UNIVERSITY OF BUCHAREST
FACULTY OF CHEMISTRY
DOCTORAL SCHOOL OF CHEMISTRY

DOCTORAL THESIS

CONTRIBUTIONS TO THE IDENTIFICATION, SEPARATION AND QUANTIFICATION OF SOME ACTIVE CONSTITUENTS FROM PLANTS OF PHARMACEUTICAL INTEREST

- SUMMARY -

Ph. D. student,
Iuliana Psegalinschi
(Married Stoicescu)

Doctoral advisor,
Prof. Dr. Camelia Bala

2013

CONTENTS

The numbering of chapters, figures, tables and bibliographic indications corresponds to the one from the Doctorate Thesis.

INTRODUCTION

Lately there can be observed the interest of scientific community towards the medicinal plants, due to their properties for the maintenance of health and especially because they represent inexhaustible sources of raw material for the preparation of pharmaceutical compounds.

Within this thesis, we aimed to study the *Trapa natans* L. Plant species from the Danube Delta region.

The selection of research theme took into consideration the following:

- The scientific world's recent and constant concern to identify new sources of bioactive compounds in the composition of medicinal plants and to take advantage of the traditional medicine's remedies. Also, the importance of vegetal origin sources is known worldwide: over 50% of medicines prescribed on the American continent comprise vegetal origin substances, and European countries such as Germany and France represent 30% of the plant-based products' market;
- The previous researches outline the presence of some active principles in the plant's fruit, which could have antioxidant and antimicrobial activity;
- At international level, the aquatic and aerial parts of *Trapa natans* L. species are not fructified; at national level, as far as we know, any systematic research study has not been elaborated until now.

The aim of this thesis was the identification, separation, and the qualitative and quantitative determination of some active principles from the *Trapa natans* L. species, which have certain therapeutic potential, and also to validate the analytical methods used in this purpose.

The researches' objectives were:

- To establish the identity, purity and quality of vegetal products obtained from *Trapa natans* L. species;
- To obtain some extracts from the plant's aquatic and aerial part, from the fruits' pulp and pericarp, in order to obtain a concentrate of active principles;
- The separation and determination of active principles content from the extracts obtained from the aquatic and aerial part, from the fruits' pulp and pericarp of the studied species;
- The elaboration and validation of a HPLC method for the identification, separation and determination of the active principles from *Trapa natans* L. species;

- The characterization of the effects of active principles, which were identified, separated and quantified from the extracts obtained from all component parts of the studied species;
- The assessment of cytotoxic and mutagen action of these extracts concentrated in active principles.

The doctorate thesis consists of a literature part and an experimental part, the latter comprising the original contributions made during the research for the elaboration of this thesis.

The first part presents the literature data regarding the *Trapa natans* L. species (chapter 1), the current stage of the pharmacochemical and pharmacological researches and the significance of this species, scientifically proven (chapter 2); chapter 3 presents the theoretical data regarding the extraction techniques used to obtain the vegetal extracts, the importance of studied phenolic compounds, the methods used to identify them, the separation and qualitative and quantitative determination. A short presentation of the analysis techniques we used and notions for the validation of analytical methods are also given in this chapter.

The original part of the present work presents data regarding the obtainment and the quality control of vegetal raw material (chapter 4), the preparation of hydro-alcoholic extracts from the aquatic and aerial part, from the fruits' pulp and pericarp through Soxhlet extraction and maceration (chapter 5), as well as the quantitative determination of content of total polyphenols through the spectrophotometric method (chapter 6).

The specialty literature gives us little data regarding the methods for the separation, identification, qualitative and quantitative determination of phenolic-type compounds in various species of *Trapaceea* gender [32,67]. Although numerous articles mentioned the presence of phenolic compounds responsible for certain effects on the human body, there is no data regarding the identification of phenolic compounds from the aerial part of *Trapa natans* L. species.

Thus, chapter 7, the most comprehensive one, presents the application of a standardized HPLC method for the identification, separation, qualitative and quantitative determination of gallic, methylgallic, chlorogenic, caffeic, ferulic acids and of E-resveratrol; the validation of the HPLC method and the application of this standardized method for the identification, separation, qualitative and quantitative determination of phenolic compounds from the hydro-alcoholic extracts obtained from the *Trapa natans* species are also presented in this chapter.

The phenolic compounds were identified and qualitatively determined based on the retention times specific to each phenolic acid and by comparing the absorption spectres for each phenolic compound from the chromatograms given by each solution of the component parts of the species obtained with a mixture of pure compounds with the absorption spectres for each of

those from the chromatograms given by each solution obtained without such mixture, at the wavelengths at which these compounds present maximum absorption levels. The quantitative determination of 3-O-methylgallic, chlorogenic, caffeic and ferulic acids was done based on the calibration curves figured in subchapter 7.1.4.3.

The identification, separation and qualitative and quantitative determination of phenolic compounds from the analysed solution is performed by comparing the chromatograms of the analysed solution with a mixture of 6 pure phenolic compounds (Figures 7.52, 7.54, 7.56), of that with mixture of 4 pure phenolic compounds (Figures 7.53, 7.55), and that without mixture of pure compounds.

