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PhD THESIS

Comparative pharmacobotanical study of some species belonging to Cucurbitaceae family

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Université de Liège

Dedication

Dedicated to my parents, my sister and all those that, with a word, a fact or a thought, have managed to heal any possible wound...

LIST OF PUBLICATIONS

Articles published *in extenso* as a result of the doctoral research

1. Rus M, **Ielciu I,** Păltinean R, Vlase L, Ştefănescu C, Crişan G. Morphological and histo-anatomical study of *Bryonia alba* L. (Cucurbitaceae). Not Bot Horti Agrobo 2015; 43(1): 47-52. *ISI Impact factor – 0.451 (publication included in Chapter 1).*

2. **Ielciu I,** Frédérich M, Tits M, Angenot L, Păltinean R, Cieckiewicz E, Crişan G, Vlase L. *Bryonia alba* L. and *Ecballium elaterium* (L.) A. Rich. – Two related species of the Cucurbitaceae family with important pharmaceutical potential. Farmacia 2016; 64(3): 323-332. *ISI Impact factor – 1.162 (publication included in the State of the art).*

3. **Ielciu I,** Vlase L, Frédérich M, Hanganu D, Păltinean R, Cieckiewicz E, Olah NK, Gheldiu AM, Crişan G. Polyphenolic profile and biological activities of the leaves and aerial parts of *Echinocystis lobata* (Michx.) Torr. et A. Gray (Cucurbitaceae). Farmacia 2017; 65(2): 179-183. *ISI Impact factor – 1.162 (publication included in Study 2).*

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ABBREVIATIONS USED IN THE TEXT

^{1}H-NMR	Proton Nuclear Magnetic Resonance
^{13}C-NMR	Carbon Nuclear Magnetic Resonance
9th Eur Ph	European Pharmacopoeia, 9th edition
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
ACN	Acetonitrile
APAD	3-AcetylPyridine Adenine Dinucleotide
APG IV	Angiosperm Phyllogeny Group IV
BA	*Bryonia alba* L.
CIRM	Center for Interdisciplinary Research on Medicines
CL	ChemiLuminescence
CUPRAC	CUPric ion Reducing Antioxidant Capacity
DAD	Diode Array Detector
DMEM	Dulbecco's Modified Eagle's Medium
DPBAE	DiphenylBoric acid-2-Aminoethyl Ester
DPPH	2,2-DiPhenyl-1-PicrylHydrazyl
DMSO	DiMethyl SulfOxide
dvp	Dried vegetal powder
EE	*Ecballium elaterium* (L.) A. Rich.
EL	*Echinocystis lobata* (Michx.) Torr. et A. Grey
EtOH	Ethanol
EPR	Electron Paramagnetic Resonance
FBS	Fœtal Bovine Serum
FRAP	Ferric Reducing Ability of Plasma
HBSS	Hank's Balanced Salt Solution
HPLC	High Performance Liquid Chromatography
HRP	HorseRadish Peroxidase
IC_{50}	The concentration that caused 50% cell growth inhibition
IMDM	Iscove's Modified Dulbecco's Medium
L-glut	L-glutamine
MeOH	Methanol
MRM	Multiple Reaction Monitoring
MS	Mass Spectrometry
NBT	NitroTetrazolium Blue
PBS	Phosphate Buffer Saline
PEG	Poly(Ethylene Glycol)
PES	Phenazine EthoSulfate
PenStrep	Penicilin-Streptomycin
PMA	Phorbol 12-myristate 13-acetate

PrepHPLC	Preparative High Performance Liquid Chromatography
PrepTLC	Preparative Thin Layer Chromatography
RF	Flora of the Popular Republic of Romania (Romanian Flora)
ROS	Reactive Oxygen Species
RNS	Reactive Nitrogen Species
RPMI	Roswell Park Memorial Institute
RPM	Rotation Per Minute
SEM	Scanning Electronic Microscopy
SNPAC	Silver NanoParticles Antioxidant Capacity
TEAC	Trolox Equivalent Antioxidant Capacity
TFA	TriFluorAcetic Acid
TLC	Thin Layer Chromatography
TRIS Buffer	Tris(hydroxymethyl)aminomethane
R_t	Retention time
ULg	University of Liège
UV	UltraViolet
WST-1	Cell Proliferation Reagent WST-1

INTRODUCTION

Cucurbitaceae family is one of the most important families worldwide, that contains the most well-known and used plants for human alimentation, such as pumpkins, melons, watermelons or cucumbers (1–3). Species that provide them belong to the genera *Cucurbita*, *Cucumis* or *Citrullus* and are cultivated all over the world in crops for their fruits or seeds, that not only represent the basis of human alimentation (4), but also represent important material for other industries (e.g. pharmaceutical industry, cosmetics industry) (5). Besides their economical uses, plants belonging to this family are also known for their medicinal purposes, which have drawn the attention of researchers worldwide, making therefore these plants important targets for the research in the domain of plants with significant pharmaceutical potential (1).

Among the plants that belong to Cucurbitaceae family, there are species lesser known and used, due to different reasons, especially related to their toxicity. *Bryonia alba* L., *Echinocystis lobata* (Michx.) Torr. et A. Gray and *Ecballium elaterium* (L.) A. Rich. are three species belonging to Cucurbitaceae family, spontaneous in the Romanian flora (6–9), that are lesser studied by scientists, but which have nevertheless proved significant activities in traditional medicine or homeopathy and appear therefore as important research subjects.

Bryonia alba L. species is known in traditional and homeopathic medicine for its antipyretic, diaphoretic, anti-inflammatory, anti-infectious, anti-rheumatic, analgesic, laxative, purgative, relaxing smooth muscle properties (10–12). *Echinocystis lobata* (Michx.) Torr. et A. Gray is a species which is used in traditonal medicine for headaches, for the analgesic effect or as a tonic drink for stomach pain, kidney diseases, rheumatism, chills and fever (13,14). *Ecballium elaterium* (L.) A. Rich. is a species known for its anti-inflammatory, anti-bacterial, analgesic, laxative, purgative, trypsin inhibitor and cytotoxic activities.

The main purpose of the present thesis is focused on bringing a novel complete approach on these three plants belonging to Cucurbitaceae family, lesser known and used. The thesis aims to perform a comparative pharmacobotanical study of these species, by offering a complete description of each of them, starting from the morphological and anatomical point of view and ending with the assesement of their biological activities. An important part of the thesis is focused on the phytochemical study of the compounds in the composition of the species. It is especially the class of flavonoids that is the most studied in the thesis. This is one of the most important points of novelty of the thesis, taken into consideration the fact that this class of compounds is lesser studied for these plants. At the same time, it is also an important support point for another purpose of the thesis, which is to bring evidence for the fact

that these species are less toxic than it is thought. In this way, the thesis is aimed to bring originality an novelty on the three species, by presenting them in a different way than existant approaches do.

Methods used for the study of the three species are mostly related to microscopic techniques for the botanical study. Regarding the phytochemical study, spectrophotometric, chromatographic and spectroscopic techniques were used for the identification, quantification and isolation of the flavonoidic compounds. For the assesement of the biological activities, anti-plasmodial, cytotoxicity, antioxidant and anti-inflammatory tests were performed. All of these allowed to achieve a characterization from various points of view for each of the species mentioned above.

All the results in the present study will contribute to the current knowledge on medicinal plants with therapeutical potential, offering a comprehensive assesement on three species belonging to Cucurbitaceae family that have proven important potential as medicinal plants. Perspectives of this study concern the complete elucidation of the phytochemical profile of the three species and testing the activity of the isolated compounds for various biological activities as anti-parasitic, anti-proliferative and anti-inflammatory. Further studies may even be directed towards the elucidation of the mechanism of action of the main compounds responsible for these activities. The thesis opens important pathways to follow in order to provide the necessary arguments for the introduction in therapy of novel molecules from plants in order to treat different pathologies.

An important part of the studies was performed at the Laboratory of Pharmacognosy of the University of Liège, Belgium. I am extremely grateful to Professor Michel Frédérich for making me feel welcome in the laboratory he leads, for his continuous scientific supervision and support. I am also grateful to Professor Luc Angenot for his patience, his scientific advices, his continuous support and encouragements that motivated me from the begining of my placement in Liège. I am grateful to Assistant Professor Ewa Cieckiewicz and Professor Monique Tits for the scientific guidance and assistance throughout my doctoral project, for their support and continuous encouragements. I express special thanks and all my gratitude to Delphine Etienne and Assistant Professor Allison Ledoux not only for their continuous support and guidance in the laboratory, for their key contributions to my study, but also for showing me their friendship and providing me the motivation which guided me everyday. I am also thankful to Annélise Bordignon and Myriam Alorchi for their friendship and support in the laboratory.

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STATE OF THE ART

1. Botanical data regarding Cucurbitaceae family

1.1. Taxonomic classification

The Angiosperm Phylogeny Group, European Flora and Romanian Flora arrange Cucurbitaceae family in the Cucurbitales order (6,7,15). According to APG IV, which relies more on molecular and genetic data, this order is included in the Rosiidae clade (15), while in the classification of the Romanian Flora it is included in the Dilleniidae sub-class (7,8), based on the morphological features of the plants belonging to it. These taxonomic systems include therefore the Cucurbitaceae family in the systematic units that are mentioned in Table I.

Table I. Taxonomic classification of the Cucurbitaceae family according to different taxonomic systems (6–9,15)

APG IV	European and Romanian Flora classification
Kingdom: Plantae **(unranked): Angiosperms** **(unranked): Eudicots** **(unranked): Rosids** **Order: Cucurbitales** **Family: Cucurbitaceae**	**Kingdom:** Plantae **Sub-kingdom:** Cormobionta **Phyllum:** Magnoliophyta (Angiospermatophyta) **Class:** Magnoliatae (Dicotiledonatae) **Sub-Class:** Dilleniidae **Order:** Cucurbitales **Family:** Cucurbitaceae

The Cucurbitales order is a taxonomic class with a worldwide distribution, having striking heterogeneity in species diversity. It comprises almost 2600 species, that are placed in around 110 genera and it is divided in seven families, that are mostly found in temperate climates:

- Anisophylleaceae (29–40 species);
- Begoniaceae (1400 species);
- Coriariaceae (15 species);
- Corynocarpaceae (6 species);
- **Cucurbitaceae (800 species);**
- Datiscaceae (2 species);
- Tetramelaceae (2 species) (3,16).

More that half of the species of the Cucurbitales order belong to the genus *Begonia*, of the Begoniaceae family (2 genera with approximately 1400 species). The rest of the species are distributed among the other families, but the largest of all appears to be the Cucurbitaceae family, that comprises over 100 genera and 800 species. Other families of the order are smaller and comprise lesser species (3).

Cucurbitaceae family is traditionally divided in two sub-families: the Nhandiroboideae, with 19 genera and almost 60 species and the Cucurbitoideae, with 111 genera and more than 740 species. This classification is mostly related to their morphological characters and it is confirmed by phylogenetic studies (2).

Molecular and phylogenetic studies bring scientifically important data that support the relationship between species of the family. Schaefer and Renner divide the Cucurbitaceae family into 15 tribes, according to their morphological features, ecology, number of genera and species and geographic distribution. It is the only classification that could be establised for the family as existing morphological and molecular data

cannot support the division of the family into equivalent clades as subfamilies (Fig.1) (3).

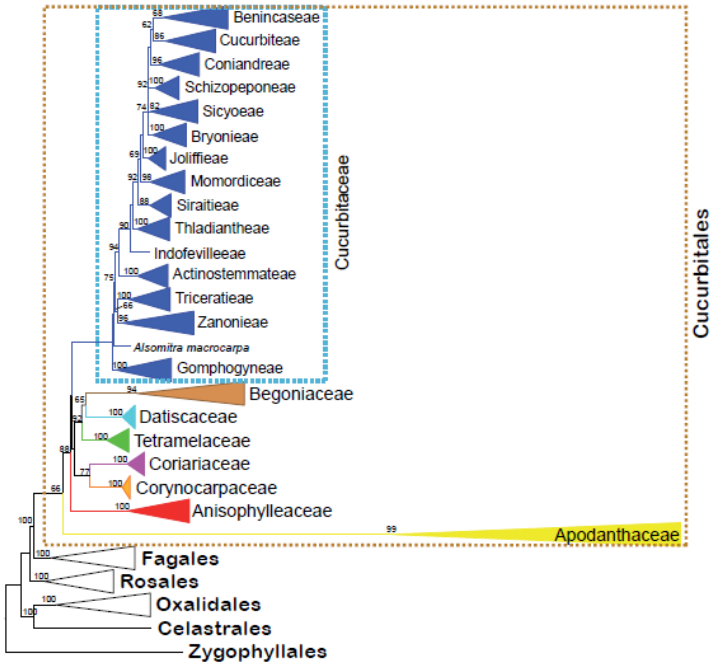


Fig.1. Simplified version of the tree obtained from 14 combined nuclear, plastid, and mitochondrial markers for 664 species of Cucurbitales plus eleven outgroups (3)

1.2. Botanical description of the Cucurbitaceae family

Cucurbitaceae family comprises 130 genera, with approximately 800 species, that are found mostly in the tropical and subtropical regions, in the South Eastern Asia, Western Africa, Madagascar and Mexico (Fig.2) (3,6,15). They are mostly climbing herbs, by tendrils, which are simple or branched (6–9).

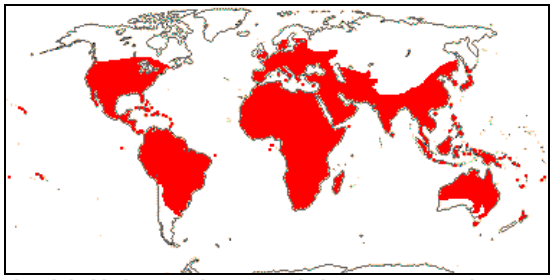


Fig.2. Map showing the global habitat of the species belonging to Cucurbitaceae family (15)

Most of the plants of this family have tendrils, which represent modified stems. Their way of coiling and the fact of being simple or branched is a taxonomically important feature. In some species, tendrils are lost (e.g. *Ecballium elaterium*) (17).

Leaves are simple, altern and palmately veined, entire or lobed, without stipules and with well-developed petioles (7–9,17,18).

Sexual system is an important feature of the plants belonging to this family. The distribution of the two sexual systems appears to be equal. Very few species are bisexual. Shifts between the sexual systems (monoecy and dioecy) can occur also within genera (e.g. *Bryonia*, *Luffa*), but also within species (e.g. *Ecballium*) (3). Flowers are radially symmetrical (actinomorphic), in inflorescences of various types, placed generally at the bottom of the branches or leaves (7–9). Calyx and corolla tipically with 5-6 elements, more or less united. Corolla is generally yellow, white-yellowish, of different forms (infundibuliform, rotate, campanulate). Male flowers have an androecium with 5 stamens, generally reunited in 3 bundles, rarely free stamens, while female flowers have the gynoecium with inferior ovary, 3-5 loci and multiple ovules. Placentation is parietal (3). Fruits are usually fleshy, berry-like. Seeds are compressed. Several species of the family have edible fruits (e.g. *Cucurbita*, *Cucumis*, *Sechium*, *Lagenaria*). Morphological characters of some Cucurbitaceae species can be found in Fig.3.

Flower formula: ♀*$K_{(5-6)}C_{(5-6)}\bar{G}_{3-5}$ (female flower)
♂ *$K_{(5-6)}C_{(5-6)}A_{(2)+(2)+1}$ (male flower)

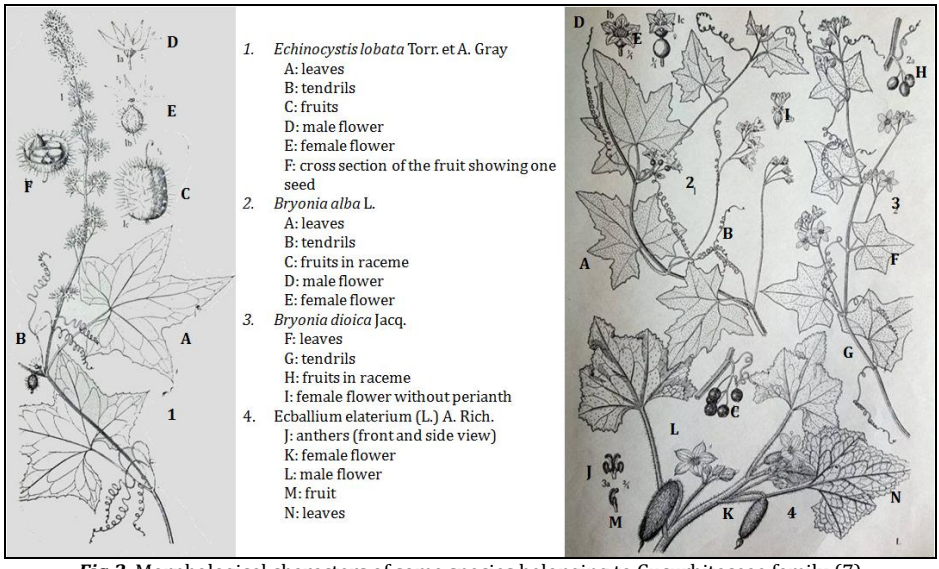


Fig.3. Morphological characters of some species belonging to Cucurbitaceae family (7)

1.3. Cucurbitaceae species from the European and Romanian flora

Cucurbitaceae species can be found naturalized in Europe in different areas, especially in the South, Central and Eastern part of the continent. As an important part of the species of this family are used for different economic purposes, most of these species are cultivated, especially in the Southern part of the continent (6).

The species that vegetate in the European and Romanian flora appear to be the same ones, with few differences, especially in the *Cucumis* and *Cucurbita* genera. The only species that appears to be cultivated only in Romania is *Luffa cylindrica* (L.) Roem.

Table II offers a comparative review of the Cucurbitaceae species found in the European flora and in the Romanian country.

Among the Cucurbitaceae species found in Europe and in the Romanian country, it appears that only *Bryonia dioica* Jacq. has created some confusion among botanists. Tutin *et al.* in the European Flora cite the presence of one species, *Bryonia cretica* L., with 3 subspecies, namely *cretica*, *dioica* and *acuta* (6), including though the species *B. dioica* as a subspecies on *B. cretica*. Romanian sources cite the presence of *Bryonia dioica* Jacq. (7,8), whilst recent sources cite the presence of *Bryonia cretica* L. subsp. *dioica* (9), following probably the classification of the European Flora. The presence of *B. dioica* in the Romanian country still remains uncertain as it is recorded as doubtful by Săvulescu *et al.* in an older Romanian flora (7). In a more recent flora of Romania, Ciocârlan *et al.* describe though the presence of the species in Arad, Radna and Bucharest (8). Studies performed by Volz and Renner in 2008 and 2009 state that it is uncertain if its presence is cited because it escaped from the Botanical Garden in Bucharest (10,11). It is therefore suggested that *Bryonia alba* L. can be the only species found in the Romanian country. To the best of our knowledge, the presence of the species in Romania was not yet confirmed by botanists, despite all bibliographic sources that state its presence.

Table II. Species of the Cucurbitaceae family that are found in Romanian and European flora (6,7)

Genus	European flora	Romanian flora	Type of species
Thladiantha	*Thladiantha dubia* Bge.	*Thladiantha dubia* Bge.	Naturalized
Luffa	-	*Luffa cylindrica* (L.) Roem.	Cultivated
Bryonia	*Bryonia alba* L.	*Bryonia alba* L.	Naturalized
	Bryonia cretica L. • subsp. *cretica* • subsp. *dioica* • subsp. *acuta*	*Bryonia dioica* Jacq.	Naturalized
Ecballium	*Ecballium elaterium* (L.) A. Rich.	*Ecballium elaterium* (L.) A. Rich.	Naturalized
Citrullus	*Citrullus lanatus* (Thunb.) Mansf.	*Citrullus lanatus* (Thunb.) Mansf.	Cultivated
	Citrullus colocynthis (L.) Schrad.	*Citrullus colocynthis* (L.) Schrad.	Naturalized and cultivated
Cucumis	*Cucumis sativus* L.	*Cucumis sativus* L.	Cultivated
	Cucumis melo L.	*Cucumis melo* L.	Cultivated
	Cucumis myriocarpus Naudin.	-	Naturalized
Lagenaria	*Lagenaria siceraria* (Molina) Standl.	*Lagenaria siceraria* (Molina) Standl.	Cultivated
Cucurbita	*Cucurbita moschata* Duch.	*Cucurbita moschata* Duch.	Cultivated
	Cucurbita maxima Duch.	*Cucurbita maxima* Duch.	Cultivated
	Cucurbita pepo L.	*Cucurbita pepo* L.	Cultivated
	Cucurbita ficifolia Bouché	*Cucurbita ficifolia* Bouché	Cultivated
	Cucurbita mixta Pangalo	-	Cultivated
Echinocystis	*Echinocystis lobata* Torr. Et A.Gray	*Echinocystis lobata* Torr. Et A.Gray	Naturalized
Sicyos	*Sicyos angulatus* L.	*Sicyos angulatus* L.	Naturalized

1.4. Morphological differentiation of the Cucurbitaceae species

Among the 130 genera of the Cucurbitaceae family, more than 50 contain a single species. This fact explains the difficulty of deducing relationship between the members of the family from morphological features. There are, though, important characters that represent the basis of the differentiation between the species belonging to this family. They are especially related to the features that are the most representative for the plants of this family (2,10,17).

The most important criterion of differentiation between the species of the Cucurbitaceae is related to the sexual system. Although it is not a morphological feature, it is an important criterion that separates the species of this family. Most of the species that belong to Cucurbitales order present unisexual flowers and can be dioecious, monoecious, andromonoecious or androdioecious. This order is the most well-known clade that present frequency of the dioecious species. Among the families that present the largest number of species, Begoniaceae (1400 spp.) and Cucurbitaceae (800 spp.), there are only species that have unisexual flowers. In the Cucurbitaceae, the vast majority of the species are dioecious, only a few are monoecious (16). Shifts between dioecy and monoecy are a subject largely discussed by authors and their phylogenetic studies of distribution of the species suggest that dioecy may be the ancestral condition (2,3,17). Concerning flower morphology, the symmetry is generally actinomorphic. The calyx and corolla are generally pentamerous, rarely hexamerous. Petals have different shapes and colors, that may vary from white, to yellow and orange. The hypanthium is derived from the basis of the petals and sepals and the area where the receptacle inserts. For male flowers, it is the point where the stamens begin to arise or where they are inserted at different levels. In female flowers, it includes the ovary wall.

Leaf morphology is however more clear criterion of differentiation between the species of the family. It plays an important role for these plants. Leaf features generally refer to the external structure of the leaves, but they can also comprise some other important anatomical characters generally found on the surface of the leaves, the trichomes, that represent important botanic characteristics for plants belonging to this family. The trichomes in Cucurbitaceae present various forms, thay may vary from unicellular to multicellular, conical to elongated, smooth to ridges, with or with-out flattened disk at base and cystolithic appendages, thin to thick walled, curved at apices to blunt. There is a large diversity in the trichomes of the Cucurbitaceae family and this appears as an important criterion used to solve taxonomic problems, that may concern especially classification into smaller taxonomic classes (17,19). It is especially in the powder form that these microscopic characters can help to differentiate the different species of the family (20).

At the same time, the other morphological features, fruits and tendrils are also key features for these plants: fruits because plants from this family are widely known for their large-sized fruits and tendrils because Cucurbitaceae plants are also widely known as climbing plants, by means of their tendrils (2,17).

Morphology of Cucurbitacee fruits is variable. Typically, fruits have many seeds, they are rarely one-seeded. Berries and berries-derived fruits are the most common type of fruit that is found for these plants. They generally present a hard shell and are called gourd or pepo (*Citrullus*, *Cucumis*, *Cucurbita*), or leathery with a fibrous

mesocarp (*Sicyos*). For the species that are commercially important, the fruit is a hard-shelled berry, that can reach high dimensions (up to 1m in diameter). This is also a modification of these species that help them withstand the seasonally dry habitats, where they are generally naturalized. Hard-shelled fruits store water for long periods, protecting thereby the seed, that can mature, even after the stems are dry or have died off. Some berries are dehiscent and even explosive, either by keeping the fruit on the plant, or by separating from the peduncle and ejecting seed by elastic contraction. Other types of fruits that are found are capsules, achenes and geocarpic fruits (17).

Pollen structure has been used as an additional criteria for the diagnose of some species (2). The pollen of Cucurbitaceae is covered with an oily pollen kit, whose color varies from yellow to orange, depending on the species (17).

European and Romanian flora offer, therefore, a dichotomous key for the differentiation of these species, based on these features. Fig. 4 comprises a simple and fast dichotomous key for the determination of the genera belonging to Cucurbitaceae family (6–8).

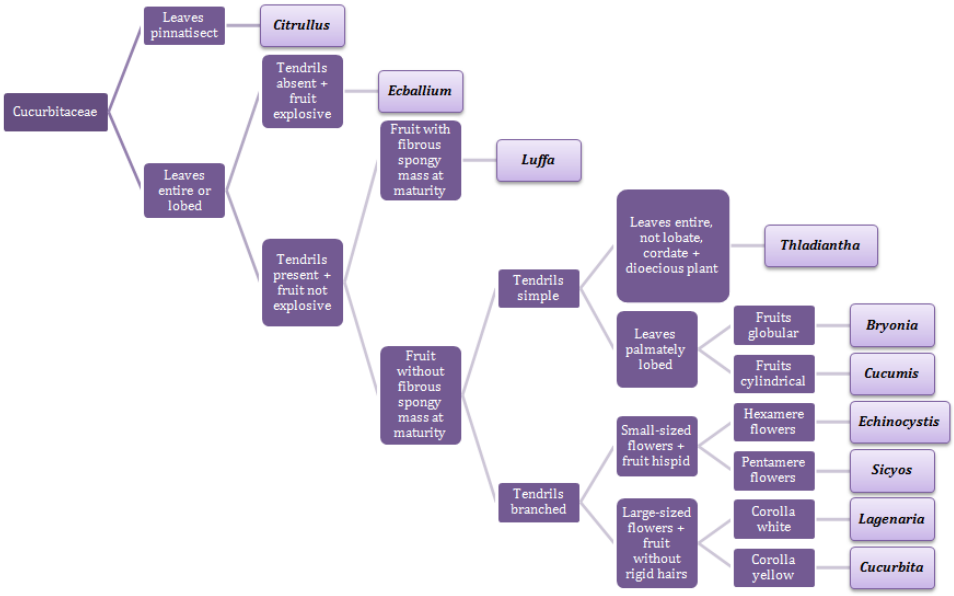


Fig.4. Dichotomous key to the determination of genera belonging to Cucurbitaceae (6–8)

1.5. Cucurbitaceae species from the Romanian flora

The present PhD thesis is focused on the study of three species belonging to Cucurbitaceae family, namely *Bryonia alba* L., *Echinocystis lobata* (Michx.) Torr. et A. Gray and *Ecballium elaterium* (L.) A. Rich. The following chapters are thus dedicated to perform a review on the knowledge until present on the three species.

The only species of the three that can be found on another name in some scientific sources is *Echinocystis lobata* (Michx.) Torr. et A. Gray, which Romanian flora cites as *Echinocystis echinata* (Mühl.) Britt. The two names appear as synonyms, but the name accepted for the species remains *E. lobata*.

In the Romanian country, each of these species is found in different areas of the country. The only one that is spread across the whole country is *B. alba*, while the other ones have more restricted areas of distribution. *E. elaterium* is the species that has the most restricted area of distribution, in the Romanian territory being only found in the South Eastern part, on litoral sands, in the Constanţa and Tulcea counties. On the other side, *E. lobata* is found in the North Western part of the country (7–9). Fig. 5. comprises the geographical distribution of the three species in the Romanian country.

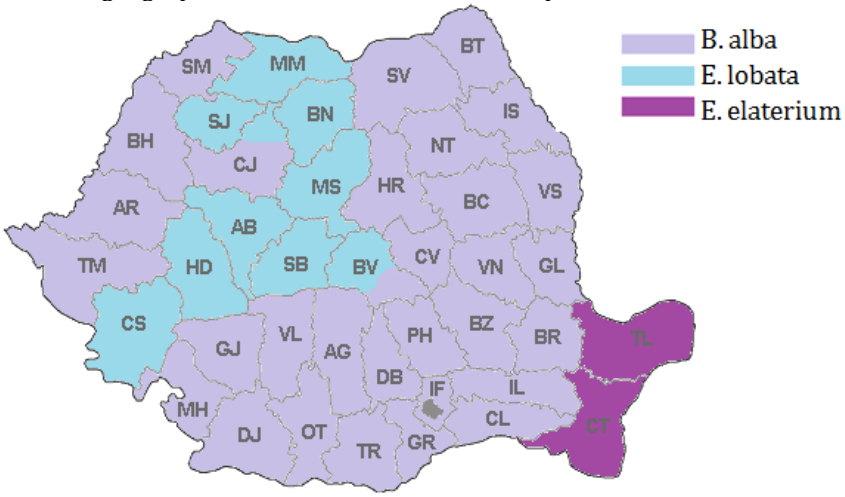


Fig.5. Geographic distribution on the Romanian country of the three species belonging to Cucurbitaceae family (7–9)

1.5.1. *Bryonia alba* L.

Bryonia alba L. is a monoecious species, which can be found in the Central part of the European continent, with some extensions that reach also to Northern, Southern and Eastern Europe and even to Kazakhstan, North Africa, Canary Islands or Central Asia (3,10,11) (Fig. 6). It prefers dried soils, rocky slopes in the mountainous areas, dry riverbeds or even sand dunes. It can also be found at forest margins and disturbed ground, semi-deserts and dry bushland (17).

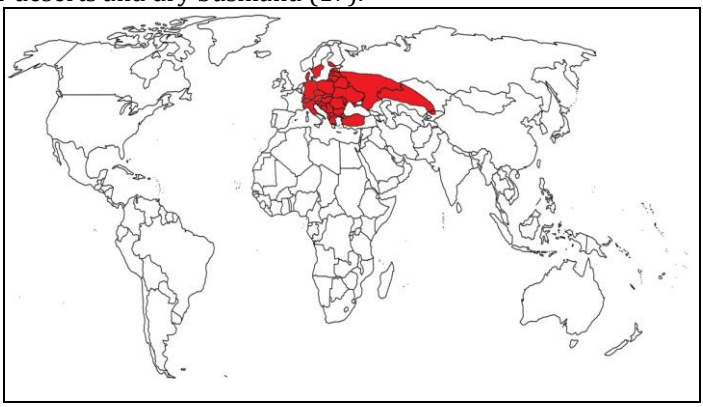


Fig.6. Geographic distribution of *B. alba* (21)

B. alba is a perennial monoecious species, having a thick and fleshy tuberous root, which helps it storing the water underground (7). Because of this special adaptation, it is a resistant species, especially to difficult external conditions (10). It is a climbing species, by simple tendrils. Stems are nodous, pubescent. Leaves are ovately cordate to triangular, with lobes that are unequaly sinuate and dentate, the middle one longer than the lateral ones, scabrous because of short trichomes. Flowers small and are found in axillary, racemose panicles or sub-umbellate fascicles. Receptacles is campanulate. Calyx is campanulate, corolla is radially campanulate, rotate, each one having 5 elements, with deep divisions. Male flowers are found in racemes with long peduncles. Female flowers have short peduncles and are found in similar inflorescences. Both type of flowers are colored in greenish white. Stamens have short filaments and are united in 3 bundles, of which 2 are formed of 2 stamens and the third is formed by one stamen. Ovary is globular, with 3 loci. Style is elongate, stigmas 2-lobed. It has many ovules. Fruits are fleshy berries, smooth, that in maturity become black. Seeds are compressed, ellipsoid (6–8,17) (Fig.7).

Scientific sources cite the presence of 10 species in the *Bryonia* genus. There are few morphological differences that can be found between the *Bryonia alba* L. species and the *Bryonia dioica* Jacq. species, both cited in the Romanian flora. Differences appear mostly in the sexual system, *B. dioica* being, as it name states, a dioecious species. Another significant difference is related to fruits, which for *B. dioica* are red at maturity. However, its presence remains uncertain, as even Romanian flora doubts its presence. It seems, though, that the only *Bryonia* species found on the Romanian territory is *B. alba*.

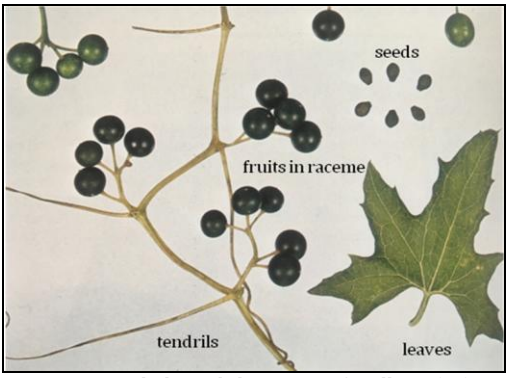


Fig.7. Morphological characters of *B.alba* L. (12)

1.5.2. *Echinocystis lobata* (Michx.) Torr. et A. Gray

Echinocystis lobata (Michx.) Torr. et A. Gray is a monoecious naturalized species, that vegetates in Central and South Eastern Europe, North America and Canada, in moist soils, forests, open areas, in thickets, along roadsides and in other disturbed areas (13,14,17).

It is a herbaceous climbing species, having branched tendrils, with 3-5 branches (18). Leaves are palmately lobed, with 5 lobes, dentate and scabrous. Flowers are white, small-sized, generally hexameric. Male flowers are arranged in racemes, while female flowers are solitary (very rarely in pairs), generally placed at the bottom of the leaves, together with the male flowers racemes. Receptacle is tube flat. It has 6 sepals, 6 petals, corolla rotate, white. Androecium has 3 stamens, inserted near the center of

the tube, with very short filaments. The ovary is globose, having 2 loci with 2 ovules and is setiformely hirsute. Style is very short, with stigma capitate. Fruits are globular, swollen, glabrous and are fleshy pepo, with surface covered in spines that are thickened at their base. Fruit is apically dehiscent. In the fibrous and reticulate placenta, 4 seeds can be found (6–8,17) (Fig.8).

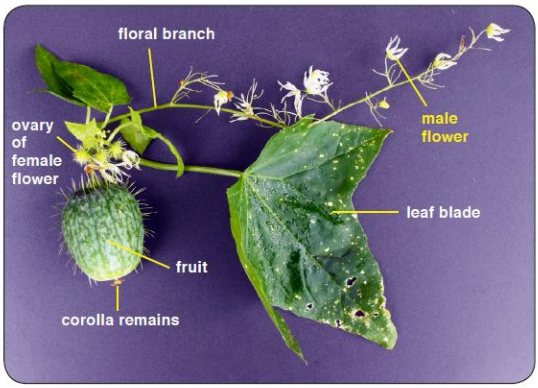


Fig.8. Morphological characters of *E.lobata* (12)

1.5.3 . *Ecballium elaterium* (L.) A. Rich.

Ecballium elaterium (L.) A. Rich. is the only species of the *Ecballium* genus. Despite this fact, it is divided in two subspecies, having different sexual systems: subspecies *dioicum*, which is dioecious and subspecies *elaterium*, which is monoecious. The study performed by Costich and Meagher in 1992 showed that there is a global allopatic distribution of the two subspecies, strongly correlated with the genetic differences and with with their sexual system. The general habitat of the species includes litoral and disturbed sands from Northern Spain, to Southern Europe and to the Mediterranean, North Africa and Southwest Asia (Fig.9). Because of this type of habitat, it has developed morpho-anatomical features especially in the root and leaf, to help it withstand stressful external conditions.

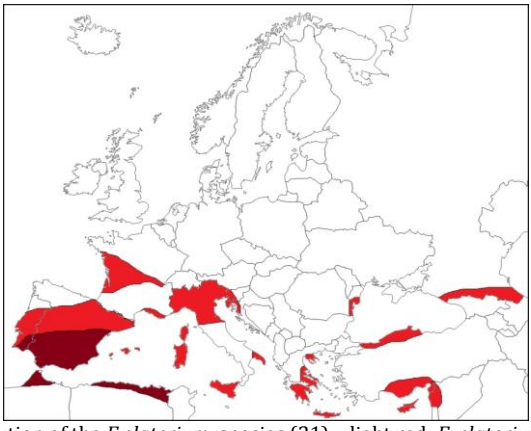


Fig.9. Geographic distribution of the *E.elaterium* species (21) – light red: *E. elaterium* ssp. *elaterium* and dark red: *E. elaterium* ssp. *dioicum*

E. elaterium is a small-sized annual and monoecious or dioecious species, with tuberous root, thick stems and without tendrils. Leaves are thick and triangular, deeply cordate, dentate, sometimes undulate, almost entire, with the superior face greener. Both surfaces of the leaves are covered in short conical rough trichomes, very frequent. Flowers are pale greenish yellow, disposed at the same axil, the male flowers in racemes. Female flowers are solitary. Receptacle is short and campanulate. Calyx and corolla of male flowers have each 5 elements, that are elliptical or lanceolate, mucronate at the tip. Androecium is composed of 5 stamens, with short filaments, inserted near the center of the tube, united by 2, and the fifth one is free. Corolla of female flowers is campanulate or almost rotate. Gynoecium has inferior ovary, oblong, hispid, with loci that contain several ovules. Style is short. The fruit is ellipsoidal, hispid, scabrous and watery. At maturity, it comes off the peduncle and by the basilar aperture, the liquid and the seeds are discarded with velocity, by elastic contraction, on relatively small distances (22). It contains many seeds, that are compressed (18) (Fig.10).

Fig.10. Morphological characters (left) (23) and an exploding fruit (right) (24) of *E. elaterium* (L.) A. Rich

1.6. Histo-anatomical characters of the Cucurbitaceae species

Study of the morphological and histo-anatomical features of vegetal species have a great importance since they represent the first step towards understanding the biological activity of these species. It is essential to study these characters for the correct description of the species, of its vegetal product, for the identification of substitutions and adulterations with species without biological activity, but also for the correct localization of the active principles at a cellular level (25).

Cucurbitaceae family is widely known for the presence of bicollateral vascular bundles in the anatomical structure of the vegetal organs, which represent an important diagnostic criterion. They are found especially in the structure of stems and of all the structures that are derived from it: petioles, tendrils, flower and fruit peduncles. Anatomical structures are generally protected by rings of sclerenchyma, that in maturity can become discontinous. Collenchyma is also frequently found, surrounding anatomical structures of herbaceous plants, that have ribs containing this mechanical tissue. The collenchyma and sclerenchyma are frequently separated by strings of assimilatory tissue, that can often go to the epidermis. Vascular bundles that are mostly bicollateral, can be found in the cortex, frequently arranged in two rings,

that are separated by strings of ground parenchyma. Successive cambia can be found between the xylem and phloem, being, at the same time, the one that is responsible for the formation of secondary rays (17,26).

Regarding the other morphological features of differentiation, they concern especially the structure of the leaves. The family is known for the presence of the multicellular trichomes, of different types, on the surface of the leaves. Important anatomical features are also concerning the leaves and are represented by stomata, which can be found on both sides of the leaf, being frequently raised above the surface of the epidermis (26).

Structure of the roots depends on their particular type. Tuberous roots generally present a fundamental parenchyma, that embedded the other elements, lesser developed, especially vascular bundles. It may often present anomalous anatomical structure, by the presence of different anatomical features (e.g. succesive rings of growth) (17,26).

Scientific literature offers few information on the morpho-anatomical features of the three species belonging to Cucurbitaceae family. It is especially *E. lobata* that lacks any information in this area. Data for *B. alba* and *E. elaterium* concern mostly histo-anatomical studies of the vegetative organs.

1.6.1. *Bryonia alba* L.

Data on histo-anatomical characterisation of *B. alba* was first published by Toma and Rugină in 1998 and revealed histo-anatomical structure of the vegetative organs (root, stem, leaves) (27). Other studies on the anatomy of the species are performed by Manvi and Ganesh in 2011 and concern especially the structure of the root and the localisation of ergastic amorphous principles (starch grains). Fig.11 comprises the anatomical features that are described by these authors. The root of the plant appears to be the most important vegetative organ, because it contains the most important active principles, cucurbitacins (28,29). This is the probably the main reason for the histo-anatomical studies studies that are directed towards the elucidation of its structure (22).

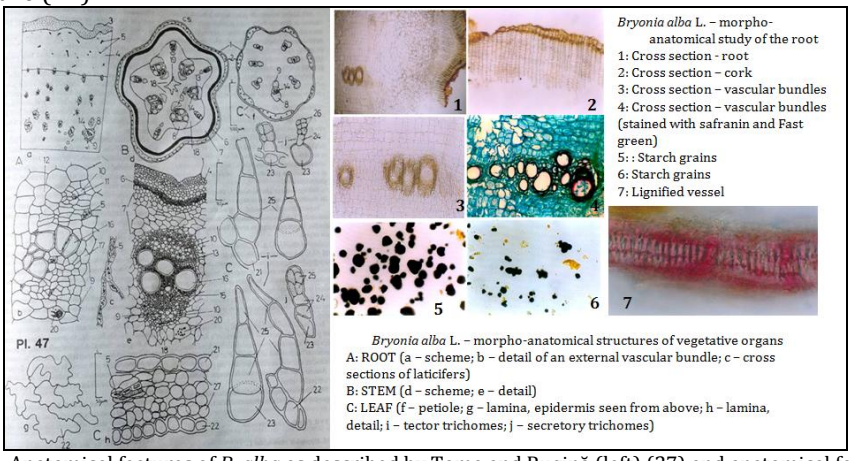
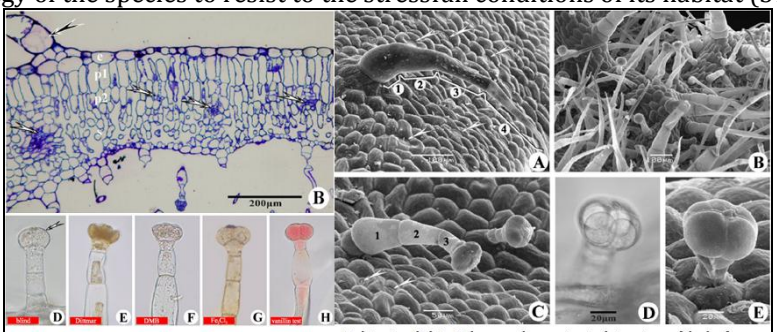


Fig.11. Anatomical features of *B. alba* as described by Toma and Rugină (left) (27) and anatomical features of the root of *B. alba* as described by Manvi and Ganesh (up, right) (30)

1.6.2. *Ecballium elaterium* (L.) A. Rich.

Histo-anatomical studies on *E. elaterium* provide data on the leaf structure and on the secretory trichomes found on the surface of the leaf (31) (Fig.12). As suggested by other authors, trichomes are important taxonomic criteria used to microscopically differentiate the species of Cucurbitaceae family (19). It is also suggested by Christodoulakis *et al.* that not only trichomes, but also leaf structure is responsible for the strategy of the species to resist to the stressfull conditions of its habitat (31).

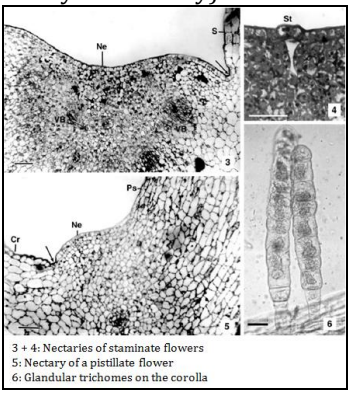


E.elaterium (L.) A. Rich– morpho-anatomical structure of the leaf (optic microscopy)
B: Cross section through the leaf
D, E, F, G, H: Secretory trichomes stained with different reagents for the main classes of active principles

E.elaterium (L.) A. Rich– morpho-anatomical structure of the leaf (electronic microscopy)
A: Tector trichome structure
B: General view on the surface of the leaf (tector and secretory trichomes)
C, D, E: Secretory trichomes

Fig.12. Anatomical characters of *E. elaterium* as described by Christodoulakis *et al.* (31)

Other microscopic studies on *E. elaterium* describe the nectaries of the species, for both the staminate and pistilate flowers. Nectary was found to be well developed in staminate flowers, while in pistilate flowers, it was reduced. Also, the study proved the implication of some small insects found in relatively large amount in pistilate flowers (attracted by the nectary) are involved in the process of pollination (32) (Fig.13, left).



3 + 4: Nectaries of staminate flowers
5: Nectary of a pistillate flower
6: Glandular trichomes on the corolla

Fig.13. Structure of the nectaries of *E. elaterium* as described by Fahn (32)(left) and anatomical tructure of the stems of *E. elaterium* as described by Metcalfe and Chalk (26) (right)

Not least, Metcalfe and Chalk describe the structure of the stem of *E. elaterium*, as being typical for the plants that belong to Cucurbitaceae family (Fig.13, right). Other observation that are made by the same authors concern the structure of the root, which can be an anomalous one, presenting succesive rings of growth, that arise from the pericycle (26).

