"Dunărea de Jos" University of Galaţi

Doctoral School of Fundamental Sciences and Engineering

DOCTORAL THESIS

Chemical characterization and study of the biological activity of some compounds present in the *Nymphaea alba* species from the Danube Delta Biosphere Reserve

PhD student,

Mihaela Cudălbeanu

Scientific leader,

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Series C: Chemistry No. 3

Galaţi, 2022

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Content

Introduction

Plants have always been a food source, but also the "nature pharmacy" for humans and animals, which contain a wealth of biologically active compounds. Thus, many remedies used since immemorial time in traditional medicine have been highlighted by studying the chemical composition, compounds with biologically active properties which demonstrated their pharmacological properties. The Danube Delta, as a result of the interaction between the Danube River and the Black Sea, is of particular scientific interest due to its renewable natural resources. The current conditions existing in the Danube Delta have led to the development of diversified aquatic vegetation, particularly rich in species. Among them is the *Nymphaea alba* (*N. alba*) species [1, 2].

Various secondary metabolites from the class of peptides, alkaloids, flavonoids, phenolic acids, terpenes, steroids and tannins have been discovered to be present in aquatic plants. These compounds are widely used as antioxidant, anti-inflammatory, antitumor agents, but also as antibiotics and antivirals [3, 4]. Numerous studies show that about 80 % of the world's population also uses traditional medicine, so researchers study biologically active compounds from natural sources and obtain new semi-synthesis products, therapeutically more potent. Over the years these natural compounds have been identified themselves as valuable tools in the fight against both chronic and infectious diseases. Aquatic plants have a long and rich history in this area, the species of the *Nymphaeaceae* family being used in traditional medicine to prevent, improve and/or treat various diseases such as diabetes, cardiovascular diseases, inflammatory diseases, tumor diseases and others [5, 6, 7, 8]. The therapeutic potential of these aquatic species has been recognized in the pharmaceutical industry due to the importance of rich content of biological compounds, used in obtaining drugs for treatment of tropical and orphan diseases [9].

It is scientifically proven that aquatic plants contain chemical compounds with biologically active properties. The *N. alba* species has a rich content of phytochemical compounds from the class of polyphenols, including phenolic acids, flavonoids, tannins, but also alkaloids, terpenoids, sterols and other classes of organic compounds [5-8, 10, 11, 12]. In the last research studies, carried out *in vitro*, results are successfully presented regarding the strong antioxidant and antibacterial potential due to the polyphenolic compounds present in the *N. alba* species, among them being gallic acid, protocatechuic acid, syringic acid, quercetin, apigenin or luteolin [7, 8, 11, 13, 14, 15]. From the *N. alba* species, several compounds belonging to the class of polyphenols were separated by different analytical techniques [6, 7]. Of these, flavonoids exhibit a variety of functions in plants, mainly helping to increase them during physiological stress and provide photo-protection through their antioxidant activity [16, 17]. Flavonoids are considered to be the most prominent class of secondary metabolites of floating aquatic species [18]. Among these, quercetin, naringin and apigenin have been studied for their antitumor activity proving active against ovarian tumors [19].

Therefore, due to their potential to produce bioactive compounds, the study of aquatic plants, is still an area of interest to identify new potential and valuable sources of compounds with pharmacological properties [4, 20]. Thus, the **purpose** of this doctoral thesis is to highlight the *N. alba* aquatic plant from the Danube Delta Biosphere Reserve, from the perspective of highlighting the important chemical compounds, the discovery of new biologically active properties, but also

the discovery of new organic molecules with importance for the pharmaceutical industry or the food industry, bringing benefits to the human society and contributing to the improvement of the quality of life.

The doctoral thesis entitled "*The chemical characterization and the study of the biological activity of some compounds present in the Nymphaea alba species from the Danube Delta Biosphere Reserve*" includes ten chapters, a chapter in which the current state of knowledge is presented and which includes data from the specialized literature on the chemical composition of the studied water lily species as well as its various biologically active properties, respectively nine chapters in which the original contribution on the chemical composition and biological properties of the *N. alba* aquatic species is presented for the first time.

The doctoral thesis represents a complex and novel study that had as main objectives:

- Extraction using different methods and separation for the first time of biologically active compounds from different anatomical parts (fruit, flower, leaf, stem and root) from *N. alba* species from the Danube Delta Biosphere Reserve;
- The use of modern analysis techniques, gas chromatography and liquid chromatography, coupled with mass spectrometry, for the separation and identification for the first time of the biologically active compounds from the fruit, flower, leaf, stem and root of *N. alba* species from the Danube Delta Biosphere Reserve;
- Identification and quantification of important classes of organic compounds (chlorophylls, carotenoids, polyphenols) from fruit, flower, leaf, stem and root of *N. alba* species from the Danube Delta Biosphere Reserve, by spectrophotometric methods;
- Identification and quantification of some macro and microelements from fruit, flower, leaf, strain and root of *N. alba* species from the Danube Delta Biosphere Reserve, by the ICP-OES and AAS technique;
- Evaluation for the first time of the antioxidant potential of the various fruit, flower, leaf, strain and root extracts of *N. alba* species from the Danube Delta Biosphere Reserve, by different spectrophotometric methods (DPPH, ABTS, FRAP, BCB) and electrochemical (CV);
- Evaluation for the first time of the antibacterial and antifungal properties of biologically active compounds from different anatomical parts of *N. alba* species from the Danube Delta Biosphere Reserve;
- Evaluation for the first time of the toxic and cytotoxic potential of biologically active compounds from different anatomical parts of *N. alba* species from the Danube Delta Biosphere Reserve, on the germination of wheat seeds and healthy human cells;
- Evaluation for the first time of the antitumor properties of biologically active compounds from different anatomical parts of *N. alba* species from the Danube Delta Biosphere Reserve;
- AuNPs sono-biosynthesis using *N. alba* extracts and characterization and evaluation of the biological properties of the nanoparticles obtained.

The originality of the doctoral thesis is evident by analyzing the data from the specialized literature in which no ample study has been found so far on the analysis of the chemical composition and the active biological potential of the five anatomical parts of the *N. alba* species (fruit, flower, leaf, stem and root) from the Danube Delta Biosphere Reserve, and for the first time obtaining through biosynthesis AuNPs with biologically active properties.

PART I. CURRENT STATE OF KNOWLEDGE

Chapter 1 Characterization of the studied aquatic species

1.2 Description of the aquatic species

The *N. alba* or *N. occidentalis* species, also known as the European white water lily or white water rose (Figura 1.1), is one of the most attractive aquatic plants in the *Nymphaea* genus. The *N. alba* species is a perennial aquatic plant belong to the *Nymphaea* subgenus, *Nymphaea* genus, *Nymphaeoideae* subfamily, *Nymphaeaceae* family and *Nymphaeales* order [6-8, 10-13, 21, 22, 23].

Figure.1.1. Nymphaea alba species from the Danube Delta Biosphere

1.3 Chemical composition of the aquatic species

Bioactive compounds present in species of the *Nymphaea* genus belong to several classes of organic compounds, such as phenolic acids, flavonoids, proanthocyanidins, anthocyanidins, tannins, lignans, alkaloids, anthraquinones, saponins, terpenoids, sterols, esters, saccharides (reducing sugars and g polysaccharides), amino acids and proteins, higher aliphatic hydrocarbons, being scientifically proven that different component parts of plants contain different chemical compounds [24, 25, 26, 27, 28].

1.4 Importance of the studied aquatic species

1.4.1 Applications in traditional medicine

Previous research has shown that *Nymphaea* species are used in traditional medicine to treat a variety of ailments such as acne, lymphadenopathy, ulcerative colitis, burns, tumors, colds, coughs, dermatoses, diarrhea, dysentery, flu, freckles, boils. , gonorrhea, sore throat, leukorrhea, nephrosis, toothache, stomatitis, tuberculosis, uterus, vaginosis, diabetes or cardiovascular disease. Certain water lily species have tranquilizing and antiparasitic effects [10, 29, 30, 31].

1.4.2 Pharmacological properties

The *Nymphaeaceae* family is an important source of antioxidants, being known for its antibacterial, antitumor, anthelmintic, lipid-lowering, antihepatotoxic and antidiabetic properties [5, 6, 14, 32, 33, 34]. Some *in vivo* studies of *N. alba* species have shown that this plant has various biological activities, such as antidiabetic, analgesic, antidiarrheal, hepatoprotective, antioxidant and anti-inflammatory, inhibiting α glucosidase, inhibiting oxidative stress, anxiolytic activity and anticarcinogenic effect. *In vitro* studies have successfully reported important antioxidant and antibacterial activities [7, 8, 11, 13-15].

PART II. PERSONAL CONTRIBUTIONS

Doctoral thesis objectives

The general objective of the doctoral thesis "Chemical characterization and study of the biological activity of some compounds present in the *Nymphaea alba* species from the Danube Delta Biosphere Reserve" was to identify and characterize chemical compounds from different anatomical parts of water lily, such as fruit, flower, leaf, stem and root and highlighting of biological activities of biologically active compounds identified in the *N. alba* species.

The specific objectives proposed were the following:

- Plant material sampling from the Danube Delta Biosphere Reserve and preliminary preparation of samples;
- Characterization of plant material using a Zeiss Axio Observer Z1 inverted microscope;
- Extraction of biologically active compounds from *N. alba* species by three extraction methods: ultrasound extraction, multiple extraction using Soxhlet extractor and simple mechanical agitation extraction;
- Chromatographic analysis of biologically active compounds of *N. alba* species by gas chromatography coupled with mass spectrometry (GC-MS);
- Chromatographic analysis of biologically active compounds of *N. alba* species by high performance liquid chromatography coupled with diode array detector (HPLC-DAD) and high performance liquid chromatography coupled with mass spectrometry (LC-MS/MS);
- Identification and quantification of classes of organic compounds with biologically active properties of *N. alba* species by *in vitro* microspectrophotometric determinations;
- Determination of the macro and microelements total content using an inductively coupled plasma optical emission spectrometer (ICP-OES) and flame (FAAS) and graphite furnace (GFAAS) atomic absorption spectrophotometer;
- Evaluation of the antioxidant potential of biologically active compounds of *N. alba* species, by microspectrophotometric methods for inhibiting free radicals, such as DPPH, $ABTS^{\bullet+}$ and FRAP, β-carotene bleaching, but also by electrochemical methods, such as cyclic voltammetry (CV);
- Evaluation of the antimicrobial properties of biologically active compounds of *N. alba* species by *in vitro* methods of inhibiting Gram-positive and Gram-negative bacteria, respectively different *Candida* species;
- Determination of the toxic and cytotoxic potential of biologically active compounds of *N. alba* species by germination tests and *in vitro* cytotoxicity assays on healthy fibroblasts cells and ovarian, mammary, prostatic and leukemic tumor cells;
- AuNPs biosynthesis using *N. alba* extracts and evaluation of the biological activity of nanoparticles, such as antioxidant, antibacterial and antitumor activity.

Chapter 2 Extraction of biologically active compounds from the *N. alba* species

2.4 Results and discussions

2.4.1 Study area

The measures for the protection and conservation of biological diversity in the Danube Delta Biosphere Reserve and, finally, the chemical investigations carried out on the potential habitats for the growth of aquatic plant species, make this area the most suitable habitat for the growth of the studied aquatic species, such as *N. alba* species. Of all the deltaic aquatic ecosystems, the Somova-Parcheș Aquatic Complex was chosen as the study area.

2.4.2 Sampling and preparation of plant material

An important step in carrying out the research study and the proposed objectives is the taking of plant samples. Samples of plant material were taken during the maximum development period of the *N. alba* species (Figure 2.3).

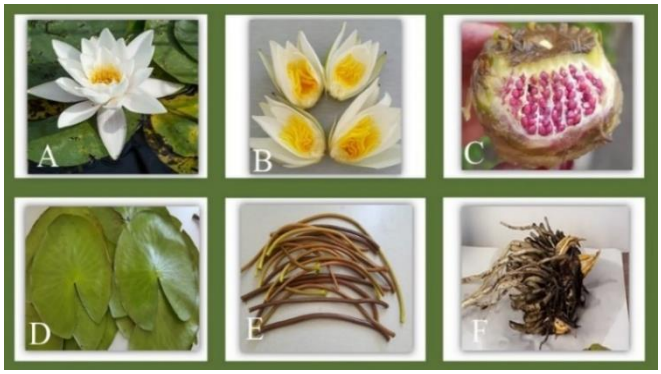


Figure 2.3.. Anatomical parts of N. alba species: A - flower, B - stamens, C - fruit and seeds, D - leaf, E - stem, F - root and rhizomes

2.4.3 Microscopic images of plant material

In order to achieve a broader characterization of the studied aquatic plant, 3D microscopic images of the fresh samples of flower, leaf, stem and root of the *N. alba* species were recorded, these being presented in Figure 2.5. Microscopic analysis of the cross-section through the flower showed the typical pollen granules, spherical or ellipsoidal, yellow, non-transparent, with a granular ornamented surface and diameters between 30 - 36 μm (Figure 2.5a). The cross-section through the leaf shows to the upper epidermis the compact palisade tissue, with pro-enzymatic cells, with reduced intercellular spaces, and towards the lower epidermis it presents lacunar tissue with parenchymal cells, isodiametric, with large intercellular spaces (gaps), and from place to place can be observed and numerous hairs (Figure 2.5b). Microscopic images of cross-sections through the stem (Figure 2.5c) and root (Figure 2.5d) show large polygonal or globular cells in the cortical parenchyma, with a secondary modified cell wall of the sclerenchyme type, with large vacuoles pushing peripherally, both cytoplasmic constituents and and the core.

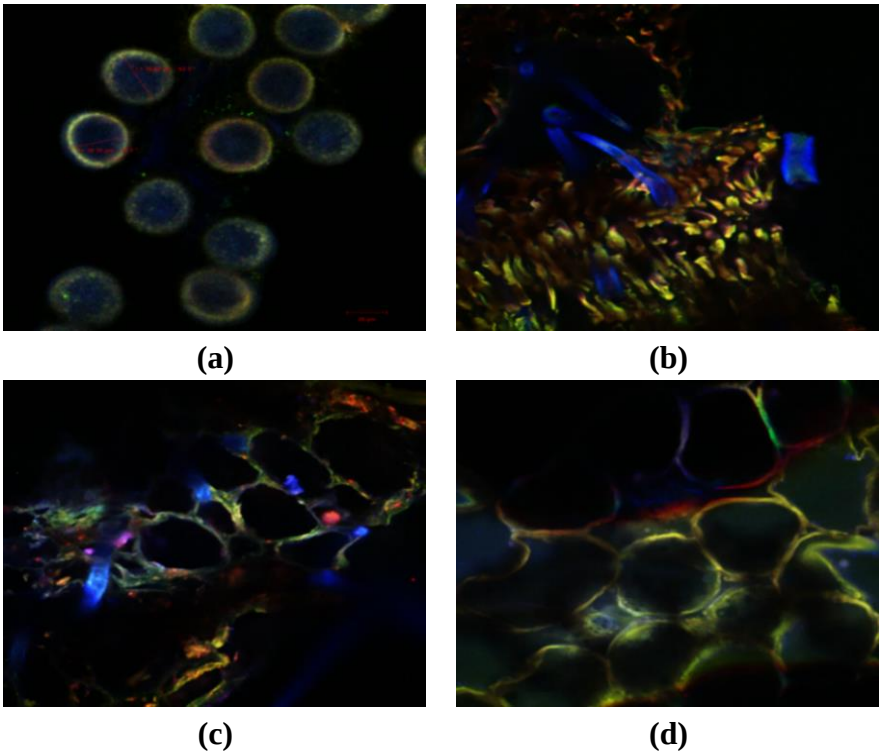


Figure 2.5. CLSM images of pollen granules (a) and cross-sections through the leaf (b), stem (c) and root (d) of N. alba species

2.4.4 Extraction of biologically active compounds from *N. alba* different anatomical parts

Non-polar solvents (petroleum ether and cyclohexane) were used for the extraction of biologically active lipophilic compounds and polar solvents (methanol, ethanol and ultrapure water) were used for the extraction of biologically active hydrophilic compounds from various anatomical parts of *N. alba* species. In order to successfully carry out the solvent extraction of natural organic compounds from various anatomical parts of *N. alba* species, such as fruit, flower, leaf, stem and root, three extraction methods were used: simple ultrasound assisted extraction (US), multiple extraction using Soxhlet extractor (ES) and simple mechanical agitation extraction (EM).

Table 2.1. Extraction yield for obtaining extracts from N. alba different anatomical parts by different extraction methods

Type of plant material	Type of solvent/ extraction*	Extract mass (g)	Yield (%)
Fruit (FR)	EP-ES	0.15±0.02	0.75±0.02
	CH-EM	0.68±0.15	0.68±0.15
	M-US	0.51±0.10	2.53±0.10
	M-ES	0.46±0.20	2.31±0.20
	E-EM	1.97±0.42	1.97±0.42
	A-EM	5.67±0.69	5.67±0.69
Flower (FL)	EP-ES	0.24±0.02	1.18±0.02
	CH-EM	1.29±0.30	1.29±0.30
	M-US	3.23±0.72	16.16±0.72
	M-ES	2.99±0.51	14.99±0.51
	E-EM	13.44±1.41	13.44±1.41

Type of plant material	Type of solvent/ extraction*	Extract mass (g)	Yield (%)
	A-EM	10.78±0.99	10.78±0.99
Leaf (F)	EP-ES	0.42±0.03	2.12±0.03
	CH-EM	1.95±0.29	1.95±0.29
	M-US	2.66±0.26	13.29±0.26
	M-ES	2.45±0.85	12.24±0.85
	E-EM	8.54±1.07	8.54±1.47
	A-EM	9.98±1.47	9.98±1.47
Stem (T)	EP-ES	0.26±0.01	1.32±0.01
	CH-EM	0.99±0.10	0.99±0.10
	M-US	2.76±1.21	13.79±1.21
	M-ES	2.70±0.61	13.54±0.61
	E-EM	9.52±1.21	9.52±1.21
	A-EM	9.75±0.92	9.75±0.92
Root (R)	EP-ES	0.06±0.01	0.31±0.01
	CH-EM	0.19±0.01	0.19±0.01
	M-US	5.55±0.85	27.75±0.85
	M-ES	5.45±1.15	27.24±1.15
	E-EM	24.80±2.07	24.80±2.07
	A-EM	8.45±0.77	8.45±0.77

*EP - petroleum ether; CH - cyclohexane; M - methanol, E - ethanol; A - ultrapure water. US - simple ultrasound assisted extraction; ES - multiple extraction using Soxhlet extractor; EM - simple mechanical agitation extraction. The results represent the average of the three determinations ± standard deviation.

Extract mass and extraction yield are shown in Table 2.1. It was found that a much larger amount of natural compounds was extracted with the methanol organic solvent compared to the ethanol organic solvent. The largest amount of natural compounds was extracted from the flower and root of the *N. alba* species, when used as a solvent methanol.

2.4.5 Separation steps of biologically active compounds from *N. alba* root extract

Separation and isolation of biologically active compounds of *N. alba* species was performed for R-E-EM extract, which took place in seven stages. Following the separation steps, 16 representative fractions were obtained. The results of the CSS analyzes obtained after the separation of the *N. alba* R-E-EM extract revealed the presence of polyphenolic compounds [35]. For further analysis, four of the 16 representative fractions, denoted F2, F5, F50 and F51, were used to prove the biological properties of the presented compounds.

2.5 Partial conclusions

The sampling of *N. alba* species was carried out from Rotundu Lake, Somova-Parcheș Aquatic Complex, Danube Delta Biosphere Reserve, which has special environmental conditions for the development of submerged aquatic species. For each taken specimen, representative subsamples were made according to the anatomical parts of the plant, namely fruit, flower, leaf, stem and root.

The characterization of *N. alba* samples, performed by CLSM microscopy, highlighted in the cross-section, the spherical or ellipsoidal pollen granules, yellow, non-transparent, with granulated ornamented surface and diameters between 30 - 36 µm. The leaf cross-section showed compact palisade tissue, with pro-enzymatic cells, with reduced intercellular spaces, as well as

lacunar tissue with parenchymal cells, isodiametric, with large intercellular spaces (gaps). The stem and root cross-sections showed polygonal or globular cells in the cortical parenchyma, with a secondary modified cell wall of the sclerenchyma type, with large vacuoles that push peripherally, both the cytoplasmic constituents and the nucleus.

