups of the glucopyranosyl unit, 1400-1300 cm^{-1} (CH deformation vibrations) and at 1030-1082 cm^{-1} (C-O-C and C-OH stretching vibrations [Şamli et al., 2014]. The deconvolution of the FTIR spectrum of β-CD in the range of 3600-3000 cm^{-1} shows 9 peaks at 3558 cm^{-1} attributed to OH from the hydrophobic β-CD cavity and the other peaks are caused by valence vibrations of intermolecular bonds of primary hydroxyls (3500 and 3448 cm^{-1}) and intramolecular bonds of secondary hydroxyl groups (3379, 3310, 3248, 3130, 3194, 3064 cm^{-1}) [Roik and Belyakova, 2011].

FTIR spectra of the HP-βCD showed important absorption bands at 3418, 3352 and 3170 cm^{-1} of O-H stretching vibrations, 2930 cm^{-1} of C-H stretching vibrations, at 1374, 1359, 1339, 1324, 1299, 1277, 1257 and 1240 cm^{-1} due to the different vibrations of C-H groups and at 1154, 1085, 1036 cm^{-1} for C-O stretching vibrations. The bands at 1464, 1277 and 850 cm^{-1} are a consequence of the hydrogen bonds formation between primary and secondary OH groups of HP-βCD located at 3418, 3352 and 3170 cm^{-1} and these hydrogen bonds produced new vibration bands at 3523 and 3299 cm^{-1}. The other bands from 3600-3050 cm^{-1} interval are specific for the water molecules which are located inside the cavity of cyclodextrin. Comparing the deconvoluted spectrum of HES with those of HP-βCD in the 1400-1200 cm^{-1} interval, HES presented a sub-band at 1380 cm^{-1}, most likely derived from the absorption bands induced by the deformation vibrations of C-H group linked to the primary hydroxyl group of HES.

In the FTIR spectra of the complexes few differences exist if we compare to those of HES/diosmin, β-CD and HP-β-CD [Corciovă et al., 2014; 2015a; 2015b].

Characterization of inclusion process by ^{1}H-NMR spectroscopy

One of the most used method to reveal the interaction between cyclodextrins (as a host) and HES (as a guest) is ^{1}H-NMR spectroscopy, showing the protons' displacement signals of both host and guest molecules [Zoppi et al., 2013].

Table 1. 6. presents the movements of the proton signals from the methoxy group and the protons of the aromatic ring substituted with the methoxy group from HES, in the absence and the presence of β-CD, HP-βCD, using a HES:β-CD/HP-βCD molar ratio of 1:1 and 1:3.

Table 1. 6. The chemical shifts of the interest signals for HES in the absence and presence of cyclodextrins

δ (ppm)	**HES**	**Molar ratio HES-βCD**		**Molar ratio HES-HPβCD**	
		1:1	**1:3**	**1:1**	**1:3**
OCH_3	3.7821	3.7814	3.7819	3.7819	3.7812
Substituted aromatic ring with OCH_3	6.8969-6.9629	6.8963-6.9625	6.8966-6.9628	6.8965-6.9626	6.8959-6.9621

Due to the presence of glucopyranosyl units in both components (HES and β-CD) which are responsible for the inclusion complex formation, those shifted peaks are undistinguishable. Moreover, it is unlikely that the hydrophilic glucopyranosyl unit of HES to penetrate the hydrophobic cavity of CD, therefore we considered the shifts of the methoxy, CH_2, CH and phenyl protons of HES for further studies.

The comparative ^{1}H-NMR spectra of β-CD, HP-βCD, HES, and their mixture at different molar ratio are shown in Figure 1.9.

Analyzing the chemical structure of HES we can suppose the formation of two types of inclusion complexes with a more or less probability, depending on the organic radical of HES, hydroxy-methoxyphenyl part and glucopyranosyl unit, respectively.

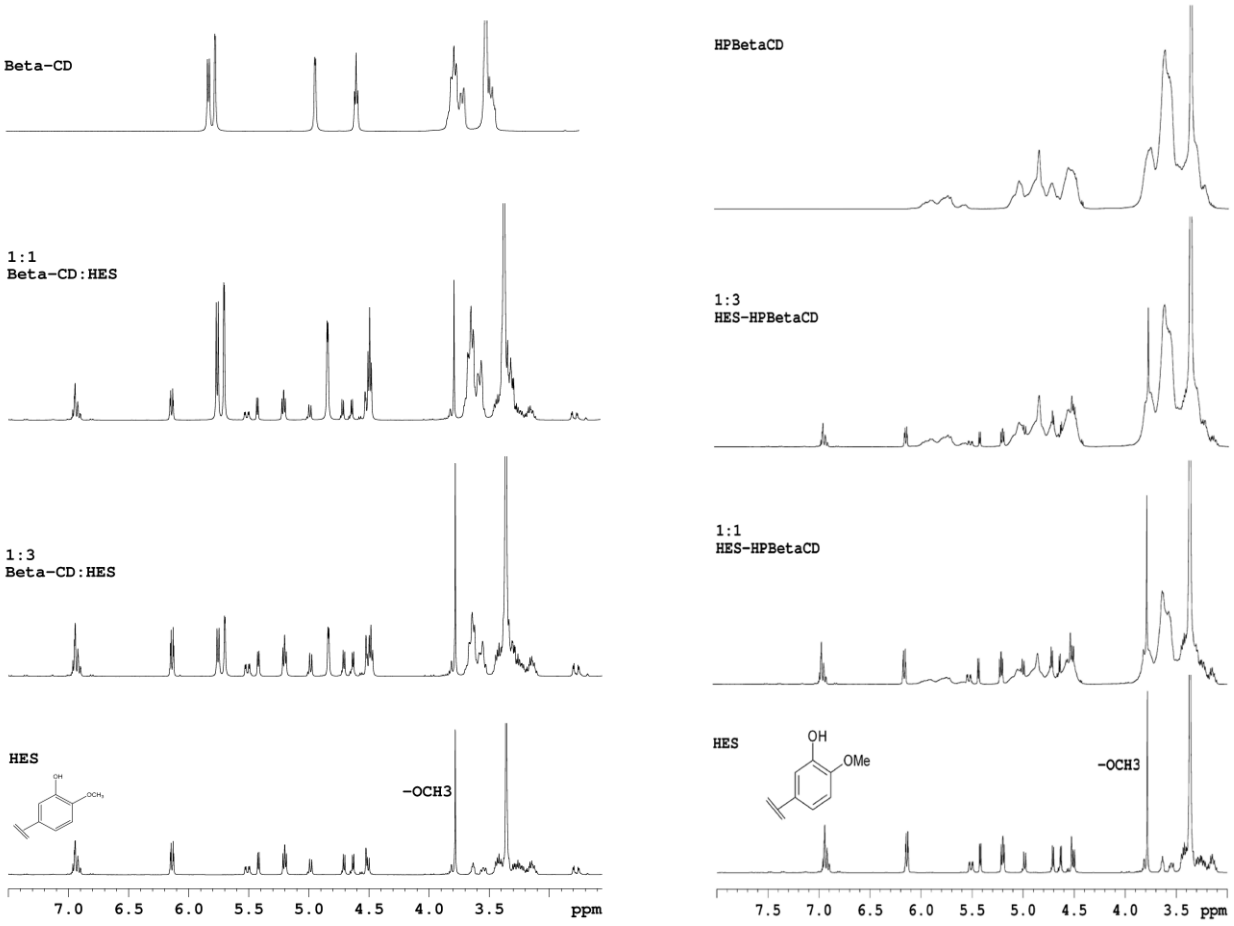


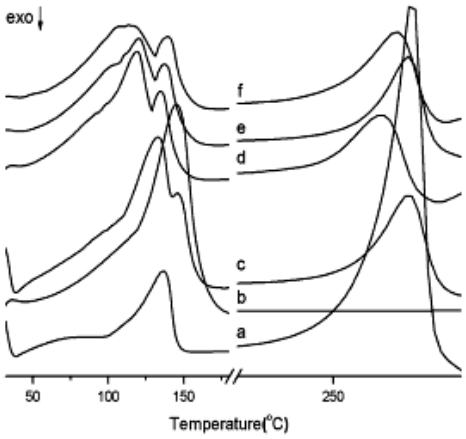
Figure 1. 9. ^{1}H-NMR spectra of β-CD, HP-βCD, HES and their mixture at different molar ratio

It should be noted that studying the inclusion phenomenon of a drug into the cyclodextrin cavity, shifts of H_3 and H_5 of β-CD should be observed [Spulber et al., 2008].

From the ^{1}H-NMR spectra of CD, as a function of HES concentration, it can be observed small values of protons' movements of methoxy and phenyl protons from -Ph-OCH_3 radical of HES between 1:3 to 1:1 molar ratios of HES:CD, while all the other peaks remain almost unchanged [Corciovă et al., 2014; 2015a].

Characterization of inclusion process by DSC

Another technique used for the characterisation of inclusion compounds is DSC, by comparing the curves and observing the differences between free substances and inclusion compounds that may occur by phase transformations during heating. The occurring of the complexation is usually evidenced by the decreased intensities or disappearance of the drug melting temperature. Figure 1. 10. presents the DSC curves of cyclodextrins, HES and inclusion compounds.



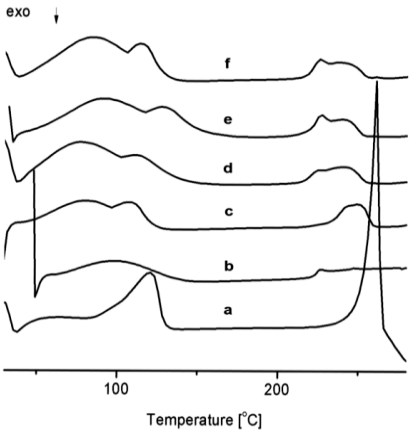


Figure 1. 10.

DSC curves of HES (a); β-CD (b); physical mixture (c); and the inclusion compounds KN (d); CV (e); L (f)

DSC curves of: HES (a); HP–βCD (b); physical mixture (c); and the inclusion compounds KN (d); CV (e); L (f)

In the case of HES, a dehydration peak is observed around 135 ^{0}C and the melting peak at 262 ^{0}C. β-CD exhibits an intense and sharp endothermic peak at 145 ^{0}C corresponding to loss of crystallized water from its cavity. Both dehydration peaks appear in the rest of the compounds DSC curves. However, in the case of the three inclusion compounds, the β-CD dehydration peak is strongly reduced in intensity and significantly shifted to the lower temperature domains, with the following values: 113 ^{0}C for KN, 120 ^{0}C for CV and 118 ^{0}C for L. Also, the HES dehydration peak in the inclusion compounds is reduced in intensity without exhibiting significant shifts (133 ^{0}C for L, 136 ^{0}C for CV and 138 ^{0}C for KN) compared to that of free HES dehydration (135 ^{0}C) [Corciovă et al., 2015a].

HP–βCD shows a broad endothermic peak at 97 ^{0}C, corresponding to the water elimination from the cyclodextrin cavity. In the inclusion complexes, the dehydration peak of HP–βCD is shifted to lower temperature domains, being the first indication that a host-guest interaction occurred. In the case of HES-HP–βCD inclusion complexes, the HES dehydration peak also appears in the rest of the compounds, with reduced intensity, exhibiting significant shifts compared to that of pure HES dehydration [Corciovă et al., 2014]. The two glycosidic moieties in the HES structure may couple with the hydrophilic exterior and/or the hydroxypropyl moiety of the HP–βCD, *via* OH groups, with hydrogen bond formation and the rest of the HES molecule entering in the HP–βCD cavity. HP–βCD exhibited an endothermic transition centered at 224 ^{0}C, attributed to a glass transition (T_g), aspect which is consistent with the reported literature [Zheng and Chow, 2009]. It is a known fact that the T_g of an amorphous compound is usually sensitive to the presence of the foreign molecules if significant physical host–guest molecules interactions can be observed. In this sense, one may observe that in the inclusion compounds the T_g of HP–βCD shifts significantly to different temperature domains and broadens its intensity. The displacement and broadening of the T_g corresponds to an increase in amorphous phase upon complexation. Furthermore, the melting peak of pure HES disappears and a new endothermic peak appears in the inclusion compound, slightly overlapping with the T_g,

indicating possible inclusion complex formation or an interaction between HES and HP–βCD molecule in solid state due to the formation of a new amorphous phase [De Paula et al., 2007].

Characterization of inclusion process by MS analysis

Prior to the analysis of inclusion complex, was performed the analysis of diosmin in the mass range 100-700 m/z. We noticed the presence of signal 607.8 corresponding to the total mass. MS/MS evaluation at a collision energy of 75eV produced further fragmentation, so fragments are observed at 284.76, 5-hydroxy-2-(3-hydroxy-4-methoxyphenyl)-4H-1-benzopyranone, and 175.75 (theoretical mass 176), 5-hydroxy-1-benzopyranone. With a larger error D-glucopyranosyl was identified at mass 149.67 (d = -2.67) [Colombo et al., 2008].

Discussions

The modifications in UV spectra can indicate the possibility of inclusion complex formation. The maximum absorbance shift can be attributed to the HES transfer or to a part of its molecule from the solution to the cyclodextrin cavity, with the formation of weak interactions (hydrogen type bonds, van deer Waals forces, hydrophobic interactions, etc) [Corciovă et al., 2015a; 2015b].

IR spectroscopy can provide information on the formation of complexes because the specific absorption bands of the free components are generally shifted or their intensities are modified [Roik and Belyakova, 2011].

In case of HES-β-CD inclusion compounds, concerning the 1800-1500 cm^{-1} interval, a new shoulder at 1733 cm^{-1} was observed due to the presence of HES into cyclodextrin cavity. The vibration absorption bands of the C-O-C bonds in the range 1200-1030 cm^{-1} are extended in the inclusion complex as compared to HES and β-CD spectra. Furthermore, the absorption bands of the C=C bonds from phenyl rings of HES are slightly shifted at 1609, 1593 and 1574 cm^{-1}.

The fitting curves of the FTIR spectra of the complexes obtained by different techniques are similar and the disappearance of the band of OH groups from β-CD cavity was observed. The complexes presented 4 shifts of lower intensity in the fitting curves at 3416, 3320, 3221, 3083 cm^{-1} due to the intermolecular bonds of primary hydroxyls and intramolecular secondary hydroxyls of the glucopyranosyl units. In addition, the complexes presented an up-shifted peak at 3253 cm^{-1} and a down-shifted peak at 3201 of the hydroxyl groups connected to the different phenyl rings. The intense peak at 3462 cm^{-1} was attributed to the stretching vibrations, at high-frequency, of the C=O group at 1731 cm^{-1} (2 x 1731 = 3462 cm^{-1}). All these results confirmed the formation of the inclusion complex between β-CD and HES by any method of preparation [Corciovă et al., 2015a].

In the FTIR spectra of the inclusion complexes, the specific bands of HP-βCD and HES are modified and reduced in their intensities due to the decreasing of the number of water molecules involved in the hydrogen bonding formation and maybe due to the formation of hydrogen bonds between HES and cyclodextrin, suggesting that the water molecules from the cavity of cyclodextrin were replaced by HES. This observation is sustained by the relevant changes in the deconvoluted FTIR spectra of L inclusion complex in 3600-3000 cm^{-1} interval.

For the deconvoluted spectra of inclusion complexes, it can observe 8 sub-bands that reflect the contribution of HP-βCD and HES compounds in their structures. In the case of the

deconvoluted FTIR spectra of HES-HP-βCD inclusion complexes, the OH vibrations caused by the activation lead to different interactions and also to a connectivity of the involved bonds, which was materialized into a transition to a higher frequency, which demonstrates a less structured compound. The presence of the stretching vibrations of C-O-C in 1250-1300 cm^{-1} interval is indicated by a relative extended and decreased intensity in the inclusion complexes (due to the asymmetric stretching vibrations of C–O–C group). It should be noted that, also, the band from 1277 cm^{-1} which is attributed to C=O group from HES is present in a decreased intensity, as compared with those of HES showing that the C=O group is present in a different environment. The bands from 1200-1250 cm^{-1} interval are assigned to the stretching and deformation vibrations of the ether units. The changes in the vibrational frequencies could be attributed to the decreasing of the inter-atomic bonds as a consequence of an altered environment that induces the formation of new intermolecular hydrogen bonds (as an example between HES and HP-βCD). Also, monitoring the characteristic bands for the vibrations located at around 1340 cm^{-1} and around 1370 cm^{-1} from the FTIR deconvoluted spectra, we can observe the appearance of some changes that seems to indicate the formation of a new supramolecular structure [Corciovă et al., 2014].

In case of diosmin, FTIR spectrum of co-precipitation sample is distinguished by the asymmetry of signal 3410 cm^{-1} that masks OH groups on the surface of the substrate, the shift to 3410 cm^{-1} being also associated with the hydrogen bonds that were created inside the complex. The presence of diosmin is further confirmed by the characteristic signals, 1661, 1610 1501, 1448 cm^{-1}. The substrate is still highlighted by the intense band at 1070 cm^{-1} [García et al., 2014]. Co-evaporation is similar to the co-precipitation sample, but the absorption maxima from 1660 to 1448 cm^{-1} show a lower intensity, confirming the inclusion.

The ^{1}H-NMR spectra and the chemical shifts of the interest signals for HES in the absence and presence of cyclodextrins indicate an inclusion phenomenon of the -Ph-OCH_3 part into cyclodextrin cavity.

Since HES possesses two glycosidic entities, one must not exclude the possibility of their coupling to the hydrophilic exterior of the β-CD, *via* OH groups, by hydrogen bonds formation, while the rest of the molecule enters the β-CD cavity demonstrated, also, by ^{1}H-NMR and FTIR methods.

The complexation phenomenon is generally evidenced by the disappearance or decrease in intensity of the guest molecule melting peak in the inclusion compound. This aspect was observed by DSC, accompanied by a displacement of the inclusion compounds melting peaks compared to that of the pure uncomplexed guest molecule [Şamli et al., 2014].

In the MS spectrum of the diosmin inclusion complex formed by coprecipitation method, ion 2119.94 is distinguished. It can be an adduct (diosmin + HP-β-CD) which has theoretical mass 2071 at equimolar ratio. For further confirmation, a residual signal is present at 606.9 that belong to residual diosmin. High intensity is determined by the higher ionization ability of the substance than of the complex. Similarly, in case of complex formed by co-evaporation method, the signal with mass 2137 is distinguished, whose probability is associated with the mass of theoretical complex 2071. The difference may be associated with Na adduct, since sodium acetate (20 mM) and methanol (50:50) is the dispersion medium [García et al., 2014].

Conclusions

Inclusion complexes of HES with β-CD and HP-βCD were prepared by co-evaporation, kneading and lyophilization. Also, inclusion complexes of diosmin and HP-β-CD were prepared by co-evaporation and co-precipitation. The molar ratio used was 1:1.

The complex formation has been studied by different methods. In case of HES the inclusion complex formation was demonstrated by ^{1}H-NMR, FTIR, DSC and UV-Vis methods and in case of diosmin were used FTIR and MS analysis. The complexes obtained have physico-chemical characteristics differentiating them from parent substances.

The results obtained by different characterization techniques clearly indicate that the methods used for preparation lead to formation of complexes between HES/diosmin and cyclodextrins used. The preliminary data allows to hope that complexation of HES/diosmin with cyclodextrins will lead to a better biological efficacy.

1. 2. 4. Cyclodextrin drug inclusion complexes: *in vitro* approache

Materials and methods

Materials: For analyses were used the inclusion compounds obtained and characterized in previous chapter, compared to parent substances.

In vitro studies: In vitro analysis includes dissolution studies, testing the antioxidant activity and the antimicrobial activity.

In vitro dissolution studies of HES/diosmin and their inclusion compounds with cyclodextrins were carried out using an USP paddle apparatus. Samples containing the same amount of HES/diosmin (free and inclusion compounds) were transferred in dissolution medium, simulated gastric fluid - 0.1 N hydrochloric acid solution, pH 1.2 and simulated intestinal fluid - phosphate buffer, pH 6.8, and then stirring at 100 rpm and at a temperature of 37 ± 0.5 ^{0}C. Aliquots were withdrawn at fixed time intervals in 10-120 minutes range, by replacing each withdrawn aliquot with the same volume of dissolution medium. The HES/diosmin concentration was determined by UV measurements at 286 (HES) / 257 (diosmin) nm against a blank containing the used medium. Suitable constructed standard curves were used. The dissolution diagrams were drawn by plotting mean values of cumulative dissolution of HES/diosmin *vs* time [Samal et al., 2012; Corciovă et al., 2014; 2015a; 2015b].

In vitro antioxidant activity of the prepared compounds *vs* HES and cyclodextrins was evaluated by three methods: determination of the inhibition of lipoxygenase activity (modified Malterud method), 2, 2 diphenyl-picryl-hydrazyl (DPPH) radical scavenging activity and determination of the reducing capacity. In case of diosmin was used only the method of the inhibition of lipoxygenase activity [Corciovă et al., 2014; 2015a].

Determination of free radicals scavenger activity – DPPH (2, 2 diphenyl-picryl-hydrazyl) assay [Malterud and Rydland, 2000]: Briefly - sample (HES, β-CD, HP-β-CD and their inclusion compounds solutions in DMSO) was treated with 4 mg % DPPH methanolic solution and after 10 minutes the absorbance's degree of decrease was determined at 517 nm. The scavenger activity was calculated according to equation 8:

$$\%\ \text{scavenger activity} = \frac{(A_{DPPH} - A_{DPPH\text{-}S})}{A_{DPPH}} \text{x}\, 100 \quad (8)$$

where: A_{DPPH} - DPPH absorbance; $A_{DPPH\text{-}S}$ - DPPH absorbance treated with sample after 10 minutes.

For samples that achieved 50 % scavenger capacity the IC_{50} value was calculated, expressed in mM HES in final solution.

Determination of the reducing capacity [Oyazu, 1986]: Briefly, the sample solution (HES, β-CD, HP-β-CD and their inclusion compounds) was treated with pH 6.6 phosphate buffer and 1 % potassium ferricyanide. The mixture was maintained for 20 minutes at 50 ^{0}C, cooled, treated with 10 % trichloroacetic acid, after which the supernatant was separated and treated with 0.1 % $FeCl_3$ and the absorbance was recorded at 700 nm. For samples where the final solution absorbance was above 0.5, the IC_{50} value was determined. The reducing capacity was calculated based on a formula that uses the absorbance and the concentration of the solutions that showed the absorbance just under 0.5 and over 0.5.

Determination of the inhibition of lipoxygenase activity [Hatano et al., 1988] – Briefly, a specific quantity of pH 9 borate buffer lipoxygenase was treated with sample solution (HES/diosmin, β-CD, HP-β-CD and their inclusion compounds) in DMSO, then, after 10 minutes at room temperature linoleic acid in 0.16 mM borate buffer pH 9 were added. The absorbance was recorded at 234 nm. The ability to inhibit the lipoxygenase activity was calculated according to equation 9:

$$\%\ \text{activity} = \frac{(A_E - A_{EI})}{A_E} \text{x}\, 100 \quad (9)$$

where: A_E - the difference between the absorbance of the enzyme without inhibitor at second 90 and the absorbance of the same solution at second 30; A_{EI} - the difference between the absorbance of the enzyme with inhibitor at second 90 and the absorbance of the same solution at second 30.

For samples that achieved 50 % inhibition activity, the IC_{50} values were calculated and are expressed in mM HES/diosmin in final solution.

The qualitative antimicrobial assay of the samples was carried out by the agar diffusion method [Danciu et al., 2014; Valnet et al., 1978]. The method can be useful for the initial screening of the compounds' antimicrobial activity.

The samples taken into work were: HES/diosmin, β-CD, HP-β-CD and their inclusion compounds solutions in DMSO.

The test organisms in case of HES and its inclusion compounds were *Staphylococcus aureus (S. aureus)* ATCC (American Type Culture Collection) 25923, *Escherichia coli* (*E. coli*) ATCC 25922 and *Candida albicans* (*C. albicans*) ATCC 10231.

The test microorganisms in case of diosmin and its inclusion compounds were: *S. aureus* ATCC 25923, *S. lutea* ATCC 9341, *B.cereus* ATCC 14579, *C. glabrata* ATCC MYA 2950, *C. parapsilosis* ATCC 22019.

The antimicrobial tests were carried out using a suspension containing the overnight culture of bacteria (~ 10^8 cfu/ml) and yeast (~ 10^7 cfu/ml). The cultures were inoculated in soft

Mueller Hinton agar (for bacteria) and Sabourand maltose agar medium (for *C. albicans*). The molten agar containing the microbial culture was transferred in a sterile Petri dish. Wells of 6 mm in diameter were placed on the surface of previously seeded agar plates and were filled with 100 µl samples. The bacterial growth inhibition zones around wells were measured after overnight incubation at 37 ^{0}C. The antibacterial activity of the investigated compounds was compared with the inhibition zone obtained with a 25 mg ampicillin disc and 30 mg chloramphenicol disc, placed on plates at the same time as the samples. Antifungal activity was compared to a 100 mg nystatin disc. Each microorganism was tested in triplicate [Corciovă et al., 2014; 2015a; 2015b].

Results

- ***In vitro dissolution studies***

The dissolution profiles of free HES and its inclusion compounds with β-CD and HP-β-CD prepared by different methods are presented in a specific period of time (120 minutes) and under physiological conditions, such as simulated gastric fluid pH 1.2 (Figure 1.11. a, 1.11. b) and simulated intestinal fluid pH 6.8 (Figure 1.11. c, 1.11. d).

In vitro dissolution profile of diosmin and inclusion compounds in the same condition is represented in Figure 1. 12.

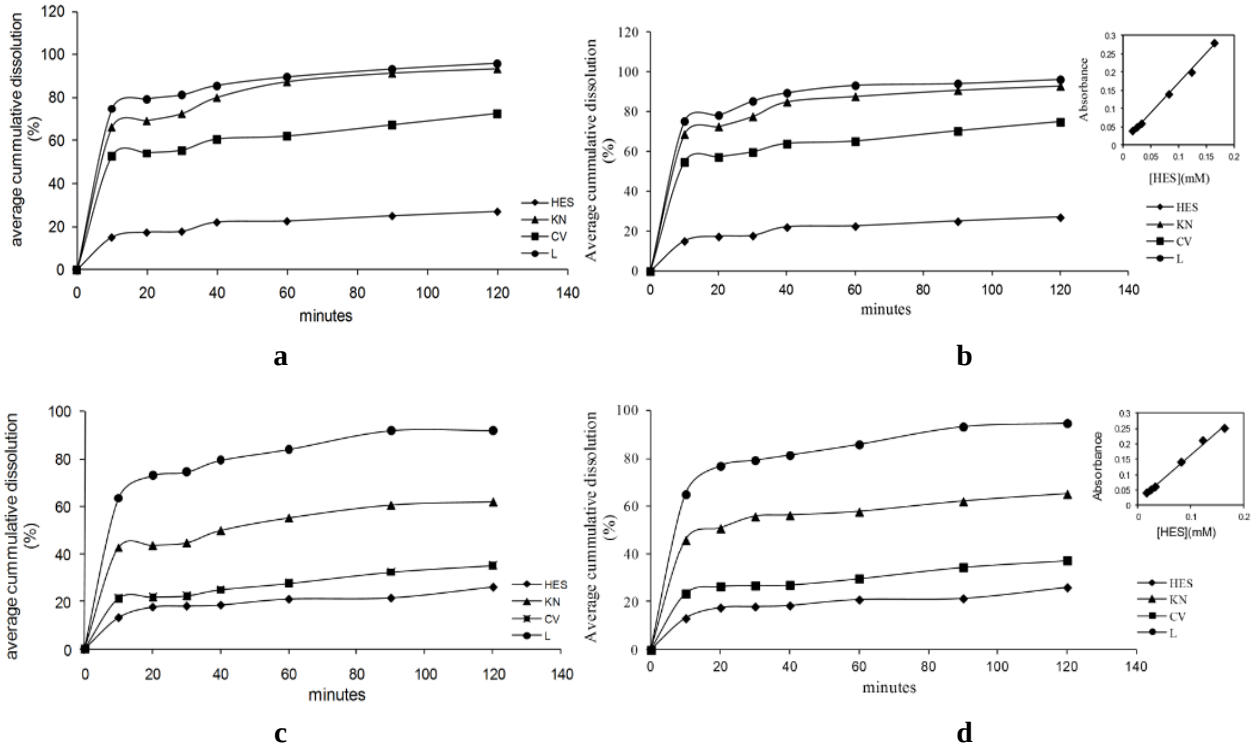


Figure 1.11. *In vitro* dissolution profile of HES and its inclusion compounds, depending on time, with:
a. β-CD, **b.** HP-βCD in 0.1 N HCl, Inset: HES etalon curves in 0.1 N HCl
c. β-CD, **d.** HP-βCD in phosphate buffer, Inset: HES etalon curves in phosphate buffer at pH = 6.8

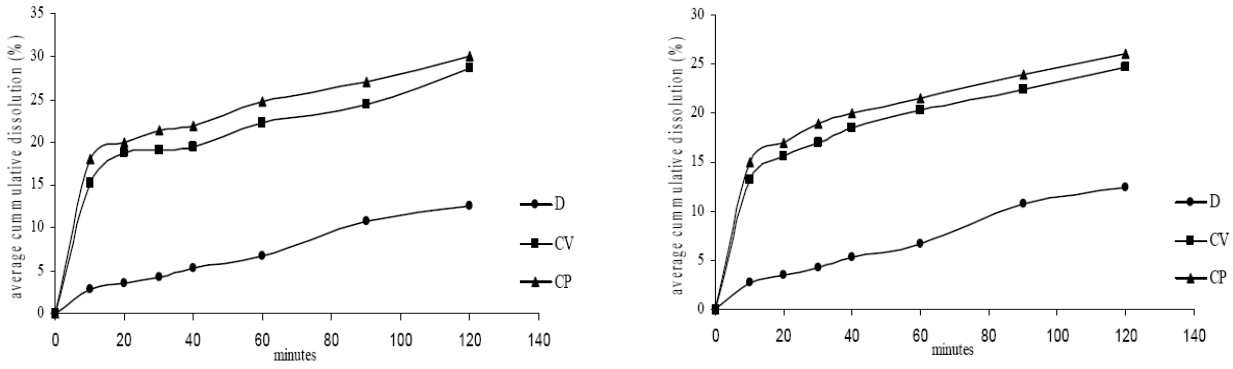


Figure 1. 12. *In vitro* dissolution profile of diosmin and inclusion compounds, at **a.** pH 1.2 and **b**. pH 6.8

All inclusion compounds had an improved dissolution rate compared to that of parent HES/diosmin.

