GRIGORE T. POPA UNIVERSITY OF
MEDICINE AND PHARMACY IASI

HABILITATION THESIS

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Pharmaceutical substances with therapeutic potential: technological characterization and optimization

-Habilitation thesis-

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CONTENTS

ABSTRACT

A quality education builds on personal development, representing the starting point of a successful career. Such a career requires a high quality education system.

The profound changes that we live at the social, economic, political, cultural and technological level influence the educational system. In order to meet the new requirements, teachers must be well trained, as well as in permanent training and development, so that they can deliver quality education to students, residents and PhD students.

The Habilitation Thesis *"Pharmaceutical substances with therapeutic potential: technological characterization and optimization"* is a complete picture of my professional development and briefly presents the most important researches in the field of Pharmacy, during the almost twenty years after being awarded with the PhD title. The thesis is as an important step in the recognition of my scientific, teaching and academic abilities and proves that I am capable of proficient transfer of knowledge to the next generation.

Also, it is a starting point for the future PhD students' activity in the field of Pharmacy, who wants to begin the research studies by preparing a doctoral thesis.

The habilitation thesis entitled *"Pharmaceutical substances with therapeutic potential: technological optimization"* 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 scientific achievements of the postdoctoral period. **Section II** presents future projects regarding the scientific, professional and academic areas which will focus on continuing these directions, but also on expanding of the interest area, in line with the progress of science. **Section III** includes the list of references used in the thesis.

In the first part of the thesis there is a synthesis of professional and scientific activity, from the beginning to date. The teaching activity follows the steps of professional development, ranging from research assistant to associate professor, and includes courses and practical lessons of "Drug Industry and Pharmaceutical Biotechnology" for the Pharmacy students in the Romanian and English teaching sections, courses and practical lessons of "Pharmaceutical Management and Marketing" for the Pharmacy students in the Romanian and English teaching sections and the optional course "Opportunities for the Pharmacists" for the Pharmacy students in the Romanian teaching sections. Also, didactic activity included courses and practical lessons of "Pharmaceutical equipments and machinery", "Pharmaceutical Biotechnologies", "Industrial Syntheses (Fundamental Chemical Processes and Technological Processes of Drug Synthesis)" within the Pharmaceutical Industry and Cosmetics Residency program at the Faculty of Pharmacy Iași (2004-2008), as well as courses, practical lessons and seminars of "Genetics and Pharmacogenetics" for the master students from the Advanced Medical Biotechnology Master program at the "Grigore T. Popa" Iași University of Medicine and Pharmacy (2009-2014).

Scientific acquisitions of the postdoctoral period include over 50 scientific papers published *in extenso*, of which 17 are found in ISI quoted journals and 22 are BDI indexed, 5 books (3 as first author, 2 co-authored) and 1 Invention Patent (Pelloid extract toothpaste). The cumulative impact factor for the first author papers is over 18, and the Hirsch index is 6, according to ISI Web of Science, Core Collection, Thomson Reuters.

Also, during this period I was involved in 3 research grants involving the scientific area of the habilitation thesis (1 as a director).

Section I is centered on two research directions. The first research direction entitled "Framework for the development of pharmaceutical substances with therapeutic potential" focuses on the obtaining of new synthetic and semi-synthetic compounds with therapeutic potential, their phisico-chemical and spectral characterization as well as the *in vitro* investigation to highlight the expected therapeutic action. Design, synthesis and characterization of new compounds were accomplished by using a different type of chemical reactions according to starting materials, reaction conditions, the desire molecule structure. Physico-chemical characterization was performed through molecular formula, molecular weight, solubilities in different solvents, yields and melting point determinations. The *in vitro* investigations involved the antioxidant and anti-inflammatory potential determination using various modern methods agreed by scientific literature.

The second research direction, "Continuous quest for pharmaceutical industrial process improvement", presents the results of the improving methods for the separation of active substances from nonconforming drugs in order to recover them due to their high cost. Also, we present the results obtained in the development, validation and application of new auxiliary substances for various pharmaceutical forms, so as to broaden the range of substances used at industrial level in drugs formulation.

The microparticles incorporating classical active substances obtaining, in order to improve their pharmacokinetic, pharmacological and pharmacotoxicological properties are also presented in this research direction. Last but not least, my contribution is also presented in terms of wastewater management. The wastes generated by pharmaceutical companies have increased concerns about environmental and human safety. These wastewaters containing secondary products, impurities, catalysts, solvents, etc. are a thorny issue for the pharmaceutical industry, which has to comply with "green chemistry" and "clean technologies" policies, in order to balance lower environmental impact with more sustainable and competitive drugs production.

Section II covers the development plans of scientific, professional and academic career which will focus on expanding the research directions.

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

REZUMAT

O educaţie de calitate pune bazele dezvoltării personale, reprezentând punctul de pornire al unei cariere profesionale de success. Pentru a avea o astfel de carieră este nevoie de un sistem de învăţământ de înaltă calitate.

Schimbările profunde pe care le trăim la nivel social, economic, politic, cultural, tehnologic influenţează şi sistemul de învăţământ. Pentru a face faţă noilor exigenţe, cadrele didactice trebuie să fie bine pregătite, precum și într-o permanentă formare şi dezvoltare pentru a putea transmite studenţilor, rezidenţilor, doctoranzilor o educaţie de calitate.

Teza de abilitare cu titlul "*Substanţe farmaceutice cu potenţial terapeutic: caracterizarea și optimizarea tehnologiilor de obținere*", reprezintă o imagine completă asupra devenirii mele profesionale şi prezintă pe scurt cele mai importante cercetări în domeniul Farmaciei, pe parcursul celor aproape douazeci de ani scurşi de la finalizarea tezei de doctorat. Este un pas important în recunoaşterea abilităţilor mele ştiinţifice, didactice şi academic și dovedește că sunt capabilă să transfer eficient cunoștințele accumulate către generația următoare.

Totodată este un punct de plecare pentru viitoarea activitate de îndrumare a doctoranzilor în Domeniul Farmacie, care îşi doresc o aprofundare a activităţii de cercetare, după finalizarea studiilor universitare, prin realizarea unei teze de doctorat.

Teza este alcătuită conform recomandărilor Consiliul Naţional de Atestare a Titlurilor, Diplomelor şi Certificatelor Universitare (CNATDCU) din trei secțiuni principale: **Secțiunea I** reprezentată de realizările ştiințifice din perioada postdoctorală; **Secțiunea II** reprezentată de planurile de dezvoltare viitoare, care se vor axa pe continuarea direcțiilor menționate, dar și pe extinderea ariei de interes, în acord cu progresul științei; **Secțiunea III** reprezentată de lista referințelor bibliografice utilizate în teză.

În partea introductivă este prezentată o sinteză a activităţii profesionale şi ştiinţifice, de la începutul carierei până în prezent. Activitatea didactică urmărește etapele evoluției profesionale, pornind de la preparator până la conferenţiar şi include cursuri şi lucrări practice de "Industria Medicamentelor şi Biotehnologii Farmaceutice" pentru studenții Facultății de Farmacie, seriile în limba română şi engleză, cursuri şi lucrări practice de "Management şi Marketing Farmaceutic" pentru studenții Facultății de Farmacie, seriile în limba română şi engleză şi cursul opţional de "Oportunităţi în profesia de farmacist" pentru studenții Facultății de Farmacie, seriile în limba română. De asemenea, activitatea didactică a inclus ore de curs şi laborator de "Utilaje şi aparatură folosite în industria farmaceutică", "Biotehnologii farmaceutice", "Sinteze industriale (procese chimice fundamentale şi procese tehnologice de sinteză a medicamentelor) în cadrul *Rezidenţiatului de Industrie Farmaceutică şi Cosmetică* la Facultatea de Farmacie Iaşi (în perioada 2004-2008), precum şi ore de curs, lucrări practice şi seminar de "Genetică şi Farmacogenetică", pentru masteranzii de la *Masteratul Biotehnologii medicale avansate* din cadrul U.M.F. "Grigore T. Popa" Iaşi (2009-2014).

Cercetarea din perioada postdoctorală s-a concretizat în peste 50 de lucrări științifice publicate *in extenso*, dintre care 17 sunt în reviste cotate ISI și 22 sunt indexate BDI, 5 cărţi (3 ca prim-autor, 2 cărți co-autor) și 1 Brevet de Invenție (Pastă de dinți cu pelloid extract). Factorul cumulat de impact pentru lucrările ca autor principal este peste 18, iar indicele Hirsch este 6 conform ISI Web of Science, Core Collection, Thomson Reuters.

De asemenea, în această perioadă am fost implicată în 3 granturi de cercetare, pe domeniile tezei de abilitare (1 în calitate de director).

Secţiunea I este centrată pe două direcţii de cercetare. Prima direcție de cercetare intitulată ”*Dezvoltarea substanțelor farmaceutice cu potențial terapeutic*” se axează pe obținerea de noi compuși de sinteză și de semi-sinteză cu potențial terapeutic, caracterizarea acestora precum și investigarea *in vitro* pentru evidențierea acțiunii terapeutice scontate.

Noile substanțe active au fost sintetizate prin diferite tehnici, iar structurile lor au fost confirmate prin metode fizico-chimice (determinarea formulei moleculare, a masei moleculare, a solubilităților în diverși solvenți, a randamentelor de reacție, a punctelor de topire) și spectrale (UV-VIS, IR, ^{1}H-RMN). Investigarea *in vitro* a presupus determinarea acțiunii antioxidante, apelând la metodele moderne agreate de literatura științifică, precum și determinarea acțiunii anti-inflamatoare, utilizând teste *in vitro* relevante.

A doua direcție de cercetare, intitulată ”*Preocupări continui pentru îmbunătăţirea proceselor din industria farmaceutică*” prezintă rezultatele cerectărilor privind îmbunătățirea proceselor de separare a substanțelor active din forme farmaceutice necoforme, cu scopul recuperării acestora. De asemenea, sunt prezentate rezultatele obținute în dezvoltarea, validarea și utilizarea de noi substanțe auxiliare pentru diverse forme farmaceutice, astfel încât să se lărgească paleta de substanțe utilizate la nivel industrial în formularea medicamentelor. Obținerea de microparticule care înglobează substanțe active clasice cu scopul îmbunătățirii performanțelor farmacocinetice, farmacologice și farmacotoxicologice sunt de asenmenea prezentate în această a doua directive de cercetare. Nu în ultimul rând, sunt prezentate contribuțiile mele în ce privește managementul apelor industriale uzate, rezultate în urma proceselor tehnologice de obținere a medicamentelor. Aceste ape industriale uzate ce conțin produși secundari, impurități, catalizatori, solvenți etc. reprezintă o problemă spinoasă pentru industria farmaceutică, ce trebuie să se conformeze politicilor de ”green chemistry”, de utilizare a ”tehnologiilor curate”, pentru a nu polua mediul și în același timp să producă competitiv medicamente.

Secţiunea II cuprinde planurile de evoluție şi dezvoltare a carierei științifice, profesionale şi academice care continua și extinde direcțiile de cercetare.

Secţiunea III include lista referințelor bibliografice care susțin datele prezentate în teza de abilitare.

OVERVIEW OF THE PROFESSIONAL, SCIENTIFIC AND ACADEMIC CAREER

Nowadays to be a successful teacher imply a permanent evolution, a continuously learning, a daily updating to scientific and technical acquisitions in order to transfer your knowledge to the next generation. This is the way for teachers to give students/residents/ postgraduate students a complete insight on the field they teach.

In the context of a fulminant evolution of society and in a competitive services market, universities need to adapt to the needs of the market and achieve an efficient educational management. This can be accomplished with the help of highly qualified teaching staff forming competitive and adaptable specialists on the market.

Professional achievements

After graduating the Faculty of Pharmacy from the "Grigore T. Popa" University of Medicine and Pharmacy Iași in September 1993, I worked for several months as a pharmacist during an internship at the "Sf. Maria" Hospital Iaşi and in April 1994 I passed the exam for research assistant in the *Drugs Industry and Pharmaceutical Biotechnology* department. This exam confirmed me also as a resident in the General Pharmacy specialty.

I became a specialist pharmacist in General Pharmacy specialty in 1996 and a primary pharmacist in General Pharmacy specialty in 2001.

I continued through examination as an Assistant Professor from March 1998, Lecturer from March 2004 and Associate Professor since October 2014.

During all this time I carried out my teaching activity in the *Drugs Industry and Pharmaceutical Biotechnology* department, and from October 2017 also in the *Pharmaceutical Management and Marketing* department.

From the position of teaching staff member I have participated in the preparation and development of practical lessons and I taught courses for 5th year pharmacy students at Drugs Industry and Pharmaceutical Biotechnology, and Pharmaceutical Management and Marketing disciplines. From October 2016 I introduced an optional course for 3rd year pharmacy students entitled *Opportunities for Pharmacists*, which have great success through students. Since October 2015 I taught all these practical lessons and courses for pharmacy students in the English Program.

During 2004-2008 I had developed teaching activities which includes practical lessons and courses in "Pharmaceutical Industry and Cosmetics" Residency program of the following modules: *Pharmaceutical equipments and machinery, Pharmaceutical Biotechnologies, Industrial Syntheses (Fundamental Chemical Processes and Technological Processes of Drug Synthesis).* At the same time, I coordinated the industrial practice of the residents registered in

this residency program at U.M.F. ”Grigore T. Popa” Iași, guiding them and distributing them to productive units: Antibiotice S.A. Iași, Mark Pharmaceutics SRL Iași, Ceta Impex SRL Sibiu etc.

During 2009-2014 I was co-opted in the Advanced Biotechnology Master's team at the Faculty of Medical Bioengineering. I taught courses, developed practical lessons and seminars for the *Genetics and Pharmacogenetics* discipline for the 1st year postgraduate students.

At all programs guided by me I supervise bachelor’s theses and also coordinated dissertations.

I completed a master's degree in Management field, "Medical and pharmaceutical health services Management” in 2018 at "Grigore T. Popa" University of Medicine and Pharmacy Iași, which was very useful for my teaching/leadership activity.

In July 2016 I was accepted at my second specialty, Clinical Pharmacy, being now resident in the 3rd year.

At the same time, from 2011 I am working as senior pharmacist in a Hospital Pharmacy at ”Elena Doamna” Obstetrics-Gynecology Clinical Hospital from Iași, practicing successfully, conscientiously and devotedly the pharmacist profession.

I am an active member in important Scientific Societies: Romanian Society of Pharmacy Science, Romanian Association of Algesiology, International Association for the Study of the Pain, European Association of Hospital Pharmacists, Society of Physicians and Naturalists etc.

During this period, I have attended and finished training courses regarding pharmaceutical technology, microparticles obtaining techniques, management, quality audit, quality assurance, health and safety at work.

Throughout the teaching activity carried out so far, I have regarded the instructive-educational process as a whole. That is why I was looking to be close to students, residents and master students, to inspire love for the profession of pharmacist, for work and for scientific research. All my teaching activity has always had an educational side in the spirit of fairness, honesty, punctuality, conscientiousness, and professional deontology.

Scientific achievements

I completed my doctoral studies at "Grigore T. Popa" University of Medicine and Pharmacy Iasi, *Doctoral Advisor, Professor Victor Năstase PhD*, with the thesis entitled "*New synthesized compounds with preservative action in food and pharmaceutical field*", for which I was conferred the title of *Doctor in Pharmacy domain*, by Ord MEC no. 3876/ 19.05.2004 (Diploma series C, No. 0010213).

Taking into account the knowledge gained during doctoral studies I continued my research activity, which allowed me to accumulate skills and expertise in the field of synthesis, semi-synthesis and biosynthesis of new biologically active derivatives belonging to different therapeutic classes, in the field of physico-chemical analysis and structure confirmation by spectral methods of the pharmaceutical substances, in the field of *in vitro* and *in vivo* studies for

therapeutic potential confirmation of the new molecules, and also in the field of pharmaceutical industrial processes optimization.

The results of the postdoctoral scientific research have been published in articles indexed by the Web of Science Core Collection and in other international databases. I have also disseminated the results at local, national and international congresses, conferences, seminars and workshops. My scientific research results can be quantified according to Thomson ISI Web of Science Core Collection database as follows: indexed full papers by international databases-23; indexed full paper in journals with IF-17; sum of times cited-75; sum of citations, without self-citations-64; H-index-6.

During 2014-2015 I was conducted a project funded by the "Grigore T. Popa" University of Medicine and Pharmacy through its Internal Grants Program, project number 29244/20.12.2013 entitled *"Research on the development of novel heterocyclic derivatives with anti-inflammatory potential"*.

Between 2007 and 2011, I participated as part of the team, in two research grants:

- Project PNCDI II, Program 4, Partnership in prioritary domain, Complex projects (PC) - *Complex characterization of active cytostatic extracts from Claviceps purpurea strains obtained by parasexual hybridization biotechnologies for use in veterinary* (contract no. 62-065/2008) (partner 1 – "Grigore T. Popa" University of Medicine and Pharmacy Iași), funded period 2008-2011;
- Project PNCDI II, Program 4, Partnership in prioritary domain, Complex projects (PC) – *New controlled release nitrogen-donating therapeutic systems (*contract no. 41017/2007) (partner 1 – "Grigore T. Popa" University of Medicine and Pharmacy Iași), funded period 2007-2010.

To help the specialists in pharmaceutical, chemical and medical field understanding some aspects concerning pharmaceutical industry, biotechnology and drug synthesis, I have been published several books as principal author or co-author:

- Cătălina Daniela Stan, *Basic Industrial and Laboratory methods for active substances obtaining*, Editura "Gr.T. Popa" Publisher, U.M.F. Iaşi, 2015;
- Cătălina Daniela Stan, *Biotech drugs*, Ed. "Gr. T. Popa" U.M.F. Iaşi 2011;
- Cătălina Daniela Stan, *Pharmaceutical Biotechnology. Antibiotics*, Ed. Ars Longa, Iaşi, 2007;
-Cătălina Daniela Stan, Maria Drăgan, *Pharmaceutical substances synthesis and biosynthesis. Industrial and lab techniques*. Ed. "Gr. T. Popa" U.M.F. Iaşi, Iaşi, 2013;
-Dumitrache M., Cătălina Daniela Stan, *Drugs industry and Pharmaceutical Biotechnology*, Lit. U.M.F. "Gr.T.Popa" Iaşi, 2000.

I have been a reviewer for various articles in ISI Farmacia journal. Also, in 2015 I have been a scientific reviewer for a doctoral thesis "*Research regarding new heterocyclic compounds with biologic potential*", *Doctoral Advisor, Professor Lenuța Profire PhD*, of doctoral student Wolszleger Maria. Moreover, during the postdoctoral period, I was a member of scientific guidance commission of two doctoral thesis.

Also, in 2010 with my co-workers I published one Invention Patent (”*Pelloid extract toothpaste*”), number 122831/30.03.2010, published in BOPI no. 3/2010.

Academic achievements

During all this time, I was deeply involved in various academic activities:

- coordinator of the Drugs Industry and Pharmaceutical Biotechnology department since 2004;
- coordinator of the Pharmaceutical Management and Marketing department since 2017;
- member of the Faculty Council (2008-2012; 2016-present);
- member of the Senat of University (2012-2016);
- member of Pharmaceutical Science II Department Council (2012-present);
- internal auditor for the Quality Management System from 2008;
- coordinator for Faculty health and safety at work (2012-2018);
- president of the Faculty Committee CEAC (2016-present);
- contact person for ARACIS periodical evaluation of the Faculty study programs in 2018;
- member of the Professional committee for the Bachelor exam (since 2009),
- member of the Professional committee for the residency exam (2015);
- member of various committees for the admission exam, specialist/primary pharmacist exams, dissertation, research assistant/assistant professor/lecturer/associate professor exams;
- member of the Organizing Committee and Scientific Committee of national scientific congresses.

SECTION I

Scientific achievements

I. Framework for the development of pharmaceutical substances with therapeutic potential

I.1. State of the art

The increasing average age of population, the growing number of patients, and the lack of long-term effective remedies push ahead the need for novel tools against various diseases. Nowadays, very few tools are available for preventing or counteracting the progression of such diseases.

Therefore, it is understandable why all the specialists in the medical and pharmaceutical field have a growing interest to develop new strategies for synthesis, semi-synthesis and chemical modification of natural occurring compounds.

A natural compound is a chemical entity, formed by a naturally occurring living organism with pharmacological properties (Feher et al., 2003).

Natural therapeutic agents are prepared from compounds found occurring in nature, which contain active components from plants, microbes, minerals and animals. The most used natural medicine source is plants, due to their chemical and structural diversity and the biodiversity of their components (Mathur and Hoskins, 2017).

Developed countries aspire as more than 60% of all medicines to be natural products or secondary metabolites (Eddershaw et al., 2000). Nowadays, natural products continue to produce additional clinical candidates and medicinal compounds, being vital in pharmaceutical and biotechnology industries. Although natural products have been vital in drug discovery, recently pharmaceutical companies have substantially reduced their natural product research (Lahlou, 2013).

Generally, the therapeutic agents based upon naturally occurring molecules are a mixture of complex therapeutic compounds (Mathur and Hoskins, 2017). Therapeutic agents are considered as natural, synthetic, or semi-synthetic dependent on the source from which they were generated.

The distinctive structural diversity of natural compounds is advantageous during the production of combinatorial libraries using semi-synthetic methods. Semi-synthetic therapeutic agents are a hybrid of natural and synthetic sources. Semi-synthetic therapeutic agents are

generally produced by transforming natural sources into final products via chemical reactions (Cragg and Newman, 2013).

The expansion of novel and advanced technologies such as metagenomics, combinatorial chemistry, high throughput screening, automate separation methods in drug discovery has transformed the screening of natural products due to their chemical and structural diversity and biodiversity (Patwardhan et al., 2004).

Even if not all natural products can be completely synthesized, with the help of advanced drug discovery, the pharmaceutical and biotech industries still use natural raw material in order to obtain new entities or derivatives of these.

The obtaining of semi-synthetic or synthetic compounds allows for improving the biological activities of the natural molecules, which are unmodified, so they can be used as a better alternative. The final goal is to extend therapeutic properties of the original/natural molecule, to ensure the safety of the compound, to maximize the benefit–risk ratio, to minimize the adverse events, to reduce the effective doses and so to reduce the undesired toxic effects of pharmaceutical substances, including carcinogenic and teratogenic ones (Lenardao et al., 2015, Zanforlin et al., 2017). In addition, these derivatives have to produce physiological and pharmacological effects within living cells, have more interaction with proteins, enzymes and other biological molecules and so have a better therapeutic index.

The purpose of drug discovery processes is to establish the most promising lead compounds, which may be used as a therapeutic agent and facilitated with treating medical conditions, including infections, cancer, nervous system diseases, high blood pressure and metabolic diseases (Lahlou, 2013; Mathur and Hoskins, 2017).

The use of new synthetic or semi-synthetic molecules must be in agreement with the green chemistry, this beeing a new philosophy, which aims to minimize the collateral effects caused by the chemical activity to the environment (Lenardao et al., 2003; Lenardao et al., 2015).

On the other hand, synthetic compounds are chemical substances, including those essential to human life, which can be characterized in many ways. They are utilized for a variety of purposes, have proliferated over the last half-century as synthetic methodology and production technology have developed to highly sophisticated levels.

A popular view holds that natural substances are superior to synthetic substances with respect to their effects on human health. A rational perspective view holds that synthetic compounds have safety, efficacy, pharmacokinetics, therapeutic properties and chemical stability as a result of combinatorial chemistry (Topless et al., 2002).

Continuous improvements in synthetic methodology have provided access to a vast array of synthetic substances with therapeutic potential, which can interact with possible targets of diseases. Chemical synthesis and structure–activity relationship (SAR) are usually done in an effort to produce an improved drug substance with better therapeutic properties (Topless et al., 2002).

Nowadays, many of the most successful and important drug substances are derived through synthetic chemistry.

Drug design process involves high throughput screening in order to obtain the lead compounds against specific targets. Many lead compounds are not selective enough for their target molecule, so in order to improve their selectivity, researchers modify these compounds as per the expected structure-activity relationships (Pascolutti and Quinn, 2014). If the modifications increase the selectivity, the promising compounds move to *in vitro* and *in vivo* testing.

My research interest and activities in the field of development of new pharmaceutical substances with therapeutic potential were extensive, considering several topics, but in the following pages I will focus on my relevant results derived from the above-mentioned research theme, since their results have already been disseminated in European and international congresses and/or published in ISI/BDI journals.

The studies in this research direction were part of a Research Grant *"Research on the development of novel heterocyclic derivatives with anti-inflammatory potential"*, funded by the "Grigore T. Popa" University of Medicine and Pharmacy through its Internal Grants Program, project number 29244/20.12.2013 and several other published papers.

- Drăgan M, Stan CD, Iacob A, Profire L. Assessment of *in vitro* antioxidant and antiinflammatory activities of new azetidin-2-one derivatives of ferulic acid. *Farmacia* 2016; 64(5): 717-721.
- Drăgan M, Stan CD, Pânzariu A, Profire L. Evaluation of anti-inflammatory potential of some new ferulic acid derivatives. *Farmacia* 2016; 64(2): 194-197.
- Stan CD, Drăgan M, Iacob AT, Profire L. Assessment of *in vitro* antioxidant activity of some new ferulic acid derivatives. *Rev Med Chir Soc Med Nat Iaşi* 2016; 120(3): 727-731**.**
- Stan CD, Drăgan M, Pânzariu A, Profire L. Evaluation of the synthesis and structure of new azetidin-2-ones of ferrulic acid. *Rev Med Chir Soc Med Nat Iaşi* 2016; 120(2): 434-438.
- Tătărîngă G, Stan CD, Mircea C, Jitareanu A, Zbancioc AM. Antioxidant evaluation of some coumarin derivatives. *Farmacia* 2016; 64(4): 533-538.
- Wolszleger (Drăgan) M, Stan CD, Pânzariu A, Jităreanu A, Profire L. New thiazolidine-4-ones of ferulic acid with antioxidant potential. *Farmacia* 2015; 63(1): 150-154.
- Tătărîngă G, Stan CD, Zbancioc, AM., Jitareanu A, Tuchiluş A. Preliminary screening of biological activities of some new Schiff bases of isatin. *Farmacia* 2014; 62(1): 14-22.

I.2. Design, synthesis and characterization of ferulic acid derivatives

In the present days we are witnessing to a growing interest of researchers in the use of antioxidants in reducing oxidative stress.

Development of new compounds including in their structure biologically active entities, especially heterocyclic systems is a major concern for researchers.

Ferulic acid (4-hydroxy-3-methoxy-cinnamic acid) is a phenolic compound found in plant cell walls as a component of lignocelluloses. It plays a key role in the plants self-preservation mechanism, ensures cell wall rigidity, protection against microbial invasion as well as sun damage protection (Zdunska et al., 2018).

Ferulic acid (FA) was isolated in 1866 from *Ferula foetida*, its name beeing based on the botanical name of plant. It is a phenolic derivative of cinnamic acid present in plant cell wall components as covalent side chains (Zhao and Moghadasian, 2008). It has been found in vegetables, fruits, flowers, coffee, cereals both in free and conjugated forms. Mainly, the conjugated forms are esters with the specific polysaccharides, alcohols, sterols, acids etc.

A lot of studies indicate that ferulic acid possesses antioxidant, antihyperlipidemic, antimicrobial, anticarcinogenic, radioprotective, and hepatoprotective effects. Ferulic acid blocks the peroxidation of lipids from membranes and by this way will protect cells and organelles against oxidation, and generally is important for the prevention of many human diseases (Baskaran et al., 2010; Yang et al., 2018).

Due to its structure, ferulic acid presents the ability to interrupt the propagation of free radical chain reactions, provide attack sites for free radicals and ensure protection against lipid peroxidation (Sangeeta et al., 2015; Sanga et al., 2017). Due to its antioxidant effect, ferulic acid may protect DNA and lipids from oxidation by reactive oxygen species and could be useful in the prevention and treatment of several oxidative stress-related disorders including Alzheimer's disease, diabetes mellitus, cancer, hypertension, and atherosclerosis (Anselmi et al., 2004; Wang et al., 2005).

At the same time the involvement of oxidative stress in inflammation suggest that ferulic acid may also be effective in the treatment of inflammatory diseases. Moreover, the specific structure gives ferulic acid a strong UV absorption ability, making it a powerful skin protecting agent (Zhao and Moghadasian, 2008).

New hydrazone derivatives of ferulic acid, obtained through chemical synthesis, could have antioxidant potential, being used in the protection of biological systems from oxidative stress (Rasras et al., 2010).

Thiazolidine-4-one derivatives represent one of the most studied class of heterocyclic organic compounds because of their important biological effects. The first argument related to the importance of this heterocycle is penicillin and its derivatives that contain the thiazolidine ring in the beta-lactam structure.

The compounds with thiazolidine structure have been reported for their antioxidant, antitumor, antitubercular, antiinflammatory and analgesic effects (Isloor et al., 2013; Sala et al., 2013). The studies of structure-activity relationship have highlighted the influence of the substituents at C_2, N_3 and C_5 on biological properties of the thiazolidine-4-ones. It is known that electron-donating groups at N_3 improve the antiviral activity and the electron withdrawing groups at N_3 prints simultaneously antibacterial and antiinflammatory properties (Shelke et al., 2012; Apostolidis et al., 2013).

It is well known that the azetidin-2-one ring is the common structure of beta-lactam antibiotics such as penicillins, cephalosporins, carbapenems, monobactams and nocardicine used as chemotherapeutic agents in the treatment of infectious diseases (Cervellati et al., 2013; Stan et al., 2016). Recent studies assign to this heterocyclic ring many other biological effects: hypoglycemic, anti-tumor, anti-HIV, anti-inflammatory, analgesic and antioxidant actions (Galletti et al., 2011).

The β-lactam heterocycles are substances which drew attention and interest of scientists for their potent antibacterial activity (Arya et al., 2014). Nowadays, the research in this area is stimulated due to the development of bacterial resistance to the β-lactam antibiotics. Therefore, novel functionalized β-lactams are being explored (Hakami et al., 2017). The new interest has been focused on the synthesis and modification of β-lactam ring in order to obtain new derivatives with diverse biological activities.

Since cyclic amide (lactam) have similar structure with vitamin C, which is a cyclic ester (lactone), it is challenging to study the antioxidant properties and also the antimicrobial properties of derivatives which includes in their structure lactam heterocycle.

Drug design process involves chemical/biochemical synthesis and semi-shynthesis, pysico-chemical characterization of the new molecules and spectral confirmation of the proposed structures. All these steps are mandatory in order to obtain the lead compounds against specific targets. Further, the new compounds support modifications to achieve selectivity.

Design, synthesis and characterization of new compounds are accomplished by using a different type of chemical reactions according to starting materials, reaction conditions, the structure of the desire molecule etc.

Due to these differences in approaching synthesis I will present the obtaining methods for the new molecules by grouping them depending on the parent molecule.

Research in this area has focused on:

- design and synthesis of thiazolidine-4-one derivatives of ferulic acid by different techniques;
- design and synthesis of azetidin-2-one derivatives of ferulic acid by different techniques;
- physico-chemical characterization of all the derivatives of ferulic acid by modern methods of analysis.

I.2.1. Design, synthesis and characterization of new thiazolidine-4-ones of ferulic acid

Personal contribution to knowledge in this research field is presented in following published paper:

- Wolszleger (Drăgan) M, Stan CD, Pânzariu A, Jităreanu A, Profire L. New thiazolidine-4-ones of ferulic acid with antioxidant potential. *Farmacia* 2015; 63(1): 150-154.

The semi-synthesis of new thiazolidine-4-one derivatives of ferulic acid have been performed in one step ("one-pot") by cyclization of ferulic acid hydrazide with different aromatic aldehydes and mercaptoacetic acid. The structural modulation of the ferulic acid molecule was performed in order to obtain better antioxidant effects for the semi-synthetic compounds than the starting molecule.

The synthesis of the designed compounds was performed in several steps:

- ✓ the obtaining of ferulic acid chloride by reacting of ferulic acid with thionyl chloride;
- ✓ the reaction between the ferulic acid chloride and hydrazine hydrate 98% to obtain the ferulic acid hydrazide;
- ✓ the condensation of ferrulic acid hydrazide with various benzaldehydes (2-hydroxy/2-nitro/4-nitro/2-methoxy/4-chloro/4-fluoro/4-bromo/2,3-dihydroxy/2,6-dichloro/4-dimethylamino/4-hydroxy-3-methoxi-benzaldehyde)and mercaptoacetic acid in exces resulting the corresponding thiazolidine-4-ones.

The synthesized compounds were characterized and structure validated by FT-IR spectra and ^{1}H-NMR spectra.

I.2.1.1. Materials and methods

Substances, reagents and solvents

The aromatic aldehydes (2-hydroxy/2-nitro/4-nitro/2-methoxy/4-chloro/4-fluoro/4-bromo/2,3-dihydroxy/2,6-dichloro/4-dimethylamino/4-hydroxy-3-methoxi-benzaldehyde), mercaptoacetic acid and organic solvents (*p.a*. quality) were purchased from Sigma Aldrich Company and Fluka Company.

All the solvents and the reagents were used without prior purification. TLC plates silica gel 60 F_{254} from Merck Company were used for monitoring of the chemical reactions.

Synthesis of thiazolidine-4-ones of ferulic acid

The hydrazide of ferulic acid (3) has reacted with different aromatic aldehydes and mercaptoacetic acid in excess to give the corresponding thiazolidine-4-ones (4a-l). The ferulic

acid hydrazide (3) was obtained starting from ferrulic acid (1) which was converted to corresponding chloride (2) and then reacted with hydrazine hydrate (Figure 1).

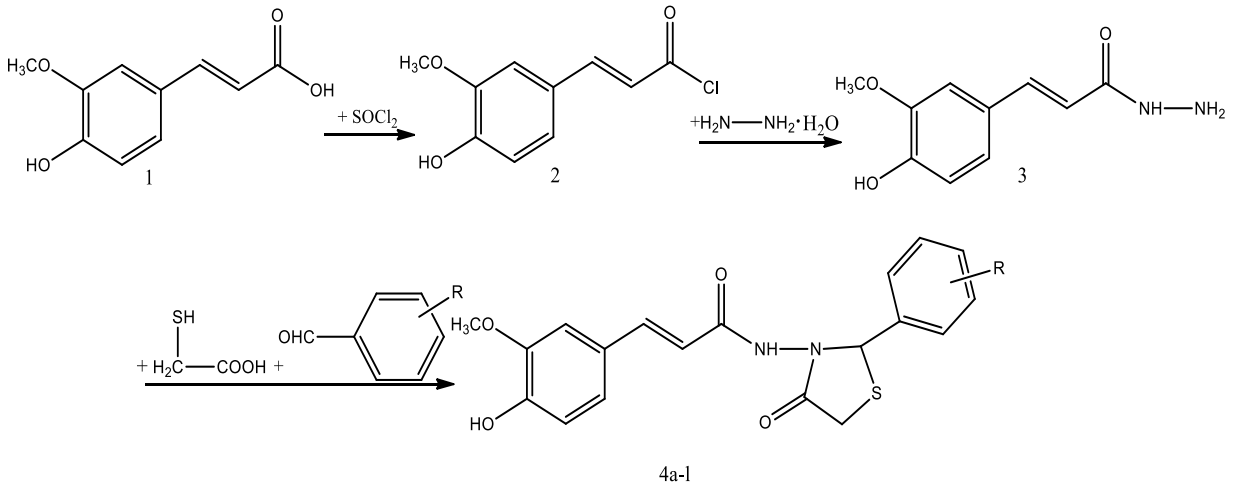


Figure 1. The synthesis of new thiazolidin-4-one derivatives of ferulic acid

General procedure for the synthesis of ferulic acid thiazolidine-4-one

To a solution of ferulic acid hydrazide (0.5 g, 2.3 mmol) in freshly distilled toluene, aromatic aldehydes (4.6 mmol) were added under inert atmosphere. The mixture was stirred for 5 min and mercaptoacetic acid 98% (0.5 mL, 6.9 mmol) was added and then it was heated at 110-120°C for 36-46 hours until completion of the reaction (TLC monitoring, using dichloromethane: methanol - 5:0.1, v/v, UV light at 254 nm).

The mixture was neutralized with saturated solution of sodium bicarbonate and extracted with ethyl acetate (2 x 25 mL). The organic layer was separated and washed with hydrochloric acid 1M and then with saturated solution of sodium chloride.

Finally, the organic layer was dried over $MgSO_4$ and filtered. The solvent was removed under reduced pressure. The residue was precipitated in petroleum ether (Wolszleger et al., 2014; Wolszleger et al., 2015).

Physicochemical characterization

The thiazolidine-4-one derivatives of ferulic acid were physicochemically characterized by melting point, yield, molecular formula, relative mass, solubility in water and other organic solvents.

The melting point was determined by using Buchi M 565 (Buchi, Switzerland).

The reaction was monitored by TLC on 60 F254 silica gel plates, and spots were visualized by UV light at 254 nm.

Characterization by spectral methods

The FT-IR spectra were recorded on ABB-MB 3000 FT-IR MIRacle^{TM} Single Bounce ATR-crystal ZnSe, over a 500-4000 cm^{-1} range, after 16 scans at a resolution of 4 cm^{-1}. The spectra processing was carried out with the Horizon MB^{TM} FTIR Software.

The ^{1}H-NMR spectra were obtained on a Bruker Avance 400 MHz Spectrometer using tetramethylsilane as internal standard and deuterated dimethyl sulfoxide (DMSO-d6) as solvent. The chemical shifts were recorded in δ values (ppm).

I.2.1.2. Results

Physico-chemical and spectral characterization

The ferulic acid thiazolidine-4-ones are crystalline powders, colored from light yellow to bright brown, very slightly soluble in DMFA (dimethylformamide), DMSO, and acetone, sparingly soluble in absolute ethanol, methanol, chloroform, dioxane and insoluble in distilled water and diethyl ether. The physico-chemical characteristics are listed in Table 1.

Table 1. Physico-chemical characteristics of ferulic acid thiazolidin-4-ones

No	R	Mol. weight	Yield η (%)	M.p. (^{0}C)
4a	-H	370.24	15.96	102
4b	-Cl(4)	404.06	31.63	190
4c	-F(4)	388.09	19.95	108-110
4d	-Br(4)	449.32	41.24	174-176
4e	-NO_2(4)	415.08	9.31	98-100
4f	-NO_2(2)	415.08	36.31	140-142
4g	-OCH_3(2)	415.08	36.31	140-142
4h	-OH(2)	400.45	29.01	118-120
4i	-Cl(2,6)	386.42	50.97	213-215
4j	-$N(CH_3)_2$(4)	439.31	82.82	229-230
4k	-OH(2,3)	413.14	30.94	160
4l	-OH(4)-OCH_3(3)	402.42	11.13	212

In the FT-IR spectra the appearance of the stretching band of the C=O of thiazolidine-4-one at 1620-1691 cm^{-1}, together with the characteristic absorption band of C-S at 633-694 cm^{-1} confirms the success of the cyclization reaction and the formation of thiazolidine system.

The characteristic bands of the aromatic ring appeared in the range of 3078-2841 cm^{-1}, and the phenyl ring substituents were observed at 1157 cm^{-1} (C-F), 764 cm^{-1} (C-Cl), 1516, 1337 cm^{-1} (NO_2), 3483 cm^{-1} (OH), <650 cm^{-1} (C-Br).

The formation of the thiazolidine-4-one system has also been proved by the NMR data. In the ^{1}H-NMR spectra the proton of CH (SCHN) resonates as a singlet, between 5.92-5.68 ppm and the protons of the methylene group (-CH_2-S) appear as doublet between 3.38-3.27 ppm. The spectral characteristics are listed in Table 2.