2. Phytochemical data on the three species belonging to Cucurbitaceae family

2.1. Introduction

The Cucurbitaceae family is one of the most economically important families of flowering plants and due to this fact it has always been in the first line of the attention of researchers worldwide. Some of the Cucurbitaceae species are widely known to have economical importance, mostly for human nutrition. Among these species, the melons, the watermelons, the cucumbers and the pumpkins are the most frequently found in crops all over the world, have been cultivated for years in a variety of environmental conditions for their content in nutrients and are largely consumed as vegetables worldwide (33). Cucurbitaceae crops include squash or pumpkin (*Cucurbita maxima*), zucchini (*Cucurbita pepo* subsp. *pepo*), bitter gourd (*Momordica charantia*), watermelon (*Citrullus lanatus* (Thunb.) Matsum. et Nakai) or melon (*Cucumis melo* L.) and cucumber (*Cucumis sativus* L.) (5). Although aerial and underground parts present small differences, cucurbits are known for an important range of fruit characteristics. These fruits can be consumed in mature or immature forms, as such, fresh or in different combinations, but also cooked. The benefit for human health is significant, not only they bring important nutrients, but also may be useful for different disorders (e.g. constipation, digestion troubles, bad blood circulation). Not only fruits are consumed from these plants, but also seeds, flowers and sometimes even roots, for other purposes related to different pathologies (1).

Some of the species of this family have different uses in other areas, not related to human nutrition. For example, *Luffa* sp. are used as sponges and *Lagenaria siceraria* Molina (Standl.) (bottle gourd, calabash) is used frequently as raw material for utensils, containers of musical instruments (5,17).

The use of the Cucurbitaceae species in these areas is largely known and cited but there are scientific sources that cite also cite the possible use of these plants as medicinal species (1), despite their well-known toxicity (4). Traditional medicine also describes the use of some of these species for the treatment of some disorders (5,22).

There is no herbal drug that is assigned to species of the family in modern allopathic pahamacopoeias. More ancient sources cite the use of different parts of some species for their medicinal uses, generally due to their cucurbitacin content. Thus, it is largely known the use as drastic purgatives for the colocynth seeds (*Citrullus colocynthis* (L.) Schrad), the juice of the fruits of wild cucumber (*Ecballium elaterium* (L.) A. Rich) and the bryony root (*Bryonia cretica* subsp. *dioica* (Jacq.) Tutin) (5,22,34). Some of these species and their herbal products are still used nowadays, especially in homeopathy (e.g. *B.dioica* and *B.alba*) (5). Some homeopathic and allopathic pharmacopoeias assign monographs to some species belonging to the family: e.g. Brazilian Homeopathic Pharmacopoeia assigns a monograph to the roots of *B.alba*, used to obtain a mother tincture (35). In the French Pharmacopoeia, from 1989, a monograph for the bryony species used for homeopathic preparations (*B.dioica* and *B.alba*) is still in place (36). In fact, the species *B.dioica* also had a monograph in the French Pharmacopoeia until 1884, when it was withdrawn as it was proved to cause severe diarrhea and intestinal haemorrhage (5). The Belgian Pharmacopoeia also

contained such a monograph until 1885 (37). Other Pharmacopoeias (e.g. Russian Pharmacopoeia) assign monographs for the seeds of *Cucurbita pepo* L. or other species of the *Cucurbita* genus, used for their antihelmintic properties (38). European Medicines Agency offers an assessment report on *Cucurbita pepo* L. seeds, which presents the phytopharmaceuticals available on market nowadays with this herbal product (39).

On the other side, scientific studies are performed on various parts of some species belonging to the family and are evaluating the content in different active principles and their possible involvement in different biological activities (40). At the same time, there are traditional uses for plants belonging to this family and the use of different parts of these species is described for the treatment of different disorders (41). To the best of our knowledge, no vegetal medicinal product for a species belonging to this family is officially known until present, despite the occasional use of different parts of the plants for different purposes.

2.2. Cucurbitacins

Plants belonging to Cucurbitaceae family have drawn the attention of researchers for their composition in bioactive principles (1). The most well-known and cited compounds in the composition of the species are cucurbitacins (42).

Cucurbitacins belong to the class of tetracyclic triterpenoids, being highly oxygenated compounds (4,40,43–46). There are 16 genins of cucurbitacins that are known until present (A-S) and classifications include them in 12 classes, in which they are grouped according to the molecular variation on the genin (Fig.14.) (46). Based on these aglycones, hundreds of heterosides have been discovered.

The basic structure of cucurbitacins is named cucurbita-5-ene (Fig.14). It is a tetracyclic nucleus skeleton, also known as 9-β-methyl-19-nor-lanosta-5-ene or 19-(10→9-β)-abeo-5-alpha-lanostane (IUPAC name). This skeleton can be modified by oxygen-containing groups and by double bonds to produce the 16 known genins of cucurbitacins (4,43). Most of the cucurbitacins are found as glucosides, having the glycosidic part linked to the carbon in the position number 2 (2-O-β-glucosides) (43).

The first cucurbitacin that was discovered was cucurbitacin E, isolated in 1831 as a crystalline substance and named α-elaterin, as it was isolated from *Ecballium elaterium*. It has only received the name of cucurbitacin E more than 100 years after its first isolation and after other cucurbitacins were discovered and described (4).

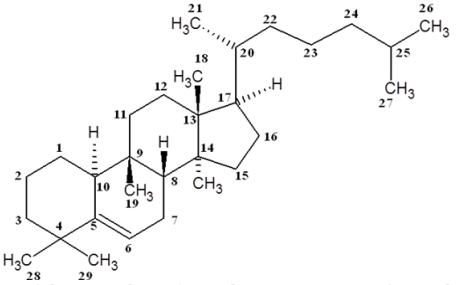


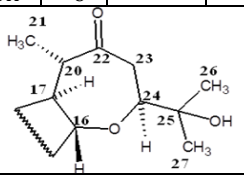
Fig.14. Structure of the cucurbita-5-ene, basic structure of cucurbitacins

With the exception of cucurbitacin A, J and K, all the cucurbitacins genins could be isolated from species belonging to the Cucurbitaceae family. The most commonly

found cucurbitacins in plants of this family are cucurbitacin B and D. A special class of cucurbitacins are the momordicosides and they have been isolated from *Momordica charantia* L. The difference between momordicosides and cucurbitacins appears in C_{19}, where the hydroxyl group found in some cucurbitacins, has been oxidized to an aldehyde group.

The 16 aglycones were called cucurbitacins and the difference between them has been marked by adding a letter from A to S, that signifies the chronological order of their discovery and isolation from plants (4) (Table III).

Table. III. Structure of the cucurbitacins (4,43)

Compound	Modification of the basic structure										
	1-2	**2**	**3**	**11**	**16**	**19**	**20**	**22**	**23-24**	**24**	**25**
Cucurbitacin A	-	-OH	=O	=O	-OH	-OH	-OH	=O	=	-	-OAc
Cucurbitacin B	-	-OH	=O	=O	-OH	-	-OH	=O	=	-	-OAc
Cucurbitacin C	-	-	-OH	=O	-OH	-OH	-OH	=O	=	-	-OAc
Cucurbitacin D	-	-OH	=O	=O	-OH	-	-OH	=O	=	-	-OH
Cucurbitacin E	=	-OH	=O	=O	-OH	-	-OH	=O	=	-	-OAc
Cucurbitacin F	-	-OH	-OH	=O	-OH	-	-OH	=O	=	-	-OH
Cucurbitacin G = Cucurbitacin H	-	-OH	=O	=O	-OH	-	-OH	=O	-	-OH	-OH
Cucurbitacin I	=	-OH	=O	=O	-OH	-	-OH	=O	=	-	-OH
Cucurbitacin J	=	-OH	=O	=O	-OH	-	-OH	=O	-	-OH	-OH
Cucurbitacin K	=	-OH	=O	=O	-OH	-	-OH	=O	-	-OH	-OH
Cucurbitacin L	=	-OH	=O	=O	-OH	-	-OH	=O	-	-	-OH
Cucurbitacin O	-	-OH	-OH	=O	-OH	-	-OH	=O	=	-	-OH
Cucurbitacin P	-	-OH	-OH	=O	-OH	-	-OH	=O	-	-	-OH
Cucurbitacin Q	-	-OH	-OH	=O	-OH	-	-OH	=O	=	-	-OAc
Cucurbitacin R	-	-OH	=O	=O	-OH	-	-OH	=O	-	-	-OH
Cucurbitacin S	=	-OH	=O	=O							

Concerning the presence of cucurbitacins in the three species that are studied in this PhD thesis, only the two related species, *B. alba* and *E. elaterium* have been extensively studied for their content in cucurbitacins. There is no scientific evidence on their presence in the composition of *E. lobata*, to the best of our knowledge (21).

The most important bioactivities that are cited for *B. alba* are due to its content in cucurbitacins. The most significant activity is cited for the roots of the species, which is the part that is the most studied (28,29,47–51). Aglycones, especially cucurbitacin B, D, E, I and their derivatives, but also cucurbitacins J, K, L and their derivatives are cited in the composition of the roots of the species (29,47) and are proved to show cytotoxic or antitumor activities (28,47). The most active compounds from this point of view proved to be cucurbitacin B, D, E and I, but the other ones also exhibited a moderate activity (28).

As its older relative, *E. elaterium* is mostly known for its cucurbitacin content, that is proved to be significant and can provide the species several bioactivites. This species is the one that provided the first known cucurbitacin, elaterin (cucurbitacin E). It was isolated for the first time from the fruit juice of *E. elaterium*. Due to this reason,

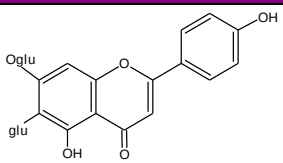
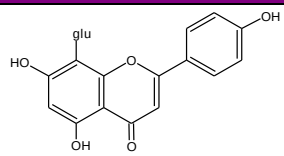
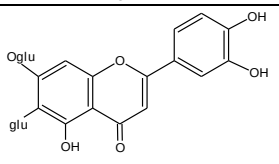
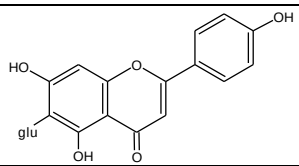
E. elaterium is considered an "indicator plant" among the species that have important amounts of cucurbitacins in their content (52). From all the organs of the species, the ones that were proved to contain the highest amounts of cucurbitacins were the fruits, followed by the stems and leaves (53).

2.3. Flavonoids

Flavonoids are a class of compounds that are widely spread in plant kingdom, being responsible of several important biological activities (54). Plants belonging to Cucurbitaceae family are also known for their content in flavonoids (55–59), even if they are not the most frequently found class of compounds in the composition of these species. Nevertheless, they may be responsible for various other biological activities, that may inhance or act synergistically with other compounds. Flavonoids are largely known as effective antioxidants, a lot of studies being recently directed towards the elucidation of the mechanisms that are involved in this bioactivity. Other biological activities that flavonoids may assign to plants they belong to are the anti-allergic, anti-inflammatory, anti-viral, anti-proliferative and anti-carcinogenic. At present, flavonoids are considered to be secondary, non-essential dietary factors, with an important role and documented relevance in human health and for the treatment of some disorders. Flavonoids in Cucurbitaceae may increase the nutritional importance of these plants, but may also be useful to justify their potential as medicinal plants.

There are few studies that deal with the composition of flavonoids in the three species belonging to Cucurbitaceae family that are studied in this thesis. For instance, flavonoids in *B. alba* are described by Krauze-Baranowska *et al.*, who elucidated the presence of C-glucosides of apigenin in the aerial part of the species. Consequenlty, saponarin, isovitexin, vitexin and a luteolin derivative, lutonarin (Table IV) were found in the composition of the species (60,61).

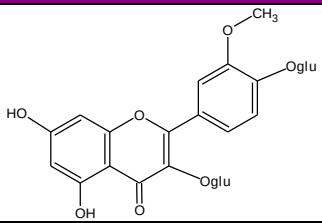
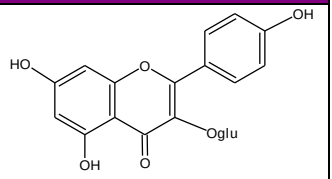
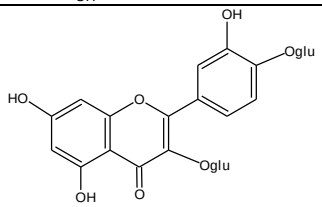
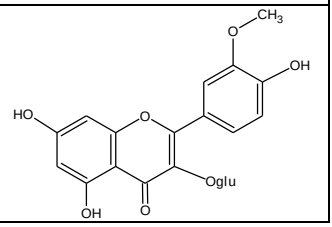
Table. IV. Structure of the flavonoids in the composition of *B. alba* (60,61)

Compound	Structure	Compound	Structure
Saponarin = isovitexin-7-O-glucoside		**Vitexin = apigenin-8-C-glucoside**	
Lutonarin = luteolin-6-O-glucoside-7-C-glucoside		**Isovitexin = apigenin-6-C-glucoside**	

The same authors were the ones that investigated the flavonoids found in the aerial parts of *E. lobata*. Quercetin and isorhamnetin derivatives were found (Table V). The study mentions the presence of a compound with an identity that changes during the study. Taken into consideration the close structures of kaempferol-3-O-glucoside (astragalin) and quercetin-3-O-glucoside (isoquercitrin), which differ by the presence of a hydroxyl group in position 3', the authors cite the presence of both compounds in

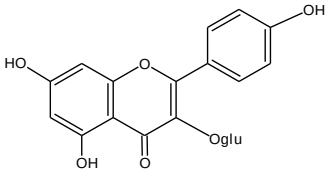
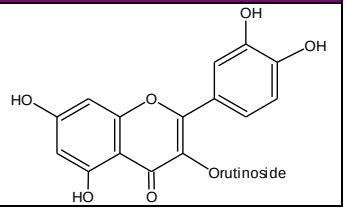
the study, the real identity of the compound being unclear. It is likely that the isolated compound was the kaempferol derivative (astragalin), as the aglycone produced by the acid hydrolysis which is performed by authors proved to be kaempferol (62) (Table V).

Table. V. Structure of the flavonoids in the composition of *E.lobata* (62)

Compound	Structure	Compound	Structure
1 = isorhamnetin derivative		**3 = kaempferol derivative (kaempferol-3-O-glucoside = astragalin)**	
2 = quercetin derivative		**4 = isorhamnetin derivative**	

The species that has proved to be the poorest in flavonoidic compounds is *E. elaterium*. Quercetin glucosides were only found in the composition of flower (63), fruit or leaves (64–67). Compounds that were found are kaempferol-3-O-glucoside and quercetin-3-O-rutinoside (rutin) (Table VI).

Table. VI. Structure of the flavonoids in the composition of *E. elaterium* (63,65,66)

Compound	Structure	Compound	Structure
Kaempferol – 3-O-glucoside		**Rutin**	

2.4. Other compounds

In the composition of *B. alba*, besides cucurbitacins and flavonoids, there are also other compounds, which can increase the biological activity or which can act synergistically to increase the biological activity. Table VII contains the most important compounds that are found in the composition of *B. alba*.

Table. VII. Active principles found in the composition of *B. alba*

Compounds	Part of the species	Study
Sterols	Roots	(51)
Lectins	Roots	(48)
Amino-acids	Roots	(50)
Trihydroxyoctadecadienoic acids	Roots	(49,68)
Lipids	Roots	(69)

E. lobata is a species on which there is few scientific evidence in literature, especially concerning the active principles composition. There are studies that state the presence of enzymes (70) and trypsin inhibitors (71). It is a species often used as a model for different studies of anatomy and morphology or ecology (72).

E. elaterium contains, besides cucurbitacins, other important compounds, which can enhance its biological activities (73). Table VIII offers a review of these compounds.

Table. VIII. Active principles found in the composition of *E. elaterium*

Compounds	Part of the species	Study
Sterols	Roots, leaves, stems, flowers, fruits and seeds	(64,74–76)
Fatty acids, lipids and oils	Roots, leaves, stems, flowers, fruits and seeds	(64,74,75,77)
Essential oils	Leaves, fruits and seeds	(78,79)
Carotenoids	Roots, leaves, stems, flowers, fruits and seeds	(64)
Soluble sugars (sucrose, raffinose)	Leaves	(64,77,80)
Trypsin inhibitors	Seeds	(81)
Tannins	Leaves, flowers	(64)
Alkaloids	Roots, leaves, stems, flowers, fruits and seeds	(64)
Amino-acids and amino-acids decarboxylases	Seeds	(82,83)

2.5. Toxicity of the three species belonging to Cucurbitaceae family

Toxicity of these plants is clearly due to their cucurbitacin content. Scientific literature often cite cucurbitacins as highly toxic compounds. Some of these compounds can rapidly cause death, while others are strongly purgative and cause death after more long periods of time. Toxicity of cucurbitacins is highly related to the lack of selectivity in the cytotoxic activity. The most poisonous triterpenes appear to be cucurbitacins B, D, E and I, being the most cytotoxic *in vitro*. It appears that structural requirements are needed for exhibiting these toxic effects: for the laxative effect, acetylation of the position 15 and the double bond in the 23-24 position, while for the abolition of this effect the esterification of the OH in the 16 position is needed (84).

Cucurbitacins appear in important amounts in plants used for human nutrition (4). There is no clear evidence concerning the presence of cucurbitacins as glycosides and/or as aglycones in these plants. Obviously, during food processing or during the consumption of fresh plants, there are enzymes as cellular β-glycosidases from the plant that will cleave the glycosidic bond and liberate the aglycone. The bond can be also cleaved in the colon, by the microflora that is present. Until present, to the best of our knowledge, there are no studies available on the kinetics of cucurbitacins. Data on the absorption, distribution, metabolism and excretion of cucurbitacin glycosides or cucurbitacin aglycones are not available. It is only clear that there is a link between the bioavailability of cucurbitacins with the delay of appearance of toxic symptomes, the administration route and the amount that is administered (4).

Regarding the cucurbitacins from plants that are not edible, scattered studies on their in vitro and in vivo toxicity exist, but still no conclusive data on their toxicokinetics (28,29,85,86).

3. Pharmacological data on the three species belonging to Cucurbitaceae family

3.1. *Bryonia alba* L.

Medicinal uses of the species *B. alba* have been recorded for centuries. It is a species that is formerly used as a drastic purgative, having monographs in pharmacopoeias in Belgium and France (37). Today there is a considerable market for *Bryonia* preparations, although effectiveness remains contested. The species is mostly known for its use in homeopathy. It is especially the roots that are used for homeopathic preparations, that are used to induce dryness of the mucous membranes in high doses (87) and as laxatives and purgatives (10), for the antiinflamatory effect in pathologic inflammatory sympthoms of the serous tissues, or for the treatment of cough, pneumonia and rheumatism (5,22,30,35,88). Pathogenesis of the species for its use in homeopathy is found in Table IX.

Table. IX. Pathogenesis of *B. alba* (89,90)

Symptoms/Systems concerned	Description
Fever	Progressive start; continuous evolution
Headaches	Frontal layout, with fever syndromes, progressive start, with congestion; suborbital localisation; in point or that radiate towards the front or the neck; exacerbated in motion, ameliorated by tightening the head with a strip, by absolute immobility or by local cold if they are migraines, local heat if they are arthritic or neuralgic; meningitis and encephalitis
Physical apparence	Motionless, lying on the painful side, asking for silence, sweating that relieves pain; sadness, anxiety, aversion to the company of others; tenacity, obstinacy, aversion for change
Respiratory system	Thoracic „side stitch" which is ameliorated by strong pressure or by lying on the painful side, exacerbated by respiration and cough; rhinitis with dry mucous membranes; repeated epistaxis; dry cough, causing pain when passing from cold to heat, exacerbated when speaking
Digestive system	Excessive thirst for long periods and high quantities of cold water; dry mouth, with bitter taste, which disappears when drinking water; dry tongue and lips, with cracks; high appetite, desire of eating meat, intolerance for vegetables; toothaches that are ameliorated by high pressure on the maxillary; sensation of fullness, weight, stones in the stomach, which is dilated; stomachaches that ameliorate when drinking hot drinks; nausea when moving; stretched abdominal wall, sensitive to touch, but ameliorated by high pressure; painful flatulence; constipation with hard dry stools
Diabetes	Intensive long-lasting thirst
Musculoskeletal system	Pains and inflammation of joints, tendons, ligaments, muscles; progressive start and continuous evolution; pains in points, that ameliorate with pressure, exacerbated at movement; inflammatory arthritis, arthrosis; headaches exacerbated by head movements, with vertigo; back pains with painful points in the thorax; back pains ameliorated on a hard surface with contention; swelling of joints; trigeminal neuralgia exacerbated by speaking, eating, high pressure
Hormonal problems	Mastitis with heavy breasts, pains that ameliorate with pressure; ovarian isolated liquid cysts; ovarian pains, especially in the right side, that ameliorate in an inclined position or by immobility;
Other syndromes	Glaucoma or eye pain in point; dry skin but face and hairy skin very seborrheic; headaches and vertigos exacerbated by movements

Other sources may also cite, besides the homeopathic uses, the use for veterinary purposes, that are almost the same as the ones described for humans: e.g. as drastic purgatives and diuretics, with doses that depend on the size of the animals (5,91).

Beside its use in homeopathy (35), recent studies have shown its potential as a medicinal plant, by the analysis of active principles and their involvement in different pathologies. In this regard, trihydroxyoctadecadienoic acids (class of trihydroxy fatty acids) found in the composition of the roots were proved to exhibit hypoglycemic activity (92) or even to improve major abnormalities that are typical in severe diabetes mellitus (49). Same compounds were proved to exhibit preventive atherogenic or antiatherosclerotic activities (68).

Besides, the extract of the roots of *B. alba* has proved other protective activities for different biological systems. For instance, it has proved hepatoprotective activity (30) or even the protective activity for human cells against endogenous oxidative DNA damage, although the ability of protection towards the exogenous oxidative DNA damage could not be demonstrated (93).

Roots of *B. alba* are also known for an adaptogen activity, which is associated with the content of cucurbitacins (especially cucurbitacin R diglucoside). Studies prove that roots of the species may enhance the sensitivity level to stress, due to the effects of eicosanoids and corticosteroids (94). Other studies assign to the roots adaptogen activity, due to the fact that it has proved to increase level of nitric oxide (NO) in humans (95), molecule which is responsible for the adaptation of the human organism to heavy physical exercise.

The same NO that is is responsible for numerous inflammatory processes is inhibited by *B. alba* roots in its formation process. The inhibition is due to the activity of its inducible enzyme, iNOS (inducible NO synthetase), which may be a possible explanation for the mechanism of action for the anti-inflammatory effect (96). All these effects are due to the cucurbitacins and their derivatives, compounds that are found in the roots of the species.

Clearly, the most important activity that is described for this species is the cytotoxic one and it is clearly due to the presence of cucurbitacins that are present, especially in the composition of roots (29). Besides the cytotoxic activity which is assigned to these roots by cucurbitacins, it has been proved that they may also exhibit significant anti-tumor activities. The most active cucurbitacins *in vitro* and *in vivo* proved to be the aglycones B, D, E and I. They proved not only to be active on cancerous cell lines, but also they inhibited the growth of tumors *in vivo*. This is the main criterion that is assessed in the study of the antitumor activity, but it may proved not to be enough in the larger context of treatment of cancerous diseases (28).

3.2. *Echinocystis lobata* (Michx.) Torr. et A. Gray

E. lobata is a species that is lesser studied in the scientific literature. It is a species that is known for its use in etnopharmacy as poultice for headaches or for menstrual disorders, rheumatisms, chills, fevers, kidney disorders or for stomach troubles (14,62). The compounds that are cited in the composition of the species may prove important pharmacological activity (97). To the best of our knowledge, no studies on a potential biological activity of the species is found.

3.3. *Ecballium elaterium* (L.) A. Rich.

Several biological activities are assigned to the species *E. elaterium*. Among these activities, the anti-microbial (98–101), anti-inflammatory (102,103), antioxidant (104) or cytotoxic (75,76,85,104–108) are the most well-known. Undiluted fruit juice is the part that is the most often studied. It has proved mostly cytotoxic and genotoxic effect, on *Allium cepa* root tip cells (77). Compounds that proved to be responsible for these activities are cucurbitacins and specifically one of the cucurbitacins isolated from *E. elaterium*, that has proved significant cytotoxic activity: cucurbitacin E (85,106–108). The same cucurbitacin is also studied for other biological activities as the immuno-modulatory effect (105,109), which can synergistically act in order to enhance the anti-cancer properties of this compound. At the same time, it is a compound that has minor cytotoxic effects on normal cell lines (106). Cucurbitacin E is not the only compound that is found in different part of the species. Other cucurbitacins, as cucurbitacin B, D or I can also be found (86,110).

E. elaterium is described in traditional medicine for the treatment of jaundice in newborns, as nasal drops obtained from the fruit juice (111–113). Despite this fact, there are sources that consider it a highly toxic plant. Toxicity seems to be due to its juice, which acts on different levels: respiratory, cardiac and gastric systems, but also on the mucous membranes (23,73,77,114–121). Some sources also cite the administration of the fruit juice to rats with surgically induced jaundice. This produced a decrease in serum bilirubin concentration, due to the content of cucurbitacin B, D and E and consequently may increase the binding of domain specific ligands, as for exemple ibuprofen and bilirubin (122,123).

Other traditional uses of the species are for the treatment of otitis, sinusitis, malarial fever, headaches or sinusitis and rheumatism (73,77,124,125). The great majority of the scientific sources cite the use of the juice of *E. elaterium* as a drastic purgative. This juice obtained from the fruits before ripening was largely used in England, but it was withdrawn as it caused gastroenteritis and congestion of all organs if administered on a hypodermic route, with general leukocytosis (5,22). English Pharmacopoeia contained a monograph of the *Ecballium* juice until 1914 (5).

In homeopathy, it is sometimes used for the treatment of various pathologies or disorders (Table X). Pathogenesis of the species is similar to the one of *B. alba*, although it is lesser used in homeopathy than it.

Table. X. Pathogenesis of *E. elaterium* (90)

Symptom/ Systems concerned	Description
Fever	Bouts of fever with chills, intermittently, without clear frequency
Physical apparence	Need to stretch, need of calm; the most frequent symptoms are located in the right side of the body
Diarrhea	Acute or subacute diarrhea, with with watery stools, abundent and explosive; abdominal pains, intermittent and sharp
Neuralgia	Neuralgic pains that are exacerbated by humidity and cold, situated especially in the right side of the body
Other symptoms	Decrease of urine volume

The species may also exhibit protective activities. Agil, Dubeau and their coworkers suggested in their studies the hepatoprotective and neuroprotective

activity (126–128). The neuroprotective activity is cited to be related to cucurbitacin E, isolated from the species (127). Another protective activity is exhibited on sepsis-induced lung injury in rats (129). Protective activity of the species can also occur due to its ability to reduce abdominal adhesion. This could be an useful tool for the anti-inflammatory activity in the prevention of postoperative peritoneal adhesion (130).

Probably the most well-known and frequently studied activity is the anti-trypsin one. This activity is actually due to the presence of a specific polypeptide, actually a protease inhibitor, which specifically acts against trypsin. It is one of the most potent trypsin inhibitor that is known (73). This molecule may be an important target for the isolation of small peptide molecules that bind with affinity to acceptor molecules (131). It is called EETI-II (*Ecballium elaterium* trypsin inhibitor) and it has a rigid molecular scaffold (Fig.15.) (132). It has the ability to increase its binding with high affinity to integrin receptor associated in tumors by a simple mutation in its loops. A radiolabelled version of this knottin peptide can help to the development of non-invasive imaging of integrin expression in humans (133–136). It is a target for protein engineering, which may find important uses both in the therapy of cancer (137), but also in the synthesis of modified derivatives that may prove potent inhibitory activity against pancreatic elastase, chymotrypsin and human leucocyte elastase (138).

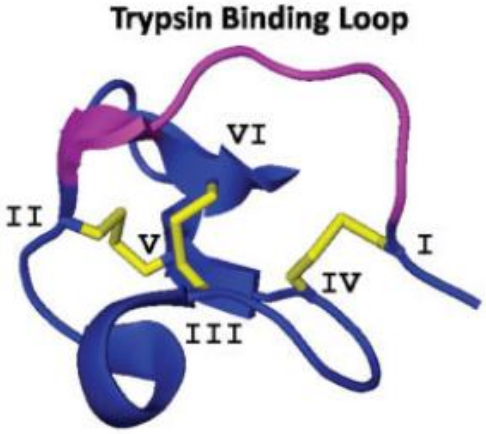


Fig.15. Structure of the EETI-II (137)

Besides the pharmaceutical uses of this species, there are other uses, that concern the defense mechanisms of the plants, which is also due to the presence of cucurbitacins, especially cucurbitacin D and cucurbitacin I. Scientific literature cites the activity against *Botrytis cinerea* Pers., by the inhibition of laccase formation (139,140). Villalobos and coworkers also proved that the extract from the leaves of *E. elaterium* can exhibit potent anti-insect activity (141). This is an important argument for the use of the plant extracts as herbicides, to control the germination and growth of some other plant species (79). Essential oils obtained from the species could also play a role in the allelopathic effect (142).

PERSONAL CONTRIBUTIONS

1. Work hypothesis

The aim of the thesis is to increase knowledge on three species belonging to Cucurbitaceae family, more specifically *Bryonia alba* L., *Echinocystis lobata (Michx*.) Torr. et A. Gray and *Ecballium elaterium* (L.) A. Rich. It is divided in three major sections, that are meant to offer a complete description of these species from several points of view. The starting point of the study was the premise that plants belonging to Cucurbitaceae family that are not used for human nutrition and are lesser known for their use in medicine may be important sources of potentially active compounds for the treatment of different disorders.

Botanical, phytochemical and pharmacological studies of these species were performed. Botanical studies are meant to offer a correct identification of the species, which is the departure point for all other studies. The most important compounds found in the composition of these plants are cucurbitacins and flavonoids. Taken into consideration the fact that in the last years cucurbitacins have known an important increase in the attention of researchers worldwide, the studies in the present thesis are directed towards the characterization of other compounds, lesser studied in the presence of these plants, flavonoids. At the same time, biological activities that are assessed in the studies of the present thesis are described for the first time in scientific literature and may bring an important way for the orientation of future studies, that may bring important evidence on the potential of these plants as medicinal species. All of these can bring an overview on these species and may offer enough argument in order to classify these species as potentially medicinal plants.

2. General methodology

2.1. Plant material

Each species, after harvesting, was separated in its main components, represented by vegetal and reproductive organs, in order to study the possible differences between them, as scientific literature provides incomplete data on the part that is the richest in active compounds. All species were harvested from the Romanian spontaneous flora and identified at the Department of Pharmaceutical Botany of "Iuliu Haţieganu" University of Medicine and Pharmacy Cluj-Napoca, where voucher specimens are deposited (Table XI).

Table XI. Voucher numbers for the three Cucurbitaceae species

Species	Voucher number
Bryonia alba L.	105.3.1.1-23
Echinocystis lobata (Michx.) Torr. et A.Gray	105.8.1.1-18
Ecballium elaterium (L.) A.Rich.	105.2.1.1-3

2.2. General methodology

Identification of the botanic characters was performed especially by microscopic examination, with the exception of the macroscopic characters, that were

carefully analyzed with a magnifying glass or a stereomicroscope. Magnifying glass was used for the general view of the features of each plant, while stereomicroscope allowed a better observation of the trichomes on the surface of the organs of each plant. Cross sections of the vegetative and reproductive organs were analyzed by an optical microscope and photos were taken using a digital camera. Concerning the sectioning techniques, depending on the type of plant material, the classical and histological techniques were used. The direct classical sectioning through the vegetal material with a manual microtome was used for organs that had an adequate size for sectioning (e.g. stems, petioles, roots), while the histological technique of mounting into paraffin was used for organs that can not be sectioned in the classical way, because of their small sizes (e.g. leaves, ovaries, peduncles, tendrils). Sections were stained using the double technique of coloring, for cellulose and lignin. Sections obtained by classical technique were colored using the mixture of alum carmine (stains cellulose in purple-red) and iodine green (stains lignin in green), while sections obtained by histological technique were stained using toluidin blue (stains cellulose in purple-blue and lignin in green).

Phytochemical analysis of flavonoids was performed by chromatographic and spectroscopic methods. Methanolic extracts of the parts that were proved to be the richest in flavonoids were obtained by maceration and subjected to a HPLC-DAD analysis for the identification of compounds. HPLC-MS screening of polyphenols was also performed. Isolation of compounds was achieved by means of preparative HPLC-DAD methods and structural identification of the flavonoidic compounds was performed by MS, ^{1}H-NMR and ^{13}C-NMR techniques. For identification of flavonoidic and polyphenolic totals, spectrophotometric methods were used. HPLC-DAD methods of quantification of compounds was also performed.

Biological activities that were tested are the anti-plasmodial, cytotoxic and anti-oxidant ones. *In vitro* tests of the anti-plasmodial and cytotoxic activities showed values of the IC_{50} for the tested samples higher than 50 μg/mL. Samples were tested on two *Plasmodium falciparum* strains (W2 - cloroquine resistant and 3D7 - cloroquine sensitive) and on two types of cell lines: cancerous (A549 - lung cancer and HeLa - cervical cancer) and healthy ones (WI38 lung fibroblasts). The anti-oxidant activity of extracts was tested by chemical assays, as DPPH, CUPRAC, FRAP, TEAC, EPR and SNPAC assays. The global anti-oxidant activity was assessed to prove the anti-catalytic and anti-enzymatic activity of the samples. Anti-catalytic activity was assessed on a peroxidase extracted from horseradish. The global antioxidant activity was assessed on neutrophils isolated from equine blood *ex vivo*. Anti-enzymatic assays targeted the NADPH enzyme and were performed on a model of human monocytes that are transformed in macrophages (HL-60). All of these tests were aimed to prove the possible involvement of the samples in the redox processes that are also involved in inflammation. These assays showed a selective activity of the samples for the anti-oxidant processes, that may also have implications in the anti-inflammatory activity. It is also sugested, by comparison of the results for the tests of total extracts with the tests of isolated flavonoids and of corresponding references, that especially the anti-oxidant and anti-inflammatory activity is due to the flavonoidic composition of the species. Taken into consideration the fact that anti-oxidant activity is largely known to be due to the polyphenolic composition, it becomes clear that the flavonoidic and polyphenolic compounds assign the species new biological activities, not previously tested by authors for these species.

3. Study 1. Comparative botanical researches of three species belonging to Cucurbitaceae family

3.1. Introduction

Botanical studies are aimed to describe the morphology and anatomy of a species, in order to facilitate its proper taxonomic classification. Moreover, such studies are aimed to provide necessary informations to avoid the possible substitutions or even adulterations of the vegetal products with species having the same features, but different biological activities or even with species with known or unknown toxicity. They can also help for the localization of the most important active principles at a cellular level, in different organs of the species, which can be helpful in the case of new possible vegetal medicinal species, lacking information about the localization of the most important bioactive compounds (143).

Analysis of botanical features must be directed on two different levels. Firstly, macroscopic studies must be performed in order to properly determine the identity of the species, which can subsequently help for the correct taxonomic classification of the species. Afterwards, it is important to provide a microscopic study of the species in order to confirm its identity by comparison with similar species from the same taxonomic classes. As a first step, microscopic studies must be performed on the grinded powder, to establish the features of the powder, as a possible vegetal material. This microscopic study is a method described by the 9th Eur Ph for the examination of the powder of herbal drugs. Data provided by the microscopic study must be completed by a proper histo-anatomical study of the most important parts of the species, namely vegetative and reproductive organs. Thus, the complete description of the species and a correct identification can be provided for each species. All of these represent the first step towards a better understanding of the possible bioactivities of vegetal species, as they can provide the necessary information to further develop pathways for more detailed studies.

Cucurbitaceae plants (also known as cucurbits) are widely known for specific morpho-anatomical features that concern especially the leaves, the tendrils and the sexual organs. It appears therefore important to highlight the specific characteristics of these parts of the species to be able to provide information about their taxonomic classification.

3.2. Work hypothesis

The aim of the present study was to perform the complete botanic description of the three species belonging to Cucurbitaceae family. In this regard, the macroscopic and microscopic features of the main parts of each species were analyzed. Vegetative and reproductive organs of *Bryonia alba* L., *Echinocystis lobata (Michx*.) Torr. et A. Gray and *Ecballium elaterium* (L.) A. Rich. were studied by specific methods. A special attention was given to one of the most specific features of the species belonging to the Cucurbitaceae family, trichomes, that can be found on the inferior part of the leaves.

3.3. Materials and methods

Samples of plant material on which the studies were performed are found in Table XII.

Table XII. Samples analyzed for microscopic and macroscopic studies

Species	Harvesting time and place
Bryonia alba L.	Cluj-Napoca, 10.07.2014
Echinocystis lobata (Michx.) Torr. et A.Gray	Aiud, 19.08.2014 Deva, 23.08.2014
Ecballium elaterium (L.) A.Rich.	Constanța, 05.09.2014

Macroscopic study was performed by an attentive examination of each species, using special tools (magnifying glass, stereomicroscope). Photos of the main vegetative and reproductive organs were taken. Studies were performed especially on fresh vegetal material, in order to properly identify the specific features. A Motic K-500L stereomicroscope, connected to a MoticCam Pro 205A digital camera was used for stereomicroscopic analysis, in order to study especially the trichomes on the surface of the leaves of these plants (144).

Microscopic study was performed by optical and electronic microscopy. Optical microscopy was used both for the study of the anatomy of vegetal species but also for the study of the powder belonging to them. On the other side, the electronic microscopy was used for the specific scanning of the trichomes on the inferior surface of the leaf of *B. alba*.

Study of the powder was performed by methods described by the 9th Eur Ph for the microscopic examination of herbal drugs, after an appropriate grinding of the plant material dried in avance. Samples were grinded using a Grindomix knife mill to obtain the powder, which was studied with the optical microscope, using chloral hydrate 80% (m/V), glycerol, potassium iodide and lactic acid as mounting media (20).

Study of the anatomical features was performed by the classical tehnique of sectioning and by the histological technique of mounting into paraffin on preserved vegetal material. For the classical technique, vegetal material was cross-sectioned using a Nahita 501 manual microtome. Sections were stained using alum carmine (stains cellulose in purple-red) and malachite or iodine green (stains lignin in green) and studied using an Olympus CX31 optical microscope equipped with a digital camera. To better observe the trichomes on the inferior face of the leaves, superficial sections were performed. For the histological tehnique, vegetal material was fixed, dehydrated, passed into xylene and poured in paraffin cubes, which were sectioned using a Microtec CUT 4050 microtome. Sections were stained with toluidin blue (stains cellulose in purple-blue and lignin in green) and studied with the optical microscope. All vegetal material containing compounds interfering the microscopic analysis (e.g. root sections containing a large amount of starch grains, leaves sections containing chlorophyll) were previously clarified by heating with a chloralhydrate solution 5% (m/V), which allowed the precipitation of compounds that prevent the proper observation of microscopic features (25).

Scanning electronic microscopy analysis were performed on *B. alba* fresh vegetal material, namely on the inferior epidermis of the leaves, in order to investigate the type of trichomes present on this part of the leaf. The samples were studied under

vacuum, using a JEOL JSM 5510 LV Scanning Electronic Microscope, after a vacuum metalizing technique.

3.4. Results

3.4.1. Macroscopic study of three species belonging to Cucurbitaceae family

Morphological study of the vegetative and reproductive organs of the three species belonging to Cucurbitaceae family confirmed the macroscopic features of each of them, as described by the existing scientific literature (6–9). Thus, the identity of each species could be confirmed (Fig.16-18).

Fig 16. Macroscopic characters of *B. alba*

Fig.17. Macroscopic characters of *E. lobata*

Fig.18. Macroscopic characters of *E. elaterium*

3.4.2. Comparative microscopic study of three species belonging to Cucurbitaceae family

3.4.2.1. Comparative microscopic study of the powder of three species belonging to Cucurbitaceae family

The powder of different organs of each species revealed different parts of the anatomical structure of the part they belong to (Fig.19-24). Reagents used for the microscopic examination were the lactic acid reagent and the chloral hydrate solution.

***a) Bryonia alba* L.**

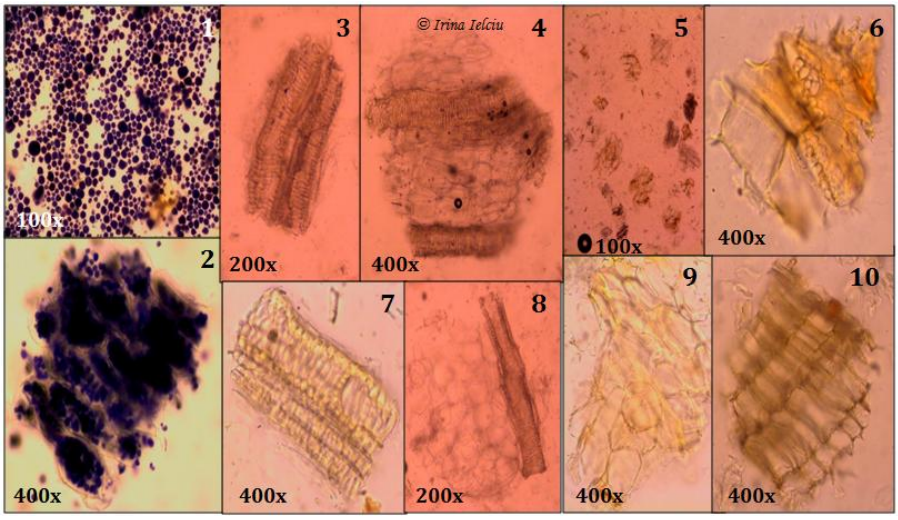


Fig.19. Microscopic characters of the powder of the roots of *B. alba*

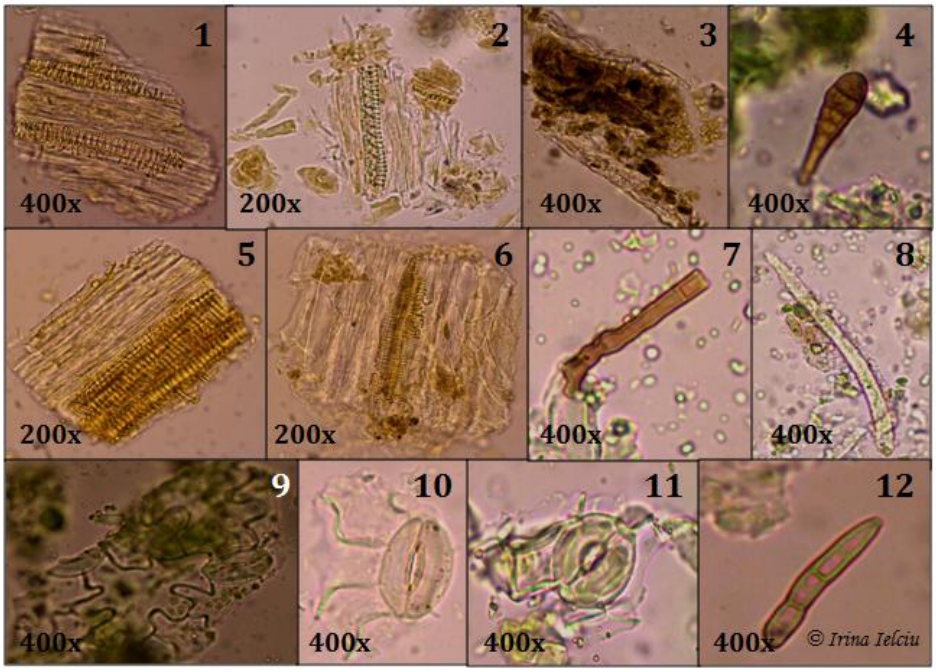


Fig.20. Microscopic characters of the powder of the leaves of *B. alba*

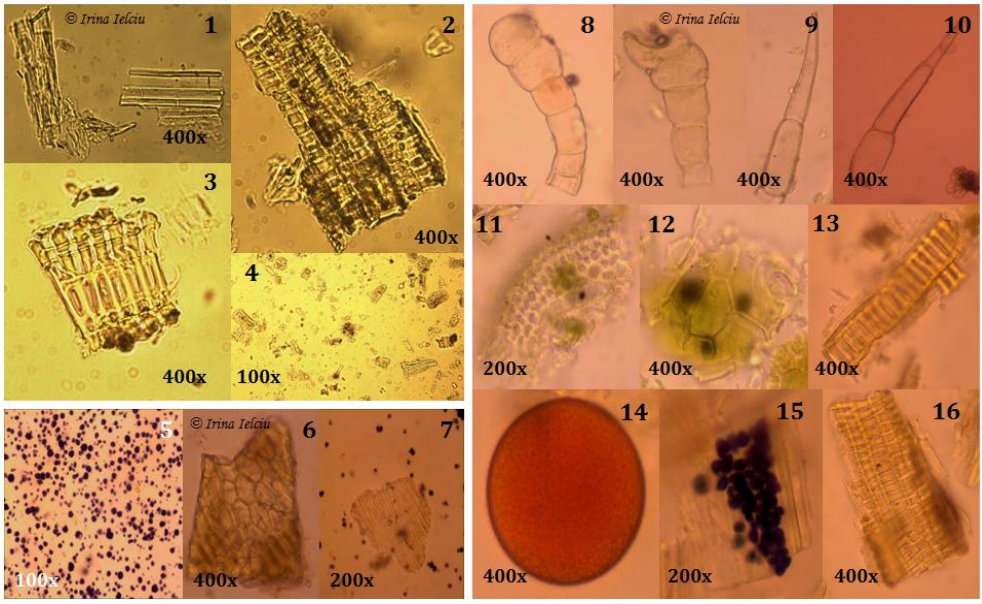


Fig.21. Microscopic characters of the powder of the stems (1-4), fruits (5-7) and of the aerial parts (8-16) of *B. alba*

b) *Echinocystis lobata* (Michx.) Torr. et A. Gray

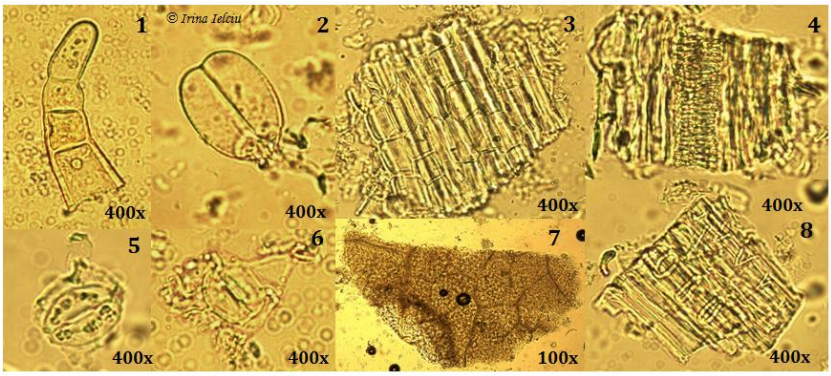


Fig.22. Microscopic characters of the powder of the leaves of *E.lobata*

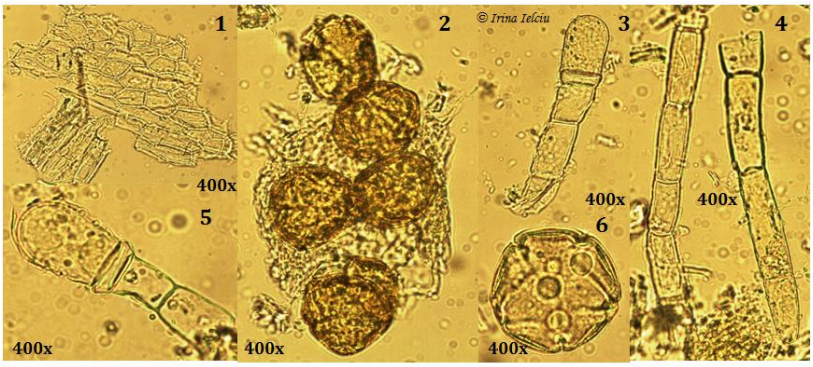


Fig.23. Microscopic characters of the powder of the flowers of *E.lobata*

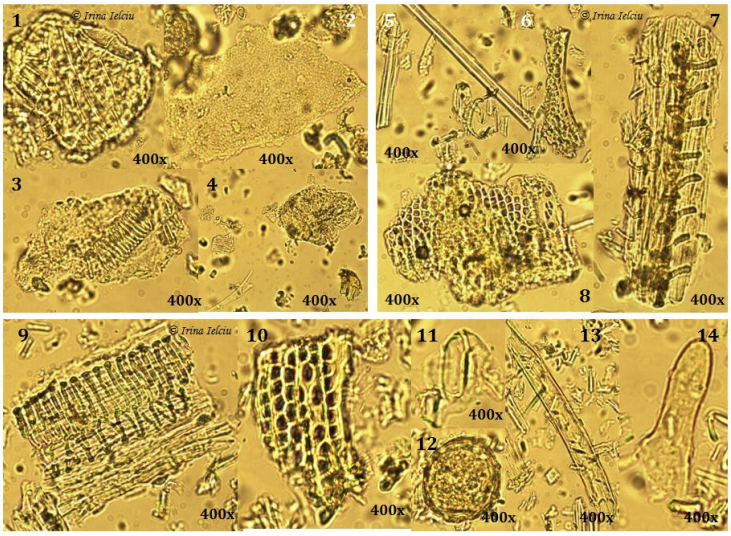


Fig.24. Microscopic characters of the powder of the fruits (1-4), stems (5-8) and of the aerial parts (9-14) of *E. lobata*

3.4.2.2. Comparative histo-anatomical study of three species belonging to Cucurbitaceae family

a) Bryonia alba L.