It should be mentioned that the enhancement of the solubility of HES depends on the method applied for obtaining inclusion complexes: the best dissolution profile was recorded for lyophilized sample, followed by kneading and co-evaporation samples. In both cases of cyclodextrins, the best rate of dissolution was observed for the lyophilization method [Corciovă et al., 2014; 2015a].

In case of diosmin, it can be observed that the profile of dissolution has similar values for both types of preparation methods [Corciovă et al., 2015b].

For both cases, HES and diosmin inclusion complexes, the best rate of dissolution was observed in acid medium.

- ***Antioxidant activity***

First method is based on the reduction of DPPH radical (purple) to diphenyl - picrylhydrazine (yellow) by HES and cyclodextrins which contain hydroxyl groups, with a progressive reduction of the absorbance at 517 nm.

The DPPH radical scavenging activity expressed in percentage of HES/cyclodextrin inhibition, and their prepared compounds, is presented in Figure 1. 13. DPPH radical scavenger activities and reducing capacity were tested at seven different concentrations, in range 0.5118 – 32.756 mM, for each sample, using as control HES and cyclodextrins.

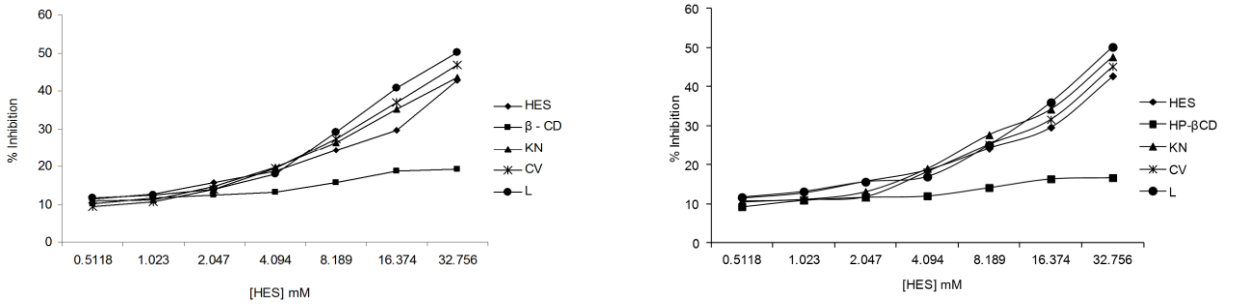


Figure 1. 13. DPPH radical scavenging activities of tested compounds, as a function of HES concentration

An increase in HES's scavenger capacity with increasing its concentration and an improvement in the antioxidant properties of the inclusion complexes can be observed, in all cases. The IC_{50} values are presented in Table 1. 7.

Table 1.7. IC_{50} values (mM) for the analyzed compounds, by DPPH radical scavenging method

Complex	KN	CV	L	β-CD	HP-βCD	HES
HES-β-CD	-	-	3.233 ± 0,171	-	-	-
HES-HP-β-CD	-	-	3.251 ± 0.12	-	-	

The IC_{50} value could be calculated only for the inclusion compound obtained by lyophilization, because for the other samples the maximum concentration taken into work (32.756 mM) did not demonstrate an inhibition greater than 50 %. Thus, we can say that, the antioxidant activity of HES improved after the interaction with cyclodextrin, by this antioxidant method, only in case of lyophilization method.

Another method used for antioxidant activity was the determination of reducing capacity. This method is based on the ability of the substances to reduce the potassium ferricyanide to potassium ferrocyanide which reacts with the ferric ion to form a blue colored complex with maximum absorbance at 700 nm.

Figure 1. 14. presents the recorded absorbance depending on samples concentration. For this method we used seven different concentrations, in range of 0.5118 – 32.756 mM.

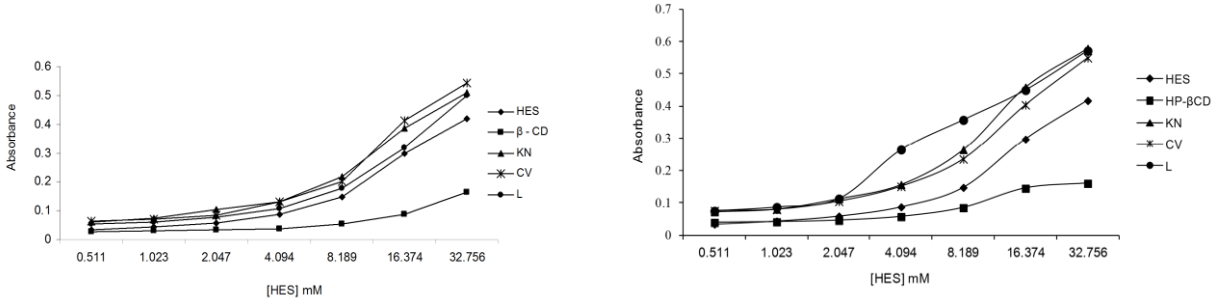


Figure 1. 14. Determination of reducing ability of the investigated compounds, as a function of HES concentration

In this case the HES from the inclusion complexes showed a more powerful antioxidant activity compared to free HES. This increase in the reducing capacity could be explained by the increment of HES's solubility after its inclusion into cyclodextrin cavity [Gharibzahedi et al., 2014]. Table 1. 8. presents the IC_{50} values for the analyzed compounds, by determination of reducing ability method.

Table 1. 8. IC_{50} values (mM) for the analyzed compounds, by determination of reducing ability

Complex	KN	CV	L	β-CD	HP-βCD	HES
HES-β-CD	3.567 ± 0.016	3.123 ± 0.037	3.547 ± 0.046	-	-	-
HES-HP-β-CD	2.501 ± 0.021	3.088 ± 0.011	2.627 ± 0.015	-	-	-

For the same concentrations of the parent substances, as in complexes, the 0.5 value of the absorbance was not reached and thus we could not calculate the IC_{50} value. For all complexation methods, the formed complexes gave absorbances over 0.5 which allowed the determination of IC_{50}. The complexes obtained with HP-β-CD have a better ability to reduce the ferric ion.

The capacity of inhibition of lipoxygenase activity was used both for HES and diosmin inclusion compounds. This method consists in the ability of HES/diosmin and its inclusion compounds to block the action of lipoxygenase which catalyses the oxidation of linoleic acid. The assay was performed using a spectrophotometric method at five different concentrations which varied between 0.5118-8.189 mM for each sample contains HES and in 312.5-10000 μg/ml range for diosmin, using as control HES/diosmin and cyclodextrins.

Figure 1. 15. presents the capacity of inhibition of lipoxygenase activity for HES, cyclodextrins and their prepared compounds and Figure 1. 16. shows the ability of diosmin samples to inhibit the activity of lipoxygenase, depending on the concentration.

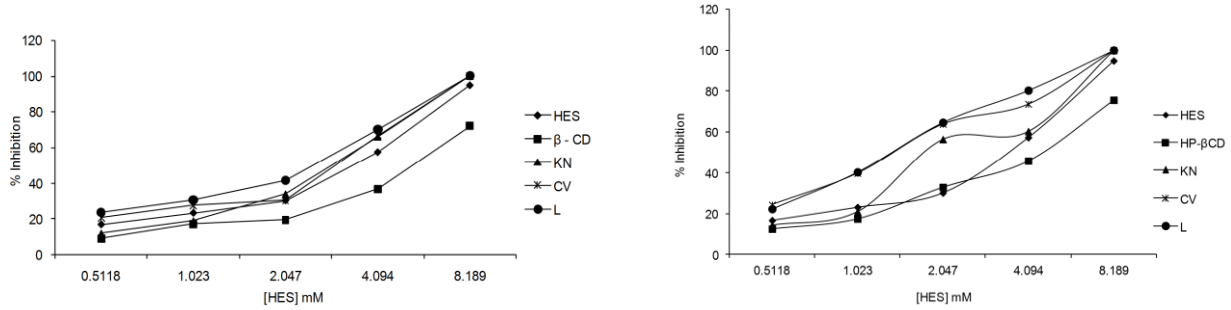


Figure 1. 15. The capacity of inhibition of lipoxygenase activity presented by HES, cyclodextrins and their complexes as a function of HES concentration

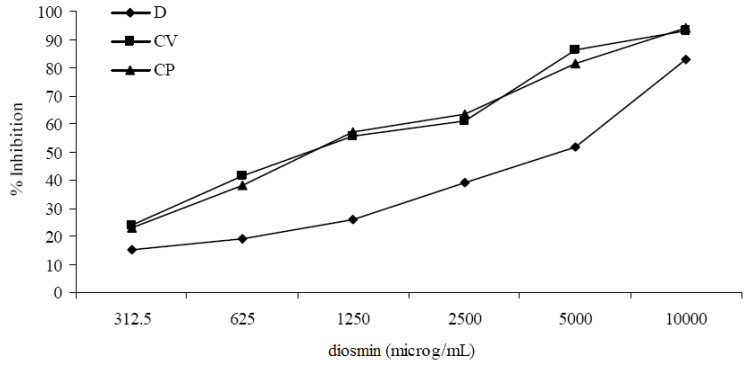


Figure 1. 16. The capacity of inhibition of lipoxygenase activity presented by diosmin and their complexes as a function of diosmin concentration

Lipoxygenase activity is influenced by the increase in HES/diosmin concentration. It can be seen that the HES/diosmin included into the cyclodextrin cavity shows a more pronounced capacity to inhibit the lipoxygenase compared to parent substances. This can be explained by the fact that the OH groups of HES/diosmin and cyclodextrins block the iron ions, knowing that the action of the enzyme depends on the oxidation state of iron which is involved in the redox reaction [Rackova et al., 2007].

In case of HES, Table 1. 9. presents the IC_{50} values for the analyzed compounds, by inhibition of lipoxygenase activity method.

Table 1. 9. IC_{50} values (mM) for the analyzed compounds, by inhibition of lipoxygenase activity method

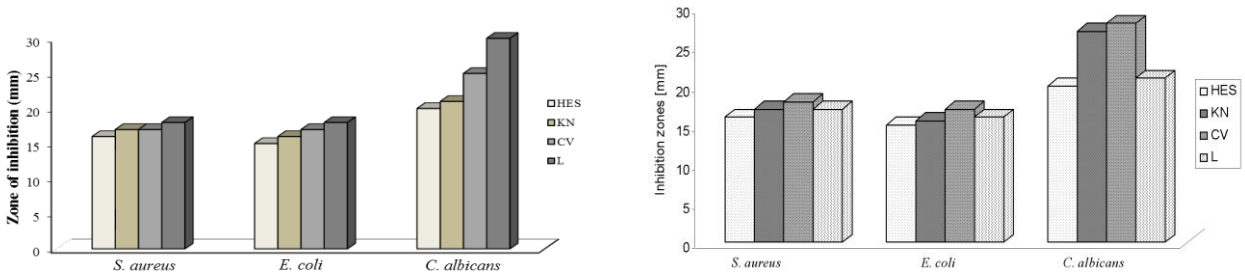
Complex	KN	CV	L	β-CD	HP-βCD	HES
HES-β-CD	0.0484 ± 0.69	0.0495 ± 0.66	0.0422 ± 0.74	0.088 ± 1.40	-	0.0565 ± 0.80
HES-HP-β-CD	0.0301 ± 0.55	0.0229 ± 0.46	0.0224 ± 0.02	-	0.0314 ± 0.33	

By comparing the results obtained for HES, the inclusion complexes have better inhibitory activity, as reflected by the lower IC_{50} value. The use of HP-β-CD and β-CD determine the improvement of lipoxygenase inhibition exerted by HES.

In case of diosmin, the capacity to inhibit lipoxygenase increased from 76.50 ± 3.89 μg/ml for free diosmin to 15.73 ± 0.57 μg/ml (co-precipitation) and 16.106 ± 0.586 μg/ml (co-evaporation), showing a 4-fold increase in activity in case of diosmin inclusion in HP-β-CD. Between the inclusions compounds obtained, the differences are insignificant.

- ***Antimicrobial activity***

HES/diosmin and their inclusion compounds were tested for antimicrobial activity against some Gram negative and Gram positive bacteria and against some fungus, the results being presented in Figure 1.17. for HES and its inclusion compounds and in Figure 1. 18. for diosmin and its inclusion compounds.



a **b**

Figure 1. 17. Zone of inhibition (mm) for HES and its inclusion compounds with **a.** β-CD and **b.** HP-β-CD

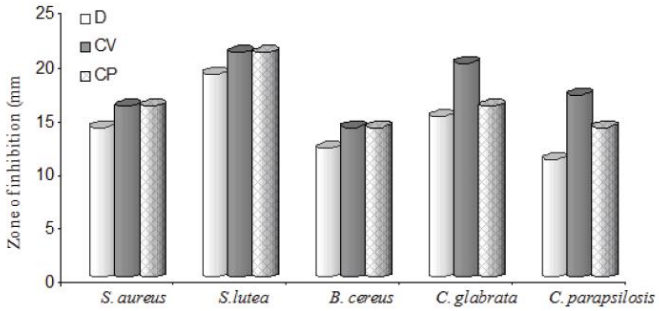


Figure 1. 18. Zone of inhibition (mm) for diosmin and its inclusion compounds

When comparing the antimicrobial activity of inclusion compounds *vs* free HES/diosmin, against all tested microorganisms, the inclusion compounds showed higher activity

Depending on the microorganisms tested, the antifungal activity was higher than antibacterial activity, in case of HES and its inclusion compounds [Corciovă et al., 2014; 2015a].

In case of diosmin and its inclusion compounds, the antifungal activity was influenced by the method of preparation, as demonstrated by the size of the inhibition zone [Corciovă et al., 2015b].

Discussions

- ***In vitro dissolution studies***

In vitro dissolution studies are important in both quality control purposes and drug development because they provide important information about the percent of HES/diosmin dissolved from inclusion compounds in a specific time and under physiological conditions, like simulated gastric fluid and simulated intestinal fluid.

The dissolution rate was higher for the compounds obtained with HP-βCD, compared to those obtained with other cyclodextrins. HP-βCD exhibits surfactant properties, being hydrophilic on the external surface, resulting in a decrease of the interfacial tension between HES/diosmin and the dissolution medium, thus results an improvement in the dissolution rate. Also, the HP-βCD has a higher solubility (generated by the presence of hydroxypropyl radical) than the one of βCD or sulfated-βCD, yielding to more soluble inclusion compounds. Increasing the rate of dissolution in an acid medium can lead to an increased release of HES/diosmin and to an improved bioavailability of the compound at gastric level [Corciovă et al., 2014; 2015a; 2015b].

- ***Antioxidant activity***

The 2,2-diphenyl-1- picril-hydrazyl radical is reduced using compounds which can yield the hydrogen atoms of the hydroxyl groups. In this case, both HES and β-CD have OH groups, but hydrogen atoms' mobility is much less than that of other compounds with known reducing properties such as polyphenol carboxylic acids. HES:β-CD inclusion complexes determine the improvement of their reducing properties, but to a lesser extent compared with the observed effects in other antioxidant tests. When HES is conjugated with HP-βCD, hydrogen bonds can be formed and these weaken the covalent bonds between hydrogen and oxygen from the OH groups, which in turn would make the donation of hydrogen from the HES's OH groups more easy [Gharibzahedi et al., 2014].

The reduction of ferric ion is performed using proton and electron donor groups such as hydroxyl group from HES and in combination with β-CD and HP-β-CD, the ability to release electrons and protons is improved, as evidenced by the increase in the reducing capacity compared to the basic compound. Reduction of ferric ion to ferrous ion is important in the biological environment because it has oxidant capacity and causes oxidant species of which the most important are the oxygen reactive species.

15-lipoxygenase is a heminic enzyme from oxidoreductases class which is widely distributed in the body. The enzyme is capable of catalyzing the oxidation of unsaturated fatty acids to form lipid peroxides that have high chemical reactivity and can enhance the oxidation

reactions of other molecules with biological role, such as proteins, nucleic acids and other lipid compounds. Studies have shown that excessive activity of this enzyme could be involved in the occurrence of pathological processes such as atherosclerosis, diabetes, inflammatory phenomena [Stavniichuk et al., 2010]. This enzyme has an important role in the biosynthesis of leukotrienes which are involved in numerous inflammatory and allergic diseases. The products resulting from the lipoxygenase activities like hydroperoxyeicosatetraenoic acid, hydroxyeicosatetraenoic acid, leukotrienes and lipoxins are involved in the development of diseases such as rheumatoid arthritis, psoriasis, asthmatic reactions and glomerular nephritis [Kumaraswamy and Satish, 2008]. Enhanced activity of the enzyme at cerebral level increases the size of amyloid plaques in Alzheimer's disease [Yang et al., 2010]. Enzyme contains iron and the catalytic activity depends by the reversible transformation of Fe^{3+} to Fe^{2+}. Hydroxyl groups from complexes have the ability to block the reversible transformation of iron and finally will reduce or block enzyme activity.

The analyzed compounds' lipoxygenase inhibitory effect can be explained through the prevention of the substrate's access (linoleic acid) to the enzyme's active center given the bigger size of the inclusion compounds compared to HES/diosmin.

- ***Antimicrobial activity***

Regarding the inclusion complexes with HES, the tested compounds, obtained by different techniques, presented a similar pattern for antibacterial activity *S. aureus* and *E. coli* but a higher activity was recorded against *C. albicans.* The differences between antimicrobial activities depending on microorganisms tested can be explained by the bacterial wall composition. The cell wall of *E. coli* consists of an outer layer of phospholipids, liposaccharides and lipoproteins while the inner layer consists of a thin layer of peptidoglycan which makes it harder to be permeated by HES. The cell wall of *C. albicans* contains perpendicularly aligned polysaccharidic fibrils with a hydrophobic surface and two classes of proteins that are covalently attached to that network of structural fibrillar polysaccharides. This structure probably makes it easy to be penetrated by HES [Chaffin, 2008]. In case of diosmin and its inclusion compounds, the best activity was observed against *S. lutea*.

Also, the method of preparation affects the complexes' solubility therefore influencing HES/diosmin's availability to the tissue. In the case of inclusion compounds of HES with β-CD the antibacterial activity decreased in the following order: lyophilization > co-evaporation > kneading and in the case of the complexes of HES with HP-βCD the best activity was obtained by co-evaporation. In case of diosmin, the inclusion compounds obtained by co-evaporation showed an increased activity. This may be attributed to the enhanced solubility of the inclusion compounds, so HES/diosmin could be more available for membrane transportation and become more available for specific tissues and could present an increased antibacterial potential [Panda and Singh, 2014].

Conclusions

The complexes were tested *in vitro* compared with the parent substance by dissolution test in mediums similar to physiological conditions, antioxidant and antimicrobial activities.

The dissolution profile increased in both mediums (simulated gastric and intestinal), being higher in case of acid medium.

In all cases, determination of the antioxidant activity of the analyzed compounds is proportional with their concentration level. The antioxidant activity of the prepared compounds increased after the formation of inclusion compounds, probably due to the increasing solubility of the compounds and therefore increasing the ability to inhibit the lipoxygenase activity, to trap free radicals and to reduce the ferric ions.

The antimicrobial activity for the inclusion compounds obtained proved to be more efficient than parent substances, also due probably to improved solubility, thus increasing the amount of compound that crosses the microbial membrane. The differences between antibacterial and antifungal activity can be explained by the different composition of the cell wall.

In vitro studies demonstrated that the inclusion compounds' antibacterial, antioxidant and dissolution profiles were enhanced, depending on the method of inclusion or type of cyclodextrins, thereby increasing the possibility of using the inclusion compounds in various pharmaceutical preparations in order to improve pharmacological effects.

1. 3. Design and characterization of inclusion complexes with antifungal agents

The largest and most commonly used classes of antifungal agents are imidazole. Propiconazole nitrate (PCZH-NO_3), a propiconazole derivative, is a synthetic antifungal agent with action against pathogenic yeasts and with a low toxicity compared to azole drugs. Its disadvantage is low solubility, which also leads to low bioavailability. Thus, it has been proposed to prepare, characterize and test new host-guest systems using sulfated-β-CD, SBE7-β-CD and MCT-β-CD [Minea et al., 2016].

Materials and methods

Materials: For obtaining inclusion complexes with PCZH-NO_3 were used cyclodextrins like: sulfated-β-CD (β-CD-SNa), SBE7-β-CD, and MCT-β-CD.

Characterization of inclusion compounds: After the synthesis of inclusion compounds by the freeze-drying method, ^{1}H-NMR, 2D Roesy NMR and DSC were used for physico-chemical characterization. Also, the antifungal activity of the inclusion compounds were tested on some clinical isolates such as *C. albicans* and *C. glabrata,* and the minimum inhibitory concentrations (MICs) were determined. The methods and the results are described in paper [Minea et al., 2016].

The *in vitro* dissolution profiles of the inclusion complexes prepared and characterized were determined in the conditions which were described in Chapter 1.2.4.

Results

The dissolution rate profiles of the inclusion compounds compared to the pure drug were represented graphically by plotting the cumulative amount of PCZH-NO3 dissolved *vs.* time (minutes) (Figure 1. 19.).

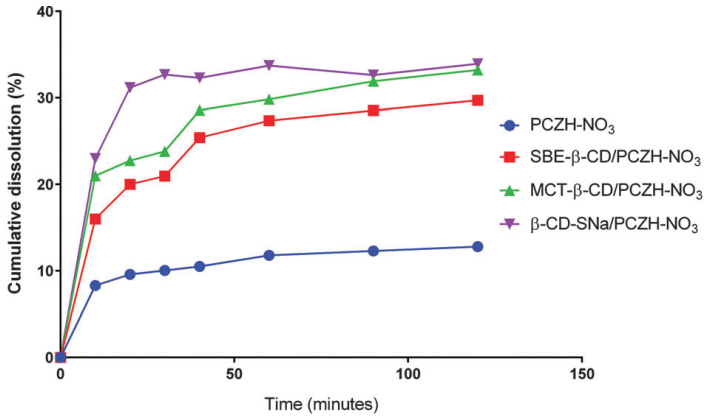


Figure 1. 19. The dissolution profile of the free drug PCZH-NO_3 compared to the inclusion complexes: SBE7-β-CD/PCZH-NO_3, MCT-β-CD/PCZH-NO_3 and β-CD-SNa/PCZH-NO_3, at pH 6.8

The dissolution studies in physiological medium with pH = 6.8 showed an improved dissolution rate of PCZH-NO_3 in the inclusion complexes compared to the free drug [Minea et al., 2016].

Discussions

One of the current trends in public health is the development of new systems to combat fungal infections, knowing that resistance rates are rising. The bioavailability of a substance can depends by some factors, among which the properties of formulation type.

Dissolution studies are routine tests in the pharmaceutical industry to effectively assess the *in vitro* and *in vivo* behavior of tested compounds. So, the dissolution profiles were performed on PCZH-NO_3 and its inclusion compounds with different cyclodextrins.

In all cases of inclusion complexes the dissolution profile increases which can be explained by the influence of cyclodextrins on drug wettability, reducing the interfacial tension between sample and dissolution medium [Rubim et al., 2017], particle size reduction, formation of inclusion complexes with a higher solubility in water. The interactions between the hydrophobic part of PCZH-NO_3 and the apolar cavity of cyclodextrins determine its transfer into the cavity, thereby increasing its affinity for water and thus increasing dissolution [Dua et al., 2011].

The dissolution depends on cyclodextrin used and decreases in the following order: β-CD-SNa/PCZH-NO_3 > MCT-β-CD/PCZH-NO_3 > SBE7-β-CD/PCZH-NO_3 > PCZH-NO_3. This can be explained by a better interaction between PCZH-NO_3 and cyclodextrin. The influence of cyclodextrin type on dissolution rate underlines the importance of appropriate choice of carriers.

Conclusions

Among others, the purpose was to improve the dissolution rate of PCZH-NO_3, an antifungal agent active against pathogenic yeasts and with a low toxicity compared to azole drugs, but with low solubility.

The prepared compounds presented a better dissolution profile compared to parent substance. The greatest improvement *in vitro* dissolution test was observed with the inclusion complex prepared with β-CD-SNa, so the cyclodextrin used played an important role in the dissolution behavior of the drug.

Taking into account these results, it can be said that the process of inclusion effectively enhanced the solubility of PCZH-NO_3, which consequently can increase its bioavailability and might improve its pharmaceutical potential.

1. 4. Design and characterization of multifunctional magnetic cargo-complexes with antioxidant agents

Another way with applications in medicine, which brings important contributions in an attractive research field is the obtaining of multifunctional magnetic cargo-complexes with active substances. The advantages of such a formulation include the production of nanoparticles, a relatively lower protein binding, higher efficacy of the loaded active substance, a controlled release of the bioactive substance, and the possibility of engineering the physical properties. By loading core-shell magnetic nanoparticles with active substances, the magnetic properties of the core can be combined with the biological activity of the active substance, offering both the possibility of orientation and the therapeutic action. In this context, the study proposed the synthesis and characterization of a core-shell-magnetic nanoparticles loaded with an inclusion complex such as protocatehuic acid (PCA, 3,4-dihydroxybenzoic acid)-SBE7-β-CD [Lungoci et al., 2019].

Materials and methods

Materials: used were PCA, polyethylenimine (PEI) and SBE7-β-CD

Methods: Initially were obtained - the inclusion compound SBE7-β-CD/PCA by lyophilization and magnetite nanoparticles (MNP) by co-precipitation; the next stage included coverage of MNP with PEI, resulting MPEI; the last step included on the one hand the loading of PCA in the shell of MPEI, resulting MPEI-PCA, and on the other hand loading the SBE7-β-CD/PCA in the shell of MPEI, resulting in MPEI-SBEβCD/PCA

For characterization of SBE7-β-CD/PCA inclusion compound were used ^{1}H-NMR and DSC analysis, for characterization of magnetic system were used: FTIR, X-ray diffraction (XRD) and Transmission electron microscopy (TEM), also, described by Lungoci et al. [Lungoci et al., 2019].

The antioxidant activity of functionalized magnetite nanoparticles was measured using DPPH (2,2-diphenyl-1-picrylhydrazyl) method already described in Chapter 1. 2. 4. and free radicals scavenger activity by ABTS assay. Briefly, ABTS method: to the sample taken into work is added ABTS radical generated by the reaction of 2,2'-azido-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt and persulfate potassium and the absorbance was registered at 734 nm, at 30 ^{0}C up to 6 minutes. Depending on antioxidant capacity, ABTS discoloration occurs and scavenger activity is calculated [Re et al., 1999].

Results

The steps in synthesis of multifunctional magnetic cargo-complexes are presented in Figure 1. 20.

$$Fe^{2+} + 2Fe^{3+} + 8HO^{-} \rightarrow Fe_3O_4 + 4H_2O$$

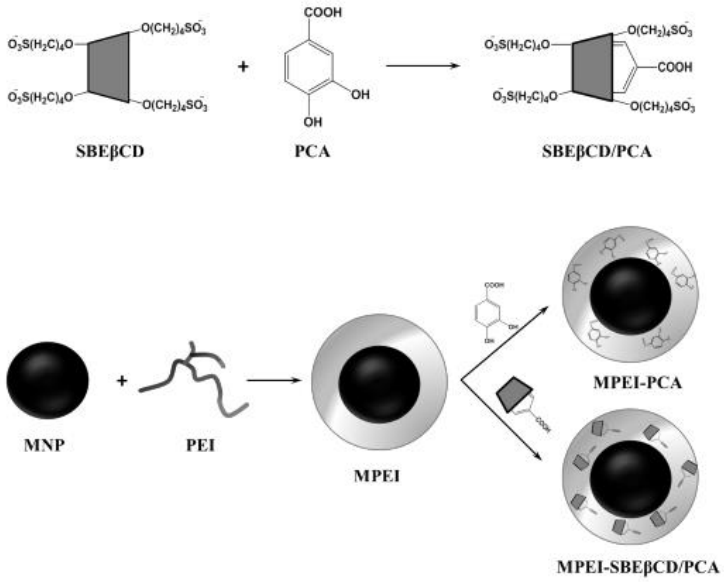


Figure 1. 20. Schematic representation of nanoconjugates synthesis [Lungoci et al., 2019]

The results for physico-chemical characterization of inclusion compound SBE7-β-CD/PCA, by ^{1}H-NMR and DSC were presented by Lungoci et al., observing the chemical shifts of the same protons in the inclusion compounds, and a decrease and a shift of the melting point of PCA.

For confirmation of crystalline nature of magnetite nanoparticles XRD analysis was used, observing the main reflection planes. The key features of FTIR spectra of MPEI, MPEI-PCA and MPEI- SBEβCD/PCA conjugates are presented in paper.

DSC curve for PEI presents a glass transition temperature which shifts in MPEI, MPEI-SBE-βCD. In DSC curves of MPEIPCA and MPEI- SBEβCD/PCA, beside the shift of glass glass transition temperature, is observed a decrease of melting point corresponding to PCA. The TEM analysis shows that the particles of MPEI-PCA and MPEI-SBEβCD/PCA conjugates are spherical, in 10-15 nm range and with aggregation tendency. These aspects are presented by Lungoci et al.

In order to measure free radical scavenging activity of free PCA, SBEβCD/PCA inclusion complex, MPEI-PCA and MPEI- SBEβCD/PCA, bleaching property of the DPPH (2,2-diphenyl-1-picrylhydrazyl) was used. A freshly prepared DPPH solution exhibits a deep purple colour with an absorption maximum at 517 nm which generally fades when antioxidant molecules reduce DPPH free radicals and convert them into a yellow product, resulting in a decrease in absorbance at 517 nm band. Optical absorbance values have permitted to calculate the % of inhibition for each used concentration of antioxidant which is presented in Figure 1. 21.