Table 2. Spectral characteristics of ferulic acid thiazolidin-4-ones

No	FT-IR characteristic band (cm^{-1})	^{1}H-NMR signals (ppm)
4a	3225 (-NH-), 3001 (N-CH-S) 2962 (CHAr), 1690 (C=O cyclic), 1219 (C-N), 694 (C-S-C)	3.73 (s, 3H), 3.38 (s, 2H), 5.02 (d, 1H), 5.92 (d, 1H), 6.84 (d, 1H), 7.55 (d, 1H), 6.57-6.69 (m, 3H), 7.06-7.14 (m, 5H), 8.01 (s, 1H)
4b	3217 (-NH-), 2939 (N-CH-S), 1674 (C=O cyclic), 1620 (-CO-NH-), 1219 (C-N), 764 (C-Cl), 633 (C-S-C)	3.75 (s, 3H), 3.28 (s, 2H), 5.08 (d, 1H), 5.82 (d, 1H), 6.74 (d, 1H), 7.52 (d, 1H), 6.61-6.67 (m, 3H), 7.02-7.15 (m, 4H), 8.04 (s, 1H)
4c	3225 (-NH-), 2962 (CHAr), 2932 (N-CH-S), 1674 (C=O cyclic), 1157 (C-F), 640 (C-S-C)	3.72 (s, 3H), 3.31 (s, 2H), 5.04 (d, 1H), 5.86 (d, 1H), 6.71 (d, 1H), 7.51 (d, 1H), 6.59-6.65 (m, 3H), 7.04-7.14 (m, 4H), 8.02 (s, 1H)
4d	3232 (-NH-), 3024 (CHAr), 2978 (N-CH-S), 1688 (C=O cyclic), 1221 (C-N), 731 (C-S-C), <650 (C-Br)	3.69 (s, 3H), 3.29 (s, 2H), 5.01 (d, 1H), 5.79 (d, 1H), 6.89 (d, 1H), 7.54 (d, 1H), 6.61-6.67 (m, 3H), 7.10-7.21 (m, 4H), 8.12 (s, 1H)
4e	3258 (-NH-), 3078 (CHAr), 2932 (N-CH-S), 1682 (C=O cyclic), 1516, 1342 (NO_2)	3.71 (s, 3H), 3.31 (s, 2H), 5.05 (d, 1H), 5.68 (d, 1H), 6.84 (d, 1H), 7.49 (d, 1H), 6.59-6.63 (m, 3H), 7.09-7.18 (m, 4H), 8.07 (s, 1H)
4f	3107 (-NH-), 3074 (CHAr), 2920 (N-CH-S), 1684 (C=O cyclic), 1516, 1337 (NO_2), 648 (C-S-C)	3.68 (s, 3H), 3.27 (s, 2H), 5.04 (d, 1H), 5.74 (d, 1H), 6.91 (d, 1H), 7.51 (d, 1H), 6.64-6.72 (m, 3H), 7.14-7.20 (m, 4H), 8.06 (s, 1H)
4g	2964(N-CH-S), 2841 (CHAr), 1684 (C=O cyclic), 667 (C-S-C)	3.73 (s, 6H), 3.34 (s, 2H), 5.01 (d, 1H), 5.81 (d, 1H), 6.86 (d, 1H), 7.52 (d, 1H), 6.57-6.70 (m, 7H), 8.06 (s, 1H)
4h	3045 (-NH-), 2974(CHAr), 2922 (N-CH-S), 1620 (C=O cyclic), 683 (C-S-C)	3.71 (s, 3H), 3.31 (s, 2H), 5.04 (d, 2H), 5.76 (d, 1H), 6.81 (d, 1H), 7.54 (d, 1H), 6.52-6.76 (m, 7H), 8.02 (s, 1H)
4i	2986 (N-CH-S), 2955 (CHAr), 1680 (C=O cyclic), 779 (C-Cl), 704 (C-S-C)	3.67 (s, 3H), 3.31 (s, 2H), 5.01 (d, 1H), 5.75 (d, 1H), 6.84 (d, 1H), 7.52 (d, 1H), 6.51-6.72 (m, 6H), 8.05 (s, 1H)
4j	2918 (N-CH-S), 2854 (CHAr), 1691 (C=O cyclic), 690 (C-S-C)	2.85 (s, 6H) 3.71 (s, 3H), 3.28 (s, 2H), 5.04 (d, 1H), 5.71 (d, 1H), 6.81 (d, 1H), 7.49 (d, 1H), 6.54-6.78 (m, 7H), 8.02 (s, 1H)
4k	3483 (OH), 3408 (-NH-), 2951 (CHAr), 2924 (N-CH-S), 1624 (C=O cyclic), 1223 (C-N), 658 (C-S-C)	3.71 (s, 3H), 3.35 (s, 2H), 5.04 (d, 3H), 5.89 (d, 1H), 6.82 (d, 1H), 7.53 (d, 1H), 6.57-6.78 (m, 6H), 8.01 (s, 1H)
4l	3022 (-NH-), 2957 (CHAr), 2926 (N-CH-S), 1676 (C=O cyclic), 1624 (CO-NH-) 1271 (C-N), 683 (C-S-C)	3.73 (s, 6H), 3.32 (s, 2H), 5.01 (d, 2H), 5.82 (d, 1H), 6.87 (d, 1H), 7.48 (d, 1H), 6.51-6.81 (m, 6H), 8.03 (s, 1H)

I.2.1.3. Discussions

The thiazolidin-4-ones are thiazolidine derivatives which chemically exhibits a ketone group at the 4-position. The thiazolidine ring is a pentaatomic heterocycle which contains at the 1-position the sulfur atom and at the 3-position nitrogen atom.

Thiazolidin-4-one derivatives synthesis can be accomplished by various methods, which are based on cyclization reactions.

Among these methods we can mention: reaction of a bases compound with an aldehyde and thioglycolic acid (Bolli et al., 2010; Desai et al., 2011), reaction of isothiocyanates with primary amines (Desai et al., 2013; Bedane et al., 2015), aldol condensation in which 5-unsubstituted thiazolidin-4-ones react with various benzaldehydes (Deep et al., 2010).

The formation of the 1,3-thiazolin-4-one ring is favored by removing the water resulting from the condensation and cyclization reaction by azeotropic distillation.

Recently there were reported novel thiazolidin-4-ones derivatives as potential anti-inflammatory, antiviral and anti-HIV agents (Suthar et al., 20143; Murugesan et al., 2014; Gaikwad et al., 2015).

In our research structural modifications of ferulic acid molecule led to 12 new ferulic acid thiazolidin-4-ones (4a-l). Thiazolidin-4-one derivatives were obtained by condensation of ferulic acid hydrazide with different aromatic aldehydes (R = H, 4-chlor/4-fluor/4-brom/4-nitro/2-nitro/2-methoxi/2-hydroxi/2,6-dichlor/4-dimetil-amino/2,3-dihydroxi/4-hydroxi-3-metoxi) and mercaptoacetic acid in excess.

As is cited in the literature (Romagnoli et al., 2010; Komor et al., 2018), the synthesis of intermediate and final derivatives was monitored by TLC on 60 F254 silica gel plates. We used as solvent systems ethyl acetate: methanol: acetone: water and dichloromethane: methanol in different proportions, depending on the polarity of the compounds.

Visualization of the spots on the chromatograms was done in UV light at the wavelength of 254 nm.

Thiazolidin-4-ones are solid compounds, which generally have low melting points and melt with decomposition. Derivatives lacking aryl or alkyl substituents are relatively water soluble (Öcal et al., 2003; Küçükgüzel et al., 2006; Tatar et al., 2015).

Our obtained compounds are crystalline powders, colored in shades of yellow to brown-red. Establishing optimal synthetic conditions led to yields ranging from 9.31 to 82.82%.

The structure confirmation of the thiazolidin-4-one derivatives can be accomplished by IR and ^{1}H-NMR.

In infrared, characteristic bands for the cyclic keto group occur around the values 1655-1760 cm^{-1}, and for the NH group the characteristic bands occur around the values 3100-3400 cm^{-1}. The characteristic spectrum for the C-S bond appears in the range 600-700 cm^{-1}.

In the ^{1}H-NMR spectra the thiazolidin-4-ones are identified by the signals characteristic of the protons of the groups -S-CH_2, -CO-NH- and -CH-S-. The methylene group may appear as

two distinct signals, given by the two protons, in the range of 3.61-4.13 ppm, the methine at frequencies ranging from 5.86-6.35 ppm and the NH proton at values in region 10,97-11.81 ppm. (Pawar and Mulwand, 2004; Markovic et al., 2005).

Condensation of ferulic acid hydrazide with thioglycolic acid and various aromatic aldehydes gave the corresponding thiazolidin-4-one derivatives (4a-1). This cyclization was confirmed by the appearance in the IR spectrum of the C-S bonding bands and the ketone group from the thiazolidine-4-one ring.

The C-S valence vibration, identified as a weak intensity absorption band, occurred in the range of 650-704 cm^{-1} and the cyclic keto group was highlighted in the range 1620-1715 cm^{-1}.

Compared to the ferulic acid hydrazide spectrum, the disappearance of the characteristic bands of the $-NH_2$ group, revealed at the 3304 cm^{-1}, 3252 cm^{-1}, could be observed, and the appearance of the absorption band characteristic for amide group -CO-NH- in the side chain occurred in the range of 1512 cm^{-1} to 1684 cm^{-1}.

In addition, the other peaks of IR spectra prove the structure of thiazolidin-4-one derivatives.

Confirmation of the structure of thiazolidin-4-one (4a-1) derivatives is supported also by the ^{1}H-NMR proton signals from the thiazolidin-4-one sequences, the aromatic nucleus and the ferulic acid.

In the ^{1}H-NMR spectra, the signals due to C<u>H</u>-N (methine) proton were observed at 5.74-5.95 ppm in the form of a singlet or double-ended signal with a coupling constant of 1.05-2.5 Hz or multiplet having integral 1.

The signals due to C<u>H$_2$</u>S (thiomethilen) proton were observed at 3.62-4.03 ppm in the form of a doublet, doublet of doublets, doublet of triplet, or multiplet, having the integral 2.

The signals due to 2-position aromatic ring of the thiazolidin-4-one derivatives protons overlapped the signals of aromatic protons in the ferulic acid structure and were identified at 6.64-8.33 ppm. Protons chemical displacements were influenced by substituents on the aromatic ring.

The signal due to OC<u>H$_3$</u> (methoxy) protons, characteristic for ferulic acid structure, was identified at 3.69-4.04 ppm in the form of a singlet having the integral 3.

I.2.1.4. Conclusions

There have been synthesized 12 new thiazolidin-4-ones of ferulic acid and optimal conditions of reaction were established. The synthesized compounds have been characterized by their physical constants (melting point, yield, molecular formula, molecular weight, solubility in different organic solvents) and the chemical structure was proved using FT-IR and ^{1}H-NMR spectroscopy.

I.2.2. Design, synthesis and characterization of new azetidin-2-ones of ferulic acid

Personal contribution to knowledge in this research field is presented in following published paper:

- Stan CD, Drăgan M, Pânzariu A, Profire L. Evaluation of the synthesis and structure of new azetidin-2-ones of ferrulic acid. *Rev Med Chir Soc Med Nat Iaşi* 2016; 120(2): 434-438.

The synthesis of new azetidin-2-one of ferulic acid was performed starting from ferulic acid hydrazide which formed by condensation with different aromatic aldehydes (2-hydroxy-/2-nitro-/4-chloro-/4-fluoro-/4-bromo-benzaldehyde) the corresponding hydrazones. These hydrazones were cyclized with chloroacethyl chloride in the presence of triethylamine to obtain the corresponding azetidin-2-ones.

The synthesis of the designed compounds was performed in several steps:

- ✓ the obtaining of ferulic acid chloride by reacting of ferulic acid with thionyl chloride;
- ✓ the reaction between the ferulic acid chloride and hydrazine hydrate 98% to obtain the ferulic acid hydrazide;
- ✓ the condensation of ferrulic acid hydrazide with various aromatic aldehydes (2-hydroxy-/2-nitro-/4-chloro-/4-fluoro-/4-bromo-benzaldehyde) and with chloroacethyl chloride in the presence of triethylamine, resulting the corresponding azetidin-2-ones.

The synthesized compounds were characterized and structure validated by FT-IR spectra and ^{1}H-NMR spectra.

I.2.2.1. Materials and methods

Substances, reagents and solvents

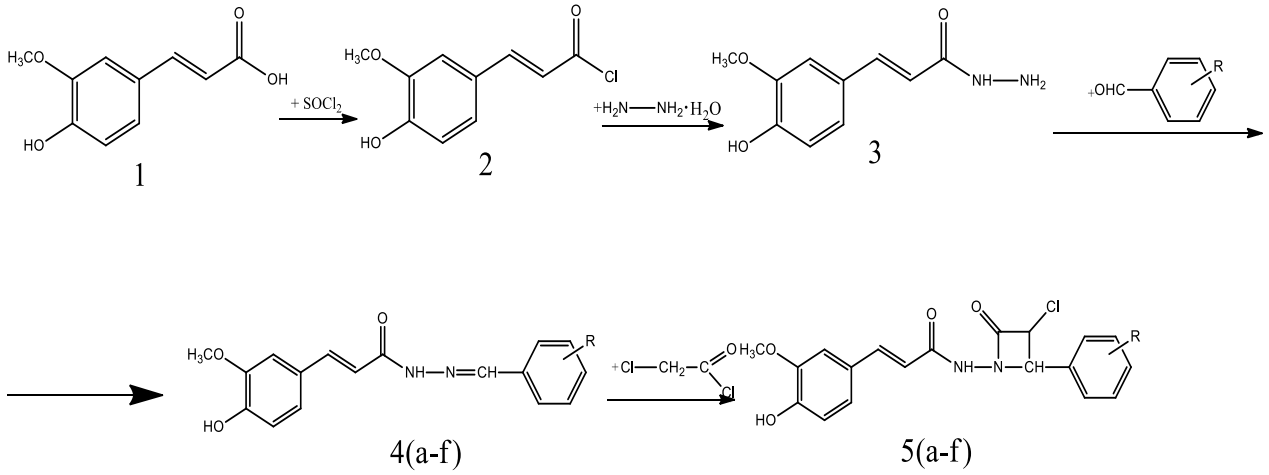
Ferulic acid, thionyl chloride, hydrazine hydrate, aromatic aldehydes (R=H/2-hydroxy-/2-nitro-/4-chloro-/4-fluoro-/4-bromo-benzaldehyde), chloroacetyl chloride, triethylamine, organic solvents (*p.a.* quality) were purchased from Sigma Aldrich Company and Fluka Company. All reagents and solvents were used without previous purification.

For monitoring the reaction, TLC silica gel 60 F_{254} plates, purchased from Merck Company, were used.

Synthesis of the azetidin-2-one derivatives of ferulic acid

Ferulic acid was reacted with thionyl chloride to obtain the ferulic acid chloride that was next treated with hydrazine hydrate 98% to obtain ferulic acid hydrazide. By condensation with different aromatic aldehydes (R=H/2-hydroxy-/2-nitro-/4-chloro-/4-fluoro-/4-bromo-benzaldehyde) the corresponding hydrazones (4a-f) were obtained.

These hydrazones were cyclized with chloroacethyl chloride in freshly distilled toluene medium and in the presence of triethylamine to obtain the corresponding azetidin-2-ones (5a-f) (Figure 2).



Compound	-R
5a	-H
5b	-OH(2)
5c	-NO_2(2)
5d	-Cl(4)
5e	-F(4)
5f	-Br(4)

Figure 2. Azetidin-2-one derivatives of ferulic acid synthesis

General procedure for azetidin-2-ones of ferulic acid synthesis

Over 0.025 moles (5 g) of ferulic acid and under continuous stirring 0.15 moled os thionyl chloride were added. The reaction mixture was maintained at 50^0C for 4 hours and the excess of thionyl chloride was removed by rotary evaporation.

In the second step, over the ferulic acid chloride (0.05 moles) 0.25 moles of hydrazine hydrate 98% were added. The reaction mixture was maintained on ice bath for 2 hours and then at room temperature for another 2 hours, under continuous stirring. Then 15 mL of cold water were added and the precipitate was dried at room temperature.

Finally 0.015 moles (3 g) of ferulic acid hydrazide with a few drops of glacial acetic acid and 0.015 moles of aromatic aldehydes (2-hydroxy-/2-nitro-/4-chloro-/4-fluoro-/4-bromo-benzaldehyde) were refluxed for 13-20 hours in absolute ethanol. The reaction was monitored by TLC, using ethyl acetate:methanol:acetone:water (5:2:2:1) as solvent system.

In the last step, the 0.0015 moles (0.5 g) of hydrazone with 0.0022 moles of chloroacethyl chloride, in the presence of triethylamine, were refluxed for 15-20 hours in toluene. The reaction was monitored by TLC, using dichloromethane:methanol (9:1) as solvent system.

The obtained precipitate was washed with 1M hydrochloric acid, and the organic layer was dried on anhydrous magnesium sulphate and then washed with petroleum ether (Wolszleger et al., 2014; Wolszleger et al., 2016; Stan et al., 2016).

Physicochemical characterization

The azetidin-2-one derivatives of ferulic acid were physicochemically characterized by melting point, yield, molecular formula, relative mass, solubility in water and other organic solvents.

The melting point was determined by using Buchi M 565 (Buchi, Switzerland).

The reaction was monitored by TLC on 60 F254 silica gel plates, and spots were visualized by UV light at 254 nm.

Characterization by spectral methods

The IR spectra of the synthesized derivatives were recorded using a Fourier transform spectrometer, ABB-MB 3000 FT-IR MIRacle^{TM} Single Bounce ATR-crystal ZnSe, and the studied spectral range was between 4000–500 cm^{-1}, at a resolution of 4 cm^{-1}, 16 scans being carried out for each sample. The spectra were interpreted using the Horizon MB^{TM} FT–IR software.

For ^{1}H-RMN, the samples were dissolved in hexadeuterio-dimethyl- sulfoxide (DMSO-d_6) and the *Bruker 400 MHz NMR spectrometer* (Germany) was used. The spectral signals were automatically integrated and the chemical shift values were expressed in parts per million (ppm) relative to tetramethylsilane (TMS) as an internal standard. The spectra were interpreted using MestReNova software.

I.2.2.2. Results

Physico-chemical and spectral characterization

The azetidin-2-one derivatives of ferulic acid are crystalline powders, colored in different shades of brown, very slightly soluble in dimethylsulfoxide (DMSO) and dimethylformamide (DMF), partially soluble in absolute ethanol, methanol, chloroform, acetone, dioxane and insoluble in distilled water, benzene and ethyl ether.

The optimal reaction conditions for obtaining high yield and high purity compounds were established. The physicochemical characteristics are listed in Table 3.

Table 3. Physicochemical characteristics of azetidin-2-one derivatives of ferulic acid

Comp.	R	Physicochemical characteristics			
		Molecular formula	Molecular weight	Yield (%)	Melting point (^{0}C)
5a	-H	$C_{19}H_{17}ClN_2O_4$	372.80	24.00	72
5b	-OH(2)	$C_{19}H_{17}ClN_2O_5$	388.80	25.93	107-109
5c	- NO_2(2)	$C_{19}H_{16}ClN_3O_6$	417.80	17.46	123
5d	-Cl(4)	$C_{19}H_{16}Cl_2N_2O_4$	407.25	39.90	176
5e	-F(4)	$C_{19}H_{16}ClFN_2O_4$	390.79	39.10	150
5f	-Br(4)	$C_{19}H_{16}BrClN_2O_4$	450.00	31.12	89

The FT-IR spectra revealed the presence of C-O stretching band, the characteristic absorption band of CH-N and the characteristic absorption band of CH-Cl. These data confirm the structure of derivatives and the successful azetidine ring formation.

The structure of derivatives is also supported by ^{1}H-NMR spectra. The apparent resonance multiplicity is described as s (singlet), d (doublet), m (multiplet), dd (double doublet), and td (triple doublet) signal. The spectral characteristics are listed in Table 4.

Table 4. Spectral characteristics of azetidin-2-one derivatives of ferulic acid

Comp.	FT-IR characteristic band (cm^{-1})	^{1}H-NMR (400 MHz, DMSO-d_6, δ ppm)
5a	3190 (NH), 2966, 1587, 839 (aromatic ring), 2839, 2773, 1447, 1402 (CH aliphatic), 1724 (C=O cyclic), 1651 (CO-NH), 1587 (C=N), 1394 (CH-N), 1255 (C-N), 723 (CH-Cl)	3.74 (s, 3H, OC<u>H</u>$_3$), 5.04 (d, 1H, C<u>H</u>-Cl), 5.34 (d, 1H, Ar-C<u>H</u>-N), 6.79-6.82 (m, 1H, Ar-H), 6.98-7.12 (m, 2H, Ar-H), 7.39-7.43 (m, 1H, Ar-H), 7.48-7.52 (m, 1H, Ar-H), 7.54-7.60 (m, 3H, Ar-H), 7.65-7.68 (m, 2H, Ar-H)
5b	3065 (NH, OH), 2974, 1627, 1524, 893 (aromatic ring), 2845, 2754, 1487, 1387 (CH aliphatic), 1732 (C=O cyclic), 1572 (CO-NH), 1524 (C=N), 1380 (CH-N), 1269 (C-N), 1196 (C-O), 690 (CH-Cl)	3.81 (s, 3H, OC<u>H</u>$_3$), 5.02 (d, 1H, C<u>H</u>-Cl), 5.32 (d, 1H, Ar-C<u>H</u>-N), 6.55-6.59 (m, 1H, Ar-H), 6.80-6.87 (m, 1H, Ar-H), 7.00-7.08 (m, 1H, Ar-H), 7.20-7.29 (m, 2H, Ar-H), 7.33-7.35 (m, 1H, Ar-H), 7.60-7.65 (m, 1H, Ar-H), 7.72-7.79 (m, 1H, Ar-H), 7.94 (s, 1H, Ar-H)
5c	3250 (NH), 2943, 1724, 1510, 851 (aromatic ring), 2895, 2856, 1471, 1419, (CH aliphatic), 1728 (C=O cyclic), 1628 (CO-NH), 1607 (C=N), 1387 (CH-N), 1232 (C-N), 1570, 1342 (NO_2), 709 (CH-Cl)	3.83 (s, 3H, OC<u>H</u>$_3$), 5.32 (d, 1H, C<u>H</u>-Cl), 5.54 (d, 1H, Ar-C<u>H</u>-N), 6.69-6.75 (m, 1H, Ar-H), 6.88-6.93 (m, 1H, Ar-H), 6.99 (dd, 1H, Ar-H), 7.16 (s, 1H, Ar-H), 7.35-7.43 (m, 1H, Ar-H), 7.59-7.64 (m, 1H, Ar-H), 7.91-7.98 (m, 2H, Ar-H), 8.09 (dd, 1H, Ar-H)
5d	3254 (NH), 2945, 1724, 1510,	3.82 (s, 3H, OC<u>H</u>$_3$), 4.92 (d, 1H, C<u>H</u>-Cl),

	862 (aromatic ring), 2901, 2847, 1470, 1402 (CH aliphatic), 1734 (C=O cyclic), 1595 (CO-NH), 1510 (C=N), 1396 (CH-N), 1230 (C-N), 814 (C-Cl), 703 (CH-Cl)	5,24 (d, 1H, Ar-C<u>H</u>-N), 6.80-6.85 (m, 2H, Ar-H), 6.95-7.04 (m, 1H, Ar-H), 7.19 (s, 1H, Ar-H), 7.37 (dd, 1H, Ar-H), 7.52 (dd, 2H, Ar-H), 7.77 (dd, 2H, Ar-H)
5e	3252 (NH), 2945, 1680, 1506, 825 (aromatic ring), 2847, 1470, 1419 (CH aliphatic), 1730 (C=O cyclic), 1634 (CO-NH), 1601 (C=N), 1384 (CH-N), 1227 (C-N), 1095 (C-F), 714 (CH-Cl)	3.83 (s, 3H, OC<u>H</u>$_3$), 4.94 (d, 1H, C<u>H</u>-Cl), 5.26 (d, 1H, Ar-C<u>H</u>-N), 6.79-6.89 (m, 2H, Ar-H), 6.99-7.03 (m, 1H, Ar-H), 7.11 (s, 1H, Ar-H), 7.29-7.37 (m, 3H, Ar-H), 7.81 (td, 2H, Ar-H)
5f	3252 (NH), 2943, 1724, 1510, 836 (aromatic ring), 2893, 2837, 1472, 1419 (CH aliphatic), 1726 (C=O cyclic), 1583 (CO-NH), 1562 (C=N), 1386 (CH-N), 1232 (C-N), 858, (C-Br), 724 (CH-Cl)	3.85 (s, 3H, OC<u>H</u>$_3$), 4.95 (d, 1H, C<u>H</u>-Cl), 5.28 (d, 1H, Ar-C<u>H</u>-N), 6.77-6.85 (m, 2H, Ar-H), 6.93-6.99 (m, 1H, Ar-H), 7.13 (s, 1H, Ar-H), 7.30-7.37 (m, 1H, Ar-H), 7.58-7.63 (m, 2H, Ar-H), 7.72-7.75 (m, 2H, Ar-H)

I.2.2.3. Discussions

Some of the most studied classes of heterocyclic compounds are azetidin-2-ones because of the multitude of their biological effects they have: antimicrobial, anti-inflammatory, antioxidant, antitumoral etc. (Galletti and Giacomini, 2011; Cervellati et al., 2013).

Azetidin-2-ones represent versatile building blocks in heterocyclic chemistry. Since the discovery of penicillins and cephalosporins as the most successful antibiotics, azetidin-2-ones have been the subject of regular discussion and investigation (Piens et al., 2016).

Furthermore, the β-lactam scaffold has found new pharmaceutical applications other than its use as antibiotics. The ring strain of the β-lactam skeleton facilitates ring-opening reactions and this unique property has been exploited for the synthesis of a variety of medicinally active compounds (Kamath and Ojima, 2012; Gupta and Halve, 2015).

The azetidin-2-ones synthesis and evaluation has always drawn the attention of researchers over the years (Zarei et al., 2011; Samadhiya et al., 2012). Moreover, due to their β-lactamase inhibitory action, azetidin-2-ones based heterocycles represent an attractive target of modern organic synthesis (Galletti et al., 2014; Kayarmar et al., 2017).

Recently O'Boyle reported the synthesis of a series of azetidin-2-ones via the Staudinger and Reformatsky reactions. Several azetidin-2-ones substituted at positions 2, 3 and 4 of the azetidinone ring scaffold were synthesised and evaluated for antiproliferative, cytotoxic and tubulin binding activity. In these compounds, the agent combretastatin A-4 is linked with the azetidin-2-one ring in order to enhance the antiproliferative effects (O'Boyle et al., 2011).

In our study, structural modifications of the ferulic acid molecule led to six new azetidin-2-one derivatives of ferulic acid (5a-f).

The reaction was performed by reacting ferulic acid with thionyl chloride in order to obtain the ferulic acid chloride that was next treated with hydrazine hydrate 98% to obtain ferulic acid hydrazide. By condensation with different aromatic aldehydes (R=H/2-hydroxy-/2-nitro-/4-chloro-/4-fluoro-/4-bromo-benzaldehyde) the corresponding hydrazones were obtained which were cyclized with chloroacethyl chloride in freshly distilled toluene medium and in the presence of triethylamine to obtain the corresponding azetidin-2-ones (5a-f).

The reaction was monitored by TLC on 60 F254 silica gel plates, and spots were visualized by UV light at 254 nm, as is mentioned in literature (Komor et al., 2018).

The obtained azetidin-2-one derivatives of ferulic acid are crystalline powders, colored in different shades of brown, very slightly soluble in dimethylsulfoxide (DMSO) and dimethylformamide (DMF), partially soluble in absolute ethanol, methanol, chloroform, acetone, dioxane and insoluble in distilled water, benzene and ethyl ether.

Establishing optimal conditions for synthesis there were obtained yields ranging from 17.46% to 39.90 %.

Several IR and FTIR methods have been reported for the characterization of the azetidin-2-ne ring formation (Cervellati et al., 2013; Al-khazraji et al., 2016; Giacomini et al., 2017; Kou et al., 2018).

The FT-IR spectra of our azetidin-2-one derivatives confirmed the cyclization by the appearance in the IR spectrum of the C=O stretching band cyclic at 1724-1734 cm^{-1}. The IR spectra also display the characteristic absorption band of CH-N at 1380-1396 cm^{-1} and the characteristic absorption band of CH-Cl in the region 690-724 cm^{-1}. Is worth to mention that the other peaks of IR spectra proved the structure of azetidin-2-ones.

In the ferulic acid hydrazide spectrum appear the characteristic bands of the $-NH_2$ group revealed at the 3304 cm^{-1}, 3252 cm^{-1}. The disappearence of this characteristic bands could be observed and the appearance of the absorption band characteristic for amide group -CO-NH- in the range of 1572 cm^{-1} to 1634 cm^{-1} and the appearance of CH-Cl in the range of 690 cm^{-1} to 724 cm^{-1}. These data confirm the structure of derivatives and the successful azetidine ring formation.

In a literature survey I found numerous studies using ^{1}H-NMR spectra to confirm the structure of several azetidin-2-ones derivatives (Samadhiya et al., 2012; Lokhandwala and Parekh, 2014; Salman et al., 2014; Gawande and Khadsan, 2014).

The structure of our derivatives is also supported by ^{1}H-NMR spectra. The apparent resonance multiplicity is described as s (singlet), d (doublet), m (multiplet), dd (double doublet), and td (triple doublet) signal.

The signals of C<u>H</u>-Cl protons resonate as a doublet in a range of 4.92–5.04 ppm and the protons linked by the N from azetidine ring (Ar-C<u>H</u>-N) exhibited a doublet at 5.24–5.54 ppm. Protons chemical displacements were influenced by substituents on the aromatic ring.

I.2.2.4. Conclusions

A general and convenient method was established for the synthesis of new azetidin-2-one derivatives of ferulic acid. Thus, six new azetidin-2-one derivatives of ferulic acid were synthesized. The general synthesis was optimized in order to gain great yields and advanced purity for the obtained derivatives.

The new derivatives were characterized by their physical constants (melting point, yield, molecular formula, molecular weight, solubility in different organic solvents).

The chemical structure was confirmed by FT-IR and ^{1}H-NMR spectroscopy.

All the obtained data confirm the cyclization of the ferulic acid with various aromatic aldehydes and established the azetidine ring formation.

I.3. Design, synthesis and characterization of isatin derivatives

Isatin, known as 1H-indole-2,3-dione, is an indole derivative containing keto group at position 2 and 3 of the ring.

Isatin was first synthesized by Erdman and Laurent in 1841 by the oxidation of indigo dye with nitric acid and chromic acid. The compound is found in many plants, such as *Isatis tinctoria*, *Calanthe discolor* and in *Couroupita guianensis* (Vandana et al., 2017).

Substituted isatins are also found in plants, for example the melosatin alkaloids (methoxy phenylpentyl isatins) in *Melochia tomentosa* (Mathur and Nain, 2014). Derivatives as 6-(3'-methylbuten-2'-yl) isatin was isolated from *Streptomyces albus* and 5-(3'-methylbuten-2'-yl) isatin was isolated from *Chaetomium globosum*. Isatin has also been found to be a component of coal tar (Mathur and Nain, 2014).

Isatin it is also an endogenous compound that is widely distributed in mammalian tissues and body fluids. It was also found in humans, as it is a metabolic derivative of adrenaline, beeing identified in urine, blood and tissue (Bhardwaj et al., 2010).

Isatins are synthetically versatile substrates, beeing useful for the synthesis of a large variety of heterocyclic compounds and as a raw material for drug synthesis. Thus, isatin is a biologically validated starting point for the design and synthesis of chemical libraries directed at different targets (Grewal and Panel, 2014).

Recently, there were reported biological activities exhibited by isatin derivatives, like anticancer, antibacterial, antidiabetic, antifungal, antiviral, anti-inflammatory and others (Grewal, 2014). Special attention has been paid to their anticancer activity and various anticancer targets such as histone deacetylase, carbonic anhydrase, tyrosine kinase, and tubulin have been investigated (Varun et al., 2019).

On the other hand, Schiff bases are characterized by the presence of –N=CH– (imine group or azomethine group) which is important for their biological activities.

Schiff bases have been reported to possess a variety of biological activities including anticancer, anticonvulsivant, antiproliferative, antioxidant, antiglycation, antimicrobial, antimalarial, antifungal, antiviral and cytotoxic activities (Batra et al., 2014; Anush et al., 2018; Kaczmarek et al., 2018).

Schiff bases of isatin are used for their pharmaceutical properties. Oxindole isatin derivatives are an important class of heterocyclic compounds endowed of interesting pharmacological and biological activities such as antimicrobial, antitumor, antitubercular, antimalaria, anti-HIV and antibacterial activities. As a class of important 3,3-disubstituted oxindoles, 3-substituted 3-hydroxyindolin-2-ones have attracted considerable attention because of their diverse biological activities (Moradi et al., 2017).

In recent years, Schiff bases of isatin are reported to exhibit broad–spectrum chemotherapeutic properties such as antiviral, antitubercular, antifungal and antibacterial activities (Bekircan and Bektas, 2008; Bhardwaj et al., 2010).

Research in this area has focused on:

- design and synthesis of a new series of isatin containing 4-aminoantipyrine ring by different techniques;
- physico-chemical characterization of all new schiff bases of isatin by modern methods of analysis.

Personal contribution to knowledge in this research field is presented in following published paper:

- Tătărîngă G, Stan CD, Zbancioc, AM., Jitareanu A, Tuchiluş A. Preliminary screening of biological activities of some new Schiff bases of isatin. *Farmacia* 2014; 62(1): 14-22.

I.3.1. Materials and methods

Substances, reagents and solvents

5-Chloro-isatin, 5-nitro-isatin were purchased from Sigma Aldrich and 4-amino-antipyrin was purchased from Fluka. The others isatin and its substituted derivatives were obtained by cyclization of the appropriate isonitrosoacetanilide.

All reagents and solvents were used without previous purification.

For monitoring the reaction, TLC silica gel 60 F_{254} plates, purchased from Merck Company, were used.

Procedure for the preparation of isatin, 5-methyl-isatin and 7-methyl-isatin

Sulphuric acid was warmed in a flask and powdered appropriate isonitrosoacetanilide was added at such a rate so as to maintain the temperature between 60-70^{0}C. After the addition

of isonitroso compound was completed, the temperature was raised to 80^0C and maintained to this value for 10 minutes. The reaction was monitored by TLC, using dichloromethane- methanol in a ratio of 9:1 as solvent system.

Then, the mixture was cooled and poured on crushed ice. After 30 minutes, the solid was filtered, dried and purified (Tătărîngă et al., 2014).

Procedure for the preparation of 1-methyl-isatin

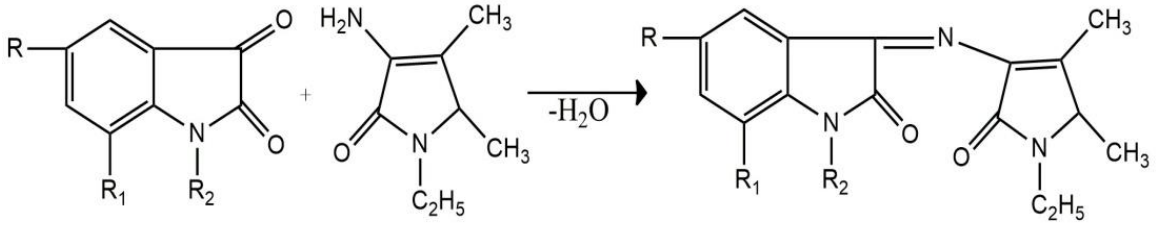
5 gm of isatin was dissolved in dilute sodium hydroxide solution, then 0.62 ml of di-methyl sulphate was added. Then whole contents were refluxed in water bath for approximately 50 minutes. After refluxing, the mixture was poured into beaker and cooled in ice. Then the content was evaporated on water bath and dried (Tătărîngă et al., 2014).

The purity of the new synthesized derivative was checked by thin-layer chromatography (TLC) and was carried out on precoated Silica Gel 60 F_{254} plates using a dichloromethane - methanol 9:1 system.

General procedure for the preparation of the Schiff bases

0.01 mols of isatin/1-methyl-isatin/5-methyl-isatin/7-methyl-isatin and 0.01 mols of 4-aminoantipyrine were dissolved in ethanol containing a few drops of acetic acid. The mixture was then heated on a steam bath for 2 hours. The reaction was monitored by TLC, using dichloromethane-methanol in a ratio of 9:1 as solvent system.

After standing at room temperature, the crystalline compounds were separated by filtration, dried and recrystallised from a mixture of solvents ethanol : water in a ratio of 1:1 (Tătărîngă et al., 2014). The general procedure for the obtaining of Schiff bases of isatins is presented in Figure 3.



Comp.	R	R_1	R_2
1a	-H	-H	-H
1b	$-CH_3$	-H	-H
1c	-H	$-CH_3$	-H
1d	-Cl	-H	-H
1e	$-NO_2$	-H	-H
1f	-H	-H	$-CH_3$

Figure 3. Schiff bases of isatins general synthesis

Physicochemical characterization

Melting points were determined using an electro thermal Melting Point apparatus and were uncorrected.

The purity of the new synthesized derivatives was checked by thin-layer chromatography (TLC) and was carried out on precoated Silica Gel 60 F_{254} plates using a dichloromethane : methanol system in a ratio of 9:1.

Reversed phase thin-layer chromatography was performed on silica gel plates impregnated with 5% (v/v) liquid paraffin in light petroleum ether. Reversed-phase thin-layer chromatographic parameters, RM values were determined from corresponding retention factors, Rf values using the equation:

$$RM = \log [(1/Rf) -1]$$

Characterization by spectral methods

Elemental analysis was performed on an Elemental Exeter Analytical CE 440 Analyzer.

The IR spectra were recorded on a FTIR Shimadzu Prestige 8400s spectrophotometer.

^{1}H-NMR spectra were recorded on a Bruker Avance DRX- 400 spectrometer at 400 MHz in dimethyl sulfoxide (DMSO)–d_6 using tetramethylsilane (TMS) as an internal reference.

I.3.2. Results

Physico-chemical and spectral characterization

The new synthesized compounds are crystalline powders, colored in different shades of orange. The optimal reaction conditions for obtaining high yield and high purity compounds were established. The physicochemical characteristics are listed in Table 5.

Table 5. Physicochemical characteristics of Schiff bases of isatins

Comp.	Molecular formula	Molecular weight	Yield (%)	Melting point (^{0}C)	Rf
1a	$C_{19}H_{16}O_2N_4$	332	50	163-164	0.238
1b	$C_{20}H_{18}O_2N_4$	346	72	154-156	0.267
1c	$C_{20}H_{18}O_2N_4$	346	49	241-242	0.252
1d	$C_{19}H_{15}O_2N_4Cl$	366.5	49	256-257	0.257
1e	$C_{19}H_{15}O_2N_5$	345	25	302	0.242
1f	$C_{20}H_{18}O_2N_4$	346	50	218-220	0.222

The new synthesized compounds were characterized by elemental analysis, IR spectroscopy and ^{1}H-NMR spectra. All the elemental and spectral data were in accordance with the proposed structures. The spectral characteristics are listed in Table 6.

Table 6. Spectral characteristics of Schiff bases of isatins

Comp	FT-IR characteristic band (cm^{-1})	^{1}H-NMR signals (DMSO-d_6, δ ppm)	Elemental analysis		
			C% (calc/found)	H% (calc/found)	N% (calc/found)
1a	1634(C=N), 1732 (C=O), 3380 (NH)	-	68.67/68.51	4.81/4.75	16.86/16.80
1b	1624 (C=N), 1727 (C=O), 2930 (CH_3), 3200 (NH)	-	69.36/69.05	5.2/4.98	16.18/16.16
1c	1623 (C=N), 1730 (C=O), 2918 (CH_3), 3188 (NH)	-	69.36/69.32	5.2/5.00	16.18/16.16
1d	702 (C-Cl), 1641 (C=N), 1728 (C=O), 3434 (NH)	2.42(3H, s), 3.30 (3H, s), 6.88-6.86 (1H, d), 7.43-7.36 (5H, m), 7.59-7.55 (2H), 10.85 (1H, s)	62.21/62.2	4.09/4.01	15.27/15.15
1e	1417, 1500 (NO_2), 1622 (C=N), 1735 (C=O), 3431 (NH)	2.50(3H, s), 3.42 (3H, s), 7.06-7.03 (1H, t), 7.44-7.42 (2H, d), 7.48-7.46 (2H, d), 7.61-7.57 (2H),7.99 (1H, s), 8.28-8.25 (1H, d)	60.47/60.38	3.97/3.91	18.56/18.5
1f	1650 (C=N), 1735 (C=O), 2920 (NH)	-	69.36/69.88	5.2/5.37	16.18/16.81

I.3.3. Discussions

Isatin is considered a important class of bioactive compounds exhibiting antibacterial and antiproliferative activity (Chohan et al., 2004) and Schiff bases of isatin analogous have anti-smallpox capabilities (Konkel et al., 2006). Isatin derivatives reported to show antiviral, anti-inflammatory, analgesic and anticonvulsant activities (El-Faham et al., 2015).

Isatin is a versatile substance able to participate in a broad range of synthetic reactions, leading to its extensive use as a precursor molecule in pharmaceutical industry.

The isatin moiety also shows important chemical reactions such as oxidation, ring expansion, Friedel–Crafts reaction and aldol condensation (Varun et al., 2019).

The keto group at position 2 and at position 3 can enter into addition reactions at the C=O bond and into condensation reactions. Through the primary amine group, these compounds could performe N-alkylation and N-acylation and Mannich and Michael reactions (Bekircan and Bektas, 2008). By these reactions, several viable biologically compounds are produced with various activities such as antibacterial, antifungal, antiviral, anti-HIV, anticancer, anti-inflammatory and anticonvulsant (Kumar et al., 2014).

Recent studies present different methods for novel Schiff base of isatin derivatives synthesis, such as: condensation of imesatin with different aromatic aldehydes (Chinnasamy et al., 2010), substituting different aromatic aldehydes at the 3rd position and linking different molecules at N1 position to obtaine Mannich base which are treated with different aromatic aldehydes in the presence of glacial acetic acid (Prakash and Raja, 2013), condensation reaction of N-acetyl, N-benzyl and N-benzoyl isatins with chelating agent dithiooxamide and their complexes which gave Schiff base ligands (Abdul-Ghani and Khaleel, 2009).

Our new synthesized Schiff bases of isatin derivatives were obtained through condensation of isatin/1-methyl-isatin/5-methyl-isatin/7-methyl-isatin with 4-aminoantipyrine in ethanol containing a few drops of acetic acid. The compounds are crystalline powders, colored in different shades of orange. The optimal reaction conditions for obtaining high yield and high purity compounds were established. The yields are ranging from 49% to 72%.

The spectral data of the novel derivatives were in good agreement with the reported data (Singh et al., 2010; Shibinskaya et al., 2010; Chen et al., 2011; Chen et al., 2012; Clay et al., 2012; El-Faham et al., 2014).

All the elemental and spectral data were in accordance with the proposed structures. The important diagnostic bands in the IR spectra were assigned and the bands positions are complied.

The IR spectra showed absorption band at 1622-1650 cm^{-1} indicated the stretching vibration of C=N (Schiff bases) which confirms to the condensation of reactants. The C=O stretching vibration appeared at 1727-1735 cm^{-1} which indicates the condensation of reactants.

For the 1e derivative the absorption band for C-Cl stretching band group appeared at at 1417-1500 cm^{-1} and for 1d derivative the characteristic absorption band of CH-Cl in the region 702 cm^{-1}. The other peaks of IR spectra prove the structure of Schiff's base derivatives.

Elemental analysisis is the classical method to obtain information about the elemental composition of an unknown substance (Sellergren and Hall, 2001).

Elemental analysis of Schiff bases of isatins clearly indicates that the found carbon content was close to that calculated the sum of carbon (C), hydrogen (H) and nitrogen (N) was greater than 90%, for all the derivatives investigated. The elemental analysis of Schiff bases of isatins indicates the proportionate change in the atomic percentage of the various atoms (C, H, and N), which further confirms the conjugation of the chemical moieties.

The chemical shifts are influenced by deshielding due to reduced electron density (due electronegative atoms). The integral measures the area of the peak, which is is proportional to the number of H that the peak represents.

NMR spectra of all the obtained products are in good agreement with their proposed structures.

In the ^{1}H-NMR spectra, the signals due to aromatic protons were observed at 6.86-8.28 ppm, the signals due to NH protons were observed at 10.85 ppm in the form of a singlet signal, the signals due to CH_3 protons were observed at 2.42 and 2.50 ppm respectively in the form of a singlet signal. Protons chemical displacements were influenced by substituents on the aromatic ring.