To successfully perform the extraction of biologically active natural organic compounds from various anatomical parts of *N. alba* species, such as fruit, flower, leaf, stem and root, three extraction methods were used: simple ultrasound assisted extraction, multiple extraction using Soxhlet extractor and simple mechanical agitation extraction. A total of 30 extracts were obtained, and the largest amount of natural compounds was extracted with the methanol organic solvent compared to the ethanol organic solvent.

Separation and isolation of biologically active compounds of *N. alba* species was performed for R-E-EM extract, which took place in seven stages. A total of 16 representative fractions were obtained, and the CSS analysis of the fractions revealed the presence of polyphenolic compounds. Four of the 16 representative fractions were used in subsequent analyzes to be thoroughly analyzed and to prove the biological properties of the secondary metabolites present in the *N. alba* species.

Chapter 3 Analysis of biologically active compounds from the *N. alba* species by gas chromatography - mass spectrometry

3.4 Results and discussions

GC-MS analysis was performed for EP-ES and CH EM lipophilic extracts, obtained from different anatomical parts of *N. alba* species (fruit, flower, leaf, stem and root). Figure 3.1 shows the GC-MS chromatogram of the *N. alba* FR-EP-ES extract, in which 11 differentiated chemical compounds were identified based on the analysis of mass spectra and accessing the existing spectral database. The compounds identified, in order of retention time, were: L-ascorbyl 2,6-dipalmitate (11.578 min), dibutyl phthalate (11.635 min), n-heneicosan (12.240 min), octadecanoic acid (stearic) (12.533 min), n-tetracosan (13.545 min), n-pentacosan (13.980 min), 2-monopalmitoyl-sn-glycerol (1,3-dihydroxypropan-2-yl hexadecanoate) (14.036 min), n-nonacosan (15.001 min), 1-stearoyl-sn-glycerol (2.3-dihydroxypropyl octadecanoate) (15.143 min), vitamin E (19.976 min) and γ-sitosterol (22.102 min). The structural elucidation of the separated and identified compounds in the GC-MS chromatogram was performed by comparison with the mass spectra in the spectral library (Table 3.1) and the application of molecular fragmentation rules.

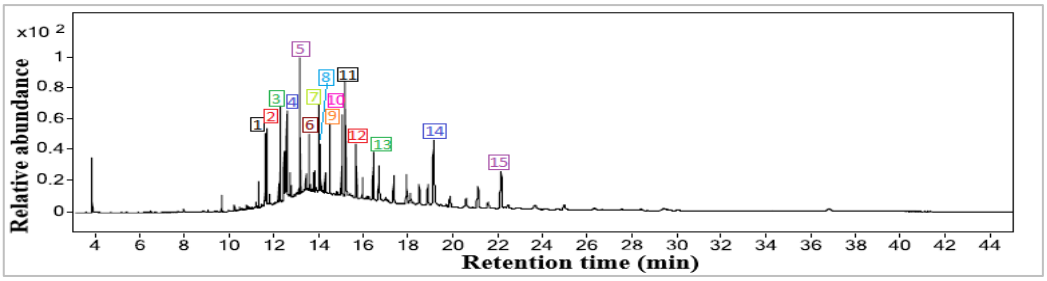


Figure 3.1. GC-MS chromatogram of N. alba FR-EP-ES extract

Thus, compound 1 (11.578 min) was identified as **L-ascorbyl 2,6-dipalmitate**, an ester of vitamin C with the molecular formula $C_{38}H_{68}O_8$. Because ascorbic acid is a thermally unstable molecule, the molecular ion present is that of hexadecanoic acid (m/z = 256), due to the loss of the $C_6H_8O_6$ fragment from the molecular ion (Figure 3.2).

Compound 11 (22.102 min) with the molecular formula $C_{29}H_{52}O_2$ (m/z = 432) was identified as **γ-sitosterol**, a phytosterol present in all *Nymphaea* species. The molecular ion M^+ = 414 and a series of molecular fragments indicating the presence of γ-sitosterol in the analyzed ether extract were identified (Figure 3.12).

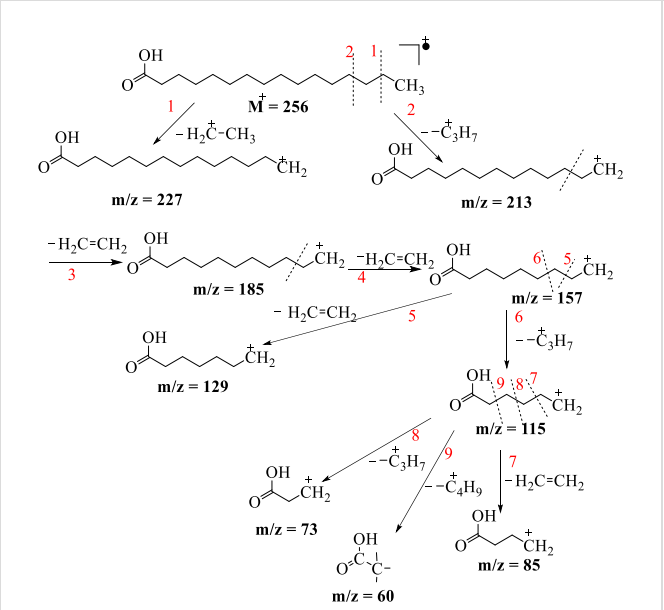


Figure 3.2. Fagmentation mechanism of hexadecanoic acid from L-ascorbyl 2,6-dipalmitate

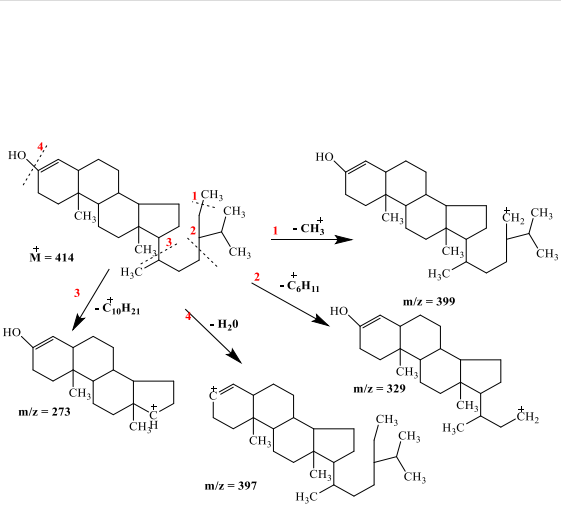


Figure 3.12. Proposed fragmentation mechanism for γ-sitosterol

Table 3.1. Spectral data of the organic compounds identified in the N. alba FR-EP-ES extract by GC-MS analysis. The table shows the retention time, the chemical name of the compound, the molecular mass, the molecular formula, the molecular ion and the molecular fragments

Nr. crt.	RT[a] (min)	Chemical name of the compound	MW[b] (g/mol)	Molecular formula	M^{+c} (m/z)	Molecular fragments (m/z)
1	11.578	L-ascorbyl 2,6-dipalmitate	652.9	$C_{38}H_{68}O_8$	256.2	55.1; 57.1; 60.1; 69.1; 71.1; 73.1; 85.1; 129.1; 213.2; 256.2
2	11.635	dibutyl phthalate	278.34	$C_{16}H_{22}O_4$	278.2	56.1; 57.1; 93; 104; 105; 121; 149.1; 150; 205.1; 223.1
3	12.240	n-heneicosan	296.6	$C_{21}H_{44}$	269.4	55.1; 57.1; 69.1; 70.1; 71.1; 83.1; 85.1; 99.1; 113.1; 127.1
4	12.533	octadecanoic acid	285.5	$C_{18}H_{36}O_2$	284.3	55.1; 57.1; 60.1; 69.1; 71.1; 73.1; 83.1; 129.1; 185.1; 284.3
5	13.545	n-tetracosan	338.7	$C_{24}H_{50}$	324.4	55.1; 57.1; 69.1; 71.1; 83.1; 85.1; 97.1; 99.1; 113.1; 127.1

Nr. crt.	RT[a] (min)	Chemical name of the compound	MW[b] (g/mol)	Molecular formula	M+[c] (m/z)	Molecular fragments (m/z)
6	13.980	n-pentacosan	352.7	$C_{25}H_{52}$	352.4	55.1; 57.1; 69.1 71.1; 83.1; 85.1; 97.1; 99.1; 113.1; 127.1
7	14.036	2-monopalmitoyl-sn-glycerol	568.9	$C_{35}H_{68}O_5$	287.1	55.1; 57.1; 69.1; 71.1; 74.1; 84.1; 98.1; 134 239.2; 287.1
8	15.001	n-nonacosan	408.8	$C_{29}H_{60}$	380.5	55.1; 57.1; 69.1; 71.1; 83.1; 85.1; 97.1; 99.1; 113.1; 127.1
9	15.143	1-stearoyl-sn-glycerol	358.5	$C_{21}H_{42}O_4$	327.3	55.1; 57.1; 71.1; 74.1; 84.1; 97.1; 98.1; 112.1; 134.1; 267.3
10	19.076	vitamin E	430.7	$C_{29}H_{50}O_2$	430.4	55.1; 57.1; 121.1; 164.1; 165.1; 166.1; 205.1; 430.4; 431.4; 432.5
11	22,102	γ-sitosterol	432.7	$C_{29}H_{52}O_2$	414.4	57.1; 81.1; 95.1; 105.1; 107.1; 145.1; 213.2; 303.3; 329.3; 414.4

[a]RT - retention time; [b]MW - molecular weight; [c]M + - molecular ion.

3.5 Partial conclusions

GC-MS analysis of *N. alba* EP-ES and CH-EM extracts identified a total of 71 volatile organic compounds, which were mainly hydrocarbons and oxygenated derivatives, fatty acids and esters of fatty acids, terpenoids and steroids. A total of 11 organic compounds were identified and characterized in the FR-EP-ES extract, while a total number of six organic compounds were identified and characterized in the FL-EP-ES extract, respectively in the T-ES-EP extract a total of seven organic compounds were identified and characterized, and only four volatile compounds were identified and characterized in F-EP-ES and R-EP-ES extracts. A total of 32 volatile organic compounds were separated, identified and characterized in the *N. alba* EP-ES extracts. At the same time, a higher number of organic compounds were identified and characterized in the *N. alba* CH-EM extracts than in the *N. alba* EP-ES extracts. Out of a total of 39 organic compounds, the highest number of volatile organic compounds were identified in FR-EP-ES and FR-CH-EM extracts.

Chapter 4 Analysis of biologically active compounds from the *N. alba* species by high - performance liquid chromatography

4.4 Results and discussions

4.4.1 Separation, identification and quantification of biologically active compounds by HPLC-DAD technique

To identify and quantify the bioactive compounds in *N. alba* M-US, M-ES, E-EM and A-EM extracts obtained from different anatomical parts (fruit, flower, leaf, stem and root), but also from F2, F5, F50 and F51 fractions (obtained after fractionation of RE-EM extract), 14 reference compounds were used (tannic acid, gallic acid, (+) catechin, caffeic acid, chlorogenic acid, (-)

epicatechin, p-coumaric, daidzein, hyperoside, rutin, naringin, quercetin, naringenin and genistein). To identify polyphenolic compounds, the samples were analyzed at seven different λ wavelengths (230, 250, 280, 300, 320 and 370) nm [36, 37]. Quantification of the polyphenolic compounds identified in the *N. alba* extracts and fractions was performed at λ_{max} of each reference compound [38, 39, 40].

The data presented in Figure 4.2 show the maximum concentration of each polyphenolic compound identified in the M-US extracts. The highest amount of caffeic acid was identified in the FL-M-US extract (1.83±0.09 mg/kg, Figure 4.1b). Gallic acid, rutin and naringin were found in the highest amounts in the R-M-US extract, with a concentration of 3.02±0.04 mg/kg, 10.48±0.11 mg/kg, respectively 17.75±0.10 mg/kg (Figure 4.1e). p-Coumaric acid was identified with the highest concentration (2.56±0.05 mg/kg) in the *N. alba* FR-M-ES extract. The naringin is the most abundant flavonoid compound in *N. alba* FL-M-ES and R-M-ES extracts, with a concentration of 22.97±1.19 mg/kg and respectively, 40.56±2.38 mg/kg. Rutin glycoside was identified in the highest concentration in F-M-ES (6.32±0.59 mg/kg) and R-M-ES (11.41±0.87 mg/kg) extracts.

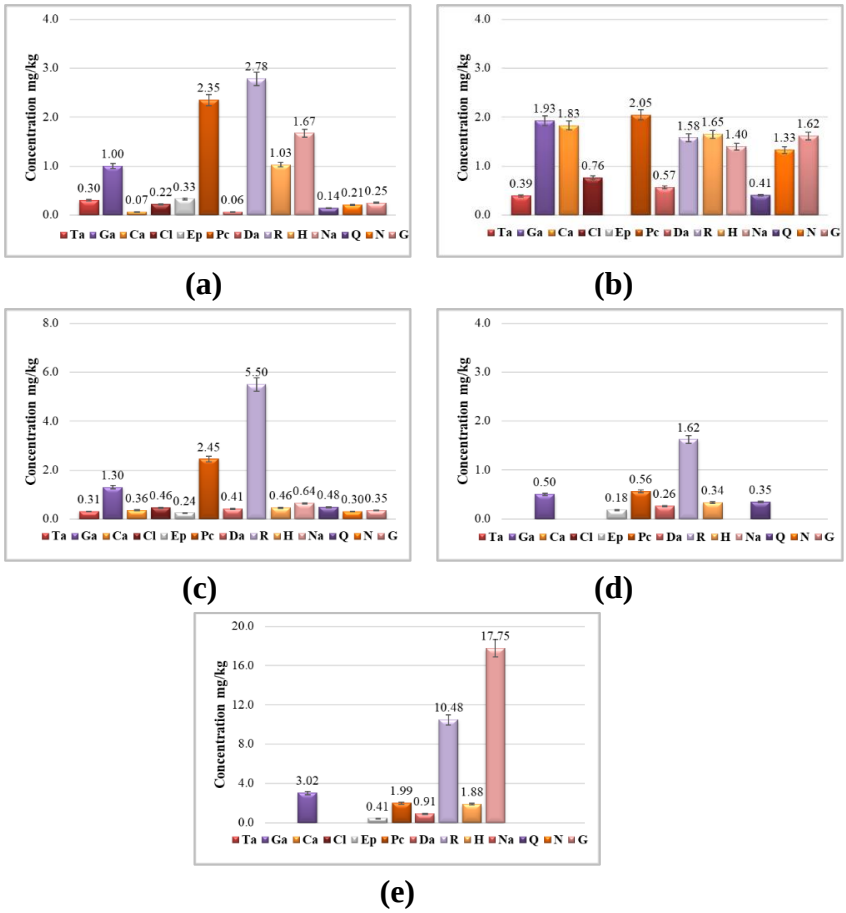


Figure 4.2. Identification and quantification of polyphenolic compounds in N. alba extracts: (a) FR-M-US, (b) FL-M-US, (c) F-M-US, (d) T-M-US and (e) R-M-US. Ta - tannic acid, Ga - gallic acid, Ca - caffeic acid, Cl - chlorogenic acid, Ep - epicatechin, Pc - p-coumaric acid, Da - daidzein, R - rutin, H - hyperoside, Na - naringin, Q - quercetin , N - naringenin and G – genistein

The same flavonoid naringin is present in the largest amount in the *N. alba* FR-E-EM and FL-E-EM extracts with a concentration of 4.24±0.23 mg/kg, respectively 6.04±0.64 mg/kg. The

highest amount of gallic acid was identified in the R-E-EM extract, with a concentration of 2.05±0.15 mg/kg. E-EM extraction favored the extraction of epicatechin, which is present with a concentration of 8.00±1.02 mg/kg in TE-EM extract and with a concentration of 19.11±2.19 mg/kg in the RE-EM extract. As with T-M-US and R-M-US extracts, chlorogenic acid was not identified in T-E-EM and R-E-EM extracts.

The naringin flavonoid is present in the largest amount in the *N. alba* F2, F5, F50 and F51 fractions, with a concentration of 2.47±0.31 mg/kg (Figure 4.4a), 1.07±0, 08 mg/kg (Figure 4.4b), 3.42±0.28 mg/kg (Figure 4.4c) and respectively, 0.96±0.03 mg/kg (Figure 4.4d). The highest amount of polyphenolic compounds was identified in the F2 fraction, and the highest number of polyphenolic compounds was found in the F5 fraction.

4.4.2 Separation, identification and confirmation of biologically active compounds by the LC-MS/MS technique

To identify and to confirm the polyphenolic compounds from *N. alba* M-US extracts, 10 reference compounds (caffeic acid, chlorogenic acid, p-coumaric acid, vanillic acid, (+) catechin, (-) epicatechin, naringin, naringenin, quercetin and rutin) were used, which were confirmed in MS by direct infusion. In the MRM mode 17 reference compounds were added (tannic acid, gallic acid, ferulic acid, ellagic acid, quinic acid, ellagic acid pentoside, ellagic acid rhamnoside, a cinnamic acid derivative, corilagin, kaempferol, castaline, orientin, apigenin, luteolin, brevifoline, HHDP acid hexoside and an unidentified compound/gerannin), using data from the literature on ion molecular and molecular transitions (Table 4.2) [41].