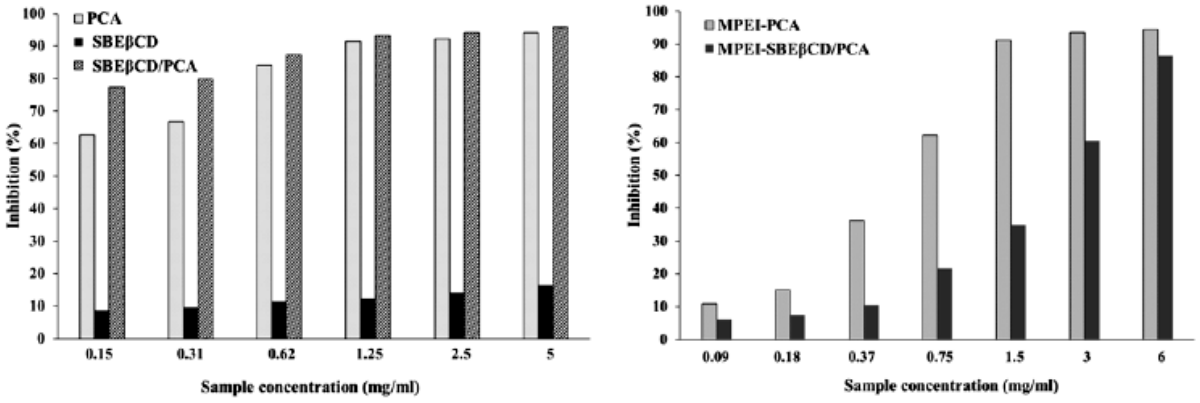


Figure 1. 21. Antioxidant activity of PCA in different formulations by DPPH assay

Free radical scavenging activity of samples was determined by ABTS radical cation decolorization assay. The results are shown in Figure 1. 22.

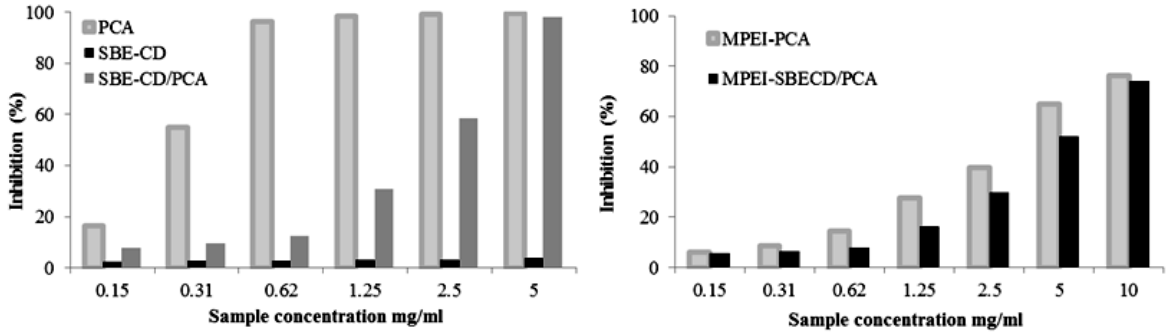


Figure 1. 22. Antioxidant activity of PCA in different formulations by ABTS method

Data obtained revealed that the both types of the loaded nanoparticles, containing different formulations of PCA (free form and its inclusion complex), presented scavenger properties and in a concentration dependent manner, the samples containing PCA showing more than 50 % scavenging activity, in the concentration range taken into work [Lungoci et al., 2019].

Discussions

After the preparation by co-precipitation of Fe_3O_4 magnetite particles, which represents the core of the therapeutic system, with the property of releasing the active substance target, must be coated with a biocompatible outer shell to stabilize them. So, was used PEI, that acts as a binding agent between hydrophobic substances and nanoparticles to make them hydrophilic and bioavailable. In order to obtain a very good functioning of the nanoparticles, PCA have been attached as inclusion compound with modified cyclodextrin. This determines the release of the active substance in two steps: in the first step, the inclusion complex is released from the PEI polymer that coating the nanoparticles, and in the second step the substance will be released from the cyclodextrin cavity at the desired site due to the magnetic properties of the core.

By recording the shift of the proton signals of the PCA it can be said that the substance is included in the cyclodextrin cavity in several possible orientations in a dynamic way and by the complementary shift of the proton signals of the cyclodextrin it is suggested a cyclodextrin

conformational effort in order to adapt the molecular geometry in a supramolecular state of its inclusion compound. Modification from the DSC heating curve of inclusion compounds demonstrates new solid phase formation by complexation. The main reflection planes from XRD can be indexed to the Fe_3O_4 inverse cubic spinel structure. By observed modification in DSC curves we can confirm the structures of prepared compounds for each step.

The PCA antioxidant property is well known and the results indicate that PCA exerted radical-scavenging action by both mechanisms of donating hydrogen atom and electron.

Apparently, the antioxidant capacity of MPEI-PCA is higher than for MPEI-SBEβCD/PCA but this is explained by the fact that PCA content is higher in MPEI-PCA than in MPEI-SBEβCD/PCA. This fact can be explained by different loaded formulation of PCA (when PCA is loaded as its free form as compared with its inclusion complex) inducing different entrapment/releasing mechanisms into/from PEI layer.

The small differences in the results obtained by the two methods used can be explained by the different principles: in the DPPH test an antioxidant react with a stable free radical, and in ABTS method a radical cation is scavenged by the antioxidant, so could be generate different interactions and thus behavioral variations [Lungoci et al., 2019].

Conclusions

The study describes the synthesis of a magnetic cargo-complex, in several stages: the obtaining of inclusion compound SBE7-β-CD/PCA and MNP, the obtaining of MPEI and the preparation of MPEI-PCA and MPEI-SBEβCD/PCA.

By using PEI as a shell of nanoparticles capable of loading inclusion complexes of chemically modified cyclodextrins with anionic groups such as SBEβCD can be a good strategy for using MPEI to load and transport other active principles that are unstable and insoluble in water and last but not least MPEIs are biocompatible and can be guided under the influence of a magnetic field at the desired site.

CHAPTER 2. RESEARCH ON THE ANALYSIS OF PHARMACEUTICAL PRODUCTS AND NATURAL BIOACTIVE COMPOUNDS

2. 1. Background

Ensuring the quality of medicines is one of the most important aspects of pharmaceutical domain, and the products must accomplish strict conditions on: quality, efficacy and safety.

For this purpose, the quality control of the raw materials, intermediate products, finished products, impurities, degradation products is a very important process and a variety of analytical procedures (titrimetric, spectrometric, chromatographic, electro-analytical, etc.), each with its advantages and disadvantages, can be used.

One of the quality parameters that must be analyzed is the quantification of the active substance, the pharmaceutical products being useful if they contain the amount of active substance writing on the package leaflet. Also, it should take into account that at various stages of development, transport, storage there is a risk of degradation of the substance with the appearance of impurities, degradation products that will need to be detected and quantified. For this reason, the choice of the analytical method plays an important role [Siddiqui et al., 2017].

Choosing the right method of analysis is a challenge for any analyst pharmacist who will need to take into account his knowledge of the physico-chemical properties of the drug, the quality parameters appropriate to each pharmaceutical form, the possibilities of the laboratory they are working in, etc.

On the other hand, it is well known fact that plant extracts contain a great number of pharmacologically active compounds which together form the phytocomplex. Each substance in the phytocomplex contributes to the overall therapeutic effect of the extract.

Thus, qualitatively and/or quantitatively compounds of different classes have been evaluated, such as: polyphenolic compounds, phytoestrogens and phytosterols.

Polyphenolic compounds constitute one of the largest, most widely spread and functionally important groups of secondary metabolites found in plants. These substances and flavonoids in particular, have attracted great interest due to their antioxidant capacity which confers them a valuable therapeutic potential in treating free-radical mediated diseases [Ivănescu et al., 2016]. Methoxilated flavones are valuable bioactive compounds with numerous therapeutic properties, mainly antitumor, anti-inflammatory, antioxidant, antimicrobial and anti-ulcer [Li et al., 2015; Yoon et al., 2011].

Phytoestrogens are naturally occurring substances and are defined as plant compounds that have estrogen – like biological activity [Cos et al., 2003].

Phytosterols are naturally occurring substances found in greater quantity in higher plants, especially in vegetable oils. They are used in the semisynthesis of steroid hormones and vitamin D [Khalaf et al., 2011]. They reduce total and LDL cholesterol levels in plasma by inhibiting its absorption from the small intestine and lower the atherosclerotic risk and offer protection against cardiovascular diseases [Calpe-Berdiel et al., 2009]. Plant sterols have anti-inflammatory and immunomodulatory properties [Bouic, 2002]. Also, they manifest a protective activity against cancer, decreasing the risks of breast, prostate and colon cancer [Jones and Abu Mweis, 2009].

The plants and natural products studied were: *Tilia cordata* – linden, *Chamomilla recutita* – chamomile, *Achillea millefolium* – yarrow, *Cynara scolymus* - artichoke, *Salvia*

officinalis - sage, *Mentha piperita* – peppermint, C*rataegus monogyna* – hawthorn, *Hypericum perforatum* – St. John's wort, *Phyllantus amarus*, *Glycyrrhiza glabra* – licorice, *Artemisia annua*, *Artemisia absinthium* and *Artemisia vulgaris* – wormwood, *Tanacetum vulgare* – tansy, *Tanacetum corymbosum* - corymbflower tansy and *Tanacetum macrophyllum* - rayed tansy and propolis.

Some of these plants are commonly used in Romanian households for different conditions, such as: digestive disorders (*Mentha piperita*), infections and inflammations (*Chamomilla recutita*), infections and inflammations of the oral cavity (*Salvia officinalis*), gastrointestinal disorders, wounds and inflammations, dysmenorrhea (*Achillea millefolium*), cardiovascular diseases and anxiety (*Crataegus monogyna*), insomnia, common colds, cough, fever, flu (*Tilia cordata*), depression, wounds, burns, ulcers, biliary dyskinesia (*Hypericum perforatum*), hepato-biliary disorders (*Cynara scolymus*) [Corciovă et al., 2013].

Phyllanthus amarus exhibits numerous pharmacological activities, like antioxidant [Lim and Murtijaya, 2007], antinociceptive [Itoro et al., 2013], anti-leptospiral [Chandan et al., 2012], anticonvulsant, anti-inflammatory, analgesic, antiulcer, anticancer, cardioprotective and antimicrobial activity [Corciovă et al., 2018c].

Propolis or "bee glue" is a resinous product that honey bees, *Apis mellifera* L., gather from tree buds and accumulate in the hives [Bankova et al., 2002]. The phytochemical complex contained in propolis determines many therapeutic actions, like: antibacterial, antifungal, antitumor, anti-inflammatory, antiviral, antioxidant, immunomodulatory, antiprotozoal, analgesic/anesthetic [Silva-Carvalho et al., 2015].

Glycyrrhiza glabra L. is widely used in therapy for its pharmacological actions (anti-inflammatory, antimicrobial, antioxidant, anticancer, immunomodulatory, hepatoprotective, antiviral, antispasmodic, demulcent, diuretic, emollient, expectorant, mild estrogenic, tonic and cardioprotective) and also as an ingredient of soft drinks [Khalaf et al., 2012]

Artemisia annua L., *Artemisia absinthium* L. and *Artemisia vulgaris* L. are widespread in nature and often used in the treatment of various diseases, such as hepatitis, inflammation, bacterial and fungal infections, cancer, malaria, helminthiasis and other parasitic infections [Bora and Sharma, 2011].

Tanacetum vulgare has inflammatory, anti-ulcer, anthelmintic, antiviral, antibacterial, vasorelaxant and insect-repellent activities. The less studied *Tanacetum corymbosum* and *Tanacetum macrophyllum* have antimicrobial, anticoagulant and anti-fibrinolytic activities [Kumar and Tyagi, 2013].

So, for the analysis of different compounds from pharmaceutical products and plants were used spectral methods like UV-Vis spectroscopy, Atomic Absorption Spectrometry (AAS) and chromatographic methods like HPLC-DAD, HPLC-DAD-MS and LC-MS.

This research direction has been materialized by publishing the following articles:

- ✓ Corciovă A. Spectrophotometric method for determination of bupivacaine hydrochloride in pharmaceutical preparations. *Eur. Chem. Bull*. 2013; 2(8): 554-557.
- ✓ Corciovă A. Validated colorimetric assay of clonidine hydrochloride from pharmaceutical preparations. *Iran. J. Pharm. Res.* 2016; 15 (1): 149-156.

- ✓ Corciovă A, Ciobanu C. Determinarea spectrofotometrică a codeinei din comprimate. *Sănătate Publică, Economie şi Management în Medicină.* 2014; 6(57): 31-33.
- ✓ Corciovă A, Ciobanu C. Zinc quantification in selected pharmaceutical products by two analytical methods. *Stud. Univ. Babes-Bolyai, Chem*. 2015; 60(4): 67-76.
- ✓ Corciovă A, Ciobanu C, Ivănescu B. Comparative study of polyphenol content from commonly used natural products using UV-Vis spectroscopy. *Bulletin of the Transilvania University of Braşov, Series II: Forestry, Wood Industry, Agricultural Food Engineering*. 2013; 6(55)2: 93-98.
- ✓ Corciovă A, Ivănescu B. Determination of caffeine content from different pharmaceutical and natural products. *Eur. Chem. Bull*. 2016; 5(4): 138-141.
- ✓ Corciovă A, Ivănescu B. Comparative quantification of escin from different products. *Eur. Chem. Bull.* 2017; 6(9): 400-404.
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2. 2. Spectroscopic methods with applications in pharmaceutical analysis

Spectroscopic methods can be considered as rapid techniques of analysis, which don't require complicated equipment or large amounts of sample to be analyzed. The analytical challenges associated with the sample matrix make selection of the method and sample processing technique the keys to the success of an analysis. Among spectroscopic methods, we have used UV-Vis spectroscopy and Atomic Absorption Spectroscopy (AAS).

UV-Vis spectroscopy is one of the classic methods of analysis, a simple, fast and relatively inexpensive technique. It is based on radiation absorption, the most important phenomenon that occurs when an electromagnetic radiation interacts with the molecules of a sample. Applications of UV-Vis spectroscopy are quite extensive, among which we mention the determination of impurities in organic molecules, the elucidation of the structure of organic molecules, the identification and quantification of compounds that absorb the radiation in this domain.

In many practical situations problems arise with the identification and quantification of small quantities of metal ions in samples of different origins, which by conventional analysis methods can't be solved because they don't have adequate sensitivity. For example, the need to analyze inorganic elements in pharmaceutical products and the detection and quantification of metals in vegetal samples are becoming increasingly important, both in terms of product quality and patient safety prospects. Thus AAS is a relatively simple solution that can be applied in qualitative and quantitative analysis of mineral substances. AAS is a widely used and accepted technique for a variety of samples, including pharmaceuticals, biological, clinical, ecological, food and geological samples with good accuracy and acceptable precision.

2. 2. 1. Spectroscopic methods used for pharmaceutical products analysis

2. 2. 1. 1. Development and validation of UV-Vis methods for application in quality control of pharmaceutical products

The development and validation of an analytical method is a permanent objective in the research, assurance and quality control departments.

An analytical method is developed to test a defined characteristic of the substance according to established acceptance criteria. The choice of methodology must be based on the scope and application domain of the analytical method.

The methodology for developing an analytical method is a complex process in which the steps and working techniques are presented. It may include: preparation of samples, standards, reagents, the equipment used and their working parameters, generation of the calibration curve, calculation formulas, etc.

By validating the method, it must be demonstrated that the analytical procedure is appropriate for the purpose for which it was performed. Various international regulatory agencies have set standards and working protocols to validate the methods used. Among these we mention the ICH (The International Conference on Harmonization) guidelines which provide the validation parameters of a method, among which: linearity, accuracy, precision (repeatability and intermediate precision), limit of detection, limit of quantitation [ICH, 2005].

In this part we developed and validated some UV-Vis methods for clonidine hydrochloride, bupivacaine and zinc to be applied in the quality control of pharmaceutical products, using as equipment a Jasco V 530 double beam UV-Vis spectrophotometer, 1.0 cm quartz cells, scan speed of 1000 nm min^{-1}, scan range of 200-800 nm, fixed slit width of 2 nm.

● **First study** described the development and validation of two methods for clonidine hydrochloride assay, based on the ion pair extraction technique, using thymol blue and bromophenol blue [Corciovă, 2016]. The ion pair extraction method has received considerable interest for quantitative determination of drugs because is simple, cheap, sensitive and rapid unlike other methods that are modern but involve complex instruments which may not be available in most laboratories.

Materials and methods

For development method were used: working solutions (0.5-7.5 μg/ml) of clonidine hydrochloride, obtained by diluting the stock solution (100 μg/ml), bromophenol blue solution (0.1 g %) and thymol blue solution (0.1 g %) in methanol.

The method was applied for clonidine hydrochloride tablets contained 0.15 mg drug, purchased from the pharmacy (two commercial products)

A specific quantity from each working solutions was mixed into a separating funnel with acetate buffer solution of pH = 3 and bromophenol blue solution/thymol blue solution respectively. The complex was extracted twice, for 5 minutes each time, with chloroform, passed through anhydrous sodium sulphate and completed with chloroform and the absorbance was measured at the wavelength of maximum absorption 448 nm (method with bromophenol blue solution) and 418 nm (method with thymol blue solution), after 30 minutes, *vs* a blank solution prepared in similar conditions [Corciovă, 2016].

Results

First, were establish the working conditions, by selection of the solvents for extraction of the complex, pH, reaction time, concentration and quantity of the dye.

The solvents used were CCl_4, chloroform and dichloromethane, but because the maximum of absorbance were obtained with chloroform, it was choose as extraction solvent.

The pH of solutions was adjusted to pH = 3, pH = 4.6 and pH = 5. After extraction, the absorbances were registrated at different time intervals.

The optimal stabilities of the formed complexes were obtained at pH = 3 for both dyes and the optimum reaction time was for method with bromophenol blue solution after 30 minutes and 20 minutes for thymol blue solution method, respectively. For the determination of the influence of the dye concentration were used three values in 0.05 – 0.5 % range and for the establishing of the dye quantity were used different quantities, in 0.5 – 2.5 ml range. Analyzing the results, further were used: 0.1 % concentration for both dyes, 1 ml for bromophenol blue solution and 2 ml for thymol blue solution.

For the validation of methods we studied the linearity, repeatability, method precision and accuracy.

For linearity three determinations for each concentration were made and the mean value of the absorbance's read at 448 nm/418 nm was calculated.

The calibration curves were obtained by plotting the mean value of the clonidine hydrochloride absorbance's *vs* clonidine hydrochloride concentrations (μg/ml) and in Table 2. 1. are presented the statistical data regarding clonidine hydrochloride determination.

Table 2. 1. Statistical data regarding clonidine hydrochloride determination

Statistical data	**Method with bromophenol blue solution**	**Method with thymol blue solution**
Person Coefficient (r^2)	0.999	0.9995
Standard Error	0.012978	0.003889
Intercept	0.4628	0.6213
Slope	0.1245	0.0528

Limit of detection	0.039115	0.011721
Limit of quantification	0.11853	0.035519

For determination of repeatability were used solutions with concentrations of 1.5 μg/mL in six replicates. The absorbances were measured and then the average, SD (Standard deviation) and % RSD (Relative Standard Deviation) were calculated. % RSD was found to be 0.5587 and 0.6420 respectively, which is lower than the maximum 2 % proposed by the european standards, so the system is considered to be precise.

To investigate the method precision, solutions of three concentration levels were chosen and three determinations were made for each of them, in the same day, at different times and in three consecutive days.

The mean recovery of the tests made in the same day for the method with bromophenol blue solution was in the range 99.00-100.24 %, with SD = 0.379-0.468 and % RSD = 0.38-0.469 and for the method with thymol blue solution was in the range 99.01-100.88, with SD = 0.556-0.59 and % RSD = 0.556-0.60.

The RSD values in 3 different days were in the range 0.42-0.61 for the bromophenol blue solution method and in the range 0.57-0.65 for thymol blue solution method.

As it can be seen the RSD values are lower than maximum 5 % proposed by the European standards, therefore the method is accurate.

The applicability of the two methods was demonstrated on two samples from pharmacy (Sample 1 and Sample 2), in 3 consecutive days, in 3 replicates each day. Table 2. 2. presents the average of the 3 replicates of mg clonidine hydrochloride/tablet found and % recovery, considering that the concentration declared is 0.15 mg/tablet.

Table 2. 2. Clonidine hydrochloride concentrations found in samples

	Method with bromophenol blue solution			
	Sample 1		**Sample 2**	
	mg found /tablet ± SD	**% Recovery**	**mg found /tablet ± SD**	**% Recovery**
Day 1	0.1490 ± 0.003	99.33	0.1471 ± 0.031	98.06
Day 2	0.1499 ± 0.012	99.93	0.1475 ± 0.007	98.33
Day 3	0.1502 ± 0.029	100.13	0.1486 ± 0.005	99.06
	Method with thymol blue solution			
	Sample 1		**Sample 2**	
	mg found /tablet ± SD	**% Recovery**	**mg found /tablet ± SD**	**% Recovery**
Day 1	0.1495 ± 0.026	99.66	0.1472 ± 0.008	98.13
Day 2	0.1503 ± 0.015	100.2	0.1477 ± 0.006	98.46
Day 3	0.1492 ± 0.079	99.46	0.1478 ± 0.017	98.53

The recovery of active substance varies between 98.06 and 100.13% without interferences from the excipients [Corciovă, 2016].

● **Second study** was to develop and validate a spectrophotometric method for the determination of bupivacaine in pharmaceutical preparation [Corciovă, 2013].

Materials and methods

From the stock standard solution (2 mg/ml) were made dilutions to obtain solutions in 0.1-1 mg/ml concentration range. The samples were prepared by dilution of pharmaceutical preparation that contains 5 mg/ml bupivacaine hydrochloride.

Results

Linearity was determined by three determinations for each concentration and the mean value of the absorbance read at 262 nm was calculated. The calibration curve was constructed by plotting absorbances *vs* bupivacaine hydrochloride concentrations (mg/ml) and the statistical data were calculated and presented in Table 2. 3.

Table 2. 3. Statistical data regarding bupivacaine hydrochloride determination

Statistical data	Values
Person Coefficient (r^2)	0.99981
Standard Error	0.004735
Intercept	0.00796
Slope	1.0949
Limit of detection	0.014269
Limit of quantification	0.04324

The system precision was evaluated by registration the absorbance at 0.6 mg/ml in 9 replicates, calculating the SD and % RSD. The RSD was found to be 0.8175 % which is lower than the maximum 2 % proposed by the european standards, therefore the system is considered to be precise.

Method precision was investigated on ± 20 % range compared to a reference concentration, for each value 3 determinations were performed. Studies were carried out in the same day at different hours and in 3 consecutive days. The % recovery, mean, SD and % RSD were calculated.

The mean recovery in the same day was in the 99.58 – 100.39 % range and with % RSD = 0.46 – 0.81. The % RSD values in different days were in the 0.82 – 1.01 range. In both cases the RSD values are lower than maximum 5 % proposed by the european standards, therefore the method is accurate.

We investigated the pharmaceutical samples in 3 consecutive days, in 9 replicates each day. The results are presented in Table 2. 4.

Table 2. 4. Bupivacaine hydrochloride concentrations found in samples

	Day 1		Day 2		Day 3	
Concentration declared /ampoule mg/ml	**Concentration found/ ampoule mg/ml**	**% Recovery**	**Concentration found/ ampoule mg/ml**	**% Recovery**	**Concentration found/ ampoule mg/ml**	**% Recovery**
5 mg/ml	5.0937	101.8759	5.01433	100.2867	5.0627	101.2549
	4.9905	99.81186	4.9869	99.7387	5.0700	101.401
	5.0965	101.9306	5.0508	101.0174	4.9686	99.3734

	5.0636	101.2731	4.9412	98.8255	5.0371	100.7434
	5.0682	101.3644	4.9321	98.6428	5.0326	100.6521
	4.9759	99.5196	4.9960	99.9214	5.0179	100.3598
	5.0362	100.7251	5.0417	100.8347	4.9695	99.3917
	5.0344	100.6886	4.9960	99.9214	4.9869	99.7388
	5.0316	100.6338	5.0691	101.3827	5.03991	100.7982
Average	5.04346	100.86925	5.0031	100.0635	5.0182	100.4126
SD	0.04182	0.83642	0.0467	0.9348	0.0378	0.7566
% RSD	0.8292	0.82921	0.9342	0.9342	0.7539	0.7535

● **Third study** presents the development and validation of determination of zinc content in 3 pharmaceutical preparations from Romanian market, by using the reaction with dithizone [Corciovă and Ciobanu, 2015].

Materials and methods

Initially were prepared: a stock solution which contains 200 mg/L zinc sulphate ($ZnSO_4 \cdot 7H_2O$) in acid medium, alkaline ammonium citrate solution 25 % prepared by dissolving dibasic ammonium citrate in water and 28 % ammonium hydroxide and dithizone solution 0. 01 %.

The samples analyzed were purchased from the local pharmacies and are three types of tablets containing 15 mg zinc/tablet, 10 mg zinc/tablet and 5 mg zinc/tablet

The solutions were treated in a separating funnel with alkaline ammonium citrate solution and 0.01 % dithizone and then repeated extractions with chloroform were performed until the color of the formed complex disappeared. The absorbance was determined at 516 nm against a blank containing dithizone.

Results

The conditions for the spectrophotometric method were optimized and the reaction between zinc and alkaline dithizone is presented in Figure 2. 1, when a red complex (soluble in chloroform) is formed.

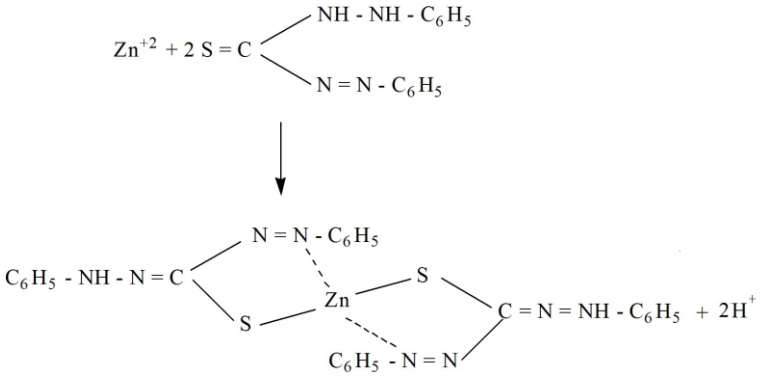


Figure 2. 1. Reaction of zinc with dithizone [Shar and Bhanger, 2001]

To establish the linearity, were prepared five dilutions in 0.5 - 10 mg/L range and the calibration curve was constructed as concentration *vs.* absorbance Table 2. 5. shows the statistical data regarding zinc determinations.

Table 2. 5. Statistical data and validation parameters for zinc determination

Statistical parameters	Values
Slope	1.3502
Intercept	0.0927
Standard error	0.0174
Regression coefficient (r^2)	0.9991
Limit of detection (mg/L)	0.4257
Limit of quantification (mg/L)	1.2901

To determine the accuracy of the method, 3 concentration levels were used and for each level 3 determinations were performed; the concentrations were calculated using the calibration curve equation. The accuracy and reliability of the proposed methods were evaluated by recovery studies of standard addition method (Table 2. 6.).

Table 2. 6. Accuracy data for the proposed methods (n = 9)

Added conc. (mg/L)	Absorbance ± SD	Recovered conc. (mg/L) ± SD	% Recovery ± SD
-	0.0015 ± 0.0001	-	-
2.500	0.4303 ± 0.0019	2.499 ± 0.100	100.0 ± 0.57
5.000	0.7631 ± 0.0033	4.976 ± 0.030	99.52 ± 0.64
7.500	1.1090 ± 0.0014	7.526 ± 0.070	100.3 ± 1.00

The average recovery was in 99.52-100.3 % range.

Precision of the method was evaluated as repeatability (intraday variation) and intermediate precision (interday variation). The repeatability studies were carried out by analyzing samples containing 5 mg/L concentration for six times in the same day. Intermediate precision (Table 2. 7.) was determined by analyzing three concentrations (2.5 mg/L, 5.0 mg/L and 7.5 mg/L) daily for three days.

The % RSD values were less than 2, indicating the precision under the same operating conditions over a short period of time.

Table 2. 7. Intermediate precision study (n = 3)

Concentration (mg/L)			Average (mg/L) ± SD	% RSD
Day 1	Day 2	Day 3		
2.504	2.497	2.479	2.493 ± 0.010	0.517
4.953	4.960	4.958	4.957 ± 0.000	0.072
7.596	7.551	7.434	7.527 ± 0.080	1.110

With respect to the intermediate precision study, the values for RSD were within the acceptable limits recommended by the international guidelines.

The method was applied for the determination of zinc in tablets. Table 2. 8. shows the average of 3 determinations for each analyzed sample, expressed in mg zinc/tablet and their recovery values, in three consecutive days.

Table 2. 8. Zinc concentrations found in analyzed samples

Samples		mg found/tablet ± SD	% Recovery
15 mg zinc/tablet	Day 1	14.86 ± 0.72	99.06
	Day 2	15.20 ± 0.22	101.33
	Day 3	14.92 ± 0.68	99.46
10 mg zinc/tablet	Day 1	9.92 ± 0. 82	99.20
	Day 2	9.97 ± 0.75	99.70
	Day 3	10.11 ± 0.90	101.1
5 mg zinc/tablet	Day 1	5.09 ± 0.54	101.8
	Day 2	5.01 ± 0.39	100.2
	Day 3	4.98 ± 0.61	99.60

Discussions

Clonidine, *N*-(2,6-dichlorophenyl)-4,5-dihydro-1*H*-imidazol-2-amine, is an imidazolinic derivative and exists as a mesomeric compound. It is used in moderate and severe forms of hypertension, for prophylaxis of migraine and other forms of vascular headache recurrence, to treat menopausal flushing [Karachalios, 1986] and in Tourette syndrome (especially for tics) [Leckman et al., 1991]. In an extended release form, FDA approved it to treat attention-deficit hyperactivity disorder (ADHD), from 2010 [Palumbo et al., 2008]. The officialized analysis methods for clonidine hydrochloride are: a potentiometric titrimetric assay for drug determination according to European Pharmacopoeia and British Pharmacopoeia [EPh. 7th ed; BPh., 2015], and a spectrophotometric method based on the reaction with bromothymol blue for clonidine quantification in tablets, as described in British Pharmacopoeia [BPh., 2015].

The literature presents several methods for the determination of clonidine hydrochloride from tablets like: HPLC, capillary electrophoresis, potentiometric, spectrophotometric titration in non-aqueous different solvents and a few colorimetric methods [Corciovă, 2016]. Our study described the development of two methods for clonidine hydrochloride quantification based on the ion pair extraction technique. After validation, the methods were used for quantification of clonidine hydrochloride in two commercial samples (tablets).

The results obtained in the 3 days comply with the limits imposed by the regulations of RPh. 10th edition, Monography - *Compressi* [± 10%] [RPh. 1993] and the recoveries of active substance are in agreement with their respective label claim. Also, the results were compared with those obtained by the spectrophotometric method stipulated in European Pharmacopoeia and the differences were insignificant.