I.3.4. Conclusions

In view of biological importance of the two moieties (isatin and 4-aminoantipyrine) it was planned to synthesize a new series of isatin containing 4-aminoantipyrine ring and to validate the structure of the newly compounds.

Thereby some new Schiff bases (six compounds) derived from isatin were synthesized. All the elemental and spectral data were in accordance with the proposed structures, so the ^{1}H-NMR, elemental analysis and IR spectra confirmed the structure of the synthesized compounds.

I.4. *In vitro* antioxidant evaluation of synthetic and semi-synthetic pharmaceutical substances with therapeutic potential

Antioxidant compounds neutralize free radicals and are very important health-protecting agents, reducing the risk for chronic diseases. One mechanism through which this is achieved is by donating hydrogen to free radicals, removing the odd electron feature and reducing them to non-reactive species.

In recent years many papers reported the potential beneficial effects of different free-radical scavenger antioxidant polyphenols as preventive agents against human neoplastic diseases (Andreani et al., 2010).

The antioxidant effects of ferulic acid have been assessed in many studies and the researchers found different mechanisms that explain its protective effects.

In vitro or *in vivo* ferulic acid forms a phenoxy radical with low energy which generates a more stable hybrid resonance structure. The phenoxy radical is unable to initiate or propagate a radical chain reaction. During this process the hydrogen atom easily pass from the ferulic acid to radicals. This property explains the antioxidant effect of ferulic acid and its ability to stabilize organic or inorganic radicals (Paiva et al., 2013).

On the other hand, hydroxyl groups on the benzene ring near to unsaturated C-C double bond explain the higher mobility of hydrogen that is easily released and pass to oxidative compounds (Krishnan et al., 2013).

Into the liver cells ferulic acid acts as an indirect antioxidant compound and up-regulates the heme oxygenase-biliverdin reductase system with bilirubin synthesis that acts as an endogenous free radical scavenger (Mancuso et al., 2009).

Other group of compounds evaluated in order to establish *in vitro* antioxidant properties were isatin and coumarin derivatives. Isatin and isatin derivatives have some important biological activities such as anticancer, antibacterial, antidiabetic, antifungal, antiviral, and anti-inflammatory effects (Premanathan et al., 2012). Special attention has been paid to their anticancer activity and various anticancer targets such as histone deacetylase, carbonic anhydrase, tyrosine kinase, and tubulin have been investigated (Varun et al., 2019).

Derivatives of isatin that include an aniline bearing selected substituents showed a good chemical antioxidant activity. Prakash et al., reported some novel isatin derivatives and analogs with antioxidant activity (Prakash et al., 2011).

Schiff bases are used as substrates for the preparation of biologically active compounds. Moreover, Schiff bases derived from various heterocycles have been reported to possess anti-fungal, anti-cancer, cytotoxic and anti-convulsant activities (Chinnasamy et al., 2010).

Schiff bases of isatin have been reported to exhibit a spectrum of properties such as antioxidant, antiviral, antitubercular, antifungal, antibacterial, anti-inflammatory, anti-convulsant and anti-depressant activities (Bekircan and Bektas, 2008; Bhardwaj et al., 2010).

Not at least, coumarin and coumarin-related compounds have proved for many years to have significant therapeutic potential. The development of coumarins as antioxidant agents has attracted much attention in recent years. Coumarins afford an opportunity for the discovery of new antioxidants with novel mechanisms of action (Kostova et al., 2011).

Various moieties when combined with coumarin can produce same or different effects but with different potencies (Al-Majedy et al., 2017).

Many coumarin derivatives have special abilities to scavenge reactive oxygen species and to influence processes involving free radical-injury (Patel et al, 2011; Shinde et al., 2014). Free radical scavenging activity of the coumarin compounds is concentration dependent.

The styryl carbonyl group in the structure of coumarin was found to be very important in scavenging reactive oxygen species, contributing to the prevention of oxidative damage caused by free radicals.

In vitro antioxidant evaluation of semi-synthetic and synthetic compounds represents one important step for biological characterisation of these. The antioxidant properties are determined by using a group of tests based on different chemical mechanism in order to find specific way of action that is applicable in biological systems and finally will reduce the costs of *in vivo* tests.

The most used tests are: the determination of antioxidant capacity, the ferric reducing power test, the iron chelating test, the radical scavenging tests, the inhibition of 15-lipoxygenase test, and the inhibition of nitric oxide test. Some of these have been used to characterise our synthetic or semi-synthetic compounds.

Personal contribution to knowledge in this research field is presented in following published papers:

- Drăgan M, Stan CD, Iacob A, Profire L. Assessment of *in vitro* antioxidant and antiinflammatory activities of new azetidin-2-one derivatives of ferulic acid. *Farmacia* 2016; 64(5): 717-721.
- Stan CD, Drăgan M, Iacob AT, Profire L. Assessment of *in vitro* antioxidant activity of some new ferulic acid derivatives. *Rev Med Chir Soc Med Nat Iaşi* 2016; 120(3): 727-731**.**
- Tătărîngă G, Stan CD, Mircea C, Jitareanu A, Zbancioc AM. Antioxidant evaluation of some coumarin derivatives. *Farmacia* 2016; 64(4): 533-538.
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I.4.1. Materials and methods

There were investigated the antioxidant capacity, through different assays, of various derivatives of ferulic acid (such as: hydrazone, thiazolidin-4-one and azetidin-2-one derivatives), of various derivatives of isatins and of various new coumarine synthetic compounds.

Determination of total antioxidant capacity

The total antioxidant capacity of the synthesized compounds was evaluated using phosphomolybdenum method following the protocol described in literature (Cacic et al., 2010) with some minor modifications. The method is based on the reduction of Mo^{+6} to Mo^{+5} by the tested compounds followed by the formation of the phosphate/Mo^{+5} green complex at acidic pH. The absorbance was read at a wavelength of 695 nm against a blank consisting of 2000 µL reagent and 200 µL DMSO. For each compound it was calculated effective concentration 50 (EC_{50}) by linear regression and ascorbic acid (2 mg/mL) was used as a positive control (Wolszleger et al., 2014).

All determinations were performed in triplicate and the results were expressed as arithmetic average ± standard deviation (SD).

The ferric reducing power

The ferric reducing power assay is based on the determination of the coloured compound formed by mixing the sample with potassium ferricyanide and ferric chloride. Reducing power of the synthesized compounds was measured by the method described in the literature (Melo-Silveira et al., 2012) with some modifications.

Absorbance was read at the wavelength of 700 nm against a blank (the reaction mixture without the sample). For each compound it was calculated effective concentration 50 (EC_{50}) by linear regression and ascorbic acid (2 mg/mL) was used as a positive control(Wolszleger et al., 2014; Tătărîngă et al., 2014; Tătărîngă et al., 2016).

All determinations were performed in triplicate and the results were expressed as arithmetic average ± standard deviation (SD).

The DPPH Radical Scavenging Assay

Reducing compounds stabilize 2,2-diphenyl-1-picril-hidrazil (light purple) radical (DPPH) to yellow diphenyl picrazine with decreasing of the absorbance value at 517 nm. The tested compounds were dissolved in DMSO to obtain a stock solution of 2 mg/mL according to the procedure from the literature (Tătărîngă et al., 2014), with minor modifications.

The absorbance was measured at 517 nm against a blank solution (methanol). The radical scavenging ability (I%) was calculted according to the following equation:

$$I\% = (A0 - At / A0) \times 100$$

where A0 is the absorbance of 0.1 mM DPPH methanolic solution and At is the absorbance after 30 minutes of the same solution mixed with the sample.

For each compound the effective concentration 50 (EC50) was calculated by linear regression analysis and ascorbic acid (2 mg/mL) was used as positive control.

All tests were performed in triplicate and the results were expressed as arithmetic average ± standard deviation (SD) (Wolszleger et al., 2015; Tătărîngă et al., 2016).

The ABTS Radical Scavenging Assay

The ABTS cation radical generation was performed by treating the solution of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (7 mM) with ammonium persulfate (2.45 mM). The ABTS solution was mixed with sample at different concentrations and the absorbance was measured at 734 nm. The radical scavenging ability was calculated according to the following equation:

$$I\% = (A0 - At / A0) \times 100$$

where A0 is the absorbance before adding the sample and At is the absorbance after 6 min of reaction.

For each sample the effective concentration (EC_{50}) was calculated by linear regression analysis and ascorbic acid (2 mg/mL) was used as positive control.

All tests were performed in triplicate and the results were expressed as arithmetic average ± standard deviation (Dragostin et al., 2013; Drăgan et al., 2016).

NO inhibition assay

Nitric oxides radicals were generated from a sodium nitroprusside solution (10 mM) and ascorbic acid was used as standard compound. NO inhibition was determined by the method described in the literature (Sunday et al., 2014) with some modifications.

The absorbance was measured at 546 nm (Tătărîngă et al., 2016). All measurements were done in triplicates and mean values were calculated.

The % inhibition of nitric oxide radical was calculated using the following equation:

$$\% \text{ inhibition} = \{(Abs_{control} - Abs_{sample})\}/(Abs_{control}) \times 100$$

where $Abs_{control}$ is the absorbance of the NO radical solution and Abs_{sample} is the absorbance of NO radical solution + tested compound at different concentrations.

The inhibitory concentration 50 (IC_{50}) of the tested compounds represent the concentration that inhibit 50% of the NO radicals and is obtained from the standard curve compared to that of standard (ascorbic acid).

I.4.2. Results

Total antioxidant capacity

Analysing the antioxidant capacity of the **thiazolidine-4-ones of ferulic acid** (Figure 4 and Table 7) it can be observed that the most active compound was 5i (R=2,3-diOH), with EC_{50} = 6.60±0.12 µg/mL, which means that it is about 4 times more active than ferulic acid (EC_{50}=26.64±0.01 µg/mL) and comparable with ascorbic acid (EC_{50}=5.12±0.17 µg/mL) (Stan et al., 2016).

A good antioxidant activity also showed compounds: 5j (R=4-OH, 3-OCH_3, EC_{50}=22.76±0.26 µg/mL), 5a (R=H, EC_{50}=23.06±0.02 µg/mL), and 5b (EC_{50}=25.11±0.03 µg/mL).

Their activity being comparable with the activity of ferulic acid (Stan et al., 2016).

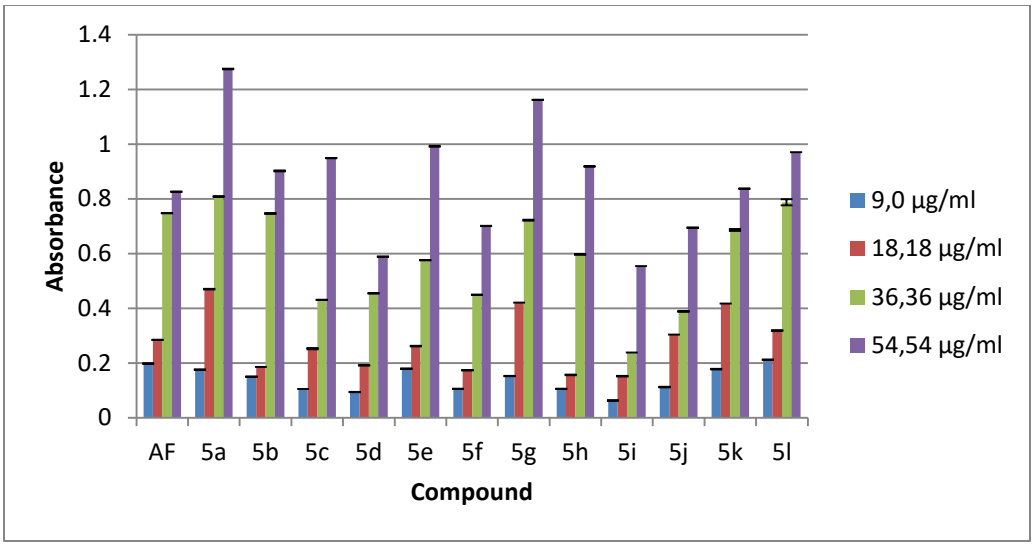

Figure 4. The absorbance value of ferulic acid hydrazones at different concentrations

Table 7. Total antioxidant activity of the ferulic acid thiazolidin-4-one derivatives

Comp	**EC_{50} µg/mL***
5a	23.06 ± 0.02
5b	25.11 ± 0.03
5c	30.37 ± 0.01
5d	39.21 ± 0.04
5e	31.17 ± 0.04
5f	31.00 ± 0.03
5g	49.54 ± 0.02
5h	39.39 ± 0.01
5i	06.60 ± 0.12
5j	22.76 ± 0.26
Ferulic acid	26.64 ± 0.01
Ascorbic acid	05.12 ± 0.17

***** the EC_{50} values are the average of three determinations ± standard deviation

The ferric reducing power

Fe (III) reduction is often used as an indicator of electron donating activity. In the reducing power assay, antioxidants with electron-donating abilities reduce ferricyanide to ferrocyanide by donating an electron. The amount of ferrocyanide is monitored by measuring the formation of Perl's Prussian blue at 700 nm. Increasing the absorbance at 700 nm indicates an increase in the reducing ability. Within this assay, EC_{50} values are the effective concentrations at which the absorbance is 0.5.

The antioxidant evaluation by this test has been applied for ferulic acid derivatives, coumarin derivatives, and Schiff bases of isatin.

Nine classes of **coumarin derivatives** were tested, all these compounds have been chosen depending on physico-chemical properties. Finally 27 compounds were included:
- the condensation products of 4-methyl and 4-propyl-7-hydroxy-coumarin (Ia-b) with bromethylacetate (IIa-b) and then their corresponding aceto-hydrazides (IIIa-b) and acids (VIIa-b),
- potassium dithiocarbazate derivatives (IVa-b) and their S-methyl analogues (Va-b),
- two categories of coumarin derivatives with other heterocyclic rings - thiadiazole (VIa-b) and isatin (IXa-b).

The reducing power of the tested coumarin derivatives was not so important comparing with ascorbic acid and the results are presented in Table 8.

Table 8. The reducing power of the investigated substances

Compound	EC_{50}	Compound	EC_{50}
Ia	>> 1mg/mL	VIIb	>> 1 mg/mL
Ib	>> 1mg/mL	VIIb1	> 1 mg/mL
IIa	>> 1mg/mL	VIIb2	>> 1 mg/mL
IIb	>> 1mg/mL	VIIIa	>> 1 mg/mL
IIIa	0.176 mg/mL	VIIIb	>> 1 mg/mL
IVa	0.627 mg/mL	IXa	>> 1 mg/mL
IVb	1.02 mg/mL	IXa1	>> 1 mg/mL
Va	0.422 mg/mL	IXa2	> 1 mg/mL
Vb	> 1 mg/mL	IXa3	>> 1 mg/mL
VIa	>> 1 mg/mL	IXb	>> 1 mg/mL
VIb	> 1 mg/mL	IXb1	>> 1 mg/mL
VIIa	>> 1 mg/mL	IXb2	>> 1 mg/mL
VIIa1	> 1 mg/mL	IXb3	>> 1 mg/mL
VIIa2	>> 1 mg/mL	**Ascorbic acid 1 mg/mL**	0.049 mg/mL

The only substances that were moderately active were the hydrazide derivatives IIIa, IVa, IVb and Va, but their activity was lower than that exhibited by the reference substance (ascorbic acid).

The calculated values for EC_{50} for the most active coumarin derivatives are shown in Figure 5. This method could not be applied to other coumarin derivatives due to the precipitation reaction during performing this test.

The **Schiff bases of isatins** have also reducing properties, and the results were expressed in mmols ascorbic acid equivalents (Figure 6). In the N-unsubstituted group, the compound 1c has the best reducing action, and it is followed in this respect by the methylate derivative in the fifth position. The compounds that have in the fifth position electron attracting groups have the lowest reducing powery (1d, 1e). The substitution on the nitrogen atom with a methyl group, favourably influenced the reducing power, and this was evident for the 1f compound that had a reducing power superior to the unmethylated at nitrogen derivative 1a

(Tătărîngă et al., 2014).

The weakest inhibitory activity was observed in the 1d and 1e compounds probably due to electron withdrawing nature of the substituent.

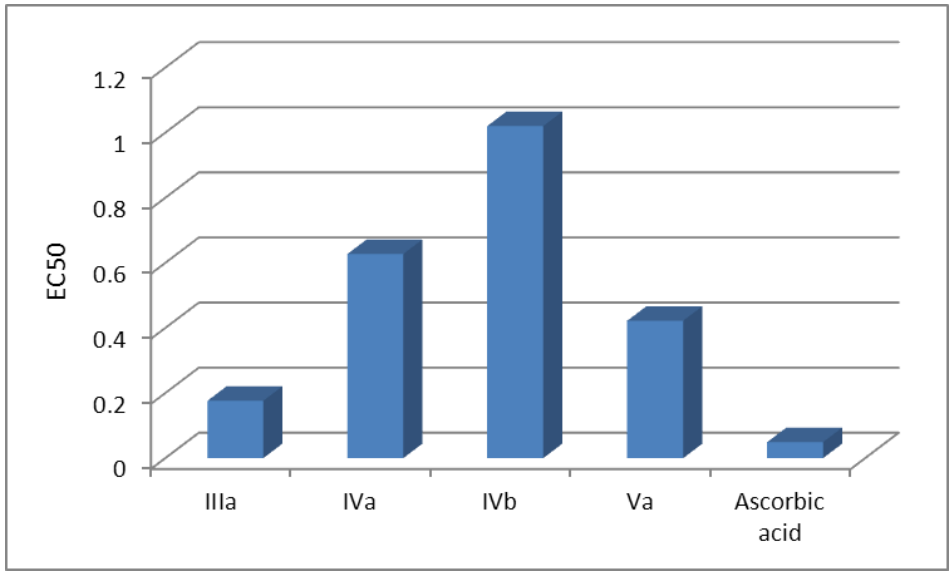

Figure 5. EC_{50} values for the most active coumarin derivatives in the reducing power assay

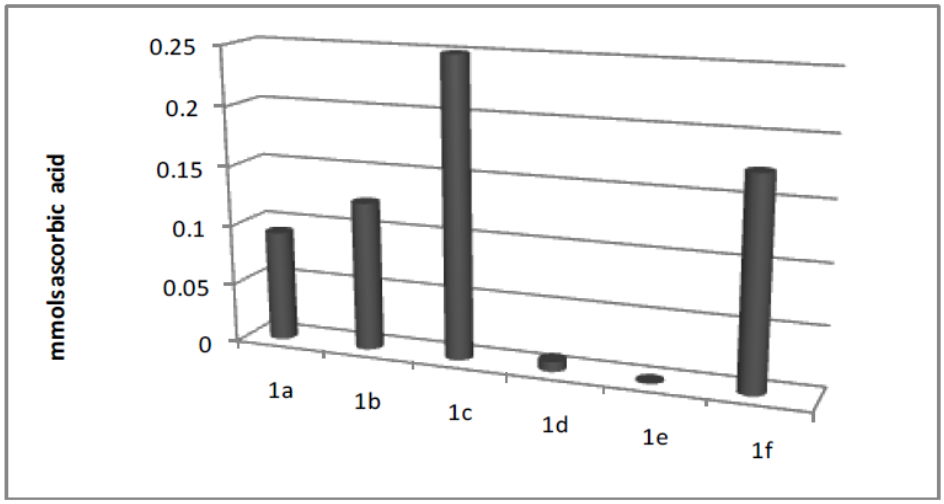

Figure 6. Reducing power of Schiff bases of isatins compared with ascorbic acid solutions

As it can be seen in Table 9 and Figure 6, for the **thiazolidine-4-ones of ferulic acid** series the most active compound was 5i (R=2,3-diOH), with the EC_{50} = 0.0899±0.01 mg/mL, which means that it is about 4.2 times more active than ferulic acid (EC_{50}=0.3812±0.09 mg/mL) and comparable with ascorbic acid (EC_{50}=0.0516±0.01 mg/mL). A good antioxidant activity showed

also 5h (R=4-N(CH_3)$_2$, EC_{50}=0.2571±0.04 mg/mL), 5d (R=4-NO_2, EC_{50}=0.2937±0.02 mg/mL) and 5e (R=2-NO_2, EC_{50}=0.3357±0.09 mg/mL) derivatives, their activity being comparable with the activity of ferulic acid (Stan et al., 2016).

Table 9. Ferring reducing power (EC_{50} mg/mL) of the ferulic acid thiazolidin-4-one derivatives

Comp	**EC_{50} mg/mL***
5a	0.3716 ± 0.03
5b	1.7269 ± 0.15
5c	0.4228 ± 0.09
5d	0.2937 ± 0.02
5e	0.3357 ± 0.09
5f	1.2106 ± 0.33
5g	0.6923 ± 0.13
5h	0.2571 ± 0.04
5i	0.0899 ±0.01
5j	0.3710 ± 0.06
5l	0,37106 ± 0,05
ferulic acid	0.3812 ± 0.09
ascorbic acid	0.0516 ± 0.01

*the EC_{50} values are the average of three determinations ± standard deviation (n=3, p<0.05)

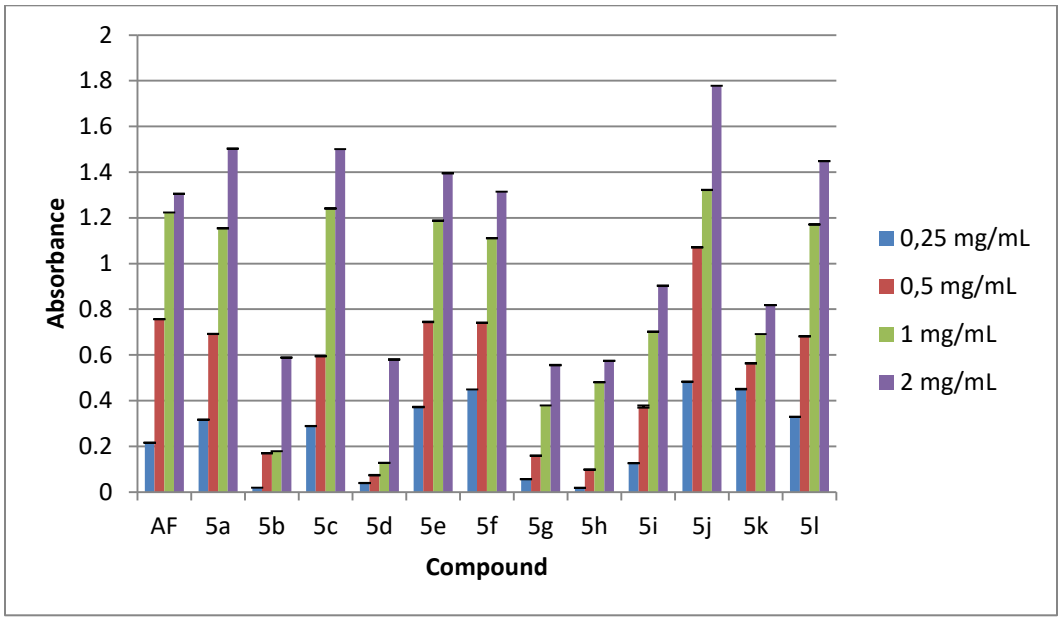

Figure 6. Absorbance value of thiazolidin-4-one derivatives of ferulic acid at different concentrations

The DPPH Radical Scavenging Assay

DPPH scavenging assay is an antioxidant test used to establish the capacity of organic compound to stabilize radicals. This radical is obtained by chemical synthesis and has similar properties with radicals produced in lived organisms.

The results obtained in the **azetidin-2-one derivatives of ferulic acid series** (Table 10) support that the most active compounds are 1e (EC_{50} = 3.81±0.35 µg/mL) and 1d EC_{50} =5.48±0.03 µg/mL), these compounds being about 9 times and respectively 6 times more active than ferulic acid (34.21±0.05 µg/mL) and comparable with ascorbic acid (EC_{50} = 5.21±0.02 µg/mL). A good antioxidant activity was showed also by 1f (EC_{50} = 25.21±0.28 µg/mL), its activity being comparable with ferulic acid (Drăgan et al., 2016).

Table 10. The DPPH radical scavenging ability (EC_{50}, µg/mL) of azetidin-2-one derivatives

Comp	R	EC_{50} (µg/mL)*
1a	-H	35.31 ± 0.70
1b	-OH(2)	47.31 ± 0.25
1c	-NO_2(2)	55.35 ± 0.40
1d	-Cl(4)	5.48 ± 0.03
1e	-F(4)	3.81 ± 0.35
1f	-Br(4)	25.21 ± 0.28
Ascorbic acid		5.21± 0.02
Ferulic acid		34.21 ± 0.05

*the EC_{50} values are the average of three determinations ± standard deviation (n = 3, p<0.05)

Out of the tested **coumarin derivatives**, the most active DPPH free radicals scavengers were the coumarin hydrazide derivatives (IIIa-b, IVa-b, Va-b). The activities of IVa and Va were similar to that of the standard (reference substance, ascorbic acid), the inhibition percentage being over 90%, the introduction of sulphur atoms in the molecule having a positive influence on the scavenging potential (Tătărîngă et al., 2016).

Substances IIIa, IVa, Va, containing a methyl group, were slightly more active than their analogues with propyl radicals (Figure 7).

A possible mechanism that can explain the antioxidant effect of the coumarin hydrazide derivatives is related to the keto-enol forms of the substances, the enol group being capable to easily donate the hydrogen and electrons with stabilisation of the DPPH radical (Tătărîngă et al., 2016).

The ability to release a hydrogen atom or an electron is reduced for **Schiff bases of isatins** compared to control, 0.1 mM ascorbic acid, except for the 1c substance, that in the investigated concentration of 2 mM has a greater DPPH scavenger activity (96.29%) (Figure 8). The other compounds exhibited DPPH scavenger activity in the following order: 1c > 1d > 1b > 1a > 1f > 1e (Tătărîngă et al., 2014).

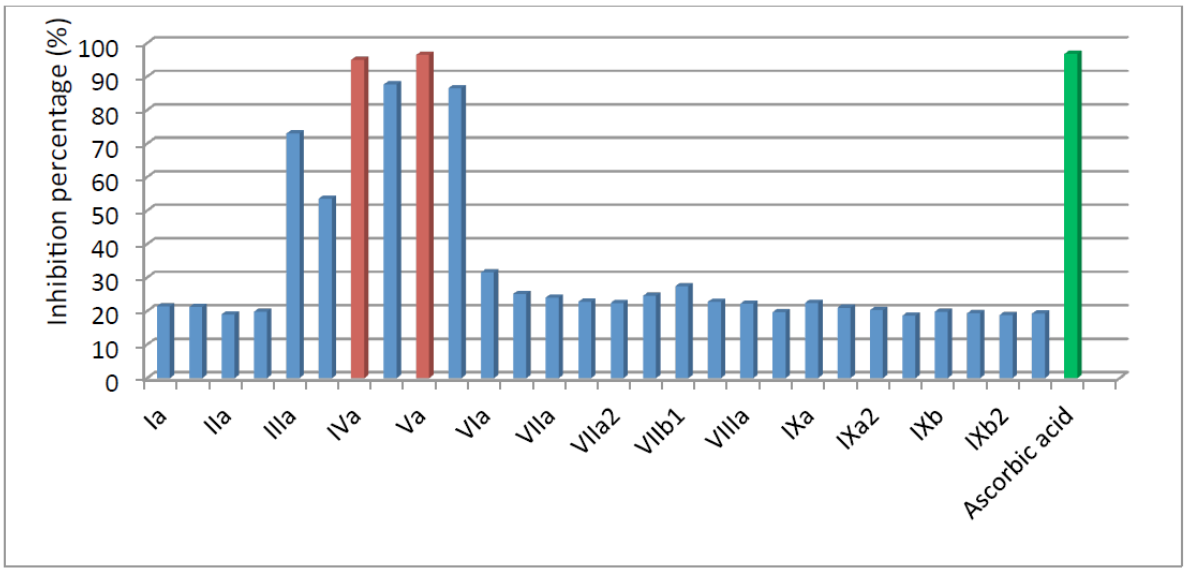

Figure 7. The DPPH inhibition percentages for the investigated substances (1mg/mL)

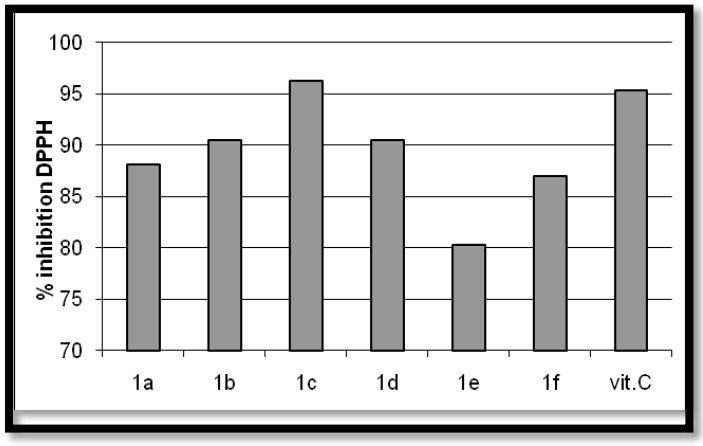

Figure 8. Scavenging effect of isatins derivatives and vitamin C on DPPH radical

On the **thiazolidine-4-ones of ferulic acid** group it was observed that the most favourable influence was exerted by -OH(2,3), -OH(4),OCH_3(3) radicals (Figure 9 and Table 11). The corresponding compounds 5k and 5l were 8.84 and 3.17 times respectively more active than ferulic acid. Moreover the compound 5k was 1.76 times more active than ascorbic acid used as positive control. A good antioxidant effect was showed also by the compounds 5c (R=4-F), 5e (R=4-NO2) and 5i (R=2,6-diCl), which presented an effect comparable with that of ferulic acid (Wolszleger et.al., 2015).

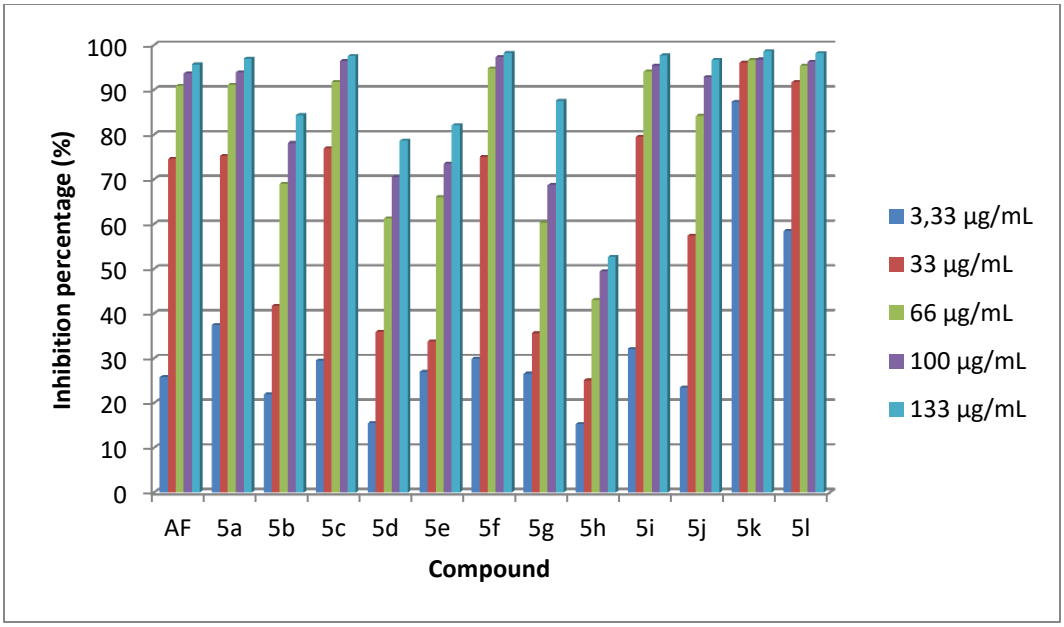

Figure 9. The DPPH inhibition percentages for the tested thiazolidine-4-ones of ferulic acid at different concentrations

Table 11. The DPPH radical scavenging ability of tested thiazolidine-4-ones of ferulic acid

Comp	R	EC_{50} (µg/mL)*
5a	-H	29,24 ± 0,60
5b	-Cl(4)	53,95 ± 0,20
5c	-F(4)	24,25 ± 0,35
5d	-Br(4)	65,08 ± 0,20
5e	$-NO_2$(2)	57,75 ± 0,20
5f	$-NO_2$(4)	24,56 ± 0,17
5g	$-OCH_3$(2)	64,28 ± 0,14
5h	-OH(2)	126,57 ± 0,48
5i	-Cl (2,6)	29,98 ± 0,22
5j	$-N(CH_3)_2$ (4)	32,85 ± 0,38
5k	-OH(2,3)	3,52 ± 0,01
5l	-OH(4),OCH_3(3)	9,79 ± 0,64
ascorbic acid		6,21 ± 0,023
ferulic acid		31,13 ± 0,08

*the EC_{50} values are the average of three determinations ± standard deviation (n = 3, p<0.05)

The ABTS Radical Scavenging Assay

The ability of **azetidin-2-one derivatives of ferulic acid** to scavenge the radical cation $ABTS^{\cdot+}$ is monitored, compounds 1e and 1d showed a better scavenging activity than ferulic acid, being 1.7 times (1e, EC_{50} = 6.05±0.02 µg/mL) and respectively 1.5 times (1d, EC_{50} = 7.23±0.05 µg/mL) more active. Moreover their activity was comparable with ascorbic acid at the

same concentration (Table 12). A good antioxidant activity was showed also by 1f (EC_{50}=10.17±0.04 μg/mL), its activity being comparable with ferulic acid (Drăgan et al., 2016).

Table 12. The ABTS radical scavenging ability (EC_{50}, μg/mL) of azetidin-2-one derivatives

Compound	R	EC_{50} (μg/mL)*
1a	-H	11.12 ± 0.05
1b	-OH(2)	26.07 ± 0.04
1c	-NO_2(2)	28.14 ± 0.03
1d	-Cl(4)	7.23 ± 0.05
1e	-F(4)	6.05 ± 0.02
1f	-Br(4)	10.17 ± 0.04
ascorbic acid		5.23 ± 0.06
ferulic acid		10.49 ± 0.03

*the EC_{50} values are the average of three determinations ± standard deviation (n = 3, p<0.05)

In the **thiazolidine-4-ones of ferulic acid** group (Figure 10, Table 13) it was observed that the compounds 5a, 5c, 5f, 5i, 5k, and 5l were more active than ferulic acid. Related to the influence of the radical which substituted the aromatic ring it was found that the most favourable influence was exerted by 2,3-OH (5k), 4-OH-3-OCH3, the corresponding compounds 5k and 5l being 8.16 and 5.21 times respectively more active than ferrulic acid.

Table 13. The EC_{50} (μg/mL) values for derivatives

Comp	R	EC_{50} (μg/mL)*
5a	-H	8,29 ± 0,08
5b	-Cl(4)	11,04 ± 0,08
5c	-F(4)	6,58 ± 0,07
5d	-Br(4)	12,07 ± 0,07
5e	-NO_2(2)	14,05 ± 0,04
5f	-NO_2(4)	6,62 ± 0,07
5g	-OCH_3(2)	17,50 ± 0,06
5h	-OH(2)	12,29 ± 0,03
5i	-Cl(2,6)	6,08 ± 0,02
5j	- $N(CH_3)_2$(4)	11,41 ± 0,02
5k	-OH(2,3)	1,27 ± 0,01
5l	-OH(4),-OCH_3(3)	1,99 ± 0,01
ascorbic acid		5,08 ± 0,03
ferulic acid		10,37 ± 0,02

*the EC_{50} values are the average of three determinations ± standard deviation (n = 3, p<0.05)

Moreover the compounds were 4 times (5k) and 2.5 times (5l) more active than ascorbic acid (AA) used as positive control. A good antioxidant effect was also showed by the compounds

5i (R = 2,6-diCl), 5c (R= -F) and 5e (R = -NO2). These compounds were 1.7, 1.57 and 1.56 times respectively more active than ferulic acid and their activity was comparable with ascorbic acid.

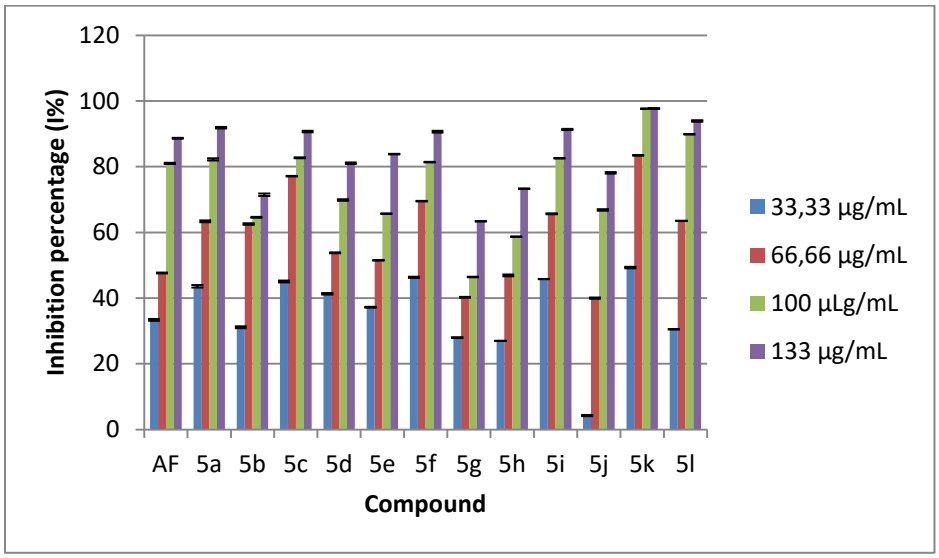

Figure 10. The ABTS radical scavenging activity of thiazolidine-4-ones of ferulic acid (% inhibition) at different concentrations

NO inhibition assay

Nitric oxide (NO) is involved in oxidative processes when it's concentration exceed the normal values. Most of the investigated **coumarine derivatives** were moderate NO inhibitors (Figure 11).

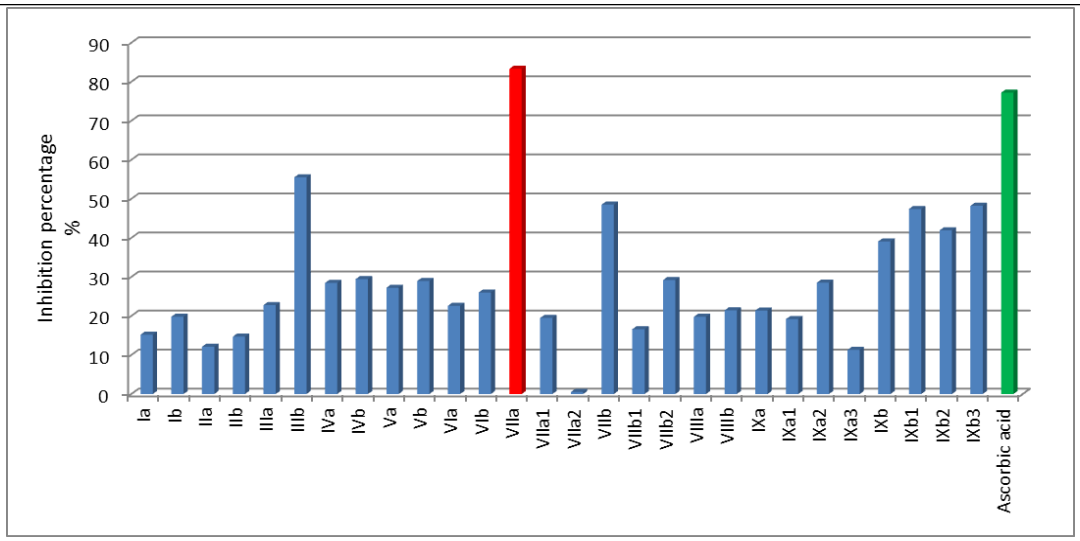

Figure 11. The NO inhibition activity of the tested coumarine derivatives

A good NO inhibition activity was exhibited by compound IIIb (2-(2-oxo-4-propyl-2H-chromen-7-yloxy)acetohydrazide) and its condensation products with isatin and three substituted isatin derivatives (7-methyl, 5-chloro and 5-nitro isatin). The presence of a methoxy group in position 7 on the coumarin ring had a positive influence on the NO inhibition potential. The activity of 4-methyl-7-methoxy coumarin was remarkable, exceeding the inhibitory potential of ascorbic acid, used as a reference substance (Tătărîngă et al., 2016).

I.4.3. Discusions

In vitro antioxidant evaluation of natural, semisynthetic, and synthetic compounds represents a mandatory step in order to evaluate the biological properties of these. Many tests have been developed during the time based on oxido-reduction reaction and they are applied depending on the chemical properties of tested compound.

The good results obtained by one test doesn't indicate always the antioxidant effects in biological system. For this reason is necessary to evaluate *in vitro* antioxidant properties of compounds by using tests based on different chemical mechanisms.

In our studies we analysed the antioxidant properties of synthesized compounds at least by two tests.

Ferulic acid is a natural compound from phenol acids group with a hydroxyl group able to donate hydrogen and electrons during redox process. Extracts from medicinal plants that contain ferulic acid are used in traditional medicine to treat many diseases. These facts represent the start point of our studies regarding ferulic acid and we tried to improve the biological properties by its transformation in derivatives with good effects and the lowest toxicity (Stan et al., 2016).

The total antioxidant capacity test revealed that most hydrazones are more active than ferulic acid, but in the thiazolidin-4-one derivatives group the effect is not so important. The both groups of compounds are less active than ascorbic acid (Wolszleger et al., 2014).

We observe that the transformation of ferulic acid to a new derivative with more than one hydroxyl groups will improve the antioxidant capacity and the EC50 value is nearest to that one for ascorbic acid.

Reducing power test evaluates the capacity of compounds to transform Fe^{3+} to Fe^{2+} and to protect against oxidative action of ferric ions. Fe^{3+} represents the most important form of iron in serum that is fixed especially on transferrin. In excess this ion induces oxidation of macromolecules, reacts with hydrogen peroxide, and produces peroxyl radicals with peroxidation of lipids and proteins.