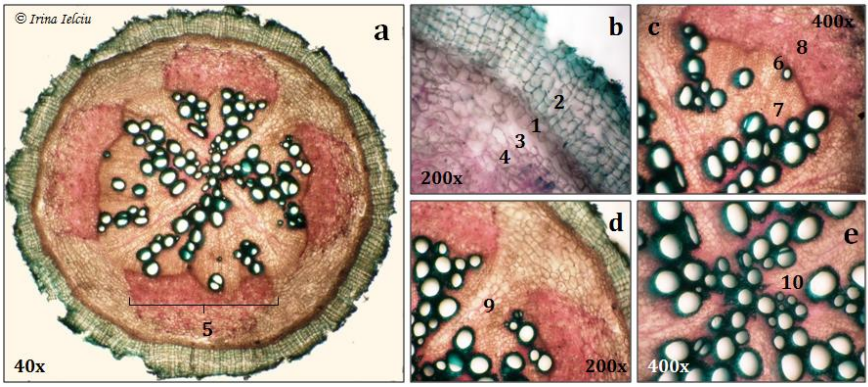


Fig.25. Microscopic observation of the roots of *B. alba* (staining reagent: alum carmine and malachite green)

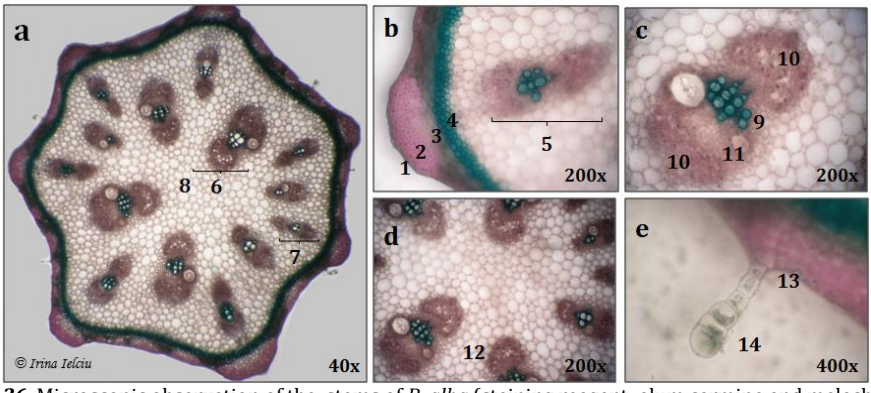


Fig.26. Microscopic observation of the stems of *B. alba* (staining reagent: alum carmine and malachite green)

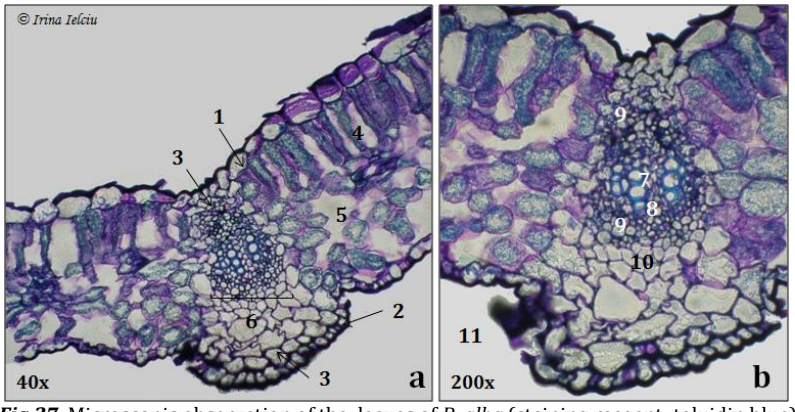


Fig.27. Microscopic observation of the leaves of *B. alba* (staining reagent: toluidin blue)

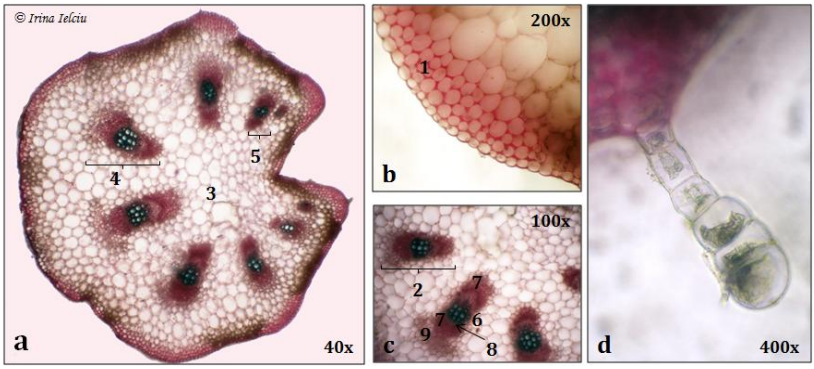


Fig.28. Microscopic observation of the petioles of *B. alba* (staining reagent: alum carmine and malachite green)

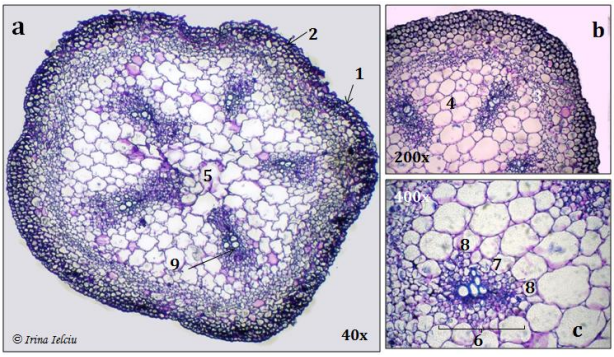


Fig.29. Microscopic observation of the tendrils of *B. alba* (staining reagent: toluidin blue)

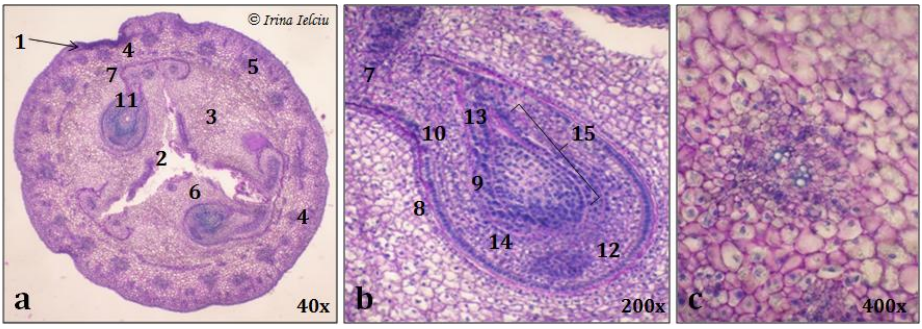


Fig.30. Microscopic observation of the ovary (a) and ovule (b) of *B. alba* (staining reagent: toluidin blue)

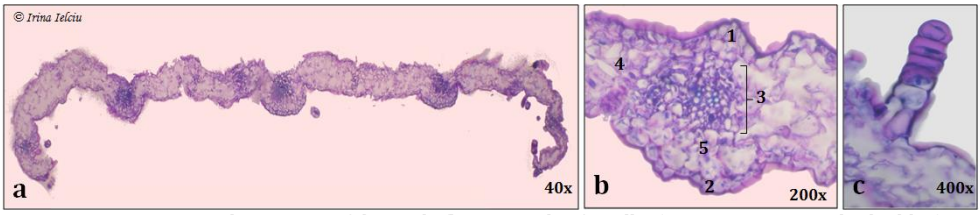


Fig.31. Microscopic observation of the male flower petals of *B. alba* (staining reagent: toluidin blue)

b) *Echinocystis lobata* (Michx.) Torr. et A. Gray

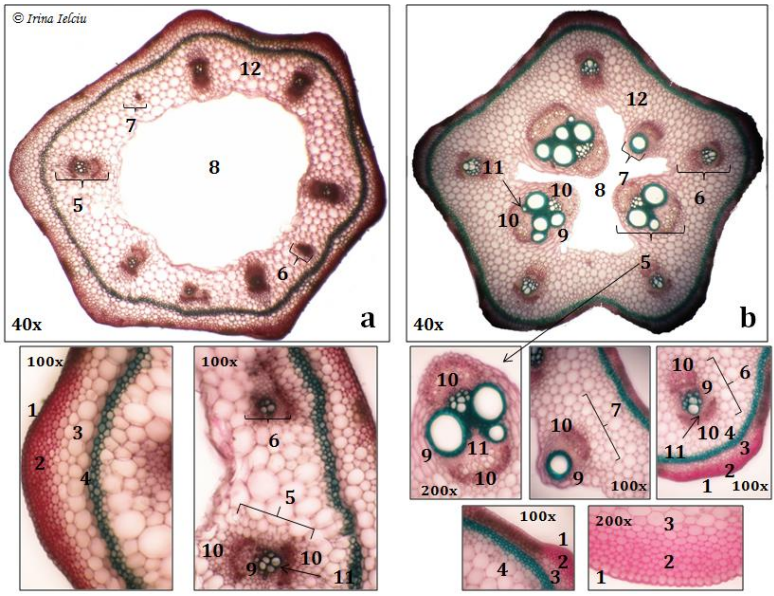


Fig.32. Microscopic observation of the stems of *E. lobata* (staining reagent: alum carmine and iodine green)

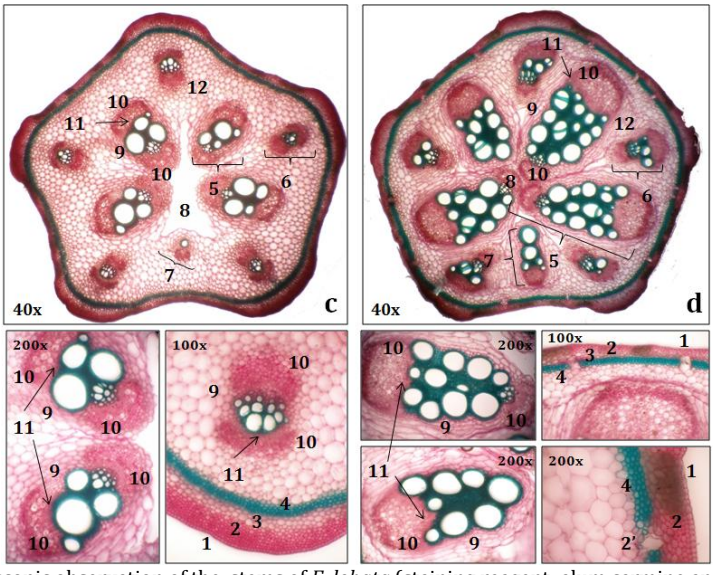


Fig.33. Microscopic observation of the stems of *E. lobata* (staining reagent: alum carmine and iodine green)

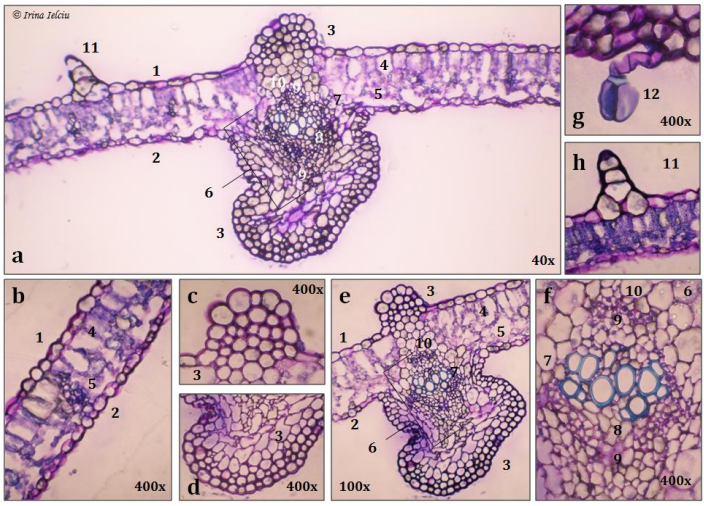


Fig.34. Microscopic observation of the leaves of *E. lobata* (staining reagent: toluidin blue)

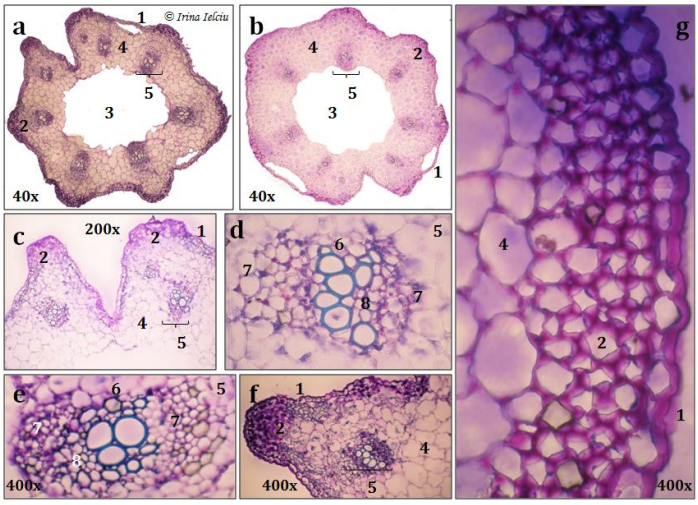


Fig.35. Microscopic observation of the petioles of *E. lobata* (staining reagent: toluidin blue)

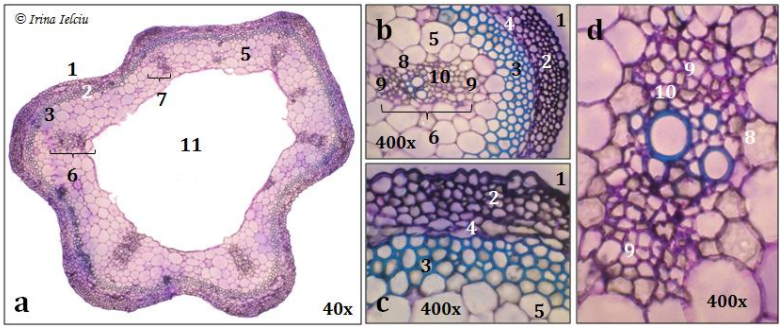


Fig.36. Microscopic observation of the tendrils of *E. lobata* (staining reagent: toluidin blue)

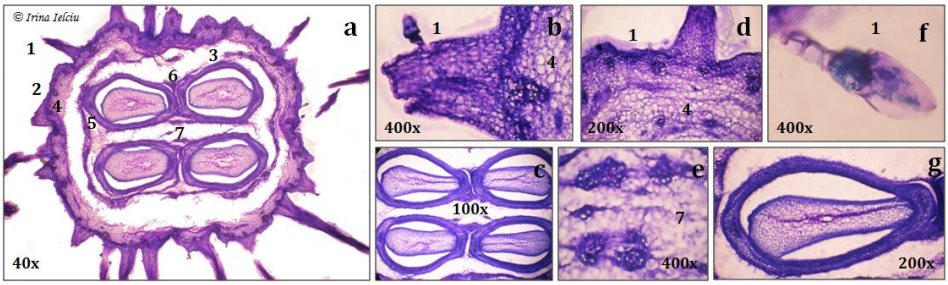


Fig.37. Microscopic observation of the ovary of *E. lobata* (staining reagent: toluidin blue)

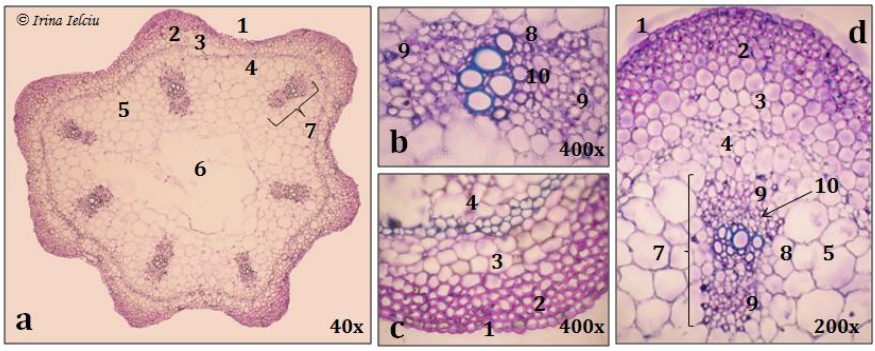


Fig.38. Microscopic observation of the flower peduncle of *E. lobata* (staining reagent: toluidin blue)

c) *Ecballium elaterium* (L.) A. Rich.

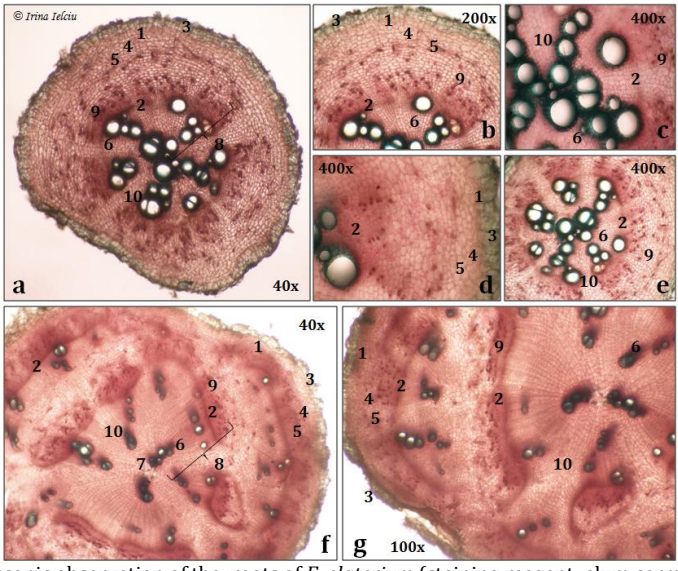


Fig.39. Microscopic observation of the roots of *E. elaterium* (staining reagent: alum carmine and iodine green)

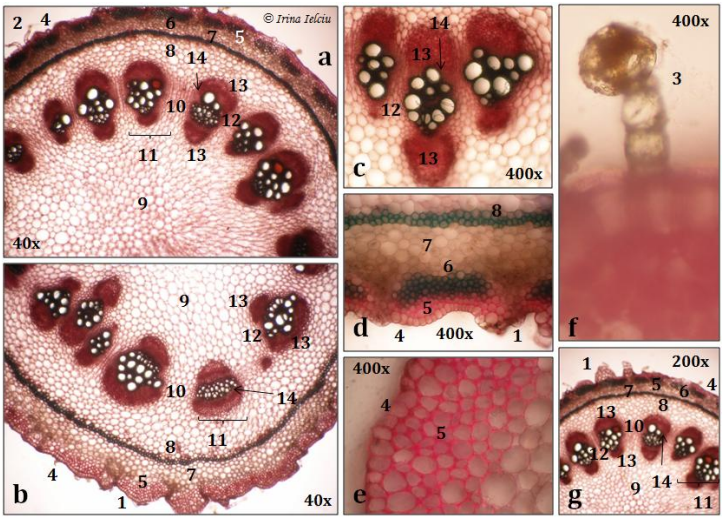


Fig.40. Microscopic observation of the stems of *E. elaterium* (staining reagent: alum carmine and iodine green)

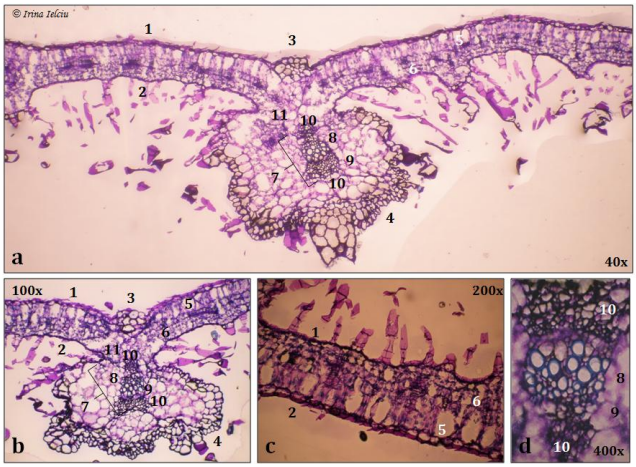


Fig.41. Microscopic observation of the leaves of *E. elaterium* (staining reagent: toluidin blue)

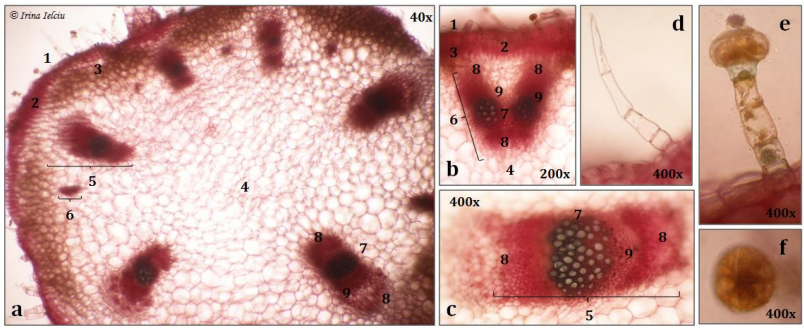


Fig.42. Microscopic observation of the petioles of *E. elaterium* (staining reagent: alum carmine and iodine green)

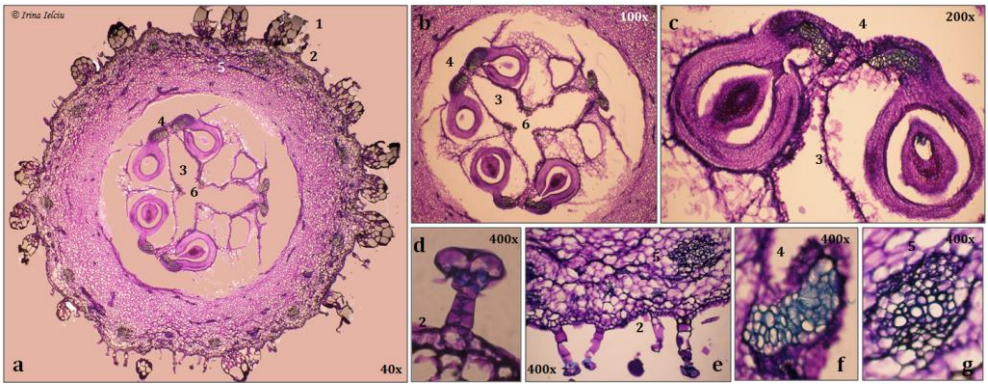


Fig.43. Microscopic observation of the ovary of *E. elaterium* (staining reagent: toluidin blue)

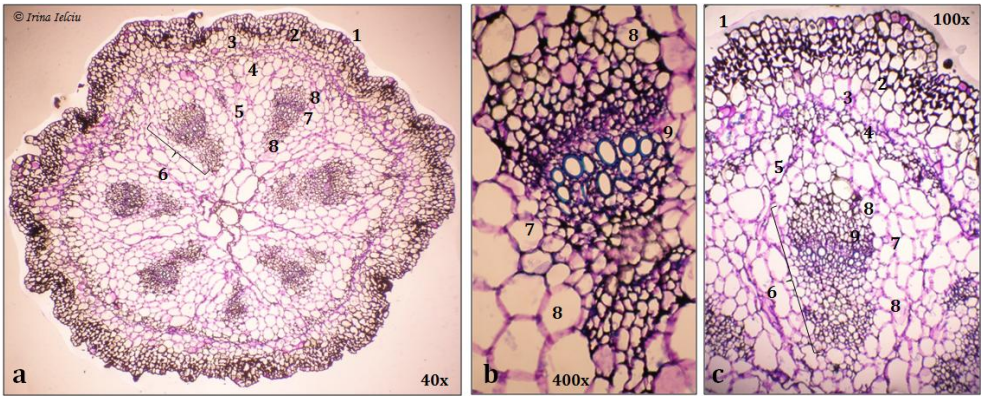


Fig.44. Microscopic observation of the fruit peduncle of *E. elaterium* (staining reagent: toluidin blue)

3.4.3. Comparative study of the trichomes on the surface of the leaves of three species belonging to Cucurbitaceae family

The most important taxonomic characters for the plants belonging to Cucurbitaceae family are related to the sexual system (androecium and gynoecium morphology), but also to the type of tendril branching or from fruit morphology (17). It is not only for these specific characters that cucurbits are known, but also for the trichome morphology, that proved to have significant consequences for the taxonomic classification of these plants (19). Trichomes are found on the surface of the aerial vegetative and reproductive organs of the three Cucurbitaceae species studied in this thesis (Fig. 45-56).

a) *Bryonia alba* L.

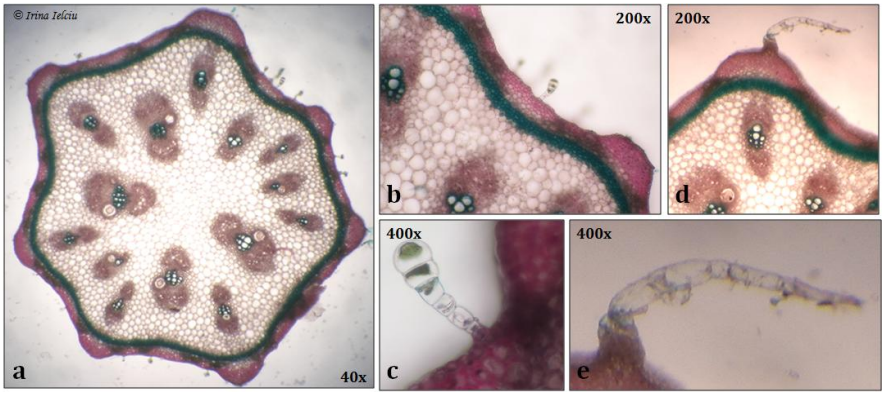


Fig.45. Microscopic observation of the trichomes on the stems of *B. alba* (staining reagent: alum carmine and iodine green)

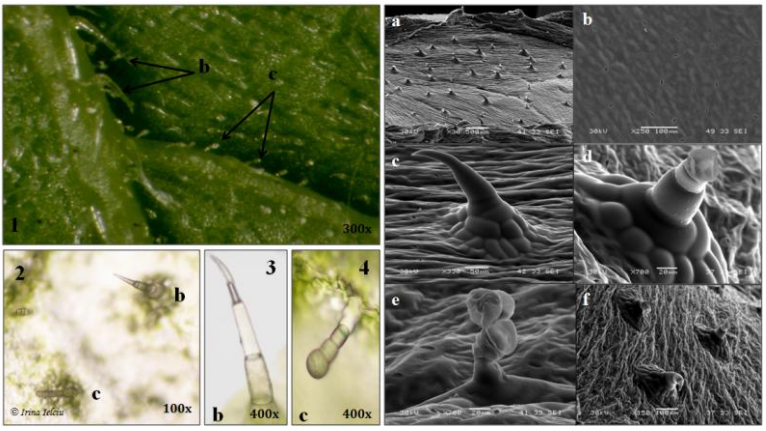


Fig.46. Microscopic observation of the trichomes on the leaves of *B. alba* (Left up: 1 - stereomicroscopy; 2 – microscopic observation; Right: SEM)

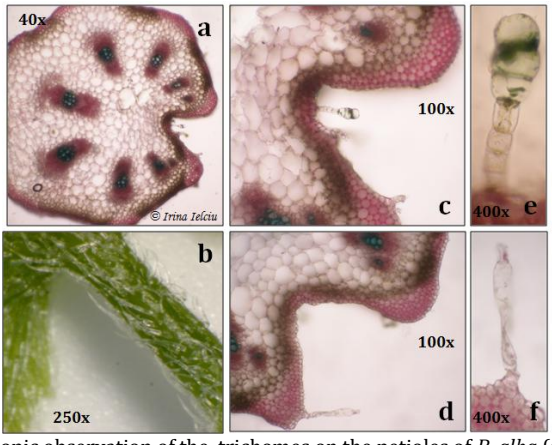


Fig.47. Microscopic observation of the trichomes on the petioles of *B. alba* (a,c,d,e,f – optical microscopy: staining reagent = alum carmine and malachite green; b –stereomicroscopy)

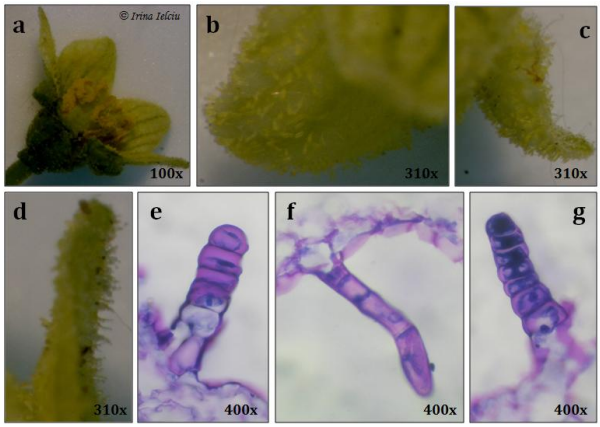


Fig.48. Microscopic observation of the trichomes on the male flower petals of *B. alba* – a-d: stereomicroscopy; e-g: 400x microscopy

b) *Echinocystis lobata* (Michx.) Torr. et A. Gray

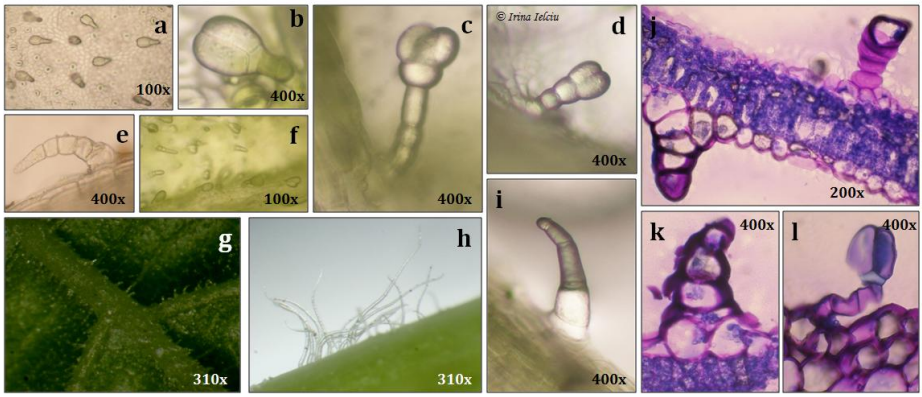


Fig.49. Microscopic observation of the trichomes on the leaves of *E. lobata* – a-i: microscopy; j-l: stereomicroscopy

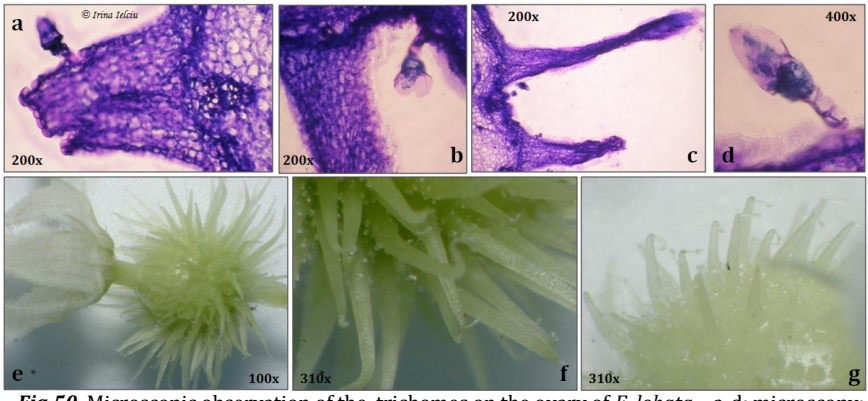


Fig.50. Microscopic observation of the trichomes on the ovary of *E. lobata* – a-d: microscopy; e-g: stereomicroscopy

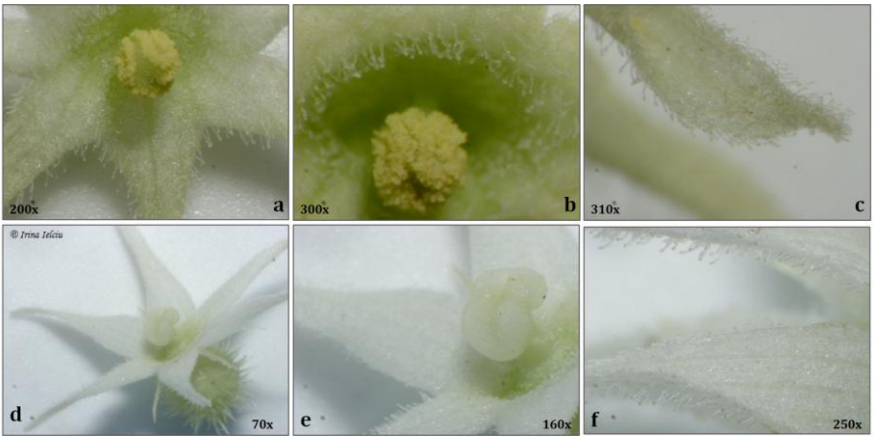


Fig.51. Stereomicroscopic observation of the trichomes on the male (up) and female (down) flower petals of *E. lobata*

c) *Ecballium elaterium* (L.) A. Rich.

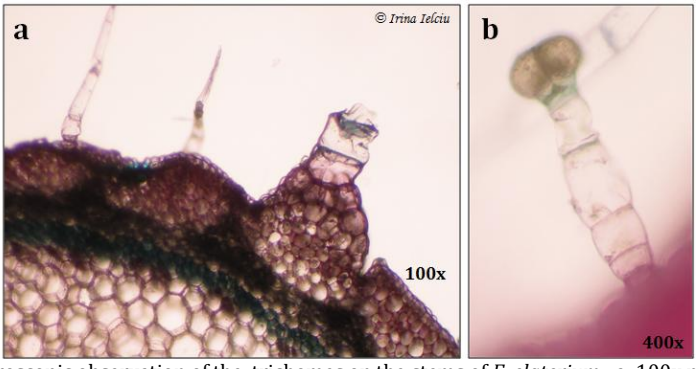


Fig.52. Microscopic observation of the trichomes on the stems of *E. elaterium*– a: 100x microscopic structural detail; b: 400x microscopic structural details

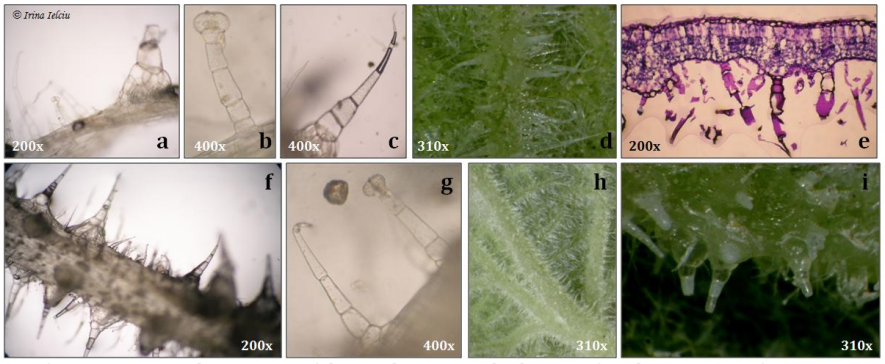


Fig.53. Microscopic observation of the trichomes on the leaves of *E. elaterium*– a,e,f: 200x microscopic structural detail; b,c,g: 400x microscopic structural details; d,h,i: 310x stereomicroscopic details

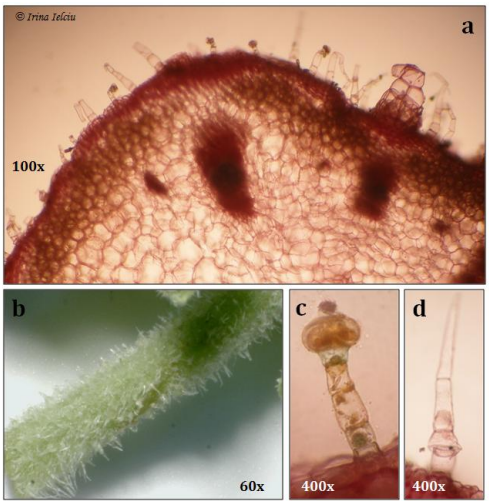


Fig.54. Microscopic observation of the trichomes on the petioles of *E. elaterium*– a,c,d: microscopic; b: stereomicroscopy

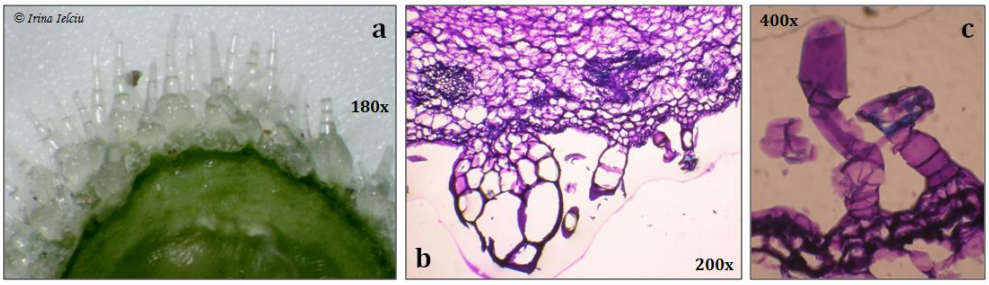


Fig.55. Microscopic observation of the trichomes on the ovary of *E. elaterium*– a: stereomicroscopy; b,c: microscopy

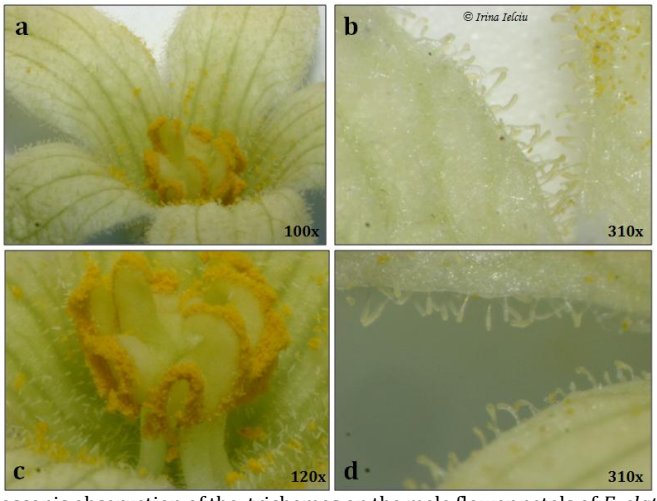


Fig.56. Microscopic observation of the trichomes on the male flower petals of *E. elaterium*– a-d: stereomicroscopy

3.5. Discussions

3.5.1. Microscopic study of the powder of three species belonging to Cucurbitaceae family

***a) Bryonia alba* L.**

Powder of the *roots* show the presence of large amounts of starch, stained in purple with the lactic reagent (Fig.19.1-2). Other elements such as xylem vessels (Fig.19.3-4,7-8), cortex, parenchyma and cork cells (Fig.19.5-6, 9-10) could also be found in the powder of roots. In the powder of *leaves*, the most frequently found elements were tector and secretory trichomes (Fig.20.4, 7-8, 12). Fragments of xylem vessels (Fig.20.1-2, 5-6), mesophyll (Fig.20.3) and stomata (Fig.20.9-11) were also noticed. Powder of the *fruits* shows large amounts of starch (Fig.21.5), but also fragments of pericarp (Fig.21.6-7). Powder of *stems* shows fragments of the main parts of their structure (Fig.21.4): e.g. parts of the mechanic tissues that inhance the mechanic protection of the structure towards external factors - collenchyma (Fig.21.2), but also of the fundamental tissues - parenchyma (Fig.21.1). Fragments of xyllem vessels can also be found (Fig.21.3). The powder of *aerial parts* contains fragments of each of the constituent parts: non-glandular and glandular trichomes belonging to the leaves (Fig.21.8-10), collenchyma from stems (Fig.21.11), pericarp fragments from fruits, with starch grains (Fig.21.12,15), xyllem vessels from different other organs (Fig.21.13,16) and pollen grains from flowers (Fig.21.14).

b) *Echinocystis lobata* (Michx.) Torr. et A. Gray

In the powder of the *leaves* some fragments of the different types of trichomes can frequently be found: tector (Fig.22.1) and secretory (Fig.22.2) trichomes. Other elements that are found are stomata (Fig.22.5,6) and fragments of different tissues that are found in the structure of the leaf: epidermis and parenchyma (Fig.22.3,7) and xylem vessels (Fig.22.4,8). Similarly, powder of the *male inflorescences* contains fragments of glandular trichomes (Fig.23.3-5), but also pollen grains (Fig.23.2,6) and some fragments of parenchyma (Fig.23.1). In the powder of the *fruits*, fragments of pericarp are most frequently found (Fig.24.1,2,4), but also some xylem vessels, included in fundamental parenchyma (Fig.24.3). Powder of the *stems* also shows the presence of xylem vessels (Fig.24.7), but also of mechanical (Fig.24.6,8) and fundamental (Fig.24.5) tissues. The powder of the *aerial parts* assembles the elements of all of the above organs: tector and secretory trichomes (Fig.24.13,14) and stomata (Fig.24.11) from the leaves, pollen grains from flowers (Fig.24.12), mechanical tissues (Fig.24.10) from stems and xylem vessels (Fig.24.9) from different other parts.

3.5.2. Microscopic histo-anatomical study of three species belonging to Cucurbitaceae family

Analysis of the main vegetative (root, stem, leaves) and reproductive (ovary, male flower petal, fruit, fruit peduncles) organs was performed by using the classical technique of sectioning and the histological technique of mounting into paraffin. For the classic technique, the double staining technique was used, in order to highlight at

the same time cellulose (stained in purple red by alum carmine) and lignine (stained in green by malachite green and iodine green). Histological technique used a single staining reagent, toluidin blue, for the simultaneous staining of cellulose (purple blue) and lignin (green). The appropriate technique was chosen for each type of vegetal material, taking into consideration its structure and characteristics. Thus, roots, stems and petioles were sectioned classically, where the sizes of the fragments of each organ allowed. Leaves, tendrils, ovaries, petals and flower peduncles were mounted into paraffin and subsequently sectioned with a manual microtome.