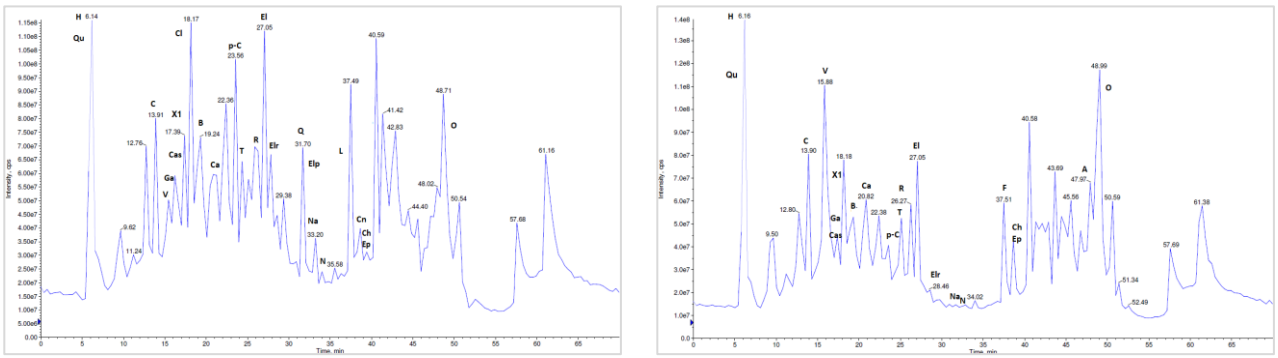
Table 4.2. General spectral data of the reference compounds used in the identification of polyphenolic compounds in N. alba M-US extracts by the LC-MS/MS technique

Reference compounds	T_R[a] (min)	Molecular formula	M^{+c}	$[M-H]^{-b}$ (m/z) Molecular fragments (AR %[d])	Mass error (ppm)	Bibliographical references
HHPD[e]	6.15	-	481	301 (50), 463 (40)	-1.85	7, 42, 43
Chinic acid	6.16	$C_7H_{12}O_6$	191	127 (33), 173 (50)	-2.21	7
Vanilla acid	15.35	$C_8H_8O_4$	167	123 (30), 125 (100), 152 (10)	-0.91	43
Gallic acid	16.50	$C_7H_6O_5$	169	125 (100)	–3.42	7, 43, 44
Castalina	16.93	$C_{27}H_{20}O_{18}$	631	301 (100), 299 (37)	-1.52	42
X_1[f]	17.29	-	-	613 (50), 301 (100), 631 (30)	-2.03	-
Chlorogenic acid	18.05	$C_{16}H_{18}O_9$	355	163 (70)	-4.52	45
Corilagin	18.17	$C_{27}H_{22}O_{18}$	633	301 (100), 589 (10)	–0.76	42
Brevifolin	19.53	$C_{10}H_{12}O_4$	247	203 (75), 175 (20)	-4.28	7, 42
Caffeic acid	20.37	$C_9H_8O_4$	181	163 (95)	-3.98	45
p-Coumaric acid	23.85	$C_9H_8O_3$	163	145 (30), 119 (10), 103 (30), 89 (10), 127 (8)	-1.96	46
Tannic acid	24.61	$C_{76}H_{52}O_{46}$	183	123 (100)	-2.45	44
Rutin	26.16	$C_{27}H_{30}O_{16}$	609	301 (100)	-2.50	45
Elagic acid	27.31	$C_{14}H_6O_8$	301	257 (100), 229 (50)	-0.10	7, 43
Elagic acid rhamnoside	27.80	$C_{20}H_{16}O_{12}$	447	359 (50), 403 (30) 385 (10), 315 (5), 301 (7), 275 (100)	-0.52	42
Quercetin	31.94	$C_{15}H_{10}O_7$	301	151 (70)	-0.37	45

Reference compounds	T_R[a] (min)	Molecular formula	M^{+}[c]	$[M-H]^{-}$[b] (m/z) Molecular fragments (AR %[d])	Mass error (ppm)	Bibliographical references
Elagic acid pentoside	32.07	$C_{19}H_{14}O_{12}$	433	291 (21), 405 (57), 301 (90), 275 (8), 247 (40), 229 (5)	-2.51	42, 43
Naringenin	32.46	$C_{15}H_{12}O_5$	271	151 (75), 177 (100), 165 (52), 107 (18), 125 (25)	-1.98	43
Naringin	34.30	$C_{27}H_{32}O_{14}$	579	151 (50), 119 (6), 271 (100)	-2.68	47
Kaempferol	36.60	$C_{15}H_{10}O_6$	285	241 (67), 217 (100)	-3.98	7
Luteolin	36.92	$C_{15}H_{10}O_6$	285	257 (40), 241 (100)	-4.58	42, 45
Ferulic acid	38.09	$C_{10}H_{10}O_4$	193	177 (7), 149 (50), 145 (100), 117 (32), 89 (62)	-2.98	46
Cinnamic acid derivative	38.65	-	329	197 (50), 239 (35), 169 (100)	-0.58	42
(+) Catechin	39.67	$C_{15}H_{14}O_6$x H_2O	289	245 (50), 205 (100), 179 (20), 261 (42)	-0.37	7, 43, 44
(-) Epicatechin	39.67	$C_{15}H_{14}O_6$	289	245 (50), 205, (100) 179 (20), 261 (40)	-2.63	7, 43
Apigenin	47.80	$C_{15}H_{10}O_5$	269	223 (50), 179 (100)	-3.04	42, 45
Orientina	49.74	$C_{21}H_{20}O_{11}$	447	403 (100), 233 (50)	-1.61	42

[a]RT - retention time; [b][M-H]$^-$ - negative ionization; [c]M^+ - molecular ion; [d]AR - relative abundance; [e]HHDP - diphenyl hexahydroxyl acid hexoside, [f]X_1 - unidentified compound/gerannin.

LC-MS/MS qualitative analysis of *N. alba* M-US extracts indicated and confirmed 27 compounds with polyphenolic structure (Figure 4.11).



(**a**) (**b**)

Figure 4.11. Chromatograms obtained on LC-MS/MS separation of N. alba M-US extracts (a) F-M-US and (b) R-M-ES. H - HHDP, Qn - quinic acid, C - corilagin, V - vanillic acid, Cas - castaline, Ga - gallic acid, X_1 - fragment of gerannin/unidentified compound, Ca - caffeic acid, pC - p-coumaric acid, T - tannic acid, R - rutin, El - ellagic acid, Elr - ellagic acid rhamnoside, Elp - ellagic acid pentoside, Cn - cinnamic acid derivative, Na - naringin, N - naringenin, F - ferulic acid, Ch - catechin, Ep - epicatechin, Cl - chlorogenic acid, Q - quercetin, A - apigenin, L - luteolin, B - brevifoline, K - kaempferol and O – orientin

4.5 Partial conclusions

A total of 27 polyphenolic compounds (HHDP acid hexoside, quinic acid, corilagin, vanillic acid, castaline, gallic acid, gerannin, caffeic acid, p-coumaric acid, tannic acid, rutin, ellagic acid, ellagic acid rhamnoside, ellagic acid pentoside, cinnamic acid derivative, naringin, naringenin, ferulic acid, (+) catechin, (-) epicatechin, chlorogenic acid, quercetin, apigenin,

luteolin, brevifolin, kaempferol and orientin) were identified and reported for the first time in *N. alba* FL-M-US, F-M-US, T-M-US and R-M-US extracts.

The highest amount of polyphenolic compounds was quantified in *N. alba* root extracts (RM-US, RM-ES, RE-EM), and the lowest amount of polyphenolic compounds found in *N. alba* stem extracts (TM-US, TM-ES, TE-EM). CH-EM fractional extraction favored the extraction of epicatechin, present in the *N. alba* FR-A-EM, FL-A-EM and F-A-EM extracts. Naringin is present in the largest amount in *N. alba* FL-M-ES and R-M-ES extracts. The highest concentration of rutin was identified in R-M-ES and R-M-US extracts. The highest amount of gallic acid was identified in the R-E-EM extract, and the highest amount of caffeic acid was identified in the FL-M-US extract. p-Coumaric acid was identified with the highest concentration in the FR-M-ES extract.

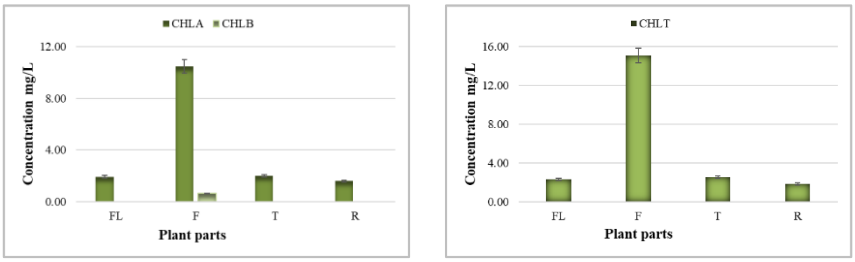
Results obtained in this subchapter were partially published in the scientific article "*Exploring New Antioxidant and Mineral Compounds from Nymphaea alba Wild-Grown in Danube Delta Biosphere*", Cudalbeanu & all, Molecules 2018.

Chapter 5 Identification and quantification of organic compound classes with biologically active properties form the *N. alba* species

5.4 Results and discussions

5.4.1 Total chlorophyll pigment contents

Chlorophyll a, chlorophyll b and total chlorophyll content were determined spectrophotometrically from fresh samples of flower, leaf, stem and root of the *N. alba* species (Figure 5.3). The highest amount of CHLA was found in the *N. alba* leaf sample, with a concentration of 10.48±1.02 mg/L. The lowest amount of CHLA was found in the *N. alba* root sample, with a concentration of 1.6±0.65 mg/L. The highest amount of CHLT was found in the *N. alba* leaf sample (15.06±1.13 mg/L). The lowest amount of CHLT was determined in the *N. alba* root sample (1.87±0.97 mg/L).



(a) **(b)**

Figure 5.3. Determination of chlorophyll a and chlorophyll b (a) and total chlorophyll (b) in the N. alba flower, leaf, stem and root. FL - flower, F - leaf, T - stem, R - root, CHLA - chlorophyll a, CHLB - chlorophyll b and CHLT - total chlorophyll

5.4.2 Total carotenoid contents

The amount of carotenoids was determined from dried and ground samples of the *N. alba* flower, leaf and stem using a linear β-carotene curve, and the results were expressed in milligrams β-carotene equivalent per gram of sample (mgEqβC/g). The highest amount of carotenoids was

found in the flower and leaf samples, with concentrations of 0.39±0.02 mgβC/g sample and respectively, 0.37±0.01 mgβC/g sample (Table 5.1).

Table 5.1. The total carotenoid content of different anatomical parts of the N. alba species

Plant parts*	**FL**	**F**	**T**
mgβC/g sample	0.39±0.02	0.37±0.01	0.29±0.01

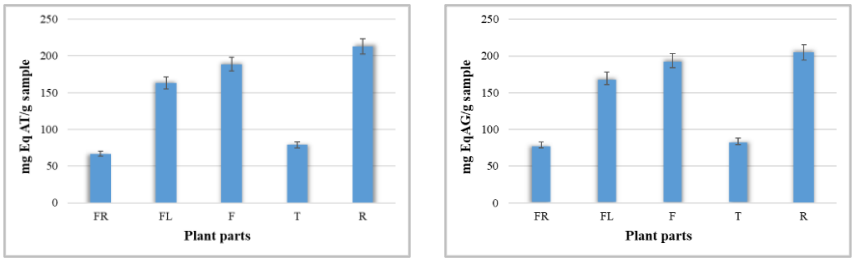
*FL – flower, F – leaf, T – stem.

5.4.3 Total polyphenol contents

5.4.3.1 Phenolic acids

In determining the total phenolic acid content of M-US, M-ES and E-EM extracts, obtained from *N. alba* various anatomical parts, such as fruit, flower, leaf, stem and root, tannic acid and gallic acid calibration curves were used and the results were expressed in tannic acid milligrams equivalent per gram of sample (mgEqAT/g sample), respectively gallic acid milligrams equivalent per gram of sample (mgEqAG/g sample).

Figure 5.6 shows the total phenolic acid content of *N. alba* M-US extracts. The highest amount of phenolic acids was identified in the R-M-US extract, with values of 212.79±2.50 mgEqAT/g sample, respectively 204.84±3.33 mgEqAG/g sample and in the F-M-US extract, with values of 188.52±5.60 mgEqAT/g sample, respectively 193.52±6.25 mgEqAG/g sample.



(a) **(b)**

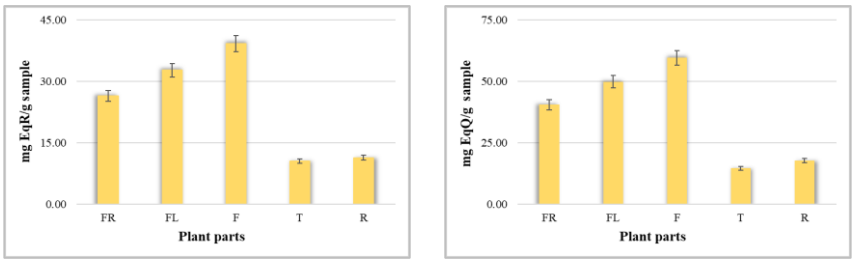
Figure 5.6. Total phenolic acid content of N. alba M-US extracts: (a) mgEqAT/g sample, (b) mgEqAG/g sample. FR - fruit, FL - flower, F - leaf, T – stem and R – root

The total phenolic acid content determined from *N. alba* M-ES extracts showed higher amounts than the total phenolic acid content determined from M-US extracts. A higher amount of phenolic acids was determined in R-M-ES, F-M-ES and FL-M-ES extracts, with concentrations in the range 205.79±5.09 to 395.75±4.14 mgEqAT/g sample, respectively 226.31±4.75 to 375.60±3.87 mgEqAG/g sample. In optimizing the extraction methods of biologically active compounds present in different anatomical parts of *N. alba* species, E-EM extraction proved to be a success. *N-alba* FR-E-EM, FL-E-EM, F-E-EM, T-E-EM and R-E-EM extracts provide the highest phenolic acid content compared to the other extraction methods used. The highest amount of phenolic acids was quantified in the R-E-EM sample, with a concentration of 606.35±5.26 mgEqAT/g sample, respectively 572.16±4.91 mgEqAG/g sample, and in the F-E-EM extract, with a concentration of 440.65±4.30 mgEqAT/g sample, respectively 456.17±6.28 mgEqAG/g sample. *N. alba* FR-E-EM and T-E-EM extracts have the lowest phenolic acid content, similar to *N. alba* FR-M-US, FR-M-ES, T-M-US and T-E-ES extracts.

5.4.3.2 Flavonoids

The total flavonoid content of M-US, M-ES and E-EM extracts, obtained from *N. alba* various anatomical parts, such as fruit, flower, leaf, stem and root, was determined by microspectrophotometric analysis, in the presence of $AlCl_3$, using rutin and quercetin calibration curves, and the results were expressed in rutin equivalent milligrams per gram of sample (mgEqR/g sample) and quercetin milligrams equivalent per gram of sample (mgEqQ/g sample). The total flavonoid content determined from *N. alba* M-US extracts is shown in Figure 5.10. The highest amount of flavonoids is present in the F-M-US sample, with a value of 39.23±1.91 mgEqR/g sample, respectively 59.63±2.87 mgEqQ/g sample. The T-M-US extract shows the lowest amount of flavonoids, with a value of 10.59±1.25 mgEqR/g sample, respectively 14.67±0.83 mgEqQ/g sample.

After removal the lipophilic fraction, the amount of flavonoids present in M-ES extracts is higher than in M-US extracts, showing values of total flavonoid content ranging from 11.97±0.75 to 46.45±0.39 mgEqR/g sample, respectively from 16.24±0.59 to 79.51±5.62 mgEqQ/g sample. In the case of E-EM extracts, the highest flavonoid content was determined for F-E-EM extract (69.93±1.23 mgEqR/g sample, respectively 85.67±4.56 mgEqQ/g sample), followed by FL-E-EM extract (51.87±2.05 mgEqR/g sample, respectively 63.58±1.85 mgEqQ/g sample) and FR-E-EM extract (40.06±0.95 mgEqR/g sample, respectively 46.70±2.92 mgEqQ/g sample).



(a) **(b)**

Figure 5.10. Total flavonoid content of N. alba M-US extracts: (a) mgEqR/g sample, (b) mgEqQ/g sample. FR - fruit, FL - flower, F - leaf, T – stem and R – root

5.4.3.3 Condensed tannins

The total content of condensed tannins in M-US, M-ES and E-EM extracts, obtained from *N. alba* different anatomical parts, was determined microspectrophotometrically, in the presence of vanillin and HCl (Table 5.2). Catechin was used as a reference standard and the results were expressed in catechin milligrams equivalent per gram of sample (mgEqC/g).

Table 5.2. Total content of condensed tannins present in N. alba extracts

Extraction method	Anatomical parts of the *N. alba* species*				
	FR	FL	F	T	R
	mgEqC/g sample or unidentified (-)				
M-US	4.64±0.11	-	0.65±0.10	28.74±1.25	14.52±0.42
M-ES	4.85±0.64	-	0.88±0.21	30.97±3.30	16.87±1.75
E-EM	5.15±0.31	-	0.91±0.36	32.35±2.04	17.00±1.40

*FR - fruit, FL - flower, F - leaf, T - stem, R - root. M - methanol, E - ethanol. US - simple ultrasound assisted extraction; ES - multiple extraction using Soxhlet extractor; EM - simple mechanical agitation extraction.

The total content of condensed tannins ranged from 0.65±0.10 mgEqC/g sample to 32.35±2.04 mgEqC/g sample. The highest content of condensed tannins was determined in *N. alba* T-M-US, T-M-ES and T-E-EM extracts (M-US - 28.74±1.25 mgEqC/g sample; M-ES – 30.97±3.30 mgEqC/g sample; E-EM - 32.35±2.04 mgEqC/g sample).

5.5 Partial conclusions

In conclusion, the analysis of the main classes of compounds, primary and secondary metabolites present in the samples of the *N. alba* species, proved the presence of significant amounts of chlorophyll, carotenoids, phenolic acids, flavonoids and condensed tannins. The highest amount of CHLA and CHLT was found in the leaf sample of the *N. alba* species. The highest amount of carotenoids was found in the flower and leaf samples of the *N. alba* species. *N. alba* R-M-US, R-M-ES and R-E-EM extracts showed the highest amount of phenolic acids, and *N. alba* F-M-US, F-M-ES and F-E-EM extracts showed the highest amount of flavonoids. Condensed tannins are present in the largest amount in *N. alba* T-M-US, T-M-ES and T-E-EM extracts.

The results obtained from the identification and quantification of the main secondary metabolites (phenolic acids, flavonoids and condensed tannins) present in various *N. alba* M-US extracts were published in the scientific article "*Exploring New Antioxidant and Mineral Compounds from Nymphaea alba Wild-Grown in Danube Delta Biosphere*", Cudalbeanu & all, Molecules 2018".

Chapter 6 Macro and microelements present in *N. alba* different anatomical parts

6.4 Results and discussions

A total of 23 macro and microelements were identified and quantified in the *N. alba* flower, leaf, stem and root samples, macroelements with mean values between 24.56±1.83 - 36712.48±589.24 mg/kg, such as Al, Ca, Fe, K, Mg, Mn, Na and P (Table 6.3) and microelements with mean values between <0.10 - 64.09 ± 2.40 mg/kg, such as As, B, Ba, Cd, Co, Cr, Cu, Hg, Li, Mo, Ni, Pb, Se, Sn and Zn (Table 6.4) [40].

Samples of *N. alba* flower, leaf, stem and root indicated large amounts of macroelements, such as K (4931.32±17.76 - 10724.93±45.24 mg/kg), Mg (1954.15±24.86 - 4084.08±36.25 mg/kg), P (1762.59±14.70 - 5181.85±57.18 mg/kg) and Na (4692.36±67.73 - 36712.48±589.24 mg/kg). The root sample contains the highest amounts of Mg (4084.08±36.25 mg/kg) and Ca (8621.94±113.28 mg/kg), the flower sample has the highest K content (10724.93±45.24 mg/kg) and P (5181.95±57.18 mg/kg), and the stem sample contains the highest amount of Na (36712.48±589.24 mg/kg).

Samples of *N. alba* flower, leaf, stem and root indicated the presence of microelements, such as B, Ba, Co, Cr, Cu, Li, Ni, Pb and Zn. The microelements Co, Cr, Li, Ni and Pb were identified only in the root sample (Co - 2.04±0.12 mg/kg; Cr - 14.56±0.56 mg/kg; Li - 6.46±0.55 mg/kg; Ni - 7.55±0.57 mg/kg; Pb - 2.81±0.33 mg/kg), and B was identified in the flower

(36.28±1.28 mg/kg), leaf (32.18±1.12 mg/kg) and stem (23.06±1.22 mg/kg). Ba was identified in the leaf samples (13.47±0.81 mg/kg), stem (5.72±1.00 mg/kg) and root (55.11±2.21 mg/kg).

Table 6.3. Macroelement contents in N. alba different anatomical parts

Macroelements	Anatomical parts of the *N. alba* species mg/kg (dry substance)			
	Flower	Leaf	Stem	Root
Al	642.69±15.19	1075.76±58.73	224.96±12.10	7151.95±57.94
Ca	3817.11±69.80	8103.09±58.89	7293.97±38.07	8621.94±113.28
Fe	149.19±6.58	817.05±4.01	24.56±1.83	5396.28±20.54
K	10724.93±45.24	4931.32±17.76	6876.61±19.92	7453.42±18.85
Mg	2857.22±82.67	2057.35±68.45	1954.15±24.86	4084.08±36.25
Mn	69.17±1.72	508.36±14.63	345.79±6.25	379.02±6.96
Na	9745.81±55.99	16908.65±580.22	36712.48±589.24	4692.36±67.73
P	5181.85±57.18	2051.31±34.28	1762.59±14.70	3467.95±23.84

Table 6.4. Microelement contents in N. alba different anatomical parts

Microelements	Anatomical parts of the *N. alba* species mg/kg (dry substance)			
	Flower	Leaf	Stem	Root
As	<1.00	<1.00	<0.50	<2.00
B	36.28±1.28	32.18±1.12	23.06±1.22	<2.00
Ba	<2.00	13.47±0.81	5.72±1.00	55.11±2.21
Cd	<0.10	<0.10	<0.10	<0.10
Co	<1.00	<1.00	<1.00	2.04±0.12
Cr	<1.00	<2.00	<1.00	14.56±0.56
Cu	6.28±0.47	2.99±0.36	<1.00	10.23±1.10
Hg	<0.50	<0.50	<0.50	<0.50
Li	<1.00	<1.00	<1.00	6.46±0.55
Mo	<1.00	<1.00	<1.00	<1.00
Ni	<1.00	<2.00	<1.00	7.55±0.57
Pb	<0.50	<0.50	<0.50	2.81±0.33
Se	<1.00	<0.50	<0.50	<0.50
Sn	<2.00	<2.00	<2.00	<2.00
Zn	64.09±2.40	16.07±1.77	12.73±1.01	44.79±2.94

6.5 Partial conclusions

The results of the experimental study on the total content of macro and microelements confirm the bioaccumulation of sediment and water elements by the *N. alba* aquatic plant. Therefore, in the processes of growth and development, the *N. alba* species assimilates substantial amounts of essential elements, but also small amounts of non-essential elements.