Thiazolidin-4-one derivative coded 5i is fourth times more active than ferulic acid. Again the compounds with more than one hydroxyl group are the most active. This test is important

because ferric ions are presented in serum and cells and by controlling their concentration the oxidative processes are maintained in normal limits.

In the radical scavenging tests we observed again that the ferulic acid derivatives with more hydroxyl groups stabilised DPPH radical and ABTS cation radical. Thiazolidine-4-ones derivative coded 5k with two more hydroxyl groups than ferulic acid has the highest scavenging properties (DPPH test) and was more active than ascorbic acid (EC_{50} 3.52±0.01 µg/mL versus 6.21±0.02 µg/mL).

Scientific databases included studies about the biological effects of ferulic acid on physiological and pathological processes. Ferulic acid could stop radical chain reactions on cell membrane and protects this against harmful effects of UV-radiation. The protective effects of ferulic acid are explained by its transformation by polymerization (Santos et al., 2008; Paiva et al., 2013).

Ferulic acid has the ability to decrease inflammatory process such as acetic acid induced ulcerative colitis on rats. Doses of 20 and 40 mg/kg ferulic acid significantly ameliorate the ulcerative lesions and improve body weight after 12 days of treatment. Also, ferulic acid (40 mg/kg) decreased serum lactate dehydrogenase and alkaline phosphatase (biomarkers for intestinal inflammation) and the effect was more important than that has been induced by prednisolone (2 mg/kg). High level of lactate dehydrogenase indicates the cellular damages induced by acetic acid. Acetic acid increases the synthesis of reactive species of oxygen and modifies the values of oxidative markers such as SOD, glutathione, malondialdehyde and colonic myeloperoxidase. All these values have been significantly reduced in ferulic acid treated rats as compared with acid acetic control group. Ferulic acid acts as an antioxidant compounds and decreases the oxidative process that is an important mediator of the pathogenesis of ulcerative colitis (Kandhare et al., 2016).

Ferulic acid (80 mg/kg) ensures protective effects against acetaminophen-induced liver toxicity. Oxidative stress induced by acetaminophen (900 mg/kg) on mice is reversed by ferulic acid with increasing of antioxidant enzymes level such as superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase and glutathione-S-transferase.

In the same time ferulic acid reduces lipid peroxidation with decreasing of malondialdehyde concentration in the liver tissue near to normal level. The improvement of liver markers in mice treated with ferulic acid and acetaminophen underlines the antiinflammatory and antioxidant effects of ferulic acid on liver tissue. Liver protection induced by the ferulic acid was more important than that has been induced by silymarin (25 mg/kg). This could be explained by the free radical scavenging action of ferulic acid (Krishnan et al., 2013).

Coumarins are natural compounds with antioxidant, anti-inflammatory effects and good effects on human health, such as reducing the risk of cancer, diabetes, cardiovascular and brain diseases. They have radical scavenging effects and bloc some enzymes involved in oxidative reactions. For this reason vegetal extracts with coumarins or products that contain synthetic

coumarins are used to treat different kind of diseases involving oxidative stress (Bubols et al., 2013).

The reduced quantity of coumarins in plant products has stimulated many researchers to develop new methods of their synthesis or coumarin derivatives with improved biological effects.

Our coumarin derivatives had low ferric reducing power above that was induced by ascorbic acid. In the same time the coumarin hydrazide derivatives stabilize DPPH radical with similar effects as ascorbic acid. Free radical scavenging activity of the coumarin derivatives was concentration dependent. 4-methyl-7-methoxy coumarin has the ability to reduce NO and to protect against oxidative processes induced by this (Tătărîngă et al., 2016).

Coumarin derivatives compounds that contain 3,4-dihydroxyphenyl and 2,5-dihidroxyphenyl have more important antioxidant activity since hydrogen donation leads to formation of a stable quinoid structure. It has been reported that two hydroxyl groups in ortho position are important for antioxidant activity (Maia et al., 2012). The critical evaluation of coumarin derivatives that have been synthesized in different laboratories indicate that the hydroxyl derivatives possess the highest antioxidant and anti-inflammatory effects (Al-Majedy et al., 2017).

Istatin and its derivatives possess diverse biological effects such as antibacterial, antifungal, antiviral, anti-HIV, anti-mycobacterial, anticancer, anti-inflammatory and anticonvulsant (Lashgari et al., 2012). The chemical reactivity of istain has led many researchers to develop new methods for the synthesis of isatin derivatives with superior biological properties. Such derivatives are the Schiff bases which can be obtained using compounds of various structures, including heterocycles.

One important group of istain derivatives are Schiff bases for which Verma and colleagues revealed better anticonvulsant activity than the standard drugs phenytoin, carbamazepine and valproic acid (Verma et al. 2004). In another study Kiran and colleagues synthesized thiocarbohydrazone derivatives of isatin with good effects against DPPH and H_2O_2 scavenging activity (Kiran et al. 2013).

Our Schiff bases of isatin synthesized with antipirin have been evaluated by two antioxidant tests: DPPH scavenging and reducing power. On DPPH scavenging test the antioxidant properties were lower than ascorbic acid, excepting compound 1c that has a methyl group nearest to heterocyclic nitrogen.

Similar results have been obtained on reducing power test, were again compound 1c was the most active. All aromatic nucleus substituents that increase it electron density can also increase the antioxidant properties of the isatin derivatives, so the derivative with nitro group had the lowest antioxidant properties.

I.4.4. Conclusions

The results obtained on *in vitro* antioxidant tests underline the advantages of chemical transformation of ferulic acids and open new research area about these compounds. Is necessary to continue the evaluation by *in vivo* antioxidant test and to determine the toxic, respectively the lethal doses of the more active compounds.

Scientific databases included a lot of studies about ferulic acid, but not so many about its derivatives.

Our studies about these derivatives open a new research area and for all compounds with *in vitro* antioxidant effects is necessary to determine pharmacokinetic properties of them.

The coumarin derivatives synthesized in our studies do not have major antioxidant effects and it is necessary to develop new synthetic methods by which hydroxyl groups are introduced into these structures.

Evaluating the antioxidant action of new Schiff bases derived from isatin revealed their antioxidant properties, but generally lower compared to ascorbic acid. The intensification of the antioxidant action requires the introduction into the structure of donor groups of hydrogen and elctrons or groups that increase the reactivity of the isatin derivatives.

I.5. *In vitro* anti-inflammatory evaluation of semi-synthetic pharmaceutical substances with therapeutic potential

Recent studies have established a strong correlation between inflammation and oxidative stress. The human body is constantly aggressed by the endogenous or exogenous highly reactive free radicals, which may damage proteins, enzymes, lipids, DNA, causing inflammatory reactions and metabolic disturbances (Badarinath et al., 2010; Drăgan et al., 2016). Also, it has been shown that chronic inflammation is responsible for the increased levels of reactive oxygen species (ROS) in cells (neutrophils, monocytes, macrophages, eosinophils) and for the decreased levels of antioxidant enzymes (catalase, superoxide dismutase, glutationperoxidase). ROS are also involved in metabolic conversion of arachidonic acid to pro-inflammatory intermediates and prostaglandins, conversion which is mediated by cyclooxygenase-1 (COX-1) and lipoxygenase. The reactive oxygen species activate redox-sensitive transcription factors as nuclear factor kappa B type (NK-kB) and activator protein 1 (AP-1). Disturbances in these proteins regulation are correlated with cancer, viral infections, autoimmune diseases (Sharad et al., 2013).

In the last years, development of new compounds with heterocyclic structure in order to reduce the oxidative stress is a major concern for researchers.

In this context, special attention was focused on ferulic acid derivatives. Due to its antioxidant effect, ferulic acid may protect lipids from oxidation by ROS and could be useful in the prevention and treatment of several oxidative stress-related disorders (Nicolae et al., 2015; Drăgan et al., 2016).

Personal contribution to knowledge in this research field is presented in following published papers:

- Drăgan M, Stan CD, Iacob A, Profire L. Assessment of *in vitro* antioxidant and antiinflammatory activities of new azetidin-2-one derivatives of ferulic acid. *Farmacia* 2016; 64(5): 717-721.
- Drăgan M, Stan CD, Pânzariu A, Profire L. Evaluation of anti-inflammatory potential of some new ferulic acid derivatives. *Farmacia* 2016; 64(2): 194-197.

I.5.1. Materials and methods

There were investigated *in vitro* anti-inflammatory potential, through different assays, of various derivatives of ferulic acid, such as:

-thiazolidin-4-one derivatives of ferulic acid: 1a-H, 1b-Cl(4), 1c-F(4), 1d-NO_2(4), 1e-NO_2(2), 1f-OH(2), 1g-Cl (2,6), 1h-N(CH_3)$_2$, 1i-OH(2,3), 1j-OH(4)OCH_3(3);

-azetidin-2-one derivatives of ferulic acid: 1a-H, 1b-OH(2), 1c-NO_2(2), 1d-Cl(4), 1e-F(4), 1f-Br(4).

Reagents and solvents

The used reagents: bovine serum albumin (BSA), 2-amino-2-hydroxymethyl-propane-1,3-diol (Tris), diclofenac sodium, phosphate buffer (pH=7.4), isosaline solution (NaCl 0.85%), hyposaline solution (NaCl 0.36%), acetic acid, methanol, dimethylformamide (DMFA) were purchased from Sigma Aldrich and Fluka.

Bovine serum albumin denaturation assay

The test was performed according by the method described in the literature (Williams et al., 2008; Drăgan et al., 2016). The inhibition of protein denaturation (%) was calculated using the following formula:

$$\text{Inhibition of denaturation (\%)} = (A_{control} - A_{sample}/A_{control}) \times 100$$

where Acontrol is the absorbance of the control and Asample is the absorbance of the tested compounds. Diclofenac (0.1 mg/mL) was used as standard anti-inflammatory drug. All determinations were performed in triplicate and the values are expressed as mean ± standard deviation (SD) (Kar et al., 2012; Gondkar et al., 2013).

Human red blood cell (HRBC) membrane stabilization assay

The blood was collected from healthy volunteers and the test was performed according by the method described in the literature (Sadique et al., 1989). The erythrocytes membrane stability (%) was calculated using the following formula:

Erythrocyte membrane stability (%) = 100 - (Asample/Ablood)

where: Asample is the absorbance of the tested compound and Ablood is the absorbance of the control. Diclofenac (0.1 mg/mL) was used as standard anti-inflammatory drug. All determinations were performed in triplicate and the values are expressed as mean ± standard deviation (SD) (Popa et al., 2014; Drăgan et al., 2016).

I.5.2. Results

Bovine serum albumin denaturation assay

Recent data support that protein denaturation are among the causes of inflammatory and rheumatic diseases (Kar et al., 2012; Gondkar et al., 2013). By heating, bovine serum albumin (BSA) undergoes denaturation with the release of antigens responsible for several hypersensitivity reactions, which lead to various diseases such as glomerulonephritis, lupus erythematosus etc. Thus, the agents that can prevent the protein denaturation could be useful for inflammatory diseases.

The anti-denaturation activity of the tested thiazolidin-4-one derivatives of ferulic acid at different concentrations are presented in Figure 18. It was observed that the inhibition of BSA denaturation is increasing with the concentration, the better anti-denaturation activity being observed at 500 μg/mL concentrations.

The anti-denaturation activity of the azetidin-2-one derivatives of ferulic acid at different concentrations is presented in Figure 13. It was observed that the inhibition of BSA denaturation is increasing with the concentration, the better anti-denaturation activity being observed at 50 μg/mL concentrations.

Human red blood cell (HRBC) membrane stabilization assay

The human red blood cell (HRBC) membrane stabilization assay is, often used for assessing the anti-inflammatory effect (Drăgan et al., 2016). Exposure of HRBC to hypotonic conditions results in the lysis of the membranes, with the haemolysis and oxidation of haemoglobin (Rajakumar et al., 2011). Human erythrocytes membrane is similar to lysosomal membrane and its stability limits the inflammatory response by inhibiting the release of cellular constituents that activate neutrophils, bactericidal enzymes and proteases causing inflammation of tissue and cell damage (Kar et al., 2012; Popa et al., 2014).

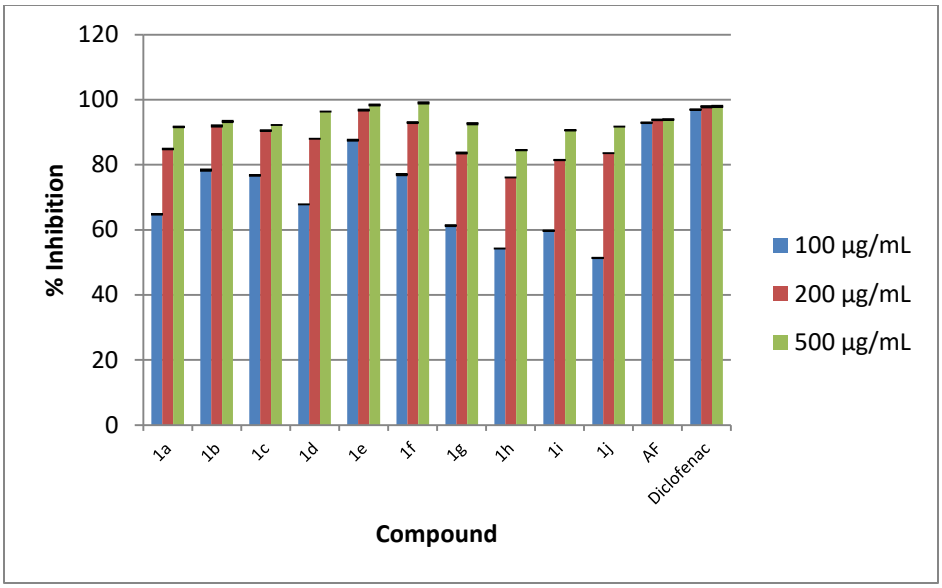

Figure 12. The BSA denaturation inhibition (%) of thiazolidin-4-one derivatives at different concentrations

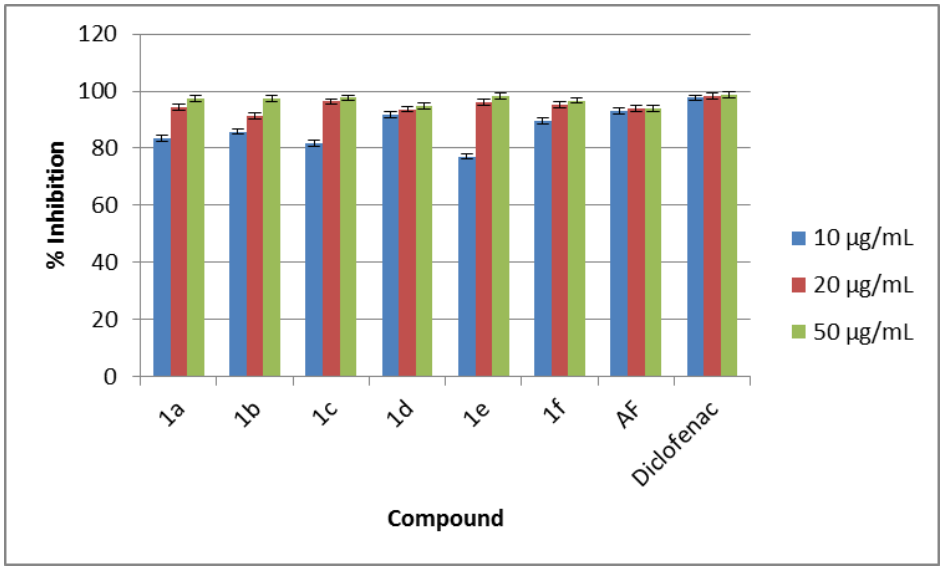

Figure 13. The BSA denaturation inhibition (%) of azetidin 2-one derivatives at different concentrations

The membrane stabilizing activity of the tested thiazolidin-4-one derivatives of ferulic acid at different concentrations (100 µg/mL, 200 µg/mL, 500 µg/mL), obtained by 10 mg/mL stoc solutions is presented in Figure 14.

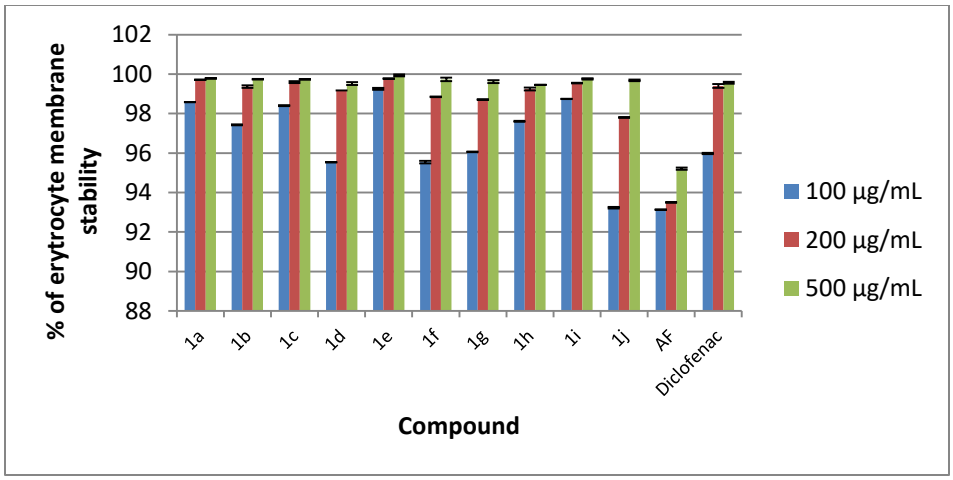

Figure 14. The erythrocyte membrane stability (%) of thiazolidin-4-one derivatives at different concentrations

For all tested compounds it was observed that protection of erythrocyte membrane was higher than ferulic acid and comparable with diclofenac for all tested concentrations, 100 µg/mL, 200 µg/mL and 500 µg/mL.

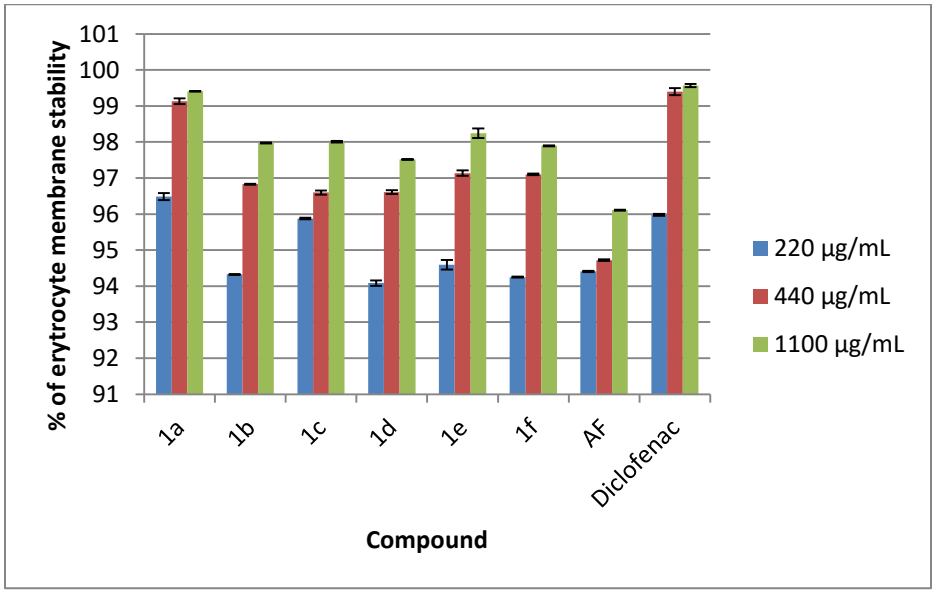

Figure 15. The erythrocyte membrane stability (%) of azetidin-2-one derivatives at different concentrations

At 500 µg/mL all the tested compounds showed a membrane protection comparable or higher than diclofenac (99.56 ± 0.045) and ferulic acid (95.20 ± 0.057) respectively.

The membrane stabilizing activity of the azetidin-2-one derivatives of ferulic acid at different concentrations is presented in Figure 15.

For all tested compounds it was observed that protection of erythrocyte membrane was higher than ferulic acid at all tested concentrations: 220 μg/mL, 440 μg/mL and 1100 μg/mL. At 1100 μg/mL all the tested compounds showed a membrane protection comparable with diclofenac (99.56±0.04%) and higher than ferulic acid (96.11±0.01%).

I.5.3. Discussions

Bovine serum albumin denaturation assay

Methods which include inhibition of protein denaturation, inhibition of oxidative phosphorylation, lysosome membrane stabilisation, platelet aggregation and erythrocyte membrane stabilization have been employed in screening drugs, compounds, herbal preparations and extracts for anti-inflammatory activities (Oyedapo et al., 2010).

The literature survey revealed that natural compounds, organic extracts and semi-synthetic derivatives of them strongly inhibited heat-induced BSA denaturation in a concentration-dependent manner (Umeti et al., 2019). For some of the investigated compounds the BSA denaturation is higher than that of the standard diclofenac.

It is considered that any compound presenting a percentage inhibition of protein denaturation higher than 20% could be studied as potential anti-inflammatory agent (Gondkar et al., 2013). Anti-inflammatory drugs as indomethacin, diclofenac, ibuprofen, acetylsalicylic acid, flufenamic acid, besides the inhibition of cyclooxygenase showed also a considerable ability to prevent the BSA denaturation at pathological pH value (Ramalingam et al., 2010).

It is noticed that the percentage inhibition of protein denaturation for the tested thiazolidin-4-one derivatives of ferulic acid (1a-j), at different concentrations (100 μg/mL, 200 μg/mL, 500 μg/mL) obtained by 10 mg/mL stoc solutions, increased with the concentration.

At 500 μg/mL concentration the most active compounds were 1e (R = 2-NO_2, I% = 98.38 ± 0.012%), 1f (R = 2-OH, I% = 99.00 ± 0.017%) and 1d (R = 4-NO_2, I% = 96.32 ± 0.009%) for which the anti-denaturation activity was comparable to diclofenac (I% = 97.88 ± 0.001), highlighting thus the importance of aromatic ring substitution from the tiazolidin-4-one derivatives in *orto* position.

For the azetidin-2-one derivatives of ferulic acid it was observed that most of them exhibited at 50 μg/mL concentration an effect comparable with diclofenac, which was used as standard.

At this concentration the most active compound was 1e (R = 4-F, I% = 98.23 ± 0.21), for which the anti-denaturation activity was comparable to diclofenac (I% = 98.61 ± 0.01). Also, compounds 1a (R = H, I% = 97.38 ± 0.02), 1b (R = 2-OH, I% = 97.38 ± 0.14), 1c (R = 2-NO_2, I% = 97.69 ± 0.20) and 1f (R = 4-Br, I% = 96.69 ± 0.11) showed an albumin anti-denaturation

activity comparable with diclofenac (I% = 98.61 ± 0.01) and higher than ferulic acid (I% = 93.88 ± 0.14) respectively, highlighting thus the importance of aromatic ring substitution.

Human red blood cell (HRBC) membrane stabilization assay

It is presented in literature that, natural compounds (Jayameena et al., 2018), organic extracts and semi-synthetic derivatives protected the human erythrocyte membrane against lysis induced by hypotonic solution and heat (Anosike et al., 2012; Atolani et al., 2018). The anti-inflammatory activity proved to be concentration dependent (Nirmala and Periyanayagam, 2010).

From the obtained results analysis it is observed that for all the studied derivatives the membrane stabilizing activity increases with concentration. The higher membrane stabilizing activity was obtained at 500 μg/mL concentrations.

For the thiazolidin-4-one derivatives of ferulic acid it was observed that for most compounds the a membrane protection was comparable or higher than diclofenac and higher than ferulic acid, at all three tested concentrations 100 μg/mL, 200 μg/mL and 500 μg/mL.

Except 1d (R = 4-NO_2), 1f (R = 2-OH), 1g (R = 2,6-diCl) and 1j (R = 4-OH, 3-OCH_3), all other compounds showed a membrane stabilizing activity higher than diclofenac at 100 μg/mL and 200 μg/mL (95,9794 ± 0,02886 at 100 μg/mL respectively 99,3987 ± 0,0950 at 200 μg/mL) and ferulic acid (93,1372 ± 0,0116 at 100 μg/mL respectively 93,4981 ± 0,0091 at 200 μg/mL).

At 500 μg/mL concentration, all the studied thiazolidin-4-one derivatives showed higher membrane stabilizing activity than diclofenac (99,5623 ± 0,0451) and ferulic acid (95,2087 ± 0,0578) respectively.

Analyzing the obtained results in the thiazolidin-4-one derivatives series it can be stated that these compounds have a more favorable effect in this *in vitro* anti-inflammatory survey. This observation supports the importance of ferulic acid structural modulation over the anti-inflammatory potential of the investigated derivatives.

For the azetidin-2-one derivatives of ferulic acid, the higher protection was observed for compound 1a (R = H) at all tested concentrations.

The *in vitro* anti-inflammatory activity investigation on new thiazolidin-4-one derivatives of ferulic acid support that the albumin anti-denaturation and erythrocyte membrane stabilizing activity of the tested compounds are depending of concentration, the best results being obtained at 500 μg/mL.

Compared with diclofenac, used as standard anti-inflamatory drug, the most active compounds were 1e (R = 2-NO_2), 1f (R = 2-OH) and 1d (R = 4-NO_2).

These compounds showed an anti-denaturation and membrane stabilizing activity comparable and higher than diclofenac at 500 μg/mL.

The *in vitro* anti-inflammatory activity investigation on new azetidin-2-one derivatives of ferulic acid showed a good albumin anti-denaturation effect for compound 1e (R = 4-F). At the concentration of 50 μg/mL its activity was comparable with diclofenac, used as standard anti-inflammatory drug.

Also, all the tested compounds showed a membrane stabilizing activity higher than ferulic acid and comparable with diclofenac, especially at 1100 μg /mL concentration.

I.5.4. Conclusions

The new obtained thiazolidin-4-one derivatives of ferulic acid showed an anti-denaturation and membrane stabilizing activity comparable and higher than diclofenac.

The anti-inflammatory activity of new azetidin-2-one derivatives of ferulic acid showed an albumin anti-denaturation effect comparable with diclofenac and a membrane stabilizing activity higher than ferulic acid and comparable with diclofenac.

II. Continuous quest for pharmaceutical industrial process improvement

II.1. State of the art

Manufacturing strategy could bring the pharmaceutical industry advantages such as consistent and improved product quality, reduced waste, lower inventory and equipment costs, easy scale-up, flexibility, and short cycle times.

Continuous manufacturing is a relatively new concept for the pharmaceutical industry which could enable chemical reactions that are difficult in batch operations and real-time release of finished products (Gerogiorgis and Jolliffe, 2015). Many batch processes have an isolation, intermediate purification, or change of solvent in them. Such steps would need to be reevaluated in order to achieve products with great quality.

Pharmaceutical companies are aware by the potential benefits of continuous manufacturing improvement, a key goal being the ability to develop a fully integrated system with robust, high-level control and automation capabilities (Badman and Trout, 2015).

The journey toward manufacturing typically starts with a single unit operation. There still remains work to be done on the upstream side to increase yields, including work needed to fully characterize all the materials going into upstream processes. Depending on their characteristics, each raw material can react differently with the drug molecule, and many raw materials going into upstream processes have not been fully characterized or optimized for the target molecule. Thus, upstream optimization needs to be done to achieve higher productivity (Mirasol, 2019).

For many decades, the manufacturing of drug products were controlled by a regulatory framework that safeguarded the quality of the final product and performed testing of batch-based operations, raw material and end-product characteristics, fixed process conditions, and in-process material (Rantanen and Khinast, 2015).

However, over the last years, there has been growing interest in increasing the safety and quality of drugs while simultaneously cutting the cost of manufacturing of pharmaceuticals by implementing manufacturing approaches (Oksanen and Muñoz, 2010). There was introduced the idea of real-time process control and real-time quality assurance in pharmaceutical manufacturing, being the basis for modern process engineering (Mascia et al., 2013; Snead and Jamison, 2015).

Science-based manufacturing of pharmaceuticals involves application of novel process, utilization of advanced data analysis techniques, process modeling and control, and molecular modeling (Ghislieri et al., 2015; Nepveux et al., 2015).

If the manufacturing processes for drug substances and drug product are robust and effective, it can be said that there was a successful technology transfer. In the pharmaceutical industry, technology transfer refers to the processes that are needed for successful progress from

drug discovery to product development (Alam and Ahmad, 2013) and means continuous information in order to maintain the product manufacturing (Feifeit, 2008).

Pilot batches may be used in the process development or optimisation stage studies. To continue developing and to optimise the manufacturing process, pilot scale batches are essential (Leuenberger, 2006). They make it possible to analyse and evaluate the difficulties and critical points of the process together with the methods most appropriate for large scale production. The production of pilot batches should assure that the product and manufacturing process are achievable on an industrial scale (Nandhakumar 2011).

The control of impurities is a critical issue in pharmaceutical industry and very strict regulations. The drugs impurities in general are classified into two types: impurities associated with the active pharmaceutical ingredients production and impurities generated during formulation and or with aging or that are related to the formulated forms (Lin, 2008). Organic impurities include unreacted starting materials, intermediates, byproducts, degradation products, reagents, ligands, and catalysts (Marchetti et al., 2014). The undesirable chemicals that remain during manufacturing, so called process-related impurities, could be generated at any of the synthetic steps in variant solvents.

Commercial-scale manufacturing of complex drug delivery systems (DDSs) using the existing technologies is challenging. An optimal drug delivery system shows an optimal therapeutic effect and a minimum of side-effects.

Unsuitable drug delivery system or the use of dosage form (drug carrier, drug delivery system, formulation, galenical vehicle, vector) that does not deliver the drug precisely, with the right quantity, in the right quality, at the right site of the body, could cause the occurrence of undesirable side-effects (Kumar et al., 2018). Using new drug delivery systems for targeting drugs could be an option that might solve these critical issues (Jahangirian et al., 2017).

Nanomaterials are nanoscale particles with sizes ranged between 1-100 nm, which could be used in drug delivery by encapsulating drugs or attaching therapeutic drugs and deliver them to target tissues more precisely with a controlled release (Patra and Baek, 2014; Rudramurthy et al., 2016). Nanoparticles exhibit unique structural, chemical, and biological properties that are obtained from materials designed at the atomic or molecular level (Mira and Siddiqui, 2014).

At all stages of clinical practices, nanoparticles have been found to be useful in acquiring information owing to their use in numerous novel assays to treat and diagnose diseases. The main benefits of these nanoparticles are associated with their surface properties; as various proteins can be affixed to the surface.

The first generation of nanoparticle-based therapy included lipid systems like liposomes and micelles, which are now FDA-approved (Haba et al., 2008).

Polymeric nanomaterials posess high biocompatibility and biodegradability properties, hence various synthetic polymers such as polyvinyl alcohol, poly-L-lactic acid, polyethylene glycol, and poly (lactic-*co*-glycolic acid), and natural polymers, such as alginate and chitosan, are extensively used in the obtaining of nanoparticles (Casettari et al., 2014).

Polymeric nanoparticles can be categorized into nanospheres and nanocapsules both of which are excellent drug delivery systems. Likewise, compact lipid nanostructures and phospholipids including liposomes and micelles are very useful in targeted drug delivery.

Recently, there have been developments in the field of delivery systems to provide therapeutic agents or natural based active compounds to its target location for treatment of various diseases (Obeid et al., 2017).

My research interest and activities in the field of pharmaceutical industrial process improvement were extensive, considering several topics, but in the following pages I will focus on my relevant results derived from the above-mentioned research theme, since their results have already been disseminated in European and international congresses and/or published in ISI/BDI journals.

The studies in this research direction were materialized in the following published papers.

- Stan CD, A. Ştefanache A, Tuchiluş C, Diaconu DE, Drăgan M, Profire L. Influence of extraction solvent on the erythromycin ethylsuccinate separation from oral suspendable powder. *Farmacia* 2011; 59(3): 396-401.
- Stan CD, Ştefanache A, Drăgan M, Corciovă AM. Development and validation of a spectrophotometric method for quantitative determination of new preservatives from pharmaceutical forms. *Rev Med Chir Soc Med Nat Iaşi* 2013; 117(4): 1014-1020.
- Stan CD, Ştefanache A, Tătărîngă G, Drăgan M, Tuchiluş CG. Microbiological evaluation and preservative efficiency of new mandelic acid derivatives in ointments. *Farmacia* 2015; 63(4): 577-580.
- Stan CD, G. Tătărîngă G, Gafiţanu C, Drăgan M, Braha S, Popescu MC, Lisă G, Ştefanache A. Preparation and characterization of niosomes containing metronidazole. *Farmacia* 2013; 61(6): 1178-1185.
- Dragostin I, Dragostin OM, Drăgan M, Stan CD, Zamfir CL. Drug Hypersensitivity Reduction Using Encapsulation Method with Chitosan-Cetirizine Derivatives. *Revista de Chimie (Bucharest)* 2018; 69(12): 3731-3735.
- Lutic D, Coromelci-Pastravanu C, Crețescu I, Poulios I, Stan CD. Photocatalytic treatment of rhodamine 6G in wastewater using photoactive ZnO. *International Journal of Photoenergy* 2012; DOI: 10.1155/2012/475131.
- Stan CD, Coromelci-Pastravanu C, Crețescu I, Drăgan M. Treatment of pesticides in wastewater by heterogeneous and homogeneous photocatalysis. *International Journal of Photoenergy* 2012; DOI: 10.1155/2012/194823.
- Samoilă P, Cojocaru C, Crețescu I, Stan CD, Nica V, Săcărescu L, Harabagiu V. Nanosized Spinel Ferrites Synthesized by Sol-Gel Autocombustion for Optimized Removal of Azo Dye from Aqueous Solution. *Journal of Nanomaterials* 2015; DOI: 10.1155/2015/713802

- Juzsakova T, Al-Jammal N, Crețescu I, Sebestyén V, Le Phuoc C, Domokos E, Rédey A, Stan CD. Case Studies for Clean Technology Development in the Chemical Industry Using Zeolite Based Catalysts. *Minerals* 2018; 8(10): 462-456.

II.2. Researchs for separation processes improving on active pharmaceutical ingredients

Erythromycin ethylsuccinate is an ester prodrug of erythromycin that is administered as oral dry suspensions or tablets. Erythromycin ethylsuccinate (EE) is produced by the S.C. "Antibiotice" S.A. as vials with oral dry suspensions. A vial contains 26 grams of powder that possesses 2.4 g of base erythromycin corresponding to 3.1 g of erythromycin ethylsuccinate, sugar (~22 g), sodium saccharin, preservatives (nipagine-nipasole ~ 1 g), flavors and sodium citrate.

In some situations, at batch quality control, the powder mixture doesn't correspond because there are some mistaken weighings, physical and microbiological contaminations etc. In these situations it`s necessary to recover EE from the powder mixture, considering the high price of the ester (~ 120 US dollars/kg).

In order to do this a solid-liquid extraction process was used, followed by the crystallization of the EE with a non-solvent.

Personal contribution to knowledge in this research field is presented in following published papers:

- Stan CD, A. Ştefanache A, Tuchiluş C, Diaconu DE, Drăgan M, Profire L. Influence of extraction solvent on the erythromycin ethylsuccinate separation from oral suspendable powder. *Farmacia* 2011; 59(3): 396-401.

II.2.1. Materials and methods

Substances, reagents and solvents

Acetone (p.a.), ethanol (p.a.), chloroform (p.a.), methanol (p.a.) and distilled water were used as solvents. An oral dry suspension of EE was used.

Erythromycin ethylsuccinate national standard (n.s.), erythromycin n.s. with an activity of 1:937 U and 2.02 % humidity, the standard solutions being achieved in methanol (p.a.). *Micrococcus luteus* ATCC 9341 was used as test micro-organism.

The solubility investigation

EE solubility in the solvents was studied according to Xth Romanian Pharmacopoeia (R.Ph.X) at 20±2^{0}C. R.Ph.X stipulates that the substance is freely soluble if 1 g of tested substance is dissolved in a quantity of solvent between 1 ml and 10 ml.

General procedure for the solid-liquid extraction process

A sample of oral dry suspension (26 g), containing the active substance and excipients, is dissolved into 60 ml of acetone, ethanol, chloroform and methanol respectively. The sugar, sodium saccharin, the flavors and sodium citrate, which are insoluble in these solvents, are filtered and the operation is repeated.

The reunited filtrates (120 ml) are evaporated on water-bath until the first crystals appear, when in the evaporating dish remains about 30 ml of concentrate. After that, 120 ml of cold distilled water, as a non-solvent, is added. Through cooling on an ice-bath EE is crystallized, then is filtered and the precipitate is washed with cold distilled water and dried in an air drift at room temperature. There were used four variants of the method (method I, II, III, IV), using different solvents.

After we established that the best solvent for solid-liquid extraction is acetone, we applied three variants of this method using different volumes of distilled water (as non-solvent) according to the concentration degree of the filtrates at their evaporation on the water-bath.

In the first variant (variant 1) we evaporate on water-bath at half of the volume and there were added 240 ml of distilled water. In the second variant (variant 2) we evaporate until the first crystals appear and there were added 120 ml of distilled water. In the third variant (variant 3) we evaporate on the water-bath up to the dry point and there were added 60 ml of distilled water (Stan et al., 2011).

Quality control for the recovered active substance

The quality control of the obtained EE was performed according to the Erythromycin ethylsuccinate monograph from X^{th} Romanian Pharmacopoeia (R.Ph. X^{th}, 1993) and 6^{th} European Pharmacopoeia (Eur.Ph. 6^{th}, 2009).

For qualitative determination we used thin-layer chromatography with silica gel G as the coating substance and a mixture of solvents as mobile phase. The EE corresponding spots of the samples must be similar in position, color and diameter to that of EE n.s. The erythromycin corresponding spots of the samples must be similar in position, color and diameter to that of erythromycin n.s. The monograph stipulates that erythromycin within the EE is no more than 5% (R.Ph. X^{th}, 1993).

The quantitative determination was achieved by microbiological assay through agar diffusion method of the EE. The microbiological activity of the EE samples were compared with the erythromycin national standard activity (1:937 U and 2.02 % humidity).

The samples correspond if the microbiological activity is at least 90.0% and no more than 110.0% of the microbiological activity of erythromycin national standard.

II.2.2. Results

The solubility investigation

The solubility determination revealed that in all tested solvents EE is freely soluble, but in acetone and chloroform is more soluble, as it can be seen in Table 14.

Table 14. The erythromycin ethylsuccinate solubility in solvents

No.	Solvent	Volume of solvent for dissolution of 1 g EE (ml)
1.	Acetone	2
2.	Ethanol	9
3.	Methanol	4
4.	Chloroform	2

Solid-liquid extraction

During the EE solid-liquid extraction from the mixture of oral dry suspension, it can be noticed that the higher quantity of the EE (2.77 g) was obtained when acetone is used as extraction solvent (Table 15). Regarding the obtained quantities of EE we can observed that the greater yield (89.35%) is when acetone is used as extraction solvent.

By dissolving the sample in these solvents the sugar remains in suspension and is filtered. In those 26 g of sample exists ~ 22 g of sugar, so we weight the obtained sugar precipitates to verify the extraction. The grater quantity of removed sugar was obtained when we used acetone as extraction solvent.

Table 15. The obtained amounts of erythromycin ethylsuccinate, removed sugar and yields using different solvents

Method and used solvent	Amounts of EE (g)				Amounts of eliminated sugar (g)				Yields (%)
	1st det.	2nd det.	3rd det.	Average of det.	1st det.	2nd det.	3rd det.	Average of det.	
I Acetone	2.88	2.62	2.81	2.77	22.1	21.9	22	22	89.35
II Ethanol	1.98	2.34	2.04	2.12	19.9	20.2	21.7	20.6	68.38
III Methanol	2.31	2.08	2.18	2.19	21.8	21.1	21.3	21.4	70.64
IV Chloroform	2.64	2.76	2.37	2.59	22	22	21.7	21.9	83.54

det. = determination

It can be noticed (Table 16) that the smallest quantity of distilled water used as non-solvent is that of variant 3 (60 ml), but the quantity of obtained EE is small (1.85 g) and the yield too (59.67%). The purification by the 2nd variant is more advantageous, since a greater quantity of EE is retrieved (2.78 g) and a greater yield is achieved (89.67%).

Table 16. The distilled water volumes used as non-solvent and the obtained amounts of EE

Variants with different volumes of water	Amounts of EE (g)				Yield (%)
	1st det.	2nd det.	3rd det.	Average of det.	
1 (240 ml)	2.43	2.27	2.59	2.43	78.38
2 (120 ml)	2.26	3.01	3.09	2.78	89.67
3 (60 ml)	0.94	2.77	1.84	1.85	59.67

det. = determination

Quality control

The qualitative determination shows that in all variants the colour, position and diameter of the erythromycin corresponding spots are no greater than the erythromycin n.s., therefore the erythromycin within the samples is no higher than 5%. The EE corresponding spots have the same colour, position and diameter as the EE n.s. spot.

The values of the Rf were calculated (Rf=6.5/11.5, Rf=0.56), therefore the samples contain EE.

From the quantitative determination (Table 17) it can be noticed that the obtained EE possess a very good activity, especially the EE obtained through the 2nd variant (926 µg/ml and 98.8%), so the samples correspond to monographs (R.Ph. Xth, 1993; Eur.Ph. 6th, 2009).

Table 17. The purified E.E. activity

Variant	EE activity (µg/ml)	EE activity (%)
Erythromycin n.s.	937	100
1	925	98.7
2	926	98.8
3	922.8	98.48

II.2.3. Discusions

Erythromycin is an orally effective antibiotic discovered from the metabolic products of a strain of *Streptomyces erythreus*, originally obtained from a soil sample collected in the Philippine archipelago.

Despite its age, erythromycin is still often prescribed, possessing a unique antibacterial activity beeing bacteriostatic by inhibiting protein synthesis, binding reversibility to the 50 S ribosomal subunits of sensitive microorganisms.

However, erythromycin can have life threatening side effects, such as QT prolongation, severe GI distress and diarrhea (Laine et al., 2000).

Erythromycin is one of the macrolide antibiotics containing a multi-constituent lactone ring to which is attached one or more deoxy sugars.