Plants of the Cucurbitaceae family are largely known for their special anatomical feature that concerns the vascular bundles, which in the case of these plants are bicollateral ones, having the xylem situated in the middle of the bundle and being surrounded by two strings of phloem on both sides. This type of vascular bundle is found in almost all vegetative and reproductive organs that are studied in this thesis, with the exception of root and leaves. All of the other anatomical features correspond to the typical anatomical features of each organ. The elements of novelty that the present study brings concern the complete and correct description of the vegetative and reproductive organs of these species, by the analysis of their anatomical structure through the photos of their structure taken with a digital camera. These features are hereby described for the first time in this manner.

a) Bryonia alba L.

Root. At the level where sections were performed, the organ presents a transition between a primary anatomical structure and a secondary one. Section has a circular shape (Fig.25.a). Secondary structure is generated by phellogen (Fig.25.b.1), a single-layered tissue that is colored in purple-red due to its cellulosic walls and has formed on its outer part the secondary cork (Fig.25.b.2) and on its internal part the phelloderm (Fig.25.b.3), which presses the primary cortex (Fig.25.b.4), that will appear less developed. The secondary cork is colored in brown-green, due to its suberified cells. Primary and secondary (phelloderm) cortexes have cellulosic walls and are colored in purple red. Vascular bundles (Fig.25.a.5) are collateral, open and disposed in the center of the anatomical structure, in a star shape. Each bundle is separated in xylem and phloem by the cambium (Fig.25.c.6), which has cellulosic walls. Xylem (Fig.25.c.7) has lignified walls, is colored in green and is situated towards the center of the structure, while phloem (Fig.25.c.8) is colored in purple red, has cellulosic walls and is situated towards the outer side. Between the vascular bundles, secondary medullary rays (Fig.25.d.9) are found as group of cells with cellulosic walls. The pith is no longer visible, being replaced by the xylem (Fig.25.e.10).

Stem. Cross section represents its primary anatomical structure and has a polygonal shape (Fig.26.a). The first layer of the structure is a typical epidermis (Fig.26.b.1), having cellulosic walls (colored in purple red), with isodiametric cells. The next zone of the anatomical structure is represented by a collenchymatic cortex (Fig.26.b.2), which is a continuous multi-layer tissue, having the walls thickened with cellulose, therefore colored in purple red. Underneath this cortex, a few strings of parenchymatous cells (Fig.26.b.3), colored in purple-red, separate it from the sclerenchymatic cortex (Fig.26.b.4), which has lignified thickenings and is colored in green. Vascular bundles (Fig.26.b.5) are bicollateral and open, having different sizes and dispositions: larger

ones (Fig.26.a.6) towards the center and smaller ones (Fig.26.a.7) towards the outer part of the section. All vascular bundles are regularly disposed in the fundamental parenchyma, a typical parenchyma, with cellulosic walls, colored in purple red (Fig.26.a.8). In the structure of each bundle, xylem (Fig.26.c.9) is found in the middle, being colored in green, due to the lignin thickenings on vessels. It is surrounded by two strings of phloem (Fig.26.c.10), which are not united, with cellulosic walls (colored in purple red). Between the external phloem and the xylem, a single layer tissue, the cambium (Fig.26.c.11) is found. All vascular bundles are separated by strings on parenchyma, colored in purple red, called medullary rays (Fig.26.d.12). From the epidermis, pluricellular secretory trichomes, with pluricellular basis (Fig.26.e.13) and 2-3 secretory cells on the top (Fig.26.e.14) can be occasionally found, together with some tector trichomes, also rarely found.

Leaf. The anatomical structure of the leaf lamina is a bifacial one (Fig.27.a). It is bordered by an upper epidermis (Fig.27.a.1) and a lower one (Fig.27.a.2) and has two protuberances, which correspond to the primary vein, more proeminent on the inferior side. Each of these protuberances contains collenchymatic tissue (Fig.27.a.3), having cell walls that are thickened with cellulose, which is colored in purple blue. Epidermis are also colored in purple blue, having cellulosic walls, with no thickenings. Below the upper epidermis, the palisade parenchyma (Fig.27.a.4) appears colored in purple blue. Immediately below it, the lacunar parenchyma (Fig.27.a.5) appears, above the lower epidermis, being also colored in the same way. The two tissues form the mesophyll and contain different amounts of chlorophyll (larger amounts in the palisade parenchyma and lower amounts in the lacunar parenchyma). The vascular bundle appears between the two protuberances (Fig.27.a.6) and is collateral and open, having xylem (Fig.27.b.7) colored in green, due to the lignin thickenings. It is situated towards the upper epidermis. The phloem (Fig.27.b.8) is colored in purple blue and it is situated towards the lower epidermis. On both sides of the vascular bundles, two sclerenchyma arches (Fig.27.b.9) appear colored in green, due to the lignin thickenings on cell walls. Vascular bundle is generally surrounded by some strings of parenchyma, that contains no chlorophyll (Fig.27.b.10). Especially on the lower epidermis, secretory and tector trichomes may appear (Fig.27.b.11).

Petiole. The cross section is laterally compressed (Fig.28.a), having semicircular shape. It is a stem-derived anatomical structure. Differences are found from the first layers of the structure, which is only protected by a collenchymatic arch (Fig.28.b.1), with cellulose thickenings, colored in purple blue. The vascular bundles (Fig.28.c.2) are disposed regularly in the anatomical structure, are bicollateral and open and are found in a fundamental parenchyma (Fig.28.a.3). There are two types of vascular bundles, larger ones (Fig.28.a.4), that are found towards the opposite part of the lateral compression and smaller ones (Fig.28.a.5), which are situated nearby the compression. Each vascular bundle has xylem (Fig.28.c.6) surrounded by phloem on both sides (Fig.28.c.7). At mature stages of the plant, arches of sclerenchyma (Fig.28.c.9) may appear near each bundle in order to increase protection. In the vascular bundle, the cambium (Fig.28.c.8) is found between the phloem situated towards the exterior of the section and the xylem. It is a single-layer tissue, colored in purple-red. Secretory multi-

seried trichomes (Fig.28.d) are sometimes on the epiderma and especially in and near the lateral compression.

Tendrils. Cross section of tendrils have circular contour (Fig.29.a) and also are a stem metamorphosis, having almost the same anatomical parts. The epidermis (Fig.29.a.1) is the first layer of cells, colored in purple blue. It is followed by the collenchymatic cortex (purple-blue) (Fig.29.a.2). In the fundamental parenchyma (purple-blue) (Fig.29.b.3), vascular bundles of the same type, bicollateral and open (Fig.29.c.6) are found and between them the medullary rays appear colored in purple-blue (Fig.29.b.4). Pith (purple-blue) (Fig.29.a.5) is found in the center of the structure. The structure of vascular bundles is the same as described for the stem: xylem (Fig.29.c.7) is colored in green and it is found in the center, being surrounded by two strings of phloem (Fig.29.c.8), colored in purple-blue. Between them, cambium (Fig.29.a.9), colored in purple-blue, is the layer that separates the exterior phloem from the xylem. Schlerenchymatic cortex is absent, if compared to the anatomical structure of the stem. Vascular bundles are less in number.

The reproductive organs. The anatomical structures described hereby are the ones of the ovary and male flower petals. *The structure of the ovary* appears as a typical one. It has 3 locules (Fig.30.a), each with two ovules (Fig.30.b), which appear more or less developed, depending on the stage. First layer of cells is represented by the external epidermis (Fig.30.a.1). The internal epidermis is difficult to be noticed, but the carpel welding line (Fig.30.a.2) can be noticed. Most of the structure is composed by a fundamental parenchyma (Fig.30.a.3). The 3 usual types of vascular bundles (marginal, median and placental) are not delimited (Fig.30.a.4-6), the placentation being parietal, as for most of the Cucurbitaceae plants. *Structure of the ovule* is anatrope (Fig.30.b). It is fixed on the ovarian locus by the funiculus (Fig.30.b.7). The external (Fig.30.b.8) and internal (Fig.30.b.9) integuments are the ones that surround the structure. The funiculus is fixed to the ovule by the hilum (Fig.30.b.10), which in this case is united with the ovule and forms the raphe (Fig.30.a.11), where the seed will come off at maturity. The chalaza (Fig.30.b.12) is the branched structure formed by the vascular system of the ovule. At the other end of the ovule, the micropyle (Fig.30.b.13) appears. The most important part of the ovule, the embryo sac (Fig.30.b.15) is found in the center of the structure, in the nucellus (Fig.30.b.14), which is the fundamental parenchyma. The whole structure is mostly colored in purple blue, except the xylem vessels of the vascular bundles, colored in green with toluidin blue. *The male flower petals* have a similar structure to the one with the leaves (Fig.31.a). They have a superior (Fig.31.b.1) and inferior (Fig.31.b.2) epidermis and several vascular bundles (Fig.31.b.3), spread in a fundamental parenchyma (Fig.31.c.4). Vascular bundles are collateral and closed, with similar structure as the ones found in the leaves, being protected by sclerenchyma arches (Fig.31.b.5). Secretory multi-seried trichomes are present (Fig.31.c).

b) *Echinocystis lobata* (Michx.) Torr. et A. Gray

Stem. Four stages in the development of the stem can be observed in Fig. 32 and Fig. 33 a-d, from a young stage (Fig.32.a), found on the top of young branches, to a mature stage (Fig.33.d.2'), found at the bottom of the branches. All structures are primary

anatomical structures and are polygonal-shaped (Fig.32 and 33.a-d). They are surrounded by an epidermis (Fig.32 and 33.a-d.1), colored in purple red and having a typical structure. All structures are protected by two different cortexes, a collenchymatic (Fig.32 and 33.a-d.2) and a sclerenchymatic one (Fig.32 an 33.a-d.4), separated by a few layers of parenchymatous cells (Fig.32 and 33.a-d.3). At mature stages, the sclerenchymatic cortex has disruptions, probably due to the development of the stem in diameter (Fig.33.d). Vascular bundles system is different, depending on the structure it belongs to. Each structure presents two types of vascular bundles, depending on their size: larger (Fig.32 and 33.a-d.5) and smaller ones (Fig.32 and 33.a-d.6). Generally, vascular bundles are bicollateral and open, but, as an abnormality, some vascular bundles are only collateral (Fig.32 and 33.a-d.7), in all structures. Younger stages of development have a large hollow pith (Fig.32 and 33.a-d.8) in the center of the structure, which is diminishing as the stems reaches its mature stages. Younger stems (Fig.32.a) have different-shaped bundles surrounding the structure, in an arranged manner around the cortexes. Bundles are disposed in an alternative manner and anatomical structure has a large central hollow pith. As the stem gets more mature, large vascular bundles start to occupy the center of the stem, diminishing the size of the hollow pith (Fig.32.b and Fig.33.c), until they occupy all the space in the center (Fig.33.d). Vascular bundles have typical structure, with xylem (Fig.32 and 33.a-d.9) colored in green and phloem (Fig.32 and 33.a-d.10) colored in purple-red. Generally, for bicollateral bundles, xylem is situated in the center of the bundle, being surrounded by two strings of phloem, while for collateral bundles, xylem is found towards the interior of the structure and phloem towards the exterior. Bicollateral bundles have cambium (Fig.32 and 33.a-d.11) between the external phloem and xylem and collateral ones between the two types of tissues, xylem and phloem. Between all these anatomical parts, a parenchymatous tissue is found, which has elongated cells between bundles and represents the medullary rays (Fig.32 and 33.a-d.12).

Leaves. Cross sections of the leaves revealed a bifacial typical structure of the leaf, which is surrounded by the two epidermis, the upper (Fig.34.a,b,e.1) and the lower one (Fig.34.a,b,e.2). The central part of the structure contain 2 protuberances that correspond to the primary vein and which contain collenchymatic tissue (Fig.34.a,c,d,e.3). Between the two epidermis, palisade (Fig.34.a,b,e.4) and lacunar (Fig.34.a,b,e.5) parenchyma are found. In the central part of the structure, between the two protuberances and surrounded by a few strings of parenchyma cells, the vascular bundle (Fig.34.a,e,f.6) is found. Structure of the bundle is collateral and closed, having xylem (Fig.34.a,f.7) towards the upper epidermis and phloem (Fig.34.a,f.8) towards the lower one. Bundle is protected by sclerenchyma arches (Fig.34.a,f.9), on both sides. Vascular bundles are surrounded by some strings of parenchyma, that does not contain clorophyll (Fig.34.a,e,f.10). The chlorophyll that gives the green color of the leaves is found in the palisade parenchyma, in larger amounts and in lacunar parenchyma, in lower amounts. Tector (Fig.34.a,h.11) and secretory (Fig.34.g.12) trichomes are found on the surface of leaves.

Petiole. The sections are semi-circular, with several protuberances and a lateral compression (Fig.35.a,b,c). It is a stem-like structure and it has similar features. The

first layer of cells is the epidermis (Fig.35.a,b,c,f,g.1), with a typical structure, but with exterior cell walls having extra cellulose thickenings, for a better protection. The structure in then surrounded by a sclerenchymatic cortex (Fig.35.a,b,c,f,g.2), which is better developed in the protuberances and in the tops of the lateral compression. The center of the section forms a hollow pith (Fig.35.a,b.3). Between the pith and the collenchymatic cortex, the fundamental parenchyma (Fig.35.a,b,c,f,g.4) occupies the whole anatomical structure. In the fundamental parenchyma, in front of each protuberance, vascular bundles (Fig.35.a-g.5) can be found, having the same structure: bicollateral and open, with xylem (Fig.35.d,e.6) in the middle and phloem (Fig.35.d,e.7) on both sides and also with cambium (Fig.35.d,e.8) between the exterior phloem and xylem.

Tendrils. The cross section is circular, with lateral protuberances (Fig.36.a) and it is surrounded by an epidermis (Fig.36.a,b,c.1) having exterior walls thickened with cellulose, for a better protection. As it is a metamorphosis of the stem, the next two zones are the same as in the case of the stems: collenchymatic (Fig.36.a,b,c.2) and sclerenchymatic (Fig.36.a,b,c.3) cortexes, separated by some strings of parenchyma cells (Fig.36.b,c.4). The next zone of the anatomical structure is the fundamental parenchyma (Fig.36.a,b,c.5), that fills the spaces between the vascular bundles, which have two different sizes: larger (Fig.36.a,b,d.6) and smaller (Fig.36.a.7) ones. The vascular bundles have a bicollateral structure, having xylem (Fig.36.b,d.8) surrounded by phloem (Fig.36.b,d.9) on the exterior and on the interior part. The cambium (Fig.36.b,d.10) is situated towards the exterior part of the structure, between the outer phloem and the xylem. The center of the section is represented by the hollow pith (Fig.36.a.11).

The reproductive organs. The only structures that allowed the sectioning were the ovary and the flower peduncle. *Ovary* is 4-carpelar, each carpel having one ovule (Fig.37.a,c,g). It has a circular contour, with several emergences on the surface (Fig.37.b,d). These emergences do not contain vascular bundles and are meant to protect the structure. On these emergences, secretory trichomes are found (Fig.37.b,f.1). The structure is also protected by an external epidermis (Fig.37.a.2). Internal epidermis is noticed (Fig.37.a.3), being the one that delimitates ovarian cavities. Fundamental parenchyma (Fig.37.a,b,d.4) contains 3 types of vascular bundles: median (Fig.37.a.5), marginal (Fig.37.a.6) and placental (Fig.37.a,e.7). Placentation is an exception from plants belonging to Cucurbitaceae family, as it is axilar. Anatomy of the ovules could not be identified in cross section. *Male flower peduncle* appears as a stem-like structure. It has almost the same anatomical zones as the tendrils and stems. From the exterior to the interior, these zones are: epidermis (Fig.38.a,c,d.1), collenchymatic cortex (Fig.38.a,c,d.2), few strings of parenchyma (Fig.38.a,c,d.3), sclerenchymatic cortex (Fig.38.a,c,d.4) and the fundamental parenchyma (Fig.38.a,d.5), that fills the spaces between all other tissues. The center of the section is represented by a hollow pith (Fig.38.a.6). In the fundamental parenchyma, vascular bundles (Fig.38.a,b,d.7) are found in front of each protuberance the anatomical structure presents. Structure of vascular bundles is the same as the one found in the stems: bicollateral and open, with xylem (Fig.38.b,d.8) in the middle,

surrounded by phloem (Fig.38.b,d.9) and cambium (Fig.38.b,d.10) between the outer phloem and xylem.

c) *Ecballium elaterium* (L.) A. Rich.

Root. Sections present circular shape (Fig.39.a). The sectioned vegetal material presented a transition towards the secondary anatomical structure (Fig.39.a) or a secondary one (Fig.39.f). The presence of phellogen (Fig.39.a,b,d,f,g.1) and cambium (Fig.39.a-g.2) determines the formation of the secondary structure. The secondary structure of the root is an anomalous one, presenting succesive rings of growth, that arise from the pericycle, as previously described by scientific literature (26). In the secondary structure, cambium is both found in the rings of growth, as in the anatomical structure. The phellogen is a tissue that is formed by a single layer of cells, which appears colored in purple-red. It has formed on the exterior the secondary cork (Fig.39.a,b,d,f,g.3) and in the interior of the structure the secondary cortex or phelloderm (Fig.39.a,b,d,f,g.4), which determines the diminishing of the primary cortex (Fig.39.a,b,d,f,g.5). The two cortexes appear in the same manner, having thick cellulosic walls and being colored in purple-red. The secondary cork has suberine on walls and is colored in brown green. The center of the structure lacks the pith, as it is replaced by secondary xylem (Fig.39.a,b,c,e,f,g.6), which is part of the vascular bundles. Secondary structure presents in the center a fundamental parenchyma (Fig.39.f.7). Vascular bundles (Fig.39.a,f.8) are collateral and open, being separated in secondary xyllem (Fig.39. a,b,c,e,f,g.6) and secondary phloem (Fig.39.a,b,c,e,f,g.9) by the cambium (Fig.39.a-g.2), a single-layered tissue with cellulosic walls. The phloem is found towards the exterior part of the cambium and it is colored in purple-red, having cellulosic walls. The xylem is found on the interior part of cambium, towards the center of the anatomical structure and has lignified cell walls, being colored in green. 4 vascular bundles are separated by medullary rays (Fig.39.a,c,e,f,g.10), with cellulosic walls and colored in purple-red.

Stem. The anatomical structure is a large sized one, so it is not included in the range that the used microscope can provide. It is however clear that the cross section has circular contour. The whole plant has setaceous hairs on all vegetative and reproductive organs, which transform on emergences at mature stages in the development of the species. Stems also have on their surface emergences, that microscopically appear as protuberances (Fig.40.b,d,g.1). Together with these, tector (Fig.40.a.2) and secretory trichomes (Fig.40.f.3) appear on the surface of the stem. The first layer of the structure is the epidermis (Fig.40.a,b,d,e,g.4). It is followed by the tissues that help for increasing the protection of the stem. Immediately after the epidermis, the collenchymatic cortex (Fig.40.fa,b,d,e,g.5) is well developed. Taken into consideration the fact that the some of the sectioned stems are at mature stages of their development, these stem presented a supplementary sclerenchyma arch (Fig.40.a,d,g.6), immediately after the collenchymatic cortex, developed to increase protection of the mature stem. Younger stages of stems lack this arches. Separated by a few strings of parenchyma cells (Fig.40.a,b,d,g.7), the sclerenchymatic cortex (Fig.40.a,b,d,g.8) appears less developed. The zone that fills the interior of the anatomical structure is the fundamental parenchyma (Fig.40.a,b,g.9). The cells of this parenchyma elongate between vascular bundles and form medullary rays

(Fig.40.a,b,g.10). Vascular bundles (Fig.40.a,b,c,g.11) are disposed in an arranged manner in a circular shape, along the sclerenchymatic cortex, separated from it by a few strings of parenchyma. Their size is different. It is noticed that, as the stem matures, small vascular bundles tend to unite, forming larger vascular bundles. The structure of the bundles is typical: bicollateral, open, with xylem (Fig.40.a,b,c,g.12) in the center and phloem (Fig.40.a,b,c,g.13) on the exterior and interior parts and cambium (Fig.40.a,b,c,g.14) towards the exterior between xylem and phloem.

Leaves. The leaf has a bifacial structure of lamina (Fig.41.a). The surface of the leaf is covered by tector and secretory trichomes (Fig.41.a,b,c). It is surrounded by two epidermis, a superior one (Fig.41.a,b,c.1) and an inferior (Fig.41.a,b,c.2) one, that present two protuberances, corresponding to the primary vein. The inferior protuberance is less developed on the superior side (Fig.41.a,b.3). Each of these protuberances contains collenchymatic tissue, that is more developed on the inferior side of the leaf (Fig.41.a,b.4). Between the two epidermis, palisade (Fig.41.a,b,c.5) and lacunar (Fig.41.a,b,c.6) parenchyma are found. In the center of the section, under the two protuberances of the primary vein, the vascular bundle (Fig.41.a,b,d.7) is found, being surrounded by some parenchyma cells. Structure of the bundle is typical: collateral and closed, with xylem (Fig.41.a,b,d.8) on top, towards the upper epidermis and phloem (Fig.41.a,b,d.9) under, towards the lower epidermis. Vascular bundle is protected by two sclerenchyma arches (Fig.41.a,b,d.10), towards the two epidermis. The vascular bundle is surrounded by strings of parenchyma without chloroplasts (Fig.41.a,b.11), while the majority of chlorophyll is contained in the pallisadic and lacunar parenchyma.

Petiole. The cross section of the petiole is semi-circular (Fig.42.a) , having no lateral compression, as other petioles. It is a hairy structure, that has on the surface both tector (Fig.42.a,d) and secretory trichomes with 4 secretory cells on top (Fig.42.a,e,f). The first layer of the anatomical structure is represented by the epidermis (Fig.42.a,b.1), being followed by a collenchymatic cortex (Fig.42.a,b.2). Immediately after, an assimilation parenchyma (Fig.42.a,b.3) is found, being followed by the fundamental parenchyma (Fig.42.a,b.4), where vascular bundles (Fig.42.a,b,c.5-6) can be found, having two sizes: larger ones (Fig.42.a,b,c.5) and smaller ones (Fig.42.a,b.6). Each vascular bundle is bicollateral and open, with xylem (Fig.42.a,b,c.7) in the middle and phloem (Fig.42.a,b,c.8) on the sides. Cambium (Fig.42.a,b,c.9) is a single-layer tissue, that is found between outer phloem and xylem.

The reproductive organs. Among all reproductive organs, only ovary and fruit peduncle allowed the sectioning. *Ovary* is 3-carpelar (Fig.43.a), with two ovules in each ovarian locus (Fig.43.a,b,c). Its surface is covered by tector and secretory trichomes (Fig.43.a,d,e), which transform in emergences (Fig.43.a.1), for a supplementary protection of the structure. The external layer is an epidermis (Fig.43.a,d,e.2). The internal epidermis (Fig.43.a,b,c.3) is noticed, but due to its fragile structure, it is easily destroyed. It is even though the one that separates ovarian cavities. The great majority of the structure is represented by a fundamental parenchyma (Fig.43.a), which contains the vascular bundle system. There are 3 types of vascular bundles: median (Fig.43.a,b,c,f.4), marginal (Fig.43.a,e,g.5) and placental (Fig.43.a,b.6). Placentation is

the same as for all Cucurbitaceae plants, parietal. The anatomy of the ovule could not be identified in cross section. *Fruit peduncle* is a metamorphosis of the stem, with similar structure: epidermis (Fig.44.a,c.1) as a first layer, followed by collenchymatic cortex (Fig.44.a,c.2), parenchymatic tissue (Fig.44.a,c.3) and sclerenchymatic cortex (Fig.44.a,c.4). In the fundamental parenchyma (Fig.44.a,c.5), vascular bundles (Fig.44.a,b,c.6) are found. They are bicollateral and open. Xylem (Fig.44.b,c.7) is found in the center, surrounded by phloem on both sides (exterior and interior) (Fig.44.b,c.8) and cambium (Fig.44.b,c.9) is found between the outer phloem and xylem.

3.5.3. Microscopic study of the trichomes of three species belonging to Cucurbitaceae family

The structure of different types of trichomes was observed by stereomicroscopy and superficial sections on the inferior epidermis of the leaf. Tector pluricellular trichomes and secretory trichomes were noticed. Their presence is not only an important microscopic feature for the plants of this family, but also represent an important taxonomic criteria, that my help for the identification of these species (Fig.45-56).

a) Bryonia alba L.

The trichomes are present on the vegetative and reproductive organs of the species. It is a pubescent species, that is known for the presence of bristly hairs on the surface of most of its organs. Microscopic analysis allowed a detailed study of the structure of all types the types of trichomes found on different parts of *B.alba*.

Stems present on their surface tector and secretory trichomes (Fig.45.a-e). The structure of these trichomes is different, depending on their type. Secretory trichomes (Fig.45.a-c) are curved on top, have pluricellular basis and 2-3 secretory cells on the top. Tector trichomes (Fig.45.a,d,e) are sharpened on top and are also pluricellular. *Leaves* are known for the presence of the trichomes on their inferior surfaces. In this case, on the surface of the leaves *of B. alba* secretory and tector trichomes are found. Secretory trichomes (Fig.46.1,2,4) have 2-3 secretory cells, of which one is curved on top and a pluricellular basis. Tector trichomes (Fig.46.1-3) are pointed on top and are also pluricellular. In order to elucidate the structure of each type of trichome, SEM analysis were performed on the inferior surface of the leaf. Pictures revealed the frequence of these trichomes on the inferior epidermis (Fig.46.a). Stomata (Fig.46.b) and the structure of each type of trichome (Fig.46.c-f) was also revealed. Tector trichomes have a pluricellurar body and a cup-like basis, also formed by multiple cells (Fig.46.c). Secretory trichomes (Fig.46.d,e) revealed 2 types of structures: first one is represented by multi-seried trichomes with unicellular basis (Fig.46.e), and the second one is represented by multi-seried trichomes with a pluri-cellular basis (Fig.46.f). Same type of trichomes are found on the *petioles* (Fig.47), but they occur with a lower frequency. Not only vegetative organs are covered with trichomes, but also reproductive ones. *Male flower petals* only have secretory trichomes, that cover all the surface of the petals (Fig.48.a-d). They are not only simple secretory trichomes (Fig.48.e,f), but also glandular ones (Fig.48.g).

b) *Echinocystis lobata* (Michx.) Torr. et A. Gray

The species is a less pubescent one, but nevertheless it presents tector and secretory trichomes on the surface of some organs. The parts that proved to be the richest in terms of trichomes were the leaves. Also, trichomes could be found on the surface of reproductive organs.

Leaves present both tector and secretory trichomes. Tector trichomes (Fig.49.e,f-k) have pluricellular structure, with curved top. On the other side, secretory trichomes present two types of structure. Their structure is almost similar, with some small differences regarding especially the size of their basis and of their secretory cells on top. Generally, they present pluricellular basis and 2-3 secretory cells on top, as all secretory cells described previously. First type is represented by secretory trichomes that have smaller basis, generally formed by one or two cells and 2-3 elongated secretory cells on top (Fig.49.a,b,d,f,g,j,l). Second type of trichomes has longer basis, formed of several cells and 2-3 secretory cells on top (Fig.49.a,c,f,g,l). *Reproductive organs* that present trichomes on their surfaces are especially flowers, male and female ones. *Ovary* has its surface covered in tector trichomes, that have a special structure and which transform into emergences (Fig.50.a-g) at mature stages of development. When it reaches at the stage of fruit, it is fully covered with thorns, that play an important role in the protection of the species. On these structures, smaller secretory trichomes can be found, having the same structure as those that are found on the leaves: pluricellular basis and 2-3 curved secretory cells on top (Fig.50.a-g). These trichomes are also found between the large emergences. *Male and female flower petals* also present on their surface secretory trichomes, probably having glandular structure (Fig.51.a-f).

c) *Ecballium elaterium* (L.) A. Rich.

This species is largely known as being a pubescent one, having on the surface of its vegetative and reproductive organs bristly hairs, of different types that increase the protection of the species. Secretory and tector trichomes can be found on these organs.

Stems have both tector and secretory trichomes on their surfaces. This species has a special type of secretory trichomes. Their structure involve a pluricellular basis and on top 4 secretory cells, situated at the same level (Fig.52.b). This type of trichomes has been previously cited by authors on the surface of the leaves (31). It appears that not only they are present on leaves, but also on stems and other organs of the plant. Tector trichomes (Fig.52.a) have pluricellular basis, are pointed on top and may transform into emergences at mature stages in the development of the species. *Leaves* of the species are coarsely haired, having both types of trichomes, secretory and tector ones (Fig.53.d-h). Secretory trichomes have the same structure as the ones on the stems, with pluricellular basis and four secretory cells on top (Fig.53.b,d-i). Tector trichomes have pointed top and are pluricellular, having pluricellular basis (Fig.53.a,c,d-i). There is a clear difference concerning the size of the trichomes and their frequency too: secretory trichomes are smaller and more rare, while tector ones are bigger and more frequently found on the epidermis. On the surface of *petioles*, both types of trichomes were also found, with quite a high frequency as the trichomes found on leaves (Fig.54.a,b). Same structure is found for secretory (Fig.54.c) and tector trichomes (Fig.54.d). *Ovary* of the species is covered in tector trichomes (Fig.55.a,b),

that are found with a high frequency and which, at maturity, develop into emergences. With a lower frequency are also found secretory trichomes (Fig.55.a,c), having the same structure as the secretory trichomes described before. On the *male flower petals*, secretory trichomes (Fig.56.a-d) are found. They have a special structure, lacking the four secretory cells on top and having curved top, with one secretory cell.

3.6. Conclusions

Botanical studies represent the first step towards the study of plants which have proven important potential as medicinal plants. Microscopic study of the powder and of the main organs brought important data concerning their features.

Elements found in the powder of each studied species confirm the morphological and histo-anatomical features of each species. Different parts of the studied structures or histological features could be identified and can help for a future identification of a possible herbal product. As the three species belonging to Cucurbitaceae family do not present a herbal medicinal product, the characters described hereby can be useful for the characterization of such a product in the possibility of its future introduction in therapy. These features are described for the first time for the three species.

Microscopic features were also described for the first time for these species, and are also useful to establish possible connections between the species belonging to this family. Anatomical structures of the vegetative (roots, stems, leaves) and reproductive (flower petals, ovary, fruits) organs could be described and appear to have similar features. Roots of *B.alba* and *E.elaterium* have similar macroscopic features, which reflects in the microscopic one (sections present the transition of a primary structure towards a secondary one). Roots of *E.lobata* have macroscopic features that did not allow sectioning. Stems have the same structure for the three species and present collenchymatic and sclerenchymatic cortexes that protect them and vascular bundles arranged orderly. Leaves are also similar and show typical bifacial structure. Petioles, tendrils (of *B.alba* and *E.lobata*) and fruit peduncles show a clear stem-derived structure, sometimes lacking sclerenchyma cortex. Sections through ovaries reveal the typical structure, both for the ovaries and for the ovules. The only exception regarding parietal placentation, specific for Cucurbitaceae species is *E.lobata*. Study of the trichomes, that are specific microscopic features for these plants, allowed to describe the structure of tector and secretory trichomes, which appear as pluri-cellular ones, having sharp (tector trichomes) heads with one cell on top or round heads (secretory trichomes), with several secretory cells on top.

There is a clear connection between the three species, which can be noticed. Bicollateral vascular bundles and presence of trichomes are the most significant. These features appear in each of the studied organs of the three species and confirm their common origin. Besides, a specific connection between *B.alba* and *E.elaterium* is confirmed, by their similar microscopic features. The connection between the two species is previously cited (21) and it is only confirmed by the study of the microscopic features of their main parts.

Therefore, data obtained in this study bring important information on *Bryonia alba* L., *Echinocystis lobata* (Michx.) Torr. et A. Gray and *Ecballium elaterium* (L.) A. Rich. and can help to establish clear features that represent the first stept towards a correct identification and description of the three species.

4. Study 2. Comparative phytochemical researches of three species belonging to Cucurbitaceae family

4.1. Introduction

After the correct identification of the species, the next step in the complete description of a species is the identification of the active compounds, that are responsible for the biological activity. The main class of compounds that is described in the present thesis are the flavonoids and polyphenols, a class of compounds that is lesser studied in the composition of these species, but which has nevertheless proved important biological activities. They may assign the species significant anti-oxidant and anti-inflammatory activities.

Tested samples in the present thesis were represented by methanolic and ethanolic extracts, obtained by the maceration of the vegetal powder with the corresponding solvent. Flavonoids in the composition of the three species belonging to Cucurbitaceae family were identified by chromatographic methods. TLC technique and a HPLC-DAD method for the identification of flavonoids were used in order to identify the main flavonoids in the three species. Where available, reference compounds were used in order to compare their parameters with each one of the detected compounds. Afterwards, the compounds were isolated by a HPLC-DAD preparative technique, that was in fact an adaptation of the analytical method. Structure identification was performed by MS and NMR techniques. Quantification of compounds in different samples collected in different stages in the development of the species was performed by HPLC-DAD and HPLC-MS techniques. The total flavonoids and total polyphenols could be quantified by spectrophotometric methods, that are provided by existing monographs in the Romanian and European Pharmacopoeias.

All performed analysis allowed to establish the part of the species that is proved to be the richest in the flavonoidic compounds. These data are not available in the scientific literature, to the best of our knowledge. At the same time, differences between samples collected in different places and at different periods of time could be established. Thus, a description of these compounds could be provided.

4.2. Work hypothesis

Potential toxicity of species belonging to Cucurbitaceae family, corresponding to the content in cucurbitacins, determined a decrease in the attention of researchers worldwide to the plants of this family, especially if the species are not edible. Recent studies have proved nevertheless that these species may contain important amounts of compounds that are not toxic and may assign them important biological activities (57–59,145). Such compounds are flavonoids and polyphenols. In this context, the present thesis is aimed to bring a description as complete as possible for the flavonoidic and polyphenolic compounds found in the three species of the Cucurbitaceae family.

4.3. Materials and methods

The harvested samples for all the species and the assays that are included in the studies of this thesis, are presented in Annex, Table I, with their harvesting date and place. Each of the assays that are performed mentions the samples that are studied.

4.3.1. Extraction procedure

4.3.1.1. Extracts used for the identification and isolation of flavonoids

Samples of plant material were grinded until reaching an appropriate degree of fineness and were subsequently extracted with methanol HiPerSolv by percolation, after 24hours of maceration. Approximately 5.00g of grinded powder of the species was subjected to the extraction procedure and extraction yields were calculated after the complete evaporation of the solvent, under reduced pressure, at 40°C.

4.3.1.2. Extracts used for the screening, identification and quantification of polyphenols

Samples of plant material were grinded until reaching an appropriate degree of fineness. The powder was subjected to a 24h maceration with 50% ethanol and subsequently sonicated for 30minutes at 70^0C. The obtained solutions were filtered and completed with the extraction solvent until reaching the initial volume. Extracts were subjected to the HPLC analysis, after being filtered on 0.45 μm membrane filters.

4.3.2. TLC method

Conditions:

- **Test solution**: extracts of the samples comprised in Table XIII and prepared as described in 4.3.1.1.

Table XIII. Samples analyzed in TLC studies

Species	Sample name	Part of the species
Bryonia alba L.	BA27	Stems
	BA40	Fruits
	BA25	Root
	BA6	Leaves
	BA7	Aerial part
Echinocystis lobata (Michx.) Torr. et A.Gray	EL73	Stems
	EL74	Leaves
	EL75	Aerial parts
	EL76	♂ flowers
	EL77	Fruits
Ecballium elaterium (L.) A.Rich.	EE82	Mature stems
	EE83	Young stems
	EE84	Leaves
	EE85	Aerial part
	EE86	Roots

- **Reference solutions:** Saponarin, Isovitexin, Vitexin 1mg/mL in Methanol HiPerSolv Chromanorm;
- **Plate:** Silica gel 60 F_{254} – precoated TLC plates (Merck, Germany);
- **Mobile phase:** Ethyl acetate: formic acid: glacial acetic acid: water (100:11:11:26 V/V/V/V);
- **Application:** 10μL as bands;

- **Development:** over a path of 10cm;
- **Drying:** in air;
- **Detection:** spray with DPBAE and PEG; examination under UV light, at 254nm and 365nm.

In order to separate the compounds forming a double peak in the composition of the flowers of *E. lobata*, a special recovering method was used, that, after the complete development of the plates allowed further analysis of the compounds present in each of the spots by the MS technique. TLC plates are obtained by the above described method. Afterwards, the compound found in the corresponding spot is collected together with the corresponding silica and sent in the MS, after the removal of the silica. Thus, the molecular peak of the compounds in the spot can be determined.

4.3.3. Spectrophotometric methods for the quantification of total flavonoids, total polyphenols and total hydroxycinnamic acids

4.3.3.1. Quantification of the total flavonoids by the method described in the European Pharmacopoeia

Quantification of the total flavonoids was assessed by the method described in the 9th edition of the European Pharmacopoeia, at the monograph *Passiflorae herba*.

Preparation of the extract: 0.200g vegetal powder was heated at reflux 60^{0}C with 40mL ethanol 60% V/V for 30min, with frequent shaking. After cooling down, the solution was filtered through a cotton in a 100.0 mL volumetric flask. The cotton and the remaining powder was re-heated at 60^{0}C with 40mL ethanol 60% V/V for 10min. After cooling down, it was filtered together with the first filtered solution in a new 100.0mL volumetric flask. After combining the two solutions, ethanol 60% V/V was added in order to reach the 100.0mL volume in the flask. Tested samples are found in Table XIV.

Preparation of the testing solution: To 5.00 mL of the extract prepared as described above, that was evaporated under reduced pressure at 40^{0}C, 10mL of a mixture of 10volumes of methanol and 100volumes of glacial acetic acid was added and 10mL of a solution containing 25g/L of boric acid and 20g/L oxalic acid in anhydrous formic acid. The solution was completed with anhydrous acetic acid at 25.0 mL in a volumetric flask.

Table XIV. Samples analyzed in HPLC studies for the quantification of saponarin and total flavonoids in samples of *B. alba*

Sample no. + part	Harvesting date	Harvesting place
BA 2 (leaves)	06.05.2014	Cluj, Parcul Feroviarilor
BA 6 (leaves)	20.05.2014	Cluj, Parcul Feroviarilor
BA 11 (leaves)	14.06.2014	Cluj, Gheorgheni
BA 23 (leaves)	10.07.2014	Cluj, Parcul Feroviarilor
BA 28 (leaves)	13.07.2014	Cluj, Mănăștur
BA 36 ((leaves)	31.07.2014	Cluj, Parcul Iulius

Preparation of the compensation liquid: To 5.00mL of the extract prepared as described above, that was evaporated under reduced pressure at 40^{0}C, 10mL of a mixture of 10volumes of methanol and 100volumes of glacial acetic acid was added

and the solution was completed with anhydrous acetic acid at 25mL in a volumetric flask.

Quantification of the total flavonoids: After 30min, the absorbance at 401nm of the testing solution was measured by comparison with the compensation liquid and total of flavonoids was calculated and expressed as vitexin equivalents (g of vitexin in 100g of dried vegetal powder), using the following expression: (A * 0.8)/m (A = absorbance at 401nm and m = mass of the herbal powder in grams). Absorbances were measured using a Hitachi U-2910 spectrophotometer.

4.3.3.2. Quantification of the total flavonoids by the method described by the Romanian Pharmacopoeia 10th edition

Quantification of the total flavonoids was assessed by the method described in the 10th edition of the Romanian Pharmacopoeia, at the monograph *Cynarae folium*. It is based on the color reaction of flavonoids with aluminium chloride.

Preparation of the extract: as described in the 4.3.1.2. for the identification and quantification of polyphenols. Tested samples can be found in Table XV.

Table XV. Samples analyzed for the quantification of total flavonoids, polyphenols and hydroxycinnamic acids in samples of the three Cucurbitaceae species

Samples	Part of the species
BA1	Stems
BA2	Leaves
BA24	Aerial part
EL46	Stems
EL47	Leaves
EL50	Flowers
EL66	Aerial part
EL69	Leaves
EL71	Flowers
EL73	Stems
EL75	Aerial part
EL77	Fruits
EE82	Mature stems
EE84	Leaves
EE85	Aerial part

Preparation of the testing solution: To 5.00 mL of each sample, 5 mL of sodium acetate 100 g/L, 3 mL of aluminum chloride 25 g/L were added and the solution was completed at 25 mL with methanol in a volumetric flask.

Preparation of the compensation liquid: To 5.00 mL of sample, 8mL of water were added and the solution was completed at 25 mL with methanol in a volumetric flask.

Quantification of the total flavonoids: The absorbance of the testing solution was measured by comparison with the compensation liquid at 430 nm, after 15 min. The amount of total flavonoids content was determined using an equation obtained from a calibration curve of rutin. Results were expressed as g rutin equivalents (RE)/100 g dried vegetal powder. Absorbances were measured using a Jasco V-530 UV-Vis spectrophotometer.

4.3.3.3. Quantification of the total polyphenols by a method inspired from the Romanian Pharmacopoeia 10th edition

Quantification of the total polyphenols was assessed by the method similar to the one described in the 10th edition of the Romanian Pharmacopoeia, at the monograph *Cynarae folium*, with some modifications. It is based on the color reaction of polyphenols with the Folin-Ciocâlteu reagent (phosphowolframmic acid).

Preparation of the extract: as described in the 4.3.1.2. for the identification and quantification of polyphenols. Tested samples can be found in Table XV.

Preparation of the testing solution: To 2.00 mL of sample, 1 mL of Folin-Ciocâlteu reagent and 10mL of distilled water were added and the solution was completed at 25.00 mL with with a solution of sodium carbonate 200 g/L in a volumetric flask. Samples were incubated in the dark for 30 min.

Preparation of the compensation liquid: To 2.00 mL of sample, water was added and the solution was completed at 25 mL in a volumetric flask.

Quantification of the total polyphenols: The absorbance of the testing solution was measured by comparison with the compensation liquid at 760 nm. The amount of total polyphenolic content was determined using an equation obtained from a calibration curve of gallic acid. Results were expressed as g gallic acid equivalents (GAE)/100 g dried vegetal powder. Absorbances were measured using a Jasco V-530 UV-Vis spectrophotometer.

4.3.3.4. Quantification of the total hydroxycinnamic acids by the method described by the European Pharmacopoeia 9th edition

Quantification of the total hydroxycinnamic acids was assessed by the method similar to the one described in the 9th edition of the European Pharmacopoeia, at the monograph *Fraxini folium*, with some modifications. It is based on the color reaction of phenols with the nitrous acid, formed by sodium nitrite and sodium molybdate in an acid solution, that form nitroso-derivatives, that isomerize in oximes, which, due to their weak acid character, are dissolved in alkaline solutions and form red compounds.

Preparation of the extract: as described in the 4.3.1.2. for the identification and quantification of polyphenols. Tested samples can be found in Table XV.

Preparation of the testing solution: To 1.00 mL of sample, 1 mL of 0.5N hydrochloric acid, 1 mL of Arnow's reagent (10 g sodium nitrite and 10 g sodium molybdate made up to 100 mL with distilled water) and 1 mL NaOH 1N were added and the solution was completed at 10 mL with water in a volumetric flask.

Preparation of the compensation liquid: To 1.00 mL of each sample, 0.5N hydrochloric acid and 1 mL NaOH 1N were added and the solution was completed at 10.00 mL with water in a volumetric flask.

Quantification of the hydroxycinnamic acids total: The absorbance of the testing solution was measured by comparison with the compensation liquid at 525 nm. The amount of total polyphenolic content was determined using an equation obtained from a calibration curve of caffeic acid. Results were expressed as g caffeic acid equivalents (CAE)/100 g dried vegetal powder. Absorbances were measured using a Jasco V-530 UV-Vis spectrophotometer.

4.3.4. HPLC-DAD method for the identification and quantification of flavonoids

Conditions:

- **Apparatus**: Hewlett Packard Agilent 1100 system, equipped with an Agilent 1100 quaternary pump, an Agilent 1100 degaser and an Agilent 1100 Automatic Liquid Sampler;
- **Test solution**: extracts (Table XVI and Table XVII), prepared as described in 4.3.1.1. dissolved in Methanol LiChrosolv and filtered by a 0.45 µm membrane;

Table XVI. Samples analyzed in HPLC studies for the identification of flavonoids

Species	Sample name	Part of the species
Bryonia alba L.	BA27	Stems
	BA40	Fruits
	BA25	Root
	BA2	Leaves
	BA3	Aerial part
Echinocystis lobata (Michx.) Torr. et A. Gray	EL73	Stems
	EL74	Leaves
	EL75	Aerial parts
	EL76	♂ flowers
	EL77	Fruits
Ecballium elaterium (L.) A. Rich.	EE82	Mature stems
	EE83	Young stems
	EE84	Leaves
	EE85	Aerial part
	EE86	Roots

Table XVII. Samples analyzed in HPLC studies for the quantification of saponarin and total flavonoids in samples of *B. alba*

Sample no. + part		Harvesting date	Harvesting place
BA 2 (leaves)	**BA 3 (aerial part)**	06.05.2014	Cluj, Parcul Feroviarilor
BA 6 (leaves)	**BA 7 (aerial part)**	20.05.2014	Cluj, Parcul Feroviarilor
BA 11 (leaves)	**BA 12 (aerial part)**	14.06.2014	Cluj, Gheorgheni
BA 23 (leaves)	**BA 24 (aerial part)**	10.07.2014	Cluj, Parcul Feroviarilor
BA 28 (leaves)	**BA 29 (aerial part)**	13.07.2014	Cluj, Mănăştur
BA 36 ((leaves)	**BA 37 (aerial part)**	31.07.2014	Cluj, Parcul Iulius

- **Reference solutions:** Saponarin, Isoorientin, Vitexin, Isovitexin 1mg/mL in Methanol LiChrosolv (Table XVIII);

Table XVIII. The reference compounds used for the identification of flavonoids and their retention times

Compound	$t_R \pm$ SD (min)	Compound	$t_R \pm$ SD (min)	Compound	$t_R \pm$ SD (min)
Saponarin	24.63 ± 0.25	**Rutin**	28.49 ± 0.32	**Hyperoside**	
Isoorientin	25.78 ± 0.25	**Isoquercitrin**	29.26 ± 0.74	**Orientin**	
Vitexin	27.10 ± 0.21	**Astragalin**	31.69 ± 0.84	**Quercitrin**	
Isovitexin	28.59 ± 0.44	**Apigenin**	45.21 ± 0.27	**Luteolin**	40.29 ± 0.30

- **Column:** Hypersil ODS C18 (250 mm x 4.6, i.d.; particle size 5 µm; Thermo Scientific);
- **Temperature:** room temperature (25°C);
- **Flow rate:** 1mL/min;
- **Injection volume:** 10µL;
- **Mobile phase:** 0.05% TFA in water (V/V) (A) and acetonitrile (B), with the gradient described in Table XIX.