Large amounts of macroelements, such as K, Mg, P and Na, have been identified in the *N. alba* flower, leaf, stem and root samples. The root sample contains the highest amounts of Mg and Ca, the flower sample has the highest K and P content, and the stem sample contains the highest amount of Na. Microelements such as B, Ba, Co, Cr, Cu, Li, Ni, Pb and Zn were identified in the *N. alba* flower, leaf, stem and root samples.The microelements Co, Cr, Li, Ni and Pb were identified only in the root sample, B was identified in the flower, leaf and stem, and Ba was identified in the leaf, stem and root samples.

The results obtained in this subchapter were published in the scientific article "*Exploring New Antioxidant and Mineral Compounds from Nymphaea alba Wild-Grown in Danube Delta Biosphere*", Cudalbeanu & all, Molecules 2018.

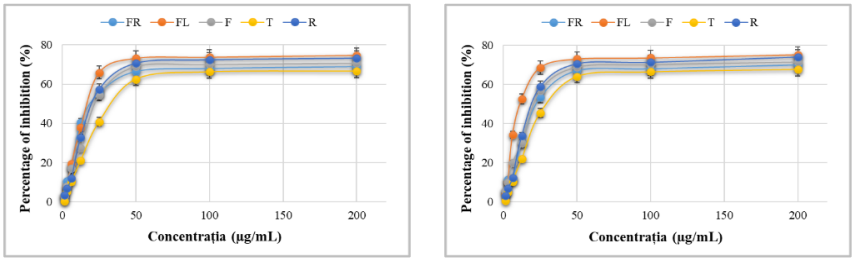
Chapter 7 Antioxidant potential of biologically active compounds from the *N. alba* species

7.4 Results and discussions

7.4.1 Antioxidant activity by free radical DPPH inhibition

The antioxidant potential, which involves the use of the DPPH free radical, was determined for M-US, M-ES and E-EM extracts, obtained from the *N. alba* different anatomical parts (fruit, flower, leaf, stem and root). Figure 7.5 shows the percentage of DPPH free radical inhibition of *N. alba* M-US extracts, obtained after 20 and 50 minutes of incubation. The highest percentage of DPPH free radical inhibition is shown by FL-M-US, RM-US and FM-US extracts (in descending order), with a percentage of 74.82±1.75 %, 73.32±0.98 %, respectively 70.81±2.01 % [40].

Regarding the percentage of DPPH free radical inhibition of *N. alba* M-ES extracts, the highest percentage of DPPH free radical inhibition is shown by FL-M-ES, RM-ES and FM-ES extracts (in order decreasing), with a percentage of 75.84±2.51 %, 74.02±1.67 %, respectively 72.41±0.78 %. *N. alba* E-EM extracts have a lower antioxidant potential than M-US and M-ES extracts because 500 µg/mL of extract concentrations were used to obtain an inhibition rate of ± 70 %, while for *N. alba* M-US and M-ES extracts were used of 200 µg/mL extract concentrations.



(a) **(b)**

Figure 7.5. Percentage of DPPH free radical inhibition of N. alba M-US extracts after 20 (a) and 50 (b) minutes of incubation. FR - fruit, FL - flower, F - leaf, T - stem and R - root

Percentage of inhibition of the *N. alba* extracts and reference compounds, obtained after 50 minutes of incubation, demonstrate that the antioxidant activity of biologically active compounds is stable over time. With regard to the percentages of DPPH free radical inhibition of the reference compounds, in ascending order, the highest percentage of inhibition is ascorbic acid, followed by trolox and quercetin, with a percentage of 91.15±2.93 %, 72.94±2.76 %, respectively 71.05±4.05 %, these being compounds known as free radical inhibitors [48, 49, 50]. IC_{50} values of the reference compounds were: ascorbic acid - 3.29±0.55 µg/mL, trolox - 5.00± 0.26 µg/mL and quercetin - 7.03±0.85 µg/mL [51, 52]. The AAI values for the reference compounds were: ascorbic acid - 15.20±0.33, trolox - 10.00±0.10, respectively quercetin - 7.11±0.53.

Table 7.1 shows the antioxidant potential of the *N. alba* M-US, M-ES and E-EM extracts, expressed as follows: IC_{50} value, equivalents of antioxidant activity and index of antioxidant activity. IC_{50} values for *N. alba* M-US extracts are as follows: FR - 17±0.50 µg/mL, FL - 17±0.98 µg/mL, F - 20±0.94 µg/mL, T - 25±0.89 µg/mL and R - 19±0.49 µg/mL. IC_{50} values for *N. alba* M-ES extracts are: FR - 18±1.08 µg/mL, FL - 15±0.73 µg/mL, F - 18±0.77 µg/mL, T - 30±1.10 µg/mL and R - 16±0.69 µg/mL. IC_{50} values for E-EM extracts are higher than M-US and M-ES extracts (FR - 30±2.27 µg/mL, FL - 26±1.13 µg/mL, F - 36±1.75 µg/mL, T - 52±3.19 µg/mL and R - 26±1.25 µg/mL), which indicates a lower antioxidant power. The experimental data show that the highest antioxidant activity, evaluated by the DPPH method, is the *N. alba* FL-M-ES extract, having the highest AAI value (3.33±0.32), respectively the *N. alba* R-M-ES extract, showing an AAI value of 3.13±0.26. *N. alba* T-M-US, T-M-ES and T-E-EM extracts showed the lowest AAI values (M-US - 2.00±0.16; M-ES - 1.67±0.64; E-MS - 0.96±0.21).

Table 7.1. Antioxidant potential of N. alba M-US, M-ES and E-EM extracts expressed as follows: IC_{50} value, equivalents of antioxidant activity and antioxidant activity index

Anatomical part*	IC_{50} (µg/mL)	mgEqT/g	mgEqAA/g	mgEqQ/g	AAI
M-US					
FR	17±0.50	36.91±0.90	31.98±4.10	70.73±6.92	2.94±0.31
FL	17±0.98	40.66±2.90	35.08±1.57	77.66±6.40	2.94±0.18
F	20±0.94	38.08±4.91	32.95±5.31	72.89±1.05	2.50±0.04
T	25±0.89	35.43±1.31	30.77±0.91	68.01±4.85	2.00±0.16
R	19±0.49	39.69±0.80	34.28±1.40	75.87±8.02	2.63±0.24
M-ES					
FR	18±1.08	36.98±4.40	32.05±1.96	70.87±7.35	2.78±0.83
FL	15±0.73	41.32±3.82	35.62±2.13	78.87±1.10	3.33±0.32
F	18±0.77	39.11±1.53	33.80±0.40	74.79±4.14	2.78±0.27
T	30±1.10	34.85±7.20	30.28±1.46	66.93±3.07	1.67±0.64
R	16±0.69	40.15±3.44	34.66±2.60	76.71±5.93	3.13±0.26
E-EM					
FR	30±2.27	14.56±0.50	12.63±1.19	27.93±1.51	1.67±0.42
FL	26±1.13	16.02±1.09	13.83±0.90	30.61±1.96	1.92±0.43
F	36±1.75	15.34±1.35	13.27±2.41	29.35±1.71	1.39±0.55
T	52±3.19	13.75±2.73	11.96±0.59	26.43±1.48	0.96±0.11
R	26±1.25	15.59±2.95	13.47±3.27	29.82±0.97	1.92±0.15

*FR - fruit, FL - flower, F - leaf, T - stem, R - root. Results were expressed as mean ± standard deviation. IC_{50} values were obtained from dose-response curves.

7.4.2 Antioxidant activity using the ABTS method

The antioxidant potential involving the use of the $ABTS^{\bullet+}$ free radical was determined for M-US, M-ES and E-EM extracts, obtained from *N. alba* different anatomical parts, such as fruit, flower, leaf, stem and root. Figure 7.9 shows the percentage of $ABTS^{\bullet+}$ free radical inhibition of *N. alba* M-US extracts after 30 and 60 minutes of incubation. The highest percentages of $ABTS^{\bullet+}$ free radical inhibition is shown by R-M-US and F-M-US extracts, with a percentage of inhibition of 78.20±0.12 %, respectively 77.01±0.73 % [53].

Regarding the percentage of $ABTS^{\bullet+}$ free radical inhibition of *N. alba* M-ES extracts, the highest percentage of $ABTS^{\bullet+}$ free radical inhibition is shown by R-M-ES and F-M-ES extracts, with a percentage of inhibition of 75.39±1.67 %, respectively 73.14±1.54 %. Also, the highest

percentage of ABTS$^{\bullet+}$ free radical inhibition of *N. alba* E-EM extracts is presented by the RM-EM and FM-EM extracts, with a percentage of inhibition of 74.39±0.99 %, respectively 73.77±2.16 %.

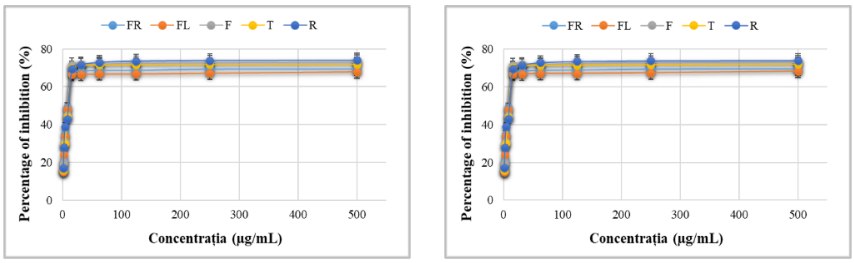


Figure 7.9. Percentage of ABTS●+ free radical inhibition of N. alba M-US extracts, after 30 (a) and 60 (b) minutes of incubation. FR - fruit, FL - flower, F - leaf, T - stem and R - root

The trolox reference compound has an percentage of inhibition of 73.87±0.79 % (250 µg/mL) and an IC_{50} value of 3.26±0.22 µg/mL [54]. Table 7.2 shows the antioxidant potential of *N. alba* M-US, M-ES and E-EM extracts, expressed as follows: IC_{50} value and trolox equivalents of antioxidant activity. IC_{50} values for *N. alba* M-US extracts are as follows: FR - 13±0.64 µg/mL, FL - 15±0.78 µg/mL, F - 12±0.11 µg/mL, T - 12±0.57 µg/mL and R - 10±0.10 µg/mL. IC_{50} values for *N. alba* M-ES extracts are: FR - 14±1.03 µg/mL, FL - 16±0.98 µg/mL, F - 13±0.23 µg/mL, T - 13±1.00 µg/mL and R - 12±0.80 µg/mL.

Table 7.2. Antioxidant potential of N. alba M-US, M-ES and E-EM extracts expressed by IC_{50} value and equivalents of antioxidant activity

Anatomical part*	IC_{50} (µg/mL)	mgEqT/g
	M-US	
FR	13±0.64	0.96±0.02
FL	15±0.78	0.91±0.01
F	12±0.11	1.31±0.03
T	12±0.57	1.04 ±0.01
R	10±0.10	1.95±0.04
	M-ES	
FR	14±1.03	0.95±0.01
FL	16±0.98	0.86±0.01
F	13±0.23	1.08±0.03
T	13±1.00	1.02±0.05
R	12±0.80	1.15±0.04
	E-EM	
FR	15±1.34	0.90±0.02
FL	17±0.54	0.84±0.01
F	14±0.23	1.04±0.03
T	15±0.64	1.02±0.02
R	12±1.10	1.10±0.02

*FR - fruit, FL - flower, F - leaf, T - stem, R - root. Results were expressed as mean ± standard deviation. IC_{50} values were obtained from dose-response curves.

IC_{50} values for *N. alba* E-EM extracts are: FR - 15±1.34 µg/mL, FL - 17±0.54 µg/mL, F - 14±0.23 µg/mL, T - 15±0.64 µg/mL and R - 12±1.10 µg/mL. F-M-US, F-M-ES and F-E-EM extracts show values of equivalents of antioxidant activity between 1.02 - 1.04 mgEqT/g, and R-

M-US, R-M-ES and R-E-EM extracts show values of equivalents of antioxidant activity between 1.10 - 1.95 mgEqT/g.

7.4.3 Antioxidant activity using BCB and FRAP methods

The DPPH and ABTS antioxidant activity tests demonstrated the antioxidant potential of *N. alba* F-M-US, F-M-ES and F-E-EM extracts, respectively of *N. alba* RM-US, RM-ES and RE-EM extracts, reason for which in the BCB and FRAP antioxidant activity tests, only the F-M-US and R-M-US extracts were analyzed, and the results obtained are presented in Table 7.3. Briefly, BCB and FRAP tests indicate the stability of the bioactive compounds present in the F-M-US and R-M-US extracts, which demonstrates that the antioxidant potentials and IC_{50} values of the extracts remain constant and act for a longer period of time [52].

Tabel 7.3. Antioxidant activity of N. alba M-US extracts evaluated by BCB and FRAP methods

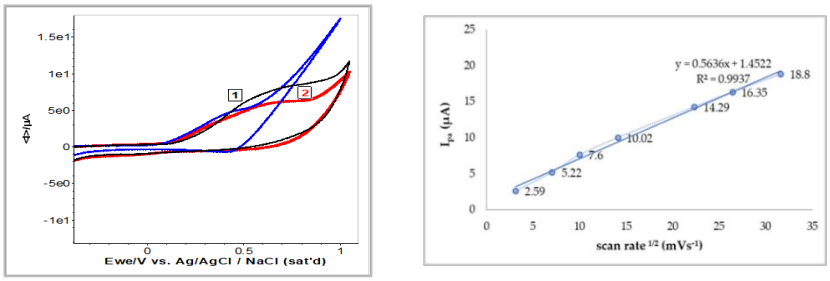
Test*	***N. alba* M-US extracts**					
	F			**R**		
	Percentage of inhibition (%)	**IC_{50} (µg/mL)**	**mgTEq/g**	**Percentage of inhibition (%)**	**IC_{50} (µg/mL)**	**mgTEq/g**
BCB	78.6±0.19	33±0.86	28.93±0.89	90.1±0.90	21±0.55	79.37±1.03
FRAP	74.6±0.13	30±0.25	2.24±0.67	79.7±0.13	13±0.15	2.40±0.98

*BCB - β-carotene bleaching process; FRAP - ferric ion reducing antioxidant power. F - leaf and R - root.

In both the BCB test and the FRAP test, the *N. alba* R-M-US extract showed higher antioxidant activity (higher percentage of inhibition, lower IC_{50} value, and higher trolox equivalents), than the *N. alba* F-M-US extracts. A percentage of inhibition of 90.1±0.90 % (BCB test) and 79.7±0.13 & (FRAP test) and an IC_{50} value of 21±0.55 µg/mL (BCB test), respectively 13±0.15 µg/mL (FRAP test) were recorded for the *N. alba* R-M-US extract. *N. alba* F-M-US extract showed a percentage of inhibition of 78.6±0.19 % (BCB test), respectively 74.6±0.13 % (FRAP test) and an IC_{50} value of 33±0.86 µg/mL (BCB test), respectively 30±0.25 µg/mL (FRAP test). The values expressed in trolox equivalents for the *N. alba* R-M-US extract were as follows: 79.37 mgTEq/g (BCB test), respectively 2.40 mgTEq/g (FRAP test), and for the *N. alba* F-M-US extract were: 28.93 µgTEq/g (BCB test) and 2.24 mgTEq/g (FRAP test).

7.4.4 Antioxidant potential by cyclic voltammetry correlated with UV-Vis

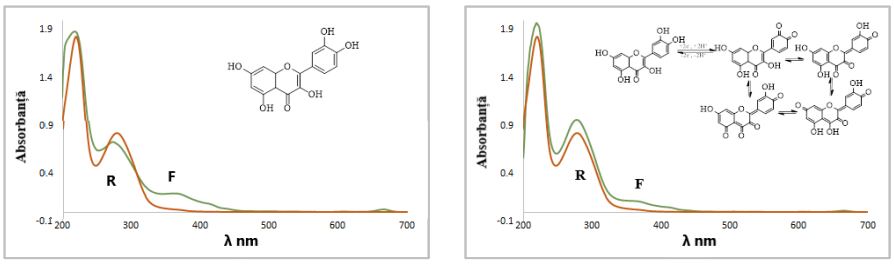
Electrochemical investigations on the antioxidant potential of the *N. alba* F-M-US and R-M-US extracts were performed using cyclic voltammetry (CV) and UV-Vis spectrophotometry. *N. alba* samples indicated a slightly positive potential from open circuit (OCV) measurements, the F-M-US sample showing an E potential of about 30 mV, more positive compared to the R-M-US sample [52]. Quercetin (10^{-3} M), the major antioxidant compound in the *N. alba* extracts, indicated a voltamogram profile similar to that of *N. alba* F-M-US and R-M-US extracts (Figure 7.13a, blue line). The redox potential ($E_{1/2}$) of the F-M-US and R-M-US extracts was 0.410 V±10 mV compared to the quercetin potential value [55]. Five consecutive voltammograms were recorded at different scanning speeds (10 - 1000 mVs^{-1}), and the current intensity increased with the scanning speed. The intensity of the anodic current varies linearly with the scanning rate due to the typical diffusion control processes (Figure 7.13b).



(a) **(b)**

Figure 7.13. Cyclic voltammograms recorded for N. alba F-M-US (1) and R-M-US (2) extracts compared to quercetin (blue line). E = ± 1V vs Ag/AgCl$_{sat.}$, Scanning speed of 100 mVs^{-1} (a). Dependence of the anodic current on the square root of the scanning speeds (b)

UV-Vis spectra of *N. alba* F-M-US and R-M-US extracts were recorded before and after electrochemical measurements (Figure 7.14). These results confirm the existence of antioxidant compounds in *N. alba* extracts, as the absorption of flavonoids varies at λ from 240 to 400 nm. λ_{max} of quercetin is at λ value of 385 nm [54], and the oxidative process of electrochemical measurements is shown in Figure 7.14b. Therefore, the observed electrochemical evaluation supports the antioxidant activity of *N. alba* extracts, which can be attributed to flavonoids, quercetin being the major component in the *N. alba* F-M-US extract [54, 56].



(a) **(b)**

Figure 7.14. UV-Vis spectra recorded for the N. alba F-M-US and R-M-US extracts before (a) and after (b) cyclic voltammetry tests

7.5 Partial conclusions

The highest antioxidant activity, evaluated by the DPPH method, is presented by the *N. alba* FL-M-ES and R-M-ES extracts. *N. alba* extracts have a strong antioxidant activity probably due to the presence of flavonoids, which could be involved in increasing their antioxidant potential, the F-M-US, F-M-ES and F-E-EM extracts, respectively FL-M-US, FL-M-ES and FL-E-EM extracts containing the highest amount of flavonoids.

The highest percentage of $ABTS^{\bullet+}$ free radical inhibition is presented by the *N. alba* R-M-US and F-M-US extracts. R-M-US, R-M-ES and R-E-EM extracts showed the highest amount of phenolic acids. The BCB and FRAP tests indicate the stability of the bioactive compounds present in the *N. alba* extracts, which demonstrates that the antioxidant potentials and IC_{50} values of the extracts remain constant and act for a longer period of time.

Results obtained in this subchapter were partially published in the scientific articles "*Exploring New Antioxidant and Mineral Compounds from Nymphaea alba Wild-Grown in the*

Danube Delta Biosphere", Cudalbeanu & all, Molecules 2018 and "*Antifungal, Antitumor and Antioxidant Potential of the Danube Delta Nymphaea alba Extracts*", Cudalbeanu & all, Antibiotics, 2020.