Erythromycin consists of the aglycone erythromolide A, the aminopryas, desosamine and the neutral soyas, cladinose and exists of a number of forms called A, B, C, D, E and F. Erythromycin and these related compounds differ from each other with respect to the fact that they have different substituents attached to the erythromycin molecule. These compounds possess the inherent chirality of the erythromycin molecule.

There are ten chiral centers in the erythromycin molecule, yielding a multitude of possible sterioisomers which can be separated, purified and characterized using high pressure liquid chromatography (US Patent, 2000).

Erythromycin ethylsuccinate is obtained by the reaction of erythromycin antibiotic or salt thereof and succinic acid monoethyl halide with the advantage of its relatively gastric acid stability.

Obtaining of EE presume solution crystallization, a process widely used in the production of pharmaceutical crystals. There have been reported very recently that during crystallization process of EE, under various conditions, the gelation phenomenon occure (Su et al., 2019). When the solute molecules in a solution self-assemble into a network structure that immobilizes the whole liquid phase, the gelation phenomena occurs. Gelation of active pharmaceutical substances is an unwanted process in industrial crystallization. Gelation can be avoided by controlling the supersaturation and selecting appropriate solvents.

Other researchers have investigated new solvent systems for the extraction of erythromycin in which octanol was used as the extractant instead of butyl acetate (Li et al., 2005), or liquid anionic-zwitterionic surfactant-based aqueous two-phase extraction (Mohamad-Aziz and Mimi-Sakinah, 2017) where the zwitterion concentration governed the erythromycin solubilization.

Other authors researched the recovery of erythromycin from aqueous solutions in single-stage and multi-stage extraction of erythromycin from an aqueous solution with a hydrophobic ionic liquid, 1-butyl-1-methylpyrrolidinium bis(trifluoromethylsulfonyl)imide as a function of pH, and high-pressure CO(2) extraction of erythromycin from the ionic liquid, as a function of pressure, temperature and presence of water (Manic et al., 2011).

But, there are very few papers in which EE separation methods are investigated (Kamarei et al., 2011), hence our study is a necessary one, especially for our local pharmaceutical industry.

Regarding the solubility determination the test revealed that even EE is freely soluble in organic solvents, in acetone and chloroform is more soluble than ethanol and methanol.

For the solid-liquid extraction, it can be noticed that the higher quantity of the EE was obtained when acetone is used as extraction solvent. Smaller quantities were obtained when chloroform, respectively methanol was used and the smallest quantities were obtained when ethanol was used. Regarding the obtained quantities of EE we can observed that the greater yield (89.35%) was achieved when acetone is used as extraction solvent. Also, the grater quantity of removed sugar was obtained when we used acetone as extraction solvent.

The mechanism for these new extraction systems is probable due the formation of a complex formed between the molecules of EE and extractant by hydrogen bonding, and the formed neutral extraction complex moves into the organic phase.

When molecular solvent such as acetone is used, the partition coefficient of EE between organic and aqueous phases is higher and consequently extraction yield is 89.35%.

Experimental results show that the new extraction systems offer technological and economic advantages owing to the low solubility of the extraction solvents in the aqueous phase. The solvent consumption of the new extraction process was lower, thus reducing energy consumption.

The purification variant, in which water was used in 120 ml ammounts (the 2nd variant) is more advantageous, since a greater quantity of EE is retrieved (2.78 g) and a greater yield is achieved (89.67%). This amount is necessary in order to recover all the EE quantities, so that active substance loss is as low as possible. This is a great advantage for the industry, because the waste water management is done with a reduce consumtio of energy and time, in order to eliminate the traces of EE, thus the procedure is an economic one.

The recovered and separated organic solutions contain EE, in accordance with the qualitative determination (Rf =0.56) and quantitative one (926 μg/ml and 98.8% activity), so that the samples correspond to Eur.Ph. 6th 2009 monography.

II.2.4. Conclusions

The separation of EE from a mixture of oral dry suspension for pediatric use can be performed by a solid-liquid extraction with acetone as extracting solvent and the EE crystallization can be performed by using water as non-solvent. The separation is achieved by evaporating the acetone concentrate until the first crystals appears, using 120 ml of water as non-solvent (1:4).

Working in such manner greater amounts of EE were obtained (2.78 g) and a greater yield is achieved (89.97%). The obtained EE possess a great purity and its microbiological activity is a very good one (98.8%).

The quantities of acetone and water used, as solvent and non-solvent respectively, to separate EE were the smallest one`s, therefore the procedure is an economic one.

Also, acetone has a lower boiling point and so its recovery is cheaper, which is an important factor from the industrial point of view.

II.3. Microbiological and analytical investigation of new auxiliary substances in pharmaceutical forms

All pharmaceutical forms contain besides the active ingredient a number of auxiliary components. The composition of auxiliary components also significantly influences the technological process conditions, structural and mechanical characteristics of drugs, their consumer properties, and cost. Depending on the physicochemical properties of active components, features of their synthesis, doses, and other factors, the auxiliary substances have to perform various functions: diluting media, preservatives, disintegrants, binders, wetting agents, lubricating agents, taste correctors, drug release dynamics regulators, coloring pigments etc (Voskoboinikova et al., 2015).

A preservative is a natural/synthetic compound that is added to pharmaceuticals to prolong their shelf life. The addition of preservatives to pharmaceutical forms, especially to those that have higher water content, is essential for avoiding alteration and degradation by microorganisms during storage (Shaikh et al., 2016).

Commonly used preservatives have potential toxicities and impact on the health caused by their repeated use: hypersensitivity, allergy, asthma, hyperactivity, neurological damage and cancer (Anand and Sati, 2017). Antimicrobial agent are such preservatives, which active against Gram positive and Gram negative bacteria, yeast and molds by cell wall inhibition, protein synthesis inhibition, DNA/RNA synthesis inhibition.

Preservatives remain necessary to prevent microbial contamination of multi-use liquid or semi-solid medicinal products, particularly from opportunistic pathogens, otherwise can result in serious patient health consequences. The optimal conditions for preservative efficacy (pH, physical and chemical stability) are rarely the same as for the product itself and as such compromises are often necessary to ensure an optimal product shelf-life (Elder and Crowley, 2012).

It is also a regulatory requirement to assess the antimicrobial efficacy of the drug product (in its final container) at the end of the product's proposed shelf-life. Activity needs to be broad spectrum, encompassing bacteria (Gram-positive and Gram-negative), yeasts, fungi and molds. An effective preservative must reduce a microbial population significantly and prevent subsequent re-growth and these effects must be both microcidal and microstatic in nature.

Ensuring the quality of pharmaceutical forms is one of the important aspects in pharmaceutical industry. All the medicines must accomplish the main criteria i.e. efficacy, quality and safety. Final product quality control is a defining step for achieving these criteria.

One of the quality parameters to be analyzed is the quantification of the auxiliary substances present in the final product, besides the quantification of the main ingredient, i.e. the active substrate, of course.

Finding the right, less expensive, reproductibile method is a real challenge for all the pharmacists that are working in research/pharmaceutical industry domain in quality lab control, or in pilot plant.

On the other hand, mandelic acid is known as an antiseptic, beeing active against some pathogens (*Staphylococcus aureus*, *E. coli*, *Proteus* sp., *Aerobacter* sp. etc.) and used in urinary infections (oral use), or dermic infections (inflammatory acne), for external use.

It`s two new derivatives, oxi-acetyl mandelic acid and oxi-propionyl mandelic acid, presents high antimicrobial activity and hence could be used as preservetives in pharmaceutical forms for external use.

Thus, we conducted a research in which we investigate the antimicrobial potential of these preservatives, there efficiency in ointments and also we developed and validate a method to determine the preservatives in these pharmaceutical forms (Stan et al., 2013; Stan et al., 2015).

II.3.1. Microbiological efficiency of new preservatives in pharmaceutical forms

The solutions, the gels, the ointments and other pharmaceutical forms can be invaded by microorganisms that determine changes in their organoleptic or physico-chemical properties (Oday and Lupuliasa, 2011). Thus, the use of the antimicrobial preservatives is required in order to impede the development of the microorganisms in the pharmaceutical forms.

Preservatives are most important substances in fighting microbial contaminations of pharmaceutical forms. However, over the past few decades, many preservatives have become less effective against certain microorganisms due to emergence of drug-resistant bacteria.

It is essential to investigate newer substances with preservative action with less resistance. Recent trends show that the discovery rate of active new molecular entities is declining (Șerban et al., 2012).

Mandelic acid is active against some bacteria (*Staphilococcus aureus, Proteus* spp., *E. coli, Aerobacter aerogenes* etc.) over a range of 350-500 mg/L (Motamedifar et al., 2014). It is a nontoxic substance which has a long history of use as an antibacterial in the treatment of urinary tract infections.

Research in this area has focused on:

- to establish the antibacterian and antifungal activity of oxi-acetyl mandelic acid and oxi-propionyl mandelic;
- to point out the minimum inhibitory concentrations (MIC);
- to highlight the preservative efficiency of these two new mandelic acid derivatives in ointments.

Personal contribution to knowledge in this research field is presented in following published papers:

- Stan CD, A. Ştefanache A, Tuchiluş C, Diaconu DE, Drăgan M, Profire L. Influence of extraction solvent on the erythromycin ethylsuccinate separation from oral suspendable powder. *Farmacia* 2011; 59(3): 396-401.

II.3.1.1. Materials and methods

Microorganisms

The antimicrobial activity was studied using Gram positive bacteria (*Staphylococcus aureus* ATCC 25923, *Sarcina lutea* ATCC 9341), Gram negative bacteria (*Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and pathogenic yeasts (*Candida albicans* ATCC 90028, *Candida glabrata* ATCC MYA 2950, *Candida parapsilosis* ATCC 22019.

Antimicrobial activity

Antimicrobial activity was evaluated by agar disc diffusion method (CLSI, 2014) with an inoculum of overnight culture, in a final concentration of about 10^5UFC/mL. Commercial available discs containing Ampicillin (25 µg/disc), Chloramphenicol (30 µg/disc) and Nystatin (100 µg /disc) were also placed on the agar surface. After incubation the diameters of inhibition zones were read in triplicate and the average was taken as final reading.

Broth microdilution method

The antimicrobial activity of the tested mandelic acid esters was quantitative determined by the broth microdilution method (CLSI, 2014). Stock solutions of the test compounds were prepared over a range of concentrations from 2000 to 1 µg/mL. The microorganisms used in this assay were *Staphylococcus aureus* ATCC 25923 and *Candida albicans* ATCC 90028. The minimum inhibitory concentrations (MIC) (µg/mL) were recorded after 24 h of incubation at 37^0C for *Staphylococcus aureus* or at 24°C for *Candida albicans* as the concentration of the compound which inhibited the visible growth of the tested microorganism.

The efficiency of preservative action in ointments

The preservative properties were determined according to European Pharmacopoeia standards (Eur.Ph., 2013).

The ointment samples were prepared according to the following formulas:

-Rp1/ cetyl alcohol 5 g, sunflower seed oil 20 g, lanoline 5 g, vaseline 20 g, oxi acetyl-mandelic acid 0.0125 g, distilled water q.s. ad 100 g;

-Rp2/ cetyl alcohol 5 g, sunflower seed oil 20 g, lanoline 5 g, vaseline 20 g, oxi-propionyl mandelic acid 0.05 g, distilled water q.s. ad 100 g;

-Rp3/ cetyl alcohol 5 g, sunflower seed oil 20 g, lanoline 5 g, vaseline 20 g, distilled water q.s. ad 100 g.

The samples were contaminated with the test microorganims *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 27853, *Candida albicans* ATCC 90028.

The ointment samples were tested over a period of 28 days in the presence and in the absence of the test microorganisms. There were gather samples at different time intervals after the inoculation (0 h, 6 h, 48 h, 7 days, 14 days, 28 days) and the viable microorganisms were counted by plate count.

II.3.1.2. Results

Antimicrobial activity

Antibacterial and antifungal potential of mandelic acid esters were assessed in terms of zone of inhibition of microbial growth (Figure 16 and Figure 17). Statistical analysis of the results included the calculation of standard deviation (Table 18 and Table 19).

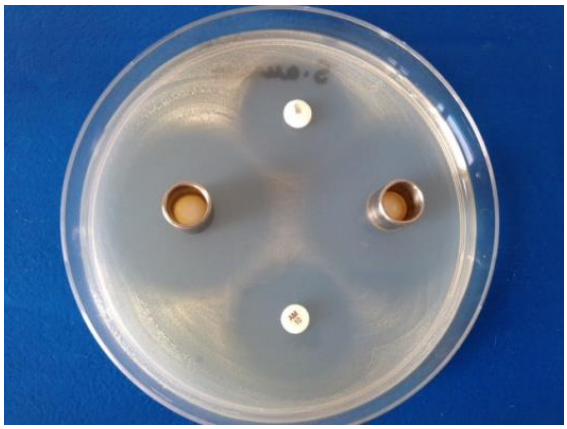

Figure 16. Antibacterial effects of oxi-acetyl mandelic acid and oxi-propionyl mandelic acid against *Staphylococcus aureus* ATCC 25923

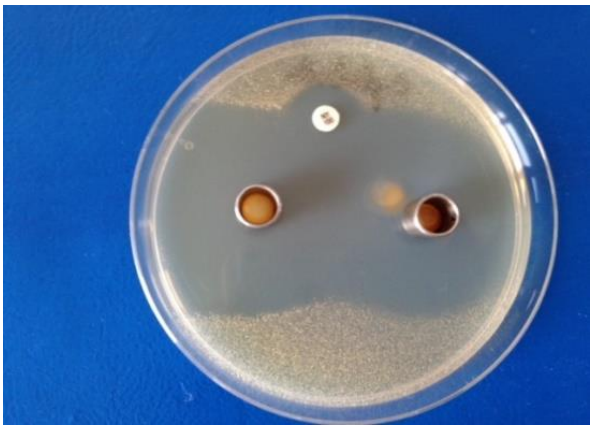

Figure 17. Antifungal effects of oxi-acetyl mandelic acid and oxi-propionyl mandelic acid against *Candida glabrata* ATCC MYA 2590

The results show that these two mandelic acid esters were found to be effective against the tested microorganisms and exert a good antibacterial and antifungal activity at lower concentrations.

Oxy-acetyl mandelic acid showed a better antimicrobial activity than oxi-propionyl mandelic acid.

The data obtained in the quantitative antimicrobial activity are presented in Table 20.

Table 18. Antibacterial effects of mandelic acid derivatives

Compounds/antibiotics	**Diameter of inhibition zone (mm) ± Standard Deviation (SD)**			
	***S. aureus* ATCC 25923**	***S. lutea* ATCC 9341**	***E. coli* ATCC 25922**	***Pseudomonas aeruginosa* ATCC 27853**
Oxi-acetyl mandelic acid	36.06±0.05	37.03±0.15	33.96±0.05	30.06±0.05
Oxi-propionyl mandelic acid	37.1±0.10	37.03±0.15	29.03±0.05	25.1±0.10
Ampicillin (25 µg/disc)	25.1±0.10	25.1±0.10	15.06±0.05	0
Chloramphenicol (30 µg/disc)	25.1±0.10	25.1±0.10	32.13±0.11	21.06±0.11

Table 19. Antifungal effects of mandelic acid derivatives

Compounds/ antibiotics	Diameter of inhibition zone (mm) ± Standard Deviation (SD)		
	Candida albicans ATCC 90028	*Candida glabrata* ATCC MYA 2590	*Candida parapsilosis* ATCC 22019
Oxi-acetyl mandelic acid	50.00±0.00	48.03±0.05	48.93±0.05
Oxi-propionyl mandelic acid	48.03±0.05	47.03±0.05	46.97±0.05
Nystatin (100 µg/disc)	17.90±0.10	19.00±0.00	20.1±0.10

Table 20. MIC values (µg/mL) of tested derivatives

Sample	Microorganism test/ MIC values (µg/mL)	
	S. aureus ATCC 25923	*C. albicans* ATCC 90028
Oxi-acetyl mandelic acid	125	150
Oxi-propionyl mandelic acid	500	500

The efficiency of preservative action in ointments

The preservative properties are considered adequate if, in the condition of the test, there is a marked reduction or no increase in the number of microorganisms in the inoculated preparation after the test period. The criteria for the evaluation of antimicrobial activity are expressed in terms of the logarithmic reduction in the number of viable microorganisms against the value obtained from the inoculum.

The efficiency of the preservative system is considered achieved if the number of viable microorganisms registers a reduction of no more than 0.1% of the initial inoculum (10^5-10^6 UFC/ml) and the number of the viable fungus colonies remains the same or diminishes in the first 4 days of the testing period (Parker, 1992).

The obtained results (Figure 18) demonstrate the antimicrobial and antifungal activity of those two tested preservatives in ointments.

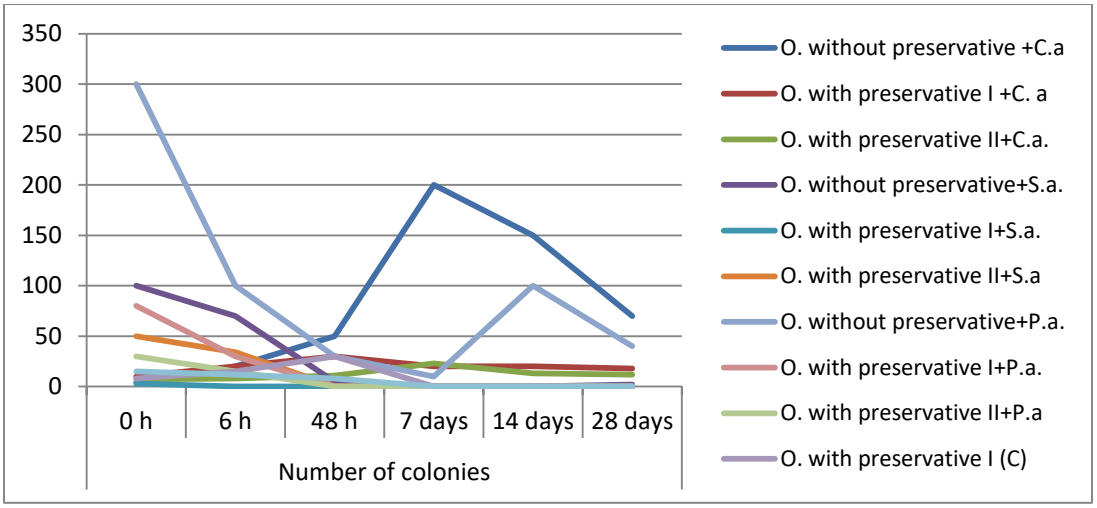

Figure 18. The preservative action evaluation of some mandelic acid esters in ointments

Legend: O=ointment; preservative I=oxi-acetyl mandelic acid; preservative II =oxi-propionyl mandelic acid; C.a.=*Candida albicans*; S.a =*Staphylococcus aureus*; P.a= *Pseudomonas aeruginosa*; C=control

II.3.1.3. Discussions

Microbial contamination and spoilage cost pharmaceutical companies huge financial loss annually through equipment malfunction, production stoppage, drug contamination, investigations and loss of energy. The target of most reputable pharmaceutical firms today is centered on determining the different sources of contamination (Eissa et al., 2014; Eissa, 2016).

The quality of product is assessed through testing and monitoring of the manufacturing activities, packaged, stored and tested as well as through sampling and analysis of the finished dosage forms (Clontz, 2008).

Most gram-positive bacteria, such as *Enterococcus* spp. *Staphylococcus aureus*, or *Streptococcus pyogenes*, survive for months on dry surfaces. Many gram-negative species, such as *Acinetobacter* spp., *Escherichia coli*, *Klebsiella* spp., *Pseudomonas aeruginosa*, *Serratia marcescens*, *Shigella* spp., can also survive for months (Kramer et al., 2006).

Ointments, creams or lotions in addition to being free from gross microbial contamination and pathogens at manufacture, should be capable of maintaining low contamination levels during use. The use of water and raw materials of suitable quality and good manufacturing practices should generally lead to the production of preparations with low microbial contamination.

Adequate preservation and the use of noninvasive increase the chances that contamination levels will remain low during storage and use of the product (Okeke and Lamikanra, 2001).

Chemical preservatives for semisolids must be carefully evaluated for their stability with regard to the other components of the formulation. No interaction between the preservative with active substances and other components of the vehicle may occure.

Preservative action appears to depend on the concentration of the free preservative in the aqueous phase.

The complexity of multi-phase products, formulated as ointments, creams or lotions mean that adequate microcidal efficacy may not be attainable. The best that can be achieved is a microstatic effect. Most preservative systems can adequately kill or attenuate growth of most organisms. Current GMP standards ensure that high standards of microbial cleanliness can be routinely achieved in finished products. Hence, the risk of contamination is probably greatest during patient use of multi-dose products (Elder and Crowley, 2012).

In general, microbial growth is optimal between pH 6-8. Outside this range, growth rate significantly declines. The product pH may reflect the intrinsic pH of the active pharmaceutical ingredient, or the specific excipients pH. Thus, pH adjustment to regions less favorable to microbial viability i.e. away from pH 6-8 must take account of competing effects on overall product quality versus the activity of the preservative system (Denyer and Hodges, 2006).

Such pH effects reflect the chemical composition of the active moiety in the preservative molecule. For instance, if activity is associated with the non-ionized moiety (acids, alcohols and phenols) the effect is usually optimal at acidic pH.

Esterification of acids can extend the pH span of activity. Efficacy could decreases at higher pH and increases with the longer alkyl chain (Johnson and Steer, 2006).

Our results show that these two mandelic acid esters were found to be effective against the tested microorganisms and exert a good antibacterial and antifungal activity at lower concentrations. Oxy-acetyl mandelic acid showed a better antimicrobial activity than oxi-propionyl mandelic acid.

On the other hand, the investigation of the efficiency of preservative action in ointments, the obtained results showed that the bacteria were killed two days after inoculation. The activity against *Candida* strains was lower, although the two preservatives were able to reduce the viable number of fungus colonies.

In the case of the samples which didn`t contain preservatives but were contaminated with the same test microorganism there was noticed the presence of the microorganisms in a variable number. Thus in the case of the samples without preservatives there was present a growth of the number of microorganisms, the microorganisms contaminating the ointment samples. The control samples which contained these preservatives but were not contaminated with test microorganisms did not present contaminants 14 days after the inoculation.

There wasn`t registered any bacterial growth of *Staphylococcus aureus, Pseudomonas aeruginosa* or other contaminants during the entire test period.

II.3.1.4. Conclusions

Two mandelic acid derivatives have shown a very effective antimicrobial action.

The two compounds, the oxi-acetyl mandelic acid and the oxi-propionyl mandelic acid, totally inhibited the Gram positive and Gram negative bacteria and diminished the number of fungal colonies two days after the inoculation of the ointment samples.

The oxi-acetyl mandelic acid and the oxi-propionyl mandelic acid proved to have a very good preservative action and could be used as antimicrobial preservatives in 0.0125 g% and respectively 0.05 g% concentrations in order to prevent proliferation or to limit microbial contamination of ointments.

II.3.2. Development and validation of spectrophotometric method for the determination of new preservatives in pharmaceutical forms

UV-VIS spectrophotometric methods are rapid techniques of analysis with large applications in the identification and quantification of compounds from pharmaceutical forms.

Spectrophotometric methods are one of the classic methods of analysis based on radiation absorbtion, beeing fast, robust, precise and universally accepted in pharmaceutical analysis.

Spectrophotometric methods present some advantages which have found their usefulness in the analysis of auxiliary substances from pharmaceutical forms: the determination is fast, small quantities are required, present a high sensitivity and offer a high degree of accuracy of the measurement results (Boyoung et al., 2016).

The development and validation of any spectroscopic methods is a complex process being an important aim in quality control for pharmaceutical industry. There are international regulatory agencies (The International Conference on Harmonization) which provide the validation parameters of a method: accuracy, precision, limit of detection, limit of quantification (ICH, 2005).

Research in this area has focused on:

- developing of a fast and sensitive spectrophotometric UV-VIS method for oxi-acetyl mandelic acid and oxi-propionyl mandelic acid determination;
- spectrophotometric method validation according to validation quides and applied to oxi-acetyl mandelic acid and oxi-propionyl mandelic acid determination (Stan et al. 2013).

Personal contribution to knowledge in this research field is presented in following published papers:

- Stan CD, Ştefanache A, Drăgan M, Corciovă AM. Development and validation of a spectrophotometric method for quantitative determination of new preservatives from pharmaceutical forms. *Rev Med Chir Soc Med Nat Iaşi* 2013; 117(4): 1014-1020.

II.3.2.1. Materials and methods

Apparatus

There were used a Jasco V530 spectrophotometer, quartz cells (1=1 cm) and IKA-Werke basic ultrasonic bath.

Materials and reagents

Oxi-acetyl mandelic acid, oxi-propionyl mandelic acid (obtained in lab), absolute ethanol pa (Merck KGaA), cetyl alcohol, lanolin, vaselin, sunflower seed oil (Redox).

The absorption spectras were recorded between 200–400 nm, and the absorbances were measured at 256 nm for oxi-acetyl mandelic acid and 258 nm for oxi-propionyl mandelic acid in a 1 cm cell versus a blank of absolute ethanol.

The developed method was validated by studying the following parameters: linearity, detection limit, quantification limit, precission, in accordance with the bioanalytical method validation guidelines.

The developed and validated method was applied to determine these two preservatives from pharmaceutical forms (ointments). According to MCI`s, the preservatives were embedded in ointments in 0.0125 g% concentration for oxi-acetyl mandelic acid and in 0.05 g% concentration for oxi-propionyl mandelic acid.

These two preservatives were extracted from ointments with gently heated absolute ethanol and after filtration the samples were spectrophotometric analyzed at 256 nm for oxi-acetyl mandelic acid and at 258 nm for oxi-propionyl mandelic acid, respectively.

The ointments were prepared by the formula:

Rp/		Rp/	
Cetyl alcohol	5 g	Cetyl alcohol	5 g
Sunflower seed oil	20 g	Sunflower seed oil	20 g
Lanolin	5 g	Lanolin	5 g
Vaseline	20 g	Vaseline	20 g
Oxi-acetyl mandelic acid	0.0125 g	Oxi-propionyl mandelic acid	0.05 g
Distilled water q.s. ad	100 g	Distilled water q.s. ad	100 g

II.3.2.2. Results

The method was validated by studying the following parameters: linearity, detection limit, quantification limit, precision.

Linearity

There were prepared stock solutions of oxi-acetyl mandelic acid, respectively oxi-propionyl mandelic acid, from which were obtained the working solutions in a concentration range of 0.1–1.5 mg/ml (for oxi-acetyl mandelic acid) and 0.1–1 mg/ml (for oxi-propionyl mandelic acid). For each concentration, three determinations were performed and the average value of absorbances was calculated for each range (Table 21).

The average values of absorbance are graphically represented according to concentrations (Figure 219 and Figure 20).

Statistical analysis of experimental data leads to regression line equations, presented in Table 22.

There were calculated the standard error of regression curve (SE), the Person correlation coefficient (r^2), the detection limit (LOD) and the quantification limit (LOQ). In Table 23 are prezented the statistical data.

In conformity with the obtained data, the developed method was linear in the range of the studied concentration.

Table 21. Absorbance values for establish the linearity of the method

No.	Oxi-acetyl mandelic acid mg/ml	Absorbance (λ=256 nm)			Average
		I	II	III	
1	0.1	0.0375	0.0322	0.0437	0.0378
2	0.3	0.1675	0.1632	0.1598	0.1635
3	0.5	0.3331	0.3129	0.3290	0.3235
4	0.7	0.4589	0.4600	0.4656	0.4615
5	0.9	0.5999	0.6231	0.6202	0.6144
6	1.1	0.7325	0.7833	0.8179	0.7779
7	1.3	0.9128	0.9323	0.9311	0.9254
8	1.5	1.1109	0.9834	1.1991	1.0978
No.	**Oxi-propionyl mandelic acid mg/ml**	**Absorbance (λ =258 nm)**			**Average**
		I	**II**	**III**	
1	0.1	0.1222	0.0982	0.1101	0.1102
2	0.3	0.2835	0.301	0.2989	0.2944
3	0.4	0.3899	0.3943	0.4101	0.3981
4	0.5	0.4952	0.4888	0.4903	0.4914
5	0.6	0.5921	0.5899	0.5932	0.5917
6	0.7	0.6854	0.7128	0.7001	0.6994
7	0.8	0.8201	0.7967	0.8024	0.8064
8	1	1.0098	1.0156	1.0167	1.0140

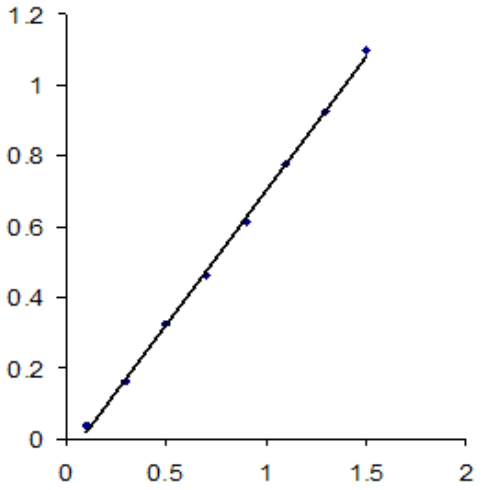

Figure 19. Regression line for oxi-acetyl mandelic acid determination

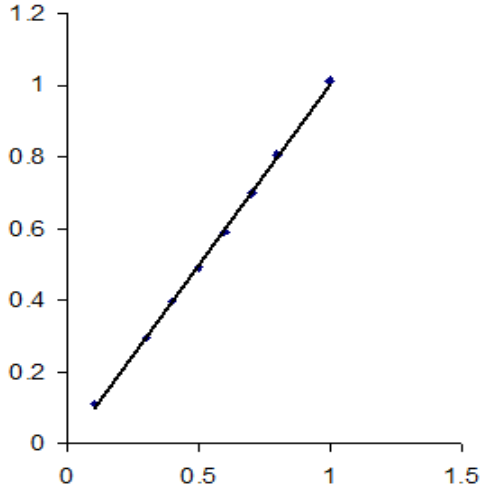

Figure 20. Regression line for oxi-propionyl mandelic acid determination

Table 22. Regression line equations for the two preservatives determination

Substance	Regression line equations
Oxi-acetyl mandelic acid	Absorbance = 0.75867x - 0.05671
Oxi-propionyl mandelic acid	Absorbance = 1.00851x - 0.00396

Table 23. Statistical data regarding the two preservatives determination

	Oxi-acetyl mandelic acid	Oxi-propionyl mandelic acid
Person coefficient (r^2)	0.99897	0.99920
Standard error	0.012876	0.008863
Intercept	- 0.05671	- 0.00396
Slope	0.75867	1.00851
Detection limit (LOD) mg/ml	0.05	0.03
Quantification limit (LOQ) mg/ml	0.17	0.08

Precision of the system and method

Precision of the system was studied for the 1.3 mg/ml concentration of oxi-acetyl mandelic acid and 0.6 mg/ml concentration of oxi-propionyl mandelic acid, for 10 assays performed in the same conditions. The results and the statistical analysis for standard deviation (SD) and relative standard deviation (RSD %) are presented in Table 24.

Table 24. System precision

	Oxi-acetyl mandelic acid	Oxi-propionyl mandelic acid
Determ.	**Absorbance**	**Absorbance**
1	0.9234	0.5917
2	0.9231	0.5998
3	0.9111	0.6001
4	0.9323	0.6013
5	0.9101	0.5928
6	0.9321	0.6032
7	0.9278	0.5925
8	0.9109	0.5922
9	0.9215	0.5912
10	0.9311	0.5922
Average	**0.92234**	**0.5957**
Standard Deviation (SD)	**0.008885**	**0.004751**
Relative standard deviation (RSD) %	**0.96328**	**0.797612**

The precision of the method was investigated in a range of 20% compared to interest concentration, grouped on a number of 3 assays in 3 concentrations levels. Using the regression line equation the concentrations and recovery were calculated (Table 25).

The analysis of the two preservatives from ointments

The presence of the oxi-acetyl mandelic acid and oxi-propionyl mandelic acid was carried out by comparing the spectras obtained after extraction with the standards ones. The obtained data, after the two preservatives quantification from the ointments were compared with the limits recommended by Xth Romanian Pharmacopoeia (Table 26).

The experimental data shows us that the concentrations of oxi-acetyl mandelic acid and oxi-propionyl mandelic acid from the studied ointments are in the recommended ranges for ointments in Xth Romanian Pharmacopoeia.

Table 25. The calculated concentrations and recovery for the two preservatives

Oxi-acetyl mandelic acid				**Oxi-propionyl mandelic acid**			
Theoretic conc. mg/ml	**Absorbance**	**Calculated conc.**	**Recovery (%)**	**Theoretic conc. mg/ml**	**Absorbance**	**Calculated conc.**	**Recovery (%)**
1.1	0.7729	1.09	99.09	0.4	0.3998	0.4003	100.07
	0.7901	1.11	100.90		0.4049	0.4054	101.35
	0.7821	1.10	100,00		0.4001	0.4006	100.15
1.3	0.9312	1,30	100.00	0.6	0.5965	0.5953	99.23
	0.9265	1.29	99.23		0.6015	0.6003	100.05
	0.9335	1.31	100.76		0.5996	0.5984	99.74
1.5	1.0801	1.49	99.33	0.8	0.8024	0.7995	99.94
	1.0930	1.51	100.66		0.7998	0.7969	99.62
	1.0850	1.50	100.00		0.8112	0.8082	101.35
Statistical data		**Average**	**99.99**	**Statistical data**		**Average**	**100.16**
		SD	**0.72634**			**SD**	**0.727444**
		RSD %	**0.726367**			**RSD %**	**0.726234**

Table 26. The calculated concentrations and recovery for the two preservatives determination from ointments

Det. no.		**Theoretical conc. ± admissible deviation FRX**	**Calculated average conc. ± standard deviation**	**Recovery %**
1.	Oxi-acetyl mandelic acid	0.0125 ± 7.5%	0.01251 ± 0.0001	100.08
2.			0.01249 ± 0.0001	99.92
3.			0.01232 ± 0.0001	98.56
1.	Oxi-propionyl mandelic acid	0.05 ± 7.5%	0.0508 ± 0.0501	101.60
2.			0.0497 ± 0.0501	99.40
3.			0.0498 ± 0.0501	99.60

II.3.2.3. Discussions

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

Regarding that, spectrofotometric procedures are simple, acurate, precise and cost-effective, they are used as a routine analysis in quality control laboratories for pharamaceutical industry.

The validation of analitical methods is required to obtain high-quality data. For the pharmaceutical industry, method validation is crucial to ensure the product quality as regards therapeutic efficacy and patient safety.

Single preservative, but more often combinations of preservatives, are commonly used in pharmaceuticals or cosmetics products to prevent alteration and degradation of the product formulations. Hence the simultaneous determination of these preservatives in pharmaceutical products is particularly important for quality assurance (Fahelelbom and El-Shabrawy, 2017).

Analytical methodologies developed for the quantification of preservatives in these pharmaceutical forms are usually designed to overcome the problems associated with interferences which are originated from other constituents (Alvarez-Rivera et al., 2018).

Several assay methods have been reported for the analysis of acids or esters preservatives in pharmaceutical products (Miralles et al., 2018).

There were been reported capillary electrophoresis methods like micellar electrokinetic chromatography and microemulsion electrokinetic chromatography to determine these preservatives in pharmaceutical products as alternatives to liquid chromatography methods (Huang et al., 2013; Wu et al., 2014; Lopez-Gazpio et al., 2014).

With regard to the analytical techniques for the determination of preservatives, reversed-phase liquid chromatography coupled with ultraviolet-visible spectrophotometry (UV/Vis) (Miralles et al., 2016; Miralles et al., 2018; Chen et al., 2018) is used. Also, high performance liquid chromatography with tandem mass spectrometry detectors is commonly used (Alvarez-Rivera et al., 2012; Wittenberg et al., 2015).

Gas chromatography (Palmer et al., 2017; Rajabi et al., 2017) and capillary electrophoresis (Ye et al., 2013; Xue et al., 2013) methods are also frequent alternatives to determine preservatives in pharmaceutical products.

Among the different types of preservatives studied in the analytical literature, parabens are by far the most frequently analyzed, whereas the number of published papers dealing with the analysis of other preservatives is more limited (Alvarez-Rivera et al., 2018).

The aim of this work was to the develop a fast and sensitive spectrophotometric UV-VIS method for oxi-acetyl mandelic acid and oxi-propionyl mandelic acid determination. The spectrophotometric method was validated according to validation quides and applied to oxi-acetyl mandelic acid and oxi-propionyl mandelic acid determination from pharmaceutical forms (ointments).

The obtained data sustained that the proposed method for oxi-acetyl mandelic acid and oxi-propionyl mandelic acid determination comply with all the validation parameters and also with the limits stipulated by the regulations (Stan et al. 2013).

In conformity with the obtained data, the developed method was linear in the range of the studied concentration (0.1–1.5 mg/ml for oxi-acetyl mandelic acid and 0.1–1 mg/ml for oxi-propionyl mandelic acid).

Processing data lead to a relative standard deviation RSD=0.96328% for oxi-acetyl mandelic acid and RSD=0.797612% for oxi-propionyl mandelic acid, compared to the maximum of 2% proposed by the European standards, so the systems are considered to be precise.

The experimental data shows us that the concentrations of oxi-acetyl mandelic acid and oxi-propionyl mandelic acid from the studied ointments are in the recommended ranges for ointments in X^{th} Romanian Pharmacopoeia.

From statistical analysis we obtained a relative standard deviation RSD=0.726367% for oxi-acetyl mandelic acid and RSD=0.726234% for oxi-propionyl mandelic acid, compared to the maximum of 5% proposed by the European standards, so the proposed method is precise.

All analytical reagents used are inexpensive, quite stable, and widely available in laboratories as well in pharmaceuticsl industry. Complex procedures are not required and the method is sutable for routine analysis in the pfase of quality control that safeguarded the quality of the final product.

In conformity with the obtained data, the developed method was linear in the range of the studied concentration, was precise and could be applied to determine these preservatives in ointments.

The method allows the quantification of the target compound in ointments with good analytical features, such as accuracy, precision and sensitivity. Moreover, the method is simple, fast, and both user and environment friendly, based on the principles of the so-called Green Analytical Chemistry.

For that reason, the proposed method is useful to perform the analytical control of ointments preserved with these two preservatives.

II.3.2.4. Conclusions

A simple, fast and sensitive UV-VIS spectrophotometric method was developed and validated for the assay of two new preservatives (oxi-acetyl mandelic acid and oxi-propionyl mandelic acid).

The validation was performed according to validation quides.

The developed and validated spectrophotometric method was applied to determine these two preservatives from pharmaceutical forms (ointments). The mean recovery in the ointments of the two preservatives was in the range of 98.56–101.60%.

II.4. Design and evaluation of drug delivery systems for targeting drugs

The search for new formulations is very expensive and the appearance of new pharmaceutical products on the market is a slow process, delayed by the severe standards of regulatory agencies.

To overcome these shortcomings the pharmaceutical industry is focused now to optimize medicines i.e. obtaining drug delivery systems to release new pharmaceutical forms on the market.

An optimal drug delivery system shows an optimal therapeutic effect and a minimum of side-effects. Using new drug delivery systems for targeting drugs could be an option that might solve these critical issues (Jahangirian et al., 2017).

For an optimal drug action, drugs molecules could be transported by a carrier to the site of action and released in order to achieve action.

Liposomes are used in the pharmaceutical industry for the transportation of diverse molecules and are among the most studied carrier system for drug delivery. They are vesicles of spherical form composed of phospholipids and steroids usually in the 50–450 nm size range (Bozzuto and Molinari, 2015).

These are considered as a better drug delivery vehicles since their membrane structure is analogous to the cell membranes and because they facilitate incorporation of drugs in them. It has also been proved that they make therapeutic compounds stable, improve their biodistribution, can be used with hydrophilic and hydrophobic drugs and are also biocompatible and biodegradable (Sercombe et al., 2015).

Niosomes (non-ionic surfactant vesicles) are vesicles systems similar to liposomes that can be used as carriers of amphiphilic and lipophilic drugs (Allen, 1998).

The chemical stability as well as the relative low cost of the materials used to prepare niosomes made these vesicles more attractive than liposomes for the industrial production, both in pharmaceutical and cosmetic applications.

The ideal material for encapsulation should have good rheological properties, good dispersing capacity of active substances, lack of chemical reactivity with encapsulated substances, the ability to complete release the active compounds, under different conditions, solubility in non-toxic solvents.

Microparticulate drug delivery systems are an interesting and promising option when developing a controlled release system.

Microparticles offer various significant advantages as drug delivery systems: an easy administration, control on the release rate of the incorporated drug (hours to months), protection of the encapsulated active agent against degradation (Singh et al., 2010).

Microencapsulation of active substances is of major interest in the controlled release range, due to the relatively light microparticle design and reproducibility of the process (Dumitriu et al., 2015; Dumitriu et al., 2016).

We obtaine niosomes with metronidazole, because targeted delivery of metronidazole via niosomes can increase efficiency of the drug in topical treatment.

Also, we obtaine new chitosan derivatives, starting from cetirizine which is well known for its antihistaminic properties, followed by the use of these new derivatives for encapsulation of drugs with allergic potential. The obtained particles lead to the reducing of the drug hypersensitivity, keeping, at the same time, the followed therapeutic effect.

II.4.1. Design, preparation and characterization of niosomes containing antibiotics

Niosomes are microscopic lamellar structures formed on a mixture of non-ionic surfactant of the alkyl or dialkyl polyglycerol ether class and cholesterol with subsequent hydration in aqueous media (Dinu Pîrvu et al., 2010). These vesicles appear to be similar in terms of the physical properties to liposomes, being prepared in the same way, but are characterized by a higher chemical stability with respect to liposomes due to the difference between surfactants and phospholipids.

Starting from these studies my research in this area eas focused on designing, preparation and characterization of niosomes with metronidazole taking into account that targeted delivery of metronidazole via niosomes can increase efficiency of the drug in topical treatment.

Personal contribution to knowledge in this research field is presented in following published papers:

- Stan CD, G. Tătărîngă G, Gafiţanu C, Drăgan M, Braha S, Popescu MC, Lisă G, Ştefanache A. Preparation and characterization of niosomes containing metronidazole. *Farmacia* 2013; 61(6): 1178-1185.