Bupivacaine, (*RS*)-1-butyl-*N*-(2,6-dimethylphenyl) piperidine-2-carboxamide, is an amide anesthetic with prolonged duration of action, indicated for the induction of local or regional anesthesia or analgesia in the surgery, oral surgery procedures, diagnostic and therapeutic procedures in obstetric interventions. Its action installs slower (5-10 min.) and anesthesia remains longer compared to other anesthetics (3-8 hours depending on dose and site of administration) [Najafianaraki et al., 2012].

The literature describes for the determination of bupivacaine hydrochloride methods like HPLC [Medenica et al., 2003] and MS [Salama and Wang, 2009]. These methods are modern, rapid, but involve complex instruments which could not be available in most of laboratories.

The results obtained showed that the proposed method for the quantification of bupivacaine hydrochloride comply with all the validation parameters. The results obtained comply with the limits stipulated by the regulations [Corciovă, 2013].

Nowadays, the use of dietary supplements based on vitamins, micro and macro elements are widespread, both young and older people using these preparations in order to compensate the deficiencies in their diet [Rock, 2007]. So maintaining an optimal level of zinc in the body is very important. For this purpose, continuous monitoring of the concentration of zinc in dietary supplements is of great importance.

Numerous analytical methods have been used for this purpose and among them we mention: spectrophotometry, spectrofluorimetry, voltammetry, chromatography, chemiluminescence, capillary electrophoresis, atomic absorption spectrometry, inductively coupled plasma mass spectrometry. Although some of these methods offer very good precision and accuracy, but they require expensive and demanding instruments. The obtained results are in accordance with the limits imposed by the RPh, BPh and EPh. The method was precise, simple, cheaper and doesn't require sophisticated equipment

Conclusions

The development and validation of an analytical method is an important step in quality control of pharmaceutical products.

The studies described the conditions for: clonidine hydrochloride assay by two spectrophotometric methods using bromophenol blue solution and thymol blue solution in acid medium, bupivacaine hydrochloride and for zinc by reaction with alkaline dithizone.

Also, the methods developed were validated by determination of linearity, accuracy, precision (repeatability and intermediate precision), limit of detection and limit of quantitation.

The methods are simple, fast, accurate, rapid, cheap, involve minimal resources and can be successfully applied for the determination of substance in pharmaceutical products.

2. 2. 1. 2. Application of UV-Vis method for quantitative control of pharmaceutical products

In this part are described three studies in which are proposed UV-Vis spectrophotometric methods for quantification of codeine, caffeine and escine from pharmaceutical products like drugs and dietary supplements purchased from pharmacy and herbal stores.

● The aim of the **first study** was to develop a simple and precise spectrophotometric method for the determination of codeine phosphate in industrial pharmaceutical preparations by ion-pair method [Corciovă and Ciobanu, 2014].

Materials and methods

The reactives used were bromophenol blue solution 0.25 g %, acetate buffer pH = 4 and the samples analysed were tablets containing codeine phosphate 15 mg/tablet.

To a specific quantity of working solutions was added acetate buffer pH = 4 and bromophenol blue solution. The obtained complex was extracted 3 times with chloroform,

shaking for 2 minutes each time. The chloroform extracts were passed through anhydrous sodium sulfate and the absorbance was recorded at 413 nm, after 5 minutes *vs* blank.

Results

First, the working conditions were established: solvent, reaction time, the concentration of dye and the quantity used, according to Figure 2. 2. a-c and the reaction between codeine phosphate and bromophenol blue are presented in Figure 2.3.

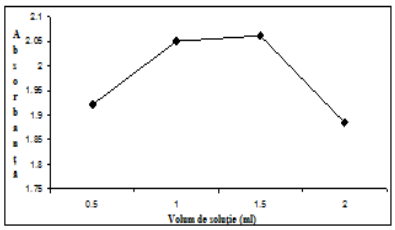


Figure 2. 2. a. Selection of the quantity of blue bromophenol solution

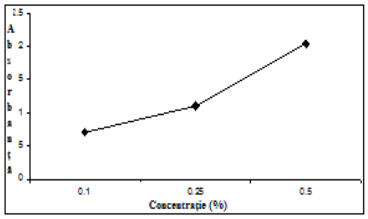


Figure 2. 2. b. Selection of the dye concentration

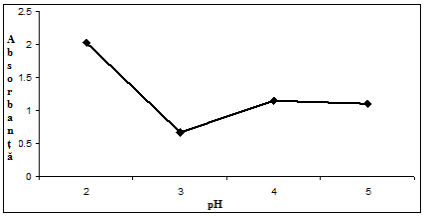


Figure 2. 2. c. Selection of pH

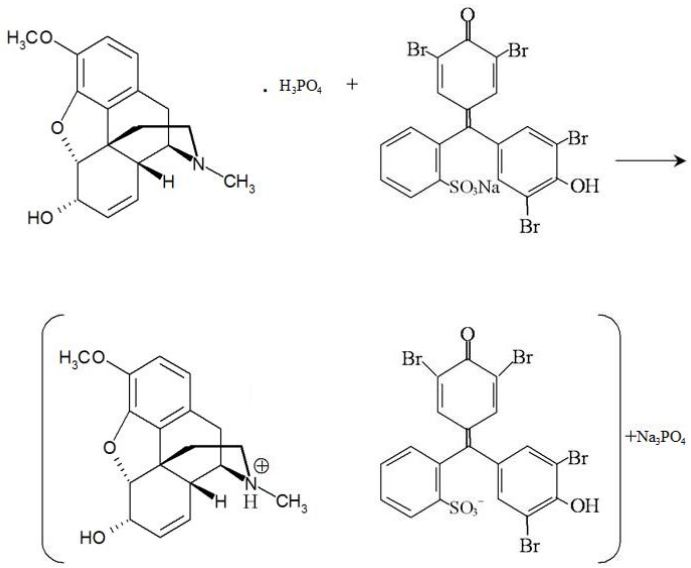


Figure 2. 3. Ion-pair complex

The results demonstrated that the most suitable solvent is chloroform. The absorbance increases with the concentration of the blue bromophenol solution but the value within the optimal range of absorbances is 0.25 %.

In case of required amount of dye, the absorbance increases to 1 ml and decreases to more than 1.5 ml, being constant in the range of 1-1.5 ml. The absorbance value at pH 2 is very high, then drops, increases and remains constant at pH 4.

The method was linear in 0.1-0.5 mg/ml range, the limit of detection was 0.036 and the limit of quantification was 0.111.

The applicability of the method has been demonstrated on tablets from pharmacy, in 3 consecutive days, in 3 replicates each day. The % recovery was in 94.26 – 96.20 % range.

● **Second study** describes the quantification of escin from dietary supplements and over the counter drugs, purchased from pharmacy and herbal stores [Corciovă and Ivănescu, 2017].

Materials and methods

The samples analysed were: Sample 1 contains dried horse-chestnut extract 250 mg/per unit expressed in 50 mg/tablet escin, butcher's broom extract ((*Ruscus aculeatus*), common bilberry (*Vaccinium myrtillus*), centella (*Centella asiatica*), vitamin C, hesperidin; Sample 2 contains dried horse-chestnut extract 200 mg/tablet, micronized diosmin, rutin trihydrate, acerola fruit extract (*Malpighia glabra*); Sample 3, gel type, contains *Aesculus hippocastanum* extract, *Ruscus aculeatus* extract, *Centella asiatica* extract and *Vaccinium myrtillus* extract; Sample 4, tincture, contains extract from horse-chestnut seeds (20 g %) in 70% (v / v) ethanol; Sample 5 contains glycerol-hydroalcohol extract of fresh horse-chestnut sprouts (45 % ethanol) (1.5 mL unit).

The quantitative analysis of saponins expressed in escin was carried out by UV-Vis spectrophotometry using the calibration curve method and the molar absorptivity method. For both methods the determinations were made in triplicate, within 3 consecutive days.

Method 1: The samples were treated with 8 % vanillin (alcoholic solution) and 72 % H_2SO_4 after which the mixture was incubated at 70 ^{0}C for 10 minutes. After a rapidly cooling on ice to room temperature, the absorbance of the solutions was measured at 560 nm. From the stock standard solution (0.1 g %), the standard scale solutions were prepared in the range 1-10 mg/L [Nguyen et al., 2015].

Method 2: The samples were treated with Folin-Ciocalteu reagent (1:10 dilution), after which 7.5 % Na_2CO_3 solution was added. The intensity of the blue colour obtained was measured at 760 nm after a reaction time of 2 h. The molar absorptivity method was applied for calculation, knowing from the literature that for escin $\varepsilon = 1.0439 \times 10^4$ $Lmol^{-1}cm^{-1}$ [Murthy and Syed, 2007].

Results

In order to determine the total saponins expressed in escin in the analysed samples, the calibration curve was design by plotting the mean values of absorbances of escin standard solutions *vs* concentrations. The statistical parameters for the analysis were presented in Table 2. 9.

Table 2. 9. Statistical data for escin determination

Statistical parameters	**Values**
Correlation Coefficient (r^2)	0.9992
Standard error	0.0101
Intercept	0.6622
Slope	0.1105
Limit of detection	0.3041
Limit of quantification	0.9214

The calibration curve has a very good linearity in the range of analysis. The system precision was determined using a 5 mg/L solution, in 6 replicates. RSD was 0.7678 %, being

lower than 2 %, the value proposed by the european standards, so we can say that the system is precise. Accuracy of the method was investigated by using three concentration levels, in triplicate. The recovery of the determination in three consecutive days was in the range of 97 – 100.6 % and the RSD values in the range 0.1149 – 0.9830 %. The RSD values were lower than 5 %, so the method is accurate. The results are presented for each sample in Tables 2.10-2. 13.

Table 2. 10. Escin mg/ tablet (Sample 1 and 2)

Stated concentration	**Method 1**			**Method 2**		
	Day 1	**Day 2**	**Day 3**	**Day 1**	**Day 2**	**Day 3**
Sample 1						
50 mg tablet^{-1}	51.12	50.87	50.78	51.01	48.97	49.78
	51.60	49.23	50.84	50.98	49.35	48.99
	51.43	49.87	50.91	50.24	49.86	50.10
Average ± SD/day	51.38 ± 0.24	49.99 ± 0.82	50.84 ± 0.06	50.74 ± 0.43	49.39 ± 0.44	49.62 ± 0.57
Average ± SD/sample	50.73 ± 0.7			49.91 ± 0.72		
Sample 2						
Not stated	39.48	38.50	39.72	38.94	38.10	38.89
	39.56	38.27	39.61	38.87	38.77	38.01
	39.87	38.19	38.48	38.25	38.12	38.23
Average ± SD/day	39.63 ± 0.2	38.32 ± 0.16	39.27± 0.68	38.68 ± 0.37	38.33 ± 0.38	38.37 ± 0.45
Average ± SD/sample	39.07± 0.67			38.46 ± 0.19		

Table 2. 11. Escin mg /100 g gel (Sample 3)

Stated Concentration	**Method 1**			**Method 2**		
	Day 1	**Day 2**	**Day 3**	**Day 1**	**Day 2**	**Day 3**
Not stated	698.73	695.67	696.28	691.67	686.28	687.67
	697.45	695.82	696.55	691.82	686.55	687.25
	697.23	695.32	697.27	691.32	686.27	687.32
Average ± SD/day	697.80 ± 0.81	695.60 ± 0.25	696.70 ± 0.51	691.60 ± 0.25	686.36 ± 0.15	687.41 ± 0.22
Average ± SD/sample	696.70 ± 1.1			688.45 ± 2.77		

Table 2. 12. Escin mg /100 g tincture (Sample 4)

Stated Concentration	**Method 1**			**Method 2**		
	Day 1	**Day 2**	**Day 3**	**Day 1**	**Day 2**	**Day 3**
Not stated	368.67	367.25	368.25	366.23	366.79	367.74
	367.26	368.01	368.15	367.76	368.27	367.10
	369.10	368.54	368.92	368.10	367.75	366.98
Average ± SD/day	368.34 ± 0.96	367.93 ± 0.64	368.44 ± 0.41	367.36 ± 0.99	367.60 ± 0.75	367.27 ± 0.40
Average ± SD/sample	368.23 ± 0.27			367.41 ± 0.17		

Table 2. 13. Escin mg/unit (1.5 mL) glycerol-hydroalcoholic extract (Sample 5)

Stated Concentration	Method 1			Method 2		
	Day 1	**Day 2**	**Day 3**	**Day 1**	**Day 2**	**Day 3**
Not stated	0.2218	0.2035	0.2156	0.2226	0.2110	0.2232
	0.2305	0.2189	0.2333	0.2145	0.2045	0.2024
	0.2018	0.2301	0.2006	0.1958	0.2232	0.2115
Average ± SD/day	0.2180 ± 0.014	0.2175 ± 0.013	0.2165 ± 0.016	0.2109 ± 0.013	0.2129 ± 0.01	0.2123 ± 0.01
Average ± SD/sample	0.2173 ± 0.007			0.2120 ± 0.001		

● Purpose of the **third study** was to determine the amount of caffeine in 5 pharmaceutical products using UV spectrophotometric methods because are cheaper, rapid, simple and approachable in scientific teaching labs of school and universities [Corciovă and Ivănescu, 2016].

Materials and methods

The samples analyzed were purchased from pharmacy - 3 types of uncoated tablets containing caffeine and: aspirin and paracetamol (Sample 1); paracetamol and propyphenazone (Sample 2); paracetamol, phenacetin and codeine (Sample 3); - 1 type of effervescent tablet containing caffeine, indomethacin and prochlorperazine (Sample 4); - 1 type of suppository containing caffeine, indomethacin and prochlorperazine (Sample 5)

Determination of caffeine content in all samples was carried out by using a UV spectrophotometric method. The specific absorbance of caffeine was used for calculation of caffeine content in tablets and the external standard method was applied for suppositories. The equations used for calculating the caffeine content were presented in the paper [Corciovă and Ivănescu, 2016]. First caffeine was extracted using special conditions in order to separate it from other active ingredients or excipients that the product may contain.

In case of Sample 1 and 2: To a quantity of powder was added water in a funnel and the mixture was stirred. Then H_2SO_4 0.05 mol/l was added and caffeine was extracted three times with chloroform by shaking every time for 5 minutes. All chloroform phases were reunited in another funnel and were extracted again three times using for every extraction NaOH 0.1 mol/l and shaking for 5 minutes. The chloroform phases were filtered through anhydrous sodium sulfate and evaporated. The residue was quantitatively transferred into a flask using HCl 0.1 mol/l. The proper dilutions were made and the absorbance was measured at 272 nm using HCl 0.1 mol/l as blank [Muntean and Imre, 2007].

In case of Sample 3: Same method was used to obtain the residue as for the previous samples; to the residue we added sodium benzoate and a small quantity of water, and then the solution containing the caffeine adduct has been processed as previous samples.

In case of Sample 4: To a quantity of powder neutralized alcohol was added. Phenolphthalein was added and the titration was started using NaOH 0.1 mol/l until a persistent pink color was obtained. After the addition of an excess of NaOH 100 g/l, the caffeine was extracted four times with chloroform and stirred for 5 minutes. The chloroform phases were filtered through anhydrous sodium sulfate and evaporated. The residue has been processed as previous samples

In case of Sample 5: An amount of suppository mass was extracted at high temperature using water and paraffin; after that, the solution was cooled and filtered in a volumetric flask, the procedure being repeated four more times using also the filter covered with residue; a specific quantity of the final solution was diluted with HCl 0.1 mol/l and the absorbance was measured at 272 nm.

Results

For each sample, the determinations were repeated three times, in three consecutive days. Table 2. 14. presents the results for determination of caffeine content in pharmaceutical products expressed in mg/tablets and mg/suppository.

Table 2. 14. Caffeine concentration in pharmaceutical products (mg/product)

	Day 1		**Day 2**		**Day 3**	
Conc. declared in samples	**Conc. found**	**% Recovery**	**Conc. found**	**% Recovery**	**Conc. found**	**% Recovery**
Sample 1 20 mg/tablet	20.49	102.45	19.89	99.45	20.23	101.15
	20.56	102.8	20.18	100.9	19.97	99.85
	20.23	101.15	19.99	99.95	20.34	101.7
Average ± SD	20.42 ± 0.17	102.13 ± 0.86	20.02 ± 0.14	100.1 ± 0.73	20.18 ± 0.19	100.9 ± 0.95
Sample 2 50 mg/tablet	51.02	102.04	49.96	99.92	50.12	100.24
	50.99	101.98	49.99	99.98	50.29	100.58
	50.97	101.94	50.01	100.02	50.07	100.14
Average ± SD	50.99 ± 0.025	101.98 ± 0.050	49.98 ± 0.025	99.97 ± 0.050	50.16 ± 0.11	100.32 ± 0.23
Sample 3 25 mg/tablet	24.88	99.52	24.87	99.48	24.68	99.52
	24.79	99.16	24.92	99.68	24.23	99.16
	24.66	98.64	24.75	99	24.76	98.64
Average ± SD	24.77 ± 0.11	99.10 ± 0.44	24.84 ± 0.08	99.38 ± 0.35	24.55 ± 0.28	99.10 ± 0.44
Sample 4 75 mg/ tablet	76.01	101.34	76.4	101.86	76.35	101.8
	76.25	101.66	76.39	101.85	75.98	101.30
	76.35	101.8	76.28	101.70	76.71	102.28
Average ± SD	76.20 ± 0.17	101.60 ± 0.23	76.35 ± 0.06	101.80 ± 0.08	76.34 ± 0.36	101.79 ± 0.48
Sample 5 150 mg/supp	148.27	98.84	147.99	98.66	148.28	98.85
	148.1	98.73	147.29	98.19	148.66	99.10
	148	98.66	147.64	98.42	148.41	98.94
Average ± SD	148.12 ± 0.13	98.74 ± 0.09	147.64 ± 0.35	98.42 ± 0.23	148.45 ± 0.19	98.96 ± 0.12

The recovery varies between 100.1 – 102.13 % (Sample 1), 99.97 – 101.98 % (Sample 2), 99.10 – 99.38 % (Sample 3), 101.60 – 101.80 % (Sample 4) and 98.42 – 98.96 % (Sample 5).

Discussions

Codeine phosphate is used for antitussive, antidiabetic and analgesic action. It is usually administered per os and is included in many pharmaceutical forms (tablets, syrups, etc.) alone or in combination with acetylsalicylic acid, paracetamol and some vasoconstricting substances. In Romania, the legislation is quite strict, according to Law no. 339/2005, codeine as a substance is included in Table II, and preparations containing codeine are included in Table III and are only issued on a special prescription.

The method applied is based on the reaction between codeine phosphate and bromophenol blue, in acidic medium, and a yellow complex, a ion-pair extractible in chloroform, is formed. The optimal reaction conditions were: chloroform, 1 ml of 0.25 % blue bromophenol solution, and pH 4.

The results for the analyzed sample with codeine phosphate comply with the limits imposed by the regulation of RPh 10^{th} edition.

Escin is a complex mixture of triterpenoid saponin glycosides, which is mainly found in *Aesculus hippocastanum* (horse-chestnut) [Zhou et al., 2009]. The actions of escin reported in various studies are anti-inflammatory, anti-edematous, venotonic, anti-cancer and antiallergic properties [Lindner et al., 2010; Sirtori, 2001]. Spectrophotometric methods, TLC, HPLC, etc. can be used for analysis of escin.

The first method is based on the reaction between oxidized triterpenoid saponins using sulphuric acid as an oxidizing agent and vanillin. The second method is based on the reduction of phosphomolybdotungstic acid from the Folin-Ciocalteu reagent by escin, in the presence of sodium carbonate, to obtain a blue product.

According to the leaflet, Sample 1 has an escin content of 50 mg/tablet. Our results meet both national and european requirements (± 7.5% according to RPh. and 5% according to EPh.).

According to the leaflet, the analysed tablets from Sample 2 contain 200 mg of horse-chestnut/tablet extract but the amount of escin mg/tablet is not specified. If we take into account the first analysed product, we can consider that the 200 mg extract contains 40 mg escin/tablet. Also, in this case we can consider that the results comply with both national and European requirements.

On Samples 3, 4 and 5, the quantity of escin is not specified in the leaflet, so no comparison can be made with the declared quantity. But, we noted that between the two methods of analysis used, the differences are insignificant [Corciovă and Ivănescu, 2017].

Caffeine ($C_8H_{10}N_4O_2$, 1, 3, 7 - trimethylxanthine) is a naturally occuring alkaloid that can be found in *Coffeae semen, Theae folium, Cacao semen, Colae semen, Mate folium* and guarana seeds. Due to its pharmacological effects, caffeine can also be found in different pharmaceutical products, associated for example with aspirin for treatment of headaches, with ergotamine for the antimigraine effect, with paracetamol and propyphenazone for pain relief or it can be used alone in the treatment of mild respiratory depression [Ogah and Obebe, 2012; Wanyika et al., 2010].

For the determination of caffeine content from different matrix, can be used: HPLC, FTIR-ATR, electrochemical detection of caffeine that uses a single-walled carbon nanotube on carbon-ceramic electrode. Most of these methods are very complex and expensive, so the purpose of our study was the quantification of caffeine from different pharmaceutical products by using different spectrophotometric methods.

The limits established by Romanian Pharmacopoeia for tablets, where deviation depends on the caffeine content in each sample, are ± 7.5 %. For suppositories the permissible deviation is ± 5 %. So the results of this study are in the recommended range [Corciovă and Ivănescu, 2016].

Conclusions

In order to verify the quality of some pharmaceutical products as drugs or supplements, codeine, caffeine and escin were quantitatively determined by simple spectrophotometric methods. Determination of codeine in the tablets was based on the reaction with bromophenol blue solution with formation of an ion-pair complex. The quantitative analysis of saponins expressed in escin was performed using the calibration curve method and the molar absorption method. Tablets, gel, tincture and glycerol-hydroalcohol extract products were analyzed as samples. For caffeine, were analyzed five products in the over the counter and dietary supplements categories by two spectrophotometric methods using calibration curve and molar absorptivity methods.

The methods used were simple, easy to use, and the results show that they can be successfully used for the determination of substances from different samples.

In the case of products which have the concentration stated on the label, the results comply with the limits imposed by the regulations. For products that did not have the concentration specified on the label, a comparison could not be made. Thus, we draw attention to the need to include the concentration of active substances on the product label, even if they are part of the category of supplements.

2. 2. 1. 3. Application of UV-Vis method for the evaluation of pharmaceutical products stability

The objective of the stability tests is to provide proofs about how the quality of the substances/pharmaceutical products varies with time or/and in different environmental conditions. Stability tests provide proofs that the quality of a substance or drug under the influence of different environmental factors does not change over time. The informations obtained can be used to provide recommendations on the handling, storage, stabilization of various substances/formulations. We studied the stability of cefixime and ceftazidime as raw materials that can be used for pharmaceutical forms.

Materials and methods

The stability for cefixime and ceftazidime raw materials was determined using an 8453 Agilent spectrophotometer.

The stability of ceftazidime was determined by a UV-Vis method, under normal storage conditions for 1 year [Palade et al., 2010a]. The method consists in: chloranilic acid and ceftazidime in 1:1 molar ratio are mixed and after 5 minute, the absorbances are registered at 520 nm [Palade et al., 2009].

For stability assessments of cefixime were used two tests: first test – the substance was exposed at different temperatures by incubation in 21-37 ^{0}C range, and at different time intervals,

absorbance was determined at 256 nm. The second test comprised stress conditions in acid medium (0.1 M HCl at 25, 50, 80 ^{0}C for 12 hours, 287 nm), alkaline medium (0.1 M NaOH at 25, 50, 80 ^{0}C, 12 hours, 287 nm) and in an oxidizing medium (0.1 M H_2O_2, at 25, 50, 80 ^{0}C for 12 h, 287 nm) [Palade et al., 2010b].

Results

For UV-Vis method, the regression line, statistical parameters, concentration of the samples and the recovery are presented in paper. The average recovery for ceftazidime was in 94.57-96.94 % range [Palade et al., 2009]. Applying the method for assessment of ceftazidime it was observed that percentage recovered after 1 year was 98.99 % [Palade et al., 2010a]. In case of cefixime, the stability was influenced by temperature and acidic/alkaline environment. The values are presented in paper [Palade et al., 2010b].

Discussions

Ceftazidime and cefixime are third-generation cephalosporins. Ceftazidime is indicated for the severe treatment of infections, respiratory tract disorders, severe infections of the ear, nose, throat, urinary tract infections, gastrointestinal, biliary and intraabdominal infections. Cefixime is a broad spectrum antibiotic that is used orally in urinary tract infections, upper and lower respiratory tract infections, acute otitis, in the treatment of *Salmonella typhy* infection [Palade et al., 2009].

The UV-Vis method used for ceftazidime stability evaluation is based on the oxidative coupling reaction by charge transfer between cephalosporin (donor) and p-chloranilic acid (acceptor) in methanol solvent and results a red colored anionic radical. The reaction is presented in Figure 2.4. [Palade et al., 2009; Palade et al., 2010a]

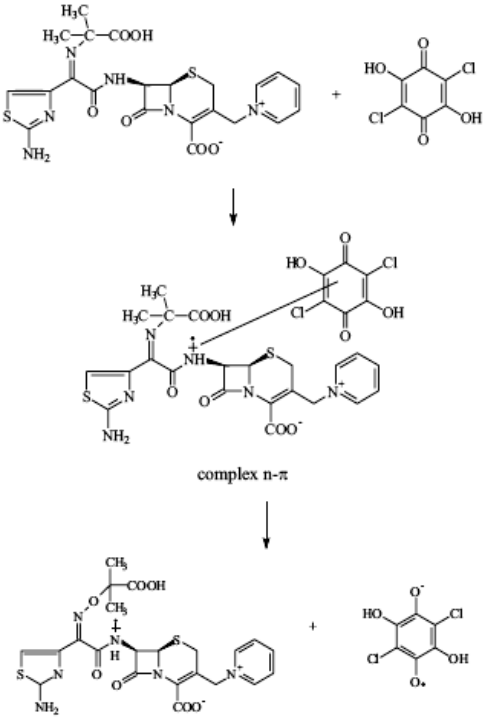


Figure 2. 4. Reaction ceftazidime with cloranilic acid [Palade et al., 2009; Palade et al., 2010a].

The method was applied for ceftazidime stability assessment and the results demonstrated that ceftazidime content decreases slightly over the period tested.

In case of stability assessment of cefixime, results obtained showed that moderate temperatures (21, 25 ^{0}C) did not affect significantly the stability, but the increase in temperature over a period of 16 hours determines a significant decrease in cefixime content. In acidic, alkaline and oxidant environment, at temperatures of 50, 80 ^{0}C, cefixime degrades more than 44 % [Palade et al., 2010b].

Conclusions

The study describes the assessment of ceftazidime stability for 1 year under normal storage conditions and the assessment of cefixime stability under stress conditions.

In case of cefixime stability the results demonstrated that is stable at environmental temperatures and degrades fast and strong in aggressive environments. For ceftazidime stability, in conditions taken into work, the substance maintained minimum content allowable over the period tested.

2. 2. 1. 4. Development and validation of AAS method for application in quality control of pharmaceutical products

Zinc is an essential constituent for many biological processes catalyzed by metalloenzymes, being involved in lipid, protein and carbohydrate metabolisms [McCall et al., 2000]. This study presents the determination of zinc content in 3 pharmaceutical preparations from Romanian market, by AAS [Corciovă and Ciobanu, 2015].

Materials and methods

Samples taken into work were three types of tablets containing 15 mg zinc/tablet, 10 mg zinc/tablet and 5 mg zinc/tablet, purchased from the local pharmacies.

The samples were treated with 65 % nitric acid and 30 % hydrogen peroxide, then was subjected to reflux boiling for 4 hours at 140 °C using a heating plate. After cooling, the content of the beakers were quantitatively transferred with water in volumetric flasks. The obtained solutions were analyzed for zinc at 213.857 nm, using flame atomic absorption spectrometer ContrAA 300, Analytic Jena, detector CCD (Charge Coupled Device).

Results

The method was validated in terms of linearity, accuracy, precision (repeatability and intermediate precision), limit of detection, limit of quantitation, according to the ICH recommendations for validation of analytical procedures [ICH, 2005].

To establish the linearity, were prepared five dilutions in 0.25 mg/L - 3 mg/L and the calibration curve was constructed (Figure 2. 5.). Table 2. 15. shows the statistical data regarding zinc determinations.

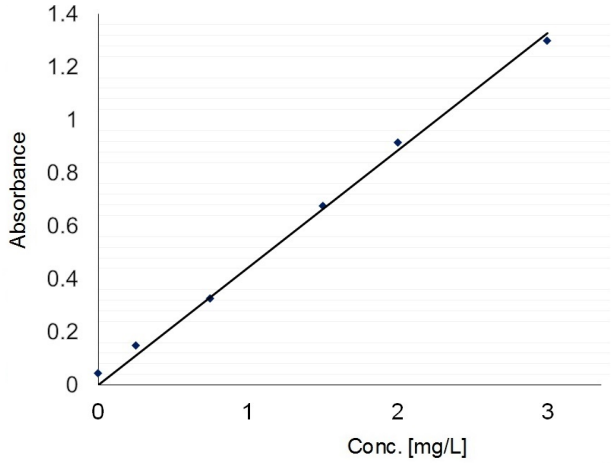


Figure 2. 5. Calibration curve for zinc determination

The results showed a good relationship over the concentration range.

Table 2. 15. Statistical data and validation parameters for zinc determination

Statistical parameters	Values
Slope	0.4091
Intercept	0.0994
Standard error	0.0041
Regression coefficient (r^2)	0.9938
Limit of detection (mg/L)	0.0078
Limit of quantification (mg/L)	0.0238

To determine the accuracy the samples were spiked with known concentrations of analyte and then wet digested and analyzed. The accuracy and reliability were evaluated by recovery studies of standard addition method (Table 2. 16.).

Table 2. 16. Accuracy data for the proposed methods (n = 9)

Added conc. (mg/L)	Absorbance ± SD	Recovered conc. (mg/L) ± SD	% Recovery ± SD
-	0.0456 ± 0.0006	-	-
0.850	0.4424 ± 0.0070	0.838 ± 0.017	98.59 ± 2.03
1.600	0.7748 ± 0.0081	1.650 ± 0.019	103.12 ± 1.23
2.400	1.0864 ± 0.0146	2.412 ± 0.035	100.5 ± 1.48

The recovery was between 98.59 and 103.12 %, therefore, any change in the active substance concentration can be accurately determined using the proposed method.