Chapter 8 Antimicrobial properties of biologically active compounds from the *N. alba* species

8.4 Results and discussions

8.4.1 Antibacterial properties of biologically active compounds

8.4.1.1 Kirby-Bauer diffusimetric method (MDKB)

The *N. alba* M-US extracts show the best inhibitory activity against Gram-negative bacterial strain *E. hormaechei* ATCC 700322, with a range of inhibition zones between 20 - 24 mm (moderate inhibitory effect). *N. alba* FR-M-US and FL-M-US extracts show 9 mm inhibition zones against the reference strain *P. aeruginosa* ATCC 27853, and *N. alba* F-M-US and R-M-US extracts 10 mm show inhibition zones against the reference strain *P. aeruginosa* 477. *N. alba* M-US extracts show low inhibitory effect against Gram-positive bacterial strain *S. aureus* ATCC 25923 (11 - 17 mm) (Table 8.1).

Table 8.1. Results obtained by measuring the inhibition zones of N. alba M-US extracts against Gram-negative and Gram-positive bacteria

Bacterial strain	**M-US extracts* *(diameter in mm)***					**Positive control *(diameter in mm)***
Gram-negative	**FR**	**FL**	**F**	**T**	**R**	**GE**
***E. coli* ATCC 25922**	-	-	11	+	11	19-26
***P. aeruginosa* ATCC 27853**	9	9	-	-	-	17-23
P. aeruginosa* 477*	**	**	10	**	10	-
***E. hormaechei* ATCC 700322**	20	20	20	21	24	-
Gram-pozitive	**FR**	**FL**	**F**	**T**	**R**	**GE**
***S. aureus* ATCC 25923**	12	15	11	14	17	19-27
S. aureus* NCTC8178*	**	**	10	**	13	-
***S. pyogenes* ATCC 19615**	-	-	-	-	-	-
***E. casseliflavus* ATCC 700327**	-	-	-	-	-	-

- No antimicrobial action. **Only F-M-US and R-M-US extacts were tested against *P. aeruginosa* 477 and *S. aureus* NCTC8178 reference strains. + at an extract concentration higher than 8 mg/mL, the extracts show antimicrobial activity. *FR - fruit, FL - flower, F - leaf, T - stem, R - root, GE - gentamicin. Values are expressed as the average of three determinations.

As in the case of *N. alba* M-US extracts, the FR-M-ES and FL-M-ES extracts show 11 mm inhibition zones against the bacterial strain *P. aeruginosa* ATCC 27853. With a moderate inhibitory effect, the *N. alba* M-ES extracts show the best inhibitory activity against Gram-

negative bacterial strain *E. hormaechei* ATCC 700322 with a range of inhibition zones between 21 - 27 mm. *N. alba* M-ES extracts have a low inhibitory effect against the bacterial *S. aureus* ATCC 25923 (12-16 mm).

In vitro evaluation of the antibacterial activity of the *N. alba* E-EM extracts against Gram-negative and Gram-positive pathogenic bacteria using MDKB showed that the extracts have a moderate inhibitory effect against Gram-negative bacterial strain *E. hormaechei* ATCC 700322 (20 - 25 mm) and low inhibitory effect against Gram-positive bacterial strain *S. aureus* ATCC 25923 (12 - 15 mm).

8.4.1.2 Minimum inhibitory concentration

N. alba M-US extracts showed MIC values of 1 mg/mL against bacterial strain *E. hormaechei* ATCC 700322. F-M-US and R-M-US extracts showed MIC values of 1 mg/, respectively 2 mg/mL, against bacterial strain *E. coli* ATCC 25922. FR-M-US and FL-M-US extracts inhibited the development of bacterial strain *P. aeruginosa* ATCC 27853, with a MIC value of 1 mg/mL. In the case of Gram-positive bacterial strains *S. aureus* ATCC 25923 and *S. aureus* NCTC8178, *N. alba* M-US extracts show MIC values of 2 mg/mL (Table 8.4).

Table 8.4. MIC values of N. alba M-US extracts against Gram-negative and Gram-positive bacteria

Bacterial strain	**M-US extract* mg/mL**					**Positive control µg/mL**
Gram-negative	**FR**	**FL**	**F**	**T**	**R**	**GE**
***E. coli* ATCC 25922**	-	-	1	-	>2	0.25-1
***P. aeruginosa* ATCC 27853**	1	1	-	-	-	0.50-2
P. aeruginosa* 477*	-	-	-	-	-	-
***E. hormaechei* ATCC 700322**	1	1	1	1	1	-
Gram-pozitive	**FR**	**FL**	**F**	**T**	**R**	**GE**
***S. aureus* ATCC 25923**	2	2	2	2	2	0.12-1
S. aureus* NCTC8178*	-	-	2	-	2	-

The experiments were performed in triplicate and the mean CMI was calculated. - no activity/the minimum inhibitory concentration has not been determined. **Only F-M-US and R-M-US extracts were tested against *P. aeruginosa* 477 and *S. aureus* NCTC8178 reference strains. *FR - fruit, FL - flower, F - leaf, T - stem, R - root, GE - gentamicin.

The best antibacterial activity of *N. alba* M-ES extracts was obtained against bacterial strains *P. aeruginosa* ATCC 27853 and *E. hormaechei* ATCC 700322 (MIC = 1 mg/mL). *S. aureus* ATCC 25923 was inhibited by *N. alba* M-ES extracts with CMI values of 2 mg/mL. As with M-US and M-ES extracts, E-EM extracts show MIC values of 1 mg/mL against Gram-negative bacterial strains *P. aeruginosa* ATCC 27853 and *E. hormaechei* ATCC 700322 and MIC values of 2 mg/mL against Gram-positive bacterial strain *S. aureus* ATCC 25923.

8.4.1.3 Minimum bactericidal / bacteriostatic concentration

The F-M-US extract has a bactericidal effect (CMB = 2 mg/mL) and the R-M-US extract has a bacteriostatic effect against bacterial strain *E. coli* ATCC 25922. *N. alba* M-US, M-ES and E-EM extracts of FR and FL have bactericidal effects against the bacterial strain *P. aeruginosa* ATCC 27853 (CMB = 2 mg/mL). *N. alba* M-US, M-ES and E-EM extracts show bactericidal effects against bacterial strains *E. hormaechei* ATCC 700322 and *S. aureus* ATCC 25923 (CMB = 2 - 4 mg/mL), only the R-E-EM fraction shows bacteriostatic effect against bacterial strain *S. aureus* ATCC 25923.

8.4.2 Antifungal properties of biologically active compounds

8.4.2.1 Concentrația minimă inhibitorie

In vitro antifungal activity (MIC) was evaluated against four reference fungal strains: *C. glabrata* CBS138, *C. albicans* SC5134, *C. parapsilosis* ATCC22019 and *C. tropicalis* ATCC750, using the *N. alba* F-M-US and R-M-US extracts, with concentrations between 0.23 - 2000 μg/mL, and FLC, with concentrations between 0.23 - 250 μg/mL, was used as reference standard [52]. *N. alba* F-M-US and R-M-US extracts were found to be active against fungal strain *C. glabrata* CBS138, with CMI values of 1.717 μg/mL, respectively 1.935 μg/mL, and for FLC with CMI value of 0.7639 μg/mL (Figure 8.3) [57].

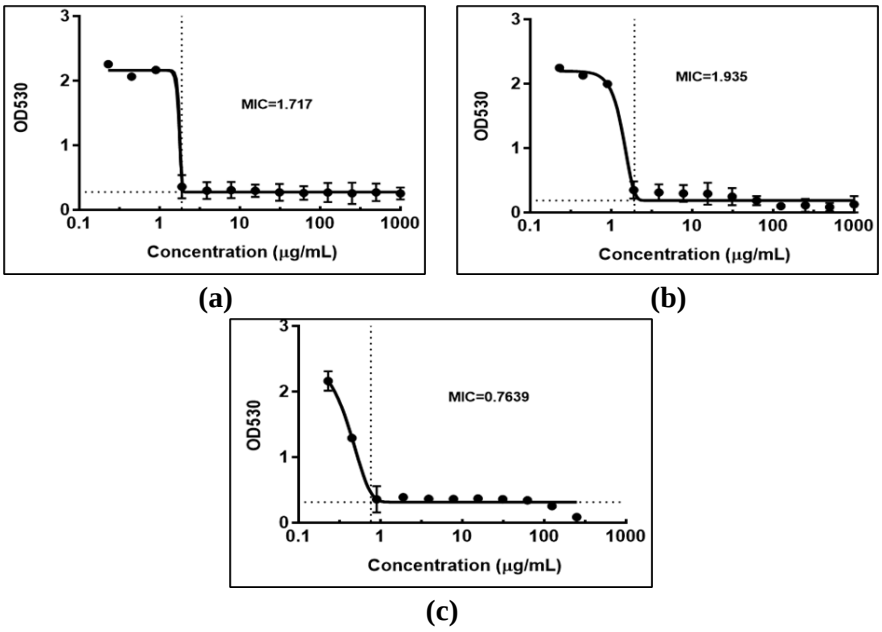
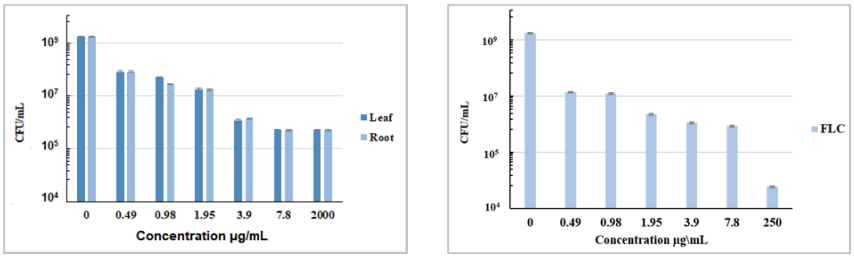


Figure 8.3. MIC values of the N. alba (a) F-M-US and (b) R-M-US extracts and (c) FLC against fungal strain C. glabrata CBS138

8.4.2.2 Minimum fungicidal/fungistatic concentration

The highest concentration, 2000 μg/mL, and also the lowest concentration, 7.8 μg/mL, of the *N. alba* F-M-US and R-M-US extracts, showed the same CFU/mL number of fungal strain *C. glabrata* CBS138. The results obtained in the determination of CFU/mL numer of fungal strain *C. glabrata* CBS138 cultured in the presence of different concentrations of FLC were also presented (Figure 8.4).



(a) **(b)**

Figure 8.4. Logarithmic values of CFU/mL of fungal strain C. glabrata CBS138, cultured in the presence of different concentrations of N alba (a) F-M-US (Leaf) and R-M-US (Root) extracts and (b) FLC

8.4.2.3 Potential interaction of the studied extracts with fluconazole

The present experimental study evaluated possible interactions between *N. alba* extracts (F-M-US and R-M-US) and FLC on the reference fungal strain *C. glabrata* CBS138, using the chessboard method and by determining the FIC index. Table 8.8 shows the average values of the FIC index associated with the type of interaction ($1<\Sigma FIC\leq 2$). The interaction of *N. alba* extracts and FLC proved to be indifferent.

Table 8.8. Interaction of N. alba M-US extracts with FLC against fungal strain C. glabrata CBS138

Fungal strain	**M-US extract**			
	F-M-US		**R-M-US**	
C. glabrata CBS138	**FIC index**	**Interaction**	**FIC index**	**Interaction**
	1.30	Indifference	1.06	Indifference

The experiments were performed in triplicate and the mean FIC value was calculated.

8.5 Partial conclusions

The *N. alba* extracts show the best inhibitory activity against Gram-negative bacterial strain *E. hormaechei* ATCC 700322 (moderate inhibitory effect). The *N. alba* M-US, M-ES and E-EM extracts of FR and FL show inhibition zones between 9 - 11 mm against the reference strain *P. aeruginosa* ATCC 27853, and *N. alba* F-M-US and R-M-US extracts show 10 mm inhibition zones against the reference strain *P. aeruginosa* 477. *N. alba* extracts have a low inhibitory effect against bacterial strain *S. aureus* ATCC 25923.

The *N. alba* M-US, M-ES and E-EM extracts showed MIC values of 1 mg/mL against Gram-negative bacterial strain *E. hormaechei* ATCC 700322 (bactericidal effects - CMB = 2 - 4 mg/mL). The *N. alba* M-US, M-ES and E-EM extracts of FR and FL showed MIC values of 1 mg/mL against Gram-negative bacterial strain *P. aeruginosa* ATCC 27853 (bactericidal effects - CMB = 2 mg/mL). The F-M-US and R-M-US extracts showed MIC values of 1 mg/mL, respectively 2 mg/mL, against the bacterial strain *E. coli* ATCC 25922, the F-M-US extracts showed bactericidal effect (CMB = 2 mg/mL) and R-M-US extracts showed bacteriostatic effect. The *N. alba* M-US, M-ES and E-EM extracts showed MIC values of 2 mg/mL against Gram-positive bacterial strain *S. aureus* ATCC 25923 (bactericidal effects - CMB = 2 - 4 mg/mL), but the R-E-EM extract showed a bacteriostatic effect against bacterial strain *S. aureus* ATCC 25923. At a concentration of 2 mg/mL, the *N alba* F-M-US and RM-US extracts showed a substantial decrease of the CFU/mL number of the bacterial strain *S. aureus* NCTC8178.

The *N. alba* F-M-US and R-M-US extracts were found to be active against fungal strain *C. glabrata* CBS138, with CMI values of 1.717 µg/mL, respectively 1.935 µg/mL, and for FLC with a CMI value of 0.7639 µg/mL. The results obtained showed that the antifungal action of *N. alba* extracts, at low concentrations, was specific on the fungal strain *C. glabrata* CBS138, having antifungal action, at much higher concentrations, on the other *Candida* fungal strain tested (*C. albicans* CB138, *C. parapsilosis* ATCC22019 and *C. tropicalis* ATCC750). The highest concentration, 2000 µg/mL and also the lowest concentration, 7.8 µg/mL, showed the same CFU/mL number of fungal strain *C. glabrata* CBS138, because the extracts have the same fungistatic action regardless of concentration used. The interaction of *N. alba* extracts (F-M-US and R-M-US) and FLC on the reference fungal strain *C. glabrata* CBS138, using the chessboard method and by determining the FIC index, proved to be indifferent.

The results obtained in the evaluation of the antifungal potential of *N. alba* M-US extracts against the fungal strain *C. glabrata* CBS138 were published in the scientific article "*Antifungal, Antitumor and Antioxidant Potential of the Danube Delta Nymphaea alba Extracts*", Cudalbeanu & all, Antibiotics 2020.

Chapter 9 Toxic and cytotoxic potential of biologically active compounds from the *N. alba* species

9.4 Results and discussions

9.4.1 Toxic effects of biologically active compounds on the wheat seeds germination (*Triticum aestivum* L.)

The toxic effect of the compounds present in the *N. alba* M-US extracts was determined using the germination test of wheat seeds (*Triticum aestivum* L.), by calculating and evaluating the most sensitive physiological parameters, such as germination percentage, relative percentage of root growth of the germinated wheat seeds, germination index and vigor index and by capturing images of the germinated wheat seeds treated and untreated with the *N. alba* M-US extracts, using CLSM. The experimental results (Table 9.1) showed that the application of different concentrations of the *N. alba* F-M-US and R-M-US extracts did not show a toxic action on the germinated wheat seeds [52].

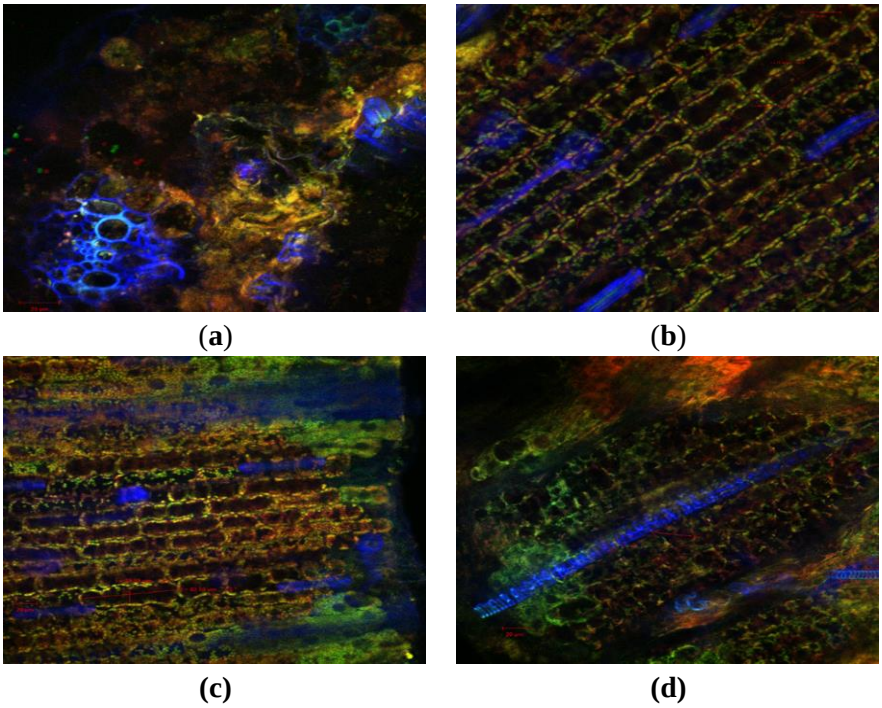
Table 9.1. Physiological parameters of germinated wheat seeds under the treatment of different concentrations of N. alba M-US extracts

Physiological parameters[a]	**Concentration (µg/mL)**				**Control (H_2O)**
	10	**100**	**500**	**1000**	
F-M-US extract					
G %	94.20±0.24	94.60±0.40	95.90±0.91	97.40±0.50	100.00±0.00
RRG %	84.60±1.20	101.13±0.87	103.43±1.13	103.55±0.97	100.00±0.00
GI	1.10±0.10	0.90±0.20	0.90±0.25	0.90±0.14	1.00±0.00
VI	120.20±0.43	146.20±0.35	117.90±0.10	113.80±0.10	66.60±0.14
TI	0.80±0.12	0.60±0.12	0.50±0.24	0.60±0.10	1.00±0.00
R-M-US extract					
G %	94.70±0.52	96.00±0.37	96.00±0.15	97.40±1.00	100.00±0.00
RRG %	69.09±0.73	91.76±1.00	94.55±0.76	100.07±0.45	100.00±0.00

Physiological parameters[a]	Concentration (µg/mL)				Control (H_2O)
	10	100	500	1000	
GI	1.40±0.10	1.00±0.21	1.00±0.50	1.00±0.45	1.00±0.00
VI	89.50±0.24	137.60±0.17	92.90±0.24	123.70±0.13	66.60±0.14
TI	0.70±0.43	0.50±0.12	0.50±0.11	0.80±0.10	1.00±0.00

Results were expressed as mean ± standard deviation. [a]G % - germination percentage, RRG % - relative percentage of root growth of the germinated wheat seeds, GI - germination index, VI - vigor index, TI - vigor index.

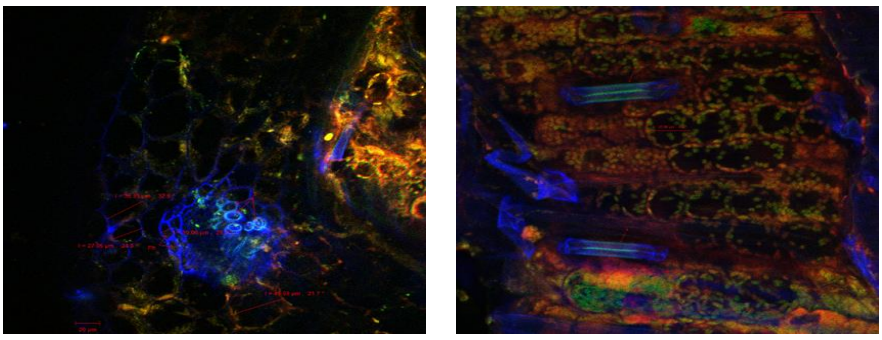
Also, the sections through the shoot of the germinated wheat seeds in the presence of *N. alba* M-US extracts showed healthy plant tissues, without morphological and structural changes, as observed by CLSM (Figures 9.2 - 9.3).