II.4.1.1. Materials and methods

Reagents

Span 40, Brij 58, Myrj 52 were purchased from Serva-Heidelberg (Germany). Metronidazole and cholesterol were purchased from Merck Co. (Germany). All other chemicals and solvents were of analytical purity. All solutions were prepared with bidistiled water. Phosphate buffer saline (PBS) was prepared as described in the Xth Romanian Pharmacopoeia.

Vesicles preparation

Niosomes were prepared using the lipid film hydration technique (Tabbakhian et al., 2006). Metronidazole, non-ionic surfactant and cholesterol were weighed as indicated in Table 27 and dissolved in chloroform/methanol system (1:2) in a 250 mL round bottom flask. There were prepared two variants of niosomes: non-ionic surfactant vesicles I (NSV I) with 1:2:2 ratio of those three components (metronidazole:non-ionic surfactant:cholesterol) and non-ionic surfactant vesicles II (NSV II) with 1:1:1 ratio of the same components.

The solvent mixture was evaporated in a rotary evaporator, under a vacuum, at 27 ± 2^0C and 70-80 rpm flask rotation, until a dry, white lipid film was obtained.

The obtained film was hydrated with 30 mL of PBS with pH = 7.4 for 4 hours, at 27 ± 2^0C and 20 rpm flask rotation, until a white suspension was obtained. The niosomal suspension was further hydrated at 4^0C, in the absence of the light, for 24 hours. The niosomes were then separated by centrifugation at 2500 rpm for 30 minutes, followed by drying in a vacuum exiccator at 25^0C. The yields were calculated.

Table 27. Composition of the niosomes

Components	HLB	NSV I			NSV II		
		P_1	P_2	P_3	P'_1	P'_2	P'_3
Metronidazole	-	1	1	1	1	1	1
Brij 58	15.8	2	-	-	1	-	-
Myrj 52	16.9	-	2	-	-	1	-
Span 40	6.7	-	-	2	-	-	1
Cholesterol	1.0	2	2	2	1	1	1

HLB – hydrophilic-lipophilic balance; NSV – non-ionic surfactant vesicles

Characterization of niosomes

The prepared niosomes were analyzed for melting points with a Melt-Temp R apparatus equipped with a digital thermometer.

The IR spectra were performed with DIGILAB Scimitar Series spectrophotometer, using KBr pellet technique.

The TG/DTG curves have been recorded with Mettler Toledo TGA-SDTA851^e derivatograph. The registrations were performed in nitrogen, with a rate of 20 mL/min., over a temperature ranging from 25^0C to 900^0C and at a heating rate of 10^0C/min. The samples weights were 3-5 mg and the operational parameters were constant for all samples in order to obtain comparable data. The processing of curves was achieved with STAR software.

Prepared niosomes were analyzed for percent drug entrapment by spectrophotometric method (X^{th} Romanian Pharmacopoeia, 1993), after the separation of free drug. We used a Jasco UV-VIS V-530 spectrophotometer and the detection was performed at λ = 277 nm, using a 0.001% standard solution of metronidazole in 0.1 M HCl. There were made by three weighing

for each of the six niosomes variants, for which were determined the absorbance. Using external standard method we determined the percent metronidazole entrapment.

II.4.1.2. Results

The physico-chemical characteristics of the obtained NSV are listed in Table 28.

Table 28. The physico-chemical and spectral characteristics of the NSV with metronidazole

NSV	Melting point (^{0}C)	Yield (%)	IR characteristic band (cm^{-1})
P_1	135-137	76.80	1350.17, 1377.1 (NO_2)
P_2	129-132	70.40	1350.17, 1465.9 (NO_2)
P_3	114-118	71.70	1377.17, 1564.27 (NO_2)
P'_1	133-136	83.20	1371.3, 1535.33 (NO_2)
P'_2	153-155	76.45	1369.46, 1537.26(NO_2)
P'_3	160-162	78.52	1371.39, 1537.26 (NO_2)

From the TG/DTG curves (Figure 21, Figure 22, Table 29 and Table 30) it can be remarked specific temperatures of decomposition for each prepared NSV. The thermic degradation of all samples is realized in I to IV steps. The prepared NSV have a particular thermal behavior compared with parent molecules. They exhibit an increased thermal stability evidenced by higher temperature than those of metronidazole.

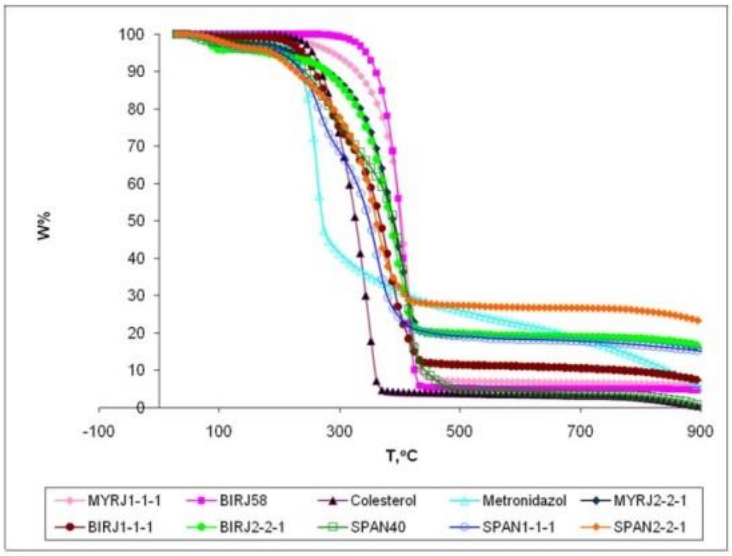

Figure 21. Thermogravimetric curves of NSV and parent substances - TG curves

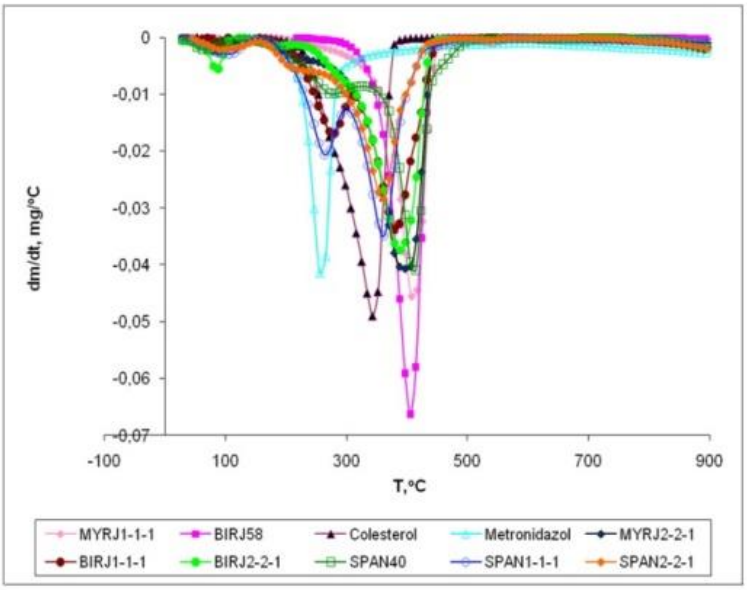

Figure 22. Thermogravimetric curves of NSV and parent substances - DTG curves

Table 29. Thermogravimetric characteristics of parent substances

Compound	Step	T_{onset}	T_{peak}	T_{endset}	Weight loss (%)	Rezidue (at 900^{0}C)
Metronidazole	I	218	260	277	73.26	0
	II	609	-	900	23.66	
Brij 58	I	349	407	428	96.04	3.96
Span 40	I	52	63	108	2.97	0
	II	212	275	375	40.79	
	III	375	412	478	56.24	
Cholesterol	I	246	346	364	100	0

Table 30. Thermogravimetric characteristics of the prepared NSV

Compound	Step	T_{onset}	T_{peak}	T_{endset}	Weight loss (%)	Rezidue (at 900^{0}C)
P_1	I	76	85	93	4.04	15.89
	II	256	386	423	80.07	
P_2	I	76	81	104	2.22	15.38
	II	326	401	428	82.40	
P_3	I	75	86	134	3.83	22.04
	II	198	360	416	74.13	
P'_1	I	229	279	298	28.31	6.99
	II	337	382	429	64.70	
P'_2	I	226	271	291	29.68	4.36
	II	335	373	429	65.96	
P'_3	I	76	103	124	3.94	14.33
	II	222	265	283	29.91	
	III	336	364	420	46.83	
	IV	420	539	900	4.99	

In Figure 23 is presented the percent metronidazole entrappement of the prepared vesicles. In Table 31 are presented the percent metronidazole entrapped average of the three determinations for each prepared NSV, the standard deviation (SD) and the relative standard deviation (RSD).

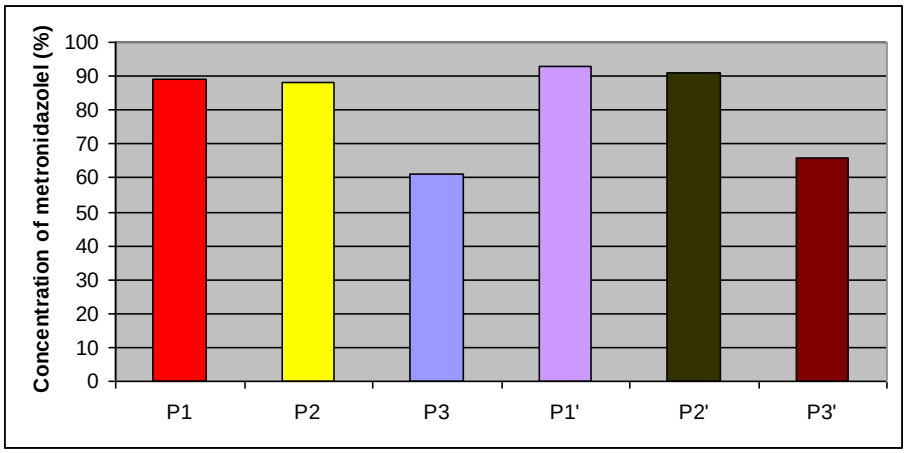

Figure 23. Percent metronidazole entrappement of the NSV

Table 31. Percent metronidazole entrappement of the NSV

Type of niosome	Percent metronidazole entrappement (%) ± SD	RSD	Type of niosome	Percent metronidazole entrappement (%) ± SD	RSD
P_1	89.03 ± 0.0416	0.0467	P'_1	92.73 ± 0.0152	0.0164
P_2	88.32 ± 0.02	0.0226	P'_2	91.22 ± 0.02	0.0219
P_3	61.32 ± 0.02	0.0326	P'_3	65.81 ± 0.02	0.0303

All characteristics of NSV shows a great metronidazole entrapment of vesicles prepared with 1:1:1 ratio of metronidazole:non-ionic surfactant:cholesterol than the vesicles prepared with 1:2:2 ratio of metronidazole:non-ionic surfactant:cholesterol.

The metronidazole entrapment decrease as follows: $P'_1 > P'_2 > P_1 > P_2 > P'_3 > P_3$. Within each niosomes range, the decrease in entrapment is as follows: Brij 58 > Myrj 52 > Span 40.

There were calculated the niosomes amount corresponding to one gram of metronidazole and the obtained results are listed in Table 32.

Table 32. The niosomes amounts corresponding to one gram of metronidazole

Sample	P_1	P_2	P_3	P'_1	P'_2	P'_3
Niosomes amounts (g)	1.1238	1.131	1.630	1.027	1.098	1.518

NSV obtained from the cetyl chain (C_{16}/surfactants) in the presence of cholesterol at 1:1 ratio, were bilayer membranes less rigid and more deformable than those obtained from stearyl chain (C_{17}/surfactants).

II.4.1.3. Discussions

Development of new drug carriers is an interesting approach since it allowed increased efficacy of antibiotic and a reduction in doses. As with most drug carriers, niosomes have been used to improve the selective delivery and the therapeutic index of antimicrobial agents (Akbari et al., 2015).

Niosomes as delivery devices have been studied with anticancer, antitubercular, anti-leishmanial, anti-inflamatory, hormonal drugs and oral vaccine (Katare et al., 2006; Cortesi et al., 2007; Achim et al., 2009).

These vesicles has been reported to decrease side effects, give sustain release and to enhance penetration of the trapped substances through skin. The reduction of the barrier properties of stratum corneum resulting from the property of vesicles as a penetration enhancer, are realized both by reducing trans-epidermal water loss and by increasing smoothness via replenishing lost skin lipids (Junginger et al., 1991).

Many drugs such as estradiol, tretinoin, dithranol, enoxacin, non-steroidal anti-inflammatory drugs, imidazoles have been successfully encapsulated in niosomes for topical application. The entrapment efficiency of the vesicles depended on alkyl chain length of non-ionic surfactants and amount of cholesterol used to prepare vesicles (Tabbakhian et al., 2006).

Ciprofloxacin loaded niosomes was formulated in order to be used as ocular prolonged-release carriers, using different molar ratios of Span 20 and Tween 20, Span 60 and Tween 60 or Span 80 and Tween 80, in combination with cholesterol (Akbari et al., 2015). Particle size of nisomes was dependent on the type of surfactant and the amount of cholesterol used in the preparation of the vesicles.

For our studies niosomes, the IR spectra revealed a band in the frequency range of 1369.46 cm^{-1} and 1535.33 cm^{-1}, for metronidazole, corresponding to nitro group vibration. Our prepared niosomes with metronidazole (NSV) display the characteristic vibrations of the nitro group in the niosomes molecule: for P_1 niosomes the intense sharp band at 1350.17 cm^{-1} and 1377.1 cm^{-1}; for P_2 niosomes the intense sharp band at 1350.17 cm^{-1} and 1465.9 cm^{-1}; for P_3 niosomes the intense sharp band at 1377.17 cm^{-1} and 1564.27 cm^{-1}; for P'_1 niosomes the intense sharp band at 1371.3 cm^{-1} and 1535.33 cm^{-1}; for P'_2 niosomes the intense sharp band at 1369.46 cm^{-1} and 1537.26 cm^{-1}; for P'_3 niosomes the intense sharp band at 1371.39 cm^{-1} and 1537.26 cm^{-1}. The IR spectra of all studied niosomes with metronidazole (NSV) revealed the presence of characteristic band for nitro group (as it can be seen in Table 28), which demonstrate that metronidazole was entrapped in the vesicles.

From the TG/DTG curves it can be seen that the temperature corresponding to the maximum mass lost rate increased from 260^0C for metronidazole to values between 360 to 539^0C for the prepared NSV. If we consider the T_{onset} a stability criterion, we can obtain a series of thermostability:

$$P_3 < P'_3 < P'_2 < P'_1 < P_1 < P_2$$

The entrapment efficiency of metronidazole in prepared niosomes was high and ranged from 61.32%-92.73%. Similar to our results, Akbari *et al*. reported an increase in ciprofloxacin liposomal encapsulation from 33 to 74%.

Increasing cholesterol content led to reducing the encapsulated drug amount (Hernandez-Borrell and Montero, 2003). This can be related to the amphipathic nature of the drug which may make the drug-bilayer interactions. In addition to entrapment in the hydrophilic compartment by protonation, there is the further possibility of the metronidazole molecule being incorporated into the niosome membrane.

All characteristics of NSV shows a great metronidazole entrapment of vesicles prepared with 1:1:1 ratio of metronidazole:non-ionic surfactant:cholesterol than the vesicles prepared with 1:2:2 ratio of metronidazole:non-ionic surfactant:cholesterol.

The metronidazole entrapment decrease as follows:

$$P'_1 > P'_2 > P_1 > P_2 > P'_3 > P_3$$

Within each niosomes range, the decrease in entrapment is as follows:

Brij 58 > Myrj 52 > Span 40

The rigidity of bilayers of Span/Brij/Myrj containing niosomes and the leaky nature of surfactants bilayers can explain the mentioned difference in metronidazole encapsulation ability. Among tested surfactants, Span 40 had lowest encapsulation efficiency. This finding is consistent with other reports (Han et al., 2002) in which the lowest colchicine entrapment efficiency of the Span formulation was explained by unsaturated status of Span causing he membrane to be more permeable. Akbari et al. also reported lower entrapment efficiency for ciprofloxacin of the Span formulation.

Surfactants with longer alkyl chains generally give larger vesicles. However, the surfactants with high hydrophilic properties and high HLB, as Brij 58, are preferable to form micelle in aqueous solutions. Surfactants with lipophilic properties, as Span 40, did not improve the solubility and the metronidazole entrapment is lower.

II.4.1.4. Conclusions

Bilayer vesicles can be prepared with the mixtures of some non-ionic surfactants and cholesterol in order to efficiently entrap metronidazole.

The IR spectra of all studied NSV revealed that metronidazole was entrapped in the vesicles. At 1:1 ratio of surfactants:cholesterol these vesicles were thermal stable than metronidazole, able to efficiently entrap metronidazole and the membrane was more deformable and yet stable.

A higher metronidazole entrapment was observed with non-ionic surfactant/cholesterol at 1:1 ratio, and the maximum metronidazole entrapment was observed with Brij 58, as non-ionic surfactant.

II.4.2. Design, development and validation of microparticles incorporating active substances

Microencapsulation of active substances is of major interest in the controlled release range, due to the relatively light microparticle design and reproducibility of the process (Dumitriu et al., 2015; Dumitriu et al., 2016).

The importance of biopolymers (chitosan type) is made evident by a multitude of studies, that have highlighted over the time, their characteristics such as: inertness and compatibility with drug substances, nontoxic, protective, antioxidant and biodegradable properties, but also the possibility of creating controlled release systems of active substances (Dragostin et al., 2017).

On the other hand, aspirin, the most used drug in the world (especially for the prevention of cardiovascular disease, but also in acute inflammation, myalgia, fever, angina or thrombosis), has some severe side effects such as prolonging bleeding time, gastric irritability, and favoring ulceration, as well as inducing hypersensitivity in some subjects.

In addition, penicillin and penicillin derivatives such as amoxicillin or ampicillin may also cause acute or subacute allergic reactions mediated by immunoglobulins E and G (Sánchez-Borges et al., 2013; Tamura et al., 2013).

Antihistamines inhibit the effects of histamine by blocking H1 receptors and have numerous clinical indications such as: allergic pathologies (rhinitis, atopic dermatitis, allergic conjunctivitis, drug hypersensitivity, urticaria, transfusion reactions), idiopathic chronic urticaria, motion sickness, vertigo and insomnia (Katselou et al., 2017; Pasko et al., 2015).

Cetirizine, an important representative of this class, is a potent antihistamine H1 of the second generation, a hydroxysin metabolite, effective in the treatment of allergic rhinitis, chronic urticaria, pollen induced asthma and atopic dermatitis. It becomes differentiated from traditional antagonists by not producing drowsiness or anticholinergic effects (Hu et al., 2015; Amelian et al., 2017).

Starting from these scientific works my research in this area eas focused on:

- the using of chemically modified chitosan via cetirizine, as a polymer matrix, in order to prepare microparticles incorporating aspirin and penicillin, substances well known for their highly allergenic properties;
- to develop and validate micro-systems based on chitosan and its chitosan-cetirizine derivatives, for reducing the drug hypersensitivity concerning the patients with extreme sensitivity to a particular active substance.

Personal contribution to knowledge in this research field is presented in following published paper:

- Dragostin I, Dragostin OM, Drăgan M, Stan CD, Zamfir CL. Drug Hypersensitivity Reduction Using Encapsulation Method with Chitosan-Cetirizine Derivatives. *Revista de Chimie (Bucharest)* 2018; 69(12): 3731-3735.

II.4.2.1. Materials and methods

General procedure for obtaining chitosan-cetirizine derivatives

1 g of cetirizine (0.0025 moles) was subjected to the esterification reaction by mechanical agitation with 2% chitosan solutions (CLMW and CMMW) in 2% acetic acid, for 24 hours, using an acid medium, as dehydrating agent. At the end of the reaction time, the products were separated by filtration, the technique being taken from the literature, with minor modifications (Sionkowska et al., 2013; Dumitriu et al., 2015).

IR spectral analysis of chitosan-cetirizine derivatives: structural confirmation

The spectra of the synthesized compounds were performed by ATR-FTIR measurements using FT-IR ABB Bomem MB-3000 (Canada) spectrometer. The spectra were recorded in the range of 4000–500 cm^{-1} with 32 scans at a resolution of 4 cm^{-1}. Spectral processing was carried out by means of a Horizon MBTM FTIR Software and GRAMS 32 Software (Galactic Industry Corporation, Salem, NH), Version 6.00.

General procedure for incorporating aspirine and peniciline G into chitosan-cetirizine-based microparticles

To optimize the method, pure chitosan microparticles have been made, and then the parameters and working conditions found, have been used and adapted to obtain the chitosan-cetirizine microparticles (CSM.CET and CSL.CET) with aspirin (ASP) and penicillin (PEN). The procedure was performed according to the literature technique (Lupașcu et al., 2015), with the necessary modifications. Their production was carried out by the method of dripping, under mechanical stirring, a method which requires exact and correct dosing of the active substances. 1 % aqueous solution of sodium tripolyphosphate (TPP) was used as crosslinker.

Microscopic characterization of the obtained microparticles

A microscopic analysis was performed using the FeiQuanta 200F Electronic Scanning Microscope (SEM), which allowed the study of surfaces of the chitosan microparticles to produce clear three-dimensional images.

Evaluation of the swelling capacity of the obtained microparticles

It was tested by determining the degree of inflation at equilibrium. The chitosan-cetirizine microparticles of penicillin and aspirin of approximately the same size were weighed for dry mass determination (Wd), after which they were left in distilled water at room temperature. Samples were taken every 15 minutes, dried with filter paper and weighed each time to determine the wet weight (Ww), the operation being repeated until the thermodynamic equilibrium was installed.

The Membrane Swelling Ratio (MSR) is calculated according to the following formula:

$$MSR\% = \frac{Ww - Wd}{Wd} \text{ x } 100$$

where: Wd is the weight of the dry sample and Ww is the weight of the wet sample at thermodynamic equilibrium. The results were plotted.

In vitro biodegradation of the obtained microparticles

The biodegradability of the microparticles was studied according to the literature using 52975 IU / mg of lysozyme as the biodegradable agent after it had previously been solubilized at 37° C in phosphate buffer pH 7.4 with 10,000 IU / mg. Until the equilibration of the swelling capacity was established, the microparticles were left in the buffer solution (pH=7.4), after which they were transferred to the lysozyme buffer and kept in the incubator at 37 ° C for 7 days, the solution being changed daily. On days 1, 4 and 7, the microparticles were removed from the buffer and weighed, following a decrease in mass by biodegradation. The estimation of the percentage of biodegradation (D%) was carried out with the following calculation formula:

$$D\% = \frac{W_0 - W_x}{W_0 \times 100}$$

where: W_0 is the mass of the microparticles, prior to incubation, at the equilibrium of the swelling capacity and Wx is the mass of microparticles after incubation on days 1, 4 and 7 (Dragostin et al., 2015).

II.4.2.2. Results

Chitosan-cetirizine derivatives (Figure 24) resulted from an esterification reaction between a hydroxyl group of chitosan and the carboxyl group of cetirizine.

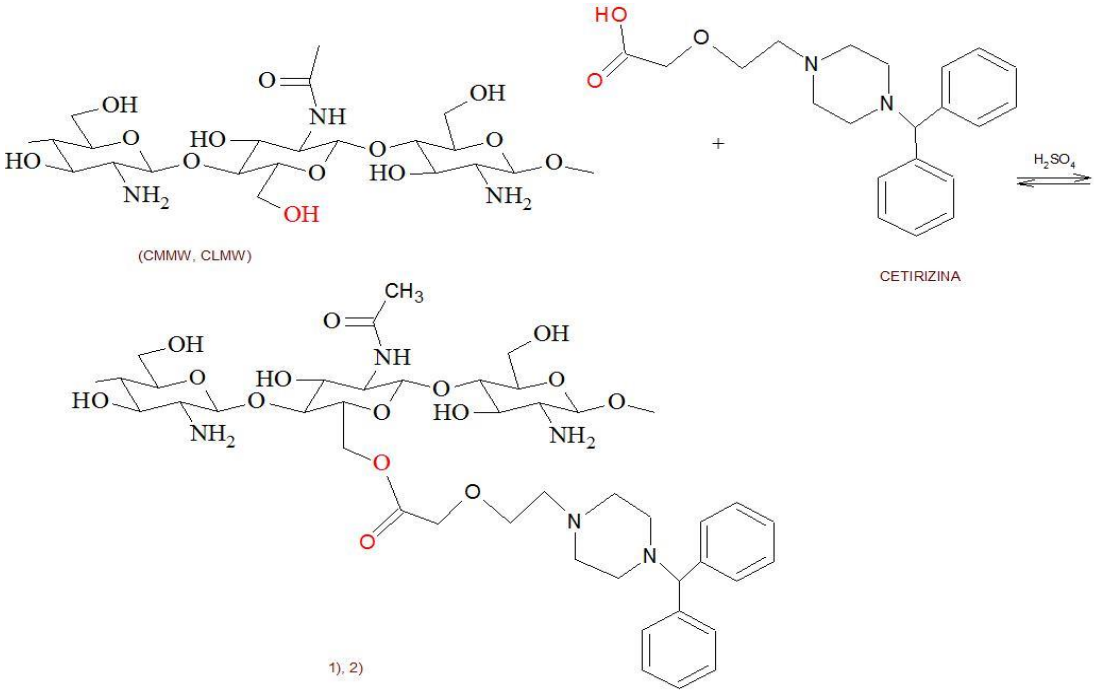

Figure 24. Esterification of chitosan with cetirizine

By IR spectroscopy, there were highlighted characteristic bands of synthesized compounds, through the glucosamine component (Figure 25). Also, the cetirizine component was identified in the spectrum (Figure 26).

The microparticles obtained, after being left stirring for 24 hours at 300 rpm, were extracted from the crosslinker solution, washed with distilled water and dried at room temperature (Figure 27).

Following the SEM analysis (Figure 28), both chitosan microparticles with aspirin and penicillin microparticles, have a rough surface of between 400-800 μm in the dry state. The presence of cracks, on their surface, is explained by the high crystallinity of encapsulated substances.

The assessment of the swelling capacity is necessary for analyzing the stability of the microparticles over time (Figure 29).

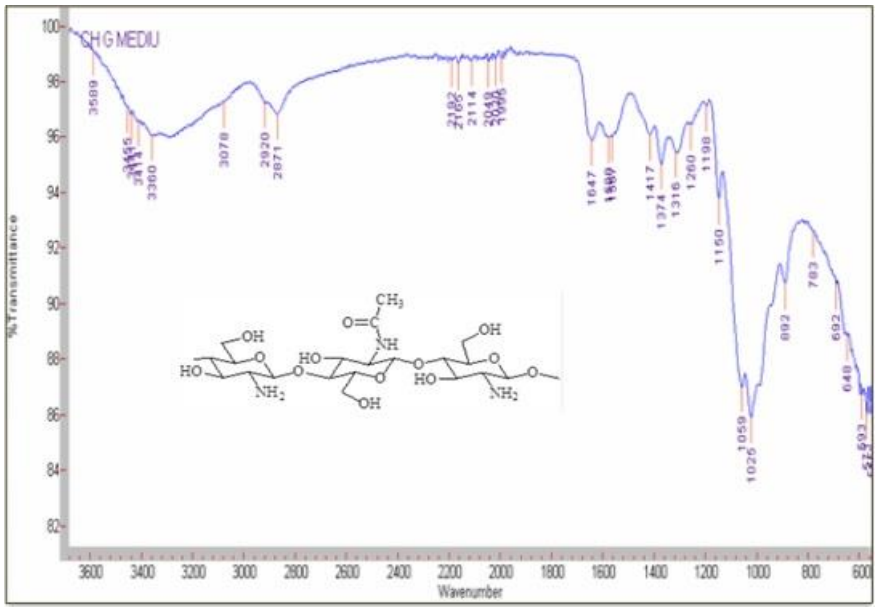

Figure 25. IR spectra of chitosan (glucosamine component highlighting)

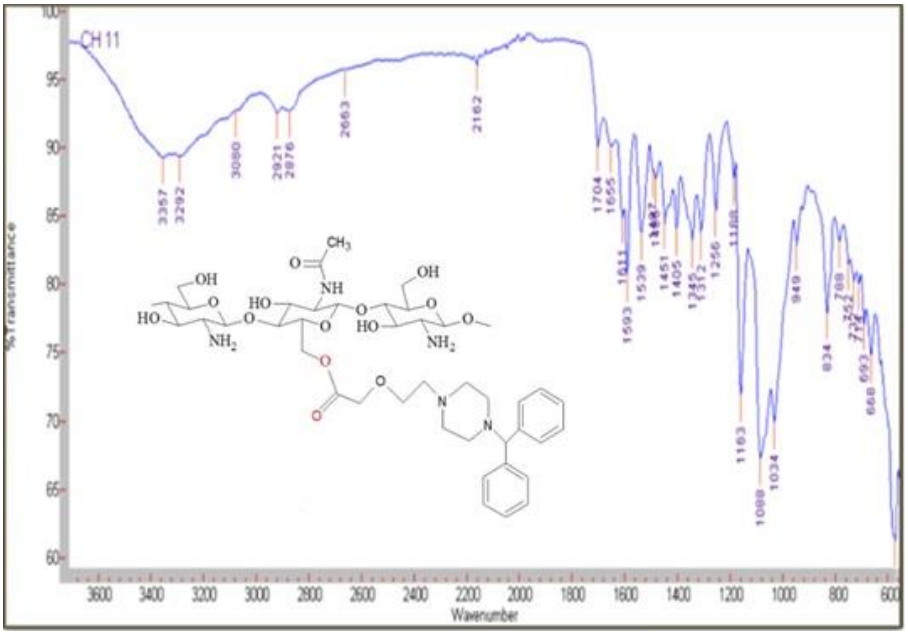

Figure 26. IR spectra of chitosan-cetirizine (cetirizine component highlighting)

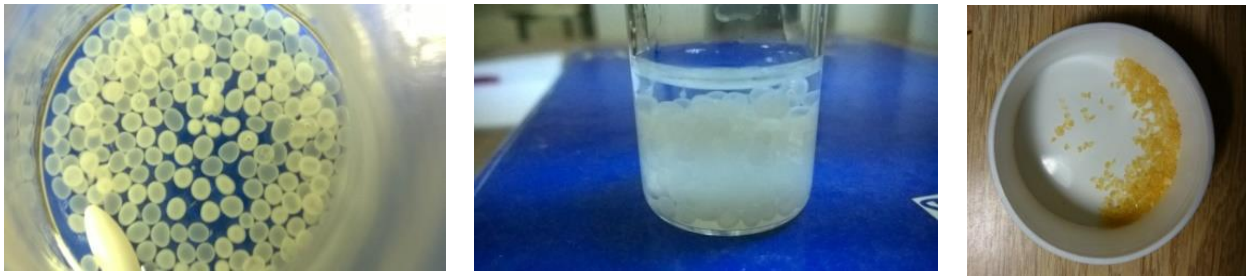

Figure 27. Macroscopic images of chitosan-cetirizine microparticles (CSM.CET-PEN)

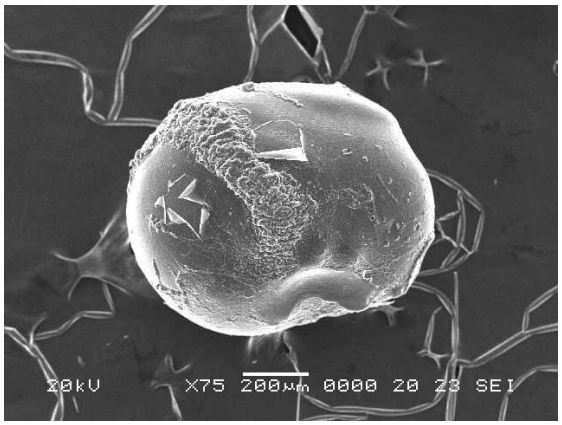

Figure 28. SEM image of chitosan-cetirizine microparticles (CSM.CET-PEN)

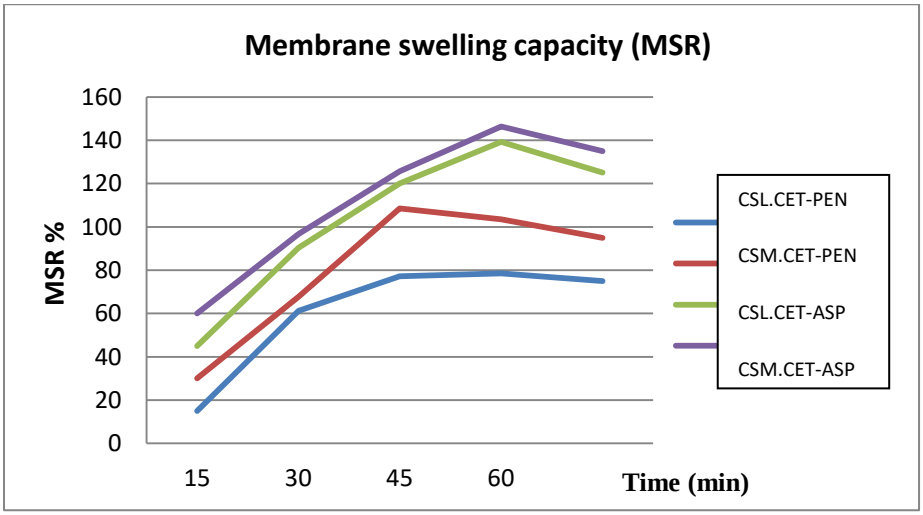

Figure 29. Graphic representation of swelling capacity

In the case of low molecular weight chitosan microparticles with penicillin (CSL.CET-PEN), the equilibrium infiltration value is 75%, calculated after 60 minutes. The highest value was found for CSM.CET-ASP microparticles, namely 135% and 125% respectively for CSL.CET-ASP, followed by CSM.CET-PEN microparticles, the swelling capacity being 95%.
The in vitro biodegradation of the four microparticles series, under lysozyme activity, was monitored for 7 days, the results being shown in Figure 30.

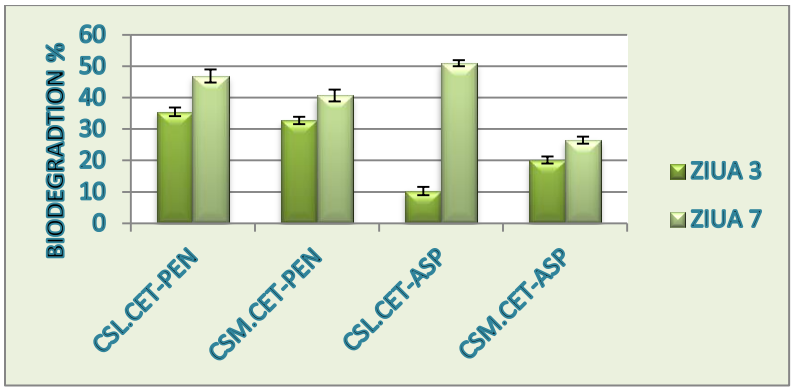

Figure 30. Biodegradation capacity of microparticles

An increase in the seventh-day biodegradation compared to the 3rd day, was found for all derivatives, the highest value being recorded for CSL.CET-ASP, respectively 50.95% versus 10.28%, on the 3rd day.

II.4.2.3. Discussions

Nanotechnology has produced an extremely important impact on nano-biomedicine and the diagnosis/treatment of disease (Vasquez et al., 2018). Nanomaterials in drug delivery can improve the cellular uptake of poorly soluble drugs and also enhance drug bioavailability at effective doses that were previously difficult to achieve (Bhavasar et al., 2017).

Nanoparticle geometry could significantly affect the rate of drug loading and drug transport. In addition, drugs wrapped by nanomaterials prolong their circulation time, thereby improving their biodistribution and pharmacodynamics (Sohail et al., 2018).

Nanomaterials modified by other bioactive molecules would exhibit distinct functions, including specific targeting, thereby facilitating in drug accumulation and better reaching its effective dose (Hoop et al., 2016).

Chitosan-based nanomaterials are widely used for continued drug release systems for various types of epithelia, including buccal, intestinal, nasal, eye and pulmonary.

There have been reported the preparation and efficacy evaluation of a isotonic solution of hydroxypropyl methylcellulose containing chitosan/sodium tripolyphosphate/hyaluronic acid nanoparticles to deliver the antibiotic ceftazidime to the eye (Silva et al., 2017).

Other researchers present nanoparticles of chitosan, alginate and pectin as potential candidates for the administration of drugs into the oral cavity (Pistone et al., 2017).

Very new investigations, reported recently the obtaining of nanoparticles of carboxymethyl chitosan to release intra-nasal carbamazepine for increasing the amount of the medication in the brain (Liu et al., 2018).

It was cited in the literature the obtaining of 5-fluorouracil hyaluronic acid-coated chitosan nanoparticles for oral administration (Jain and Jain, 2016).

On the othe hand, cetirizine is a potent second-generation H1-antihistamine, which has anti-inflammatory properties and high specific affinity for histamine H1-receptors. It is widely used to treat symptoms of allergic disease in patients of all ages. The somnolence that sometimes occurs after oral administration is dose-dependent and generally mild (Sharma et al., 2014).

Our research directions were: *(i)* the synthesis, characterization and physico-chemical and spectral (infrared spectroscopy) evaluation of certain derivatives of chitosan-cetirizine (using chitosan with low molecular weight -CLMW and medium molecular weight -CMMW); *(ii)* the preparation of microparticles based on these derivatives, in which substances with potential allergens were included: aspirin and penicillin; *(iii)* the morphological characterization of the obtained microparticles using the electronic scanning microscopy (SEM) and, last but not least, *(iv)* determining the swelling capacity of the obtained polymer matrices, and *(v)* assessing their biodegradability.

The study focused on the synthesis methods that are based on the principles of "green chemistry" with the purpose of using them to obtain chitosan derivatives with anti-allergic properties (Dumitriu et al., 2015; Dumitriu et al., 2016).

According to the literature the therapeutic efficacy is significantly influenced by the ability of the polymer membranes to retain water (Sionkowska et al., 2013; Dragostin et al., 2016).

By the esterification of the two types of medium and small chitosan, the corresponding derivatives were obtained with cetirizine (1-CSM.CET and 2-CSL.CET). The reaction products were white and homogeneous in appearance, the reaction having a yield of 85.76% -96.78%. IR spectroscopy highlighted characteristic bands of synthesized compounds, through the glucosamine component: the carbonyl group (C=O) was highlighted at the wavelength $\lambda = 1647$ cm^{-1}; the amino group (-NH-) exhibits vibrations at $\lambda = 1589$ cm^{-1}; the hydroxyl alcohol group (-OH) at $\lambda = 3300\text{-}3400$ cm^{-1}.

Also, the cetirizine component was identified in the spectrum: the aromatic nucleus is present in the form of multiple bands, the most intense of which were highlighted in the 1539-834 cm^{-1} region; the piperazine component is characterized by a very strong bond of carbon-nitrogen (C-N) in the nucleus at $\lambda = 1163$ cm^{-1}; a methylene group band CH_2 at $\lambda = 1405$ cm^{-1};

the presence of the vibration band at $\lambda = 1704$ cm^{-1} confirms the realization of the ester-CO bond.

Following the SEM analysis, both chitosan microparticles with aspirin and penicillin microparticles, have a rough surface of between 400-800 μm in the dry state. The presence of cracks, on their surface, is explained by the high crystallinity of encapsulated substances.

The assessment of the swelling capacity is necessary for analyzing the stability of the microparticles over time. Typically, polysaccharides have a strong water affinity due to the presence of a large number of hydrophilic groups, which can be easily hydrated. In the case of low molecular weight chitosan microparticles with penicillin (CSL.CET-PEN), the equilibrium infiltration value is 75%, calculated after 60 minutes. The highest value was found for CSM.CET+ASP microparticles, namely 135% and 125% respectively for CSL.CET-ASP, followed by CSM.CET-PEN microparticles, the swelling capacity being 95%.

In the seventh-day an increase biodegradation value was found for all derivatives compared to the 3rd day. The highest value on the 7th day was recorded for CSL.CET-ASP (50.95%). For medium-weight (CSM.CET-ASP), biodegradation ranges from 20.14% to 26.45%. On the other hand, in the case of penicillin, for medium-molecular weight chitosan (CSM.CET-PEN), the value increases from 32.71% (day 3) to 40.68% (day 7), while in the case of low molecular weight chitosan derivative (CSL.CET-PEN), a higher biodegradation of 35.45% -46.89% is observed.

II.4.2.4. Conclusions

It were developed chitosan-cetirizine microparticles, in which aspirin and penicillin have been incorporated (CSM.CET-PEN, CSL.CET-PEN, CSM.CET-ASP, CSL.CET-ASP), to obtain polymeric matrices able to reduce allergic reactions in patients whose immune system develops extreme sensitivity to a particular active substance.

The synthesis of chitosan-cetirizine derivatives was done properly and efficiently, proved by IR absorption spectra, which demonstrated the existence of characteristic vibrations of each functional group: the aromatic ring, the carbonyl group, the carboxyl group, the piperazine moiety and the amino group.

Also, the use of solubilized chitosan in 2% acetic acid and 1% TPP, as a crosslinker, resulted in stable, rusty and non-uniform microparticles. In addition, it was confirmed the ability of microparticles to swell by absorbing a percentage of water between 75 and 135%, and to biodegrade to a maximum of 50.95%.

II.5. Applying green chemistry by removing organic compounds from wastewater from the pharmaceutical industry

Today we know the benefits to mankind of the drugs that are used worldwide. The success of the modern pharmaceutical industry is built on the achievements of synthesis and semi-synthesis over the last century. The complex task of manufacturing pharmaceuticals has to balance current knowledge of chemical and biological processes with the regulatory pressures and escalating costs.

The expectations of modern society for improved safety, lower environmental impact, more sustainable practices and lower energy use at a fair cost place tremendous demands and responsibility on us.

Our ability to better and more rapidly profile for impurities and evaluate alternative routes is leading to new opportunities and creating better understanding.

In the last two decades it has become increasingly clear that the pharmaceutical industry faced with serious environmental problems. Many of the organic synthesis generate large amounts of waste. The pharmaceutical industry has been subjected to increasing pressure to minimize/eliminate this waste (Constable et al., 2001; Curzons et al., 2002).