Precision of the method was evaluated as repeatability (intraday variation) and intermediate precision (interday variation). The repeatability study was carried out by analyzing samples containing 1.6 mg/L concentration for six times in the same day. Intermediate precision (Table 2. 17.) was determined by analyzing three concentrations (0.85 mg/L, 1.6 mg/L, 2.4 mg/L), daily for three days.

The % RSD values was less than 2, the results indicate the precision under the same operating conditions over a short period of time.

Table 2. 17. Intermediate precision study (n = 3)

Concentration (mg/L)			Mean (mg/L) ± SD	% RSD
Day 1	Day 2	Day 3		
0.8582	0.8480	0.8162	0.8408 ± 0.021	2.605
1.6100	1.5940	1.6760	1.6266 ± 0.043	2.672
2.4070	2.3670	2.4220	2.3986 ± 0.028	1.185

With respect to the intermediate precision study, the values for RSD were within the acceptable limits recommended by the international guidelines [Corciovă and Ciobanu, 2015].

The method has been applied for the determination of zinc in tablets. Table 2. 18. shows the average of 3 determinations for each analyzed sample, expressed in mg zinc/tablet and their recovery values, in three consecutive days.

Table 2. 18. Zinc concentrations found in analyzed samples

Samples		mg found/tablet ± SD	% Recovery
15 mg zinc/tablet	Day 1	14.94 ± 0.13	99.60
	Day 2	14.77 ± 0.19	98.46
	Day 3	14.90 ± 0.19	99.33
10 mg zinc/tablet	Day 1	9.92 ± 0.04	99.20
	Day 2	9.92 ± 0.08	99.20
	Day 3	9.51 ± 0.13	95.10
5 mg zinc/tablet	Day 1	5.12 ± 0.05	102.4
	Day 2	5.04 ± 0.07	100.8
	Day 3	5.09 ± 0.09	101.8

Discussions

The obtained results are in accordance with the limits imposed by the RPh, BPh and EPh.

The method is precise and simple, more sensitive, faster and uses smaller quantities of reagents than the spectrophotometric method presented in Chapter 2.2.1.1.

Conclusions

In conclusion, it has been demonstrated that the proposed method can be successfully used for the determination of zinc in pharmaceutical preparations containing zinc as an active substance.

2. 2. 2. Spectroscopic methods used for analysis of natural bioactive compounds and plant based products

2. 2. 2. 1. Application of UV-Vis methods for quantification of natural bioactive compounds

UV-Vis spectroscopy was used for analysis of total polyphenols, flavonoids and polyphenolic carboxylic acids.

Materials and methods

Samples were constituted from different sorts of propolis from Romania and Israel, ethanolic extracts from *A. absinthium, A. annua,* and *A. vulgaris,* ethanolic and aqueous extracts from *Tilia cordata, Chamomilla recutita, Achillea millefolium, Cynara scolymus, Salvia officinalis, Mentha piperita, Crataegus monogyna, Hypericum perforatum,* methanolic extracts of *T. vulgare, T. macrophyllum* and *T. corymbosum,* alcoholic and aqueous extracts of *Lavandula angustifolia,* aqueous extracts from *Capsicum annum* (sweet pepper) and methanolic extract of *Phyllanthus amarus.* The equipment: used was a spectrophotometer Jasco V530 in a scan range of 400 - 800 nm.

Determination of total phenolic content: by the Folin-Ciocalteu method described by Singleton [Singleton and Ross, 1965, Singleton et al., 1999]. The diluted plant extract was mixed with Folin-Ciocalteu reagent (1:10 dilution) and after a period of time ranging from 30 seconds to 8 minutes, 7.5 % Na_2CO_3 solution was added. The absorbance was determined after a reaction time of 2 h at 20 ^{0}C, compared to a control blank (water plus reagent) with absorbance 0. The intensity of the blue colour obtained was measured at a wavelength of 760 nm. The total concentration of phenolic compounds in the extract was calculated by comparison with a calibration curve prepared with gallic acid in the same way.

Determination of flavonoids: by the spectrophotometric method officialised by RPh 10th ed., based on the reaction with aluminium chloride. The sample was treated with sodium acetate 100 g/l and aluminium chloride 25 g/l, and after 15 minutes the absorbance was determined at 470/415 nm using a compensation liquid obtained similarly, but the reagents were replaced with water. Flavonoid concentration was calculated using a calibration curve prepared in the same conditions (from a standard solution of rutin or quercetin)

Determination of polyphenol carboxylic acids: by a method officialised by the EPh 7th edition. To sample, was added 0.5 M hydrochloric acid, and a solution prepared by dissolving sodium nitrite (R) and sodium molybdate (R) in water (R). Then 8.5 % sodium hydroxide solution was added. The absorbance was immediately measured at 525 nm, using a solution that contains sample diluted with water (R) as compensation liquid. The results were expressed in chlorogenic acid or/and rosmarinic acid.

Results

In order to make a comparison of the real level of the polyphenols, flavonoids and total phenolic acids obtained by alcoholic extraction, and the compounds obtained by the use of the usual preparations containing them, for each product *Tiliae flos, Chamomillae flos, Salviae folium, Crataegi flos, folium et fructus, Menthae folium, Hyperici herba, Millefolii herba,*

Cynarae folium were performed three kinds of samples: alcoholic extract, infusion according to RPh 10th ed and infusion as indicated on the package [Corciovă et al., 2013].

Total phenolic content in alcoholic and aqueous extracts was expressed in mg gallic acid/g dry plant (Table 2.19.), the amount of flavonoids was expressed in mg rutin /g dry plant (Table 2.20.) and the hydroxycinnamic acids in mg chlorogenic acid/g dry plant (Table 2.21.).

Table 2. 19. Total phenolic content in aqueous and alcoholic extracts

Sample	**Total phenols [**mg gallic acid/g dry plant]		
	Alcoholic extract	**Aqueous extract RP 10th ed.**	**Aqueous extract package**
Tiliae flos	25.98	7.23	5.16
Chamomillae flos	19.71	2.75	2.70
Salviae folium	31.88	12.16	4.85
Crataegi flos, folium et fructus	35.40	9.11	5.68
Menthae folium	24.96	11.99	5.22
Hyperici herba	47.73	12.88	6.94
Millefolii herba	35.86	7.38	4.86
Cynarae folium	43.98	5.94	3.03

The amount of total polyphenols varied between 19.71 - 47.73 mg/g in alcoholic extracts, between 2.75 - 12.88 mg/g in extracts obtained according to RP 10th ed. and between 2.70 - 6.94 mg/g in extracts obtained according to package instructions [Corciovă et al., 2013].

Table. 2. 20. Flavonoid content in aqueous and alcoholic extracts

Sample	**Flavonoids** [mg rutin/g dry plant]		
	Alcoholic extract	**Aqueous extract RP 10th ed.**	**Aqueous extract package**
Tiliae flos	5.22	2.07	1.73
Chamomillae flos	4.25	1.95	1.62
Salviae folium	3.53	2.63	1.98
Crataegi flos, folium et fructus	3.26	2.2	2.08
Menthae folium	7.6	2.85	1.94
Hyperici herba	3.5	2.5	2.39
Millefolii herba	11.23	3.06	1.7
Cynarae folium	5.82	4.4	1.43

Flavonoids varied between 3.26 - 11.23 mg/g in alcoholic extracts, between 1.95-4.4 mg/g in extracts obtained according to RP 10th ed. and between 1.43-2.39 mg/g in extracts obtained according to package instructions [Corciovă et al., 2013].

Table 2. 21. Hydroxycinnamic acids content in aqueous and alcoholic extracts

Sample	Hydroxycinnamic acids [mg chlorogenic acid/g dry plant]		
	Alcoholic extract	**Aqueous extract RP 10th ed.**	**Aqueous extract package**
Tiliae flos	17.82	5.15	3.01
Chamomillae flos	7.16	1.05	0.5
Salviae folium	27.56	8.28	2.13
Crataegi flos, folium et fructus	17.23	6.33	3.15
Menthae folium	14.86	8.60	3.06
Hyperici herba	36.1	9.05	3.75
Millefolii herba	13.22	3.54	1.54
Cynarae folium	8	0.9	0.8

Hydroxycinnamic acids content was between 8 - 36.1 mg/g in alcoholic extracts, between 0.5 - 9.05 mg/g in extracts obtained according to RP 10th ed. and between 0.8 - 3.75 mg/g in extracts obtained according to package instructions [Corciovă et al., 2013].

The results obtained, in case of *Tanacetum spp*, the total phenolic content (expressed as mg gallic acid equivalents/g dry weight of sample – mg GAE/g dw), total flavonoids content (expressed as mg quercetin equivalents/g dry weight of sample - mg QE/g dw) and total phenolic acids content (expressed as mg chlorogenic acid equivalents/g dry weight of sample - mg CGAE/g dw) in aerial parts extracts of *T. vulgare* L. and *T. corymbosum* and in leaves extract of *T. macrophyllum* are presented in Table 2. 22. [Ivănescu et al., 2018].

Table 2. 22. Phenolic compounds content in *Tanacetum* extracts

Extract	Total phenolic content, mg GAE/g dw	Total flavonoids content, mg QE/g dw	Total phenolic acids content, mg CGAE/g dw
T. vulgare	26.37	1.38	0.55
T. macrophyllum	0.64	0.30	0.09
T. corymbosum	5.90	3.76	0.40

The total phenols content in the *Phyllantus amarus* extract was 5.17 mg GAE/g dry plant, total flavonoids content 0.11 mg QE/g dry plant and total phenolic acids content was 0.14 mg CAE/g dry plant [Corciovă et al., 2018c].

The total phenols content in *Artemisia spp*. was: 80.65 mg gallic acid/g dry weight plant material *(A. annua*), 41.67 mg gallic acid/g dry weight plant material (*A. vulgaris)* and 18.14 mg gallic acid/g dry weight plant material (*A. absinthium).* The flavonoids content was 13.88 mg quercetin/g dry weight plant material (*A. annua),* 10.11 mg quercetin/g dry weight plant material *(A. vulgaris)* and 3.23 mg quercetin/g dry weight plant material (*A. absinthium)* [Ivănescu et al., 2016].

For the samples obtained from propolis, the flavonoids content was 24.7 – 24.95 g quercetin % (Romania samples) and 26.53 – 31.41 quercetin g % (Israel samples) [Croci et al., 2009b].

For the sample of *Lavandulae spp.* extracts, was compared the level of hydroxycinnamic acids (Table 2.23.) obtained by alcoholic extraction and aqueous extraction (infusions) performed according to package instructions from lavender flowers (*Lavandula angustifolia* Mill. and *L. latifolia* Medik).

Table 2. 23. The content of hydroxycinnamic acids in *Lavandulae flos* commercial samples

Samples	**Chlorogenic acids (g %)**		**Rosmarinic acid (g %)**	
	Alcoholic extract	**Aqueous extract**	**Alcoholic extract**	**Aqueous extract**
L. angustifolia	10.06	5.91	4.74	2.79
L. latifolia	7.95	2.53	3.75	1.19

The amount of chlorogenic acid varied between 2.53-5.91 g % in aqueous extract and 7.95-10.06 g % in alcoholic extract. For rosmarinic acid, the amount ranged between 1.19-2.79 g % in aqueous extract and 3.75-4.74 g % in alcoholic extract [Lungu et al., 2014].

Studing the influence of fertilization type (organic, chemical and microbiological) and pepper cultivars on their total phenolic content, the amount measured ranged widely in 2.64 to 6.46 mg GAE/ 100 fresh weight, being higher in the case of microbiological fertilizer [Dimitriu et al., 2016].

Discussions

The amounts of total polyphenols found in alcoholic extracts are comparable to those reported in the literature: *Chamomillae flos* - 23.2 mg/g in 80 % ethanol [Al Bahtiti, 2012]; *Tiliae flos* - 14 mg/g in methanol extract [Demiray et al., 2009], *Cynarae folium* - 50.5 mg/g in 75 % ethanol extract [Vamanu et al., 2011], *Menthae folium* - 41.1 mg/g in 70 % ethanol extract, *Salviae folium* - 81.2 mg/g in 70 % ethanol, *Crataegus monogyna* - between 28.3 and 114.3 mg/g in methanol extract.

Regarding the total content of flavonoids, our results are similar to those reported by other authors, with differences related to the type of extract and standard substance used: *Millefolii herba* - between 13.7 and 39.7 mg/g related to apigenol [Spinarova and Petrikova, 2003], *Salviae folium* - 2.56 mg/g in 70 % ethanol extract, *Menthae folium* - between 8.4 and 8.8 mg/g in 50 % ethanol extract related to izocvercitrin, *Chamomillae flos* - 9.48 mg/g in 50 % ethanol extract related to luteolin-7-glucoside.

The literature is poorer in data regarding the total content of hydroxycinnamic acids in medicinal plants: *Menthae folium* - between 8 and 9.3 mg/g in 50 % ethanol extract related to rosmarinic acid [Aprotosoaie and Răileanu, 2013], *Chamomillae flos* -15.89 mg/g in 50 % ethanol extract, expressed as chlorogenic acid. The data reported are similar to the results of our study.

The total polyphenol and phenolic acids contents are high in *T. vulgare* and the total flavonoid content is high in *T. corymbosum*. These results can be explained by the different plant part used in this analysis (aerial parts for *T. vulgare* and *T. corymbosum vs* only leaves for *T. macrophyllum).* The total polyphenol content of tansy was close to that previously reported for a 70 % methanol extract from aerial parts (30.42 mg GAE/g), where it decreased in the order inflorescences > herbs > roots [Wegiera et al., 2012].

The literature provides different values of the total phenolic content in *Phyllanthus amarus*: 21.3 ± 3.6 mg GAE /g dry extract [Eldeen et al., 2011], 171± 15.6 mg GAE /g plant extract [Kumaran and Karunakaran, 2007], 1083 ± 66 mg GAE /100 g plant material [Lim and Murtijaya, 2007]. The values obtained by Kumaran et al. for total flavonoids content was 21.7 ± 1.4 mg rutin/g plant extract and for flavonols 18 ± 2.4 mg rutin/g plant extract [Kumaran and Karunakaran, 2007].

The total phenols and flavonoids content in extracts decreases in the following order: *A. annua* > *A. vulgaris* > *A. absinthium* [Ivănescu et al., 2016].

Differences in quantity of flavonoids in propolis are explained by geographical area and native flora of locations from which the analysed samples were collected [Croci et al., 2009b]

In case of *Lavandulae spp*. extracts, was observed that the extraction method influences the amount of rosmarinic and chlorogenic acid, which was reduced in aqueous extracts compared to the alcoholic extracts [Lungu et al., 2014]. The results are similar to those in the specialty literature that reports concentrations of rosmarinic acids in plants from *Lamiaceae* family ranging from 0 to 58.5 mg/g, quantified by HPLC methods [Shekarchi et al., 2012]. In wild plants of *L. angustifolia*, rosmarinic acid content varies between 2.31 and 4.04 mg/L depending on the solvent and method of extraction [Komes et al., 2010].

The applying of differentiated fertilization materials can influence the bioactive content in pepper fruits. The results obtained demonstrated that phenolic compounds are influenced by cultivar and fertilizers [Dimitriu et al., 2016].

Conclusions

The aim of our studies was to determine flavonoids, polyphenols and total phenolic acids in different products, like alcoholic extracts, aqueous extracts prepared accordingly to RP 10th ed. or as indicated on the packaging, and in one case to observe the influence of fertilization on phenolic compounds.

The results indicate that the chemical composition of extracts is influenced, among others, by the extraction process. In case of using the fertilizer, was observed that the microbiological method determined a higher content of total phenols, which is a useful option for organic agriculture.

2. 2. 2. 2. Application of AAS in assessing the safety in using of some plant based products

In order to assess the safety in using of plant based products, the presence of heavy metals was determined by AAS.

Materials and methods

Samples: were two lavender commercial samples of *Lavandulae flos* (L1 and L2), from different manufacturers which were mineralized by using a mixture of 65 % nitric acid and 30 % hydrogen peroxide and the infusions prepared in accordance to the leaflet instructions.

The AAS analyses: were performed on an atomic absorption spectrometer ContrAA 300: HR-CS AAS (High Resolution Atomic Absorption Spectrometry Source Continuum). The

wavelengths at which the absorbances of the solutions were recorded were: 213.85 (Zn) nm, 217.00 nm (Pb), 228.80 nm (Cd) and 324.75 nm (Cu).

Results

The zinc, copper, lead and cadmium concentrations are presented in Table 2.24.

Table 2. 24. The zinc, copper, lead and cadmium concentrations

Samples		**Concentration**			
		Zn	**Cu**	**Pb**	**Cd**
Sample L1	Vegetal material (μg/g)	31.39	10.25	< LOQ	< LOQ
	Infusion (μg/mL)	0.181	0.085	< LOQ	< LOQ
Sample L2	Vegetal material (μg/g)	23.43	10.78	< LOQ	< LOQ
	Infusion (μg/mL)	0.143	0.063	< LOQ	< LOQ

The limit of quantification was: 0.0149 μg/mL (for zinc), 0.0014 μg/mL for copper, 0.0294 μg/mL for lead and 0.0065 μg/mL for cadmium. In the samples analyzed lead and cadmium were smaller than the limit of quantification [Lungu et al., 2014].

Discussions

The concentrations of lead and cadmium are below the maximum permissible limits (10 μg/g for lead and 0.3 μg/g for cadmium) in medicinal plants [WHO, 2005].

The concentration of copper in vegetal material was in 10.25 – 10.78 μg/g range. In case of medicinal plants, the maximum permissible limits of copper has not been yet established, but China set the limit to 20 μg/g and Singapore to 150 μg/g [WHO, 2005]. The results showed that the levels of copper were well below these limits. The concentration of zinc in vegetal material varied was in 23.43 - 31.39 μg/g.

In case of infusions: zinc varied between 0.143 - 0.181 μg/mL and copper varied between 0.063 - 0.085 μg/mL. According to WHO guideline values for drinking water (2 μg/mL for copper) the results are below these values [WHO, 2011].

Conclusions

Plants have a high absorption power of chemicals in soil and atmosphere and are among the best bioindicators. The presence of heavy metals in the soil, such as Cu, Zn, Cd or Pb, can be attributed to industrial activity or to excessive use of fertilizers.

The content of heavy metals in samples of *Lavandulae flos,* vegetal material and infusion, was determined using an AAS method. The results have shown that the amount of heavy metals in plants and tea preparations is reduced so the plant products are safe to use in phytotherapy.

2. 3. Chromatographic methods with applications in pharmaceutical analysis

HPLC is one of the most widely used analytical tool for the separation and determination of substances in any sample, especially pharmaceutical, biological, food, industrial and environmental samples/products. It is used in the pharmaceutical industry in: drug discovery, drug development, production and quality assurance.

2. 3. 1. Chromatographic methods used for pharmaceutical products analysis

The most popular of the oral dosage forms are the tablets and the quantitative analysis is a step in quality control of the pharmaceutical products. In order to use simple and rapid methods we developed two HPLC-DAD methods for the determination on the one hand of diosmin and hesperidin from tables and on the other hand for determination of glibenclamide in tablets.

Materials and methods

For analysis were used tablets with diosmin and hesperidin and tablets with two concentrations of glibenclamide.

As equipment was used an Agilent 1100 Series HPLC system with a G1315A diode-array detector, G1322A vacuum degasser, G1311A quaternary pump, G1313A autosampler. The chromatographic conditions for the first case were: Zorbax SB-C18 100 mm x 3.0 mm i.d., 3.5 µm column; mobile phase a mixture of acetic acid 0.1%/methanol (60:40, v/v), flow rate of 1ml/min at 50 °C, injection volume 5 µl, UV detection at 285 nm [Corciovă et al. 2012]. Also, the method was validated.

In the second case, the chromatographic conditions were: Lichrosorb® octadecilsilan (125 mm x 4.6 mm, 5 µm) column, temperature: 25 ^{0}C, mobile phase - acetonitrile: 1.36 % potassium dihydrogenophosfate, at pH 3 (47:53 v/v) with 1.5 mL/min flow rate and 20 µL injection volume [Dorneanu et al., 2009].

Results

Figure 2. 6. presents a typical chromatogram of hesperidin and diosmin standard solutions.

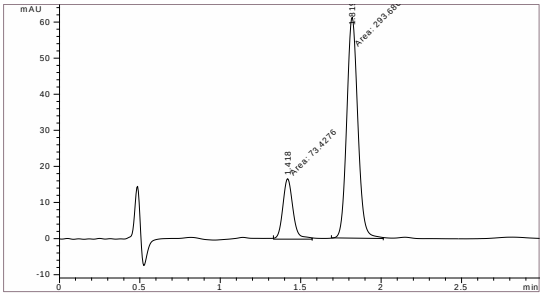


Figure 2. 6. Typical chromatogram of hesperidin and diosmin standard solutions

In order to validate the proposed method, we investigated the following validation parameters: linearity, limit of detection, limit of quantification, precision and accuracy in accordance with the bioanalytical method validation guidelines [ICH 2005].

The linearity of the method was demonstrated over the concentration range 38.82 – 90.58 μg/ml for diosmin, and 4.644 – 10.836 μg/ml for hesperidin. The statistical interpretation of the data obtained led us to the results presented in Table 2. 25.

Table 2. 25. Statistical data regarding diosmin and hesperidin determinations

Statistical data	Diosmin	Hesperidin
Person Coefficient (r^2)	0,99998	0,99997
Standard Error	0,2887	0,0002262
Intercept	-0,54	-0,4376
Slope	4,53168	9,1623
Limit of detection	0,21023	0,0000814
Limit of quantification	0,63707	0,0002468

The system precision was evaluated at concentrations of 64.7 μg/ml (n = 10) for diosmin and 7.4 μg/ml (n = 15) for hesperidin. Method precision was investigated on a range of 20 % compared to the interest concentration, grouped on a number of 5 determinations in 5 concentration levels.

The RSD was found to be 0.58089 % for diosmin and 1.18547 % for hesperidin which is lower than the maximum 2 % proposed by the european standards, therefore the system is considered to be precise.

The mean recovery for the determination of diosmin and hesperidin was in the range 99.17 – 100.85 %, with % RSD = 0.39 and 95.39 – 103.23 %, with % RSD = 2.199, respectively. The RSD values are lower than maximum 5 % proposed by the european standards, therefore the method is accurate.

Quantification of glibenclamide was achieved by measuring the peak areas corresponding to glibenclamide from the injected solutions and the recovery values obtained were in 99.95 - 104.10 % range.

Discussions

The literature describes other methods for the determination of diosmin and hesperidin in different matrices (pharmaceutical formulations, fruit juice). For exemple, in one study, is used a Spherisorb ODS1 column and a mobile phase consisting of acetic acid 2 %/acetonitrile, pH 2.58, gradient elution, when for hesperidin a retention time of 33.2 minutes was obtained [Gorinstein et al., 2006].

In another study was used a LiChrosorb RP-18 column and a mixture of methanol-water (60:40, v/v) as a mobile phase with a flow rate of 1.5 ml/min, isocratic elution and a retention time of 6 minutes was obtained [El-Shafae and El-Domiaty, 2001]. The proposed method reduced the analysis time down to 3 minutes, with an efficient separation of the analytes and the retention times obtained were 1.4 minutes for hesperidin and 1.8 minutes for diosmin.

Glibenclamide is a sulphonylurea-derived, used as antidiabetic agent in the treatment of non-insulin dependent diabetes. The run time of analysis was 6 minutes.

Conclusions

The first study presents the development and validation of a HPLC-DAD method for the determination of diosmin and hesperidin. The method is simple, reliable, accurate and precise and the chosen chromatographic conditions provided a very short analysis time, compared with other conventional methods in literature. The results obtained showed that the proposed method for the quantification of diosmin and hesperidin comply with all the validation parameters and can be successfully applied in the determination of these two substances in pharmaceutical tablets. The second study presents a HPLC method for determining the content of glibenclamide in tablets.

The results obtained demonstrated, in both cases, the applicability of the methods presented for the HPLC determination of diosmin and hesperidin and glibenclamide, respectively, being rapid methods.

2. 3. 2. Chromatographic methods used for natural bioactive compounds analysis

2. 3. 2. 1. Application of HPLC-DAD for polyphenols analysis

Materials and methods

Samples: used were ethnolic extracts of *A. absinthium* and *A. vulgaris* [Ivănescu et al., 2009]; methnolic extracts of different sorts of propolis from Romania (Grajduri-S1, Răducăneni-S2, Drânceni-S3 areas) and Israel (Kfar Lin1-S4), Kfar Lin2-S5, Iad Mordechai-S6, Tiros1-S7, Tiros2-S8, Nes Tioana-S9, Galileea Sea-S10, Oca-Ierusalim-S11, Tel-Aviv-S12 [Croci et al., 2009a].

In this studies were established the working conditions like column and composition of mobile phase for identified and quantified the polyphenols (some polyphenolic acids and flavonoids).

The method was used for analysis of polyphenolic acids - protocatechuic acid, mandelic acid, chlorogenic acid, syringic acid, vanillic acid, caffeic acid, p-coumaric acid, trans-cinnamic acid, and flavonoides - fisetin, quercetin, isorhamnetin, kaempherol from *A. absinthium and A. vulgaris* [Ivănescu et al., 2009].

Also, using the same working conditions like column and composition of mobile phase were identified and quantified 15 polyphenolic acids: benzoic acid (1), 3,4-dimethoxycinnamic acid (2), p-hydroxybenzoic acid (3), protocatechuic acid (4), chlorogenic acid (5), caffeic acid (6), o-coumaric acid (7), p-coumaric acid (8), trans-cinnamic acid (9), syringic acid (10), ferulic acid (11), gentisic acid (12), R-(-)-mandelic acid (13), S (+)-mandelic acid (14), vanillic acid (15) in propolis samples [Croci et al., 2009a].

The equipment: used was a HPLC Thermo-Surveyor system equipped with automatic sample injection and diode array detector (DAD) (the spectral detection interval was 190-360 nm).

Results

In the *A. absinthium* extract were identified and quatified syringic acid (0.4823 mg/ml), fisetin (1.5282 mg/ml), isorhamnetin (0.2574 mg/ml) and kaempherol (0.1843 mg/ml).

In the *A. vulgaris* extract were identified and quantified syringic acid (2.9640 mg/ml), fisetin (3.29.06 mg/ml), trans-cinnamic acid (0.0911 mg/ml) and quercetin (0.1559 mg/ml).

The polyphenolic acids content from propolis analysed samples (S1-S12) is presented in Figure 2.7.- 2.8.

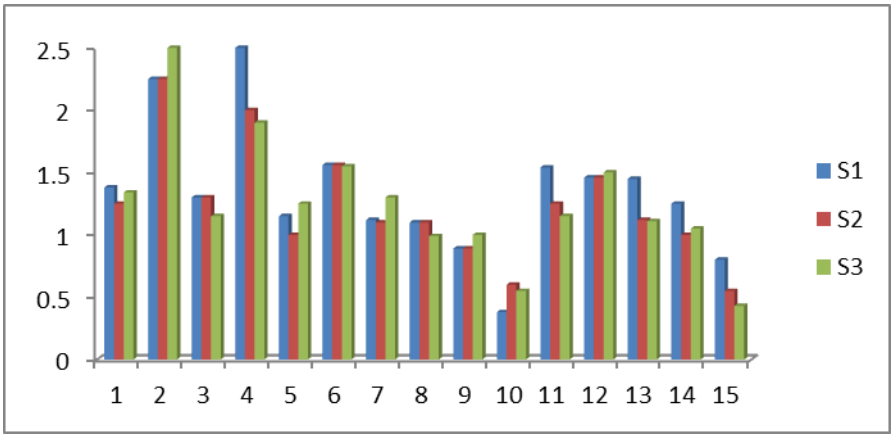


Figure 2. 7. The polyphenolic acids content in Romanian samples

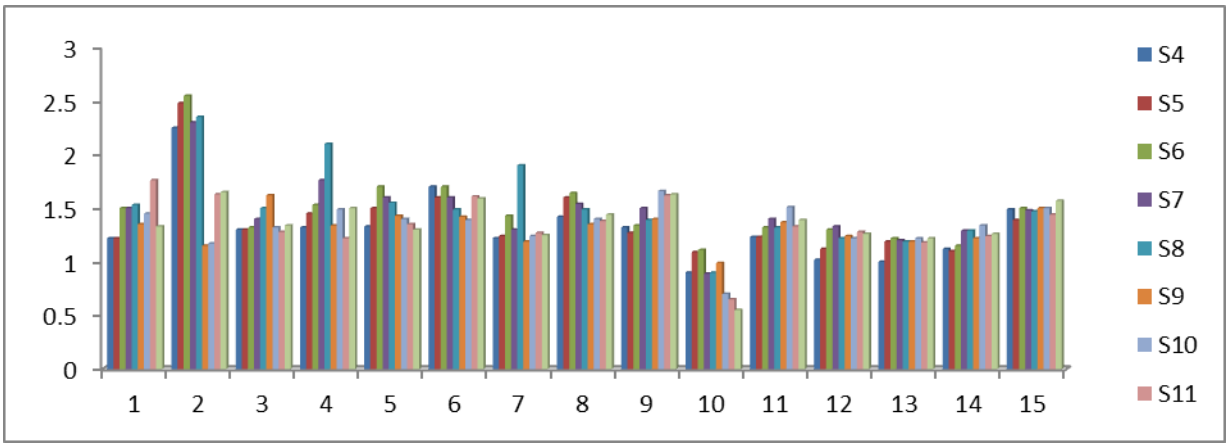


Figure 2. 8. The polyphenolic acids content in israelian samples

Discussions

Initially was used a Discovery RP-Amide - C16 column (250 x 4.6 mm, 5 µm) and then to improve the separation, were used a Zorbax Eclipse XDB – C18 column (250 x 4.6 mm, 5µm) Agilent Technologies and a Hypersil BDS - C18 column (250 x 4.6 mm, 5µm) Thermo-Fisher, the best results being obtained with the last one.

Column temperature used was 25 ^{0}C, injection volume sample 20 µl, and detection was performed at 254 nm for mandelic, chlorogenic and protocatehuic acids and 280 nm for other compounds. The mobile phase, with a flow rate of 1 mL/min was 10 % acetic acid:acetonitrile, in gradient elution (0 -30 min from 92:8 to 9:91, 30-35 min from 9:91 to 92:8).

In these conditions were established the calibration curves and the regression coefficients (> 0.996).