Table XIX. Gradient used for the analysis of flavonoids

Minute	% A	%B
0-1	100	0
1-45	97	3
45-55	60	40
55-56	40	60
56-66	40	60
66-67	100	0
67-82	100	0

- **Detection:** UV, at 350nm.
- **Quantification of saponarin:** samples were prepared by sonication of 100 mg vegetal powder in 20mL methanol, filtrated by 0.45 µm membrane and subjected to HPLC-DAD analysis, using the above described method; calibration curve (R^2=0.9999), having the equation y = 974.7x + 3.1664 (Fig.57) was plotted by representing six increasing concentrations obtained by successive injections of increasing volumes of a 0.231mg/10mL saponarin solution and their corresponding area under the curve, determined by the integration of the corresponding peak of saponarin in each sample.

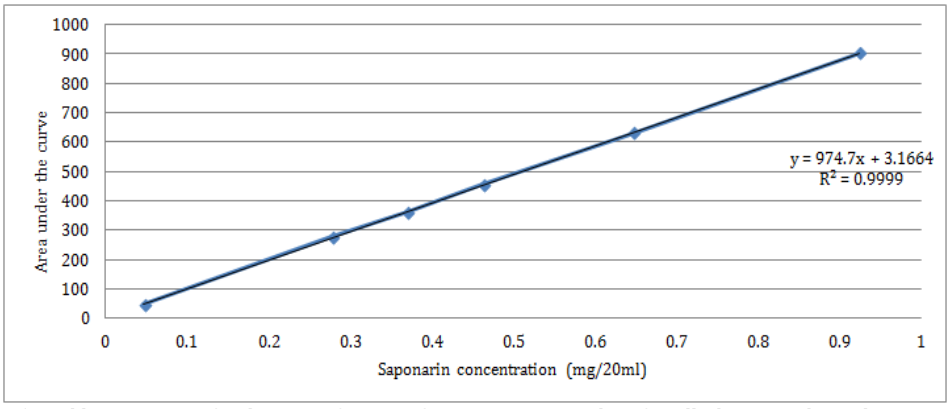


Fig.57. Calibration curve for the quantification of saponarin in samples of *B. alba* leaves and aerial parts

4.3.5. Hydrolysis of flavonoids for the identification of aglycones

In order to identify the aglycones of the flavonoids that are present in the composition of the three species, hydrolysis of the flavonoids was performed, following a hydrolysis test according to the 9th European Pharmacopoeia at the *Acaciae gummi*.

Preparation of the testing solution: To 0.100 g of herbal powder, 2mL of 100g/L solution of trifluoroacetic acid was added in a thick-walled tube. The mixture was heated at 120^0 for 1h, 2h, 3h, 4h, 5h and 6h. The solution was transfered into a flask, diluted with water and evaporated to dryness under reduced pressure. The residue was dissolved in methanol and the solution was subjected to the HPLC method, using the conditions described at 4.3.4.
Reference solutions: Saponarin, Isoorientin, Vitexin, Isovitexin, Luteolin, Apigenin 1mg/mL in Methanol LiChrosolv (Table XVIII).

4.3.6. HPLC-MS method for the screening of polyphenols

Conditions:

- **Apparatus**: Agilent 1100 HPLC Series system (Agilent, USA) equipped with degasser, binary gradient pump, column thermostat, autosampler and UV detector; the HPLC system was coupled with an Agilent 1100 mass spectrometer (LC/MSD Ion Trap VL);
- **Test solution**: extracts of the samples described in Table I from Annex, prepared as described in 4.3.1.2.;
- **Reference solutions:** the compounds found in Table XX, 1mg/mL in Methanol;

Table XX. The reference compounds used for the identification of polyphenols and their retention times

Peak	Phenolic compound	$t_R \pm$ SD (min)	Peak	Phenolic compound	$t_R \pm$ SD (min)
1	Caftaric acid *	2.10± 0.06	10	Rutoside	20.20 ± 0.15
2	Gentisic acid *	2.15± 0.07	11	Myricetin	20.70 ± 0.06
3	Caffeic acid *	5.60± 0.04	12	Fisetin	22.60 ± 0.15
4	Chlorogenic acid *	5.62± 0.05	13	Quercitrin	23.00 ± 0.13
5	p-coumaric acid	8.7± 0.08	14	Quercetol	26.80 ± 0.15
6	Ferulic acid	12.2 ± 0.10	15	Patuletine	28.70 ± 0.12
7	Sinapic acid	14.3 ± 0.10	16	Luteolin	29.10 ± 0.19
8	Hyperoside	18.60 ± 0.12	17	Kaempferol	31.60 ± 0.17
9	Isoquercitrin	19.60 ± 0.10	18	Apigenin	33.10 ± 0.15

* overlapping in UV detection, only qualitative analysis possible using MS detection

- **Column:** Zorbax SB-C18 (100 mm x 3.0 mm i.d., particle size 3.5 µm, Agilent);
- **Temperature:** 48°C
- **Flow rate:** 1mL/min;
- **Injection volume:** 5µL;
- **Mobile phase:** binary gradient, composed of methanol and acetic acid 0.1% (V/V); elution started with a linear gradient, beginning with 5% methanol and ending at 42% methanol, for 35 minutes; followed for the next 3 minutes by isocratic elution with 42% methanol;
- **Detection:** UV and MS; UV detector was set at 330 nm until 17.5 min, then at 370 nm; MS system was operated using an electrospray ion source in negative mode; for the identification of polyphenols, the MS spectra of the references

were integrated in a library; thus, by comparison of the compounds in extracts with data from library, the positive identification of compounds could be performed, based on spectral match; quantification of compounds from MS detection was performed by using the UV trace. Results were expressed as mg polyphenol/100g dried vegetal product (dvp).

4.3.7. Preparative HPLC-DAD method for the isolation of flavonoids

Separation and purification of flavonoids was performed by adapting the analytical HPLC conditions to the preparative ones, in order to obtain the compounds of interest in an appropriate amount for the correct and complete identification (146).

Conditions:

- **Apparatus**: Modular HPLC-DAD Preparative System, equipped with a Varian 218 Solvent Delivery System, a ProStar 335 Photodiode Array Detector and an automatic Varian 440LC Fraction Collector;
- **Test solution**: The dry extracts of the samples BA23, BA 6 and BA2 (Table VII), in quantities between 200-500mg, prepared as described in 4.3.1.1. dissolved in Methanol LiChrosolv® (for crude extracts) or in TFA 0.05% V/V (for blocks of fractions concentrated in compounds) and filtered through a 0.45 μm membrane;
- **Column** LichroPrep 100 RP-18;
- **Temperature:** room temperature (25°C);
- **Flow rate:** 30mL/min;
- **Injection volume:** 5mL;
- **Mobile phase:** 0.05% TFA in water (V/V) (A) and acetonitrile (B), with the gradient described in Table XXI;

Table XXI. Gradient used for the analysis of flavonoids

Minute	% A	%B
0-1	100	0
1-45	97	3
45-55	60	40
55-56	40	60
56-66	40	60

- **Detection:** UV, at 350nm; peaks corresponding to the main flavonoids were collected manually, based on the retention times obtained on the chromatograms.

4.3.8. Structural identification of flavonoids

In order to confirm and to complete data that were obtained in the HPLC-DAD and TLC analysis that were performed for the identification of the structure of flavonoidic compounds that are found in the composition of the three Cucurbitaceae species, MS and NMR (^{1}H-NMR and ^{13}C-NMR) analysis were performed.

NMR spectra were measured with a Bruker® Avance II spectrophotometer, equipped with a cryoprobe operating at 500 MHz for ^{1}H and at 125.7 MHz for ^{13}C in CD3OD solution (Euriso top®, Gyf-surYvette, France) with TMS as an internal reference. MS analyses were performed using a Thermo Scientific LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) with ESI source in positive mode (146).

4.4. Results

4.4.1. Extraction procedure

The extraction yields can be found in Table XXII and were calculated for extracts obtained as described at 4.3.1..1. by reporting the final quantity of crude extract to the departure quantity of vegetal powder.

Table XXII. Yields obtained for each of the main parts corresponding to each species

Sample	Extraction yield
BA leaves	18.54 %
BA stems	12.98 %
BA fruits	30.04 %
BA aerial parts	17.62 %
BA roots	3.27 %
EL leaves	93.76 %
EL flowers	16.26 %
EL stems	8.68 %
EL fruits	23.53 %
EL aerial parts	8.75 %
EE leaves	14.45 %
EE mature stems	18.66 %
EE young stems	17.68 %
EE aerial parts	15.93 %
EE roots	7.90 %

4.4.2. TLC analysis

The TLC analysis performed allowed to identify the plants parts that are proved to contain the highest amount and number of flavonoids (Fig.58-60).

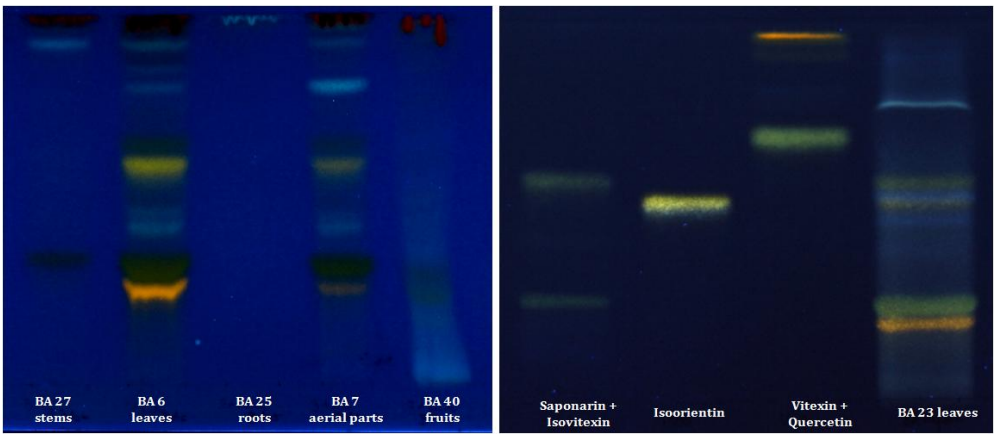


Fig.58. TLC of the main parts of *B. alba* (spray reagent: DPBAE and PEG; examination under UV light, at 365nm)

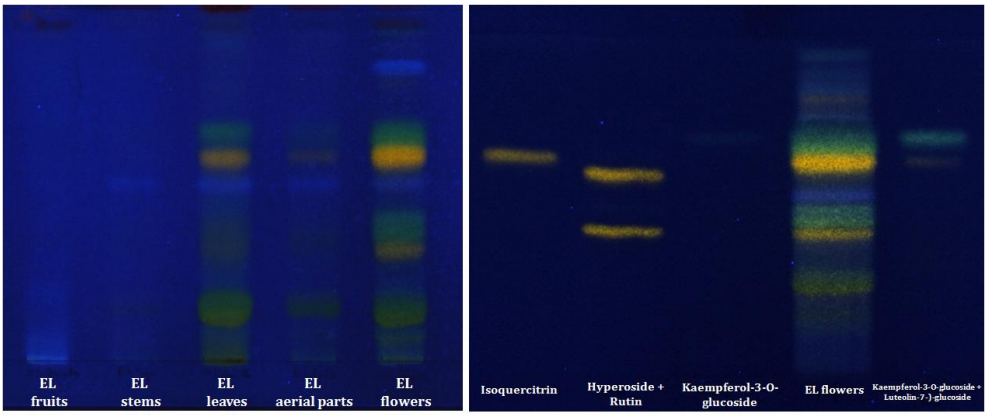


Fig.59. TLC of the main parts of *E. lobata* (spray reagent: DPBAE and PEG; examination under UV light, at 365nm)

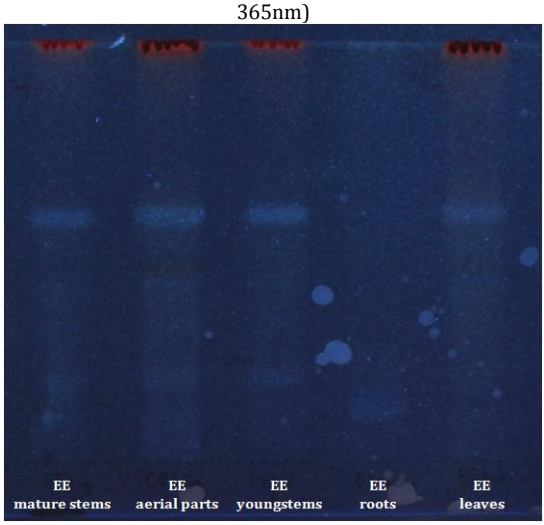


Fig.60. TLC of the main parts of *E. elaterium* (spray reagent: DPBAE and PEG; examination under UV light, at 365nm)

4.4.3. Spectrophotometric methods for the quantification of total flavonoids, polyphenols and hydroxycinnamic acids

4.4.3.1. Quantification of the total flavonoids total by the method described in the European Pharmacopoeia

As it was noticed that the leaves of the species contain the highest amounts of flavonoids, the total flavonoids content was analyzed for samples of leaves (Fig.61).

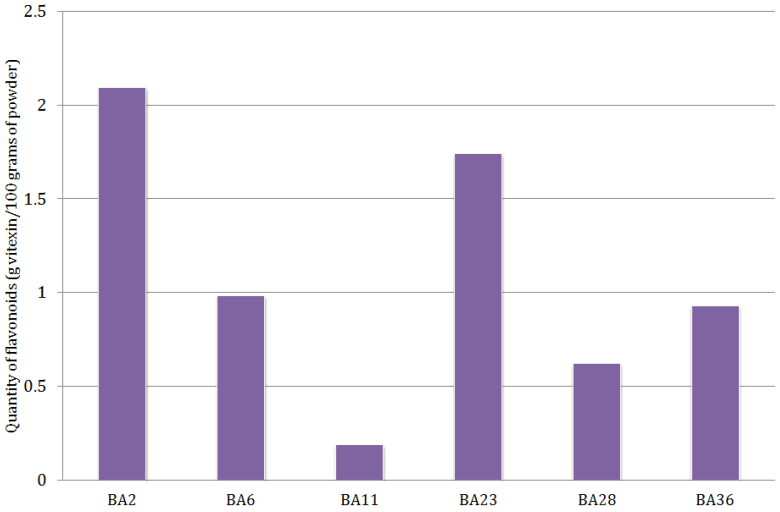


Fig.61. Quantification of the total flavonoids in samples of leaves of *B. alba*

4.4.3.2. Quantification of the total flavonoids, polyphenols and hydroxycinnamic acids by the method described by the Romanian Pharmacopoeia 10th edition and European Pharmacopoeia 9th edition

Selection of the samples on which assays were performed was made in order to offer a general idea on the main parts of each species that are supposed to contain the flavonoidic compounds. Therefore, mainly samples of stems, leaves and aerial parts were tested for the totals flavonoids, polyphenols and hydroxycinnamic acids. Total flavonoids were quantified by the method described in the Romanian Pharmacopoeia 10th edition. The species that proved to contain the highest amounts of flavonoidic and polyphenolic compounds proved to be *E. lobata*. The lowest amounts of compounds were found for *E. elaterium*. Results for the quantification of these totals are found in Table XXIII.

Table XXIII. Quantification of the flavonoidic and polyphenolic totals in samples belonging to the three species

Samples	Polyphenols (g GAE/100g dvp)	Flavonoids (g RE/100g dvp)
BA1	0.64 ± 0.19	0.40 ± 0.13
BA2	3.65 ± 0.39	3.79 ± 0.64
BA24	1.12 ± 0.31	1.47 ± 0.94
EL46	0.90 ± 0.12	0.30 ± 0.06
EL47	1.89 ± 0.45	1.15 ± 0.53
EL50	3.23 ± 0.39	3.75 ± 0.37
EL66	3.35 ± 0.25	1.86 ± 0.59
EL69	3.21 ± 0.12	2.17 ± 0.09
EL71	3.04 ± 0.18	2.89 ± 0.11
EL73	1.21±0.09	0.19 ± 0.05
EL75	4.41 ± 0.52	2.20 ± 0.37
EL77	3.03 ± 0.45	0.13 ± 0.07
EE82	0.02 ± 0.31	0.18 ± 1.17
EE84	0.03 ± 0.79	0.09 ± 1.14
EE85	0.11 ± 0.61	0.23 ± 0.73

The performed assays reported not detectable amounts of hydroxycinnamic acids in the composition of the tested samples.

4.4.4. HPLC-DAD method for the identification and quantification of flavonoids

a) Bryonia alba L.

Identification and quantification of the flavonoids present in the composition of the species was performed on methanolic extracts, prepared as described at 4.3.1.1. and dissolved in methanol. Some flavonoids had to be purified by prep-HPLC, and, in this context, 400-500mg of crude extract were injected into the preparative system. From this amount, fractions of 200-300mg concentrated in the compounds of interest were isolated and subsequently subjected to another preparative analysis that allowed the purification of the main flavonoids.

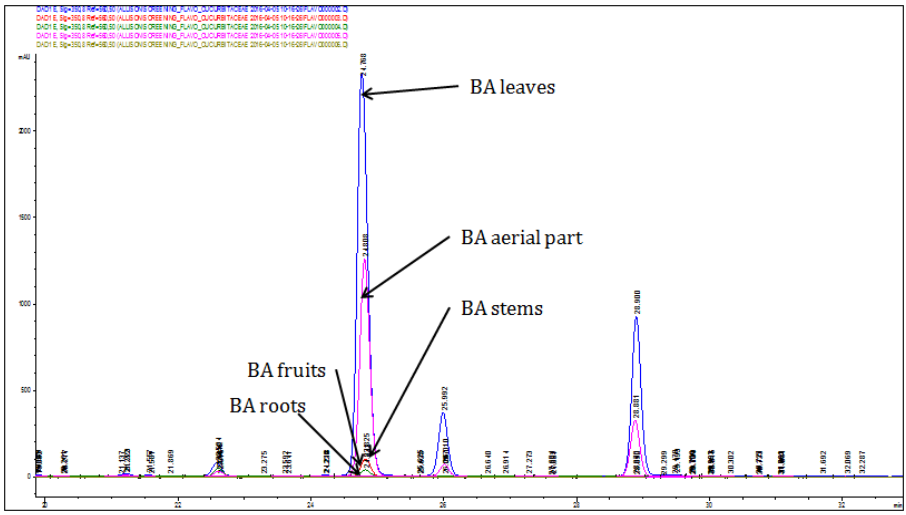


Fig.62. HPLC-DAD chromatogram showing the profile of flavonoids in different parts of *B. alba*

In the composition of the aerial parts, previous studies cite the presence of four flavonoidic compounds: saponarin, vitexin, isovitexin and lutonarin (60,61). Studies performed in the present thesis revealed that only saponarin, lutonarin and isovitexin are present in the composition of the aerial parts.

The present study showed that the parts that proved to be richer in flavonoidic compounds are leaves (Fig.62). The composition of the aerial parts and the leaves was similar, so the analysis of identification and quantification of compounds were further performed on leaves, after the analysis of different samples, collected at different periods of time, which allowed to find the samples that are richer in compounds (Fig.63).

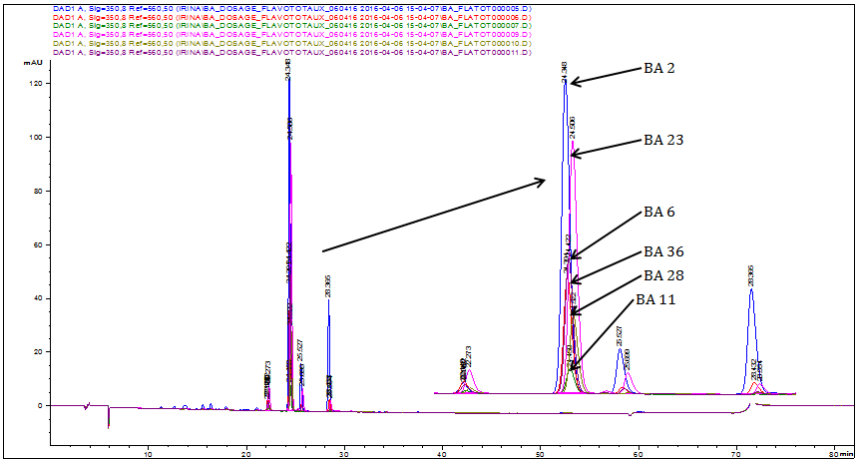


Fig.63. HPLC-DAD chromatogram showing the profile of flavonoids in different samples of leaves of *B. alba*, the parts that proved to be the richest in flavonoids

The main compound that was found in the composition of the leaves was saponarin. The identification of saponarin (also identified by TLC in Fig.58) was performed by comparison with the chromatographic parameters of a reference. The retention time and UV spectrum of the main compound in the extract of leaves of *B. alba* present the same values and profile as the ones that are obtained for the reference saponarin (Fig.64).

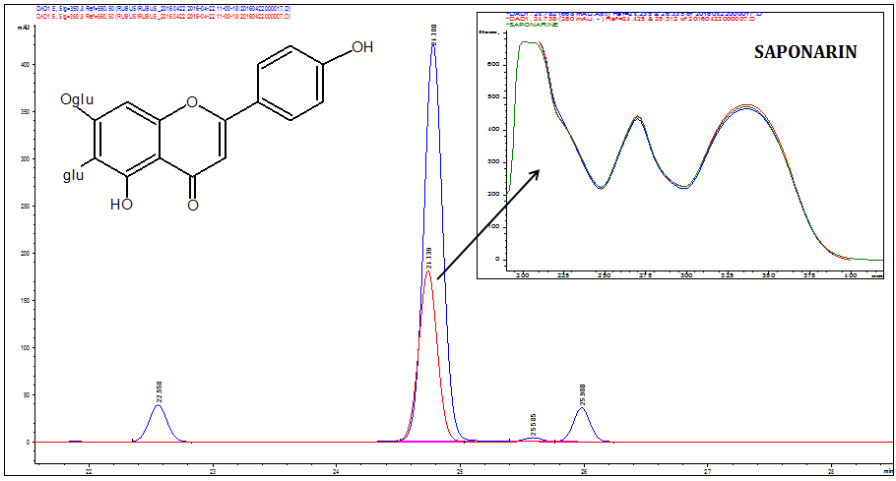


Fig.64. HPLC-DAD identification and UV spectra of saponarin in the leaves of *B. alba*, comparison of the compound found in the extract (blue), in the reference compound (red) and in the data basis with flavonoids spectra of the Laboratory of Pharmacognosy of the ULg (green)

After the identification of the compound, the saponarin, as main compound, was quantified in different samples of the leaves, that were collected at different moments in the development of the species and in different places, in order to establish the differences between the quantity of saponarin (Fig.65).

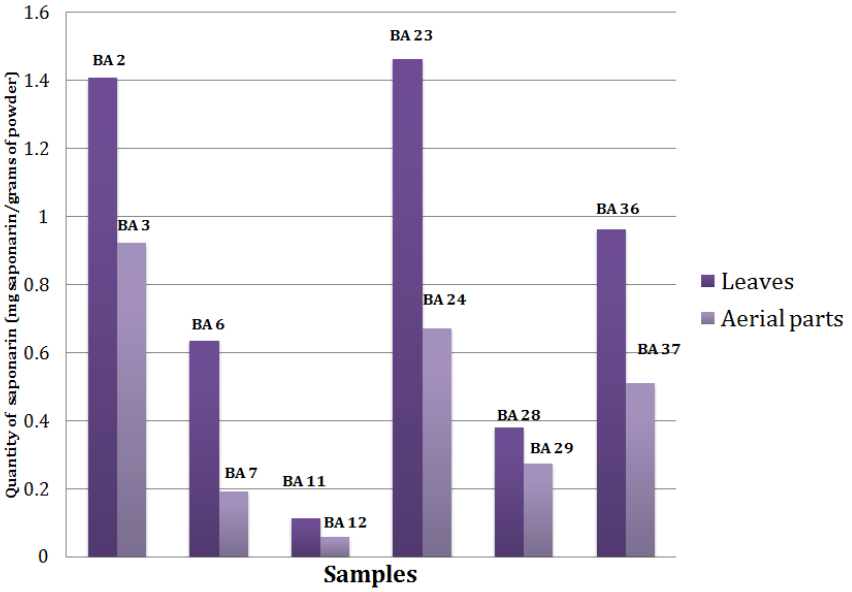


Fig.65. HPLC-DAD histograms showing the quantification of saponarin in different samples of leaves and aerial parts of *B. alba*

The other compounds that were found in the composition of the leaves of the species are isovitexin and lutonarin. Identification of isovitexin was also performed by comparison with a reference (Fig.66).

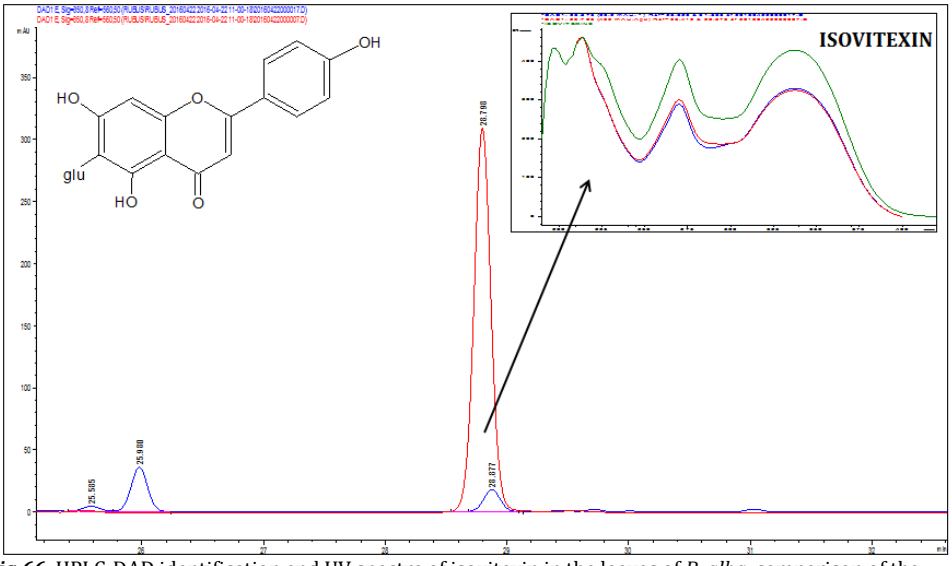


Fig.66. HPLC-DAD identification and UV spectra of isovitexin in the leaves of *B. alba*, comparison of the compound found in the extract (blue), in the reference compound (red) and in the data basis with flavonoids spectra of the Laboratory of Pharmacognosy of the ULg (green)

HPLC-DAD identification of lutonarin (Fig.67) was difficult as no reference was available for comparison. MS and NMR analysis helped to confirm the identity of the compound.

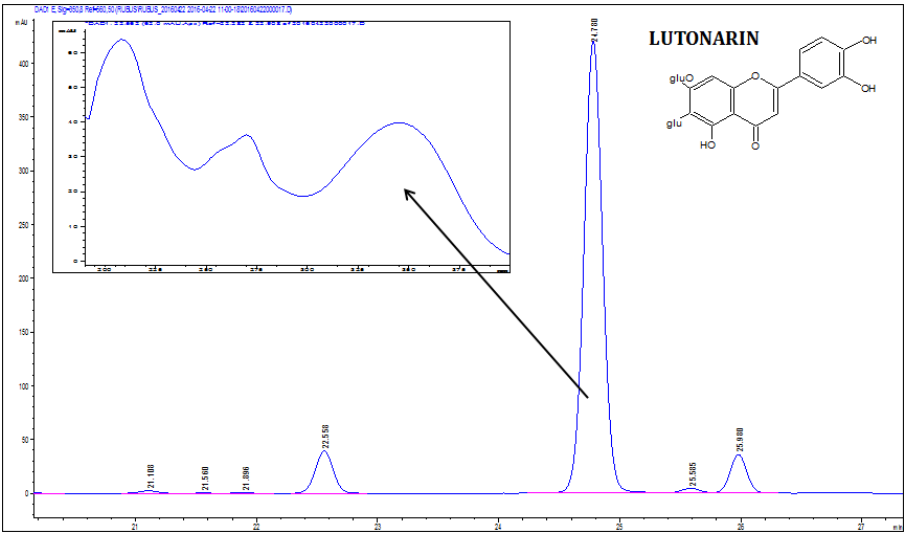


Fig.67. HPLC-DAD identification of the compound that is supposed to be lutonarin in the leaves of *B. alba*

The other compound that was cited in the composition of the species is vitexin. Its presence was not confirmed in the present study. On the other hand, a new compound, never cited before in the composition of *B.alba* is isoorientin, which was identified by means of TLC (Fig.58), HPLC-DAD (Fig.68) and spectroscopic techniques. The confirmation of its structure is the novelty that this study brings.

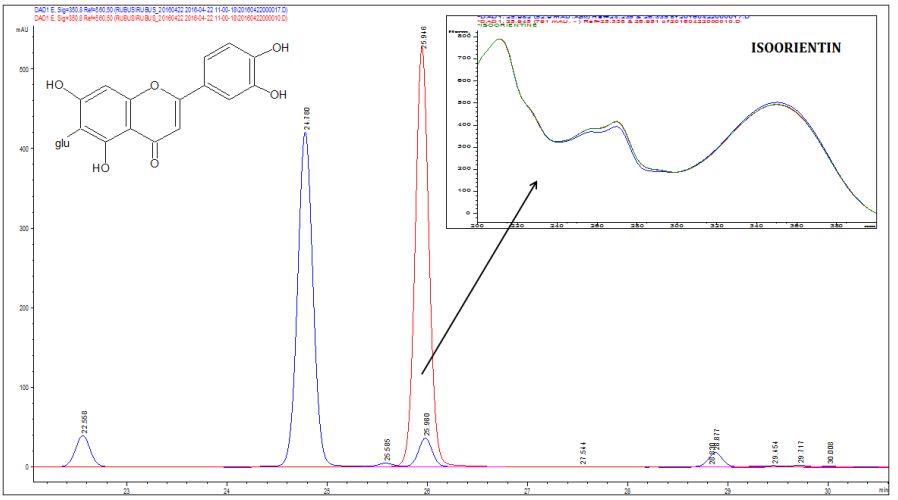


Fig.68. HPLC-DAD identification and UV spectra of isoorientin in the leaves of *B. alba*, comparison of the compound found in the extract (blue), in the reference compound (red) and in the data basis with flavonoids spectra of the Laboratory of Pharmacognosy of the ULg (green)

b) Echinocystis lobata (Michx.) Torr. et A. Gray

Identification of the flavonoids present in the composition of the species was performed on methanolic extracts, prepared as described at 4.3.1.1. and dissolved in methanol, in order to reach a concentration of 1mg/mL.

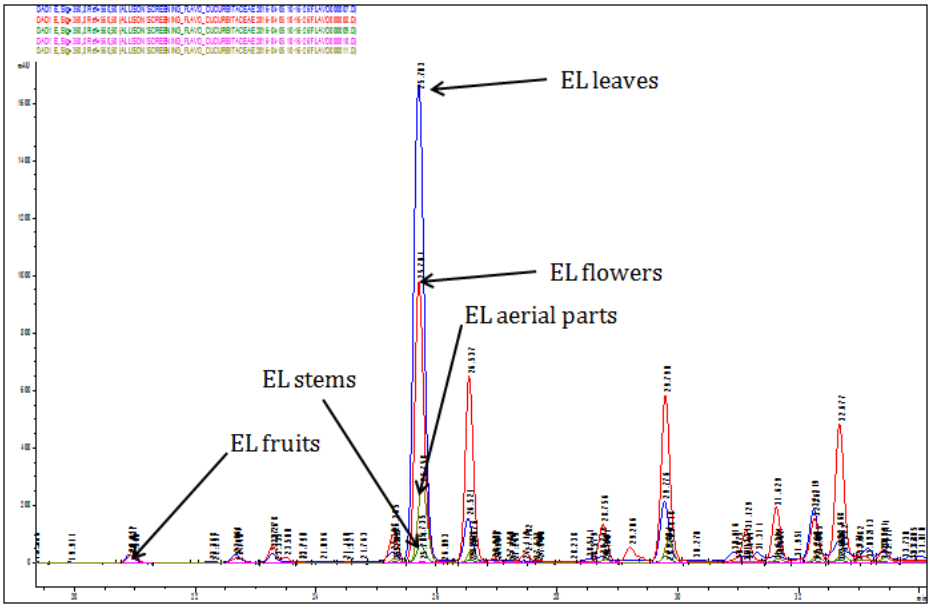


Fig.69. HPLC-DAD chromatogram showing the profile of flavonoids in different parts of *E. lobata*

Existing studies on the flavonoidic compounds in the composition of the species cite the presence of quercetin and isorhamnetin derivatives. One of the compounds changes is identity, being cited both as kaempferol-3-O-glucoside (astragalin) and as quercetin-3-O-glucoside (isoquercitrin). These compounds have similar structure, their only difference is the presence of a hydroxyl group in position 3'. The compound that is being identified by the authors seems to be actually the kaempferol derivative (astragalin) (62). These studies were performed on the aerial parts of this species.

In the present study, the parts that proved to be the richest in flavonoidic compounds were the male flowers (Fig.69). The flavonoidic compounds that were identified in the composition of the species were both kaempferol-3-O-glucoside (astragalin) (Fig.73) and as well as quercetin-3-O-glucoside (isoquercitrin) (Fig.72). Both compounds could be identified by comparison with reference solutions. Another compound that was identified in the composition of the species is rutin. The parameters of the compound that is found in the extract proved to be the same as the ones obtained for a reference solution (Fig.71). Nevertheless, the main peak seems to contain two flavonoidic compounds, as the peak profile corresponding to it seems to be not symmetric, and the corresponding UV spectra also show different profiles (Fig.70). Performed HPLC-DAD analysis could not allow the separation of the two flavonoidic compounds from this peak, neither in the described technical conditions, nor by changing various parameters.

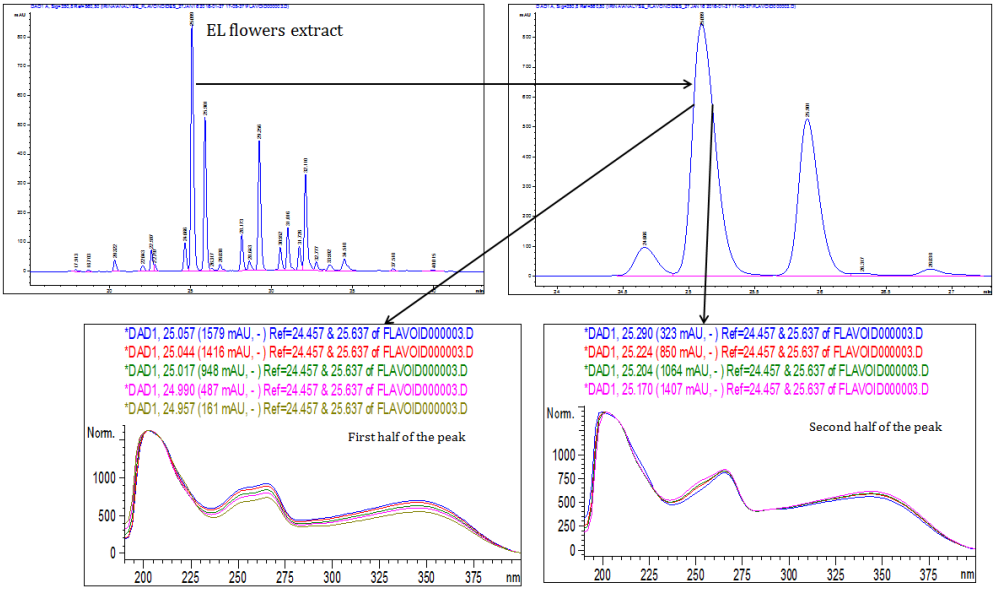


Fig.70. HPLC-DAD chromatogram showing the profile of the main compound in the flowers of *E. lobata*

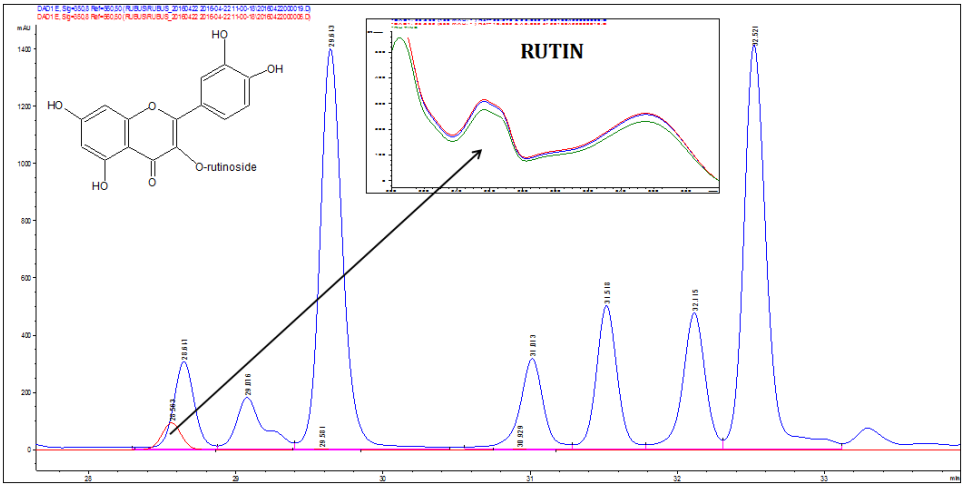


Fig.71. HPLC-DAD identification and UV spectra of rutin in the leaves of *E. lobata*, comparison of the compound found in the extract (blue), in the reference compound (red) and in the data basis with flavonoids spectra of the Laboratory of Pharmacognosy of the ULg (green)

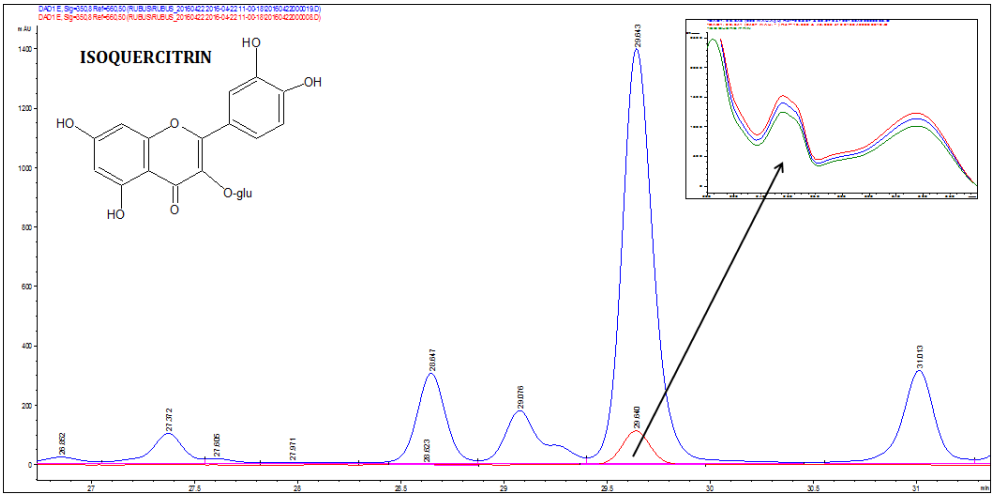


Fig.72. HPLC-DAD identification and UV spectra of isoquercitrin in the leaves of *E. lobata*, comparison of the compound found in the extract (blue), in the reference compound (red) and in the data basis with flavonoids spectra of the Laboratory of Pharmacognosy of the ULg (green)

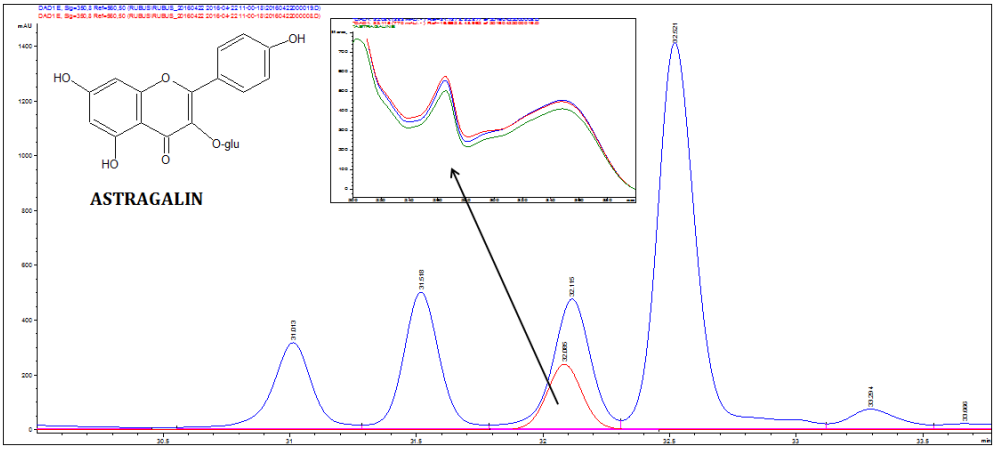


Fig.73. HPLC--DAD identification and UV spectra of astragalin in the leaves of *E. lobata*, comparison of the compound found in the extract (blue), in the reference compound (red) and in the data basis with flavonoids spectra of the Laboratory of Pharmacognosy of the ULg (green)

c) *Ecballium elaterium* (L.) A. Rich.

Identification of the flavonoids present in the composition of the species was performed on methanolic extracts, prepared as described at 4.3.1.1. and dissolved in methanol, in order to reach a concentration of 1mg/mL.

Scientific literature cites the presence of quercetin glucosides, more specifically kaempferol-3-O-glucoside and quercetin-3-O-rutinoside (rutin), in the composition of flowers (63), fruits or leaves (64–67).

The analysis performed in the present study showed that the aerial parts were the richest in the amount of flavonoidic compounds (Fig.74), of which rutin could be identified, by comparison with a reference (Fig.75).

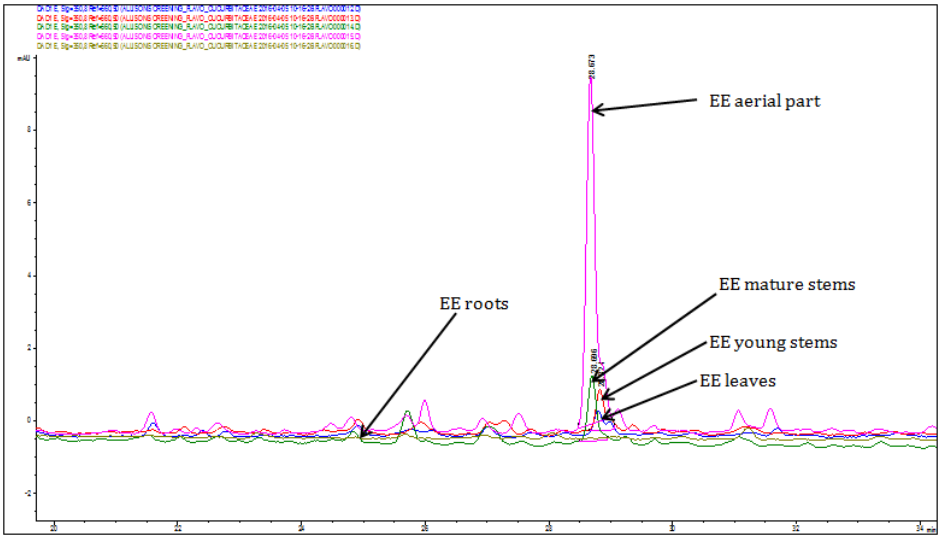


Fig.74. HPLC-DAD chromatogram showing the profile of flavonoids in different parts of *E. elaterium*

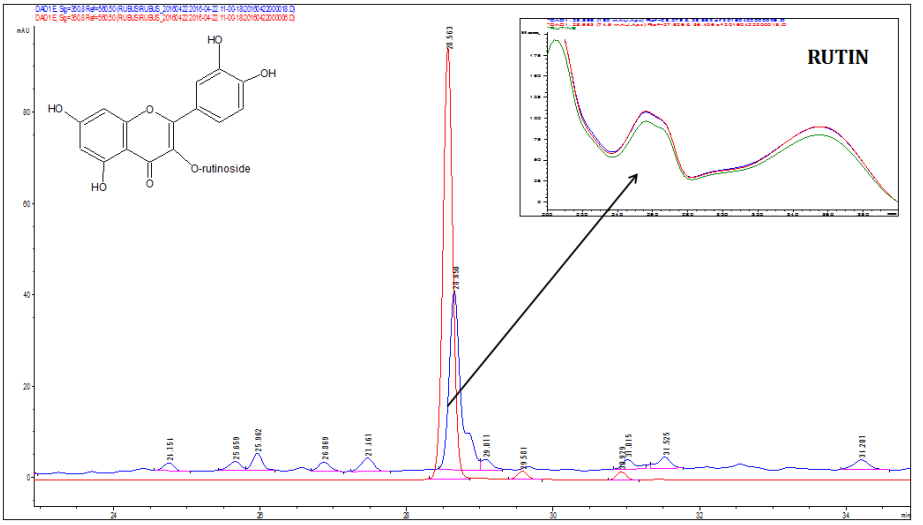


Fig.75. -DAD identification and UV spectra of isoquercitrin in the leaves of *E. elaterium*, comparison of the compound found in the extract (blue), in the reference compound (red) and in the data basis with flavonoids spectra of the Laboratory of Pharmacognosy of the ULg (green)

4.4.5. Hydrolysis of flavonoids for the identification of aglycones

For the leaves of *B. alba*, as no reference was available for the identification of the lutonarin and taken into consideration its structure that reveals a possible hydrolysis to isoorientin or even to luteolin, the hydrolysis of the compounds directly on the powder was performed, following the hydrolysis method described in the 9th edition of the European Pharmacopoeia at the monograph *Acaciae gummi*. The time of hydrolysis was varied between 1 and 6 hours, but it did not significantly influence the results of the conversion of flavonoids (Fig.76-77).