An example is provided by the manufacture of phloroglucinol, a pharmaceutical intermediate, which was produced in the mid 1980`s with the obtaining of large ammounts of waste. For every kg of phloroglucinol produced approx. 40 kg of solid waste were generated (Dunn et al., 2010). This process was discontinued as the costs associated with the disposal of waste approached or exceeded the selling price of the product.

The Green Chemistry Institute, which includs several leading pharmaceutical companies (Eli Lilly, GlaxoSmithKline, Pfizer, Merck, AstraZeneca, Schering-Plow, Johnson&Johnson) was created to use the research provided data to drive the greening of the pharmaceutical industry (Constable et al., 2002).

In pharmaceutical manufacture time to market is crucial and the advantage of classical technologies is that they can be implemented quickly. Consequently, environmentally inferior technologies are often used to meet stringent market deadlines and subsequent process changes can be prohibitive owing to problems associated with FDA approval (Rajbongshi et al., 2016).

The wastes generated by pharmaceutical companies have increased concerns about environmental and human safety. Direct releases of treated solvent wastes and accidental releases of toxic chemicals into the environment have led to the implementation of many laws and regulations.

Starting from the presented aspects my research in this area focused on:

- pharmaceutical wastes management through photocatalytic processes;
- pharmaceutical wastes management through magnetic separation and chemical transformation of byproducts.

II.5.1. Heterogeneous and homogeneous photocatalytic treatments of of wastewaters from the pharmaceutical industry

Removing organic pollutants from wastewaters is an important issue given the fact that most of them are toxic, mutagenic, and/or carcinogenic, even in low concentrations, and are a real health threat to humans, animals, as well as to the environment (Stan et al., 2012).

Heterogeneous and homogeneous photocatalytic detoxification methods have recently shown great promise for the treatment of pharmaceutical industrial wastewater (Suditu et al., 2008) due to several important advantages such as: complete mineralization or formation of more readily biodegradable intermediates when complex organic compunds are treated, no need of auxiliary chemicals, no residual formation, easily operation and maintenance of the equipment.

A very important feature of the photocatalysis is that the reaction can occur at temperature values close to ambient, therefore, the photoreactions are "greener," since the thermal energy is expensive and, most often, its production is pollutant.

Heterogeneous photocatalysis is influenced by catalyst loading, initial pollutant concentration, pH, radiant flux, aeration, the presence of other substances or impurities and photoreactor geometry. The most widely used catalyst in photoinduced processes is titanium dioxide, because it is chemically and biologically inert, photocatalytically stable, relatively easy to produce and to use, able to efficiently catalyse reactions, cheap, and without risks to the environment or humans.

Homogeneous photocatalysis uses UV radiation in combination with chemical oxidising agents such as hydrogen peroxide or ozone.

A performant photocatalyst is expected to offer adsorption sites for the organics, meaning that a high specific surface area and/or open pore structure are welcome.

Mepiquat chloride or 1,1-dimethylpiperidine chloride, also known as DPC, is a new plant growth regulator (Quintás et al., 2004). The effect of different heterogeneous and homogeneous photocatalytic systems on the oxidative degradation of mepiquat chloride in aqueous solutions was investigated. In the case of heterogeneous reactions, the influence of five factors was studied: the type of catalyst, photocatalyst concentration, pH, pesticide concentration, and the presence of H_2O_2 and/or Fe^{3+}. For homogeneous catalysis, other factors were studied: the oxidising agent and the light source.

Rhodamine 6G (R6G) is used in biotechnology and also in the biochemistry laboratories (as a diagnostic tool in medicine). Due to its high stability, it is interesting to find photocatalytic systems able to decompose R6G to smaller biodegradable species or eventually mineralize it.

There are quite many papers in the literature dealing with the photocatalytic degradation of several rhodamines, especially rhodamine B (Guo et al., 2011; Arsene et al., 2011; Ghazzala et al., 2012).

We investigated the properties of ZnO prepared in a very simple way in the photocatalytic degradation of R6G.

Personal contribution to knowledge in this research field is presented in following published papers:

- Lutic D, Coromelci-Pastravanu C, Crețescu I, Poulios I, Stan CD. Photocatalytic treatment of rhodamine 6G in wastewater using photoactive ZnO. *International Journal of Photoenergy* 2012; DOI: 10.1155/2012/475131.
- Stan CD, Coromelci-Pastravanu C, Crețescu I, Drăgan M. Treatment of pesticides in wastewater by heterogeneous and homogeneous photocatalysis. *International Journal of Photoenergy* 2012; DOI: 10.1155/2012/194823.

II.5.1.1. Materials and methods

Light Sources

A G23 Radium Ralutec UVA lamp (9W/78, 350–400 nm, ein/min) and a Radium Ralutec VIS lamp (9W/71, ein/min) were used. Actinometry was used to determine the exact radiation intensity of both lamps within the vessels. The radiation intensities obtained for the UVA and the VIS lamp were 1.3517 ein/min and 0.3517 ein/min, respectively.

Vessels and Reagents

A solution of mepiquat chloride pesticide was used and as catalysts TiO_2P-25, TiO_2UV-100, TiO_2-A, TiONa and ZnO were studied.

Procedures and Analyses

In the initial 300 mL aqueous solution of DPC (mg/L), different quantities of catalysts were added. The reaction solutions were magnetically stirred in the dark for 30 min until adsorption/desorption equilibrium was reached. The solutions were then irradiated under UV light with continuous magnetic stirring. A fixed quantity of each mepiquat chloride solution was taken at regular time intervals during the illumination period and filtered through a syringe filter to analyse the amount of pesticide remaining in the solution. DPC concentrations during the experiments were monitored by a Total Organic Carbon Analyzer (Shimadzu), in order to measure the mineralisation of the pesticide.

Preparation of ZnO

It was prepared by precipitation as zinc hydroxide, followed by calcination. Two precipitation agents were used, NaOH and urea.

The precipitation with NaOH was performed at room temperature, by the simultaneous pouring of zinc nitrate and NaOH solutions under continuous magnetic stirring in a beaker, while monitored by a Hanna HI 991003 pH-meter, to help keeping the pH value at 9. The achievement of the complete precipitation degree occurred by heating the slurry at 353 K for 16 hours.

The precipitation in the presence of urea was performed using a 5/1 molar ratio urea/Zn, by mixing the zinc nitrate solution with urea solution, at a concentration of zinc of about 0.25 moles L^{-1} in the final solution mixture. This solution was stirred at 363 K for 14 h under reflux; the final pH settled at 7.

The solids were recovered in both cases by centrifugation at 4000 rpm, washed several times with ultrapure water, dried at 353 K, then calcined 4 h at 500°C with a heating rate of 1° min^{-1}, then cooled down spontaneously.

Spectral characterization

The characterization of the solids was performed by X-ray diffraction, IR spectroscopy, scanning electron microscopy (SEM), and BET nitrogen adsorption. The XRD patterns were obtained on a Shimadzu D6000 machine, in the range of 5–70°, using CuKα radiation (λ = 1.5406 Å), in steps of 0.05°. The IR spectra were collected on an ABB MB 3000 apparatus. The scanning electron microscopy (SEM) was used to show the morphology of the synthesized solids; a SEMTescan VEGA II LSH was used in this respect.

Photocatalytic activities

The photocatalytic degradation was performed using a cylindrical glass reactor, accommodating a central quartz tube in which an Osram UV-A lamp of 9 W (wavelength range 350–400 nm, maximum at 370 nm) is placed to irradiate the dye solution. At the bottom of the reactor, a magnetic range assures the homogenization. The experiments were performed on samples of 300 mL aqueous solutions of R6G. The photocatalyst powder was dispersed in the R6G solution, magnetically stirred for 30 minutes in dark, in order to enable establishing the adsorption/desorption equilibrium, then the lamp was turned on.

Samples of 5 mL suspension were taken at specific time intervals, filtered immediately to separate completely the catalyst particles, and measured to determine the remaining concentration. The decolorization and photocatalytic degradation efficiency were calculated using the following:

$$\text{R6G removal (\%)} = C_0 - C_t/C_0 \times 100$$

where C_0 and C_t are the initial and t is time concentrations of R 6G.

II.5.1.2. Results

Five types of catalysts were investigated: TiO_2P-25, TiO_2UV-100, TiO_2-A, TiONa and ZnO. The results are plotted in Figure 31. It can be seen that the TiO_2P-25 catalyst exhibited the best behaviour in the degradation of DPC solution and was thus considered the reference catalyst. The influence of catalyst concentration was studied for the TiO_2P-25 catalyst, which was chosen as the reference, the results of the degradation experiments are depicted in Figure 32.

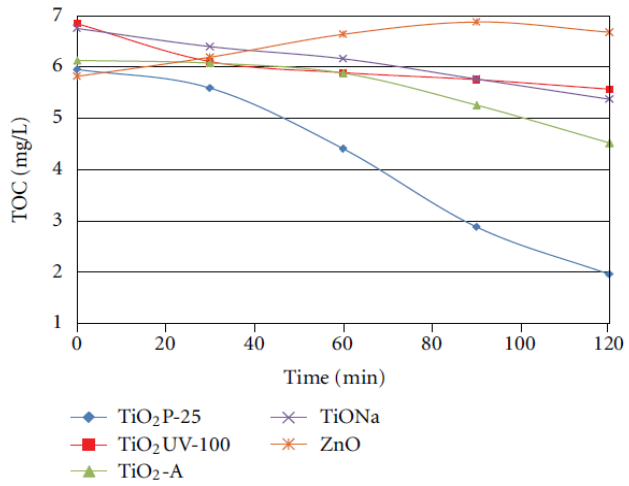

Figure 31. DPC degradation by heterogeneous photocatalysis using different catalysts

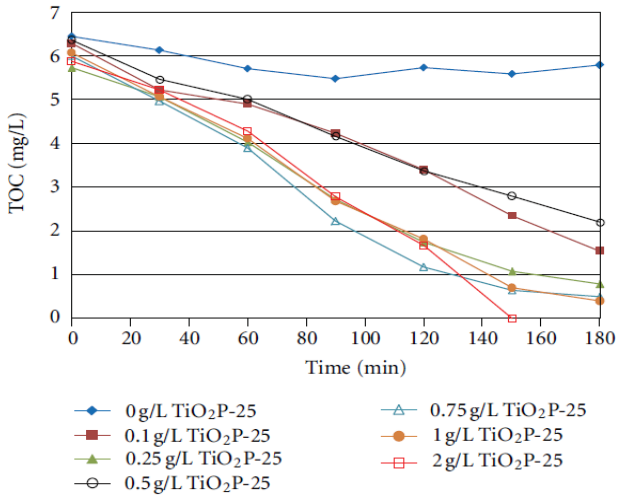

Figure 32. The TiO_2P-25 dose influence on the degradation of DPC

Figure 33 illustrates the variation in TOC/TOC(0) values over time at different pH values. The positive effect of acidic pH on DPC degradation noted in the figure can be assigned to the fact that the surface is positively charged when the solution pH is lower than 6.8, thus facilitating the photocatalytic process. The influence of the DPC concentration on the photocatalytic process was studied using the TiO_2P-25 catalyst, the results being presented in Figure 34.

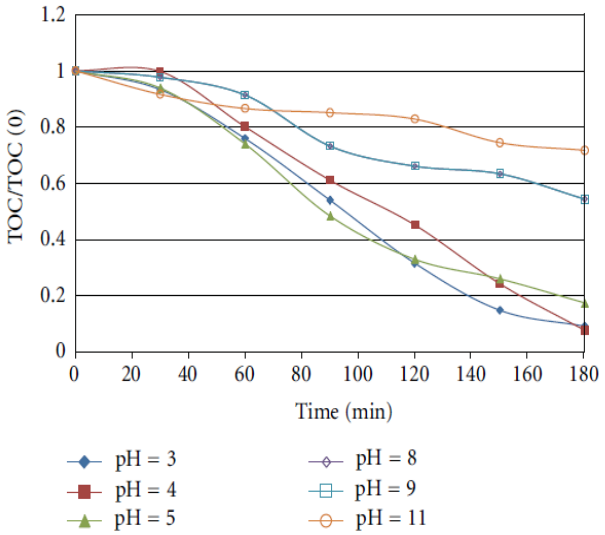

Figure 33. DPC degradation over time at different pH values of the initial solution

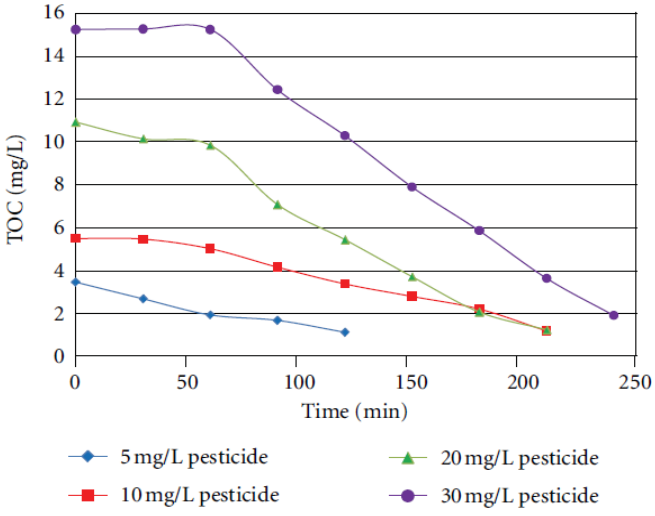

Figure 34. Behaviour of different DPC solutions concentrations during heterogeneous photocatalysis

Enhancing the photocatalysis reaction by adding H_2O_2 or Fe^{3+} in different concentrations, to the initial solution, is presented in Figure 35 and 36. The best degradation rate was obtained when the P-25 photocatalysis was used alone.

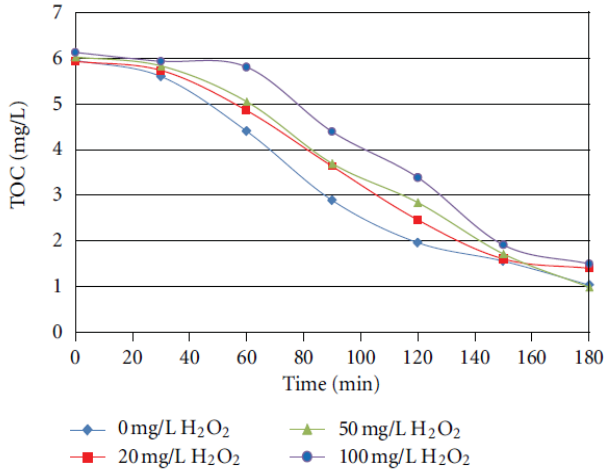

Figure 35. Heterogeneous photocatalysis of DPC in the presence of P-25 and H_2O_2

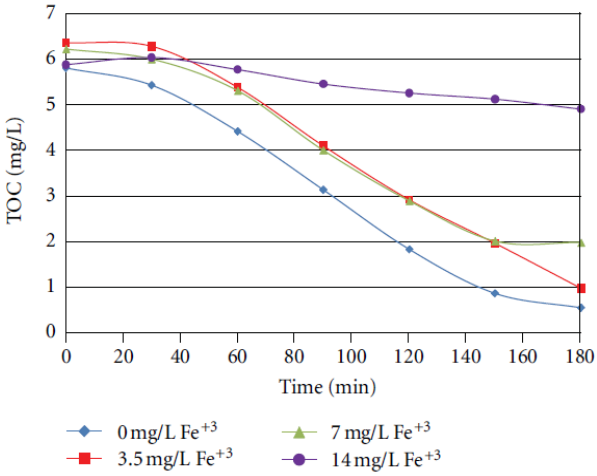

Figure 36. Heterogeneous photocatalysis of DPC in the presence of P-25 and Fe^{3+}

Homogeneous photocatalytic oxidation of the DPC was also tested using two oxidising agents: a photo-Fenton agent (H_2O_2 + Fe^{3+}) and a ferrioxalate agent (potassium oxalate + H_2O_2 + Fe^{3+}) in various concentrations, the results beeing depicted in Figures 37 and 38 respectively.

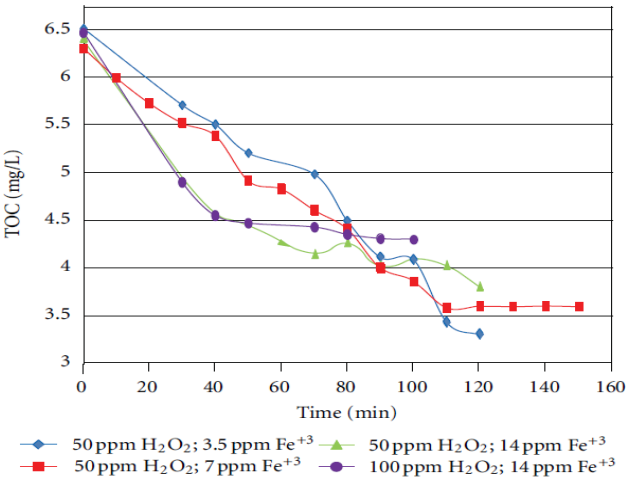

Figure 37. Homogeneous photocatalysis of DPC using the photo-Fenton agent

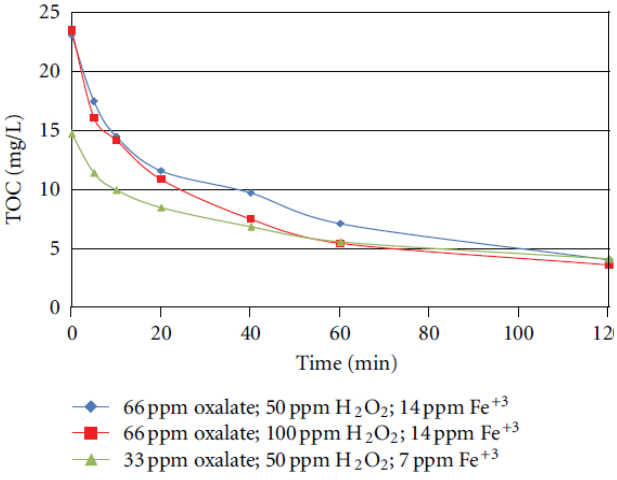

Figure 38. Homogeneous photocatalysis of DPC using the ferrioxalate agent

In the case of the photo-Fenton reagent, the best results were obtained when less oxidant was used: 50 ppm and 3.5 ppm. With the ferrioxalate reagent, an excellent degradation rate was obtained when visible light was used.

The XRD patterns of the samples ZnO-NaOH and ZnO-urea, presented in Figure 39, indicate well-crystallized solids, exhibiting the main peaks due to zinc oxide phase and no impurity phases. The IR spectra of our samples were compared with those of a commercial zinc oxide, see Figure 40. The spectra for the samples synthesized in our laboratory are quite similar and in line with the literature data.

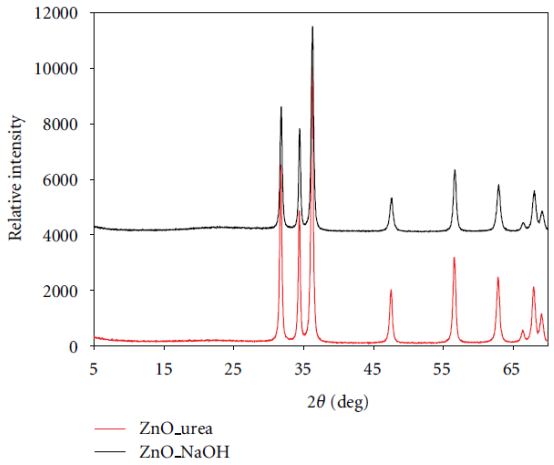

Figure 39. XRD pattern of ZnO samples

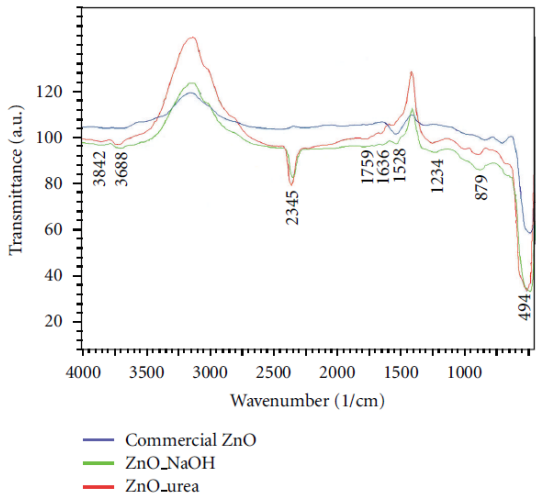

Figure 40. IR spectra of ZnO samples

The XRD pattern also allowed calculating the medium crystallite size. These particle sizes represent the dimensions of the primary particles, which associate in groups of several hundreds of nanometers, as shown on the SEM images (Figure 41a and b).

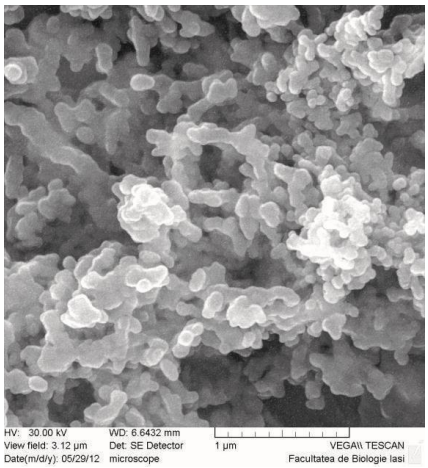

Figure 41a. SEM images of ZnO-urea

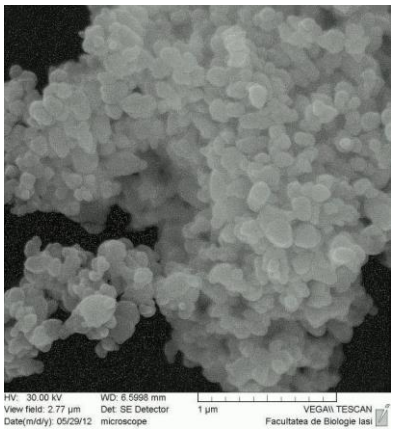

Figure 41b. SEM images of ZnO-NaOH

The BET analysis allowed the determination of the BET-specific surface areas, found to be 17.3 $m^2 g^{-1}$ for ZnO-urea and, respectively, 13.04 $m^2 g^{-1}$ for the ZnO-NaOH sample. The isotherms of type 3 indicates low porosity solids, with a very narrow hysteresis loop. No significant internal porosity is found on these samples, see Figure 42. For all the tested photocatalyst samples, the same characteristic for the removal of R6G is noticed. More than 60% of the R6G is removed from the solution in all the tested mixtures. The values of the total removal degree are similar for both samples, we admit from this point forward the 0.3 g/L as the optimal concentration value. The removal degree in time is presented in Figure 43.

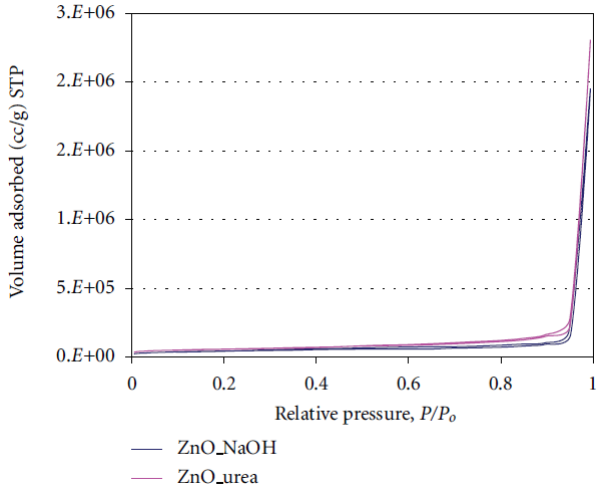

Figure 42. BET analysis results for ZnO

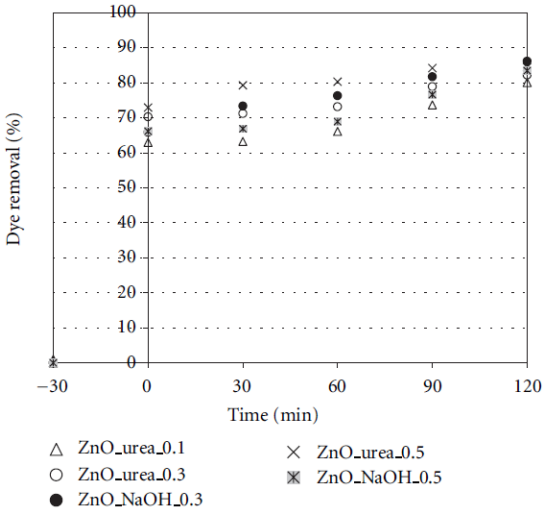

Figure 43. The influence of catalyst concentration on R6G decolorization

In order to investigate the influence of the pH role of the reaction medium on R6G decolorizing, this series of experiments was performed at pH values of 6.6, 8.5, and 10. The R6G removal in these conditions is presented in Figure 44. The R6G removal degree reaches 98% on ZnO-Urea at pH=10 in 90 minutes. This somehow unexpected behavior determined us to investigate the R6G removal by measuring the remaining concentrations at intermediate time values between up to 120 minutes of the photocatalytic reaction, Figure 45.

In order to study influence of R6G concentration on the degradation process, we find that an almost constant increase of the R6G removal degree occurs at the low value of 5 ppm on the sample ZnO-Urea, see Figure 46. After 2 hours of irradiation the removal of the R6G is practically total.

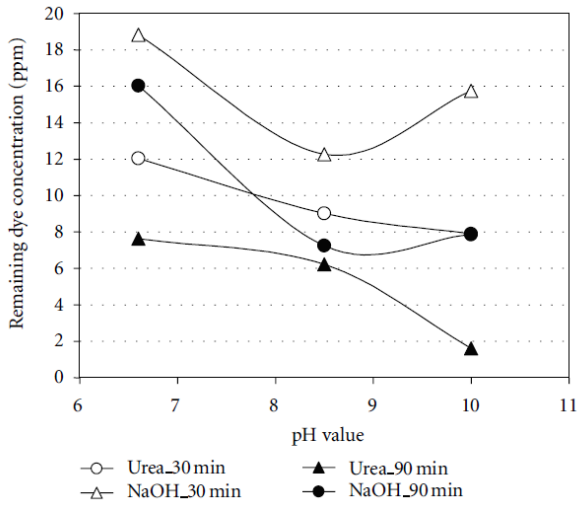

Figure 44. The pH influence on R6G decolorization after 30 and 90 minutes of photoreaction

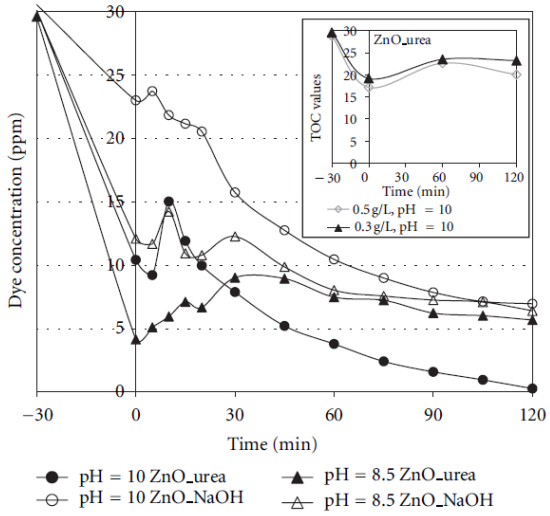

Figure 45. R6G concentration profile in time and TOC values for ZnO-urea sample, at pH = 10

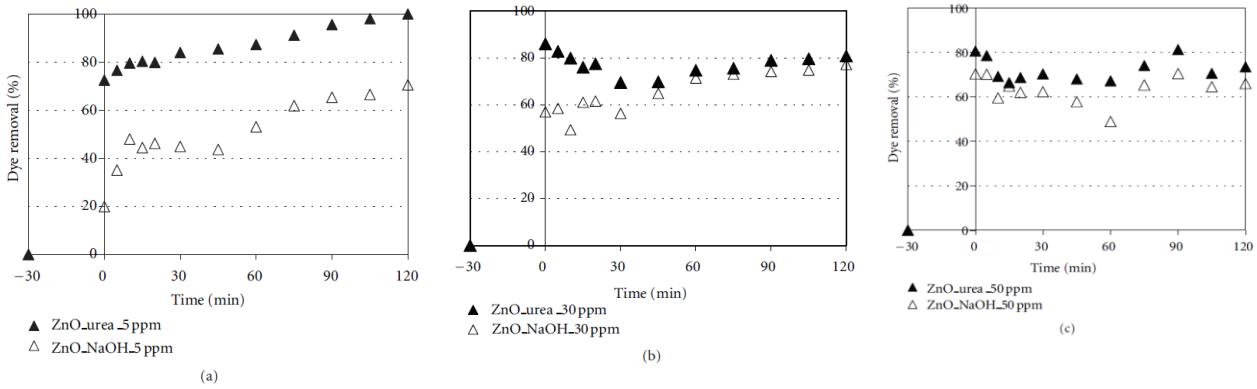

Figure 46. The R6G removal in time at 5, 30, and 50 ppm

In order to compare the performance in the photoreaction of our ZnO samples with the results obtained on Titania, we performed two experiments on both ZnO samples, Figure 47.

The R6G removal on TiO_2 indicates its different adsorptive behavior in function of the pH value. There is a clear higher adsorption degree at pH=7 than at pH=10, but the photocatalytic reaction brings quite similar removal degrees for both pH values, over 80% for both cases.

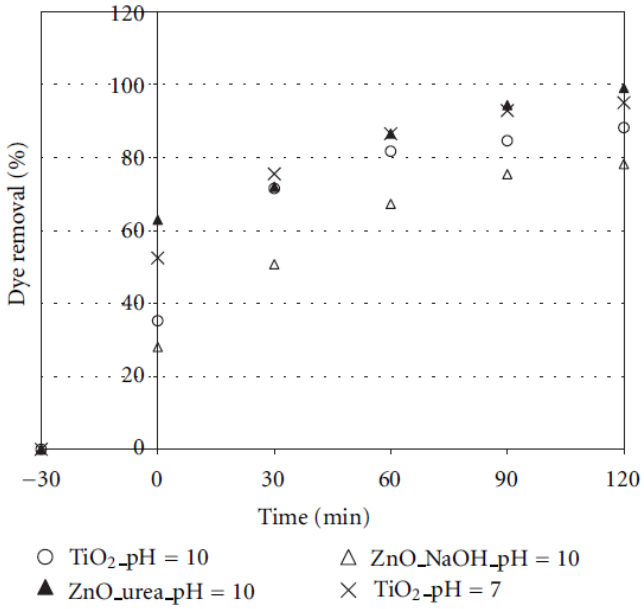

Figure 47. Comparison between R6G removal on ZnO and TiO_2

II.5.1.3. Discussions

Various technologies have been developed for the advanced treatment of pharmaceutical industry wastewater containing pollutants that cannot be removed by conventional treatment processes. Destructive oxidation methods such as heterogeneous photocatalysis (TiO_2/UV-A), ozonation, H_2O_2/UVB, photo-Fenton, and sonolysis are considered very attractive since they transform hazardous pollutants into compounds with a reduced impact on the environment (Caliman et al., 2017).

Also, among advanced oxidation processes, homogeneous and heterogeneous photochemical oxidation processes have proven their efficiency in the degradation of refractory organic pollutants (Kaniou et al., 2019).

Photocatalysis implies the acceleration of a photoinduced reaction by the presence of a catalyst. Photoinduced reactions are activated by the absorption of a photon with sufficient energy that is equal to or higher than the band-gap energy (E_{bg}) of the catalyst (Carp et al., 2004).

The most intensively species used as photocatalyst is the titanium dioxide is widely available, green, and costless. Other oxidic species such as ZnO, WO_3, Fe_2O_3, SnO_2 and combinations thereof as photocatalysts were investigated by researchers (Fujishima et al., 2000; Hashimoto et al., 2007; Nakata and Fujishima, 2012; Anandan et al., 2012; Tian et al., 2012).

Five types of catalysts were investigated in this study: TiO_2P-25, TiO_2UV-100, TiO_2-A, TiONa and ZnO.

The corresponding amounts of catalyst were added to 10 ppm solutions of DPC in order to obtain a dose of 0.5 g catalyst/L and then irradiated with UV-A light. The TiO_2P-25 catalyst

exhibited the best behaviour in the degradation of DPC and was thus considered the reference catalyst. The increase of the TOC values in the case of the ZnO catalyst might be assigned to desorption of DPC from the ZnO surface, taking into consideration that the adsorption of DPC on this material is less intense in comparison with the other photocatalysts.

The most advanced DPC degradation occurred when heterogenous photocatalysis was conducted eith a higher dose of the catalyst (TiO_2P-25). The positive effect of acidic pH on DPC degradation can be assigned to the fact that the TiO_2 surface is positively charged when the solution pH is lower than 6.8, thus facilitating the photocatalytic process (Belver et al., 2019).

The next step of this study focused on enhancing the photocatalysis reaction by adding H_2O_2 or Fe^{+3} in different concentrations to the initial solution. The DPC degradation during photocatalysis with TiO_2P-25, facilitated by H_2O_2 and Fe^{+3} respectively was investigated. The best degradation rate was obtained when the TiO_2P-25 photocatalysis was used alone. It was noted that no enhancement was obtained by adding H_2O_2 or $Fe^{+}3$ at any dose.

In the case of the photo-Fenton reagent, the best results were obtained when less oxidant was used: 50 ppm H_2O_2 and 3.5 ppm $Fe^{+}3$. When more photo-Fenton agent was used, lower values for DPC decontamination were obtained. With the ferrioxalate reagent, an excellent degradation rate was obtained when visible light was used, this being economically reliable. When using the UV-A lamp, photodegradation results were comparable with those achieved for photo-Fenton homogeneous catalysis.

On the other hand, in the case of R6G decontamination the XRD patterns of the samples ZnO-NaOH and ZnO-urea indicate well-crystallized solids, exhibiting the main peaks due to zinc oxide phase, as filed in JCPDS and no impurity phases (Barros et al., 20016). The peaks are somehow sharper and higher for the sample obtained with urea, indicating a more uniform and better-crystallized solid. The particle medium size, considering the factor K=0.94, gave values of 21.5 nm for the sample ZnO-urea and 14.4 nm for ZnO-NaOH. These particle sizes represent the dimensions of the primary particles, which associate in groups of several hundreds of nanometers, as shown on the SEM images. The grouping of the particles keeps a relatively uniform aspect of the bulk powder. The agglomerations of ZnO-urea particles are almost spherical in shape and still keep a big free volume fraction in between, which is beneficial for the organic diffusion to the solid surface during the adsorption step. The ZnO-NaOH sample consists of grain associations with more planar shape and have the tendency to agglomerate very tightly, offering a smaller interparticle free space.

The IR transmission spectra of our samples were compared with those of a commercial zinc oxide, the spectra for the samples synthesized in our laboratory are quite similar and in line with the literature data (https/, 2019). Only small differences in peaks intensities are noticed for our two samples. The peaks around 3600 cm^{-1} indicate the presence of OH groups derived from water adsorption, while the one at 1636 cm^{-1} is due to the characteristic H_2O scissoring mode (Noei et al., 2018). The peak from 2345 cm^{-1} is due to the CO_2 adsorption on the basic sites of ZnO and is missing in the commercial sample (Pragati, 2010).

The BETspecific surface areas, found to be 17.3 m^2 g^{-1} for ZnO-urea and, respectively, 13.04 m^2 g^{-1} for the ZnO-NaOH sample. The slow precipitation leads to higher specific surface areas, as expected. The isotherms of type 3 according to IUPAC, indicates low porosity solids, with a very narrow hysteresis loop. No significant internal porosity is found on these samples.

In the photocatalytic experiments it was revealed the influence of catalyst concentration. In a searching reviews process similar behavior for rhodamine B has been communicated by other researchers (Natarajan et al., 2011; Fan et al., 2012; Chen et al., 2012).

In our study for all the tested photocatalyst samples, the same characteristic for the removal of R 6G was noticed. They strongly adsorb R6G in the equilibrium period of 30 minutes before the exposure to UV light; more than 60% of the dye is removed from the solution in all the tested mixtures. The photocatalytic reaction follows a slow increasing slope. The values of the total removal degree are similar for both samples, over 80% in 2 hours of irradiation. Since the removal degree of R6G on the photocatalyst in the medium containing 0.3 g catalyst per liter of R6G solution is very close to the ones in the system with 0.5 g catalyst, we admit from this point forward the 0.3 g/L as the optimal concentration value.

In order to compare the performance in the photoreaction of our ZnO samples with the results obtained on Titania, we performed two experiments on both ZnO samples at pH=10 and on P-25 at pH values of 7 and 10, using an initial solution of 30 ppm R6G at a photocatalyst concentration of 0.3 g/L.

The R6G removal on TiO_2 indicates its different adsorptive behavior in function of the pH value. There is a clear higher adsorption degree at pH=7 than at pH=10, but the photocatalytic reaction brings quite similar removal degrees for both pH values, over 80% for both cases. The performance of the ZnO-urea is closely similar to that on P-25. Since the stability at high pH values is better for ZnO than for TiO_2, our photocatalyst could be an alternative to the well-known P-25, for the photocatalytic reactions performed in basic solutions for rebel compounds decomposition. The values show that the activity of ZnO-urea sample at alkaline pH is close to that of TiO_2 at pH values lower with about 3 units.

II.5.1.4. Conclusions

The study demonstrated the possibility of oxidative degradation of persistent organic DPC by heterogeneous photocatalysis, nearly complete degradation of DPC being achieved after about 180 minutes in the presence of an acid medium (pH=3) using a UV-A lamp and the TiO_2P-25 catalyst (0.5 g/L), for an initial DPC concentration of 10 ppm.

The remnant DPC concentrations were higher when homogeneous photocatalytic oxidation was involved, and the degradation rates were lower compared to those corresponding to the use of TiO_2 as the photocatalyst.

In this way, the remnant concentrations of DPC were below the levels for acute and chronic risk range for infants and children (6 ppm), but as a consequence of the heterogeneous

photocatalysis degradation process, this remnant concentration was about 10 times lower than the above mentioned level.

Our study succeeded in achieving the task of finding a suitable photocatalytic system to provide a DPC remnant concentration in accordance with EPA standards.

The zinc oxide prepared in urea medium led to the obtaining of a ZnO-urea sample with high capabilities of R6G removal from slightly basic aqueous media. The study of parameters influencing the decolorizing process allowed us to find the operating conditions for achieving the highest R6G removal rate: pH values over 8.5, up to 10; catalyst concentration 0.3 g/L; initial R6G concentrations higher than 30 ppm. A strong initial adsorption is noticed on ZnO samples, followed by a slow photocatalytic reaction on ZnO.

The global performance in terms of R6G removal is comparable with that obtained on P-25 Titania, known as the most typical and widely applied photocatalyst.

II.5.2. Adsorption technology and chemical transformation of byproducts from pharmaceutical industry

Cleaner production and other preventive initiatives, such as eco-auditing and green technologies, are one of the main measures being used to advance sustainable development in the pharmaceutical industrial sector (Nowotny et al., 2018). This strategy focuses on processes as conservation of energy and raw materials, elimination of toxic compounds and reducing emissions, byproducts and waste (Fu et al., 2018).

Clean technology consists of conversion of byproducts in recycled products required on the market and simultaneously solving an issue related to the management of technological residual solutions (Zhai et al., 2011; Gbededo and Liyanage, 2018).

The pharmaceutical industry converts raw materials (plants, natural organic or inorganic substances, microorganisms, cells fragments) into more than 70,000 different products used in our daily life.

Different physical, chemical and biological methods have been used to remove byproducts from industrial wastes (Vishwakarma et al., 2012; Kerkez et al., 2014).

In this line, ferrites were proposed as potential adsorbents for byproducts removal due to their appropriate physical characteristics and facile separation under external magnetic fields (Zhang et al., 2014). There were evaluated the adsorption capacity of three representative magnetic spinel ferrites with the general formula MFe_2O_4 (M=Ni, Co, Zn), obtained as nanosized particles by sol-gel autocombustion method. The adsorption of Congo-Red (CR) has been investigated via kinetics, equilibrium and thermodynamic approaches.

Also, chemical methods (catalytic processes) have been used to remove byproducts from pharmaceutical industrial wastes. A very large number of potentially useful catalysts have been used to accelerate the rate of transesterification of oils, including enzymatic bio-catalysts, homogeneous and heterogeneous acid or base catalysts. Vegetable oil, soybean oil, jatropha oil,

sunflower oil, rapeseed oil and other fats from wastes represent a promising raw material for industrial sector (Popp, et al., 2016).

Zeolites have found promising application in biomass conversion and wastewater treatment (Li et al., 2017). We focused our study on zeolite-based catalysts prepared by the impregnation method, for sunflower vegetable oil conversion from wastes. The effects of operating and processing variables such as reaction temperature and time were investigated.

Personal contribution to knowledge in this research field is presented in following published papers:

- Samoilă P, Cojocaru C, Crețescu I, Stan CD, Nica V, Săcărescu L, Harabagiu V. Nanosized Spinel Ferrites Synthesized by Sol-Gel Autocombustion for Optimized Removal of Azo Dye from Aqueous Solution. *Journal of Nanomaterials* 2015; DOI: 10.1155/2015/713802.
- Juzsakova T, Al-Jammal N, Crețescu I, Sebestyén V, Le Phuoc C, Domokos E, Rédey A, Stan CD. Case Studies for Clean Technology Development in the Chemical Industry Using Zeolite Based Catalysts. *Minerals* 2018; 8(10): 462-456.

II.5.2.1. Materials and methods

Reagents and Materials

Analytical grade $Ni(NO3)_2 \cdot 6H_2O$, $Co(NO_3)_2 \cdot 6H_2O$, $Zn(NO_3)_2 \cdot 6H_2O$, $Fe(NO_3)_3 \cdot 9H_2O$, citric acid monohydrate and Congo-Red were purchased from Sigma-Aldrich and used without further purification.