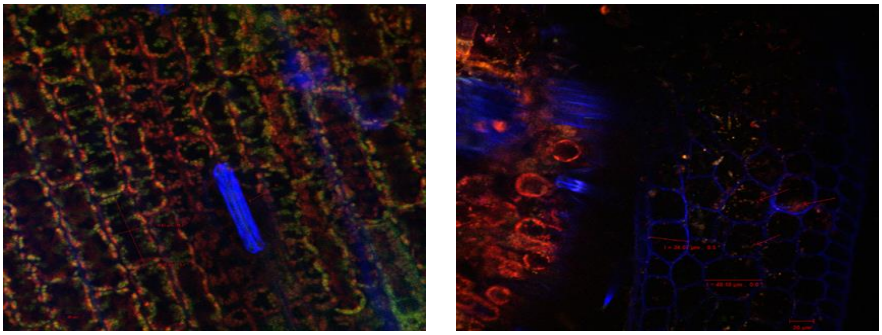
(a) **(b)**

(c) **(d)**

Figure 9.2. CLSM images of different sections of shoot wheat seeds germinated under the treatment of different concentrations of the N. alba F-M-US extract: a - 10 µg/mL, b - 100 µg/mL, c - 500 µg/mL and d – 1000 µg/mL



(a) **(b)**



(c) **(d)**

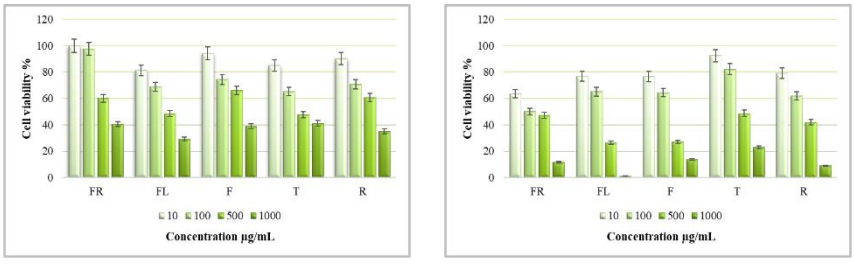
Figure 9.3. CLSM images of different sections of shoot wheat seeds germinated under the treatment of different concentrations of the N. alba R-M-US extract: a - 10 µg/mL, b - 100 µg/mL, c - 500 µg/mL and d – 1000 µg/mL

Cell differentiation was not affected by the *N. alba* extracts used. The unilayered epidermis (Figure 9.3d), the cortical parenchyma with isodiametric cells rich in chloroplasts (assimilating tissue), the pericycle separating the cortical parenchyma from the central cylinder (Figure 9.3d), and specific components of the conducting tissues (in blue): xylem (X) (Figure 9.3a, 9.2a, 9.2d) or phloem (Ph) (Figure 9.3a) can be seen. In all performed experiments, microscopic analysis of the sections showed normal epidermal formations such as numerous hairs (H) or stomata type Zea (S) (in blue), which means that *N. alba* M-US extracts allowed or even facilitated the differentiation of these structures [52].

9.4.2 Cytotoxic effects of biologically active compounds on healthy lung fibroblast cells

The cytotoxicity of the compounds present in *N. alba* extracts was evaluated using V79 healthy lung fibroblasts, and cell viability was measured by MTT assay. Extracts from different anatomical parts of the *N. alba* species (fruit, flower, leaf, stem and root), extracted by different methods (US, ES, EM) and with different solvents (petroleum ether, cyclohexane, methanol, ethanol and water), but also R-E-EM fractions (F2, F5, F50 and F51) were analyzed (Figure 9.4).

The *N. alba* EP-ES extracts showed a reduced cytotoxicity, with a cell viability greater than 30 % at a concentration of 1000 µg/mL (Figure 9.4a). Regarding the cytotoxicity of the *N. alba* CH-EM extracts, at a concentration of 1000 µg/mL, the extracts showed a percentage of cell viability of less than 15 % (Figure 9.4b). The *N. alba* FR-M-US, FL-M-US and T-M-US extracts showed a reduced cell viability at a concentration of 1000 µg/mL (Figure 9.4c) [52]. The highest cytotoxicity of the *N. alba* M-ES extracts is shown by the FL-M-ES and T-M-ES extracts, with a low percentage of cell viability (Figure 9.4d).



(a) **(b)**

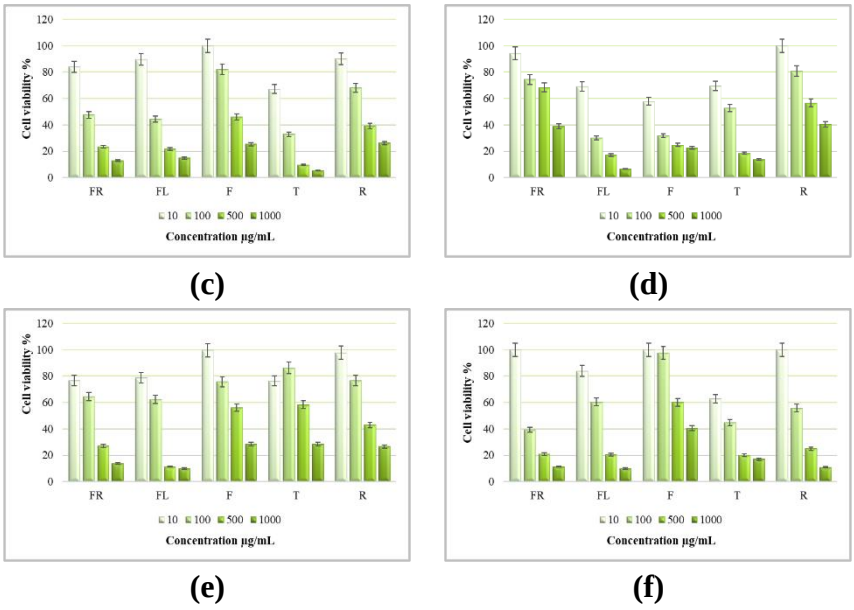


Figure 9.4. Cytotoxicity of N. alba extracts on V79 healthy lung fibroblast cells: (a) EP-ES, (b) CH-EM, (c) M-US, (d) M-ES, (e) E-EM and (f)) A-EM. FR - fruit, FL - flower, F - leaf, T - stem and R - root

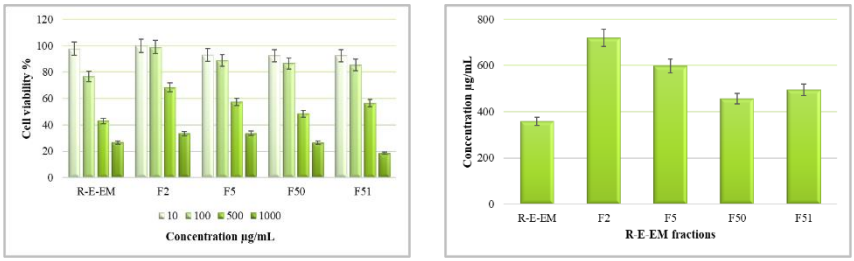
The FR-E-EM and FL-E-EM extracts showed the highest cytotoxicity at concentrations of 100 µg/mL, respectively 1000 µg/mL, (Figure 9.4e). IC_{50} values of *N. alba* extracts relative to V79 cell line are shown in Table 9.2. The results show that the *N. alba* FR, FL and R extracts showed negligible cytotoxicity on V79 healthy lung fibroblast cells, but the *N. alba* F and T extracts showed a high cytotoxicity on healthy cells.

Table 9.2. IC_{50} values of the N. alba extracts on V79 healthy lung fibroblast cells

Anatomical part[a]	***N. alba* extract**[b] **(µg/mL)**		
	EP-ES	**CH-EM**	**M-US**
FR	722.45±50.43	71.79±4.51	90.09±5.39
FL	319.98±10.46	133.22±5.73	91.63±4.80
F	757.74±29.71	133.24±6.55	367.00±50.01
T	426.23±17.21	412.66±35.40	29.72±4.47
R	567.80±20.25	158.75±7.87	281.00±59.00
	M-ES	**E-EM**	**A-EM**
FR	802.81±36.31	133.24±5.72	88.63±9.75
FL	31.72±2.39	111.08±4.17	125.97±28.44
F	19.68±1.66	473.00±55.33	722.45±10.03
T	64.04±5.17	535.03±69.50	39.14±5.23
R	646.91±55.53	357.80±14.36	143.71±3.57

[a]FR - fruit, FL - flower, F - leaf, T - stem, R - root. [b]EP - ether extracts, CH - cyclohexane extracts, M - methanolic extracts, E - ethanolic extracts, A - aqueous extracts. US - simple ultrasound assisted extraction; ES - multiple extraction using Soxhlet extractor; EM - simple mechanical agitation extraction. Data were obtained from dose-response curves.

The *N. alba* F2, F5, F50 and F51 fractions show low cytotoxicity, with a cell viability greater than 45 % at a concentration of 500 µg/mL (Figure 9.5a). IC_{50} values of the F2, F5, F50 and F51 fractions relative to the V79 cell line showed negligible cytotoxicity on V79 healthy lung fibroblast cells (Figure 9.5b).

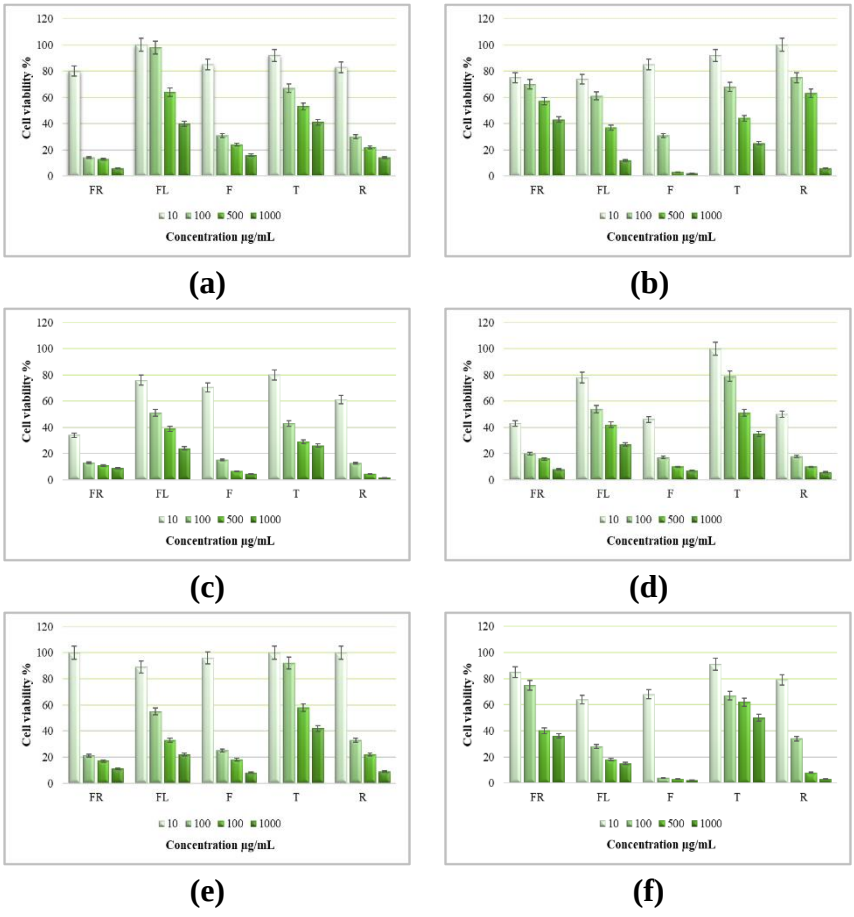


(a) **(b)**

Figure 9.5. Cytotoxicity of the N. alba F2, F5, F50 and F51 fractions on V79 healthy lung fibroblast cells

9.4.3 Cytotoxic potential of biologically active compounds on ovarian tumor cells

The *in vitro* evaluation of the cytotoxicity of *N. alba* extracts on A2780 sensitive ovarian tumor cells were realized by testing the extracts from different anatomical parts of the *N. alba* species (fruit, flower, leaf, stem and root), obtained by different methods (US, ES, EM) and with different solvents (petroleum ether, cyclohexane, methanol, ethanol and water), but also R-E-EM fractions (F2, F5, F50 and F51) (Figure 9.7).



(a) **(b)**

(c) **(d)**

(e) **(f)**

Figure 9.7. Cytotoxicity of N. alba extracts on A2780 sensitive ovarian tumor cells: (a) EP-ES, (b) CH-EM, (c) M-US, (d) M-ES, (e) E-EM and (f) A-EM. FR - fruit, FL - flower, F - leaf, T - stem and R – root

The FR-EP-ES extract has the highest percentage of cytotoxicity, with a cell viability below 10 %, at a concentration of 1000 µg/mL. Of all the *N. alba* CH-EM extracts, the F-CH-EM

extracts was found to be the most active on A2780 sensitive ovarian tumor cells (Figure 9.7b). *N. alba* M-US extracts show the highest cytotoxicity on A2780 sensitive ovarian tumor cells (Figure 9.7c). The FR-M-US extracts is the most active extract, with a cell viability of 34 % at a concentration of 10 µg/mL. The F-M-US and R-M-US extracts also proved to be active, at concentrations of 500 and 1000 µg/mL, showing a cell viability of 7 and 4 %, respectively 5 and 2 %. The *N. alba* FR-M-ES, F-M-ES and R-M-ES extracts are the most active M-ES extracts, showing considerable effects on the A2780 cell line (Figure 9.7d). The most cytotoxic *N. alba* E-EM extracts, which inhibited the development of A2780 sensitive ovarian tumor cells are the FR-E-EM, F-E-EM and R-E-EM extracts (Figure 9.7e).

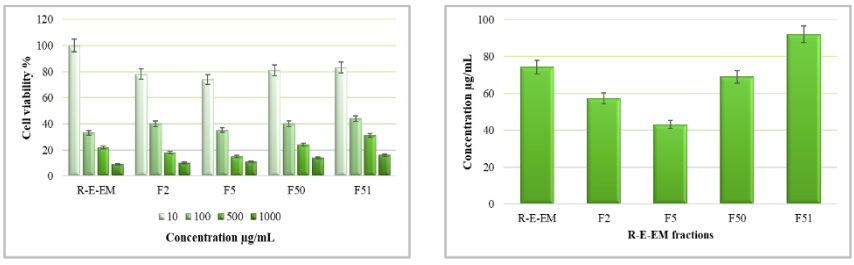
After 24 hours treatment of A2780 cell lines with *N. alba* extracts, IC_{50} values between 1.70 - 840.23 µg/mL were obtained (Table 9.3).

Table 9.3. IC_{50} values of N. alba extracts on A2780 sensitive ovarian tumor cells

Anatomical part[a]	***N. alba* extract**[b] **(µg/mL)**		
	EP-ES	**CH-EM**	**M-US**
FR	28.96±1.76	787.15±72.80	1.70±0.29
FL	751.97±10.79	127.37±36.28	122.43±3.51
F	60.52±1.84	47.48±1.71	23.20±3.00
T	524.12±10.82	291.28±10.20	90.37±6.31
R	53.81±1.63	560.03±54.91	19.40±3.80
	M-ES	**E-EM**	**A-EM**
FR	4.87±0.99	52.32±2.44	357.59±47.36
FL	161.48±22.89	156.87±30.68	25.14±3.89
F	7.22±1.59	54.17±8.68	15.73±2.67
T	502.55±42.54	714.92±18.23	840.23±61.12
R	9.73±1.76	74.21±2.51	44.73±8.20

[a]FR - fruit, FL - flower, F - leaf, T - stem, R - root. [b]EP - ether extracts, CH - cyclohexane extracts, M - methanolic extracts, E - ethanolic extracts, A - aqueous extracts. US - simple ultrasound assisted extraction; ES - multiple extraction using Soxhlet extractor; EM - simple mechanical agitation extraction. Data were obtained from dose-response curves.

The *N. alba* F2, F5, F50 and F51 fractions inhibited the development of A2780 sensitive ovarian tumor cells, both at a concentration of 1000 µg/mL and at concentrations of 100 and 500 µg/mL (Figure 9.5a). Regarding the determination of IC_{50} values, after 24 hours from the treatment of A2780 cell lines with F2, F5, F50 and F51 fractions, IC_{50} values between 43.10 - 91.95 µg/mL were obtained (Figure 9.8b).



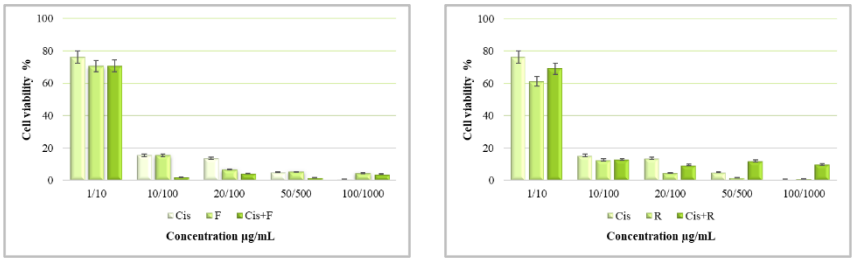
(a) **(b)**

Figure 9.8. Cytotoxicity of the N. alba F2, F5, F50 and F51 fractions on A2780 sensitive ovarian tumor cells

9.4.3.1 Potential interaction of studied biologically active compounds with cisplatin on cisplatin sensitive and resistant ovarian tumor cells

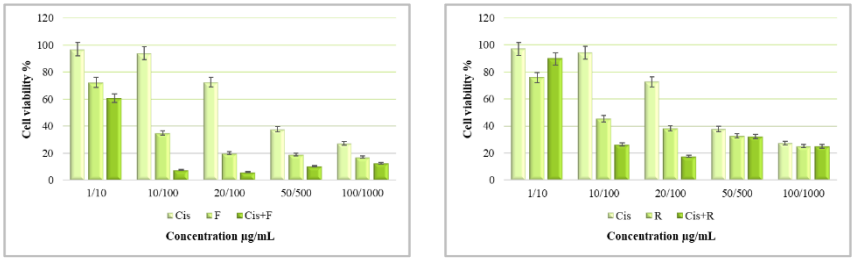
To evaluate the potential of *N. alba* extracts by combination therapy, the most active and effective extracts on A2780 cisplatin sensitive ovarian cells, with no high cytotoxicity on V79 healthy fibroblast cells were used. Thus, A2780 cisplatin sensitive and A2780cisR cisplatin resistant ovarian tumor cell lines were treated with *N. alba* F-M-US and R-M-US extracts and/or Cis for 24 hours. A ratio of 1/10, Cis/extract was used [52].

At a concentration of 100 µg/mL, the F-M-US extract has 15 % cell viability and in combination with Cis, in the ratio 10/100 Cis/extract, the cell viability decreases to a percentage of 2 % (Figure 9.10a). As with combination therapy for A2780 cisplatin sensitive ovarian tumor cells, the synergistic effect of the Cis/F-M-US extract on A2780cisR cisplatin resistant ovarian tumor cells is also present in the ratio 10/100 Cis/extract, from an initial percentage of cell viability of 35 %, the percentage of cell viability decreases to 7 % (Figure 9.11a). The R-M-US extract in combination with Cis does not have as strong potential as the F-M-US extract on A2780 cisplatin sensitive (Figure 9.10b) and A2780cisR cisplatin-resistant (Figure 9.11b) tumor cell lines.



(a) **(b)**

Figure 9.10. Cytotoxicity of N. aba F-M-US (F) and (b) R-M-US (R) extracts combined with cisplatin (Cis) on A2780 cisplatin sensitive ovarian tumor cells. Cis + F - cisplatin combined with the F-M-US extract, Cis + R - cisplatin combined with the R-M-US extract



(a) **(b)**

Figure 9.11. Cytotoxicity of N. alba (c) F-M-US (F) and (b) R-M-US (R) extracts combined with cisplatin (Cis) on A2780cisR cisplatin resistant ovarian tumor cells. Cis + F - cisplatin combined with F-M-US extract, Cis + R - cisplatin combined with R-M-US extract

The experimental results showed that *N. alba* extracts contain strong antioxidant compounds (rutin, quercetin, naringenin, naringin, apigenin, luteolin), which reduce the concentration of Cis and reduce the survival of A2780 and A2780ciR ovarian tumor cells (Table 9.4) . The synergistic interactions between the *N. alba* F-M-US and R-M-US extracts and Cis were performed by Chou's method [58]. The combination index (CI) and the affected dose fraction (Fa)

are shown in Table 9.5. The obtained results based on the combination of Cis and the *N. alba* F-M-US and R-M-US extracts indicate CI values between 0.05 and 0.5, suggested a strong synergistic effect [52].