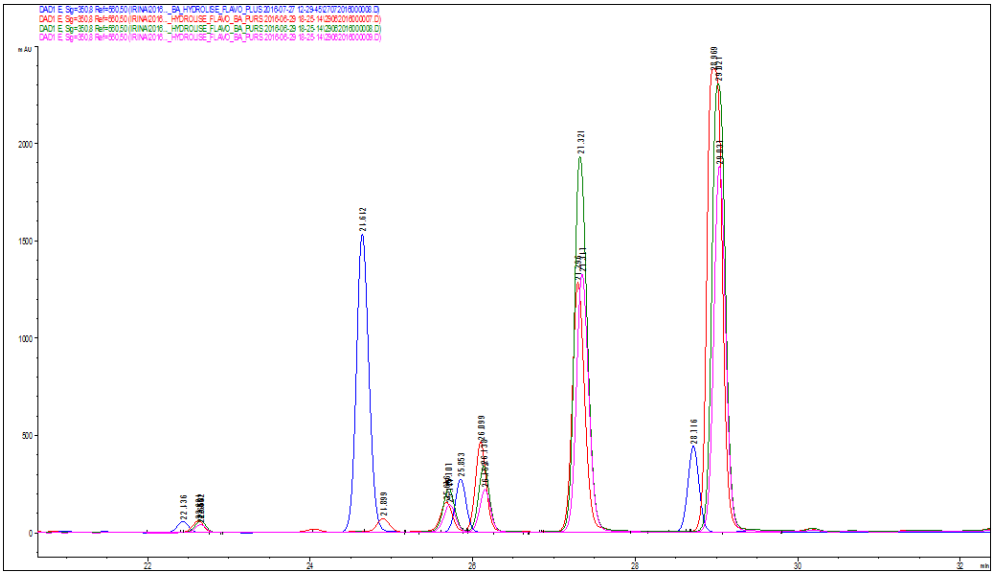


Fig.76. HPLC-DAD chromatogram showing the profile of the samples of leaves of *B. alba*, that were hydrolysed for 1h (red), 2h (green) and 3h (pink), in comparison with the extract non-hydrolysed (blue)

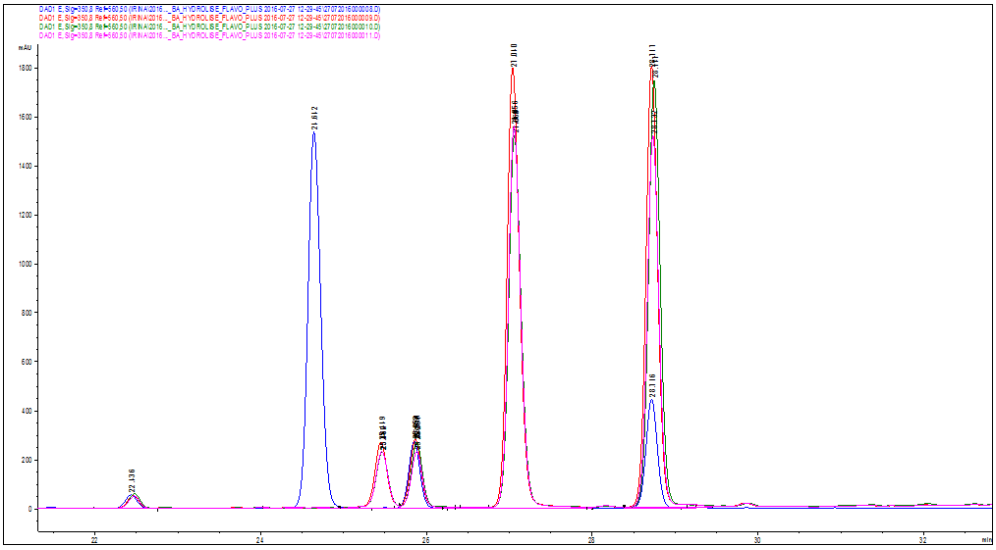


Fig.77. HPLC-DAD chromatogram showing the profile of the samples of leaves of *B. alba*, that were hydrolysed for 4h (red), 5h (green) and 6h (pink), in comparison with the extract non-hydrolysed (blue)

Some of the existing flavonoids suffered hydrolysis and were converted into other similar flavonoids, generally lacking one glucose. Other flavonoids did not loose the glucosidic part and remained non-hydrolysed. HPLC-DAD identification of flavonoids in the leaves of *B. alba* can be found in Fig.78.

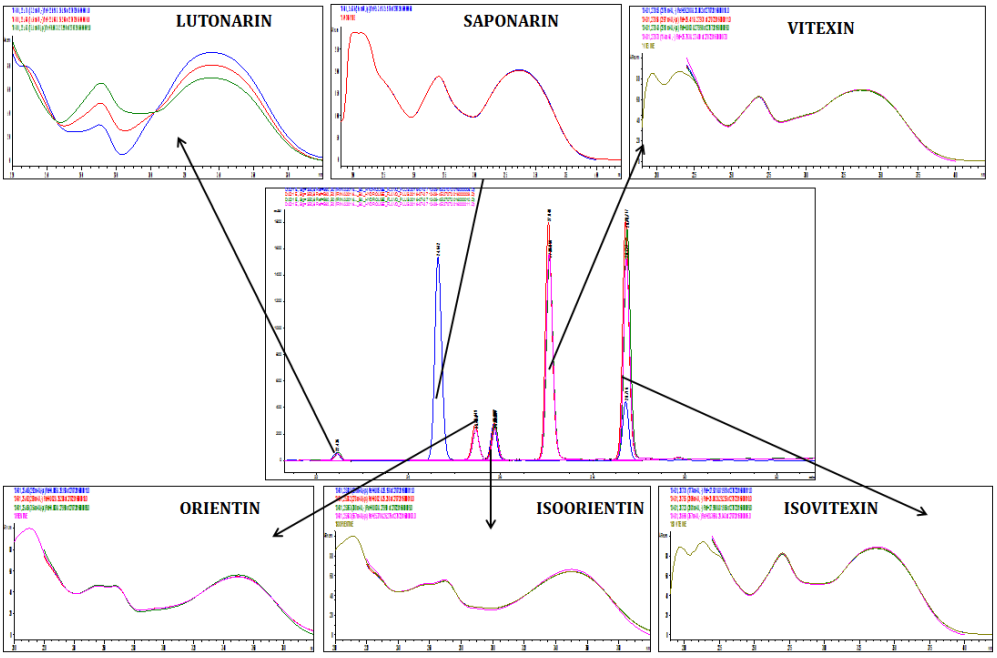


Fig.78. HPLC-DAD chromatogram showing the profile and the UV identification of the compounds that are found in the samples of leaves of *B. alba*, that were hydrolysed for 4h (red), 5h (green) and 6h (pink), in comparison with the extract non-hydrolysed (blue)

4.4.6. HPLC-MS screening of polyphenols

In order to identify the polyphenols that can be found in different samples of the three species, a HPLC-MS screening of these compounds was performed. The screening allowed to identify some of the polyphenols that are present in the tested samples and to quantify their amount in the samples. Analysis were performed on extracts that are prepared as described at 4.3.1.2. Tested samples are found in Annex, Table I. The purpose of the quantification was to notice the difference between samples, that are collected at different periods in the evolution of the species and from different places. Compounds were identified and quantified by comparing the results with the MS spectra and retention times of existing references in database of the Laboratory of Biopharmacy of the UMF Cluj. Results were expressed as mg polyphenol/100g dried vegetal product (dvp) and can be found in Table XXIV-XXXVII.

a) Bryonia alba L.

Table XXIV. Quantification of the polyphenols in samples of fruits of *B. alba*

Sample	*p*-Coumaric acid	Ferulic acid
BA 26	0.22 ± 0.17	0.11 ± 1.54
BA30	0.26 ± 1.78	ND
BA 39	ND	ND

ND=not detected, below limit of quantification; Values represent mean ± standard deviation (SD)

Table XXV. Quantification of the polyphenols in samples of leaves of *B. alba*

Sample	*p*-Coumaric acid	Ferulic acid	Isoquercitrin	Rutin	Quercitrin	Apigenin
BA 2	1.55 ± 0.17	0.26 ± 3.79	ND	ND	0.82 ± 1.56	1.48 ± 0.08
BA 6	0.23 ± 0.10	0.20 ± 1.64	ND	ND	ND	ND
BA 11	0.51 ± 1.19	0.17 ± 5.12	ND	ND	ND	ND
BA 23	0.90 ± 1.79	0.26 ± 0.05	ND	0.53 ± 1.02	0.63 ± 7.29	0.19 ± 1.19
BA 28	0.32 ± 4.29	0.12 ± 0.71	0.27 ± 6.28	0.46 ± 2.79	0.13 ± 0.12	ND
BA 36	0.38 ± 2.37	0.18 ± 2.01	ND	ND	0.28 ± 4.13	ND

ND=not detected, below limit of quantification; Values represent mean ± standard deviation (SD)

Table XXVI. Quantification of the polyphenols in samples of aerial parts of *B. alba*

Sample	*p*-Coumaric acid	Ferulic acid	Isoquercitrin	Rutin	Quercitrin	Apigenin
BA 3	1.16 ± 0.29	0.27 ± 0.07	ND	ND	0.27 ± 0.17	0.28 ± 1.97
BA 7	0.81 ± 0.27	0.41 ± 0.04	ND	0.38 ± 0.69	0.85 ± 0.18	0.09 ± 2.52
BA 12	ND	ND	ND	ND	0.32 ± 0.12	ND
BA 24	1.62 ± 0.79	0.61 ± 0.03	21.43 ± 4.59	0.52 ± 0.08	2.35 ± 1.39	0.25 ± 0.01
BA 29	0.77 ± 0.09	0.23 ± 0.17	ND	ND	ND	ND
BA 37	0.59 ± 0.27	0.19 ± 0.04	0.66 ± 0.05	ND	0.23 ± 0.06	ND

ND=not detected, below limit of quantification; Values represent mean ± standard deviation (SD)

Table XXVII. Quantification of the polyphenols in samples of roots of *B. alba*

Sample	*p*-Coumaric acid	Ferulic acid	Isoquercitrin	Rutin	Quercitrin
BA 4	0.20 ± 0.79	0.16 ± 0.49	ND	ND	ND
BA 8	0.15 ± 1.89	0.15 ± 1.19	ND	ND	ND
BA 13	0.18 ± 5.07	0.07 ± 0.72	ND	ND	ND
BA 25	0.23 ± 4.71	ND	0.39 ± 0.18	ND	0.14 ± 2.17
BA 38	0.53 ± 3.31	ND	0.16 ± 0.93	0.07 ± 0.97	0.09 ± 3.17

ND=not detected, below limit of quantification; Values represent mean ± standard deviation (SD)

Table XXVIII. Quantification of the polyphenols in samples of stems of *B. alba*

Sample	*p*-Coumaric acid	Ferulic acid	Hyperoside	Isoquercitrin	Rutin	Quercitrin
BA 1	1.29 ± 0.29	0.33 ± 0.09	ND	6.67 ± 2.58	0.17 ± 0.06	1.33 ± 1.46
BA 5	0.30 ± 0.08	0.33 ± 0.27	ND	ND	ND	0.37 ± 0.07
BA 10	0.21 ± 1.24	0.19 ± 0.01	ND	ND	ND	ND
BA 22	0.47 ± 0.73	0.19 ± 1.47	98.63 ± 4.89	2.31 ± 0.79	1.33 ± 1.02	1.69 ± 0.96
BA 27	0.47 ± 0.39	0.35 ± 1.15	ND	ND	ND	0.74 ± 0.29
BA 35	0.38 ± 0.56	0.17 ± 0.28	ND	0.68 ± 0.19	0.28 ± 0.06	0.77 ± 0.04

ND=not detected, below limit of quantification; Values represent mean ± standard deviation (SD)

b) *Echinocystis lobata* (Michx.) Torr. et A. Gray

Table XXIX. Quantification of the polyphenols in samples of flowers of *E. lobata*

Sample	*p*-Coumaric acid	Ferulic acid	Isoquercitrin	Rutin	Quercitrin	Quercetin	Kaempferol
EL 34	1.3 ± 0.08	0.57 ± 0.08	61.78 ± 0.79	27.72 ± 0.71	32.26 ± 0.27	0.71 ± 0.76	0.98 ± 0.24
EL 50	1.31± 0.18	0.51 ± 1.03	231.86 ± 1.92	106.36 ± 0.76	67.84 ± 0.74	ND	2.18 ± 0.04
EL 56	4.25 ± 0.01	0.75 ± 0.37	84.07 ± 5.83	26.31 ± 5.10	33.65 ± 5.17	2.04 ± 0.22	1.63 ± 0.09
EL 63	2.22 ± 1.22	0.29 ± .001	92.86 ± 4.37	26.50 ± 2.21	17.57 ± 0.11	0.40 ± 0.12	0.23 ± 0.07
EL 71	1.81± 0.74	0.62 ± 0.05	238.22 ± 0.54	83.06 ± 0.05	56.81 ± 0.83	5.29 ± 0.12	2.66 ± 3.02
EL 77	4.13 ± 0.08	0.30 ± 0.02	86.86 ± 5.61	22.01 ± 7.79	23.31 ± 0.77	2.52 ± 0.01	1.13 ± 0.07

ND=not detected, below limit of quantification; Values represent mean ± standard deviation (SD)

Table XXX. Quantification of polyphenols in samples of fruits of *E. lobata*

Sample	*p*-Coumaric acid	Isoquercitrin	Rutin	Quercitrin	Quercetin	Kaempferol
EL 57	0.81 ± 0.18	8.16 ± 4.39	2.75 ± 0.64	3.42 ± 1.74	0.20 ± 0.07	0.12 ± 0.01
EL 64	0.18 ± 0.79	3.88 ± 1.19	1.53 ± 0.47	1.24 ± 0.08	0.04 ± 0.01	ND
EL 72	0.36 ± 0.23	6.15 ± 1.58	2.62 ± 1.07	2.11 ± 0.98	0.08 ± 0.78	ND
EL 77	ND	8.48 ± 1.29	3.07 ± 1.91	3.34 ± 0.54	0.23 ± 0.04	ND

ND=not detected, below limit of quantification; Values represent mean ± standard deviation (SD)

Table XXXI. Quantification of polyphenols in samples of leaves of *E. lobata (1)*

Sample	Chlorogenic acid	*p*-Coumaric acid	Ferulic acid	Isoquercitrin	Rutin
EL 15	31.11 ± 4.28	17.85 ± 4.58	ND	23.96 ± 5.27	1.28 ± 0.11
EL 19	ND	ND	ND	3.30 ± 1.05	0.93 ± 0.09
EL 32	ND	19.26	ND	9.95 ± 2.09	1.27 ± 1.02
EL 42	ND	ND	0.48 ± 0.07	1.17 ± 0.53	0.32 ± 1.12
EL 47	ND	10.94 ±1.17	1.07 ± 5.01	98.96 ± 0.98	30.46 ± 4.03
EL 54	ND	11.37 ± 1.75	0.40 ± 0.08	9.85 ± 2.57	2.15 ± 0.28
EL 61	ND	12.86 ± 1.27	0.39 ± 0.28	45.05 ± 5.07	4.10 ± 1.48
EL 75	ND	15.10 ± 0.54	0.67 ± 1.95	163.02 ± 0.01	37.78 ± 1.14

ND=not detected, below limit of quantification; Values represent mean ± standard deviation (SD)

Table XXXII. Quantification of polyphenols in samples of leaves of *E. lobata (2)*

Sample	Quercitrin	Quercetin	Kaempferol	Apigenin
EL 15	33.75 ± 5.89	0.23 ± 0.04	0.14 ± 0.01	1.32 ± 1.07
EL 19	12.39 ± 1.39	ND	ND	0.76 ± 0.19
EL 32	10.63 ± 4.18	ND	0.46 ± 0.08	0.72 ± 0.24
EL 42	5.97 ± 1.74	ND	ND	ND
EL 47	118.84 ± 3.9	3.59 ± 8.65	8.51 ± 1.19	ND
EL 54	22.21 ± 5.07	ND	ND	1.76 ± 0.04
EL 61	44.33 ± 6.07	0.66 ± 0.07	4.80 ± 1.18	0.69 ± 0.73
EL 75	128.96 ± 7.77	7.30 ± 1.18	6.23 ± 5.08	ND

ND=not detected, below limit of quantification; Values represent mean ± standard deviation (SD)

Table XXXIII. Quantification of polyphenols in samples of aerial parts of *E. lobata (1)*

Samples	*p*-Coumaric acid	Ferulic acid	Sinapic acid	Hyperoside	Isoquercitrin	Rutin
EL 16	0.96 ± 0.01	ND	0.10 ± 0.02	ND	11.81 ± 5.41	1.25 ± 0.07
EL 20	6.33 ± 1.17	1.64 ± 0.02	0.16 ± 0.05	1.97 ± 0.18	11.86 ± 4.27	26.90 ± 1.43
EL 33	ND	ND	ND	ND	40.43 ± 7.82	10.74 ± 0.76
EL 33*	14.24 ± 4.71	ND	ND	ND	41.17 ± 2.62	6.45 ± 4.12
EL 43	6.32 ± 1.13	0.53 ± 0.81	0.14 ± 0.79	ND	2.55 ± 0.89	1.79 ± 2.18
EL 48	13.56 ± 0.17	ND	ND	ND	90.92 ± 0.05	9.05 ± 2.98
EL 55	6.29 ± 1.28	0.53 ± 0.13	ND	ND	58.74 ± 0.09	19.77 ± 0.61
EL 62	5.21 ± 1.61	0.62 ± 0.06	ND	ND	86.56 ± 8.37	22.56 ± 0.22
EL 66	7.62 ± 1.88	0.24 ± 1.17	ND	ND	115.86 ± 4.56	42.00 ± 7.24
EL 70	6.88 ± 2.23	0.61 ± 0.57	ND	ND	53.25 ± 1.49	11.81 ± 0.28
EL 76	22.58 ± 0.78	0.72 ± 0.28	0.05 ± 7.03	ND	82.10 ± 2.05	5.42 ± 0.17

ND=not detected, below limit of quantification; Values represent mean ± standard deviation (SD)

Table XXXIV. Quantification of polyphenols in samples of aerial parts of *E. lobata (2)*

Samples	Quercitrin	Quercetin	Kaempferol	Apigenin
EL 16	15.00 ± 1.79	0.36 ± 0.08	0.55 ± 0.07	ND
EL 20	6.47 ± 2.26	0.32 ± 0.08	1.53 ± 1.01	0.26 ± 0.09
EL 33	36.81 ± 5.57	7.07 ± 1.29	11.77 ± 2.18	0.52 ± 0.03
EL 33*	70.38 ± 1.00	7.39 ± 2.81	16.36 ± 5.01	ND
EL 43	11.51 ± 0.06	ND	1.02 ± 2.22	ND
EL 48	96.56 ± 0.39	ND	0.57 ± 0.03	ND
EL 55	36.98 ± 0.08	1.42 ± 1.11	2.13 ± 1.13	ND
EL 62	73.16 ± 1.18	2.89 ± 1.07	4.14 ± 1.11	ND
EL 66	87.09 ± 1.23	ND	0.86 ± 0.05	ND
EL 70	20.04 ± 1.11	1.09 ± 0.04	1.22 ± 1.07	ND
EL 76	69.34 ± 4.34	2.59 ± 0.01	6.95 ± 3.06	2.51 ± 5.17

ND=not detected, below limit of quantification; Values represent mean ± standard deviation (SD)

Table XXXV. Quantification of polyphenols in samples of stems of *E. lobata (1)*

Sample	*p*-Coumaric acid	Ferulic acid	Sinapic acid	Isoquercitrin	Rutin
EL 14	1.51 ± 1.02	0.46 ± 1.28	ND	2.19 ± 0.27	0.25 ± 0.06
EL 18	1.30 ± 0.66	0.39 ± 0.07	0.14 ± 0.39	4.28 ± 1.91	1.31 ± 0.57
EL 31	1.27 ± 0.22	0.52 ± 0.29	0.54 ± 0.06	6.06 ± 0.79	1.55 ± 0.48
EL 41	0.58 ± 0.04	0.17 ± 0.74	ND	1.17 ± 0.04	0.53 ± 1.17
EL 46	2.09 ± 1.55	0.12 ± 3.23	ND	29.51 ± 5.55	6.53 ± 4.22
EL 52	1.35 ± 0.12	0.21 ± 0.08	ND	20.61 ± 2.21	6.32 ± 3.33
EL 53	1.04 ± 0.75	0.21 ± 0.01	0.28 ± 0.04	15.58 ± 0.09	4.40 ± 2.37
EL 59	3.86 ± 1.28	ND	ND	2.77 ± 0.89	0.55 ± 0.33
EL 60	1.01 ± 1.11	0.41 ± 0.37	ND	9.93 ± 0.33	1.99 ± 1.04
EL 67	2.04 ± 0.29	0.37 ± 0.01	ND	11.93 ± 1.93	1.11 ± 0.03
EL 68	4.33 ± 2.23	0.45 ± 0.06	ND	22.25 ± 2.22	2.62 ± 2.27
EL 73	2.71 ± 0.27	1.42 ± 0.22	0.44 ± 0.44	27.83 ± 4.19	5.17 ± 0.21
EL 74	ND	10.70 ± 4.14	0.46 ± 1.20	19.78 ± 7.68	3.00 ± 0.30

ND=not detected, below limit of quantification; Values represent mean ± standard deviation (SD)

Table XXXVI. Quantification of polyphenols in samples of stems of *E .lobata (2)*

Sample	Quercitrin	Quercetin	Kaempferol
EL 14	1.99 ± 0.14	ND	0.05 ± 1.11
EL 18	2.82 ± 1.11	ND	0.32 ± 2.87
EL 31	1.55 ± 0.28	1.57 ± 1.14	1.10 ± 0.08
EL 41	1.73 ± 1.13	0.10 ± 0.01	0.21 ± 0.01
EL 46	11.00 ± 0.08	ND	0.14 ± 2.24
EL 52	5.52 ± 1.01	0.15 ± 1.40	0.27 ± 1.18
EL 53	6.00 ± 1.03	ND	0.22 ± 0.02
EL 59	2.01 ± 0.99	ND	ND
EL 60	0.82 ± 0.21	ND	ND
EL 67	3.00 ± 0.88	0.79 ± 0.27	0.34 ± 1.15
EL 68	3.34 ± 1.14	0.94 ± 0.24	ND
EL 73	5.32 ± 1.22	4.34 ± 2.34	2.15 ± 0.06
EL 74	34.07 ± 2.68	ND	ND

ND=not detected, below limit of quantification; Values represent mean ± standard deviation (SD)

c) Ecballium elaterium *(L.) A. Rich.*

Table XXXVII. Quantification of the polyphenols in samples of *E. elaterium*

Sample	*p*-Coumaric acid	Ferulic acid	Isoquercitrin	Rutin	Quercitrin
EE 82	5.76 ± 0.17	ND	0.35 ± 0.73	0.26 ± 0.17	0.55 ± 1.15
EE 83	3.73 ± 1.14	ND	ND	0.26 ± 0.07	ND
EE 84	ND	ND	ND	ND	ND
EE 85	3.61	0.25 ± 0.01	ND	ND	ND
EE 86	ND	ND	ND	ND	0.04 ± 0.96

ND=not detected, below limit of quantification; Values represent mean ± standard deviation (SD)

4.4.7. Preparative HPLC-DAD isolation of flavonoidic compounds

The isolation of the flavonoidic compounds was perfomed by adapting the HPLC-DAD method used for the identification of compounds to a preparative system. The isolation of compounds was performed in two stages, in order to obtain adequate quantities of each pure compound. The first stage consisted in the pre-concentration of the extract, in order to obtain blocks of fractions concentrated in the compounds of interest. Crude extract was dissolved in methanol in order to recover quantitatively the compounds of interest, due to the fact that the starting crude extract was not soluble in the mobile phase, which corresponds to the first concentration in the gradient of the isolation method. The methanolic solution was injected in the preparative system. In the used conditions, two important fractions were obtained: the solvent front, that also contains the compounds of interest, in lower amounts and the fraction concentrated in the compounds of interest (Fig.79). After the analytical HPLC-DAD confirmation of the composition of the fractions, they were re-injected in the preparative system in order to afford the flavonoidic compounds of interest (Fig.80).

a) *Bryonia alba* L.

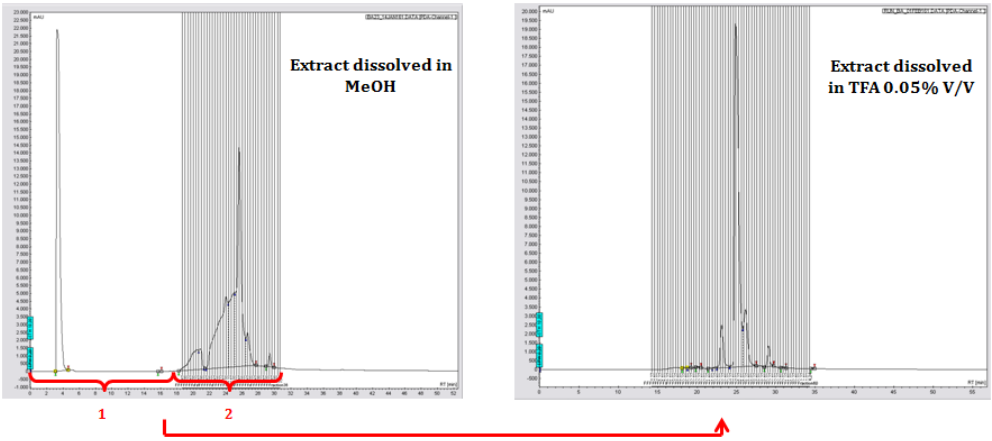


Fig.79. Isolation of compounds in samples of leaves of *B. alba*

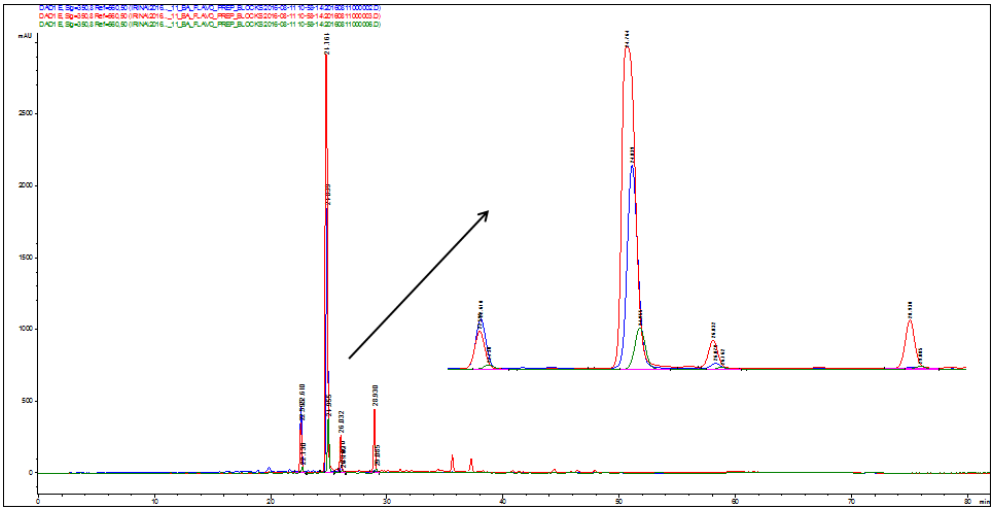


Fig.80. HPLC-DAD chromatogram showing the similar profile of fractions isolated from the leaves of *B. alba:* blue - the fraction at the beginning, corresponding to min. 0-20 in the isolation method (Fig.72.1), red – the fraction containing the compounds, corresponding to min. 20-40 in the isolation method (Fig.72.2) and green – the starting extract for the isolation of compounds

The fractions corresponding to the 4 flavonoidic compounds in the composition of the leaves of *B. alba* were collected and the confirmation of their identity was performed by HPLC-DAD. The method allowed the separation of lutonarin, saponarin, isoorientin and isovitexin. The only compound that contained more than one compound in the isolated fraction is isoorientin, that also contained traces of the main compound, saponarin (Fig.81).

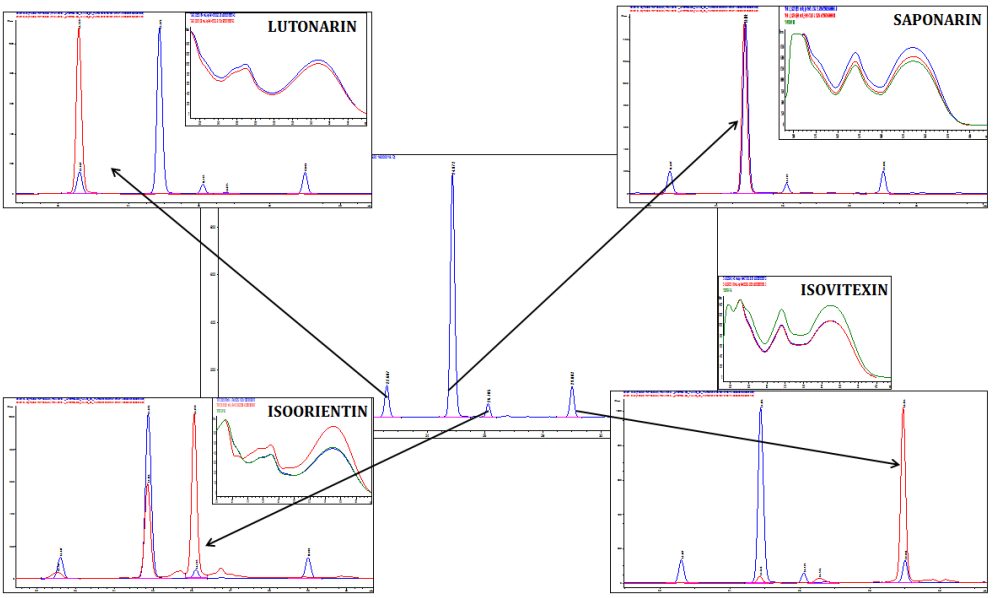


Fig.81. Isolated of compounds from the samples of leaves of *B. alba*

b) *Echinocystis lobata* (Michx.) Torr. et A. Gray

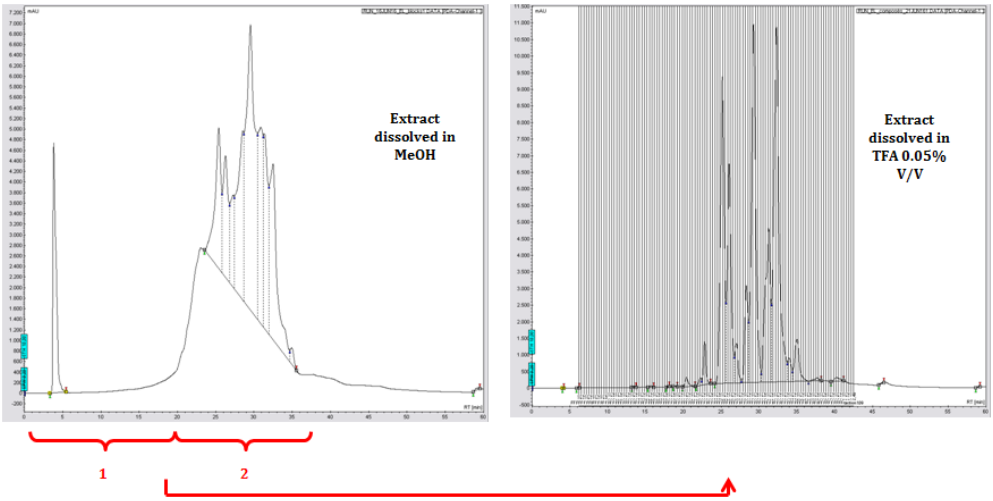


Fig.82. Isolation of compounds in samples of flowers of *E. lobata*

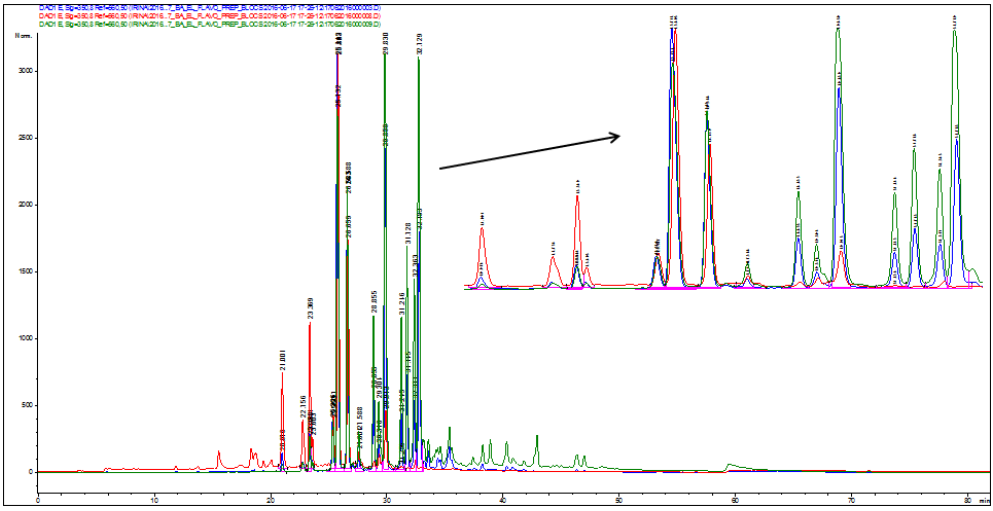


Fig.83. HPLC-DAD chromatogram showing the similar profile of fractions isolated from the flowers of *E.lobata:* blue - the fraction at the beginning, corresponding to min. 0-20 in the isolation method (Fig.75.1), red – the fraction containing the compounds, corresponding to min. 20-40 in the isolation method (Fig.75.2) and green – the departure extract for the isolation of compounds

The separation of the compounds in samples of flowers of *E. lobata* was performed in the same way as described for the leaves of *B .alba* (Fig.82). As the starting extract contained more flavonoidic compounds, the separation of compounds proved to be more difficult (Fig.84). Confirmation of the identity of compounds was also performed by HPLC-DAD (Fig.83).

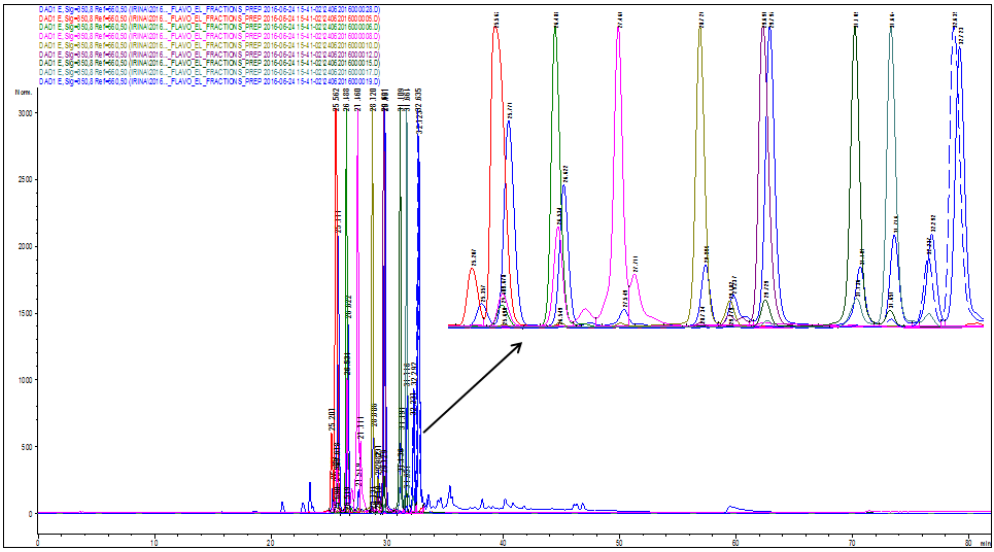


Fig.84. HPLC-DAD chromatogram showing different fractions isolated from the flowers of *E. lobata*

Regarding the main compound found in the flowers of E.lobata, whose peak is dedoubled, preparative HPLC-DAD could not afford the separation of the two compounds found in its composition, even if the used column was different from the one used in the analytical system (Fig.85).

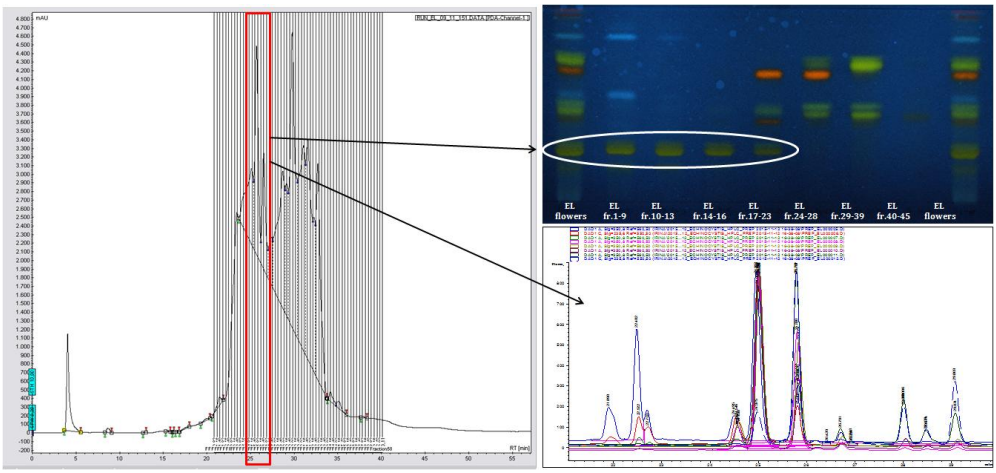


Fig.85. HPLC-DAD chromatogram showing the attempts to separate the two compounds found in the composition of the main peak obtained from the flowers of *E. lobata*

4.4.8. Structure identification of the flavonoidic compounds

The structure identification was performed by spectrometric and spectroscopic methods. Where available, reference compounds were also analyzed, in order to compare the results with the compounds that were isolated.

4.4.8.1. Mass spectrometry

a) Bryonia alba L.

The MS spectra of the isolated compounds allowed to identify the peak corresponding to the molecular weight and the fragmentation profile of each molecule (Fig.86-89).

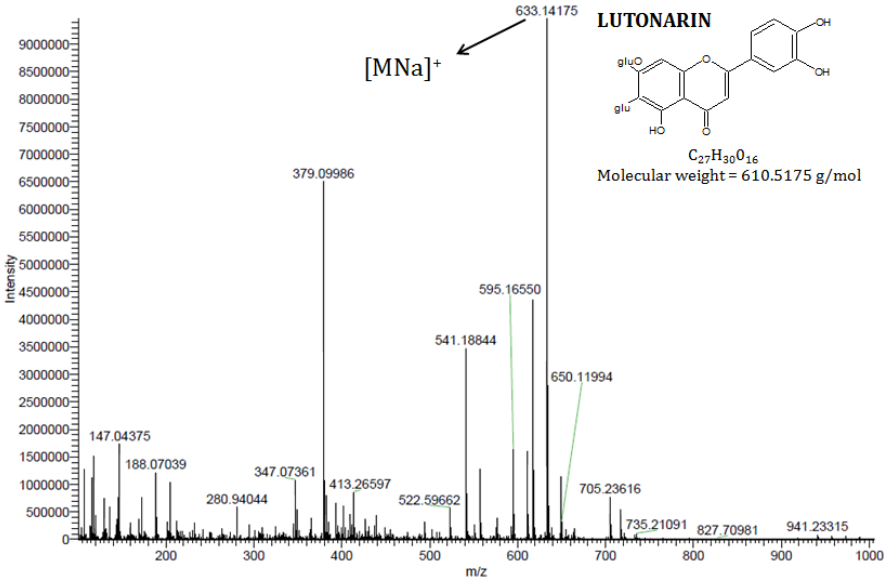


Fig.86. MS spectra of the lutonarin isolated from the leaves of *B. alba*

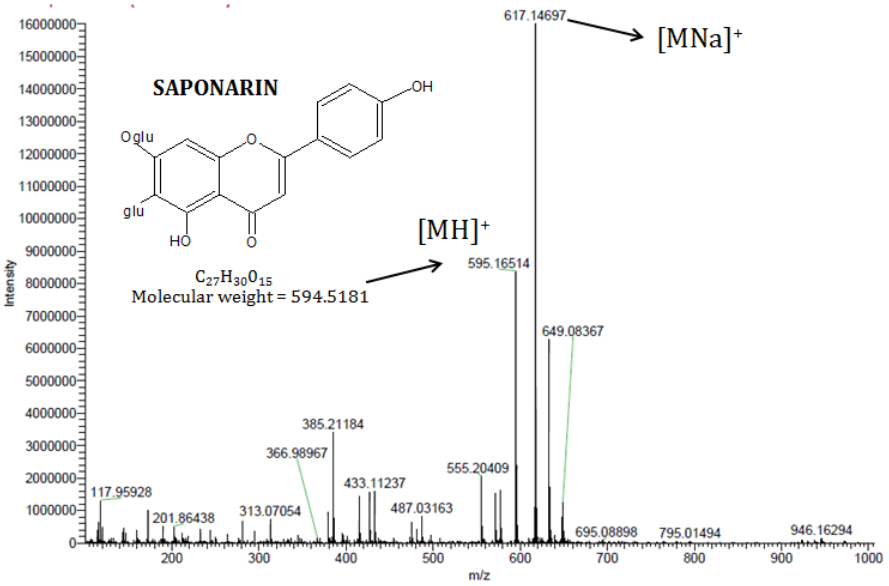


Fig.87. MS spectra of the saponarin isolated from the leaves of *B. alba*

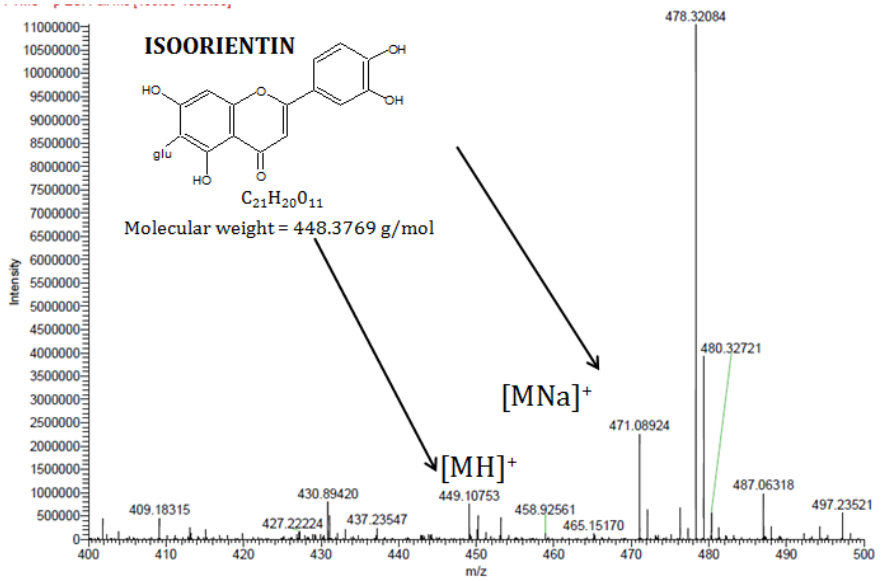


Fig.88. MS spectra of the isoorientin isolated from the leaves of *B. alba*

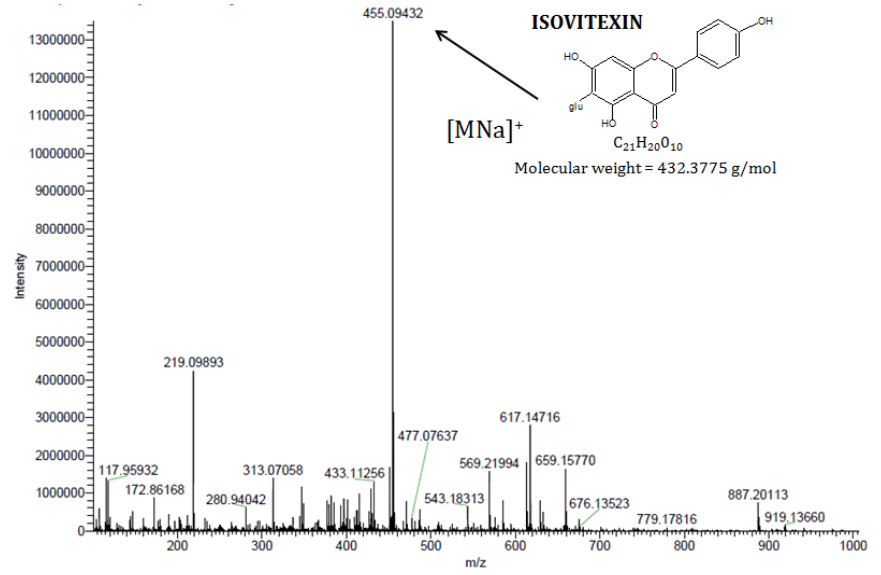


Fig.89. MS spectra of the isovitexin isolated from the leaves of *B. alba*

b) Echinocystis lobata *(Michx.) Torr. et A. Gray*

As analytical and preparative HPLC-DAD could not afford the separation of the compounds, the TLC-MS technique was used. Because of the difference of migration between the two compounds (two very close spots on TLC), this technique provided the molecular peak corresponding to the compounds, which proved to be very close. The two compounds can be positional isomers (Fig.90).