Synthesis of nanosized spinel ferrites sorbents

MFe_2O_4 (M=Ni,Co, Zn) spinel ferrite materials were prepared by sol-gel autocombustion method (Tan et al., 2012). Stoichiometric amounts of metal nitrates were dissolved in distilled water with the molar ratio $M^{2+/}Fe^{3+}$ of 1:2. The molar ratio of metallic cations to citric acid was 1:1. The aqueous solution of citric acid was added to the solution of nitrate salts and heated to 353K on a water bath under stirring until a viscous gel was formed. The gel was gradually heated to 623K when the autoignition was clearly observed. The resulting powder was sintered in two steps (at 773K/5 h and at 973 K/5 h) in order to achieve the spinel phase formation.

Characterization techniques

XRD patterns of the powder samples sintered at 973K were recorded using a Shimadzu LabX 6000 diffractometer equipped with graphite monochromator and CuKα (λ = 0.15406 nm) radiation. The samples were scanned from 20 to 80° (2θ) using a scanning rate of 0.02°/s.

The formation of spinel phase of MFe_2O_4 ferrite was monitored by infrared spectroscopy in 4000–350 cm^{-1} range using a BrukerVertex 70 FTIR spectrometer with a resolution of 2 cm^{-1} (KBr pellets technique). The morphology and microstructure of ferrite samples sintered at 973K

were investigated using Hitachi High-Tech HT 7700 transmission electron microscope, operated at 100 kV accelerating voltage in high-contrast mode.

Adsorption Experiments

A stock solution of 200mg/L was prepared by dissolving CR in distilled water. The working solutions were prepared by diluting the stock solution to the desired concentrations. The concentrations of CR in the solutions were analyzed using UV-Vis spectrophotometer (Shimadzu UV-1700 PharmaSpec) by monitoring the absorbance at the wavelength of λmax = 497 nm. The prepared ferrites were applied as adsorbents for the removal of CR from the aqueous solutions using the batch technique. For this purpose, calculated amounts of adsorbents were added to 50mL of working solutions and the samples were magnetically stirred at 500 rpm for different periods of time.

The adsorption experiments were carried out at naturally occurring pH 6.80 and at the different temperatures (i.e. 293K, 313 K, and 333 K). At the end of the adsorption tests the loaded ferrites were separated using a magnet and the resulting clear solutions were analyzed for CR concentrations. In all the experiments (screening test, kinetics, isotherms, and optimization) the adsorption capacity was determined using the following equation (Tan et al., 2012):

$$q = \frac{(C0 - C) \cdot V}{m \cdot 1000}$$

where q denotes the adsorption capacity (mg/g), $C0$ is the initial concentration of CR in solution (mg/L), C is the final concentration of CR in solution (mg/L), V is the volume of solution (mL), and m represents the weight of the adsorbent (g). In addition, the color removal efficiency Y (%) was determined by subsequent equation (Zhu et al., 2007):

$$Y = \left(1 - \frac{C}{C0}\right) \times 100$$

Transesterification of vegetable oils

A series of transesterification reaction were performed. Four different reaction temperatures were selected: 30, 40, 50 and 60^0C. The stirring speed ranged between 200-1200 rpm and the particle size of the catalyst varied between 125-500 µm. The amount of catalyst was 2.1 wt%, 4.3 wt% and 6.4 wt % at a methanol-oil molar ratio of 11.5.

The reaction time was between 1-5 h. The waste vegetable oil conversion was calculated as the amount of the converted vegetable oil related to the vegetable oil fed. The biodiesel yield was defined as the ratio of the mass of biodiesel formed to the mass of initial oil feedstock.

The zeolitic tuff was obtained from the Jordanian Natural Resources Authority (NRA),

after being collected from the Jabal-Aritayn site (30 km northeast of Azraq) south of Jordan (Al-Jammal et al., 2016).

The analytical methods and/or devices used were carried out according to international standards (Zahan and Kano, 2018). All tests were done in triplicate and the averaged values were adopted. A Varian 3300 chromatograph equipped with FID (Varian, Inc., Walnut Creek, CA, USA) was used to determine and quantify all the individual components of the used oil (fatty acid distribution). Chromatographic separation was achieved by a 15% OV-275 on Chromosorb WAW, 80/100 mesh, 2-m length, 3.17-mm outside diameter, stainless steel column type.

The influences of different parameters on the waste vegetable oil conversion and biodiesel yield were investigated as a function of the reaction temperature. Conversion tests were implemented to follow the changes in the biodiesel yield (Al-Hamamre and Yamin, 2014). The biodiesel (methyl ester) dissolves easily in methanol, forming a clear bright phase, while vegetable or animal oils and fats (triglycerides) do not dissolve in methanol. However, monoglycerides and diglycerides (partly converted vegetable oil) are partially soluble.

The mass fractions for the individual components of the waste vegetable oil were obtained from the gas chromatographic analysis. The vegetable oil used for the experiments was mainly composed of 57.1 wt % linoleic acid and 28.2 wt % oleic acid. Other saturated fatty acids that were utilized included palmitic and stearic acids.

II.5.2.2. Results

Removal of CR from industrial wastes

XRD diffraction patterns of MFe_2O_4 (M = Ni, Co, Zn) spinel ferrites are shown in Figure 48. For all the analyzed materials, the most intense peaks characteristic for spinel structure were observed at 2θ of about 35^0.

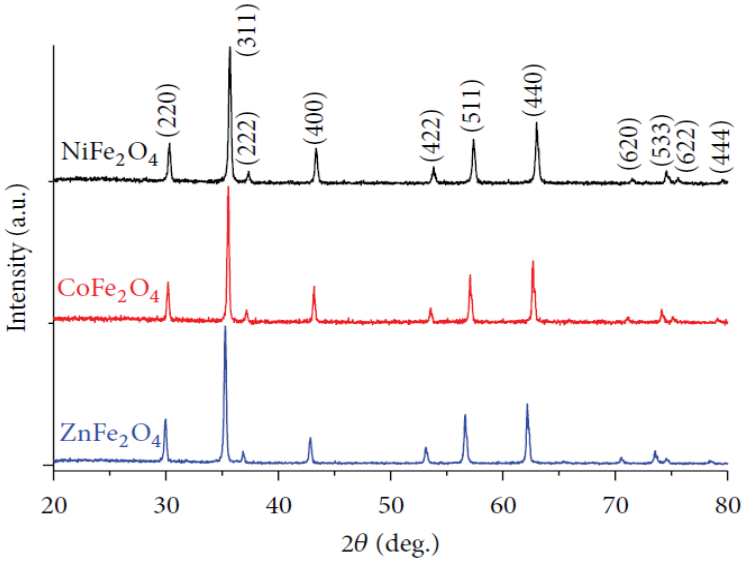

Figure 48. XRD patterns of MFe_2O_4 (M = Ni, Co, Zn) spinel ferrites

The crystallite size (D_c, nm) for each sample was calculated and the results are presented in Table 33.

Table 33. Experimental and calculated data obtained for MFe_2O_4 (M=Ni, Co, Zn) spinel ferrites

Spinel ferrite	[a] $2\theta_{311}$ (degrees)	[a] D_c (nm)	[b] $\langle d_p \rangle$ (nm)
$NiFe_2O_4$	35.68803	30.6	46
$CoFe_2O_4$	35.53905	36.7	69
$ZnFe_2O_4$	35.27044	34.4	101

[a]Diffraction angle (θ_{311}) and crystallite size (D_c), data from XRD; [b]particle size, data from TEM.

The FTIR spectra, in 4000–350 cm^{-1} range for MFe_2O_4 (M= Ni, Co, Zn) powders heated at 973K are shown in Figure 49. The FTIR spectra revealed only the specific bands for metal-oxygen stretching vibration from the tetrahedral site in 584–540 cm^{-1} range and from the octahedral site at 397–389 cm^{-1}.

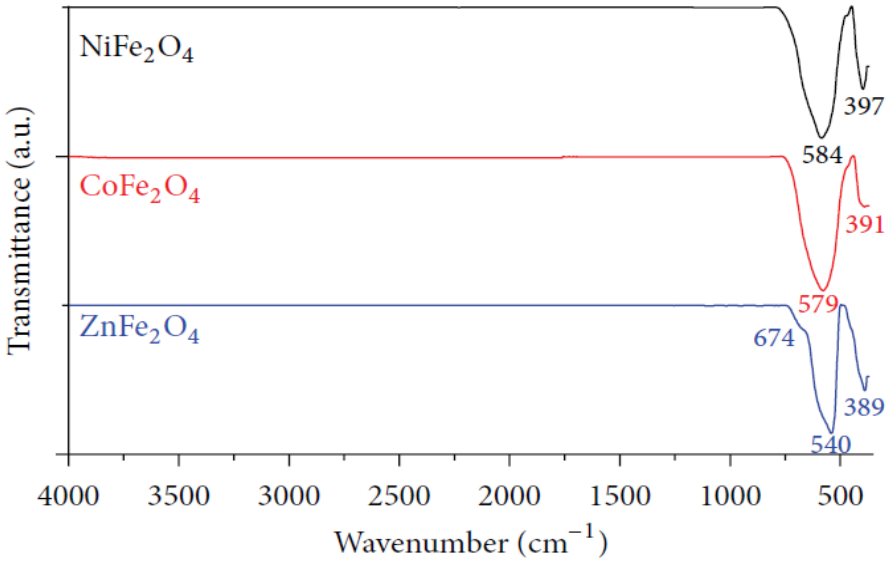

Figure 49. FTIR spectra of MFe_2O_4 (M = Ni, Co, Zn) spinel ferrites

The representative TEM micrographs of MFe_2O_4 samples are illustrated in Figure 50. The average particle sizes ($\langle dp \rangle$, nm) determined from microscopy images (TEM) are reported in Table 33. For all three studied materials, TEM images prove the formation of soft agglomerates constituted of nanosized particles with irregular (nanopolyhedra) shapes.

The obtained spinel ferrites were easily separated from aqueous solution by means of a permanent magnet. The magnetic separation of $CoFe_2O_4$ spinel ferrite from the aqueous solution is shown in Figure 50d (before separation) and Figure 50e (after magnetic separation).

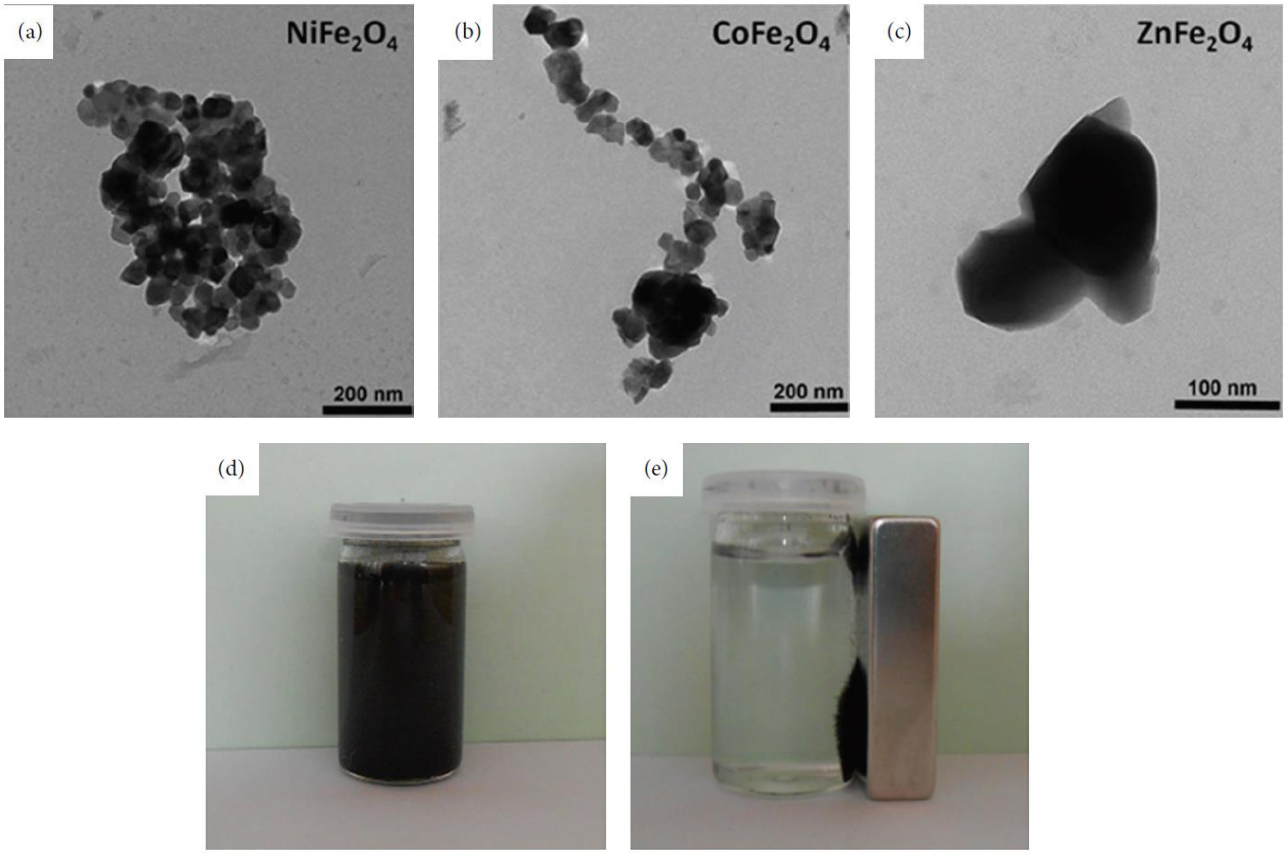

Figure 50. TEM micrographs of MFe_2O_4 (M=Ni, Co, Zn) spinel ferrites (a, b, c) and images (d, e) of magnetic separation of $CoFe_2O_4$ spinel ferrite from the aqueous solution with a permanent magnet: (d) before separation and (e) after magnetic separation

The objective of the screening test was to compare the performances of the ferrites for adsorption of Congo-Red from aqueous solutions under certain conditions. The results of the screening test are shown in Figure 51.The highest adsorption performances were attributed to the adsorbent $NiFe_2O_4$ followed by $CoFe_2O_4$.

The adsorptions of CR onto $NiFe_2O_4$ and $CoFe_2O_4$ ferrites were studied versus time at 293 K. Figure 52 shows the amount of the dye adsorbed q_t (mg/g) against the contact time t (min) for two sorbent materials that is $CoFe_2O_4$ (Figure 52a) and $NiFe_2O_4$ (Figure 52b).

The kinetic data suggest that $NiFe_2O_4$ ferrite is superior to $CoFe_2O_4$ for CR adsorption owing to higher values of adsorption capacity observed against the time.

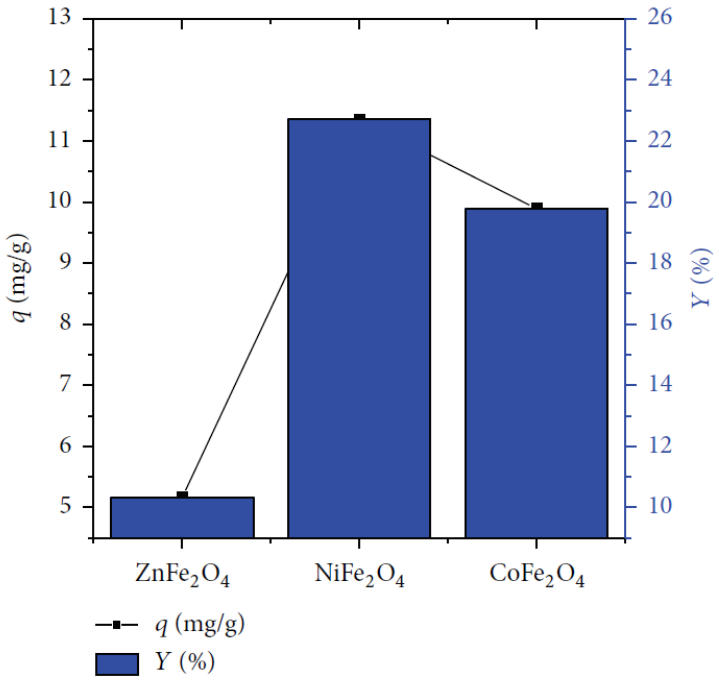

Figure 51. Screening test for CR adsorption using different ferrite adsorbents showing the adsorption capacity and color removal efficiency

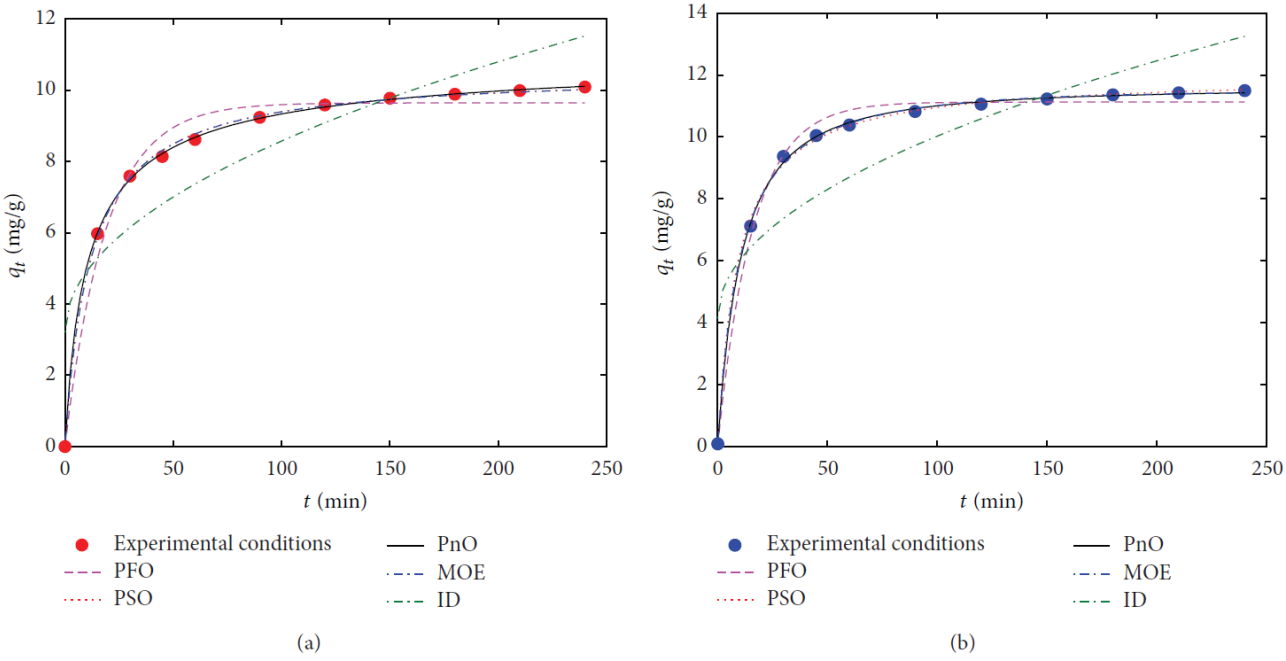

Figure 52. Adsorption kinetics data for Congo-Red removal by ferrite sorbents and comparison of kinetic models: (a) adsorbent $CoFe_2O_4$ and (b) adsorbent $NiFe_2O_4$; experimental conditions: C_0= 50mg/L, SD=1 g/L and T=293 K

Conversion of the vegetable oil from industrial wastes

The influence of several technological parameters on the conversion of the transesterification reaction and the biodiesel yield was investigated.

The measured kinematic viscosity of the used waste vegetable oil was 39.4± 0.20 mm^2/s at 40^0C, higher than the value reported in the literature (Leung et al., 2006) (35.3 mm^2/s at 40^0C).

In the present study, the peroxide value of waste vegetable oil was 13.02 mEq O_2/kg oil.

The zeolite catalyst was prepared from zeolitic tuff using two impregnation steps with heating encountered in the second step, according to the procedure described in the literature (Al-Jammal et al., 2016).

The analytical results (XRD) confirmed the presence of some salts in the raw zeolitic tuff. The specific surface area of the raw zeolitic tuff was 77 m^2/g.

The transesterification reaction was carried out using the prepared catalyst and according to the procedure described in the literature (Al-Jammal et al., 2016).

The results showed that the KOH-treated zeolitic tuff had the highest activity and therefore the further investigation refers only to this catalyst.

Transesterification can occur at different temperatures depending on the types of catalyst and alcohol used. However, high reaction temperatures accelerate the reaction (both transesterification and saponification reactions).

The influence of reaction temperature on the conversion of waste vegetable oil to biodiesel was investigated as a function of temperature.

The results are shown in Figure 53, which depicts the effect of temperature on the waste vegetable oil conversion at a methanol/waste oil molar ratio of 11.5:1 and a catalyst amount of 2.1, 4.3 and 6.4 wt %.

The increment of conversion was different depending on the amount of the catalyst that was used. The effect was more pronounced at higher catalyst amounts.

The effect of reaction temperature on biodiesel yield is shown in Figure 54 for the temperature range of 30–60^0C at 2 h reaction time and at 11.5:1 methanol/oil molar ratio.

In the presence of higher amount of catalyst (6.4 wt %), the biodiesel yield increased from 53.0–96.7% by increasing the temperature from 40–50^0C at 2 h of reaction time.

The yield decreased to 77.8% at 60^0C. Therefore, 50^0C was chosen as the optimum reaction temperature.

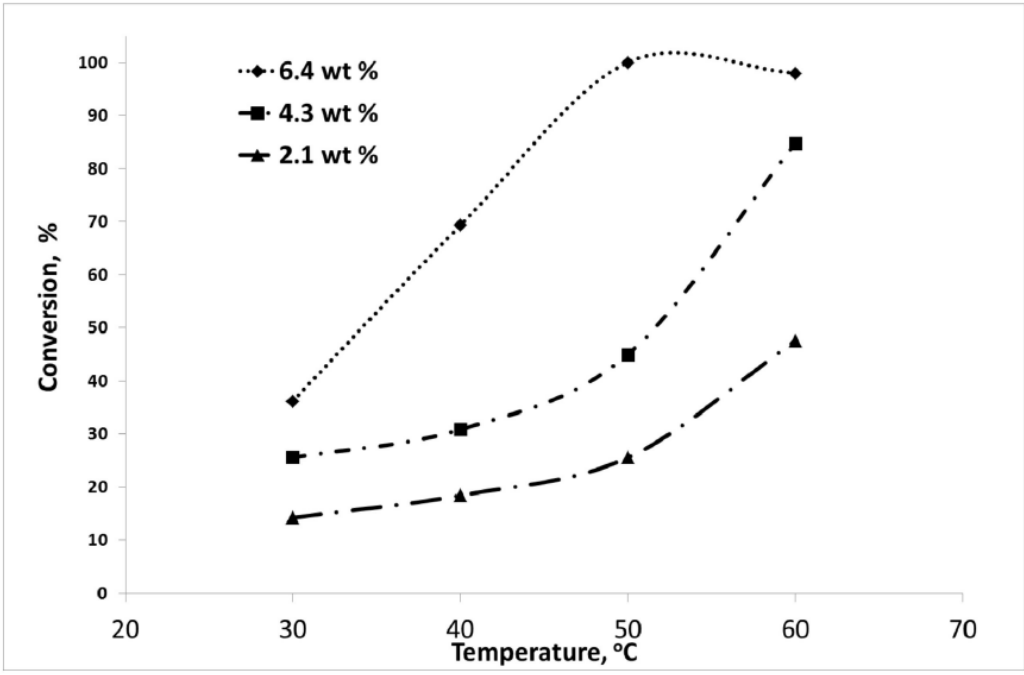

Figure 53. The effect of temperature on waste vegetable oil at 30, 40, 50 and 60°C

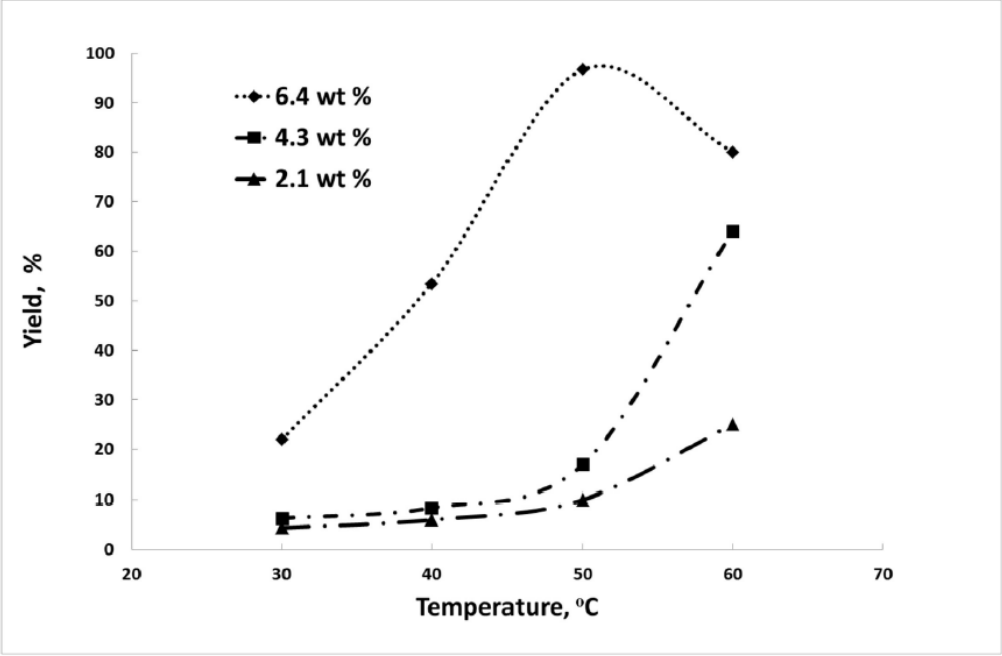

Figure 54. The effect of temperature on biodiesel yield at the at temperatures of 30, 40, 50 and 60 °C

II.5.2.3. Discussions

It is difficult to remove the dyes from the industrial waste, because dyes are not easily degradable. Congo-Red is extensively used as coloring agent indifferent industries including biomedical industries, biochemistry laboratories etc.

Different physical, chemical and biological methods have been used to remove byproducts from industrial wastes (Vishwakarma et al., 2012; Kerkez et al., 2014). Among physical methods, adsorption is very often employed for effective treatment of wastewater. The adsorption consists in attaching of soluble pollutants on a solid material (organic or inorganic support). Therefore, different sorbents such as activated carbon, coal, fly ash, clays, silica, alumina and chitosan have been employed in adsorption studies (Saroj et al., 2014; Sudha et al., 2014).

Despite their good adsorption performances, the separation and recovery of these adsorbents from heterogeneous systems still remain a drawback. Recently, the magnetic separation technology has attracted attention as a feasible alternative for traditional separation technologies such as settling, centrifugation, or membrane filtration (Khosravi and Eftekhar, 2013; Narkiewicz, 2013).

In this line, ferrites were proposed as potential adsorbents for byproducts removal due to their appropriate physical characteristics and facile separation under external magnetic fields (Zhang et al., 2014).

Our study evaluate the adsorption capacity of three magnetic spinel ferrites MFe_2O_4 (M = Ni, Co, Zn), obtained as nanosized particles by sol-gel autocombustion method. The adsorption of Congo-Red has been investigated via kinetics, equilibrium and thermodynamic approaches, paying attention to the response surface modeling and optimization of the adsorption process to enhance the removal efficiency.

As is cited in literature the the spinel ferrite were prepared by sol-gel autocombustion method (Samoila et al., 2012).

Characterization of MFe_2O_4 spinel ferrites sorbents were done by Xray diffraction patterns. For all the analyzed materials, the most intense peaks characteristic for spinel structure were observed at 2θ of about 35^0.

All peaks observed in the XRD patterns correspond to the face-centered cubic structure of pure spinels according to JCPDS cards number 44-1485, number 22-1086, and number 22-1012. The crystallite size (Dc, nm) for each sample was calculated and the values of crystallite size depend on M^{2+} cation nature. The smallest nanosized crystallite value was found for nickel ferrite (30.6nm), followed by zinc ferrite (34.4 nm), and cobalt containing ferrite (36.7 nm), respectively.

The FTIR spectra revealed only the specific bands for metal-oxygen stretching vibration from the tetrahedral site in 584‑540 cm^{-1} range and from the octahedral site at 397‑389 cm^{-1}. Careful observation of the$ZnFe_2O_4$ IR spectra shows a supplementary third absorption band at

674 cm^{-1}. The presence of this band is explained by the cation exchange between the tetrahedral and octahedral spinel sites which often occurs for zinc ferrite (Zhu et al., 2007; Slatineanu et al., 2012).

The average sizes of individual particles are of 46 nm, 69 nm, and 101nm for nickel, cobalt, and zinc ferrites, respectively (Table 33). Note that from XRD patterns only crystallites sizes can be evaluated. In turn, the microscopy techniques are employed to determine the particle sizes and morphologies (every particle is formed by a number of crystallites). In many cases there is no direct correlation between crystallite size (determined from XRD) and particle size (determined from TEM).

In the adsorbtion screening test it were obtained the performances of ferrites for adsorption of Congo-Red from aqueous solutions in different conditions. It was revealed that the highest adsorption was obtained for $NiFe_2O_4$.

The spinel ferrite $ZnFe_2O_4$ disclosed the lowest sorption performances (Figure 51). The higher adsorption capacities were correlated with the smaller particle size of ferrites determined from TEM analysis. Therefore, the spinel ferrites $NiFe_2O_4$ and $CoFe_2O_4$ with better sorption performances were used for the kinetics and isotherms studies.

From kinetic studies, the contact time has been fixed at180 min to attain the stationary adsorption (equilibrium). Figure 52 shows the amount of CR adsorbed at equilibrium versus the equilibrium concentration for both sorbent materials $CoFe_2O_4$ (Figure 52a) and $NiFe_2O_4$ (Figure 52b).

Several kinetic models have been employed in this study to fit the adsorption kinetics data, namely:

(1) pseudofirst order (PFO) kinetics (Ho, 2004),

(2) pseudosecond order (PSO) kinetics (Ho, 2006),

(3) pseudo-n-order (PnO) kinetics (Ozer, 2007; Azizian and Fallah, 2010),

(4) mixed 1,2-order (MOE) kinetics (Marczewscki, 2010; Fallah and Azizian, 2012),

(5) intraparticle diffusion (ID) kinetics (Weber and Morris, 1963; Constantin et al., 2013).

The predictions given by kinetic models have been plotted as solid, dashed and dot lines (as it can see in Figure 52). To determine the goodness-of-fit between models predictions and experimental data the average relative error (ARE) function has been calculated, as is cited in literature (Ncibi, 2008).

As one can see from Figure 52, the best-fitting kinetic data is given by PnO model. Likewise, the PSO and MOE models approximate quite well the kinetic data with an average relative error less than 1% (ARE<1). The kinetic model (PFO) fits satisfactorily the observations with the average relative errors of 4.3% and 2.6% for $CoFe_2O_4$ and $NiFe_2O_4$, respectively. The intraparticle diffusion (ID) model shows some discrepancy between the predicted data and experimental observations with the errors of 10-11%. The kinetic data suggest that $NiFe_2O_4$ ferrite is superior to $CoFe_2O_4$ for CR adsorption owing to higher values of adsorption capacity observed against the time.

In the pharmaceutical industry, clean technological processes have a positive impact on local, regional and global environmental issues (Vaccaro, 2016). The pharmaceutical/biomedical industrial sector includes companies that produce drugs, biochemicals and tools for biomedicine.

Transforming waste vegetable oil, soybean oil, jatropha oil, sunflower oil and rapeseed oil, as well as other fats, in a renewable fuel, like biodiesel, by various methods represent a clean technology (Popp et al., 2016). Waste vegetable oils represent a promising raw material for biodiesel production via the transesterification of triglycerides of oils using alcohol, in the presence of a catalyst (Loreto et al., 2005; Andrade et al., 2011). In the transesterification reaction alcohol is reacted with triglycerides using the catalyst to result in fatty acid esters and glycerin, unharmful substances.

Industrial waste management in the pharmaceutical industry is an important aspect that should be taken into consideration as the main clean technology related to the pollution prevention and improving the recycling of the waste products (Walsh et al., 2016).

The conversion of the transesterification reaction and the biodiesel yield was investigated by measuring kinematic viscosity of the used waste vegetable oil. In the literature (Leung et al., 2006) were reported values smaller than ours 39.4± 0.20 mm^2/s at 40^0C. The test result for the acid value was found to be 2.01 mg KOH/g for the waste vegetable oil. The resulting acid value of the waste vegetable oil was on the lower side compared to the maximum value recommended for oils to be used for biodiesel production by alkali transesterification. The peroxide value of waste vegetable oil was 13.02 mEq O_2/kg oil. A lower peroxide value was reported for sunflower oil (Goering and Fry, 1984), namely 10.7 mEq O_2/kg oil. For the biodiesel sample, the measured peroxide value was 44.38 mEq O_2/kg biodiesel.

The zeolite catalyst was prepared from zeolitic tuff in accord cu literature procedure (Al-Jammal et al., 2016). The specific surface area of the raw zeolitic tuff was 77 m^2/g. The KOH-treated zeolitic tuff had the highest activity.

The transesterification reaction was carried out using the prepared catalyst KOH-treated zeolitic tuff and according to the literature (Al-Jammal et al., 2016).

Transesterification can occur at different temperatures depending on the types of catalyst and alcohol used. It was observed at a catalyst amount of 2.1 and 4.3 wt % that the conversion of waste vegetable oil increased as a function of the reaction temperature. The increment of conversion was different depending on the amount of the catalyst that was used. The effect was more pronounced at higher catalyst amounts. In the case of a catalyst amount of 6.4 wt %, by increasing the reaction temperature from 30–50^0C, the conversion of waste vegetable oil increased from 35–100%. By increasing the reaction temperature to 60^0C, the oil conversion slightly decreased.

Significant difference in conversion values was observed when the amount of catalyst was increased from 2.1, to 4.3, to 6.4 wt % and the temperature was changed. This can be explained by the mutual interaction between reaction parameters. It is worthy to mention here that was proved the mutual interaction between the reaction temperature and catalyst weight and

the effect of this interaction on biodiesel yield. Such a kind of interaction among the reaction parameters was investigated by several studies and it was proven by the response surface methodology (Ayoola et al., 2017).

At higher reaction temperatures, there was a slight reduction in the biodiesel yield. It should be noted that a reaction temperature near or above the boiling point of alcohol will result in a yield decrease due to the fact that higher temperatures can accelerate the saponification reaction of glycerides before the completion of the alcoholysis. These results are in agreement with previous reported work (Dorodo, 2012; Eevera et al., 2009).

The optimized reaction temperature for the transesterification reaction obtained by previous studies was higher than the value found in this study. For example, NaX zeolite loaded with KOH was used as the base heterogeneous catalyst for the transesterification reaction, and the achieved conversion was 85.6% at 120^0C at a reaction time of 8 h (Xie et al., 2007). In fact, limited studies have been conducted to investigate catalyst performance at temperatures lower than 70^0C (Intarapong et al., 2011).

The optimal yield of biodiesel was achieved with a MeOH/oil molar ratio of 11.5, a catalyst amount of 6.4 wt % and at 50^0C reaction temperature. A biodiesel yield of approximately 96.7% was achieved when the reaction time was adjusted to 2 h.

Increasingly, industrial companies are aware of the fact that environmentally-friendly operation is a core management issue and not just a matter of compliance with regulations.

II.5.2.4. Conclusions

The nanosized spinel ferrites MFe_2O_4 (M=Ni, Co, Zn) were obtained by sol-gel autocombustion method. The obtained ferrite materials were applied for Congo-Red adsorption from aqueous solutions. The screening test revealed that the most efficient adsorbents for CR removal were $NiFe_2O_4$ followed by $CoFe_2O_4$. The maximum adsorption capacity was 14.06 mg/g for $CoFe_2O_4$ and 17.13 mg/g for $NiFe_2O_4$.

The naturally-occurring zeolites and the synthetic zeolites can be efficiently used in several processes. Therefore, special attention should to be paid to mitigating environmental pollution and to waste management in the pharmaceutical industry.

Clean technologies have larger applications in the pharmaceutical industry, with the objective of contributing to the implementation of the goals of sustainable development. In the case of pharmaceutical industrial companies the application of cleaner technologies is one of the key priorities for the future.

SECTION II

FUTURE DIRECTIONS IN SCIENTIFIC, PROFESSIONAL AND ACADEMIC ACTIVITY

Any *researcher* who aims discovery needs to be both systematic and flexible. It needs to be systematic in order that the chances of discovery are maximized and not left solely to luck or ”happy accidents”.

Keeping in mind this idea, after obtaining my PhD degree I was involved in a wide range of research topics, from which I can distinct two major directions, obtaining new molecules with therapeutic potential and pharmaceutical industrial process optimization. My future activity will be a continuation and also an extending in the research area of new drugs obtaining. I will continue to improve my research skills in order to participate in grants competitions, which allow me to update my research topics and also my equipments.

To gain better research results needs a multidisciplinary and interdisciplinary team, so futher collaboration with Research National Institutes and Universities are mandatory. Hence, I intend to extend my collaboration with colleagues and develop collaborative networks to maximize the opportunities of partnerships.

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 spectral characterization of new pharmaceutical compounds with therapeutic activity

The main objective of this direction will be the design, synthesis and characterization of new therapeutic compounds with potential biological actions such as: antioxidant, anti-inflammatory, antimicrobial, antitumor, neuroprotective.

With the help of advanced drug discovery, I will use natural, synthetic and semi-synthetic raw material in order to obtain new entities or derivatives of these. The obtaining of such compounds allows for improving the biological activities, so they can be used as a better alternative. The final goal is to extend therapeutic properties of the molecules to ensure the safety of the compound, to maximize the benefit–risk ratio, to minimize the adverse events, to reduce the effective doses and so to reduce the undesired toxic effects of pharmaceutical substances.

Drug design process involves high throughput screening in order to obtain the lead compounds against specific targets, so in order to improve their selectivity, I will modify these compounds as per the expected structure-activity relationships. I will use different design techniques to maximize the diversity of structure databases or combinatorial libraries.

In this line, nowadays, heterocyclic systems is a major concern for researchers, including the β-lactam heterocycles which drew attention and interest of scientists for their potent

antibacterial activity. The research in this area could be extended and stimulated, novel functionalized β-lactams being explored.

Structure validation is a key step in drug design process. Among the clasical techniques I will focus on novel technology, such as: ^{1}C-HNMR, thermogravimetric analysis, crystal structure validation, X-ray crystallography etc.

Future directions involve chemical farmaco-modulation by molecular *docking studies*, in order to improve biological properties of the molecules. The docking process involves two basic steps: prediction of the ligand conformation as well as its position and orientation within these sites (usually referred to as *pose*) and assessment of the binding affinity.

Biological evaluation of therapeutic potential of new designed molecules

I will focus on the clinical and biochemical investigation of the new compounds. It is very important in the drug design process of a new molecule to test its toxicity by determining the acute toxicity in animal models, methods that can be done in the near future.

Also, the *in vivo* oxidative stress evaluation will be in my attention as one of the research direction by determining the levels of catalase, superoxide dismutase, gluthatione peroxidase and malondialdehyde levels for the future new synthesized compounds.

For the compounds with antitumoral properties a powerful tool in tumoral research are tumor cell culture techniques. Traditional cell culture methods use a two-dimensional (2D) monolayer. By continuous improvements, this method has become a standard technology in life sciences at present. However, it fails to correctly imitate the architecture and microenvironments of *in vivo*, which makes 2D-cultured cells different from cells growing *in vivo* in terms of morphology, proliferation, cell-cell and cell-matrix inter-connections, signal transduction, differentiation and other aspects. In order to improve these simulations of cell microenvironments *in vivo* the 3D culture has become the next frontier of biology research and such approach could be done with the help of a multidisciplinary team.

For pharmaceutical substances with anti-inflammatory potential will be interesting to evaluate the anti-inflammatory action through carrageenan-induced rat paw acute edema model and also cotton pellet-induced granuloma model in rats. The last one test is a method used in experimental pharmacology to evaluate the proliferative and transudative component of chronic inflammation.

Biochemical and haematological parameters evaluation could be a research topic for the newly compounds, meaning: alaninaminotransferase (ALT), aspartataminotransferase (AST), lactat dehidrogenase (LDH) and bilirubine seric levels for the investigation of liver function; LDL, HDL total cholesterol and triglycerides for lipidic profile determination; investigation of kidney function – urea, uric acid and creatinine levels; harmatotological parameters evaluation.

Also, histopathological studies can be done in order to investigate the significant histological alterations of different organs for these new compounds.

Improvement and optimization of pharmaceutical industrial processes

The current trends in nanomedicine is nanoparticle obtaining. For this research direction I intend to diversify the type of the obtained nanoparticles which are non-toxic carriers for active substances.

Nanoparticles are used in the pharmaceutical industry for the transportation of diverse molecules to the site of action and released the active substance in order to achieve action. The chemical stability as well as the relative low cost of the materials used to prepare nanoparticles made these vesicles attractive for the industrial production, both in pharmaceutical and cosmetic applications. The ideal material for encapsulation should have good rheological properties, good dispersing capacity of active substances, lack of chemical reactivity with encapsulated substances, the ability to complete release the active compounds, under different conditions, solubility in non-toxic solvents.

Microencapsulation of active substances is of major interest in the controlled release range, due to the relatively light microparticle design and reproducibility of the process.

The new obtained systems will be physico-chemically characterized by various methods (UV-Vis, FT-IR, NMR, thermal methods, TEM, SEM, EDX) and then will be tested *in vitro* (the release and dissolution kinetics, their stability, various biological activity determination).

In the last decades it has become increasingly clear that the pharmaceutical industry faced with serious environmental problems. The wastes generated by pharmaceutical companies have increased concerns about environmental and human safety. My future research in this area will focus on pharmaceutical wastes management through photocatalytic/ magnetic/adsorbent processes and chemical transformation of byproducts. All these data will be used by the pharmaceutical industry to drive the greening of the pharmaceutical industry.

The obtained results will be published in ISI journals with impact factor and will be presented at national and international scientific conferences. Moreover, I want to continue to publish books at 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

As a teaching activity coordinator, the goal is to form and develop a team, in order to deliver quality education to our students in areas such as drug industry, management and marketing or other topics.

Another goal is to active involve students in the didactic and research activities, in order to stimulate them to participate at scientific activities. Also, special attention will be to provide information and to guide students/residents/master students to integrate into the market.

Briefly, my postdoctoral period may be characterized by a sustained involvement in different research topics, which I will extend in the next post-habilitation era.

SECTION III

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