Table 9.4. IC_{50} values of the N. alba F-M-US and R-M-US extracts combined with cisplatin on A2780 cisplatin sensitive and A2780cisR cisplatin resistant ovarian tumor cells

Sample[a]	A2780[b]	A2780cisR[b]
F	23.20±3.00	38.87±1.60
R	19.40±3.80	86.41±3.72
Cis	2.72±0.44	41.07±1.58
Cis+F	16.22±2.38	14.71±1.02
Cis+R	21.16±2.05	56.73±2.46

[a]F- F-M-US, R - R-M-US, Cis - cisplatin, Cis + F - cisplatin combined with the FR-M-US extract, Cis + R - cisplatin combined with the R-M-US extract. [b]A2780 - cisplatin-sensitive ovarian tumor cells, A2780cisR - cisplatin-resistant ovarian tumor cells.

Table 9.5. Potential interaction between N. alba F-M-US and R-M-US extracts and Cis on A2780 cisplatin sensitive and A2780cisR cisplatin resistant tumor cell lines

Sample concentration (µg/mL)		A2780[b]		A2780cisR[b]	
F[a]	**Cis**[a]	**Fa**[c]	**CI**[c]	**Fa**[c]	**CI**[c]
10	1	0.70	2.07	0.60	0.57
100	10	0.02	0.35	0.07	0.05
200	20	0.04	1.22	0.06	0.08
500	50	0.01	1.03	0.10	0.44
1000	100	0.03	4.85	0.12	1.20
R[a]	**Cis**[a]	**Fa**[c]	**CI**[c]	**Fa**[c]	**CI**[c]
10	1	0.69	2.25	0.89	10.03
100	10	0.12	1.52	0.26	0.30
200	20	0.09	2.25	0.17	0.30
500	50	0.12	7.64	0.32	2.28
1000	100	0.10	12.59	0.25	2.81

[a]F- F-M-US, R - R-M-US, Cis - cisplatin, Cis + F - cisplatin combined with the F-M-US extract, Cis + R - cisplatin combined with the R-M-US extract. [b]A2780 – cisplatin sensitive ovarian tumor cells, A2780cisR – cisplatin resistant ovarian tumor cells. [c]Fa - the fraction affected by the dose, CI - the combined index.

Of a total of 34 extracts of the *N. alba* evaluated, only 11 extracts were more active and effective in inhibiting the A2780 ovarian tumor cell line. A significant loss of cell viability (<50 %) indicates that the extracts have an important selectivity on tumor cells. The 11 extracts of the *N. alba* (M-US, M-ES and E-EM extracts of FR, F and R, respectively F-A-EM and R-A-EM extracts) were further studied on LNCaP prostate, MCF -7 breast and HL60 leukemia tumor cells.

9.5 Partial conclusions

Of the four tumor cell lines used, A2780 and MCF-7 appear to be more sensitive on treatment with the *N. alba* extracts. *N. alba* extracts have been found to be selective cell type and most importantly, they have a low cytotoxicity on healthy cells. In addition, it has been shown that the *N. alba* F-M-US extract used with Cis in combination therapy, in the ratio 10/100 Cis/F-M-US extract, inhibited by 10 % more the development of A2780cisR cisplatin resistant ovarian tumor cells.

In parallel, the toxicity and cytotoxicity of the *N. alba* extracts were evaluated using the germination method of wheat seeds and respectively, V79 healthy lung fibroblast cells to demonstrate the non-toxicity of these compounds. of a total of 34 extracts of the *N. alba* evaluated, 11 extracts were found to be very effective in inhibiting the development of tumor cells and also have no toxic and cytotoxic effects. The most effective extracts proved to be the F-M-US and R-M-US extracts, having antitumor potential on the four tumor cell lines tested (A2780, MCF-7, LNCaP and HL60). The *N. alba* FR-M-US, FR-M-ES, and FR-E-EM extracts have also been shown to be most effective in inhibiting the development of HL60 leukemic tumor cells.

Results obtained in this subchapter have been partially published in the scientific article "*Antifungal, Antitumor and Antioxidant Potential of the Danube Delta Nymphaea alba Extracts*", Cudalbeanu & all, Antibiotics 2020.

Chapter 10 Applications: Sono-biosynthesis of AuNPs using *N. alba* extracts

10.4 Results and discussions

10.4.1 "Green" sono-biosynthesis of AuNPs using *N. alba* root extract

Different concentrations of $HAuCl_4$ solution and *N. alba* root extract, different reaction time and different pH were used in $AuNPR_n$ biosynthesis (Table 10.1). Purified $AuNPR_n$ suspensions, redispersed in 2 mL ultrapure water were stored in the dark at room temperature [59].

Table 10.1. Reaction conditions used in $AuNPR_n$ biosynthesis

Sample	$HAuCl_4$ (mM)	R-E-EM* (mg/mL)	Reaction volum (mL)	Reaction pH	Time reaction (min)
$AuNPR_1$	1.5	5.47	15.24	7.0	10
$AuNPR_2$	1.5	7.39	20.30	6.4	10
$AuNPR_3$	1.5	7.38	20.32	8.4	40
$AuNPR_4$	2.0	8.16	18.38	7.8	40
$AuNPR_5$	3.0	5.54	15.04	7.8	40

*R-E-EM – *N. alba* root ethanolic extract obtained by simple mechanical agitation extraction.

10.4.2 Characterization of the AuNPs

The total content of phenolic acids, flavonoids and condensed tannins in the $AuNPR_n$ samples was determined using microspectrophotometric methods, while the Au content was determined by the PIXE technique. The Au/extract ratio for $AuNPR_n$ samples was also determined. As detailed in the experimental part, $AuNPR_n$ were characterized by UV-Vis spectroscopy, DLS and zeta potential measurements, ATR-FTIR spectroscopy, TEM and SEM microscopy and XRD determinations.

Phytochemical screening by microspectrophotometric methods showed the presence of secondary metabolites, phenolic acids, flavonoids and condensed tannins (Table 10.2). The results of the analysis showed that in the *N. alba* root extract, the total content of phenolic acids, flavonoids and condensed tannins is higher than those in the $AuNPR_n$ root samples due to the ability of polyphenolic compounds to form a complex with the gold nanoparticles [58].

Table 10.2. Total content of phenolic acids, flavonoids and condensed tannins in AuNPR$_n$ samples. Abbreviations: GA, gallic acid; TA, tannic acid; Q, quercetin; R, rutin; C, catechin; Eq, equivalent

Sample	Phenolic acids		Flavonoids		Condensed tannins
	(mgEqGA/g)	(mgEqTA/g)	(mgEqQ/g)	(mgEqR/g)	(mgEqC/g)
R-E-EM*	572.16±4.91	606.35±5.26	22.35±0.96	14.38±0.97	1.70±0.13
AuNPR$_1$	39.65±1.43	42.08±1.50	15.52±0.82	10.03±0.89	0.04±0.00
AuNPR$_2$	0.43±0.05	0.46±0.03	-	-	-
AuNPR$_3$	28.89±0.99	30.55±1.23	5.22±0.33	3.17±0.26	0.02±0.00
AuNPR$_4$	33.00±1.17	34.95±2.01	-	-	-
AuNPR$_5$	22.84±0.86	24.07±0.94	7.78±1.45	4.87±0.72	0.03±0.00

- Negative/unidentified. *R-E-EM – *N. alba* root ethanolic extract obtained by simple mechanical agitation extraction.

The UV-Vis spectra of AuNPR$_n$ were measured in order to determine the Surface Plasmon Resonance (SPR) wavelength and infer the shape and stability of the AuNPR$_n$. Figure 10.1 shows the UV-Vis spectra of the synthesized AuNPR$_n$, and the positions of SPR bands in these spectra are given in Table 10.3. The SPR wavelength of all of the AuNPR$_{1–5}$ synthesized by sonochemistry is in the λ range of 587 - 628 nm. The observed red shift of the SPR band relative to that of spherical nanoparticles was ascribed to the inherent anisotropic shape of nanoparticles and/or to the formation of stable nanoaggregates/agglomerates, resulting from the interaction among the spherical nanoparticles due to Oswald ripening [60, 61, 62].

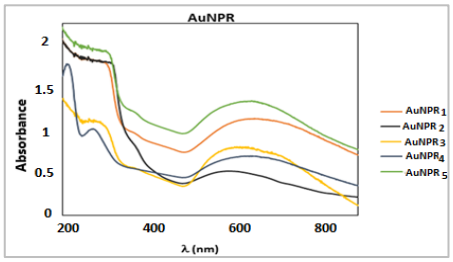


Figure 10.1. UV–Vis spectra of the AuNPR$_n$ in ultrapure water

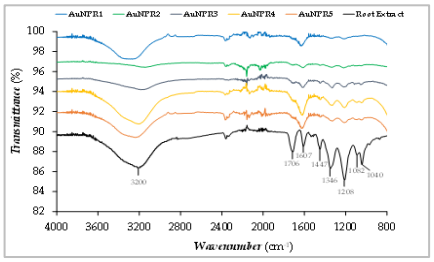


Figure 10.3. ATR-FTIR spectra of the AuNPR$_n$ samples and R-E-EM extract

The AuNPR$_3$ and AuNPR$_4$ samples also showed two absorption bands at λ <350 nm, corresponding to the polyphenolic molecules of the R-E-EM extract [63], which suggests that the polyphenols present in the R-E-EM extract act as an effective stabilizer in these two samples, which were prepared with a higher concentration of R-E-EM extracts. Thus, the observed red-shift on the absorption maxima of the SPR bands reflects the tendency of the AuNPR$_n$ to form larger nanoparticles and/or agglomerates in a solution, because the formation of aggregates would lead to obvious changes in the UV-Vis spectra [64, 65].

Table 10.3. Gold content, root extract content, hydrodynamic size, zeta potential and Au/root extract ratios of the AuNPR$_n$

Sample	[Au]* mg/mL	Ratio Au/Root extract	SPR λ_{max} (nm)	Hydrodynamic size (nm) (PDI)	Zeta potential (ζ) (mV)
AuNPR$_1$	3.05±0.15	0.56	625	280.2 (0.23)	−52±7

$AuNPR_2$	3.23±0.65	0.44	587	150.0 (0.20)	−46±7
$AuNPR_3$	2.81±0.14	0.38	618	60.7 (0.22)	−62±11
$AuNPR_4$	1.94±0.10	0.24	601	32.3 (0.35)	−56±9
$AuNPR_5$	4.04±0.20	0.73	628	209.8 (0.28)	−60±9

*The Au content was determined by the PIXE technique.

As can be seen in Table 10.3, the hydrodynamic size is strongly dependent on the ratio of Au/R-E-EM extract, with the higher ratios leading to higher hydrodynamic size values of AuNPs. The zeta potential (ζ) values of the synthesized $AuNPR_n$ samples were negative, ranging from −62 ± 11 mV; −46 ± 7mV, confirming the stability of NPs [66]. Regarding the other AuNPs formed with plant extracts, the synthesized $AuNPR_n$ sample present lower ζ values [67].

The water soluble R-E-EM fraction showed IR absorption regions characteristic of polyphenolic compounds. Almost all bands were observed for all AuNPRn samples in the same range of wave numbers [68]. However, it was observed that the 1706 cm^{-1} band suffered a significant decrease in its intensity. From ATR-FTIR data, it can be deduced that acidic groups of polyphenolic compounds remain chemically attached to the surface of $AuNPR_n$ by an acidic function, which may be due to OH groups present in polyphenolic compounds, compounds involved in reducing Au^{3+} to Au^0 ions [69]. FTIR spectra analysis also confirmed the low concentration of phenolic acids and flavonoids in the $AuNPR_2$ sample (Figura 10.3).

A PXRD analysis was conducted in order to assess the nature of the crystallinity of all of the $AuNPR_n$ samples (Figure 10.4). The PXRD pattern of $AuNPR_4$ sample (Figure 10.5) revealedthe presence of five diffraction peaks that were indexed to the (111), (200), (220), (311) and (222) lattice planes specific to the face-centered cubic (FCC) structure of metallic gold, inagreement with the Crystallography Open Database (COD 9008463). The absence of other crystalline phases confirms the pure crystalline nature of all of the $AuNPR_n$ samples [58].

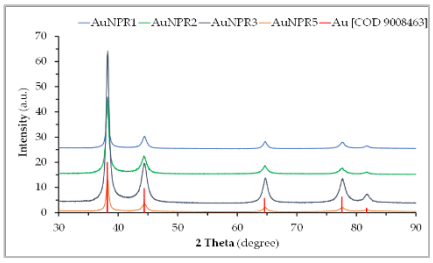


Figure 10.4. PXRD patterns of the $AuNPR_n$ samples

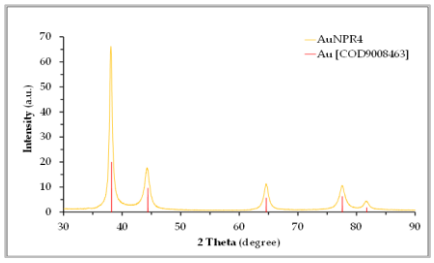


Figura 10.5. PXRD patterns of the $AuNPR_4$ samples

Although the different experimental conditions were used in the biosynthesis of the NPs, no significant differences were observed in the XRD diffractograms of the $AuNPR_n$ samples. The only changes observed were small shifts in the positions of the diffraction peaks, reflecting differences in the size of the Au nucleus. $AuNPR_n$ samples showed an average Au core size of less than 20 nm ($AuNPR_4$ <$AuNPR_3$ ≈ $AuNPR_2$ <$AuNPR_5$ ≈ $AuNPR_1$).

SEM and TEM analyzes were performed to morphologically characterize the biosynthesized AuNPRn samples, in terms of size and shape distribution. Figure 10.6 shows representative SEM and TEM images of $AuNPR_n$ samples. The images of SEM (Figure 10.6a,c,e) and TEM (Figure 10.6b,d,f) show that biosynthesized $AuNPR_n$ samples have different shapes,

coexisting in quasi-spherical, triangular and stellar shapes, among other irregular shapes, as well as a large size distribution, which confirms UV-Vis spectra data and DLS measurements [58].

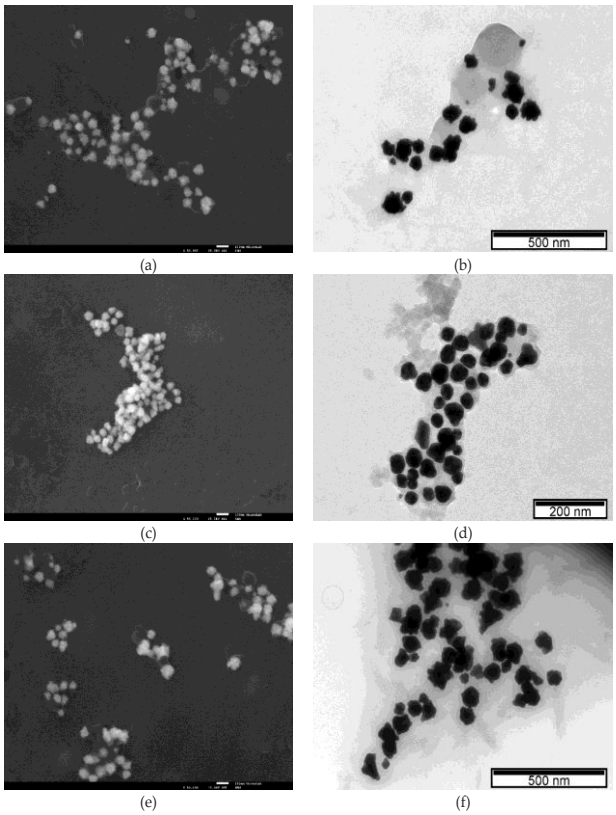


Figura 10.6. SEM and TEM images of AuNPR$_1$ (a,b), AuNPR$_4$ (c,d) and AuNPR$_5$ (e,f). SEM images were obtained at a ×50,000 magnification

The coexistence in the colloidal solution of NPs of a great diversity of morphologies, in which the NPs are surrounded by a bioorganic matrix, is a common characteristic and is attributed to a protective and stabilizing function preventing the NPs aggregation [70, 71]. The average of NPs size obtained by the TEM analysis was higher than that obtained from the analysis of the diffraction patterns. Thus, the larger the size and shape distribution of the NPs in the colloidal solution, the greater the discrepancy of the values found by these two techniques [72, 73]. Considering the size measurements of biosynthesized AuNPR$_n$ samples using *N. alba* RE-EM extract, under different reaction conditions, an order can be established depending on the technique used: DLS>TEM>XRD, according to the results for NPs synthesized using plant extracts [74, 75].

10.4.3 Biological properties of the AuNPs

10.4.3.1 Antioxidant activity of the AuNPs

A DPPH free radical assay was employed in order to evaluate the antioxidant activity of the AuNPR$_n$ samples. As can be observed in Table 10.6, the AuNPR$_n$ samples showed a more pronounced antioxidant activity than the R-E-EM extract, with the exception of AuNPR$_2$ sample, result that demonstrates once again the low content of R-E-EM extract in the AuNPR$_2$ sample [58].

Table 10.6. Antioxidant activity of the $AuNPR_n$ samples determined by the DPPH method

Sample	Percentage of inhibition	Equivalents of antioxidant activity		
		mgEqT/g	mgEqAA/g	mgEqQ/g
R-E-EM*	72.20±0.33	15.59±2.95	13.47±3.27	29.82±0.97
$AuNPR_1$	95.77±1.25	10.83±1.76	9.24±0.87	20.51±1.69
$AuNPR_2$	56.47±2.03	5.76±0.46	5.06±0.38	11.16±1.03
$AuNPR_3$	92.38±2.54	10.39±1.07	8.88±0.94	19.70±1.47
$AuNPR_4$	94.29±3.14	10.64±0.67	9.08±0.57	20.16±2.01
$AuNPR_5$	90.05±0.99	10.09±0.87	8.63±0.89	19.15±1.40

*R-E-EM – *N. alba* root ethanolic extract obtained by simple mechanical agitation extraction. Results were expressed as mean ± standard deviation. mgEqQ/g - quercetin milligrams equivalent per gram of sample; mgEqAA/g – ascorbic acid milligrams equivalent per gram of sample; mgEqT/g - trolox milligrams equivalent per gram of sample.

10.4.3.2 Antibacterial activity of the AuNPs

The antimicrobial properties of the $AuNPR_n$ samples and the gold salt $HAuCl_4$ were assessed towards the Gram-positive bacterial strain *S. aureus* Newman and the Gram-negative bacterial strain *E. coli* ATCC25922 by determining the minimum inhibitory concentration (MIC) values using the microdilution method (Table 10.7).

Tabel 10.7. Estimated MIC values of the $AuNPR_n$ and $HAuCl_4$ precursor towards bacterial strains S. aureus Newman and E. coli ATCC25922

Bacterial strain	MIC values (µgAu/mL)					
	$AuNPR_1$	**$AuNPR_2$**	**$AuNPR_3$**	**$AuNPR_4$**	**$AuNPR_5$**	**$HAuCl_4$**
***S. aureus* Newman**	200	>200	200	100	>200	50
***E. coli* ATCC25922**	>200	>200	200	200	>200	6,25

The results are the mean of three independent experiments performed with two replicates.

The Au (III) salt precursor $HAuCl_4$ exhibited a high antimicrobial activity against bacterial strains *S. aureus* Newman and *E. coli* ATCC25922, with estimated MIC values of 50 µg Au/mL and respectively, 6.25µg Au/mL. The $AuNPR_4$ samples had the highest antimicrobial activity against bacterial strain *S. aureus* Newman, with an estimated MIC value of 100 µg Au/mL. The $AuNPR_4$ sample also exhibited antimicrobial activity against bacterial strain *E. coli* ATCC25922, with an estimated MIC value of 200 µg Au/mL. These results agree with other reported results, demonstrating an inverse relationship between the antibacterial activity and the NPs size [58].