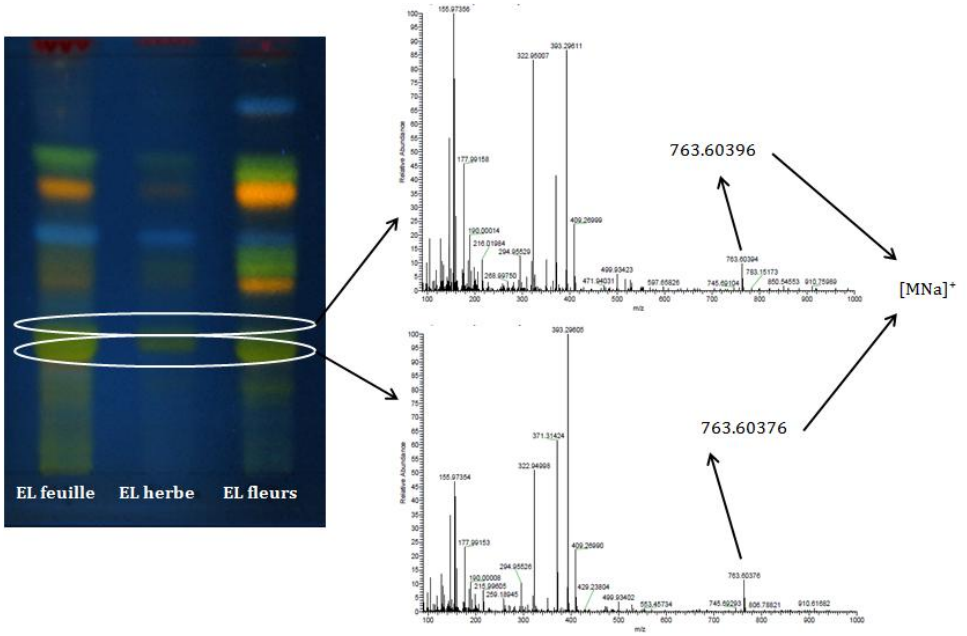


Fig.90. TLC-MS revealing the molecular weights of the two compounds found in the composition of the main peak obtained from the flowers of *E. lobata*

The two compounds could not be identified by the used techniques. Further analysis are needed in order to clearly establish their chemical identity. The mass spectrum of the two compounds provided the molecular weight, which proved to be similar for the two compounds.

4.4.8.2. Nuclear Magnetic Resonance

The NMR analysis allowed to complete the structural identification of the compounds isolated from the leaves of *B. alba*. As the isolation of compounds in the flowers of *E.lobata* was not completely performed, the NMR analysis of compounds could not be performed.

The ^{1}H-NMR, ^{13}C-NMR, COSY, HSQC, HMBC and ^{13}C-APT techniques could allow to confirm the identity of some of the isolated compounds. Where available, reference compounds were studied in the same conditions, in order to identify the similarities or differences between the isolated compounds and references, as not all the isolated compounds were pure. Each sample was dissolved in CD_3OD before being subjected to the NMR analysis. Not all of the results obtained from these techniques could be interpretable, due to various reason concerning especially purity or concentration. By means of the ones that could be interpreted, by comparison with a reference (where available), the structure identity of some of the flavonoidic compounds could be confirmed. The ^{1}H-NMR spectra of each compound is presented below (Fig.91-96), in order to completely elucidate the structure of the compounds and the othe corresponding NMR spectra are found in Annex.

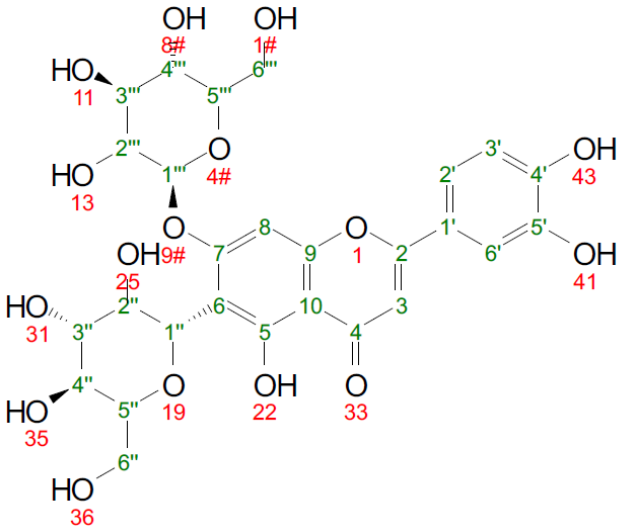


Fig.91. Structure of lutonarin isolated from the leaves of *B. alba*, with corresponding signals annotated on the structure

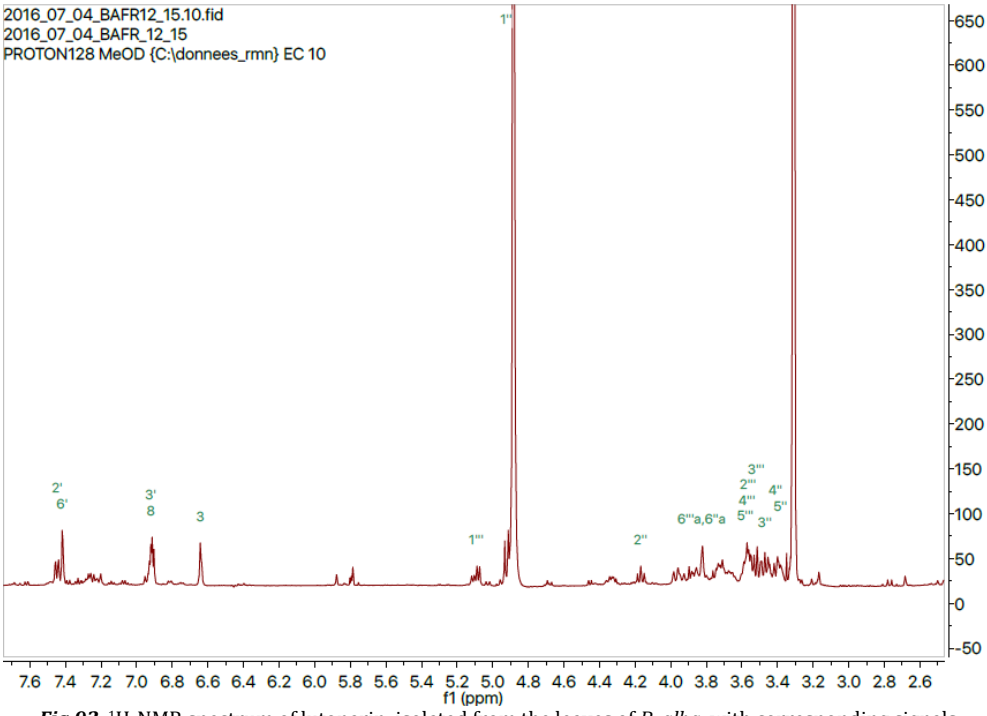


Fig.92. ^{1}H-NMR spectrum of lutonarin isolated from the leaves of *B. alba*, with corresponding signals annotated on the structure

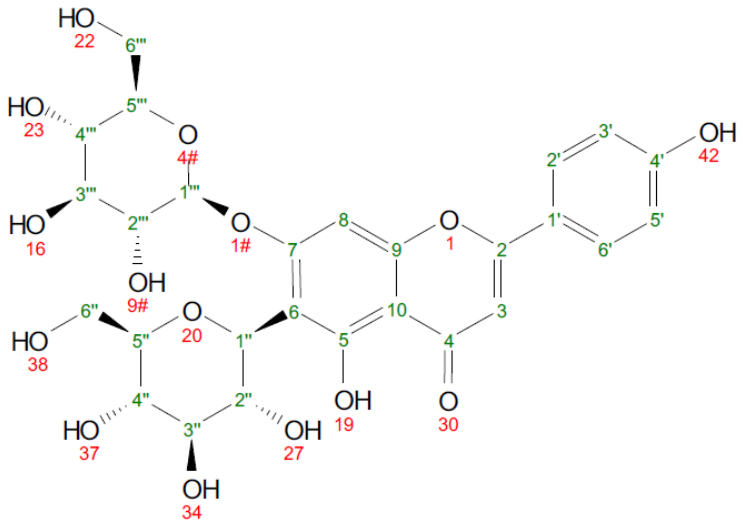


Fig.93. Structure of saponarin isolated from the leaves of *B. alba*, with corresponding signals annotated on the structure

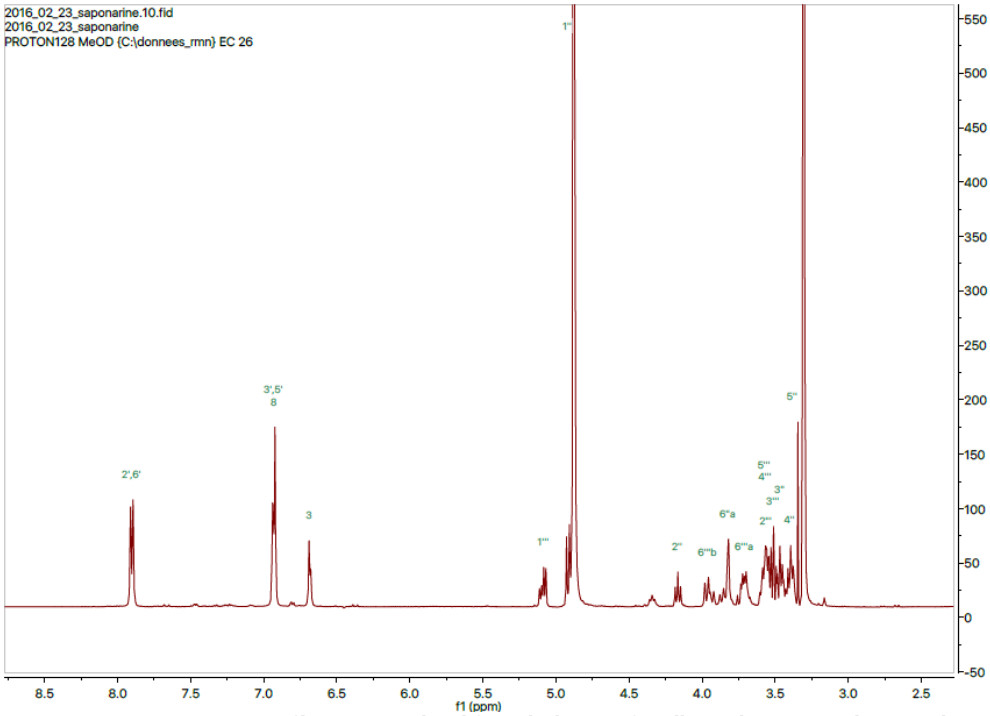


Fig.94. ^{1}H-NMR spectrum of lutonarin isolated from the leaves of *B. alba*, with corresponding signals annotated on the structure

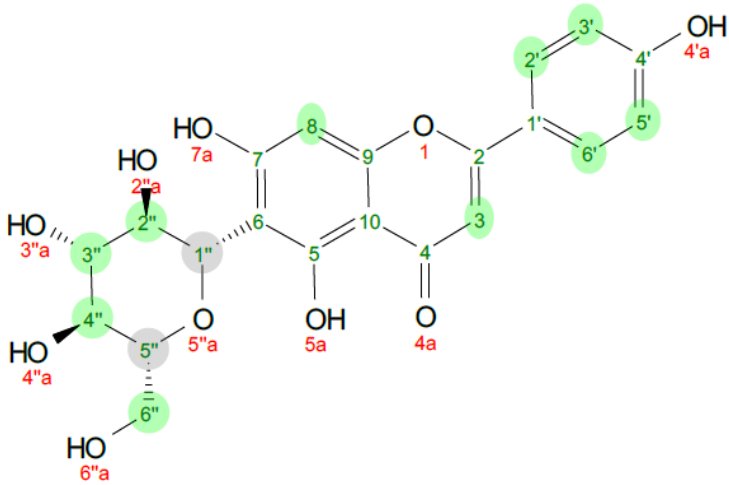


Fig.95. Structure of isovitexin isolated from the leaves of *B. alba*, with corresponding signals annotated on the structure

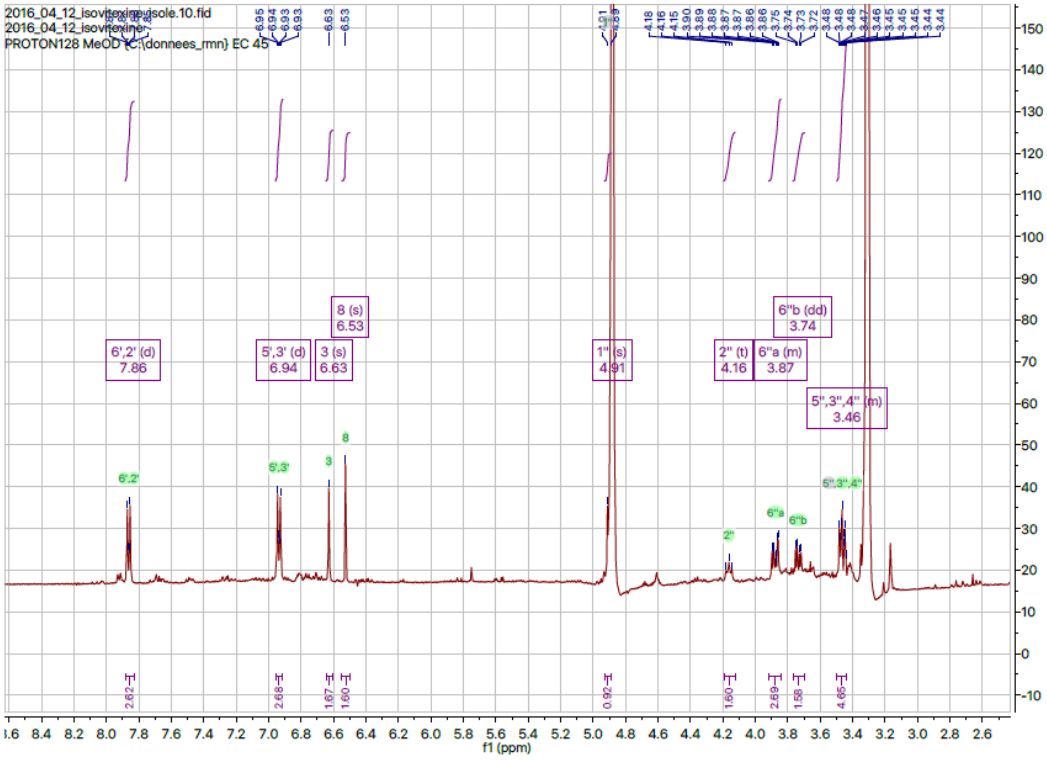


Fig.96. ^{1}H-NMR spectrum of isovitexin isolated from the leaves of *B. alba*, with corresponding signals annotated on the structure

4.5. Discussions

a) Bryonia alba L.

The **TLC analysis** which were performed on different plant parts showed that the richest parts in flavonoids are leaves and aerial parts. Moreover, these parts proved to have the same profile concerning the main flavonoids. The obtained profile in TLC provides important indications on the type of flavonoids that are present: the two green spots indicate the possible presence of two apigenin derivatives, while the other yellow spots indicate the presence of luteolin derivatives. Following the indications of existing studies, the presence of saponarin and isovitexin could be proven by comparison with the corresponding references. Furthermore, after advancing in the study, the presence of isoorientin, never cited before in the composition of the species, could be proven, also by comparison with the available reference.

The performed spectrophotometric assays concerned the quantification of total **flavonoids, polyphenols and hydroxycinnamic acids**. Determination of total flavonoids total was the only assay that was performed using 2 methods. The first method was the one described by the 9th edition of the European Pharmacopoeia, in the *Passiflorae herba* monograph. The choice of the monograph was made taken into consideration the profile of flavonoids cited by scientific literature for the samples of *B. alba*, particularly the presence of C-heterosides. As the monograph of *Passiflorae herba* expresses the total flavonoids in a typical C-heteroside, vitexin, the monograph appeared to be the most appropriate for the quantification. Total flavonoids was expressed therefore as vitexin equivalents (g of vitexin in 100g of vegetal powder). As TLC assays proved that leaves are richer in flavonoidic compounds (highest intensity of the spots), the quantification was performed on samples of leaves, that were collected in different places and at different periods of time, in order to identify the differences between these samples. The variation of compounds that was identified does not show a specific trend as an increase or decrease in the amount of compounds in a time dependent manner. The variations observed could then be explained by the differences of harvesting time and place, but also by the differences of age, *B.alba* being a perennial species.

The second method used for the quantification of the total flavonoids is described in the 10th edition of the Romanian Pharmacopoeia, in the monograph *Cynarae folium*. Furthermore, the method used for the quantification of polyphenols is also described in the 10th edition of the Romanian Pharmacopoeia, in the same monograph. Some modifications of this last method were made, compared to the original one and they concerned especially the wavelength and the way results were expressed. For the hydroxycinnamic acids, a method described by the 9th edition of the European Pharmacopoeia, at the monograph *Fraxini folium*, with some modifications was employed. The modifications also concerned the way results were expressed (as caffeic acid equivalents instead of chlorogenic acid equivalents, as the European Pharmacopoeia describes). All these methods allowed the quantification of average amounts of flavonoids and polyphenols, while hydroxycinnamic acids could not be quantified in the tested samples (not detected).

B.alba proved to contain average amounts of compounds (concentrations ranging between 0.64 ± 0.13 g GAE/100g dried vegetal product and 3.65 ± 0.39 g GAE/100g

dvp for the polyphenols and between 0.40 ± 0.13 g RE/100g dvp and 3.79 ± 0.64 g RE/100g dvp for the flavonoids). A decreasing trend in terms of concentrations can be noticed from the richest parts in flavonoidic and polyphenolic compounds (leaves) to the parts containing lesser flavonoids and polyphenols (stems). These results correlate with the results obtained in the TLC analysis, where spots corresponding to flavonoids proved to be significant only in leaves and aerial parts, whereas in stems these spots were not easily noticed.

Analytical HPLC-DAD was employed to confirm the identity of the four flavonoidic compounds that were identified in the TLC assays. Scientific literature indicates the presence of four flavonoidic compounds in the composition of the fresh aerial parts belonging to the species: saponarin, isovitexin, vitexin and lutonarin (60,61). The studies performed hereby show that the part of the plant which is the riches in flavonoidic compounds is the leaff. In fact, HPLC-DAD studies were also performed on the most important parts of the species (leaves, aerial parts, stems, fruits and roots). Results show the same profile for the tested samples, but leaves proved to contain the highest amount of these compounds. This is the reason why further studies were performed only on leaves. Selection of samples on which the isolation of the four compounds was made was also performed by HPLC-DAD. Based on previous studies, the presence of saponarin and isovitexin could be confirmed by comparison with available references. Retention times and UV spectra were compared and afforded the same values. On the contrary, the presence of vitexin could not be confirmed. The presence of lutonarin could not be confirmed by HPLC-DAD, as no reference was available. However, its retention time and UV spectrum were determined. Correlated with the color of the spot on TLC, some indications can lead towards the identification of its structure: its polarity (di-glucoside) that determines a lower retention time and the aspect of its UV spectrum that indicates a compound from the family of luteolin derivatives. A compound that was not previously described in the composition of this species but whose presence is confirmed by the present study is isoorientin. Its HPLC-DAD confirmation is also performed by comparison with a reference. Where available, data for the compounds were compared to the existing data in the databasis of the Laboratory of Pharmacognosy of the University of Liège.

As saponarin was the main compound, the quantification of its amounts in leaves and aerial parts was performed on samples collected in different periods in the development of the species and in different places. Variation of the saponarin was found to be similar to the variation of total flavonoids, performed by the method described by the 9th edition of the European Pharmacopoeia. This suggests that it is the saponarin that may be responsible for the variation of compounds in the leaves and aerial parts of the species, as the main compound. If comparing samples of leaves and aerial parts, the leaves proved each time to contain a larger amount of saponarin than aerial parts. Possible explanations for the variation of total flavonoids and of saponarin may be found in differences of pedo-climatic conditions, but also in the age of the plant.

As no reference was available for the identification of lutonarin, hydrolysis was performed on crude extracts, to identify by comparison with existing references the apparition of the genin orientin, as the quantity of lutonarin diminishes. The acid hydrolysis was performed according to the method described by the 9th European Pharmacopoeia in the *Acaciae gummi* monograph. The time of hydrolysis was modified between one and six hours and it resulted in the same profile of flavonoids in different

hydrolyzed samples. By comparison with the starting extract, the lutonarin remains unchanged in the crude extract, but other compounds were hydrolysed and were not further found in the composition of the extract. Lutonarin could not be converted in a compound with an available reference. On the contrary, the whole quantity of saponarin was transformed in isovitexin, that was futher transformed in vitexin. In fact, the departure extract does not contain vitexin, but in the samples obtained after hydrolysis the peak corresponding to vitexin has almost the same intensity as the peak of isovitexin, whose quantity clearly increases, compared to the initial extract. At the same time, another compound that is not initially found in the starting extract is orientin, that also appears in an equal quantity with isoorientin. The equilibrium that is found between isoorientin and orientin and between isovitexin and vitexin is in fact an equilibrium between two C-heterosides of luteolin and apigenin, which differ from one another only by the position of a glucose. In fact, the transformation of the compounds is the transformation of a 6-C-glucoside (apigenin-6-C-glucoside = isovitexin and luteolin-6-C-glucoside = isoorientin) in a 8-C-glucoside (apigenin-8-C-glucoside = vitexin and luteolin-8-C-glucoside = orientin). The transformation is cited by scientific literature as being a Wessely-Moser rearangement for such molecules (147). The transformations that occur in the extract are the found in Fig.95.

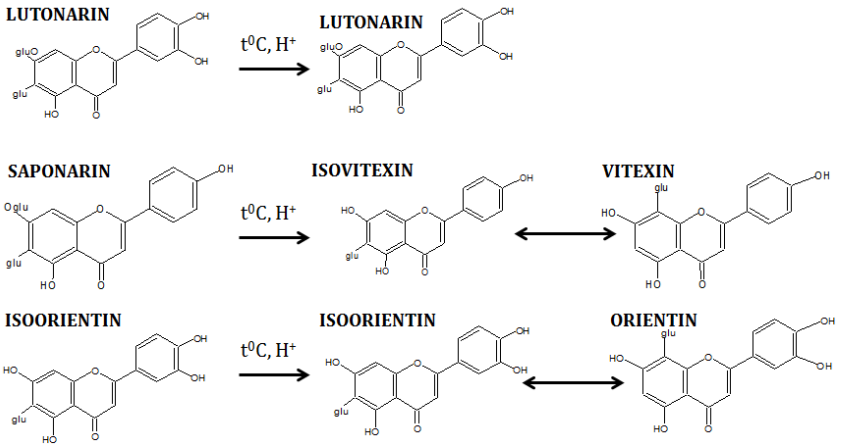


Fig.97. Transformations that occur during the hydrolysis of the flavonoids in samples of leaves of *B.alba*

The **HPLC-MS analysis** of the polyphenols was performed on samples harvested in different places and periods, in order to establish the possible differences between the amounts of compounds. Results were classified by the main parts of the species: leaves, stems, roots, fruits and aerial parts. Among these, the parts that proved to be the richest in compounds are stems, leaves and aerial parts. These samples present almost the same profile of compounds, with little differences concerning hyperoside (only found in samples of stems) and apigenin (only found in samples of leaves and aerial parts). Leaves and aerial parts showed similar profiles of compounds. Differences that appear in the amounts of compounds of these samples may be due to other parts of the plant found in the aerial part, which also contain these type of compounds (flowers, stems, tendrils etc.). Roots and fruits contain the lowest amounts and number of polyphenolic compounds.

Compounds that were found almost in all samples were *p*-coumaric acid and ferulic acid. Other compounds that could be quantified in some samples are isoquercitrin, rutin, quercitrin and apigenin. Only one sample of stems proved to contain hyperoside, in a significant amount (98.63 ± 4.89 mg/100g dvp). Amounts of polyphenols in the majority of samples were below 1mg/100g dvp. At the same time, compounds that were identified by HPLC-MS proved to be mostly O-heterosides and phenolic acids, while in the HPLC-DAD studies, C-heterosides were identified. Values that are exceeding 1mg/100g dvp are only found for isoquercitrin in aerial parts (21.43 ± 4.59 mg/100g dvp for BA24) and in stems (6.67 ± 2.58 mg/100g dvp for BA1 and 2.31 ± 0.79 mg/100g dvp). As factors involved in the collection of samples were so different, a rigorous dynamic of accumulation was difficult to establish.

As leaves were proved to contain the most important amounts of flavonoids in the HPLC-DAD assays, the isolation of flavonoids found in their composition was performed by **preparative HPLC-DAD**. The method used for the isolation was an adaptation of the analytical method and it was performed in two stages, that concerned first the isolation of a flavonoidic fraction that was subsequently re-injected in order to lad to the isolation of compounds. This procedure led to the isolation of the four flavonoidic compounds found in the samples of leaves: lutonarin, saponarin, isoorientin and isovitexin. Each fraction of these compounds proved, when subjected to analytical HPLC-DAD, to contain only one compound, except for the fraction that should contain isoorientin, which contains traces of the main compound, saponarin. Even though this fraction did not contain only one compound, isoorientin was found as the main compound in the fraction. The presence of lutonarin, saponarin and isovitexin was previously in literature discussed in the aerial parts belonging to the species, but not in the leaves. At the same time, it is the first approach of the kind that describes the presence of isoorientin in the composition of the leaves of the species. TLC assays, analytical HPLC-DAD, comparison with references and acid hydrolysis of extracts previously allowed the confirmation of several parameters of these compounds that corresponded to the references (where available). The isolated compounds were subjected therefore to analytical HPLC-DAD analysis and especially to mass spectrometry and nuclear magnetic resonance assays, in order to establish and confirm the structure.

Structure identification of the four flavonoidic compounds was firstly performed by mass spectrometry. Assays were performed on isolated compounds, by direct injection in the MS system. Thereby, the peak corresponding to the molecular weight could be established for each compound. For lutonarin, the molecular peak appears at a m/z of 633.14175, which corresponds to the sodium adduct of lutonarin. Saponarin and isovitexin showed the same spectrometric profile, with the molecular peak as the main one, both corresponding to sodium adducts: for saponarin at m/z 617.14697 and for isovitexin at m/z 455.09432. Isovitexin was the only compound for which the molecular peak was not the main one but, despite this fact, its molecular peak could still be found in the spectrum of the compound, also corresponding to a sodium aduct, at m/z 471.08924. Moreover, for saponarin and isoorientin, the $[MH]^+$ peak can be found in the spectra, at m/z 595.16514 and at m/z 449.10763 respectively.

Nuclear Magnetic Resonance was the technique that completed the structural identification of the four flavonoidic compounds. Assays were performed for isolated compounds and, when references were available, the results were compared to the

ones obtained for references. ^{1}H-NMR assays were perfored for compounds isolated from the leaves of the species and for references, for the following compounds: saponarin, isoorientin and isovitexin. Signals obtained for the two types of compounds were analyzed and allowed to complete the structural identification of each compound.

For *lutonarin*, as no reference was available, the NMR identification proved to be more difficult, even more as the compound itself wass found in lower amounts in the extract, which made its isolation more difficult. Lower concentration and possible impurities made the NMR identification more difficult. Therefore, ^{13}C-NMR and COSY spectra could not be interpreted for the isolated compound, but the HMBC and HSQC afforded data that allowed the confirmation of the aglycone (lutonarin) and the position of the two molecules of glucose on it: C-7 for the O-glucoside and C-6 for the C-heteroside. Correlating all these data with data obtained in the previous assays, it appears clear that the identity of the compound is *7-O-β-D-glucopyranoside-6-C-β-D-glucopyranoside-4',5',5-trihydroxy-flavone* (lutonarin).

For *saponarin*, reference compound was available, so identification was performed by comparison with this compound. Signals in the^{1}H-NMR spectra of the reference compound proved to be matching and perfectly superposable with the ones corresponding to the isolated compound (Fig.98). All of the other NMR analysis that were performed (especially HMBC, HSQC, as COSY spectrum could not bring important data) allowed to identify the compound as being *7-O-β-D-glucopyranoside-6-C-β-D-glucopyranoside-4',5-dihydroxy-flavone* (saponarin).

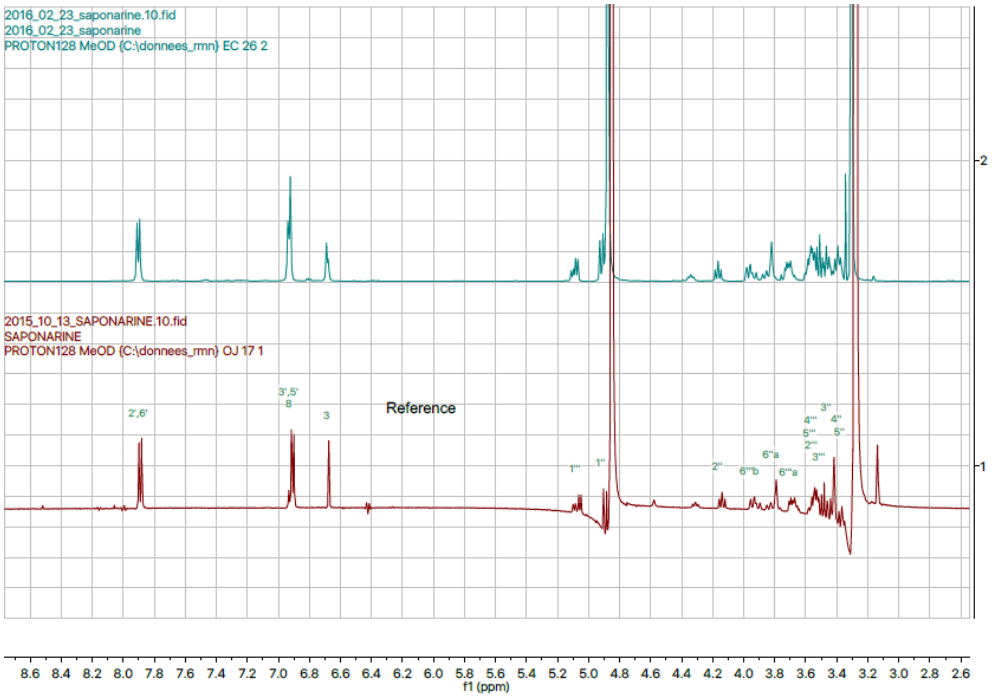


Fig.98. Superposition of the ^{1}H-NMR spectra of isolated saponarin from the leaves of *B. alba* and reference saponarin

For *isovitexin*, identification proved also easier as reference of the compound was available. Therefore, the signals in the ^{1}H-NMR spectra of the reference and of the isolated compound were compared at they proved to be perfectly matching and superposable (Fig.96). The lower concentration of the isolated compound and the possible presence of some impurities made the ^{13}C-NMR spectra difficult to interpret, but all other NMR assays (COSY, HMBC, HSQC) could afford data that allowed to confirm the identity of the compound as being *6-C-β-D-glucopyranoside-4',5,7-trihydroxy-flavone* (isovitexin).

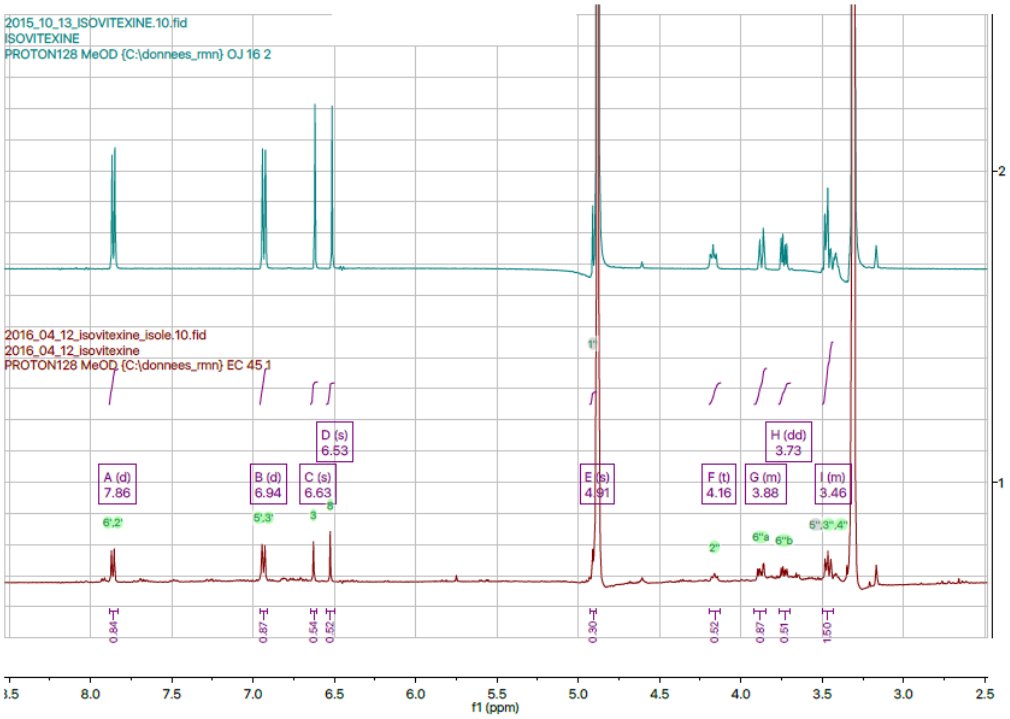


Fig.99. Superposition of the ^{1}H-NMR spectra of isolated isovitexin from the leaves of *B. alba* and reference isovitexin

For *isoorientin*, identification proved more difficult, even though the reference of the compound was available. As seen in the isolation of the compound (Fig.81), the fraction that should correspond to the pure compound contains traces of saponarin, the main compound, that is also the one that appears before it in the separation of the compounds. Even if isoorientin has proved to be the majoritary compound in the fraction, identification of the signals proved to be difficult. Despite this fact, is is clear that, by the superposition of the spectra corresponding to the reference and the one corresponding to the isolated compound that the two compounds have similar profiles of the signals in all performed analysis. Isolation of a compound that contains lesser impurities should be necessary in order to correctly assign the corresponding signals to the compound. Fig.100 contains a superposition of the spectra corresponding to the isolated fraction and the spectra corresponding to the reference, that proves the

similar profile of the two compounds. No signal could be assigned to the structure, due to the traces of saponarin, that make identification more difficult.

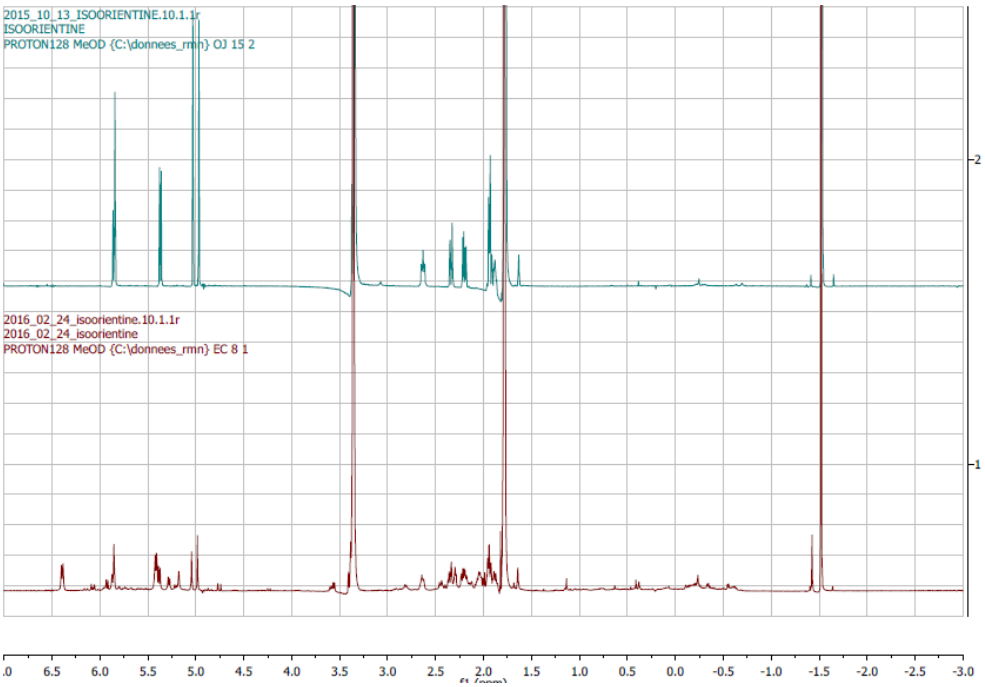


Fig.100. Superposition of the ^{1}H-NMR spectra of isolated isoorientin from the leaves of *B. alba* and reference isoorientin

b) Echinocystis lobata *(Michx.) Torr. et A. Gray*

The species that has proved to contain the largest amount of flavonoids and polyphenols is *E. lobata.* Compared to *B. alba*, the variety and amounts of flavonoids has proved each time higher for this species, even if the classes of compounds were different. The pattern of the identification of compounds was the same as for the previous species.

Scientific literature cites the presence of four flavonoidic compounds in the composition of aerial parts of the species: one quercetin derivative, one kaempferol derivative and two isorhamnetin derivatives. One of the compounds is described as being kaempferol-3-O-glucoside (astragalin), but reading the manuscript, it was not clear if the compound was not quercetin-3-O-glucoside (isoquercitrin). As on acid hydrolysis it affords kaempferol, it appears that the compound identified by these authors was astragalin (62).

TLC analysis was performed on the most important parts of the species and showed that the richest parts are the flowers, followed by the leaves and the aerial parts. All of these parts proved to have similar compounds profile. Various spots can be noticed, showing different colors and, at the same time, being very close one to another. Differences between compounds can be hardly noticed. It appears therefore difficult to establish the number of compounds in the studied parts and to establish

indications on the possible class they belong to. It is only the intensity of the spots that indicate a largest amount of compounds for the flowers. Based on the existing studies, the presence of kaempferol-3-O-glucoside (astragalin), quercetin-3-O-glucoside (isoquercitrin) and of quercetin-3-O-rutinoside (rutin) could be established, by comparison with the existing references.

The spectrophotometric assays that were performed concerned the quantification of the **total flavonoids, polyphenols and hydroxycinnamic acids**, by the methods described in the Romanian or European Pharmacopoeias. Quantification of the total flavonoids and of the polyphenols were performed by methods described by the 10th edition of the Romanian Pharmacopoeia, in the monograph *Cynarae folium*. Modifications of the method for the quantification of total polyphenols were made especially for the wavelength and the way results were expressed. Hydroxycinnamic acids were quantified by a method described by the 9th edition of the European Pharmacopoeia, at the monograph *Fraxini folium*, with some modifications concerning the expression of results (as caffeic acid equivalents). Thus, higher amounts of flavonoids and polyphenols than for the samples of *B. alba* could be quantified. Hydroxycinnamic acids could not be quantified in the samples.

The richest parts of the species in terms of flavonoids were flowers (concentrations ranging between 3.04 ± 0.18 g GAE/100 g dvp and 3.23 ± 0.39 g GAE/100 g dvp for polyphenols and 3.75 ± 0.37 g RE/100 g dvp and 2.89 ± 0.11 g RE/100 g dvp for flavonoids), followed by aerial parts (concentrations ranging between 3.35 ± 0.25 g GAE/100 g dvp and 4.41 ± 0.52 g GAE/100 g dvp for polyphenols and 1.86 ± 0.59 g RE/100 g dvp and 2.20 ± 0.37 g RE/100 g dvp for flavonoids) and leaves (concentrations ranging between 1.89 ± 0.45 g GAE/100 g dvp and 3.21 ± 0.12 g GAE/100 g dvp for polyphenols and 1.15 ± 0.53 g RE/100 g dvp and 2.17 ± 0.09 g RE/100 g dvp for flavonoids). Stems were the parts containing the lowest amounts of compounds (concentrations ranging between 0.90 ± 0.12 g GAE/100 g dvp and 1.21 ± 0.09 g GAE/100 g dvp for polyphenols and 0.30 ± 0.06 g RE/100 g dvp and 0.19 ± 0.05 g RE/100 g dvp for flavonoids). These results are in correlation with the intensity of spots obtained in TLC, even if the intensity of spots seems a little higher in leaves. As also proven on TLC, stems and fruits are the parts that contained the lowest amounts of compounds.

The next step towards the identification of flavonoidic compounds in this species was the **HPLC-DAD analysis**. Various plant parts were analyzed by the same method as samples of *B. alba*. The parts that proved richer in compounds were leaves and flowers, as previously proved by TLC and spectrophotometric assays. Despite the fact that leaves contained the highest amount of the main compound, all the other compounds were found in higher amounts in flowers. This is the main reason for which all the following studies were performed on the flowers of the species.

As suggested by the existing study in the scientific literature, which investigates the presence of flavonoids in the composition of the aerial parts, the presence of quercetin and kaempferol derivatives was studied. In the present study, it is demonstrated that flowers of the species are richer in flavonoidic compounds than aerial parts. Moreover, the present study confirms, by HPLC-DAD analysis, the presence of astragalin, rutin and isoquercitrin in the flowers of the species, by the comparison with references. Retention times and UV spectra were compared for the compounds in the flower extract, with the ones of available references. All these

informations were matched with the ones existing in the data basis of the Laboratory of Pharmacognosy of the University of Liège and with the ones obtained in the TLC assays and the presence of the three compounds could be confirmed.

Regarding the study of other compounds found in the composition of the species, attempts to identify the main peak were performed. The HPLC-DAD study showed that the peak may contain two flavonoidic compounds, as the peak and as the UV spectra of different parts of the peak were not superimposable. The performed HPLC-DAD analysis could not allow the separation of these two compounds, even if the technical parameters of the analysis were changed. These results correlate with the ones obtained in TLC, where compounds appear very close one to another and the clear distinction between spots cannot be established.

HPLC-MS analysis was performed for the screening of polyphenols in different parts of the species corresponding to samples harvested in different places and periods of time. The quantification of polyphenols was performed though in order to establish the differences according to the amounts of polyphenols found in these samples. Results were classified by the main parts of the species: flowers, leaves, stems, fruits and aerial parts. The parts that proved to be richer in compounds were flowers, followed by aerial parts and leaves. The number of compounds that were found in samples was higher in samples of stems, but their amounts appear lower than the amounts found in flowers, leaves and aerial parts. Profile of compounds appears to be quite similar in these parts of the species. *p*-Coumaric acid, ferulic acid, isoquercitrin, rutin, quercitrin, quercetin and kaempferol were found in almost all samples. Among these compounds, only isoquercitrin and rutin were also identified by HPLC-DAD and were also cited by scientific literature (62). Two samples of leaves contained chlorogenic acid in significant amounts, EL 15 (31.11 ± 4.28 mg/100g dvp) and EL 75 (15.10 ± 0.54 mg/100g dvp). Another compound that is found in some of the tested samples of leaves and aerial parts is apigenin: concentrations ranging between 0.69 ± 0.73 and 1.76 ± 0.04 mg/100g dvp for leaves and between 0.26 ± 0.09 and 2.51 ± 1.27 mg/100g dvp for aerial parts. Hyperoside was only found in one sample of aerial parts, EL20, in an amount of 1.97 ± 0.18 mg/100g dvp. The compound that is found in the highest amounts in all the tested samples is isoquercitrin. Concentrations were ranging between 1.17 ± 0.53 and 163.02 ± 0.01 mg/100g dvp for leaves, 2.55 ± 0.89 and 115.86 ± 4.56 mg/100g dvp for aerial parts and 1.17 ± 0.04 and 29.51 ± 5.55 mg/100g dvp for stems, reaching the highest values in samples of flowers (between 61.78 ± 0.79 and 238.22 ± 0.54 mg/100g dvp) and the lowest values in samples of fruits (between 3.07 ± 1.91 and 8.106 ± 4.39 mg/100g dvp). It is not only the isoquercitrin that is found in lowest amounts in fruits, but all the other compounds. A general trend that concerns the accumulation of compounds in the tested samples is noticed and it concerns the increasing of the amounts of compounds towards the period where plant reaches a mature stage of development. Further analysis are needed in order to establish a rigorous dynamic of accumulation.