After performing the analysis of obtained chromatograms, the following phenolic compounds were identified: at 216 nm, gallic acid which eludes at 1.44 min, at 324 nm, caffeic acid and ferulic acid , which eludes at 4.61 min and 8.60 min, respectively. The concentrations of caffeic and ferulic acids were calculated based on the calibration lines performed in subchapter 7.1.4.3.1, and the obtained results and the statistical processing of measured areas (mAU x sec), of the concentrations' retention times (mg/g v.p.) are shown in Table 7.3.4.

Table 7.34. The results recorded at the quantitative determination of phenolic acids from the 10% hydro-alcoholic extract obtained from the aquatic part of *Trapa natans* L. species through Soxhlet extraction

	1	2	3	4	5	6	$\overline{X}$	SD	SDx	RSD (%)	$\overline{X} \pm t_{\alpha/2}xSD_x$
						caffeic acid					
Analyt peak area (mAU x sec)	46.44	46.99	41.56	45.74	44.34	46.15	45.20	1.99	0.81	4.40	45.20 ±91.09
Experimental concentration (mg/g p.v.)	0.48	0.48	0.43	0.47	0.46	0.48	0.47	0.01	0.01	4.03	0.47 ±0.01
Retention time (min)	4.61	4.61	4.6	4.61	4.61	4.60	4.61	0.01	0.01	0.13	4.61 ± 0.01
						ferulic acid					
Analyt peak area (mAU x sec)	284.51	278.19	272.42	281.44	273.13	280.40	278.35	4.77	1.95	1.71	278.35 ±3.93
Experimental concentration (mg/g p.v.)	1.33	1.30	1.28	1.32	1.28	1.31	1.31	0.02	0.01	1.72	1.31 ± 0.01
Retention time (min)	8.60	8.62	8.58	8.61	8.57	8.62	8.60	0.01	0.01	0.23	8.60 ± 0.01

v. p. –vegetal product

We were very interested in checking the purity of the peaks of the analyts of interest. The results figured in Table 7.35 show us that the impurities do not co-eluate with the peaks of studied analyts. There were analysed the purity curves of peaks corresponding to the phenolic

compounds present in solutions (Figures 7.57.-7.60.). The purity factor of the identified analyts' peaks was higher than 95%.

In the hydro-alcoholic extract obtained from the *Trapa natans* L. species' aquatic part, through Soxhlet extraction, there were identified, separated and qualitatively determined the gallic, caffeic, and the ferulic acid, and quantitatively determined the ferulic acid (1.13 mg/g v.p.) in higher quantity compared to the caffeic acid (0.47 mg/g v.p.). The 3-O-methylgallic and chlorogenic acids were not identified.

In the hydro-alcoholic extract obtained from the *Trapa natans* L. species' aerial part, through Soxhlet extraction, there were identified, separated and qualitatively determined the gallic, caffeic, and the ferulic acid, and quantitatively determined the caffeic acid (4.64 mg/g v.p.) and the ferulic acid (2.04 mg/g v.p.). The 3-O-methylgallic acid at 216 nm, the chlorogenic acid at 324 nm and the E-resveratrol were notidentified.

In the hydro-alcoholic extract obtained from the pulp of the *Trapa natans* L. species' fruit, through Soxhlet extraction, there were identified, separated and qualitatively determined the gallic acid, 3-O-methylgallic acid, and the ferulic acid, and quantitatively determined the 3-O-methylgallic acid (0.54 mg/g v.p.) and the ferulic acid (0.09 mg/g v.p.). At the 324 nm, the chlorogenic acid and the caffeic acid were not identified, and at the 310 nm the E-resveratrol was not identified.

In the hydro-alcoholic extract obtained from the pericarp of *Trapa natans* L. species' fruit, through Soxhlet extraction, there were identified, separated and qualitatively determined the gallic acid, the caffeic, and the ferulic acid, and quantitatively determined the caffeic acid (4.25 mg/g v.p.) and the ferulic acid (1.78 mg/g v.p.). The 3-O-methylgallic acid at 216 nm, the chlorogenic acid at 324 nm and the E-resveratrol at 310 nm were not identified[390].

Amongst the compounds we detected, only the gallic acid, the 3-O-methylgallic acid and the ferulic acid had been previously cited in the specialty literature, as being present in this species' fruit pulp; they were identified, separated and quantitatively determined also through the liquid chromatography method, in different chromatographic conditions, from the methanol extracts obtained from the fruit's pulp in fresh condition, in frozen state and hot air dried samples [32].

Also, for the separation, identification and qualitative and quantitative determination of phenolic compounds, a new HPLC method was developed, which was optimized and validated. It was applied for the determination of polyphenolic compounds from the hydro-alcoholic extracts obtained through the two extraction methods.