10.4.3.3 Antitumor activity of the AuNPs

The cytotoxic effect of the biosynthesized $AuNPR_n$ samples against a cancer cell model was evaluated in order to obtain information regarding the prospective value of these NPs as chemotherapeutic agents. The cytotoxicity of AuNPs synthesized with plant extracts is dependent on the particle's physicochemical properties, namely its size, shape, surface charge and phytochemical constituents. All of them play key roles in the cellular uptake and the degree of cytotoxicity.

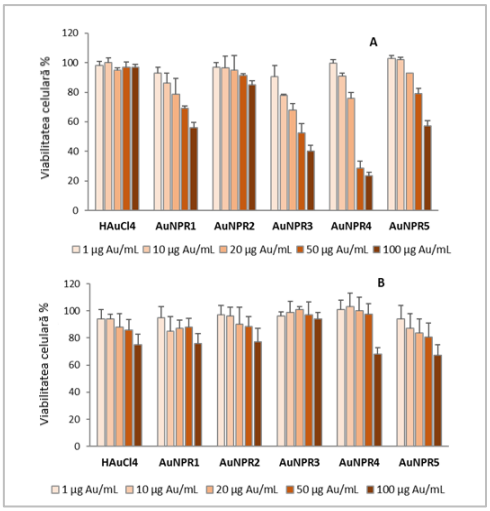


Figure 10.7. The viability of A2780 cells (A) and V79 fibroblasts (B) after 48 h of treatment with AuNPR$_n$ serial concentrations. The results are the mean±SD of two independent experiments performed with six replicates per condition

The results obtained on A2780 tumor cells, treated for 48 hours with AuNPR$_n$ serial dilutions (1 µgAu/mL to 100 µgAu/mL) and $HAuCl_4$, are shown in Figure 10.7. The results showed a concentration-dependent decrease in the viability of the A2780 cells with the increase of the AuNPR$_n$ concentration. IC_{50} values, calculated from the dose-response curves (Figure 10.7A and 7B), were 108.7±15.0 µgAu/mL, > 100 µgAu/mL, 51.9±7.7 µgAu/mL, 33.5±6.3 µgAu/mL and 136.1±15.0 µgAu/mL for AuNPR$_1$, AuNPR$_2$, AuNPR$_3$, AuNPR$_4$ and AuNPR$_5$, respectively. The $HAuCl_4$ precursor gold salt was also tested, and was without cytotoxic effect. The AuNPR$_3$ and AuNPR$_4$ samples presented the lowest IC_{50} values, which proves that the AuNPR$_3$ and AuNPR$_4$ samples are the most antitumor active of all the AuNPR$_n$ samples tested. These NPs were able to inhibit the viability of A2780 cells by 50 % at 33.5±6.3 µg Au/mL and 51.9±7.7 µgAu/mL concentrations, respectively. AuNPR$_3$ and AuNPR$_4$ samples have a smaller hydrodynamic size and coresize relative to the other NPs evaluated in this work, which might justify their enhanced anticancer activity. The cytotoxic selectivity of AuNPRn against tumor cells versus healthy cells was evaluated in V79 healthy fibroblasts. As shown in Figure 7B, a dose-dependent effect was observed even for the $HAuCl_4$ sample [58].

10.5 Partial conclusions

Experimental conditions such as reaction pH, Au/extract ratio and reaction time are important parameters to control biosynthesis, as it adjusts the size and load of AuNPs. The hydrodynamic size of AuNPR$_n$ increases with the ratio Au/RE-EM extract: a larger ratio corresponds to a larger hydrodynamic size, on the other hand the size of the Au nucleus (average crystallite size), characterized by the PXRD technique, did not show differences significant and showed a crystallite size of less than 20 nm.

All AuNPR$_n$ samples had a high negative zeta potential value from - 62±11 mV to - 46±7 mV. AuNPR$_n$ samples showed a certain heterogeneity of shape and a moderate and monodisperse distribution, as shown by the TEM and SEM analyzes. ATR-FTIR spectra indicated that AuNPR$_n$ samples interacts mainly with the hydroxyl groups present in the polyphenolic compounds in the

R-E-EM extract, which participate in the reduction of Au^{3+} to Au^{0} and the stabilization of NPs. ATR-FTIR analysis also confirmed a low concentration of phenolic acids and flavonoids in the $AuNPR_2$ sample. SEM images showed that NPs have a coating of organic material (R-E-EM extract), which has a protective and stabilizing function, preventing their aggregation. Apart from $AuNPR_2$, all $AuNPR_n$ samples had a high antioxidant capacity, superior to that obtained for R-E-EM extract, used in biosynthesis. Stabilized $AuNPR_n$ showed size-dependent antibacterial and antitumor activity, both of which increased with decreasing particle size. In fact, the highest AuNPs particle size ($AuNPR_2$ and $AuNPR_5$) were the least biologically active. In addition to size, this low biological activity certainly reflects the low content of polyphenolic compounds.

The results of this chapter have been published in the scientific article "*Sono-Biosynthesis and Characterization of AuNPs from Danube Delta Nymphaea alba Root Extracts and Their Biological Properties*", Cudalbeanu & all, Nanomaterials 2021.

Conclusions and future research directions

General conclusions

The doctoral thesis "*Chemical characterization and study of the biological activity of some compounds present in the Nymphaea alba species from the Danube Delta Biosphere Reserve*" aims was the separating and identifying the biologically active compounds present in different anatomical parts (fruit, flower, leaf, stem and root) of the water lily, *N.alba* species by applying modern analysis techniques (chromatographic coupled with mass spectrometry, microspectrophotometry and spectrometry),The *in vitro* evaluation of potential bioactive properties, such as antioxidants, antibacterial, antifungal and antitumor, but also reduction and stabilization applied in the biosynthesis of gold nanoparticles was also proved.

The biologically active compounds identified in *N. alba* species belong to several classes of organic compounds, such as phenolic acids, flavonoids, proanthocyanidins, anthocyanidins, tannins, lignans, terpenoids, sterols, esters, higher aliphatic hydrocarbons, the results demonstrated the fact that different plants component parts have different chemical composition.

The interest for this research topic is due to the fact that in the literature the *N. alba* species is presented as an important source of biologically active natural compounds, with various pharmacological properties, thus encouraging us in the in-depth research of a native *N. alba* species present in the Danube Delta Biosphere Reserve. This research contributes to the development of aquatic species in terms of the development of new methods of separation, identification and analysis of chemical compounds of interest to the pharmaceutical or food industry.

Personal conclusions

The research of this doctoral thesis was directed towards a complex study of the biologically active compounds present in different anatomical parts of the *N. alba* species. Thus, the personal, original scientific contributions and the synthesis of the experimental results are presented in the following:

- The *N. alba* species was taken from Rotundu Lake, Somova-Parcheș Aquatic Complex, Danube Delta Biosphere Reserve, a habitat that has special environmental conditions for the development of submerged aquatic species.
- Solvent extraction of natural organic compounds from various anatomical parts of the *N. alba* species, such as fruit, flower, leaf, stem and root, was performed for the first time by three extraction methods: simple ultrasound assisted extraction (US), multiple extraction with Soxhlet extractor (ES) and simple mechanical agitation extraction (EM). A total of 30 extracts were obtained, and the largest amount of natural compounds was extracted with the methanol organic solvent compared to the ethanol organic solvent.
- The separation and isolation of biologically active compounds from the *N. alba* species was performed, for the first time, for the *N. alba* R-E-EM root extract, a separation that took place in seven steps. A total of 16 fractions were obtained, and four representative fractions of the 16 fractions were used in subsequent analyzes to prove the biological properties of the secondary metabolites present in the *N. alba* species.
- GC-MS analysis of the *N. alba* lipophilic extracts (EP-ES and CH-EM) led to the identification of 71 volatile organic compounds, which were mainly long chain hydrocarbons, and oxygenated derivatives, fatty acids and esters of fatty acids, terpenoids and steroids.
- In the HPLC experimental study (HPLC-DAD and LC-MS / MS) of the *N. alba* hydrophilic extracts (M-US, M-ES and E-EM) a number of 27 polyphenolic compounds were identified and reported for the first time (HHDP, quinic acid, corilagin, vanillic acid, castaline, gallic acid, gerannin, caffeic acid, p-coumaric acid, tannic acid, rutin, ellagic acid, ellagic acid rhamnoside, ellagic acid pentoside, cinnamic acid derivative, naringenin, naringin, ferulic acid, catechin, epicatechin, chlorogenic acid, quercetin, apigenin, luteolin, brevifoline, kaempferol and orientin), compounds also known for their biologically active properties.
- The analysis of the main classes of compounds, primary and secondary metabolites present in the *N. alba* extracts, revealed the presence of significant amounts of chlorophylls, carotenoids, phenolic acids, flavonoids and condensed tannins. The results obtained are consistent with data from the literature and those obtained by HPLC-DAD and LC-MS/MS techniques and confirm the presence in *N. alba* different extracts of a high content of phenolic acids, flavonoids and condensed tannins.
- The results of the experimental study on the total content of macro and microelements confirm the bioaccumulation of sediment and water elements by the *N. alba* aquatic plant. Therefore, in the processes of growth and development, the *N. alba* species assimilates substantial amounts of essential elements, but also small amounts of non-essential elements. Large amounts of macroelements, such as K, Mg, P and Na, have been identified in the *N. alba* flower, leaf, stem and root samples. Microelements, such as B, Ba, Co, Cr, Cu, Li, Ni, Pb and Zn were identified for the first time in the *N. alba* flower, leaf, stem and root samples.
- The presence of a relatively large number of compounds in the analyzed fractions, compounds with important biologically active properties, justifies the known use of *N. alba* species in traditional medicine for the treatment and prevention of various diseases. In the current concerns about the need to discover new therapeutic agents without their adverse

toxic effects, this study evaluated, for the first time, the antioxidant potential of the *N. alba* M-US, M-ES and E-EM extracts, using DPPH, ABTS, BCB, FRAP and CV methods. The obtained results highlight the biological properties of the native *N. alba* species from the Danube Delta Biosphere Reserve and are comparable with the data from the literature, which presents plant extracts with biologically active potential containing the same type of compounds. The *N. alba* M-US, M-ES and E-EM extracts showed a strong antioxidant activity at concentrations ± 500 µg/mL, and the presence of quercetin in the *N. alba* extracts was once again confirmed by the CV technique.

- The *N. alba* extracts show antimicrobial activity against Gram-negative bacterial strains *E. hormaechei* ATCC 700322, *P. aeruginosa* ATCC 27853 and *E. coli* ATCC 25922 and against Gram-positive bacterial strains *S. aureus* ATCC 25923, *S. aureus* NCTC8178 and *S. pyogenes* ATCC 19615.
- The *N. alba* F-M-US and R-M-US extracts are active against fungal strain *C. glabrata* CBS138, with MIC values of 1.717 µg/mL, respectively 1.935 µg/mL, and for FLC with MIC value of 0.7639 µg/mL. The results obtained showed that the antifungal action of the *N. alba* extracts, at low concentrations, was specific against the fungal strain *C. glabrata* CBS138, having antifungal action, at much higher concentrations, against the other *Candida* fungal strains tested (*C. albicans* CB138 , *C. parapsilosis* ATCC22019 and *C. tropicalis* ATCC750).
- The search for new natural chemotherapeutic compounds, it was evaluated for the first time, the cytotoxicity of the *N. alba* extracts on A2780/A2780cisR ovarian, MCF-7 breast and LNCaP prostate tumor cell lines using the MTT test, but also HL60 leukemic tumor cells, using WST-8 test. Of the four tumor cell lines used, A2780 and MCF-7 appear to be more sensitive to treatment with the *N. alba* extracts. The *N. alba* extracts have been found to be selective cell type and most importantly, they have low cytotoxicity on healthy cells. The induced effect of biologically active compounds present in the *N. alba* extracts demonstrates their potential to be intersing for preclinically studied as antitumor agents.
- The *N. alba* F-M-US extract used with Cis is combination therapy, in the ratio 10/100 Cis/F-M-US extract, inhibited by 10 % more the development of A2780cisR cisplatin resistant ovarian tumor cells. In parallel, the toxicity and cytotoxicity of the *N. alba* extracts were evaluated using the germination method of wheat seeds, respectively V79 healthy lung fibroblasts.
- A total of 34 extracts of the *N. alba* were tested. The biological activity of these extracts is variable due to the different chemical composition, which depends on the extraction method, but also on the type of solvent used. Of all the extracts tested, 11 extracts were found to be very effective in inhibiting the development of tumor cells and also showed no toxic and cytotoxic effects on healthy tissues. The results obtained in the evaluation of the *in vitro* cytotoxicity of *N. alba* extracts are also correlated with the antioxidant capacity of the compounds.
- The most effective extracts proved to be the F-M-US and R-M-US extracts, having antitumor potential on the four tumor cell lines tested (A2780, MCF-7, LNCaP and HL60). The *N. alba* FR-M-US, FR-M-ES and FR-E-EM extracts have been shown to be most effective in inhibiting the development of HL60 leukemic tumor cells. In conclusion, the

antitumor potential of the compounds present in different extracts of the *N. alba* species taken from the Danube Delta Biosphere Reserve, has been demonstrated for the first time.

- For the first time, it has been shown that the *N. alba* R-E-EM extract allows the stabilization of AuNPs, obtained by a single reaction, at room temperature, using sonochemistry as a simple, cheap, efficient and environmentally friendly method. The experimental analysis focused on AuNPs biosynthesis in the presence of the *N. alba* R-E-EM extract, with dual biological, antimicrobial and antitumor activity. This multi-perspective performance, moderate selectivity and low cytotoxicity can position AuNPRn samples as effective therapeutic agents, with the added benefit of a low environmental impact.

Future research directions

Based on the experimental results obtained during the years of doctoral studies, the future research perspectives will focus on the following activities:

- Consolidation of investigations on polyphenolic compounds present in various *N. alba* anatomical parts, which showed significant antioxidant, antibacterial, antifungal and antitumor activity, through in-depth understanding of their mechanism of action, both *in vitro* and *in vivo* analyzes.
- *In vitro* and *in vivo* evaluation of the active potential of polyphenolic compounds present in different *N. alba* anatomical parts with emphasis on antidiabetic, antiviral and anti-inflammatory properties.
- Advanced purification of biologically active compounds naturally present in various *N. alba* anatomical parts, especially those with interesting biological activity.
- The *N. alba* extracts proved to be active against fungal strain *C. glabrata* CBS138, with MIC values of 1.717 µg/mL, respectively 1.935 µg/mL, so that the extension of the research for their inclusion in pharmaceutical forms (creams, gels, etc.) for external use and their testing against fungal infections will be another direction of research.
- The molecular mechanisms underlying the antitumor action of *N. alba* phytochemicals, especially on ovarian, prostate, mammary and leukemic tumor cells, are not yet very clear, and the results suggest further research *in vitro* as well as *in vivo* analysis for an understanding of the mechanisms of action.
- In future research, $AuNPR_n$ samples, especially $AuNPR_3$ and $AuNPR_4$, could be exploited as potential candidates for applications in the pharmaceutical industry due to their biological properties, such as their antitumor activity against cisplatin-sensitive ovarian tumor cells, benefiting from the same time and their antibacterial potential.
- $AuNPR_3$ and $AuNPR_4$ samples used in combination therapy with antitumor drugs or herbal medicines could improve their biological profile, *in vitro* and *in vivo*, thus preventing the development of drug resistance, reducing systemic drug toxicity and at the same time helping to improve therapeutic results.
- Biosynthesis of Au, Ag, Cu, Pt and Sn nanoparticles and obtaining thin biofilms with antibacterial and antifungal properties with possible application in the pharmaceutical and/or biomedical field.

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Research paper list

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Book chapter

1. Sorin Avramescu, Irina Fierascu, Radu Claudiu R. Fierascu, ***Mihaela Cudalbeanu***. Extraction of Natural Products from Agro-Industrial Wastes: A Green and Sustainable Approach. Chapter 4: Pressurized liquid extraction of natural products. Elsevier 2021. Accepted, in progress.

ISI published papers from the doctoral thesis

1. ***Mihaela Cudalbeanu***, David Peitinho, Francisco Silva, Rosa Marques, Teresa Pinheiro, Ana C Ferreira, Fernanda Marques, António Paulo, Catarina F Soeiro, Sílvia Andreia Sousa, Jorge Humberto Leitão, Aurel Tăbăcaru, Sorin Marius Avramescu, Rodica Mihaela Dinica, Maria Paula Cabral Campello. Sono-Biosynthesis and Characterization of AuNPs from Danube Delta *Nymphaea alba* Root Extracts and Their Biological Properties. Nanomaterials. 2021; 11(6):1562. https://doi.org/10.3390/nano11061562. ***Impact factor: 5.076***, ***Q2***.

2. ***Mihaela Cudalbeanu***, Bianca Furdui, Geta Cârâc, Vasilica Barbu, Alina Viorica Iancu, Fernanda Marques, Jorge Humberto Leitão, Sílvia Andreia Sousa, Rodica Mihaela Dinica. Antifungal, Antitumoral and Antioxidant Potential of the Danube Delta *Nymphaea alba* Extracts. Antibiotics. 2020, 9, 7; doi:10.3390/antibiotics9010007. ***Impact factor: 4.639, Q1.***

3. ***Mihaela Cudalbeanu***, Ioana Otilia Ghinea, Bianca Furdui, Durand Dah-Nouvlessounon, Robert Raclea, Teodor Costache, Iulia Elena Cucolea, Florentina Urlan, Rodica Mihaela Dinica. Exploring New Antioxidant and Mineral Compounds from *Nymphaea alba* Wild-Grown in Danube Delta Biosphere. Molecules 2018, 23(6), 1247; doi:10.3390/molecules23061247. ***Impact factor: 4.412, Q2.***

Cumulative impact factor: 14,127.

ISI published papers in collaboration

1. Costea IF, Melinte RG, Noapteş SN, ***Cudălbeanu M***, Dediu AV, Dinică RM, Cârâc G. Factors of influence for functionalised of chitosan with n-heterocyclic salt in aqueous medium. Journal of Physics: Conference Series 1960. 2021, 012001. doi:10.1088/1742-6596/1960/1/012001.

2. Ioana O. Ghinea, Maria D. Ionica Mihaila, Giorgiana-Valentina Blaga, Sorin M. Avramescu, ***Mihaela Cudalbeanu***, Simona-Florina Isticioaia, Rodica M. Dinica, Bianca Furdui. HPLC-DAD Polyphenolic Profiling and Antioxidant Activities of *Sorghum bicolor* during Germination. Agronomy 2021, 11, 417. https://doi.org/10.3390/agronomy11030417. ***Corresponding author. Impact factor: 3.417***, ***Q1***.

3. Balanescu Fanica, Maria Daniela Ionica Mihaila, Geta Cârâc, Bianca Furdui, Costel Vînătoru, Sorin Marius Avramescu, Elena Lacramioara Lisa, ***Mihaela Cudalbeanu***, Rodica Mihaela Dinica.

Flavonoid Profiles of Two New Approved Romanian *Ocimum* Hybrids. Molecules. 2020, 25, 4573. doi:10.3390/molecules25194573. ***Corresponding author. Impact factor: 4.412, Q2.***

4. Chokki Michaelle, Cheikna Zongo, Durand Dah-Nouvlessounon, ***Mihaela Cudălbeanu***, Pacôme Noumavo, Ioana Otilia Ghinea, Bianca Furdui, Aly Savadogo, Rodica Dinica, Lamine Baba-Moussa, Farid Baba-Moussa. Phytochemical screening and antimicrobial activity of *Momordica charantia* L. and *Morinda lucida* Benth extracts from Benin. African Journal of Microbiology Research. 2020, 14, 8, 426-435. doi:10.5897/AJMR2020.9347.

5. Chokki Michaelle, ***Mihaela Cudălbeanu***, Cheikna Zongo, Durand Dah-Nouvlessounon, Ioana Otilia Ghinea, Bianca Furdui, Robert Raclea, Aly Savadogo, Lamine Baba-Moussa, Sorin Marius Avramescu, Rodica Mihaela Dinica and Farid Baba-Moussa. Exploring Antioxidant and Enzymes (A-Amylase and B-Glucosidase) Inhibitory Activity of *Morinda lucida* and *Momordica charantia* Leaves from Benin. Foods. 2020, 9, 434. doi:10.3390/foods9040434. ***Impact factor: 4.350, Q1.***

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Cumulative impact factor: 15,802.

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