The retention times obtained were increasing in the following order: benzoic acid (4 min) < 3,4-dimethoxycinnamic acid (6 min) < p-hydroxybenzoic acid (6.5 min) < protocatechuic acid

(8 min) < chlorogenic acid (8.5 min) < caffeic acid (11 min) < o-coumaric acid (11.5 min) < p-coumaric acid (12 min) < trans-cinnamic acid (14 min) < syringic acid (16 min) < ferulic acid (17.5 min) < gentisic acid (19 min) < R-(-)-mandelic acid (21 min) < S (+)-mandelic acid (22 min) < vanillic acid (27 min) [Croci et al., 2009a]. In case of flavonoids, the retention times were: fisetin 11.38 min < isorhamnetol 20.2 min and kaempferol 21.5 min [Ivănescu et al., 2009].

Conclusions

The studies revealed the importance of a judicious choice of experimental conditions for identification and quantification of natural compounds from extracts.

The method used was simple and rapid and can be used for identification and quantification of polyphenolic acids and flavonoids from natural extracts.

2. 3. 2. 2. Application of HPLC-DAD-MS for polyphenols analysis

Materials and methods

Samples: analyzed were methnolic extracts of *Glycyrrhiza glabra* [Khalaf et al., 2010b], *Phyllanthus amarus* [Corciovă et al., 2018c], *A. absinthium, A. annua* and *A. vulgaris* [Ivănescu et al., 2010], *T. vulgare, T. corymbosum, T. macrophyllum* [Ivănescu et al., 2018]. For some samples, were studied the flavonoid aglycons that can be obtained by hydrolysis, in presence of 2N hydrochloric acid and heating at 80 ^{0}C.

Standards: used were polyphenolic acids – caffeic acid, chlorogenic acid, p-coumaric acid, ferulic acid, gentisic acid, sinapic acid, caftaric acid and flavonoids - kaempferol, apigenin, rutin, quercetin, quercitrin, isoquercitrin, fisetin, hyperoside, myricetin, patuletin and luteolin.

The *equipment*: Agilent 1100 HPLC Series system equipped with a G1322A degasser, a G1311A quaternary gradient pump, and a G1313A autosampler, a G1315A diode array detector system and an Agilent Ion Trap 1100 VL mass spectrometer.

For the separation was used a Zorbax SB-C18 reversed phased analytical column (100 x 3.0 mm i.d., 3.5/5 μm), at 48 ^{0}C. The mobile phase, prepared from methanol: 0.1 % acetic acid (v/v), initial with a linear gradient (started at 5 % to 42 % methanol for the first 35 minutes), followed by isocratic elution (with 42 % methanol for the next 3 minutes). The flow rate was 1 mL/min and the injection volume was 5 μL.

The DAD detection was performed at 330 nm and 370 nm.

For MS detection, the mass spectrometer was equipped with electrospray ionization interface (ESI), negative ion mode, ion source temperature 360 ^{0}C, gas: nitrogen, flow rate 12 L/min, nebulizer: nitrogen at 70 psi pressure, capillary voltage 3000 V. The analysis mode was multiple reaction monitoring (MRM) and single ion monitoring (SIM).

Results

The results from *Glycyrrhiza glabra* indicate the presence of caffeic, gentisic, p-coumaric, ferulic and sinapic acids in all samples, especially in esteric form. In the case of flavonoids, only the flavones luteolin and apigenin were present in all four samples as glycosides and free aglycons, except one sample which contains just glycosylated apigenin. None of the five

flavonols (quercetin, patuletin, kaempferol, fisetin, myricetin) or four glycosides (hyperoside, quercitrin, izoquercitrin, rutin) tested were found in any of the samples [Khalaf et al., 2010b].

The results indicate the presence of caffeic, chlorogenic, p-coumaric, ferulic and gentisic acids, quercitrin, isoquercitrin and rutin in the analyzed *Phyllanthus amarus* extract (Figure 2.9.). Among the identified compounds, p-coumaric acid, ferulic acid, isoquercitrin, quercitrin, and rutin have been quantified (Table 2.26.) [Corciovă et al., 2018c].

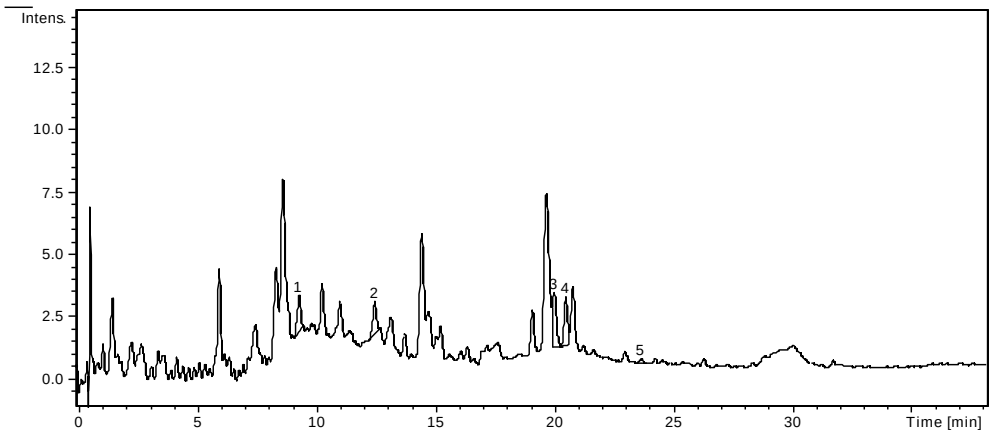


Figure 2. 9. HPLC chromatogram of *Phyllanthus amarus* methanol extract: p-coumaric acid (1); ferulic acid (2); isoquercitrin (3); rutin (4); quercitrin (5)

Table 2. 26. The concentration of phenolic compounds in *Phyllanthus amarus* (µg/g plant)

Compound	Concentration (µg/g plant)
Caffeic acid	*
Caftaric acid	-
Chlorogenic acid	*
p-coumaric acid	8.62
Ferulic acid	7.09
Fisetin	-
Gentisic acid	*
Hyperoside	-
Isoquercitrin	37.41
Quercitrin	3.65
Rutin	29.35

*compound present in sample (MS qualitative determination, UV signal < limit of quantification)

In *A. absinthium*, caffeic acid, chlorogenic acid, ferulic acid and flavonoids like hyperoside, isoquercitrin, rutin, quercetin, luteolin, apigenin were identified. Ferulic acid and kaempferol were also quantified. The sample of *A. vulgaris* presents caffeic acid, chlorogenic acid, p-coumaric acid, isoquercitrin, rutin, quercitrin and luteolin, from which p-coumaric acid and quercitrin could be quantified. In case of *A. annua*, were quantified ferulic acid, isoquercitrin, quercetin, patuletin, luteolin, kaemferol, apigenin, and identified caffeic acid, chlorogenic acid, p-coumaric acid and rutin [Ivănescu et al., 2010].

Chlorogenic acid was quantified in all sample, quercetin and luteolin in *T. vulgare* and quercitrin in *T. corymbosum.* Kaempferol and apigenin were present in *T. vulgare,* apigenin,

quercitrin and isoquercitrin in *T macrophyllum*, luteolin and apigenin were present in *T. corymbosum* [Ivănescu et al., 2018].

Discussions

For the analysis of polyphenols from samples was developed a HPLC-DAD-MS method which allows the simultaneous analysis of compounds by a single pass column. This method is based on the HPLC-DAD method already described in literature [Fodorea et al., 2005]. The method was modified to be compatible with mass spectrometry detection. So, in the composition of the mobile phase, the potassium phosphate was replaced with acetic acid. Thus, the mobile phase contains only volatile compounds, and we can introduce the eluent from the chromatographic column directly into the mass spectrometer.

First, the compounds were detected based on the wavelength corresponding to the maximum of absorbtion in UV spectrum: polyphenolic carboxylic acids at 330 nm and flavonoids and their aglicons at 370 nm. The retention times obtained were: caftaric acid (2.10 min), gentisic acid (2.15 min), caffeic acid (5.6 min), chlorogenic acid (5.6 min), p-coumaric acid (8.7 min), ferulic acid (12.2 min), sinapic acid (14.3), hyperoside (18.6 min), isoquercitrin (19.6 min), rutin (20.2 min), myricetin (20.7 min), fisetin (22.6 min), quercitrin (23.0 min), quercetin (26.8 min), patuletin (28.7 min), luteolin (29.1 min), kaempferol (31.6 min), apigenin (33.1 min). The caftaric acid-gentisic acid, respectively cafeic acid-chlorogenic were incompletely separated, in the chromatographic conditions used. So, these compounds were detected by MS.

Then, for the quantitative analysis, the calibration curves were realized.

The polyphenolic compounds analyzed contain at least one phenolic function (and one carboxyl for polyphenolic acids) so, they can be transformed into negative ions (M-H) and they can be analyzed by negative ionization. The mass spectrometer is set to isolate the interest ions and then to fragmentize them, finally recording the corresponding mass spectra. The papers present the MS analysis mode and the specific ions from the mass spectra of the polyphenols used as standards [Khalaf et al., 2010b; Ivănescu et al., 2010].

We analyzed the polyphenols from *Glycyrrhiza glabra*, and was observed the presence of p-coumaric acid, ferulic acid, sinapic acid, luteolin and apigenin, which were quantified in hydrolyzed forms [Khalaf et al., 2010b].

The results obtained for *Phyllanthus amarus* are in agree with literature data. Thus, among the identified polyphenolic compounds found in the literature, we mention hydroxybenzoic and hydroxycinnamic acid derivatives: β-glucogallin, geraniin, gallic acid, castalin; flavonols: quercetin, kaempferol and derivatives, rutin, myricetin, quercitrin; flavones: luteolin and derivatives; ellagic acid and derivatives; and other compounds like: quinic acid, brevifolin carboxylic acid, phloridzin, pinoresinol, ursolic acid [Kumar et al., 2015]. Also, Kumar et al. reported the presence of corilagin, protocatechuic acid, p-coumaric acid, caffeic acid, syringin, 4-hydroxybenzoic acid; flavanone: prunin, eriodictyol, naringenin, gentisic acid and their derivatives, gallic acid, quinic acid, ferulic acid, chrysin, betulinic acid, oleanolic acid [Kumar et al., 2017]. Also, Guha et al. has signaled the presence of caffeic acid, chlorogenic acid (caffeoylquinic acid), p-coumaric acid, isoquercitrin (quercetin-3-O-glucoside), rutin (quercetin-3-O-rutinoside) [Guha et al., 2010]. Berezi et al. identified and quantified gentisic acid (0.00006

mg %), ferulic acid (0.5958 mg %), caffeic acid (22.51 mg %) and chlorogenic acid (9.23 mg %) in an aqueous extract of *Phyllanthus amarus* [Berezi et al., 2017]. Apigenin, caftaric acid, fisetin, hyperoside, luteolin, kaempferol, luteolin, myricetin, patuletin, quercetin and sinapic acid were not present in the analyzed extract [Corciovă et al., 2018c].

From polyphenolic acids, caftaric, sinapic and gentisic acid were absent in all samples of *Artemisia spp.* In the case of flavonoids, fisetin was absent in all samples. Caffeic and chlorogenic acid were presents in all samples but couldn't be quantified. Hyperoside was present only in *A. absinthium* sample. Quercetrin was present and quantified only in *A. vulgaris* and patuletin in *A. annua* [Ivănescu et al., 2010]

Generally the results confirm other research conducted on Romanian plants on *T. vulgare*, such as the presence of quercetin, luteolin, kaempferol, apigenin [Muresan et al., 2015]. Quercitrin and isoquercitrin were identified for the first time in *T. macrophyllum* extract and apigenin was reported before [Williams et al., 1999]. In case of *T. corymbosum* luteolin was reported before [Williams et al., 1999], but apigenin, quercitrin were identified for the first time [Ivănescu et al., 2018].

Conclusions

The HPLC-DAD methods was modified to be compatible with mass spectrometry detection and in this way all the polyphenolic compounds were analyzed by a single pass column. In these conditions and in case of samples analyzed, some compounds have been identified and quantified for the first time.

2. 3. 2. 3. Application of LC-MS for bioactive substances analysis

- **Analysis of methoxylated flavones by LC-MS**

Materials and methods

Samples: methanolic extracts of *Phyllanthus amarus* [Corciovă et al., 2018c], *A. absinthium, A. annua* and *A. vulgaris* [Ivănescu et al., 2016], *T. vulgare, T. corymbosum, T. macrophyllum* [Ivănescu et al., 2018].

Standards used were: eupatilin, jaceosidin, acacetin, casticin, eupatorin and hispidulin

The methoxylated flavonoids content was determined by a LC-MS method [Mocan et al., 2016]. The equipment used was an Agilent 1100 HPLC Series system equipped with a G1322A degasser, a G1311A quaternary gradient pump, and a G1313A autosampler and an Agilent Ion Trap 1100 VL mass spectrometer. The separation was achieved using a Zorbax SB-C18 reversed-phase analytical column (100 × 3.0 mm i.d., 5 μm particle) fitted with a guard column Zorbax SB-C18, operated at 48 °C. The mobile phase consisted of 0.1 % (v/v) acetic acid and methanol with the following gradient: beginning with 45 % methanol and ending at 50 % methanol, for 8 minutes with a flow rate of 0.9 mL/min and an injection volume of 5 μL. For the MS analysis the following optimized conditions were used: ESI interface operating in negative mode, gas (nitrogen) temperature 325 °C at a flow rate of 12 L/min, nebulizer pressure 60 psi and capillary voltage 2500 V. The full identification of compounds was performed by comparing the retention times and mass spectra with those of standards in the same chromatographic

conditions. The MS was operated in the multiple reactions monitoring analysis (MRM) mode instead of single ion monitoring.

Results

In *Phyllanthus amarus* extract the major compound was hispidulin, followed by acacetin and casticin (Table 2.27.). Eupatilin, eupatorin, and jaceosidin were not present [Corciovă et al., 2018c].

Table 2. 27. The concentration of methoxylated flavones in *Phyllanthus amarus* (µg/g plant)

Compound	Concentration (µg/g plant)
Acacetin	2.7396
Casticin	0.3615
Hispidulin	6.9216

All analyzed samples of *Artemisia spp.* contain casticin, hispidulin and eupatorin, the most notably being casticin, especially in *A. annua*. Also, *A. annua* is the only species from those analyzed that contains a small quantity of eupatilin. Jaceosidin and acacetin were not found in the analyzed samples [Ivănescu et al., 2016].

T. macrophyllum contains all of the six methoxylated flavonoids analyzed, eupatilin and hispidulin are higher. *T. vulgare* contains eupatilin, acacetin, casticin and hispidulin. *T. corymbosum* contains eupatorin, casticin and hispidulin [Ivănescu et al., 2018].

Discussions

To the best of our knowledge, our study was first report of the identification and quantification of hispidulin, casticin, and acacetin in *Phyllanthus amarus* [Corciovă et al., 2018c]. Hispidulin is 4',5,7-trihydroxy-6-methoxyflavone and has been shown to have antioxidant, anti-inflammatory, antifungal, antimutagenic and antineoplastic properties [Patel and Patel, 2016]. Acacetin, 5,7-dihydroxy-4'-methoxyflavone has shown anti-inflammatory/antinociceptive activity and can be used in the treatment of pain-related diseases [Carballo-Villalobos et al., 2014]. Among the casticin activities (3', 5-dihydroxy-3, 4', 6, 7-tetramethoxyflavone) cytotoxic and immunomodulatory properties are noted [Mesaik et al., 2009].

The methoxylated flavones are bioactive compounds widely distributed in *Artemisia* genus, found generally as aglycones in the epicuticular wax so their determination is of interest from the point of view of utility of a medicinal plant.

Eupatorin and hispidulin were reported for the first time in *A. absinthium* and *A. vulgaris*, eupatilin in *A. annua* and casticin in *A. vulgaris*. Casticin, the component find in the biggest quantity, is an active compound that potentiates the antimalarial activity of artemisinin and exhibits modest antimicrobial activity against *Clostridium perfringens* [Ivarsen et al., 2014]. Also, manifest anti-inflammatory activity and antitumor activity against a large spectrum of cancer cell lines [Ivănescu et al., 2016].

Eupatilin, acacetin and casticin were identified for the first time in *T. macrophyllum*, and eupatorin in *T.corymbosum*. Jaceosidin and eupatorin found in *T vulgare* in South-America,

responsible for anti-inflammatory effect of plant [Schinella et al., 1998] are absent in our case, probably due to the existence of various chemotypes [Ivănescu et al., 2018].

Conclusions

The LC-MS method developed for determined the methoxylated flavones in plants was applied to extracts from: *Phyllantus amarus, A. annua, A. vulgaris, A. absinthium, T. vulgare, T. corymbosum* and *T. macrophyllum*. The highlighted analytes were jaceosidin, hispidulin, eupatilin, eupatorin, casticin and acacetin. In the chromatographic conditions chosen, the analytes eluted in less than 10 minutes.

Compared to other polyphenols, methoxylated flavonoids have a high oral bioavailability due to their lipophilic nature and increased metabolic stability, so can be promising therapeutic candidates [Walle, 2007].

- **Analysis of phytoestrogens by LC-MS**

Materials and methods

Samples: methanolic extracts (20 g %) of *Glycyrrhiza glabra* (S1-S4, from different zones) [Khalaf et al., 2010a], *Glycyrrhiza glabra* tincture (10 g %) [Khalaf et al., 2012]; also, the samples were hydrolyzed by treating with 6 N hydrochloric acid, at 80 ^{0}C.

Standards used: daidzin, genistin, ononin, daidzein, genistein, formononetin, glycitein and coumestrol.

For the separation was used RP Zorbax SB-C18 analytical column (100 x 3.0 mm i.d., 5μm), that operates at 50 ^{0}C. The mobile phase, with a flow rate of 1 ml/min, was prepared from methanol : 0.1 % acetic acid (v/v), and was used a gradient elution (started with 20 % methanol 2 minutes, until 10 minutes 40 %, 0.5 seconds 40 % methanol, 1 minute until 45 % methanol and then 1 second 45 % methanol). The injection volume was 5 μl. The MS was equipped with ESI interface and the settings were: negative ionization, ion source temperature 360 ^{0}C, nitrogen gas with a flow rate 12 L/min, nitrogen nebulizer at 65 psi pressure, capillary voltage 4500 V. The analysis mode was Single Ion Monitoring (SIM) (for aglycones) and Single Reaction Monitoring (SRM) (for glycosides) [Khalaf et al., 2010a; Khalaf et al., 2012].

Results

The results obtained for the methanolic extracts are presented in Table 2.28. and for tincture in Table 2.29.

Table 2. 28. Phytoestrogens content in methanolic extracts of *Glycyrrhiza glabra* (ng/ml)

Phytoestrogen	**S1**		**S2**		**S3**		**S4**	
	NH	**H**	**NH**	**H**	**NH**	**H**	**NH**	**H**
Daidzin	233.4	0.0	0.0	0.0	306.9	0.0	114.5	0.0
Genistin	-	-	-	-	-	-	-	-
Ononin	23120.1	5036.7	507.3	5055.1	13928.8	6605	21335.3	4350.7
Daidzein	2681.4	2487.6	-	3165.5	2026.6	2590.4	382.6	634.9
Glycitein	-	-	-	-	-	-	-	-

Genistein	285.1	433.6	-	193.9	180.3	330.8	69	288.3
Coumestrol	1016.1	705.4	-	252.6	432.9	320	575.5	214.9
Formononetin	7838.7	14505.3	64.2	12032.2	3919.3	14896.6	4789.8	14157

NH – non hydrolyzed sample; H – hydrolyzed sample

None of the tested vegetal product contains genistin or glycitein. Ononin and formononetin are present in all extracts, non-hydrolyzed and hydrolyzed, sometimes in a greater quantity in the hydrolyzed extract. The glycoside daidzin is found in all non-hydrolyzed extracts, except for S2 sample. Daidzein, genistein and coumestrol are present in all extracts as glycosides and free aglycones, except S2 [Khalaf et al., 2010a].

Table 2. 29. Phytoestrogens content in *Glycyrrhiza glabra* tincture (ng/ml)

Phytoestrogen	**Ticture**	
	NH	**H**
Daidzin	884.3	286.8
Genistin	0.0	251.2
Ononin	13744.6	11620.5
Daidzein	2672.3	2682.1
Glycitein	1237.7	1907.6
Genistein	513.2	531.3
Coumestrol	948.8	211.1
Formononetin	12749	10030.7

Ononin and its aglycon formononetin are found in large quantities in tincture, followed by daidzein and glycitein. There are also smaller amounts of daidzin, genistein and coumestrol. Genistin is present only in the hydrolyzed sample [Khalaf et al., 2012].

Discussions

Generally, glycosides ions lose the sugar group thus we can observe the aglycone ion, so all glycosides can be analyzed by SRM mode. Ions of aglycones didn't fragmentize efficiently, so for these compounds we applied a SIM mode analysis. So, for daidzin, genistin and ononin was apllied SRM mode and for daidzein, glycitein, genistein, coumestrol and formononetin was applied SIM mode analysis.

The abnormality regarding the presence of a greater content in the hydrolyzed extract can be explained by ineffective hydrolysis in the working conditions. Also, superior glycosides may be hydrolyzed to the monoglycoside and at the same time the later may be partially hydrolyzed. We have to take into account the fact that all compounds are polyphenols and can be degraded during hydrolysis. So, in the hydrolyzed samples the determination is only semi-quantitative [Khalaf et al., 2010a].

Also, although genistin and glycitein were not present in methanol extracts, they were quantified in tincture.

Conclusions

For the analysis of phytoestrogens like daidzin, genistin, ononin, daidzein, glycitein, genistein, coumestrol, formononetin was developed a LC-MS method.

In case of the methanolic extracts, the differences can be explained by diverse pedo-climate conditions from the four harvesting areas of the vegetal product. Also, the method of extraction influences the content of phytoestrogens.

- **Analysis of sterols by LC-MS**

Materials and methods

Samples analyzed were methanolic extracts of *Phyllanthus amarus* [Corciovă et al., 2018c], *Glycyrrhiza glabra* (S1-S4, from different zones) [Khalaf et al., 2011], *Glycyrrhiza glabra* tincture [Khalaf et al., 2012]; hexane extracts of *A. absinthium, A. annua* and *A. vulgaris* [Ivănescu et al., 2013]; methnolic extracts of *T. vulgare, T. corymbosum, T. macrophyllum* [Ivănescu et al., 2018]

Standards used were campesterol, ergosterol, β-sitosterol and stigmasterol.

For the separation we used a reversed-phased Zorbax SB-C18 analytical column (100 mm x 3.0 mm i.d., 5 μm particles) fitted with precolumn Zorbax SB-C18, both operated at 40 ^{0}C. The mobile phase was prepared from methanol and acetonitrile 30:70 (v/v), isocratic elution. The flow rate was 1 mL/min and the injection volume was 4 μL.

MS/MS detection using MRM of specific daughter ions was used for each sterol.

The MS was equipped with APCI interface and the settings were: positive ionization, nitrogen gas with a flow rate 7 L/min, ion source temperature 250 ^{0}C, nebulizer – nitrogen at 50 psi pressure, capillary voltage -4000 V.

Results

In order to quantify the four sterols from plant extracts, were constructed the extracted chromatograms for each of them, taking into account the intensity of major ions in the mass spectrum.

Concentrations of sterols found in the four *Glycyrrhiza glabra* extracts and in tincture are presented comparatively in Figure 2. 10.

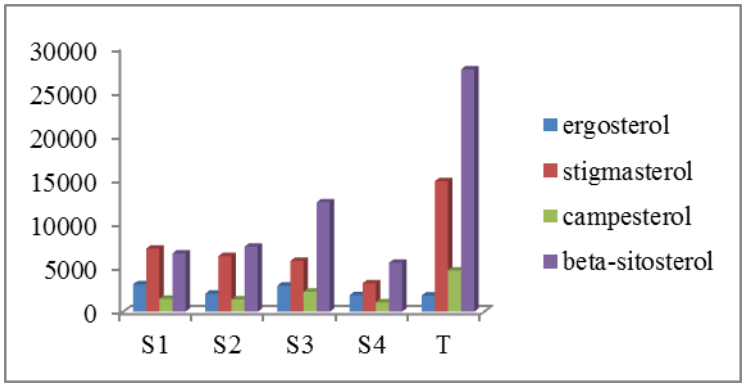


Figure 2. 10. The sterols content (ng/ml) in *Glycyrrhiza glabra* extracts (S1-S4) and in tincture (T)

In all samples, beta-sitosterol and stigmasterol are in higher amounts than ergosterol and campesterol, especially in tincture. In extracts the ergosterol quantity is higher than campesterol, although this compound is not specific to higher plants. Instead, in tincture the campesterol is in a higher quantity than ergosterol.

The concentrations of sterols found in the *Phyllanthus amarus* (µg/g plant) extract is presented in Figure 2.11.

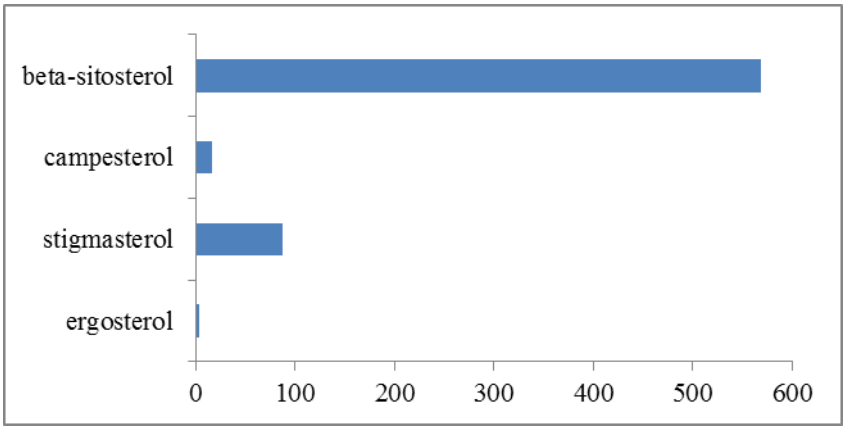


Figure 2. 11. The sterols content (µg/g plant) in *Phyllanthus amarus* extract

Stigmasterol, beta-sitosterol and campesterol were identified and quantified in the analyzed *Phyllanthus amarus* extract. Also, ergosterol was found in small amounts [Corciovă et al., 2018c].

All three species of *Artemisia* contains beta-sitosterol, stigmasterol and campesterol. From these compounds, campesterol appeared in small concentration in all species. Ergosterol has not been identified in any of the investigated species [Ivănescu et al., 2013].

All three *Tanacetum* species contain the sterols analyzed, the content decreases in order: beta-sitosterol > stigmasterol > campesterol > ergosterol [Ivănescu et al., 2018].

Discussions

The applied method of analysis is based on a published HPLC method from the literature [Sanchez-Machado et al., 2004], to which were made some changes, regarding the chromatographic column and mobile phase. As a result, compared to the method published in literature, the analysis time decreased from 30 minutes to 5 minutes without affecting the separation and peak resolution of sterols [Leucuţa et al., 2005].

Since in the ionization conditions, all sterols lose a molecule of water, ions detected by the spectrometer are always of the form $[M\text{-}H_2O^+H]^+$.

This method of analysis (also called MS/MS) is highly specific compared to the screening method, where is recorded only the intensity of the main ion and an isomer compound - with the same molecular weight - can give a false positive signal. Based on analysis of fragments of the MS spectrum, which are specific to each structure separately and are not the same for different isomers, the MS/MS method will detect only the compound of interest without interference from others. Moreover, the intensity of ions in the mass spectrum is proportional to

the concentration of the substance in the sample, so the method can also be applied for quantitative determination [Khalaf et al., 2011].

The ergosterol presence in root extracts of *Glycyrrhiza glabra* is probably due to the mycorrhizal fungi that can contribute to the biological activity of the vegetal product by the metabolites synthesized and also by increasing the glycyrrhizin concentration in roots [Sanchez-Machado et al., 2004].

Our results, regarding the sterols, are in agreement with the literature, the literature providing in *Phyllanthus amarus*, the existence of amarosterol A and B, stigmasterol, campesterol [Nahar et al., 2012] and beta-sitosterol [Yuandani et al., 2016].

The highest amount was obtained for β-sitosterol (24α-ethyl-cholesterol), which shows anti-inflammatory and analgesic activities, hypocholesterolemic, antidiabetic, antioxidant, angiogenic effects, induces apoptosis and has genotoxic, immunomodulatory, chemoprotective and chemopreventive effects [Saeidnia et al., 2014].

Literature also states that, among these three compounds, the most widespread is sitosterol, followed by campesterol and stigmasterol. In our case, sitosterol was followed by stigmasterol (Δ22-24α-ethyl-cholesterol), a compound with anti-inflammatory, anti-osteoarthritic, anti-hypercholesterolemic, cytotoxic, anti-tumor, antimutagenic and antioxidant activities [Kaur et al., 2011].

Among the activities of campesterol (24α-methyl-cholesterol), we mention the reduction of carcinogen production, which leads to the inhibition of cancer development [Woyengo et al., 2009].

The results confirm the fact that the main sterols in all three *Artemisia* species are sitosterol and stigmasterol that have intense antiviral effect [Abid Ali Khan et al., 1991] and antifungal properties [Tang et al., 2000]. Campesterol was identified and quantified for the first time in *Artemisia* species [Ivănescu et al., 2013].

The results obtained for *T. vulgare* are in agreement with the literature. Regarding the analysis of sterols in *T. macrophyllum* and *T. corymbosum*, from our knowledge, this was the first study [Ivănescu et al., 2018].

Conclusions

This investigations offer an approach to qualitative and quantitative determination of sterols in different products, by a LC-MS method. Extracts with a high amount of sterols can be taking into account in the treatment of inflammatory conditions and prevention of cancers and cardiovascular diseases.

CHAPTER 3. RESEARCH ON THE DESIGN AND CHARACTERIZATION OF DIFFERENT PLANT-MEDIATED SILVER NANOPARTICLES

3. 1. Background

Nanotechnology is a developing domain which refers to the manipulation of particles with sizes smaller than 100 nm, named nanoparticles. A special attention is attributed to zero-valent metal nanoparticles which can serve as basis for various physical and biological systems. Metallic nanoparticles have numerous biological applications and can be used as therapeutic and diagnostic agents, as carriers for targeted drug delivery, for biomolecules detection, and they can be employed in other fields too, such as food, agricultural or treatment of waste [Mashwani et al., 2015].