As the compounds we want to separate represent the acid groups, the retention of separations will increase together with the decrease of mobile phase's pH, because the

dissociation of –OH groups will be blocked and the molecules will interact pregnantly with the stationary phase based on its hydrophobicity . The method's principle was represented by the identification, separation and qualitative and quantitative determination of gallic, 3-O-methylgallic, chlorogenic, caffeic, cinnamic and ferulic acids, and of the E-resveratrol from the hydro-alcoholic solutions through the high performance liquid chromatography method with diode network detector, based on the retention times and the UV-Vis absorption spectres.

The identification, separation and qualitative and quantitative determination of phenolic compounds from the analysed solution is performed by comparing the chromatograms of the analysed solution with a mixture of pure substances made of 7 pure phenolic compounds and of the analysed solution without the mixture of pure substances.

The analyts separated through the standardized HPLC method were found in the method elaborated, but in smaller concentrations. The results concurred at the samples obtained through maceration. The advantage of this method consists in shorter time for analysis, and detection and quantification limits much more reduced compared to the standardized method.

The new proposed method is adequate for the separation and quantitative assessment of phenolic compounds from vegetal products.

The HPLC analyses, using both the method standardized in the American Pharmacopeia and the new elaborated method allowed the separation and identification of phenolic compounds from vegetal extracts based on the retention times and UV absorption spectres, and the quantitative determination based on the validated regression lines' equation.

The results obtained at the separation and quantitative determination of total polyphenols through the spectrophotometric method and the some compounds' identification, separation and batching through HPLC determined us to continue the study by quantifying the antioxidant activity of hydro-alcoholic extracts obtained from the component parts of *Trapa natans* L. plant, through the photochemiluminescence method produced by the reaction of radical species with luminal. The experimental data and their interpretation are presented in chapter 8.

Based on the obtained results and on the correlation between chemical composition and antioxidant action, we can state that the concentrates in active principles of polyphenols type extracted from the aerial part, the aquatic part, from the species' pulp or pericarp, can be used in medicine for their antioxidant action.

The determination of antimicrobial activity of studied hydro-alcoholic vegetal extracts is presented in chapter 9. The antimicrobial activity was assessed for three bacterial species, that is *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATTC 25922, *Proteus vulgaris* ATTC 13315, as well as for *Candida albicans* ATTC 10239 fungi species, using the difusimetric method in agar gel. The antibacterial activity's largest inhibition areas were obtained at the

hydro-alcoholic extracts made in Soxhlet extractor for the samples obtained from the pericarp, followed by the ones obtained from the aerial and aquatic part. Small inhibition areas, less significant, were recorded for the samples obtained from fruits' pulp. The stems most sensitive to the action of samples obtained through Soxhlet extraction were the Gram + *S. Aureus* bacteria. The Gram + stems were more sensitive to the action of the tested hydro-alcoholic extracts compared with Gram – bacteria. The obtained results regarding the antibacterial action of hydro-alcoholic extracts were comparable to those reported in specialty literature.

In order to deepen the study on the therapeutic potential of *Trapa natans* L. plant extracts, in chapter 10 there are presented the results obtained, regarding the assessment of extracts' cytotoxicity and genotoxicity on the germination of wheat caryopses. It was observed that all extracts obtained from the aquatic part, the aerial part, from the fruit's pulp and pericarp have obvious cytostatic effects. The most accentuated cytostatic response is given by the extract obtained from the aquatic part and fruit's pericarp, fact explained also by the morphological functions of the components of this plant.

There were not observed genotoxicity elements, modified chromosomes or chromosomal arches. There must be mentioned the fact that, although the divisions are reduced, all cellular cycles occur, in different proportions depending on the extract (Table 10.3), which shows that normal divisions are possible, but the signal which triggers the cellular cycle is interrupted .

Taking into consideration that the hydro-alcoholic extracts obtained from the *Trapa natans* L. species' aquatic part, aerial part, from the fruits' pulp and pericarp have antioxidant, antimicrobial and cytostatic action, we can perform further researches to obtain a standardised product with total polyphenols and polyphenolic compounds with clinically proven therapeutic effects, identified through the chromographic method. Therefore, the perspective of using these hydro-alcoholic extracts from the vegetal products obtained from *Trapa natans* L. plant in the food and pharmaceutical industry has become obvious.

THE LIST OF PAPERWORKS ELABORATED WITHIN THE THESIS

1. Simultaneous determination of phenolic acids in water caltrop by HPLC-DAD
   Iuliana Stoicescu, Antoanela Popescu, Rodica Sirbu, Camelia Bala
   Analytical Letters, **2012**, 45(17), 2519-2529.

2. Spectrophotometric method for polyphenols analysis: validation and application on Trapa natans L. species
   Iuliana Stoicescu, Antoanela Popescu, Rodica Sirbu, Cosmin Rosca, Dragoş Nicolae Doicescu, Vasile Bendic, Camelia Bala
   Revista de Chimie, **2012**, 63(9), 865-868.

3. In vitro antioxidant and antibacterial activity of Trapa natans L. Aquatic plant from Danube Delta area
   Iuliana Stoicescu, Rodica Sirbu, Ticuţa Negreanu-Pirjol, Monica Cociaşu, Doina Paula Balaban, Camelia Bala
   Revue Roumaine de Chimie, **2012**, 57(7-8), accepted in July 2012, in press.