Silver nanoparticles (AgNPs) can be prepared and stabilized by physical and chemical methods. Beside the fact that these methods raise problems of toxicity and have a relatively inadequate cost-efficiency ratio, many experimental conditions affect their size, morphology, stability and properties [Mashwani et al., 2015; Sharma et al., 2009]. Preparation of nanoparticles involves the reduction of metal ions by a reducing agent and their stabilization at zero valence state through capture by another stabilizing agent. This involves the use of chemicals that pose a potential risk to the environment and influencing the size and shape of the particles [Shimpi et al., 2015]. Generally, synthesis of nanoparticles can be accomplished by applying physical methods (ultrasonication, irradiation, microwave), chemical methods (chemical reduction, solgel method) or biological methods. The current trend is the biological synthesis of AgNPs through the use of various biological agents, such as yeasts, enzymes, bacteria, polysaccharides, algae, oligosaccharides, fungi, DNA, human cell lines [Mashwani et al., 2015; Rajeshkumar et al., 2013; Sharma et al., 2009]. Perhaps one of the most accessible, less laborious and shorter non-toxic and environmentally safe methods compared to others methods, for nanoparticles production, consists in the use of plant extracts and whole plants. [Kumar and Yadav, 2009; Shimpi et al., 2015].

The majority of plants used for the synthesis of AgNPs belong to the following families: *Acanthaceae, Amaranthaceae, Apocynaceae, Asteraceae, Burseraceae, Dioscoreaceae, Euphorbiaceae, Fabaceae, Lamiaceae, Moraceae, Myrtaceae, Poaceae, Ranunculaceae, Rutaceae, Solanaceae, and Asphodelaceae* [Mashwani et al., 2015].

Biomolecules that participate in reduction and capping of the nanoparticles are distributed in the leaves, stems, fruit, seeds, roots, etc. and are primary and secondary metabolites from plants. Primary metabolites like carbohydrates, proteins, peptides, amino acids, vitamins etc. are always present in plants and are involved in reducing and stabilizing metallic silver in nanoparticles [Anjum et al., 2016]. Some secondary metabolites with biological activities such as terpenoids, alkaloids, flavonoids, phenolic acids and other polyphenols have been reported to act as either reducing or/and stabilizing agents in AgNPs formation. The alcohols, ketones, aldehydes, and lactones of terpenoids are involved in both reduction and capping of silver nanoparticles. In adequate concentration, terpenoids could be adsorbed on the surface of AgNPs, possibly by interaction through π-electrons or carbonyl groups [Mashwani et al., 2016]. Various functional groups of flavonoids can actively chelate and reduce metal ions into nanoparticles [Makarov et al., 2014]. The hydroxyl and carbonyl groups of phenols reduced

silver ions to silver, while amines present in the extract capped the AgNPs [Asmathunisha and Kathiresan, 2013].

For the extraction of biomolecules are used solvents such as water, ethanol, 70 % ethanol, etc. Among these, water is preferred, because is a polar solvent, easy to use for active principles extraction and further is a non-toxic solvent [Halawani, 2017].

The synthesis of nanoparticles has several stages:

- preparation of the plant extract and silver salt solution
- preparation of AgNPs by mixing those two solutions in different proportions, at certain pH and temperature values and for different time periods. In the presence of plant compounds, Ag^{+} is reduced to Ag^{0}, and then oligomeric clusters are formed, which lead to AgNPs.
- AgNPs purification is carried out by centrifugation to remove the unreacted plant extract, followed by resuspension in distilled water and again centrifugation, repeatedly, to remove unwanted substances
- confirmation for obtaining the AgNPs, through different methods of analysis

For the AgNPs synthesis various parameters need to be taken into account in order to achieve a maximum yield, nanoparticles with a certain shape and size and to achieve stability.

Parameters that need to be investigated include: concentration and amount of plant extract, concentration of silver salt, pH, temperature, reaction time.

Among the methods used to characterize the AgNPs encountered in the literature, can be mention: visual inspection, UV-Vis spectroscopy, Dynamic light scattering and determination of Zeta potential, FTIR spectroscopy, X-ray Diffraction (XRD), Scanning and transmission electron microscopy (SEM and TEM), Photoluminescence spectroscopy etc. Studies have shown that the size, morphology, stability and biological properties of metallic nanoparticles are strongly influenced by the type of molecules found in plant extracts and by the experimental conditions, so designing the synthesis of AgNPs with desired characteristics is a real challenge in the production of nanoparticles from plants [Corciovă and Ivănescu, 2018].

The most important biomedical applications of silver nanoparticles were: antibacterial, antioxidant, hepatoprotective, anti-inflammatory and cytotoxic. AgNPs have been shown to be active against both Gram-positive and Gram-negative bacteria as well as fungi. Generally, the results from antioxidant activity showed significant differences between the plant aqueous extract and AgNPs due to the higher total phenolic content and total flavonoids content in the case of AgNPs. The IC_{50} values can increase in a dose-dependent manner. Abdel-Aziz et al. were tested AgNPs for cytotoxic action compared to the aqueous leaf extract. It was observed that both samples show cytotoxicity against breast cancer (MCF-7) cell lines [Abdel-Aziz et al., 2014]. The cytotoxicity of AgNPs is dose dependent and that of the plant extract is not because some of the bioconstituents may increase cell growth, while others may decrease it.

Research in this area has focused on the use of plant extracts for the preparation of AgNPs, followed by confirmation of nanoparticles generation through various methods and the evaluation of some biological activities.

This research direction has been materialized by publishing the following articles:

- ✓ Corciovă A, Burlec AF, Fifere A, Lungoci AL, Marangoci N, Turin-Moleavin I, Cioancă O, Mircea C, Tuchiluş C. Biosynthesis and characterization of silver nanoparticles containing *Quercus robur* extract. Congresul Naţional de Farmacie, ediţia a XVII-a. 2018; 32.
- ✓ Corciovă A, Ivănescu B. Biosynthesis, characterization and therapeutic applications of plant-mediated silver nanoparticles. *J. Serb. Chem. Soc*. 2018; 83(5): 515-538.
- ✓ Corciovă A, Ivănescu B, Tuchiluş C, Fifere A, Doroftei F, Lungoci AL, Marangoci N, Mircea C. Biosynthesis of AgNPs using *Tilia cordata* flowers extracts and evaluation of some biological activities. *Environ. Eng. Manage. J*. 2018; 17(12): 2957-2968.
- ✓ Corciovă A, Mircea C, Tuchiluş C, Cioancă O, Burlec AF, Ivănescu B, Vlase L, Gheldiu AM, Fifere A, Lungoci AL, Hăncianu M. Phenolic and sterolic profile of a *Phyllanthus amarus* extract and characterization of biosynthesized silver nanoparticles. *Farmacia*. 2018; 66(5): 831-838.

3. 2. Design and physico-chemical characterisation of AgNPs

Our research aims to synthesize AgNPs by using different vegetal products and to confirm the generation of the nanoparticles through various methods.

The plants used for AgNPs synthesis are: *Phyllantus amarus*, *Tilia cordata* and *Quercus robur*.

The *Phyllanthus* genus (*Phyllanthaceae* family) includes over 800 species of plants widespread in tropical and subtropical regions, including China and India [Putakala et al., 2017]. The biologically active compounds highlighted in *Phyllanthus amarus* belong to the following categories: alkaloids, ellagitannins, lignans, volatile oil, triterpenes, flavonoids, sterols [Patel et al., 2011]. *Phyllanthus amarus* pharmacological activities were mentioned in Chapter 2.

Tilia cordata (linden) is native throughout Europe and western Asia and extensively planted. The active principles in linden flowers are flavonoids, mucilage, volatile oil, phenolic acids (caffeic, p-coumaric and chlorogenic acids), amino acids (alanine, cysteine, cystine, isoleucine, phenylalanine, and serine) and proanthocyanidins. Linden flowers are diaphoretic, antispasmodic, and expectorant and are used to relieve irritation of the throat in catarrh, to treat indigestion and feverish colds, and to alleviate headaches [Bisset and Wichtl, 2001].

Quercus genus (Fagaceae) (oaks) includes over 450 species worldwide. *Quercus robur* bark contains a various biologically active compounds, among which we mention catechins and oligomeric and polymeric proanthocyanidins [Popović et al., 2013].

Materials and methods

Plant material: The analyzed sample of *Phyllanthus amarus* aerial parts and *Quercus robur* bark were purchased from retailers [Corciovă et al., 2018a; Corciovă et al., 2018c]. Linden flowers were harvested in June 2016 in Iaşi and air dried at room temperature [Corciovă et al., 2018b].

Preparation of the extracts: The extract of *Phyllantus amarus* was obtained by ultrasonication in methanol (10 % w/v) for 10 minutes, and magnetic stirring for another 10

minutes, at room temperature followed by filtration [Corciovă et al., 2018c]. For preparation of linden flowers extract 10 g of plant were sonicated for 10 minutes at 80 ^{0}C with 100 ml of distilled water, and after cooling to room temperature, the solution was filtered through and stored in the refrigerator at 4 ^{0}C until further use [Corciovă et al., 2018b], and the extract in case of *Quercus robur* was 10 g % in water, prepared initially by ultrasonication at 80 ^{0}C for 10 minutes and then by magnetic stirring for another 10 minutes, at room temperature followed by filtration [Corciovă et al., 2018a].

Synthesis of AgNPs: In case of linden extract [Corciovă et al., 2018b] and oak bark extract [Corciovă et al., 2018a] over the silver nitrate ($AgNO_3$) solution of various concentrations the extract was added dropwise in a magnetic stirrer with integrated temperature control and the reaction conditions were varied: different ratios of extract to $AgNO_3$, different pH values, different temperatures and different stirring times. The resulting mixture was centrifuged at 8000 rpm for 10 minutes in case of linden extract and at 10.000 rpm for 30 minutes in case of oak bark extract. For *Phyllantus amarus* the synthesis of AgNPs was accomplished by mixing 1 ml extract with 10 ml of 5 mM $AgNO_3$ at 600 rpm for approximately 1 hour at room temperature. AgNPs were separated by centrifugation at 10.000 rpm for 15 minutes [Corciovă et al., 2018c].

In all cases, for purification, the supernatant was removed and the obtained nanoparticles redispersed in distilled water. To remove any substance adsorbed to the surface of nanoparticles, the centrifugation process was repeated twice, and the AgNPs obtained were separated and dried.

Physico-chemical characterization of AgNPs: Initially, the reduction of $AgNO_3$ was visually monitored by color change of the reaction mixture consisting of extract and $AgNO_3$. Then, the methods used for the characterization of AgNPs were: UV-Vis spectroscopy, FTIR spectroscopy, Transmission electron microscopy (TEM), Energy dispersive X-ray analysis **(**EDX), Photon correlation spectroscopy (PCS), Electrophoretic light scattering (ELS).

To demonstrate the reduction of silver ions and the formation of AgNPs, comparative spectra of AgNPs, extract and $AgNO_3$ were recorded using a Jasco V 530 double beam UV-Vis spectrophotometer. The nanoparticles solution was monitored in 1.0 cm quartz cells at a scanning speed of 1000 nm min^{-1} and a scan range of 300-600 nm, fixed slit width of 2 nm, at room temperature.

In order to highlight the functional groups of the biomolecules responsible for the reduction, capping and stabilization of AgNPs, the extract and synthesized silver nanoparticles FTIR spectra have been studied comparatively. FTIR spectra were obtained on a Bruker Vertex 70 instrument on KBr discs in a scanning range from 4000 to 310 cm^{-1}.

TEM method was used to evaluate the morphology and dimensions of the prepared AgNPs. TEM investigations were carried out with a Hitachi High-Tech HT7700 Transmission Electron Microscope operated at a 100 kV accelerating voltage in High-Contrast Mode. The samples were prepared on carbon-coated copper grids with 300-mesh size. Microdroplets of the samples dispersed in water (0.1 %) were placed on the grids, and then the solvent was removed under vacuum.

The chemical composition of AgNPs was evaluated by EDX analysis. An EDX system available on a Quanta 200 Environmental Scanning Electron Microscope (ESEM) was used for qualitative analysis. The EDX studies were performed on samples fixed on aluminum supports at 10 mm WD (working distance), which is the stage eucentric position and the collection point of

the EDX detector at 20 KV. The EDX detector used is the Si detector - EDX silicon-drift detector enables rapid determination of elemental compositions.

The hydrodynamic diameter and zeta potential of the particles were examined on the Delsa Nano Submicron Particle Size Analyzer (Beckman Colter) that uses photon correlation spectroscopy, for determining particle size and DLS for zeta potential determination. This analyzer determines the particle size of suspensions in a range from 0.6 nm to 7 mm.

Results

Some of the factors that influence the shape, size and amount of nanoparticles obtained have been investigated in case of AgNPs obtained from linden and oak bark extracts.

The conditions for obtaining AgNPs from linden extract are detailed below. First parameter studied was the concentration of $AgNO_3$: 3 mM, 5 mM and 10 mM. The absorbances of the resulting solutions were recorded and are presented in Figure 3. 1.

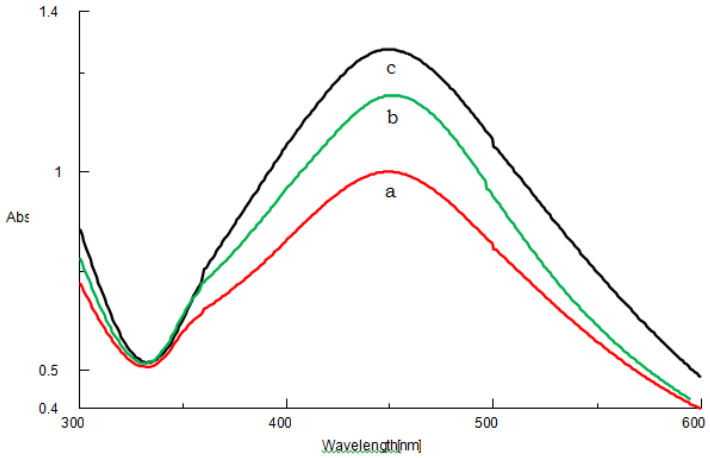


Figure 3. 1. Effect of different concentrations of $AgNO_3$ on the production of AgNPs
3 mM (a); 5 mM (b); 10 mM (c)

By increasing the concentration of $AgNO_3$, a higher amount of nanoparticles was obtained. Even though all the absorbances values were adequate, the following analyses were carried out with only 3 mM and 5 mM concentrations of $AgNO_3$, because we wanted to use smaller concentration of $AgNO_3$ and, at the same time, to be able to make a comparison of the results.

Another condition studied for synthesizing the maximum quantity of nanoparticles was the ratio of linden extract: $AgNO_3$ (3mM, 5mM) which varied as follows 5:1, 4:2, 3:3, 2:4, 1:5 (Figure 3. 2.).

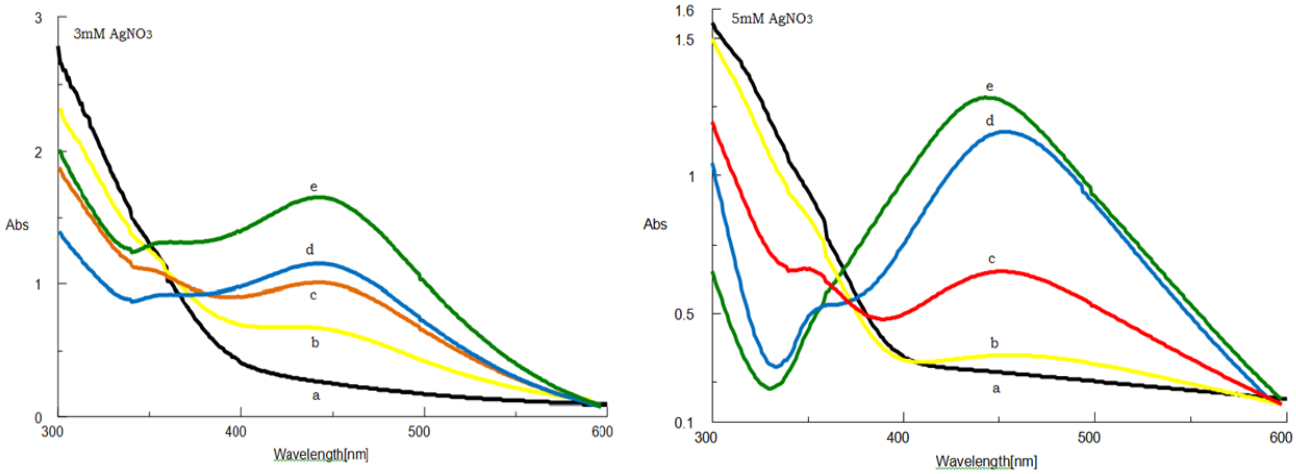


Figure 3. 2. Effect of ratio extract:$AgNO_3$ (3mM, 5mM), on production of AgNPs
5:1 (a); 4:2 (b); 3:3 (c); 2:4 (d); 1:5 (e)

For both concentrations of $AgNO_3$ solution (3 mM and 5 mM) the appearance of a characteristic peak of AgNPs formation was observed starting from a 3:3 molar ratio. Absorbance increased as the amount of $AgNO_3$ increased, the maximum absorbance of surface plasmon resonance peaks ranging from 435-460 nm. The most appropriate ratio was considered 1:5 because the solution has become darker faster compared to other samples and the surface plasmon resonance peak was highest at this ratio.

The pH of linden extract was 6. To optimize the process of producing AgNPs, the reaction medium pH was adjusted to 2 and 4 using 0.1 N HCl, and 8 and 10 using 0.1 N NaOH (Figure 3. 3.).

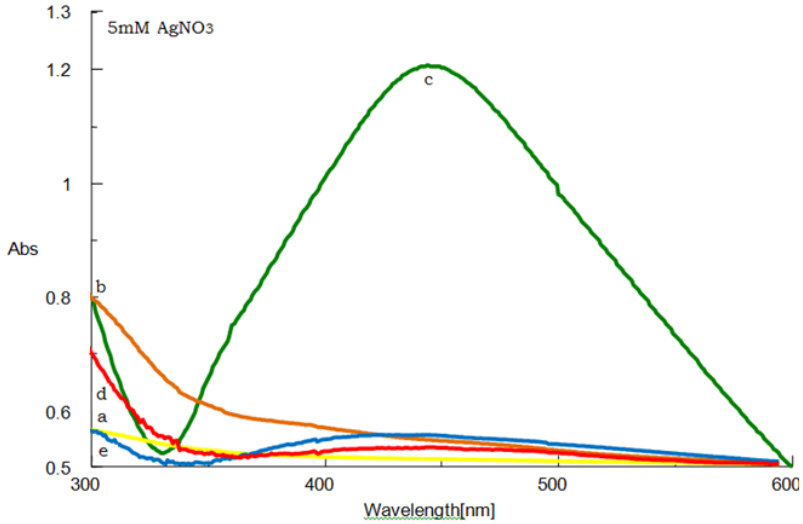


Figure 3. 3. Effect of pH on production of AgNPs: pH 2 (a); pH 4 (b); pH 6 (c); pH 8 (d); pH 10 (e)

In both cases (3 mM and 5 mM $AgNO_3$) UV-Vis spectra demonstrated that at acid pH and alkaline pH, AgNPs formation is suppressed. The optimal pH for obtaining highly dispersed nanoparticles was 6.

Also, to optimize the reaction conditions, the reaction mixture was stirred at different temperatures: 25 ^{0}C, 35 ^{0}C, 50 ^{0}C, 70 ^{0}C, 90 ^{0}C (Figure 3. 4.).

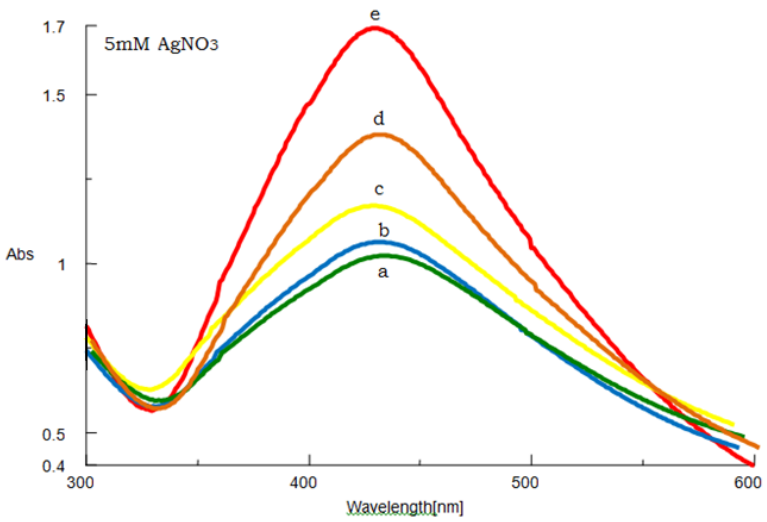


Figure 3. 4. Effect of temperature on production of AgNPs:
25 ^{0}C (a); 35 ^{0}C (b); 50 ^{0}C (c); 70 ^{0}C (d); 90 ^{0}C (e)

The absorbance increased with temperature, demonstrating an increase in the rate of production of AgNPs. Since at 25 ^{0}C was achieved a value of absorbance which demonstrates that the accuracy and precision are optimal and because this temperature is easy to attain, this temperature was selected to be further used in AgNPs synthesis.

To complete the conditions for obtaining AgNPs, the reaction was monitored in 0 – 240 minutes stirring time (Figure 3. 5.).

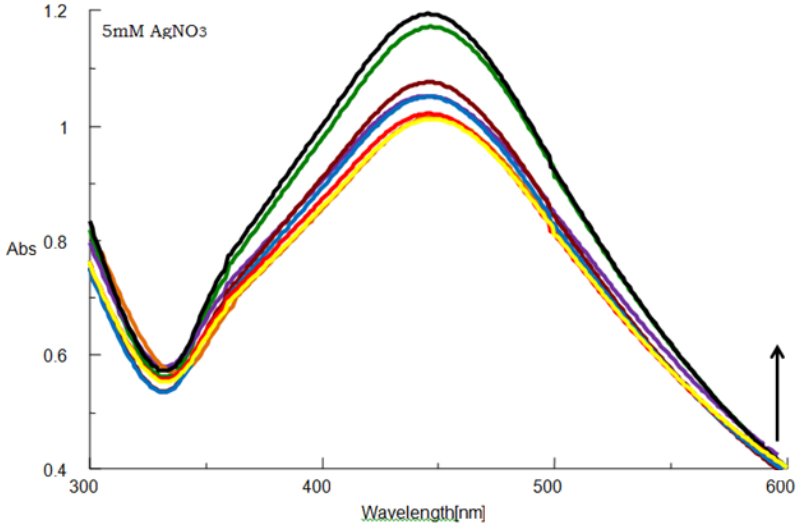


Figure 3. 5. Effect of stirring time on production of AgNPs:
15, 30, 45, 60, 90, 120, 180, 240 minutes

The absorbance increased with reaction time, and a larger amount of nanoparticles were formed. Because there were no significant differences compared to 180 and 240 minutes stirring

time, for further analysis we considered 120 minutes stirring time appropriate, in order to make the process more efficient and time-saving.

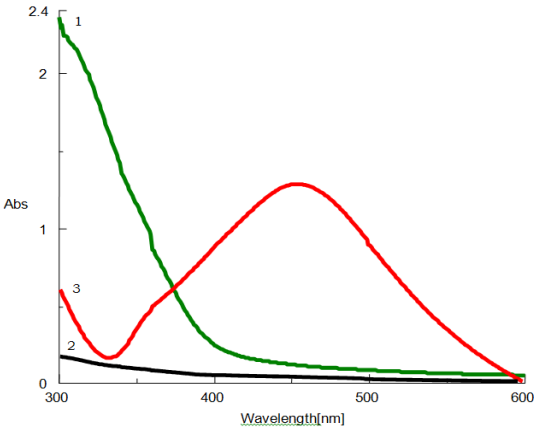
Thus, for further analysis, for obtaining AgNPs from linden extract, the next conditions were considered: 3 mM and 5 mM $AgNO_3$ (for a comparison between results), 1:5 extract: $AgNO_3$ ratio, pH 6, 25 ^{0}C and 120 minutes stirring time.

In case of oak bark extract, after studing different conditions of reaction, like various concentration of $AgNO_3$ (1mM, 3 mM, 5 mM), different ratios oak bark extract:$AgNO_3$ (1:9, 5:5, 9:1), different pH values (2 - 8), different temperatures (20 ^{0}C - 80 ^{0}C) and different stirring times (10 – 600 minutes), the conditions for AgNPs synthesis considered were: 3 mM $AgNO_3$, ratio extract: $AgNO_3$ 1: 9, pH 6, 60 ^{0}C and 90 minutes stirring time.

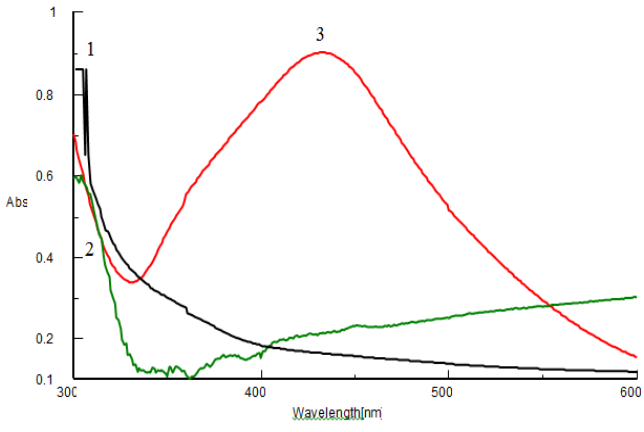
In all cases, the biosynthesis was observed in time through the change of the reaction mixture color from yellow to dark brown (Figure 3. 6.). indicating surface plasmon resonance appearance and formation of AgNPs. When using only $AgNO_3$ or extract, no color change was observed.

Figure 3. 6. Visual observation of synthesis process of AgNPs

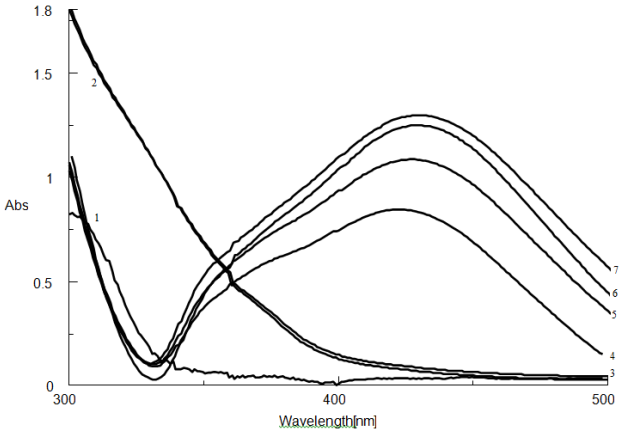
The UV-Vis spectra of the extracts, the $AgNO_3$ solution and the colloidal solutions were recorded and are presented in Figure 3.7.



a



b



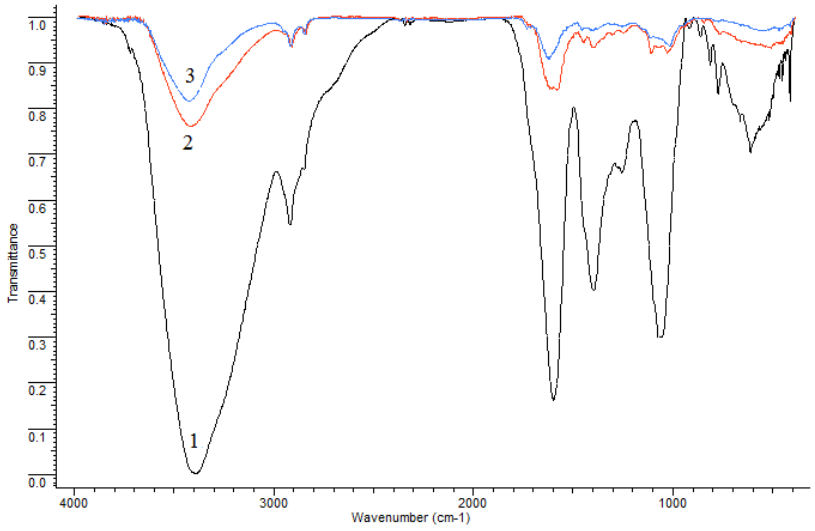
c

Figure 3. 7. Comparative UV-Vis spectra of **a.** linden extract (1), $AgNO_3$ solution (2), AgNPs (3); **b.** oak bark extract (1), $AgNO_3$ solution (2), AgNPs (3); **c.** $AgNO_3$ solution (1), *Phyllantus amarus* extract (2), the initial mixture (3) and of the colloidal solution after 10 minutes (4), 20 minutes (5), 30 minutes (6) and 60 minutes (7)

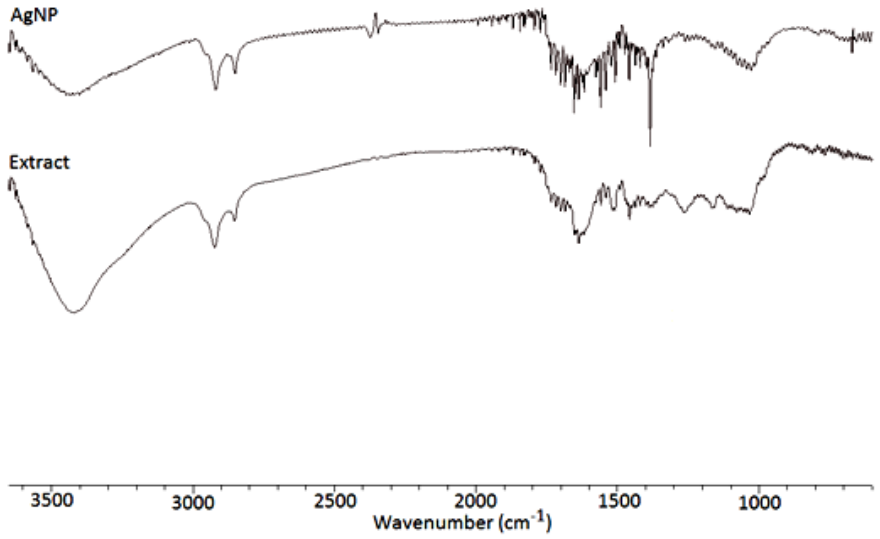
The $AgNO_3$ and extracts spectra have no absorption peaks in the investigated region. The UV-Vis spectra of the colloidal solution showed the surface plasmon resonance band, because of unique optical properties of noble metals, corresponding to AgNPs, centered at 450 nm (for linden) (Figure 3. 7.a), 432 nm (for oak bark) (Figure 3. 7.b), and 424 nm (for *Phyllantus amarus*) (Figure 3. 7.c).

Observing the synthesis over time, in case of *Phyllantus amarus*, the peak of AgNPs was evidentiated after 10 minutes, which demonstrates the start of the reduction process of Ag^+. With increasing reaction time, a rise in absorbance can be observed and after 60 minutes the peak remained unchanged, which shows that the process was complete.

FTIR analysis performed on the obtained AgNPs relative to the extract is presented in Figure 3.8.



a



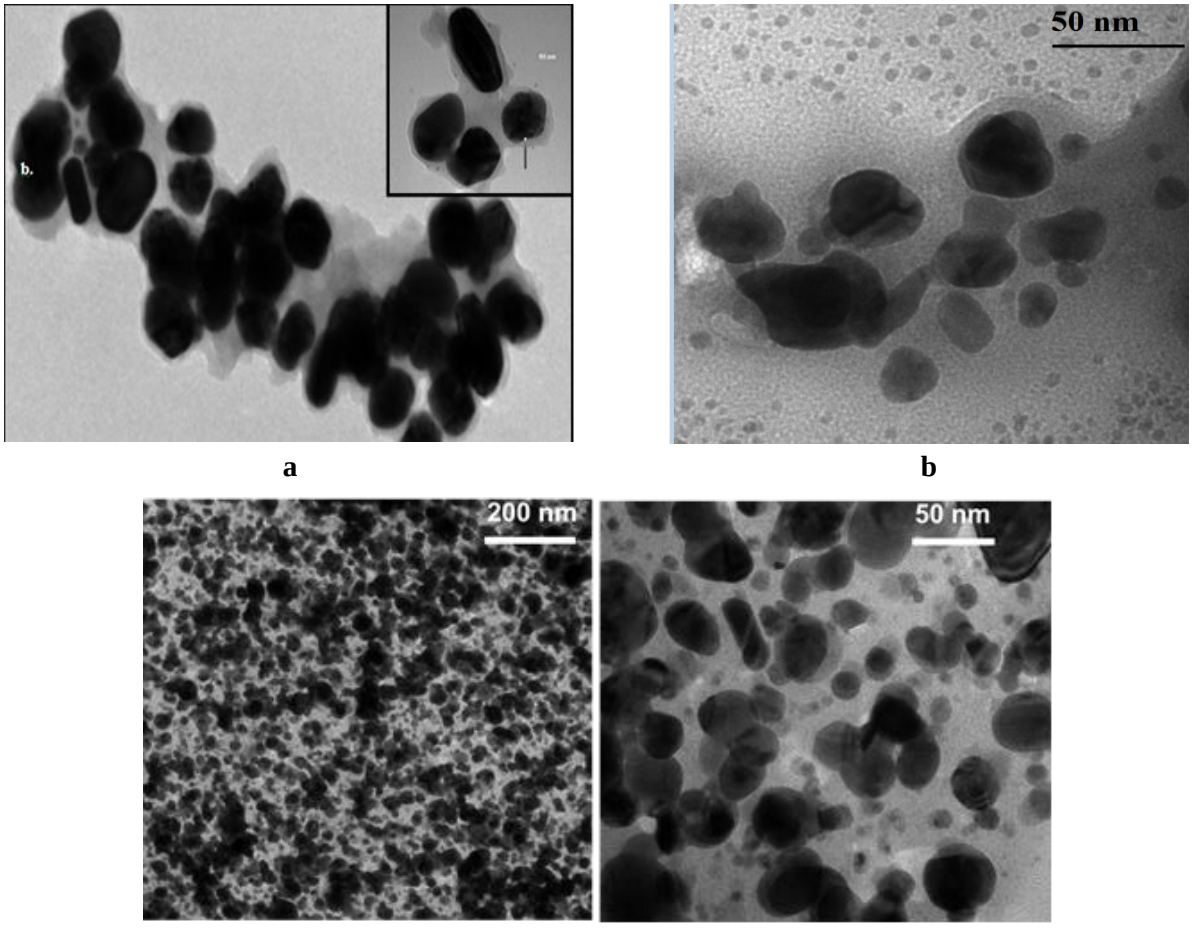
b

Figure 3.8. Comparative FTIR spectra of **a.** linden extract (1), AgNPs obtained using 3 mM $AgNO_3$ (2) and 5 mM $AgNO_3$ (3); **b.** *Phyllanthus amarus* extract and AgNPs

Overlapping spectra, a shift in peaks can be observed. So, for linden (Figure 3. 8.a) the shifted peaks are: 3401 to 3433/3433 cm^{-1}, 2925 to 2922/2923 cm^{-1}, 2855 to 2851/2851 cm^{-1}, 1603 to 1628/1619 cm^{-1}, 1405 to 1465/1465 cm^{-1}, 1078 to 1120/1117 cm^{-1}. The peaks from 1405, 1262 and 617 cm^{-1} transform in broad peaks. The peaks can be attributed as listed next: 3401 cm^{-1} to O-H stretching vibrations of polyphenols, 2925 cm^{-1} to C-H stretching bonds, 1603 cm^{-1} to amide I bonds (NH) proteins, 1405 cm^{-1} to carboxyl groups, 1078 cm^{-1} to C-O of aromatic OH group.

In case of *Phyllanthus amarus* (Figure 3. 8.b) the shifted peaks are: 3419 to 3435 cm^{-1} (OH stretching vibrations), 2923 to 2919 cm^{-1} and 2852 to 2850 cm^{-1} (alkyl C-H stretching vibrations bands), 1636 to 1653 cm^{-1} (stretching vibrations of C=C absorption), 1448 to 1458 cm^{-1} (bending frequency for cyclic $(CH_2)n$), and bending CH vibrations of flavonoids), 1026 to 1028 cm^{-1} (cycloalkane). The peak from 1273 cm^{-1} (C-O single bonds stretching vibrations) in extract spectra disappears in AgNPs spectra. Moreover, the appearance of a peak at 1383 cm^{-1} in AgNPs spectra may be interpreted as an attachment, during the synthesis, of some $-CH_2(CH_3)$ group on the surface of AgNPs.

Also, for oak bark, FTIR analysis confirmed Ag^+ reduction and AgNPs formation, secondary metabolites from the extract being used as reducing and encapsulating agents as demonstrated by TEM images (Figure 3. 9.).



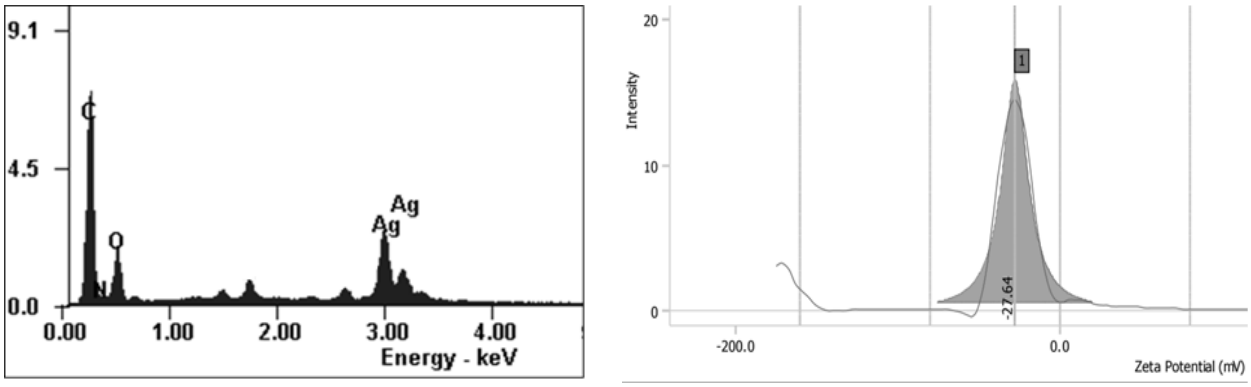
a **b**

c

Figure 3. 9. TEM images of AgNPs from **a.** linden extract; **b.** oak bark extract; **c.** *Phyllanthus amarus* extract

In all cases, most of the AgNPs displayed a spherical and a uniform shape. Particles have different sizes, and the average diameter estimated was in 10-90 nm range (Figure 3.9.a-c).

The presence of biomolecules involved in the reduction of silver ion and the presence of elemental silver are also demonstrated by EDX (Figure 3. 10.).



a **b**

Figure 3. 10. a. EDX spectra of AgNPs; **b.** Zeta potential of the AgNPs (obtained from *Phyllanthus amarus*)

In all cases, the study of the chemical composition of AgNPs, determined by EDX, confirms the presence of silver, by the significant peak at 3 keV, characteristic for elemental silver [Ibrahim, 2015]. Also, characteristic peaks are observed for carbon, oxygen that suggests the presence of biomolecules at the surface of AgNPs or in its proximity (Figure 3.10.a).

In case of AgNPs coated with linden extract, there are differences in the silver percentage, derived from the initial concentrations of the $AgNO_3$ used for nanoparticles preparation: AgNPs obtained from 5 mM $AgNO_3$ have higher Ag content and less carbon content than AgNPs obtained from 3 mM $AgNO_3$.

Generally, DLS analysis demonstrated that the average size was in a very good correlation with TEM results.

The values of zeta potential were negative. For exemple: for AgNPs obtained from linden, zeta potentials were -38.32 mV and -40.58 mV when 3 mM and 5 mM $AgNO_3$ were used [Corciovă et al., 2018b], and for AgNPs obtained from *Phyllanthus amarus*, zeta potential (Figure 3. 10.b.) was -27.64 mV [Corciovă et al., 2018c].

Discussions

By controlling some reaction parameters (such as $AgNO_3$ solution concentration, ratio extract: silver nitrate, temperature, pH, stirring time), the particle morphology can be controlled.

The easiest way to monitor the formation of AgNPs is to visualize the color change of the silver nitrate - extract mixture AgNPs [Ali M. et al., 2016]. This color change can be explained by the reduction of Ag^+ to Ag^0, via the bioactive compounds [Lu et al., 2014] like polyphenols, carbohydrates, amino acids or terpenoids from extracts used and to the excitation of surface plasmon vibrations in AgNPs.

By UV-Vis was evidenciated the wavelength corresponding to the surface plasmon resonance band, characteristic of AgNPs, in 400-450 nm range. The obtaining of a broad peak in case of AgNPs from linden and *Phyllanthus amarus* extracts indicates that the nanoparticles are polydispersed [Veerasamy et al., 2011]. The polydispersed suspension obtained can be explained by the variety of biomolecules present in extracts which have a different potential for silver ion reduction, hence the damage to nucleation and growth of nanoparticles [Ali A. et al., 2016].

The results from FTIR analysis showed that the peaks are shifted or disappeared, demonstrating that the respective groups belonging to some compounds which participate to the formation of nanoparticles. For exemple, in case of AgNPs obtaining from linden extract the compounds responsible are those containing phenolic OH, amide and carboxyl groups and in case of *Phyllanthus amarus*, the biomolecules responsible for obtaining AgNPs may belong to phenols and sterols classes.

Enhanced TEM images showed that nanoparticle edges are brighter, suggesting that nanoparticles are encapsulated by a layer of biomolecules [Ibrahim, 2015; Halawani, 2017]. This fact demonstrates that nanoparticles are not in direct contact and biomolecules prevent aggregation [He et al., 2017]. Also, the biomolecules act not only as reducing agents but also cap the nanoparticles surfaces and thus, playing, at the same time, the role of stabilizing agents [Halawani, 2017; Moldovan et al., 2016].

However, in time, a week tendency of particle agglomerations was observed, indicating a possible sedimentation [Ali A. et al., 2016]. This behavior is probably due to the evaporation of the solvent during preparation.

EDX analysis demonstrated the presence of silver and also of biomolecules that capping AgNPs. The colloidal suspension is stable due to the capping biomolecules which by negative charges determine a rejection between nanoparticles [Haider and Mehdi, 2014].

Negative values of zeta potential indicate the stability of colloidal solution [Varadavenkatesan et al., 2016] and a strong rejection between nanoparticles, demonstrating that they do not aggregate [Padalia et al., 2015].

Conclusions

Synthesis of silver nanoparticles by plants is a simple, non-toxic and eco-friendly method. For reducing the silver ions, were used: *Tilia cordata* (linden) flowers, *Phyllanthus amarus* and *Quercus robur* (oak) bark extracts.

For linden and oak cases, some parameters were investigated to optimize the synthesis method: pH, concentration of silver salt, different ratio of plant extract and silver salt, temperature and stirring time.

The formation of nanoparticles was demonstrated by the change in colour of initial mixture ($AgNO_3$ and extract), UV-Vis, FTIR, TEM, EDX, DLS. The UV-Vis spectrum shows the characteristic surface plasmon resonance peak. FTIR, TEM and EDX techniques, revealed the presence of dispersed spherical nanoparticles containing an organic shell. FTIR and EDX analysis confirmed the reduction of Ag^+, the presence of silver and the formation of AgNPs. The extracts through their secondary metabolites were used as reducing, capping and stabilizing agents. The zeta potential of synthesized AgNPs was negative, indicating the stability of the synthesized nanoparticles.

3. 3. Biological characterisation of AgNPs

Because of their properties, AgNPs can play a significant role in biology and pharmaceutical fields. So, we studied the antimicrobial and antioxidant properties of synthesized AgNPs.

3. 3. 1. *In vitro* evaluation of antimicrobial activity of AgNPs

Materials and methods

Samples: extracts and AgNPs obtained from *Phyllantus amarus*, linden, oak were dissolved in dimethylsulfoxide and ultrasonicated 20 minutes.

The antimicrobial activity of the analyzed samples was evaluated by diffusion method on agar medium. On the surface of Petri plates Mueller-Hinton agar medium (Oxoid) for bacteria and Mueller-Hinton agar medium (HiMedia) for fungi, inoculated with the suspension of the test microorganism, were placed in stainless steel cylinders with an inside diameter of 6 mm and 10 mm height, in which 100 μg of the test samples were deposited.

The test microorganisms used were: Gram positive bacteria – *S. aureus ATCC 25923,* Gram negative bacteria – *E. coli ATCC 25922*, *P. aeruginosa ATCC 27853,* and fungus – *C. albicans ATCC 10231* and *C. parapsilosis ATCC 22019.* After incubation, 24 hours at 37 ^{0}C for bacteria and 48 hours at 24 ^{0}C for fungi, the diameters of the microbial growth inhibition zones were recorded. As positive control were used discs containing ciprofloxacin (5 μg/disc) and nystatin (100 μg/disc). All the determination were performed in triplicate and the results were expressed as the mean ± SD.

Results

The antibacterial activity of the synthesized AgNPs synthesized from *Phyllanthus amarus* extract was investigated on *S. aureus* and *P. aeruginosa*. The diameter of the inhibition zones is shown in Table 3.1.

Table 3. 1. The diameters of the inhibition zones (mm) of *Phyllanthus amarus* extract and AgNPs

Sample	**Antibacterial activity**	
	S. aureus	***P. aeruginosa***
Phyllanthus amarus extract	10 ± 0	0
AgNPs	15 ± 0	14 ± 0.5

The extract exhibits antibacterial activity only against Gram-positive bacteria, while AgNPs show activity against both Gram-positive and Gram-negative bacteria, the effect on *S. aureus* being higher than in the case of the extract [Corciovă et al., 2018c].

The antimicrobial activity of the AgNPs synthesized from linden extract was tested against *S. aureus, E. coli, P. aeruginosa, C. albicans* and *C. parapsilosis*. The diameters of the inhibition zones corresponding to the test samples and standard compounds are presented in Table 3. 2.

Table 3. 2. The diameters of the inhibition zones (mm) corresponding to the samples

	Antibacterial activity			**Antifungal activity**	
	S.aureus	***E. coli***	***P. aeruginosa***	***C.albicans***	***C. parapsilosis***
Linden extract	0	0	0	0	0
AgNPs from $AgNO_3$ 3 mM	11 ± 0.0	10 ± 0.3	12 ± 0.5	15 ± 0.1	16 ± 0.0
AgNPs from $AgNO_3$ 5 mM	12 ± 0.2	12 ± 0.5	14 ± 0.2	17 ± 0.0	21± 0.3

It can be seen that AgNPs exhibit a good antibacterial and antifungal activity, in contrast to the linden extract used as control. Although the linden extract contains molecules with antimicrobial activity, such as phenolic compounds, phytosterols, and saponins, it appears that the concentration of these compounds is too low for a noticeable activity. The antimicrobial activity varied depending on the $AgNO_3$ concentration used in the synthesis of AgNPs: the diameter of the inhibition zones was higher when the 5 mM $AgNO_3$ was used, compared to 3

mM $AgNO_3$. The antifungal activity of AgNPs was more pronounced than antibacterial activity [Corciovă et al., 2018b].

Synthesized AgNPs from *Quercus robur* bark have been shown to have antibacterial activity against *S. aureus ATCC 25923, P. aeruginosa ATCC 27853* and antifungal activity against *C. parapsilosis ATCC 22019* [Corciovă et al., 2018a].

Discussions

The differences noted between antifungal and antibacterial activity can be explained by the differences between bacterial and fungal wall composition. Also, we notice a slight difference between antibacterial activity against Gram positive and Gram negative bacteria, probably due to the difference in the thickness of bacterial cell membrane and the constituents of the membrane.

The literature proposes several mechanisms for antibacterial activity of AgNPs. Because of the small size of AgNPs, they can attach to the surface of the bacterial cell membrane and/or penetrate inside the bacteria, interact with the sulfur and phosphorus proteins, leading to the destruction of membrane permeability and respiratory cell functions, ultimately destroying the cell [Kvitek et al., 2008]. Another explanation would be that by dissolving AgNPs, silver ions are strongly reactive to the surface or inside the bacterial cell, interacting with sulfur-containing proteins [Reidy et al., 2013] or with the thiol group of vital enzymes, resulting in their defective function or inactivation [Cao et al., 2010], or silver ions may interact with phosphorus-containing compounds (e.g., DNA) leading to a decrease in bacterial proliferation [Wong and Liu, 2010]. Furthermore, the association of antimicrobial activity of the nanosilver form and the presence in the extract plant of some compounds with antimicrobial properties like phenols and flavonoids contribute to the increase of the antimicrobial activity of the AgNPs, because the surface to volume ratios in the nanoparticles increases which means the maximum contact area with the bacteria [Kumar et al., 2016].

Antifungal activity can be explained by the interaction of AgNPs with compounds with phosphorus and sulfur, which causes the destruction of proteins and DNA and results the destruction of membrane integrity and cell death [Krishnaraj et al., 2012].

3. 3. 2. *In vitro* evaluation of antioxidant activity of AgNPs

Materials and methods

Samples: extracts and AgNPs obtained from *Phyllantus amarus* and linden were dissolved in dimethylsulfoxide and ultrasonicated 20 minutes.

For evaluation of antioxidant activity were used: lipoxygenase inhibition assay (AgNPs from *Phyllantus amarus* and linden), DPPH free radical scavenging assay (AgNPs from *Phyllantus amarus*), iron-chelating capacity (AgNPs from linden).

Determination of the ability of the samples to inhibit the lipoxygenase activity (modified Malterud method [Malterud and Rydland, 2000]) – 10 minutes after the samples (extract and AgNPs) are treated with lipoxygenase, linoleic acid in borate buffer pH 9 was added and the absorbance was recorded, in 30-90 seconds range at 234 nm. The ability of samples to inhibit the lipoxygenase activity was calculated.

The capacity of samples to neutralize DPPH radical - the DPPH solution was added to samples, and the absorbance was registered after 10 minutes at 517 nm [Tătăringa et al., 2016] The scavenger activity of samples was calculated.

For the method iron-chelating capacity - 0.1 M acetate buffer (pH 5.25), 2 mM ferrous sulfate were added to the sample to be analyzed, and after stirring for 10-15 seconds, 5 mM ferrozine solution was added. After 10 minutes of rest in the dark, the absorbance of the solution at 562 nm was determined against a blank prepared under the same conditions as the sample (the ferrous sulfate solution was replaced with ultrapure double distilled water). Ferrous ion chelating capacity was calculated [Venditti et al., 2010].

For the samples that achieved 50 % inhibition/scavanger activity/chelating capcity, the IC_{50} value was calculated. All the determination were performed in triplicate and the results were expressed as the mean ± standard deviation

Results

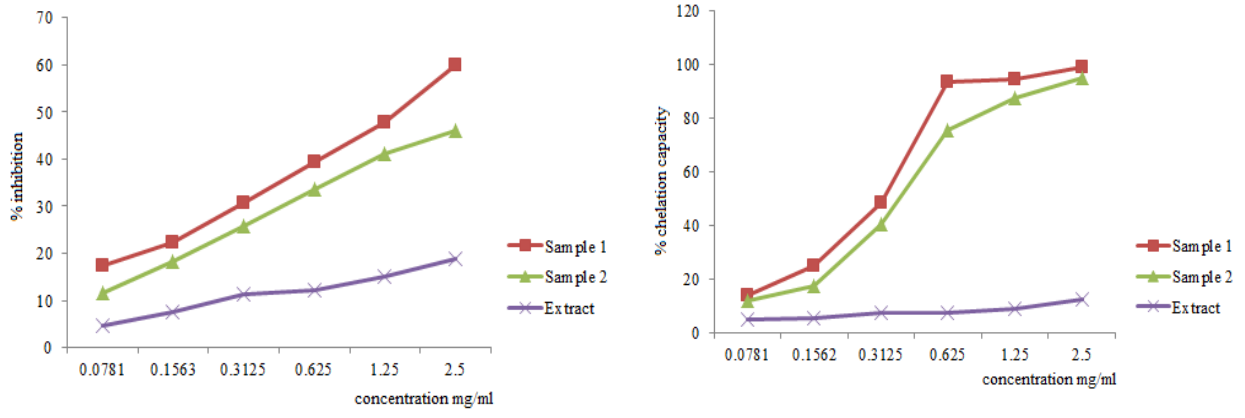
The antioxidant activity of the *Phyllanthus amarus* extract and of the synthesized AgNPs expressed as IC_{50} values are presented in Table 3. 3.

Table 3. 3. Antioxidant activities of *Phyllanthus amarus* extract and AgNPs

Sample	**Antioxidant activity**	
	DPPH free radical scavenging assay	**Lipoxygenase inhibition assay**
Phyllanthus amarus extract	12.68 ± 0.01 mg/mL	-
AgNPs	-	3.82 ± 0.07 µg/mL

It can be noticed that in the case of AgNPs the IC_{50} value cannot be calculated for the DPPH free radical scavenging assay, whereas in the case of the lipoxygenase inhibition assay IC_{50} is 3.82 ± 0.07 µg / mL.

For the samples from linden, six different concentrations were used, ranging from 0.0781 to 2.500 mg/mL. The results are shown in Figure 3. 11.



a **b**

Figure 3. 11. a. The capacity of inhibition of lipoxygenase activity and **b**. the iron-chelating capacity of AgNPs and linden extract, as a function of sample concentration

Our results showed a significant increase in the antioxidant activity of the synthesized AgNPs compared to the linden extract. Also, the values increased depending on the concentration of the analyzed sample.

Discussions

The differences between the values of antioxidant activity in case of *Phyllantus amarus* obtained by the two methods, could be explained by the different mechanisms of antioxidant activity. Thus, in the DPPH free radical scavenging assay, the 2,2-diphenyl-1-picrylhydrazyl radical requires compounds with hydroxyl groups (or other hydrogen donor groups), which have contributed to the production of AgNPs, while, in the case of lipoxygenase inhibition assay, the AgNPs lipoxygenase inhibitory effect can be explained by the high dimensions of silver nanoparticles which are encapsulated by biomolecules and in that way could prevent the access of the substrate to the active center of the enzyme. On the other hand, these nanoparticles could modify the tridimensional structure of the enzyme or the tridimensional structure of its active site.

Antioxidant activity can be explained by the higher total phenolic and flavonoid content of nanoparticles compared to that of linden extract. For samples that have a capacity of inhibition of lipoxygenase activity/iron-chelating capacity higher than 50 % in the range of tested concentrations, an IC_{50} value expressed in μg of sample/mL of final solution was calculated. Thus, in the case of the extract, it was not possible to calculate the IC_{50}. For the first method, the IC_{50} was 63.84 ± 0.03 μg/mL (Sample 1 - AgNPs obtained from 3 mM $AgNO_3$) and 75.68 ± 0.13 μg mL (Sample 2 - AgNPs obtained from 5 mM $AgNO_3$). For the second method, the IC_{50} was 282.05 ± 11.25 μg/mL (Sample 1). By comparing the results obtained with the $AgNO_3$ concentration used for synthesis, AgNPs obtained using a 3 mM concentration have been shown to have a stronger antioxidant activity. This fact was also explained by EDX spectra analysis, the content of biomolecules being higher in the case of AgNPs obtained from 3 mM $AgNO_3$.

Conclusions

In order to determine possible biological applications, we tested the antimicrobial and the antioxidant activities of AgNPs synthesized. The results demonstrated that the antimicrobial and the antioxidant activities of AgNPs synthesized were high compared to the extracts.

Also, the results demonstrated that all the extracts taken into work could be used to obtain AgNPs by green chemistry, a simple, fast, cost-effective and eco-friendly method, with an important potential in the development of some therapeutic agents.

In conclusion, the green synthesis could be an alternative way to obtain silver nanoparticles.

SECTION II.

FUTURE DIRECTIONS IN SCIENTIFIC, PROFESSIONAL AND ACADEMIC ACTIVITY

Future scientific activity development

Regarding the scientific activity, the intention is to continue and develop the research with reference to the topics analyzed so far.

Design, synthesis, physico-chemical and biological characterization of new multifunctional systems

The main objective of this direction will be the design, synthesis, characterization and testing of new therapeutic systems with potential biological actions such as antioxidant, antimicrobial and antitumor properties. It is expected that multifunctional systems will exhibit an increase in solubility and permeability of bioactive substances, which will lead to an increase in drug concentration and to an increase and prolongation of therapeutic effects in the target tissue compared to the free substance, but also to reduced side effects. Such studies are representative for current international scientific interests.

The excellent biocompatibility and unique inclusion capacity, as well as the high functionality of cyclodextrins and its derivatives make them particularly attractive for the engineering of new functional materials and for biomedical applications. Scientific literature has reported several bioactive substances-cyclodextrins complexes with improved solubility, bioavailability, tissue distribution, and therapeutic action. Therefore, a first approach would be to use cyclodextrin derivatives that have been successfully used to build supramolecular systems and to design new functional materials, with the benefits of host-guest interactions between cyclodextrin units and bioactive molecules.

At the same time, one of the objectives is to continue to obtain nanoparticles, diversifying the type of synthesized nanoparticles and creating magnetic hybrid materials of organic-inorganic type. For example, gold nanoparticles are non-toxic carriers for drug substances and their applications provide a complementary support for traditional vehicles. The combination of low toxicity, high surface area and good stability are some of the unique attributes that should provide new advantageous delivery strategies for bioactive substance. These nanoparticles can be used *in vivo* to protect the drug in the systemic circulation, to limit the access to the drug only at chosen sites and to deliver the drug at a controlled and sustained rate at the site of action.

In order to increase the stability and bioavailability of substances and to decrease the time spent in the circulatory system, avoiding first pass clearance by the reticuloendothelial system and reducing the required dose, it is possible to use nanomagnetic systems based on magnetite and a self-assembled material based on zinc-ferrite associated with cyclodextrin.

As a first step, the obtained systems will be physico-chemically characterized by various methods such as UV-Vis spectral analysis, FT-IR, NMR, thermal methods, TEM, SEM and EDX. Afterwards, these systems will be tested *in vitro*, in order to establish the release and dissolution kinetics of the active substance, the stability of the obtained systems and the

antioxidant, antimicrobial and antitumor activities, depending on the substances/plant compounds used.

This research direction fits the current trends in nanomedicine and could significantly contribute to an attractive research field related to nanoparticle obtaining. Moreover, it provides a real opportunity to create an interdisciplinary team, through collaborations with fellow teachers and researchers from the "Center for Advanced Research for Bionanoconjugates and Biopolymers" of "Petru Poni" Institute of Macromolecular Chemistry Iasi, thus creating a real basis for future project proposals.

Development and validation of simple and fast analysis methods

Regarding this area of study, the intention is to develop several methods for analysis of active compounds from different matrices such as pharmaceuticals, dietary supplements, vegetal products, biological samples.

Analytical techniques play an important role in pharmaceutical research, starting from the development stage and going further into manufacturing and marketing stages of the drug. Consequently, the development and validation of simple, rapid, economic and accurate methods of analysis is a constantly evolving theme. Therefore, the aim is to develop and validate methods that can be used for the analytical control of raw materials, intermediate products, drugs containing one or several active substances. The newly developed methods will also be applied for comparative control of commercial products containing the same active substance.

One should not forget the importance of determining the physical and chemical stability during drug formulation, thus, developing methods for identifying and quantifying impurities and degradation products is another aspect to be included in future researches.

Given the fact that drug analysis also involves the evaluation of drug release characteristics from biological samples, in addition to the development of *in vitro* dissolution studies, the wish is to establish simple methods to qualitatively and quantitatively analyze drugs and metabolites, which could be later applied in pharmacokinetic studies.

In addition, future research will also be directed towards the development of methods used to separate, identify and quantify substances with bioactive potential from plant material.

The obtained results will be valued by publishing scientific papers and through presentations held at national and international scientific conferences.

The wish is to develop new collaborations with researchers from different national and international universities, faculties and research centers and consequently, to be part of multidisciplinary research teams and of various research project competitions.

Another goal is to continue the publishing of books/book chapters of recognized national and international publishing houses and to be a peer-reviewer for various journals whenever the opportunity will present itself.

Future professional and academic activity development

From the perspective of a teaching activity coordinator, the aim is to form, and, depending on future possibilities, to develop and perfect a team, in order to set up positive professional prospects for our students in areas such as drug analysis and pharmaceutical legislation.

Regarding this aspect, the objective is to develop the study materials based on accessible scientific language, in order to facilitate understanding through explanations, examples and convincing demonstrations for the subjects covered in courses and practical lessons. Depending on the possibilities and the development of the laboratory with regard to reagents and equipment, the aim is to diversify the methods of analysis used, according to the standards of the area of study. Another goal is to create the prospect for an active involvement of the students in the didactic and research activities, which could lead to an increase in scientific interest and number of participations at student scientific events. Therefore, students will receive the necessary information for an easy access to the job market and will be trained to carry out competent scientific work and to take responsibility for their professional decisions.

At the same time, special attention will be granted to the guidance of residents, master students and all graduates interested in specializing through various education programs.

In order to ensure the exchange of ideas and good practices for institutional optimization, the collaboration with colleagues from other departments will continue and develop and an optimal communication with those from other faculties in the country will be maintained.

"Coming together is a beginning,
keeping together is progress,
and working together is success."
Henry Ford

SECTION III.

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