Isolation of the flavonoidic compounds in the flowers of the species were performed by **preparative HPLC-DAD**. Isolation of compounds was performed in two stages, that concerned the isolation of fraction that contained the compounds of interest, which was subsequently subjected to another preparative analysis in order to afford the pure flavonoidic compounds. As in the extract compounds were numerous and situated close to each other, the separation of the compounds was difficult to be

done in the described conditions. Despite this fact, fractions that were concentrated in each compound could be isolated.

For the main peak that contained two compounds, the preparative system that was used and the described conditions could not afford the separation of the two compounds. **TLC-MS technique** was used for obtaining more information on the two compounds. The technique was employed because the two compounds afforded on TLC two very close spots, so the system could be able to collect the two compounds and directly send them in the MS. Mass of the two compounds could be therefore established as being 763.60396 and 763.60376 m/z, which could correspond to a sodium adduct of a flavonoid having the molecular weight around 740. The identity of the compounds could not be established, but, as they proved to have similar molecular weight, the compounds appear as possible positional isomers.

c) *Ecballium elaterium* (L.) A. Rich.

The species that contained the lowest amount of flavonoidic compounds was *E.elaterium.***TLC assays** could not offer enough information to conclude which part could be richer in flavonoids. **Spectrophotometric assays** performed after the methods described by the European and Romanian Pharmacopoeias showed the lowest amounts of total flavonoids and polyphenols, compared to the other two species.. Concentrations were ranging between 0.02 ± 0.31 g GAE/100g dried vegetal product and 0.11 ± 0.61 g GAE/100g dvp for the polyphenols and between 0.18 ± 1.17 g RE/100g dvp and 0.23 ± 0.73 g RE/100g dvp for the flavonoids). Hydroxycinnamic acids could not be identified in the composition of the species. **HPLC-DAD analysis** showed that the aerial parts are richer in compounds. In fact, only one compound was identified in the composition of the species, rutin. The compound was identified by comparison with a reference and is it previously cited for the composition of the species (63–67). **HPLC-MS analysis** for polyphenols found the presence of *p*-coumaric acid in mature (5.76 ± 0.17 mg/100g dvp) and young (3.73 ± 1.14 mg/100g dvp) stems, of ferulic acid in aerial parts (0.25 ± 0.01 mg/100g dvp), of isoquercitrin in mature stems (0.35 ± 0.73 mg/100g dvp), of rutin in mature (0.26 ± 0.17 mg/100g dvp) and young (0.26 ± 0.07 mg/100g dvp) stems and of quercitrin in mature stems (0.55 ± 1.15 mg/100g dvp) and roots (0.04 ± 0.96 mg/100g dvp). All these values were situated under the values obtained for other species. No other analysis were performed for the identification of the compounds in this species.

4.6. Conclusions

Flavonoids present in the composition of the three species belonging to Cucurbitaceae family were studied by TLC assays and by HPLC-DAD. Elucidation of the flavonoidic structure was performed by MS and RMN techniques.

The flavonoidic profile of *B. alba* appears to be completely elucidated and different from the one that is cited in the scientific literature. The parts that proved to be the richest in compounds were the leaves. In their composition, main compound that was identified was saponarin. Other compounds that were identified were lutonarin, isovitexin and isoorientin. The only compound that was not previously cited in the composition of the leaves was isoorientin.

By comparison with *B. alba*, where C-heterosides were prevalent, in the composition of *E. lobata* the O-heterosides were prevalent. Isoquercitrin, astragalin and rutin were identified in the composition of the flowers of the species. Attempts to identify the other compounds were also perfomed, but the flavonoidic profile remains uncompletely identified. This species is the richest in flavonoidic compounds of the three species that were studied.

The last species is the one that has proved to be the poorest in flavonoidic compounds was *E. elaterium*. Only rutin was identified in the composition of the aerial parts of the species.

The results obtained in the present thesis bring important data on the species that are studied, especially from the point of view of some compounds that are not intensively studied. Flavonoids and polyphenols are a class of compounds that may assign important activities to plants, as antioxidant or anti-inflammatory. As they are lesser studied in the case of Cucurbitaceae species, the present study brings originality and may help to increase knowledge on plants that have proven important data as potential medicinal plants.

5. Study 3. Comparative pharmacological researches of three species belonging to Cucurbitaceae family

5.1. Introduction

Plants belonging to Cucurbitaceae family are widely known for their use in human nutrition (33), but in the last years they have proven a series of bioactivities that made them important targets for the research in the domain of plants with medicinal potential (1). Among the bioactivities that are mostly studied for plants belonging to this family there is the anti-inflammatory, the antioxidant and the cytotoxic one (1,4,44). Most of the existing studies assign these activities to the content of cucurbitacins (4,44), but recent studies have proved that other compounds as polyphenols found in the composition of these species may also be the ones that assign them these activities (57,145).

The three plants belonging to Cucurbitaceae family that are described by this thesis are known for some biological activities, but data remain unclear and old. For example, *B.alba* is largely known for its cytotoxic activity (28), that is assigned to its content in cucurbitacins (29). Other biological activities that are cited for the species and the compounds in its composition are the antiinflammatory (96), adaptogen (94), hepatoprotective (30) and the hypoglycemic (49,92) ones. *E. elaterium* is known for its anti-microbial (98–101), anti-inflammatory (102,103), antioxidant (104) or cytotoxic (75,76,85,104–108) activities. *E. lobata* has lesser known medicinal activities, being known especially in ethno-medicine for its use in menstrual disorders, rheumatisms, chills, fevers, kidney disorders or for stomach troubles (14) .

In this context, the present study aims to bring a new approach on the three species, bringing evidence that may support their potential as future phytopharmaceuticals. The biological activities that are assessed in this thesis for the three species are the anti-plasmodial, the cytotoxic, the antioxidant and the anti-inflammatory ones. Besides, in order to support the findings of this study, the *in vivo* toxicity of these species was tested on a zebrafish larvae model. The anti-plasmodial activity was assessed on two strains of *Plasmodium falciparum*, that are chloroquine sensitive (3D7) and chloroquine resistant (W2). Cytotoxic activity was tested on two cancerous cell lines (*A549* - lung cancer and *HeLa* - cervical cancer) and on one healthy cell line (*WI38* - fetal lung fibroblasts). Antioxidant assays that were used are the DPPH, CUPRAC, FRAP, TEAC, EPR and SNPAC assays. As plant antioxidants may act as unfavorable modulators of the catalytic activity of various enzymes, the anti-catalytic activity of the extracts obtained from the species was assessed by using the HRP model. In order to support the global anti-oxidant activity and the inhibitory activity of enzymes that are involved in the inflammatory processes, the activity on the HL-60 cells converted into macrophages and on isolated *ex vivo* equine neutrophils was assessed. Not least, in order to prove the lack of toxicity of the extracts on different organs, a zebrafish larvae model was used.

The originality of this study concerns not only in the activities that are assessed, but also in the fact that it shows that the compounds that are involved in these

activities are flavonoids, compounds that are lesser studied for these species. A supplementary reason to support this is found in some complementary work of this thesis, that evaluated the content of cucurbitacins in these plants and proved results that could not support their presence in the tested samples. Because of this reason and because of the fact that all assays were performed by comparing the isolated compounds with their available references and results proved to have similar profiles, important evidence on the involvement of flavonoids in these activities could be brought. All these allowed to establish the potential of these species and of their active compounds for the use in the treatment of certain disorders.

5.2. Work hypothesis

Plants belonging to Cucurbitaceae family are widely known for their content in cucurbitacins, which assign them important biological activities, but also certain toxicity. As these plants are lesser known and studied for their content of flavonoids and for the biological activities they may assign them, the studies performed in the present thesis are aimed to bring a different approach that may prove the important potential of these plants as possible medicinal species. The activities that are assessed in the present thesis are the anti-plasmodial, the cytotoxic, the antioxidant, the anti-catalytic and the anti-enzymatic ones. Anti-enzymatic assays concern especially enzymes that are involved in the inflammatory processes. In order to support all the findings, the plant extracts were tested for special toxicity on a model of zebrafish larvae. Thus, evidence for the potential as medicinal species for the three plants could be brought and data available in scientific literature on these plants could be enriched.

5.3. Materials and methods

Most of the biological assays that were assessed in this chapter were performed on crude extracts, prepared as described at 4.3.1.1. and dissolved in DMSO in order to reach a concentration of 10mg/mL. Tested samples can be found in Table XXXVIII. Corresponding dilutions of the departure samples extract were performed in order to establish at which concentration the respective activity is produced.

Table XXXVIII. Samples that were tested in the biological assays

Species	Sample name	Part of the species
Bryonia alba L.	BA27	Stems
	BA40	Fruits
	BA25	Root
	BA2	Leaves
	BA3	Aerial part
Echinocystis lobata (Michx.) Torr. et A. Gray	EL73	Stems
	EL74	Leaves
	EL75	Aerial parts
	EL76	♂ flowers
	EL77	Fruits
Ecballium elaterium (L.) A. Rich.	EE82	Mature stems
	EE83	Young stems
	EE84	Leaves
	EE85	Aerial part
	EE86	Roots

For the species *B. alba*, isolated flavonoidic compounds were also tested, especially for the enzymatic and catalytic assays. Results were compared to those obtained from testing in the same conditions of available references of the corresponding flavonoidic compounds. Thus, isolated saponarin, isoorientin and isovitexin and their corresponding references were tested and results could be compared in order to establish and correlate the tested activities of total extracts to these compounds. Tested references and isolated compounds were both dissolved in DMSO in order to reach a departure concentration of 10mg/mL.

5.3.1. *In vitro* anti-plasmodial assays

The continuous cultures of *Plasmodium falciparum* chloroquine-sensitive (3D7) and chloroquine-resistant (W2) strains were maintained as described by Frédérich *et al*. (148) in the RPMI medium, supplemented with 10% (V/V) human plasma, 1% (v/V) gentamicin (10mg/mL) and 10% of a solution of glucose-hypoxanthine. The host were represented by human red blood cells (A or 0, Rh+). The *in vitro* anti-plasmodial assay reproduces the erythrocytic stage in the development of the parasite. A series of eight two-fold dilution of each sample with the culture medium was put in contact with the *Plasmodium* culture in a 96-well microplate. Incubation time was 48h. Concentration of tested samples were ranging between 0.8 µg/mL and 100µg/mL. Each condition was tested in duplicate. After 48h, plates were freezed for 24h in order to stop the development of the parasite. Results were obtained by the assesssment of the parasite growth, after 24h. Plates were thawed and revelation was performed by a colorimetric assay, based on the evaluation of the plasmodial lactate dehydrogenase (pLDH), as described by Makler *et al.* (149). 20µL of each well of the test plates was transferred in new 96-well plates and 100µL of a solution of APAD (1% V/V Triton X100, 1% m/V Lithium Lactate, 0.05% m/V APAD and 0.01% m/V Saponin in Tris tampon, pH=8) was added. Plates were incubated at 37°C for 30min and aftwerwards the PES 0.05% and NBT 1% solutions were added. Absorbances were measured at 630nm after 30min incubation at at 37°C, on a multiwell scanner (Stat Fax 2100, Awareness Technology Inc). Artemisinin was used as a positive reference. Infected and uninfected erythrocytes were also used as positive and negative controls. IC_{50} values of extracts/compound were calculated by linear regression from the eight tested concentrations of each sample and represent the concentration of each sample that is needed in order to obtain 50% inhibition of parasite growth. Values of the growth inhibition were calculated in comparison with the erythrocytes that were infected but not treated with the samples (100% growth, positive control). Each samples was subjected to three independent assays (n=3) for each of the strains. Fig.101 contains the steps that are followed for the assesssment of anti-plasmodial activity of samples.

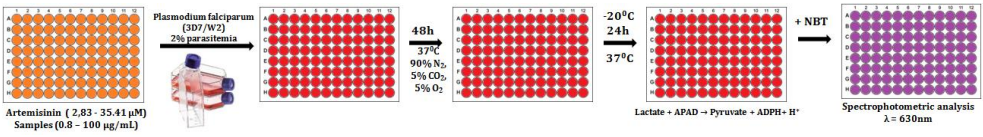


Fig.101. Anti-plasmodial assays – steps to follow for the assessment of the anti-plasmodial activity of samples

5.3.2. *In vitro* cytotoxicity assays

The *A549* (lung cancer) and the *HeLa* (cervical cancer) cell lines, as well as the *WI38* (fœtal lung fibroblasts) were maintained in continuous culture in the DMEM medium, in humid atmosphere, at 37°C and 5% CO2. Culture medium was supplemented with 10% (V/V) heat-inactivated FBS, 1% (V/V) L-glutamine 200 mM and 1% (V/V) of the following antibiotics: penicillin (100 UI/ml) and streptomycin (100 µg/ml). Each well of the 96-well cell culture microplates was seeded with a cell suspension containing a number of 8000-9000 cells, depending on the cell line (8000 cells were seeded for A549 and HeLa and 9000 cells for WI38). Plates were incubated for 24h and subsequently treated with six dilutions of the samples, containing concentrations ranging between 3.2-100 µg/mL. Cells were incubated with the samples for 48h and afterwards cell viability was assessed by adding WST-1 tetrazolium salt as a cytotoxicity indicator. The plates were emptied and the cytotoxicity indicator reagent (represented by a 1% V/V solution of WST-1 in the culture medium) was added in each well, in order to assess the number of living cells, which remained adhered on the bottom of each well. After another hour of incubation of the cells with the reagent, absorbances were measured at 450nm on a multiwell scanner (Stat Fax 2100, Awareness Technology Inc). The revelation is based on the cleavage of tetrazolium salt to formazan dye by enzymes that are present in cells. Thus, the measured absorbance is directly correlated to the number of cells that are still viable in each well. Each sample was tested in triplicate and each test was performed twice (n=2). Values of the IC_{50} for each tested sample were calculated by linear regression from the six tested concentrations of each sample and represent the concentration of each sample that is needed in order to obtain 50% inhibition of cell growth. Values of the growth inhibition were calculated in comparison with the negative control (non-treated cells, 100% viability). Fig.102 contains the steps that are followed for the assesssment of cytotoxic activity of samples.

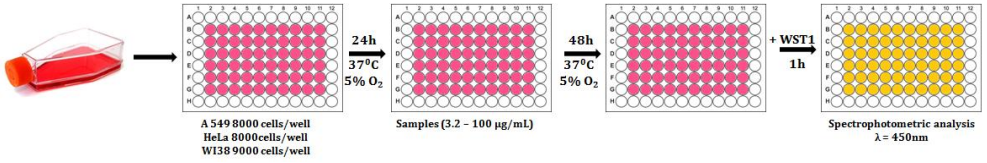


Fig.102. Cytotoxic assays – steps to follow for the assessment of the cytotoxic activity of samples

5.3.3. Antioxidant assays

Classical DPPH bleaching, TEAC, FRAP, CUPRAC SNPAC and EPR methods were used to assess the antioxidant potential of samples belonging to the three species. Assays were performed on samples prepared as described at 4.3.1.2. Table XXI contains the samples that were tested for the antioxidant potential. Different samples of the species that proved the most important antioxidant capacity, *E. lobata*, were tested, in order to establish possible corelations between the amount of compounds found in these samples and the factors that involved plant collection (pedo-climatic conditions). Choosing the samples was also performed taken into consideration the results obtained in the phytochemical assays. For the other species, only parts that proved to contain an important amount of compounds were tested (Table XXXIX).

Table XXXIX. Samples that were tested in the biological assays

Species	Sample name	Part of the species
Bryonia alba L.	BA1	Stems
	BA2	Leaves
	BA24	Aerial parts
Echinocystis lobata (Michx.) Torr. et A. Gray	EL46	Stems
	EL47	Leaves
	EL50	Flowers
	EL66	Aerial parts
	EL69	Leaves
	EL71	Flowers
	EL73	Stems
	EL75	Aerial parts
	EL77	Fruits
Ecballium elaterium (L.) A. Rich.	EE82	Mature stems
	EE84	Leaves
	EE85	Aerial part

The classical ***DPPH free radical method*** assesses the antioxidant capacity of extracts, based on the neutralization of the 2,2-diphenyl-1-picrylhydrazyl free radical (violet), which can be reduced in the presence of an antioxidant (the color is turned to yellow). The color change is in strong correlation with the antioxidant capacity and can be evaluated by a spectrophotometric determination, which was performed at 517 nm. The percentage of inhibition of the radical was expressed as IC_{50} (μg/ml), the concentration of extracts required to cause a 50% DPPH inhibition (150–153).

The ***TEAC assay*** is an antioxidant method based on the ability of an potential antioxidant molecule to reduce the 2,2-azinobis-(3-ethyl-benzothiazoline-6-sulfonic acid) free cationic radical ($ABTS^{\bullet+}$), which is a blue solution, obtained by adding potassium persulfate. The color of the solution is turned to yellow or even it can become incolor in the presence of an antioxidant. The spectrophotometric determination of absorbance correlated with the color change was performed at 734 nm. The percentage of inhibition was expressed as IC_{50} (μg/ml) and it was compared with a Trolox standard (154–156).

The ***FRAP method*** is based on the reduction of the ferric to the ferrous ion in a complex formed with iron of the radical 2,4,6-tripyridyl-s-triazine. According to the concentration of antioxidant compounds in the sample, the color of the complex is changed from light green or yellow to blue. Absorbance correlated with the color change was measured at 593nm and results were expressed as mM Trolox equivalent/100 ml extract, on the basis of a calibration curve using a Trolox standard (157–159).

CUPRAC method assesses the reduction of the copper ion (II) to the copper iron (I) in the complex neocupreine (2,9-dimethyl-1,10-phenantroline), which determines a change in the color from light green to reddish-orange. The color change is correlated with the antioxidant capacity by measuring the absorbance at 450nm. The calibration curve was plotted using concentrations of the Trolox standard and the results are expressed as mM Trolox equivalent/100 ml extract (158,160).

SNPAC method uses the spherical silver nanoparticles (SNPs), obtained from silver nitrate by reducing of Ag^{+} ions and using as surface stabilizer the trisodium citrate. It is based on the reduction of the silver ion to colloidal silver with fine silver nanoparticles suspended in solution, which occurs in the presence of the antioxidants. The color change that occurs is from pale yellow to brownish. The absorbance

correlated with the color change was measured at 423 nm and the calibration curve for standard was plotted using concentrations ranging of Trolox standard. Results are expressed as mM Trolox equivalent/100 ml extract (160,161).

EPR method uses the DPPH radical, which is added to the extract. EPR spectra is recorded after the sample is being mixed and transferred in a EPR quartz capillary. Measurements were performed on a Bruker Elexsys E500 spectrometer. The difference of reaction between antioxidant compounds in the samples and DPPH radical was expressed as integral intensity (I) (150,161,162).

For all of the above analysis that were performed in order to assess the antioxidant activity of the samples, obtained results were presented as mean values ± standard deviation (SD). All the assays were performed in triplicate. The average and the SD were calculated using the Excel software package (150,153,156,158,161,162).

5.3.4. Anti-catalytic assay

The **HRP (horseradish peroxidase) model** of assessment of the anti-catalytic activity of samples, using hydrogen peroxide as oxidizing agent and the probe L012 as chemiluminescent substrate was performed both for samples that are found in Table XXXIX and for the isolated compounds from the leaves of *B.alba*. Assays were performed on white 96-well plates, using phosphate saline buffer as a medium for the reaction. Tested concentrations of samples were ranging between 0.5 and 100 µg/mL for extracts and between 0.01 and 30 µg/mL for isolated compounds (Table XLII and XLIII). The dilutions were put in contact with the chemiluminescent probe and with the enzyme. Afterwards, after adding the oxidizing agent (H_2O_2), the chemiluminescent response was monitored for 40min at 37^0C with a Fluoroscan Ascent spectrophotometer and expressed as percentage of inhibition, using as a positive control the enzyme and the probe. Results are compared to the ones obtained for the control of the solvent (DMSO). Each condition was tested in triplicate. Negative control was represented only by the probe. Inhibition percentages were calculated for each tested concentrations and, by the linear regression of these values, the IC_{50} values were calculated. Each sample was subjected to three independent assays (n=3).

5.3.5. *In vitro* and *ex-vivo* antioxidant assays

The first type of assays that was aimed to prove the involvement of the three species in the processes that are involved in the inflammation concerns the testing of the capacity of the extracts obtained from these species to act on the **global antioxidant activity of the equine neutrophils, that are freshly isolated *ex vivo* from the blood of healthy horses and stimulated by PMA**. Production of ROS in presence of plant extracts is measured by chemiluminescence, in the presence of a luminescent probe (L012). Isolation of Equine Neutrophils was performed from horse blood, which was collected by using EDTA as anticoagulant. The blood was drawn from the jugular vein of healthy horses that do not follow any specific medical treatment. Neutrophils were isolated at room temperature in a discontinuous Percoll density gradient by centrifugation (45min, 400g). Neutrophils were collected carefully and washed two times with phosphate buffered saline (PBS). 90mL of blood from one horse was used for each batch of isolated neutrophils, which were used within 4h after isolation. The activator of neutrophils (PMA) was dissolved in DMSO and preserved at -20^0C. Before use, ultra pure water was added to each aliquot in order to obtain a

solution of 16μM with 1%DMSO. Positive and negative controls were represented by cells in PBS and PBS with the DMSO, activated with PMA and in cells in PBS without the activator. The ROS that were produced by the activated neutrophils were measured by CL, in presence of L012, a luminescent probe. Samples were incubated for 10min at 37^0C in a white 96-well microplate, with a suspension of neutrophil. 10^6 neutrophils were added in each well and the tested sample concentration ranges were between 1 and 100 μg/mL (100, 75, 50, 25, 10, 5 2.5, 2 and 1 μg/mL) both for the total extract and for the isolated products.. After 10 minutes of incubation, 25μL of $CaCl_2$ (10μM) and 10μL of the CL L012 probe were added in each well. Suspension were activated with 10μL of PMA (16μM) just before the CL measurement. The response of the neutrophils was monitored for 40min at 37^0C with a Fluoroscan Ascent spectrophotometer and expressed as the integral value of the total CL emission. Control was represented by neutrophils activated with PMS in PBS, without the samples and was considered as 100% response.

The capacity of samples to act on the cellular production of ROS in conditions that imitate the inflammation was studied in vitro by means of **a cellular model of human monocytes HL-60, activated by PMA in the presence of HRP**. The cellular line that was used is represented by a human promyelocytic cell line (HL-60) and was maintained in continuous culture in the IMDM medium, supplemented with 20% FBS, 100 U/mL penicillin/streptomycin, 1.25 mg/mL amphotericin B and 2g/L $NaHCO_3$ at at 37°C and 5% CO_2 in humidified atmosphere. Before experiments, cells were counted with Burker's cell in order to reach a number of 10^6 cells/mL and DMSO was added in order to reach a concentration of 1.25% (V/V). The DMSO allowed the transformation of the monocytes in macrophages. The cells were incubated over night at 37°C and 5% CO_2 in humidified atmosphere with 5μL of the samples, in 48-well microplates. The tested samples concentration ranges were between 1 and 100 μg/mL (100, 75, 50, 25, 10, 5 2.5, 2 and 1 μg/mL) both for the total extract and for the isolated products. The next day, the content of each well was transferred in 5mL tubes for centrifugation (300g) and centrifuged for 10 minutes at 37°C. The cell pellet was recovered with 150μL of HBSS and transferred into the corresponding wells containing 150μL HBSS. 50μL of the luminescent probe L012 was added, 50μL of $CaCl_2$ (10μM), 10μL of HRP (30μg/mL) and last 25μL of PMA were added. The volume of each well was adjusted in order to reach at 500μL. Each test was performed in the presence of HRP and in the absence of it (plates that corresponded to the assays without HRP were completed with 10μL of HBSS). In each assay, three wells were not loaded with the samples and were considered as a positive control for the ROS production. Other three wells were not activated with PMA and were considered as a negative control, to measure the basic ROS production in the absence of activation. Each sample was tested three times and each test was repeated twice. The produced fluorescence was measured for 40min at 37^0C on a Fluoroscan Ascent spectrophotometer.

5.3.6. Special toxicity assay

The **zebrafish larvae (*Danio regio*) model** was used for the assessment of the special toxicity of extracts belonging to the species *B. alba*. Tested samples were represented by the leaves (BA2), the aerial part (BA3), the stems (BA27) and the root (BA25) extracts. Samples were prepared as described at 4.3.1.1. Crude extracts were dissolved in the water used as a medium for the development of the zebrafish larvae

(deionized water with methylene blue). Zebrafish larvae were treated with the extracts dissolved in water from the development stage they arrived at 24h and were observed until the stage of 72h. Five concentrations ranging between 1µg/mL and 10^4 µg/mL were put in contact in 6-well plates with the extracts. Negative control was represented by larvae that are not treated with extracts and are developing in the water used for the dissolution of extracts. Each well contained, at the beginning of the treatment, 20 larvae at the stage of the development 24h. Treatments consisted in changing their medium with 5mL of the samples dissolved in water, every 24h. Two treatments were consequently applied, at 24 and 48h. Changes in the development of the larvae were observed using the optical microscope. The observed parameters were related to mobility, eventual morphological, cardiac (e.g. heart rate, blood circulation) or breathing changes, but also to pigmentation and loss of chorion. Changes in the development of the larvae were also observed. The number of dead larvae was counted after exposure to extracts. Results of each observation concerning the larvae treated with extracts were compared to the ones of the negative control.

5.4. Results

All the assays that were performed in this chapter allowed to assess different biological activities of the three plants belonging to Cucurbitaceae family. The obtained results were different for each species and could be correlated with the content in the compounds that were evaluated in the phytochemical studies.

5.4.1. Anti-plasmodial and cytotoxicity assays

Anti-plasmodial assays that were performed showed no activity at a starting concentration of extracts of 100µg/mL. Cytotoxicity assays showed a moderate activity for mature and young stems, but also for the roots of *E. elaterium*. The other species proved no cytotoxic activity at a starting concentration of 100µg/mL. Results for the assessment of these activities in the tested samples are found in Table XL.

Table XL. IC_{50} of the anti-plasmodial and cytotoxicity tests of the extracts obtained from the main parts of the three species (n=3)

Sample	Part	Anti-plasmodial (µg/mL)		Cytotoxic (µg/mL)		
		3D7	W2	HeLa	A549	WI38
BA27	Stems	> 50	> 50	> 50	> 50	> 50
BA40	Fruits	> 50	> 50	> 50	> 50	> 50
BA25	Roots	> 50	> 50	> 50	> 50	> 50
BA2	Leaves	> 50	> 50	> 50	> 50	> 50
BA3	Aerial parts	> 50	> 50	> 50	> 50	> 50
EL73	Stems	> 50	> 50	> 50	> 50	> 50
EL74	Leaves	> 50	> 50	> 50	> 50	> 50
EL75	Aerial part	> 50	> 50	> 50	> 50	> 50
EL76	Flowers	> 50	> 50	> 50	> 50	> 50
EL77	Fruits	> 50	> 50	> 50	> 50	> 50
EE82	Mature stems	> 50	> 50	15.28 ± 4.80	22.05 ± 0.88	20.45 ± 0.57
EE83	Young stems	> 50	> 50	22.27 ± 10.33	29.59 ± 12.12	23.09 ± 2.08
EE84	Leaves	> 50	> 50	> 50	> 50	> 50
EE85	Aerial parts	> 50	> 50	> 50	> 50	> 50
EE86	Roots	> 50	> 50	11.08 ± 4.94	12.03 ± 1.34	10.78 ± 0.27

Values represent mean ± standard deviation (SD)

5.4.2. Antioxidant assays

Several antioxidant chemical *in vitro* assays were performed in order to correlate the content of polyphenols and flavonoids with the biological activity that they are widely known for. Each of the performed assays provided different results, that could allow to establish connections between these results and the polyphenols. The selection of the samples which are tested hereby was done based on the results obtained for the quantification of these compounds. Results of the antioxidant assays that were performed can be found in Table XLI.

Table XLI. Results of the antioxidant assays that were performed for the extracts obtained from the main parts of the three species (n=3)

Sample	Method					
	DPPH / *IC50*	CUPRAC / µM TE/100 mL	FRAP / µM TE/100 mL	TEAC / *IC50*	SNPAC / µM TE/100 mL	EPR / *Integral intensity**
EL 46	394.3±0.72	230±0.75	78±1.72	36.8± 0.39	217± 5.72	423.38±3.52
EL 47	60.1±0.42	292±1.73	321± 2.76	15.7± 0.57	559± 6.52	83.68±2.11
EL 48	122.1±0.98	152±0.69	190± 0.98	18.4± 0.72	405± 2.74	285.05±2.74
EL 50	74.3±0.65	226±0.72	308± 1.95	8.2± 0.05	492± 3.22	93.34±1.76
EL 69	80.6±0.92	235±1.42	311± 2.45	14.1± 0.86	800± 4.72	93.7±3.25
EL 71	91.9±0.84	196±0.85	294± 1.25	16.5± 0.43	684± 3.69	125.08±1.90
EL 75	518.7±0.56	68±1.72	105± 1.56	46.5± 0.29	259± 1.21	354.62±2.92
EL 76	82.4±0.85	219±0.96	290± 2.75	16± 0.26	679± 3.69	86.01±0.77
EL 77	239.4±0.37	199±0.93	120± 1.45	23.9± 0.79	306± 1.72	400.99±3.41
BA 1	548.2±0.64	123±0.71	43±0.41	70,4±1.04	95±0.41	308,61±2.51
BA 2	302.1±0.41	381±2.41	110±2.40	22,6±0.49	620±3.45	555,49±3.45
BA 24	99,8±0.92	338±2.24	117±2.45	21,9±0.89	227±2.46	401,96±2.72
EE 82	2184,3±3.42	51±1.41	181±3.01	68,5±1.05	114±1.26	464,5±3.81
EE 84	1816,4±2.81	57±1.24	25±0.41	96,1±1.89	80±0.98	401,88±2.95
EE 85	1170,9±2.05	140±0.41	45±1.42	47±0.57	145±1.21	497,67±3.76

Values represent mean ± standard deviation (SD)
*DPPH Integral intensity = 668.62 ± 0.18

5.4.3. Anti-catalytic assay

The anti-catalytic activity was firstly assessed for total extracts obtained from the main parts of each species. Concentrations ranging between 0.5 and 100 µg/mL were tested in order to establish the concentration where the percentage of inhibition of the enzyme reached at 50%. For the species *B. alba*, as the identification and isolation of compounds is complete, the assessment of the anti-catalytic activity was also performed on the compounds that were isolated by preparative HPLC-DAD. The results of these assays were compared to the results obtained for available references, in order to establish the possible differences that could appear in terms of inhibition of the enzyme, but mostly to establish connections that help to assign the activity of total extracts to specific compounds that are found in their composition. Tested concentrations were ranging between 0.01 and 30 µg/mL for the isolated compounds and the available references. On basis of these inhibition percentages, the values of the IC_{50} for the enzymatic inhibition for each sample were calculated by linear regression. Table XLII contains the values that correspond to total extracts and and Table XLIII contains the values that correspond to isolated compounds and their references.

Table XLII. IC_{50} of the anti-catalytic assays of the extracts obtained from the main parts of the three species (n=3)

Sample	Part	IC_{50} (µg/mL)
BA27	Stems	25.69 ± 2.97
BA40	Fruits	17.61 ± 7.23
BA25	Roots	>50
BA2	Leaves	3.45 ± 0.66
BA3	Aerial parts	3.48 ±1.20
EL73	Stems	>50
EL74	Leaves	10.39 ± 4.80
EL75	Aerial part	34.77 ± 11.36
EL76	Flowers	11.68 ± 2.37
EL77	Fruits	>50
EE82	Mature stems	>50
EE83	Young stems	>50
EE84	Leaves	>50
EE85	Aerial parts	>50
EE86	Roots	>50

Table XLIII. IC_{50} of the anti-catalytic assays of the isolated compounds and of their corresponding references for the flavonoidic compounds in the composition of the leaves of *B.alba* (n=3)

Isolated compound	IC_{50} (µg/mL)	Reference compound	IC_{50} (µg/mL)
Saponarin	0.92 ± 0.55	Saponarin	0.79 ± 0.58
Isoorientin	7.66 ± 2.28	Isoorientin	1.59 ± 0.11
Isovitexin	2.48 ± 1.07	Isovitexin	0.57 ± 0.27
Lutonarin	4.35 ±0.05		

5.4.4. *In vitro* and *ex-vivo* antioxidant assays

The global antioxidant effect on the samples was assessed using *in vitro* and *ex vivo* methods. Thus, by justifying the involvement of the tested samples and products in the inhibition of redox processes that take place in the cell, it could be possible to establish the involvement of the samples in the inflammatory process, therefore to assign a potential anti-inflammatory activity.

The effect of the samples and products on *ex vivo* isolated equine neutrophils, freshly isolated from the blood of healthy horses was assessed on samples selected from the ones that proved to be the most active ones in the anti-catalytic assays. As shown in Table XLII, the species that has proved to be the most active was *B.alba*, as most of the extracts obtained from its parts proved IC_{50} values that correspond to the values that are considered to be corresponding for samples with promising activities. Among the parts of this species, the leaves and aerial parts proved to have the most promising potential, having the IC_{50} values of 3.45 ± 0.66 µg/mL and 3.48 ± 1.20 µg/mL. As the assays for the phytochemical composition of the species were performed on leaves and isolation of the flavonoids was performed on leaves, the total extract of leaves was chosen to be tested on leaves. Then, the isolated flavonoids were tested. Concentration ranges were between 1 and 100 µg/mL (100, 75, 50, 25, 10, 5 2.5, 2 and 1 µg/mL) both for the total extract and for the isolated products. Results were compared to the ones obtained for the solvent used for the dissolution of samples, DMSO and can be found in Fig.103.

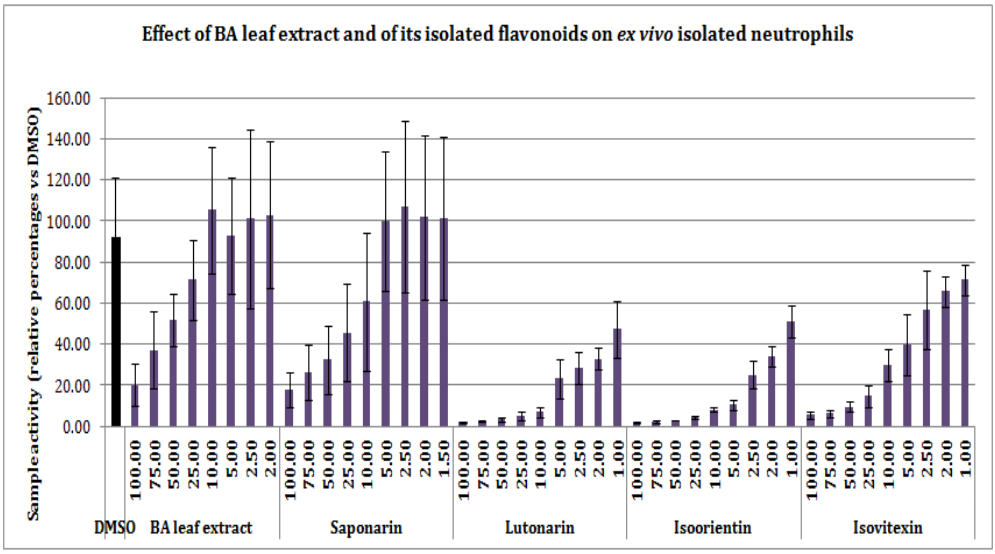


Fig.103. The results obtained for the assessment of the toxicity of leaves of *B. alba*(n=3)

The *in vitro* assay that is used to assess the inhibitory effect of samples on the enzymatic processes that are determined by ROS in the cells is performed on a model of human monocytes that are transformed on macrophages, by adding DMSO as a transformation factor. Same samples, chosen on the same principles as described above, were tested. As lower concentrations proved low inhibitory activity on neutrophils and on the HRP enzyme, they were no longer tested. The range of concentrations was therefore the following: 100, 75, 50, 25, 10, 5 and 2.5 µg/mL. Results were compared to the ones obtained for the DMSO and can be found in Fig.104.

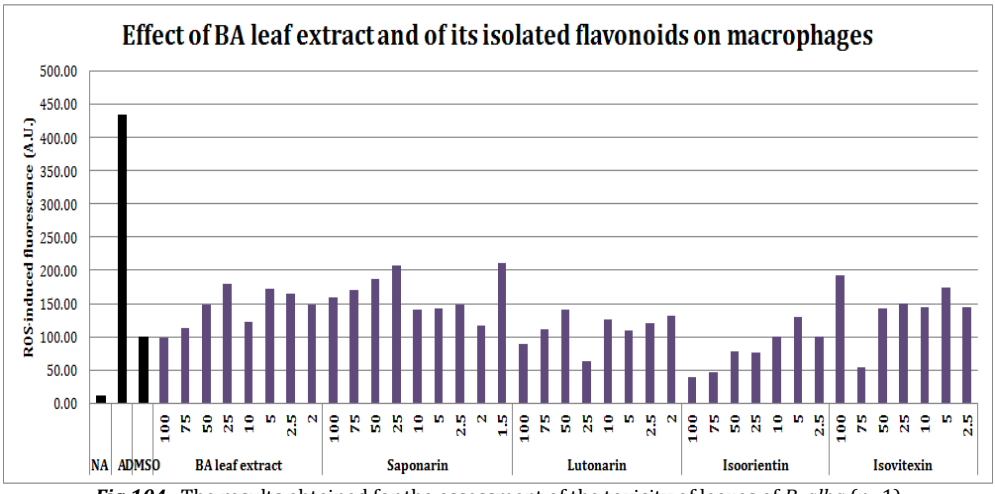


Fig.104. The results obtained for the assessment of the toxicity of leaves of *B. alba* (n=1)

5.4.5. Zebrafish toxicity assay

The zebrafish larvae model allowed to assess the *in vivo* toxicity of the extracts obtained from the main parts of *B. alba*. The parts that were tested were the leaves, the stems and the aerial parts, in order to establish their differences in terms of toxicity. The obtained results can be found in Fig.105-108. The pictures were taken when the zebrafish larvae reached the stage of development of 72h, after 2 treatments with the corresponding extracts. One representative picture of the 20 larvae that were treated from the stage of development of 24h is found in each of the 6 wells of each plate. All of the other larvae in each well were found in the same condition, having all the parameters that were observed in the same stage. Parameters that were monitored are the cardiovascular ones (heart rate, blood circulation), dermatological ones, but also motility problems and morphological changes. Tested concentrations were ranging between 1 and 10.000 µg/mL for leaves, aerial parts and stems, whereas for the roots concentrations were ranging between 0.1 and 1000 µg/mL. Concentrations for roots were different from the ones of the leaves, aerial parts and stems because of the fact that it was noticed, for the three extracts that the first levels of concentration determined the death of all embryos, so only one of these concentration was maintained. The main objective of the range of concentration was the level of 100 µg/mL, which corresponds to the higher level that is tested in the cellular assays aiming to determine the cellular toxicity of the samples. Correlation between the two types of tests may bring important data to justify the biological activity and toxicity of these species.

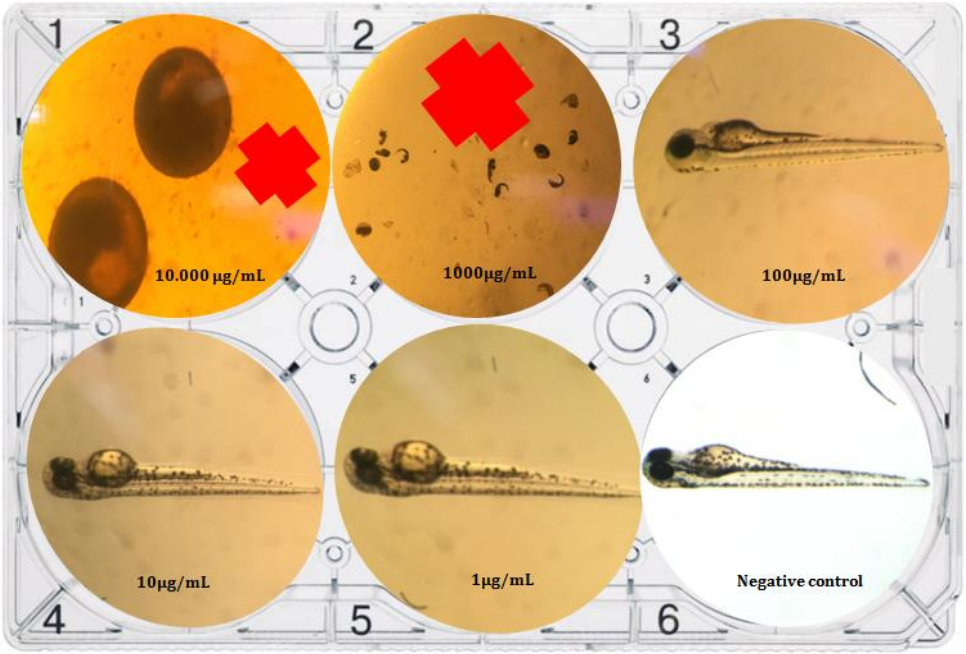


Fig.105. The results obtained for the assessment of the toxicity of leaves of *B. alba*

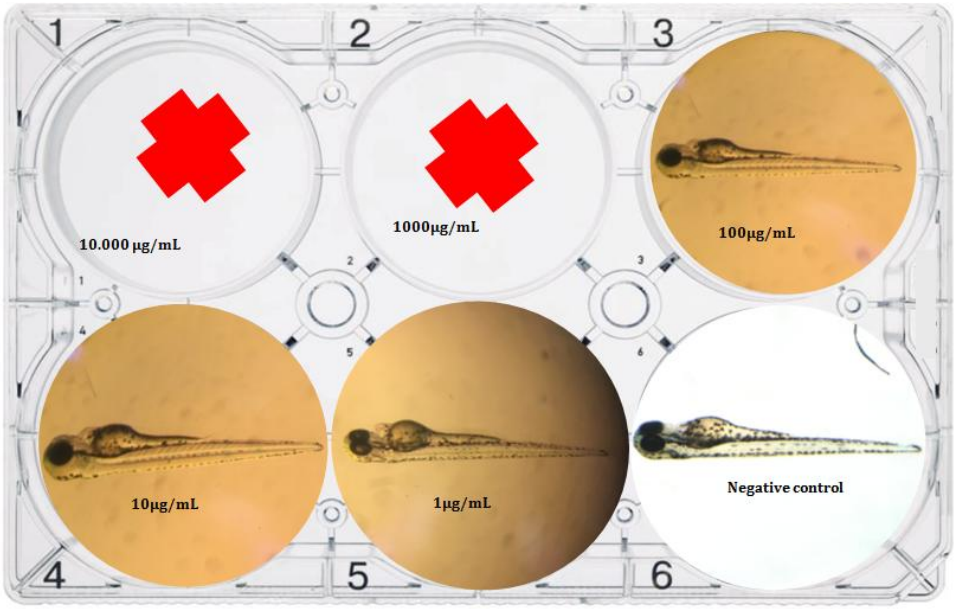


Fig.106. The results obtained for the assessment of the toxicity of aerial parts of *B. alba*

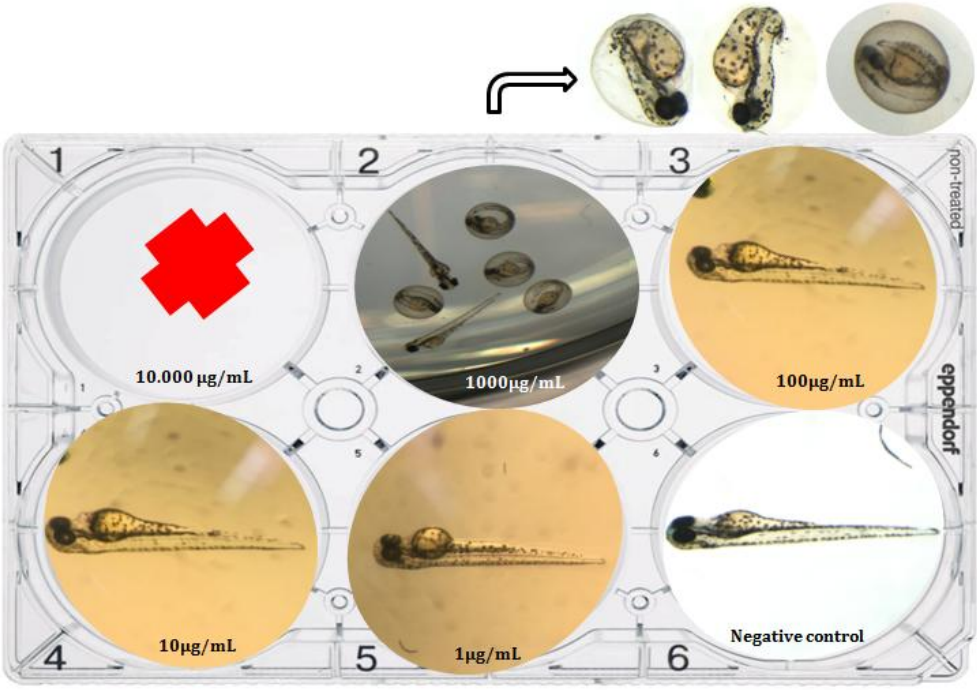


Fig.107. The results obtained for the assessment of the toxicity of stems of *B. alba*

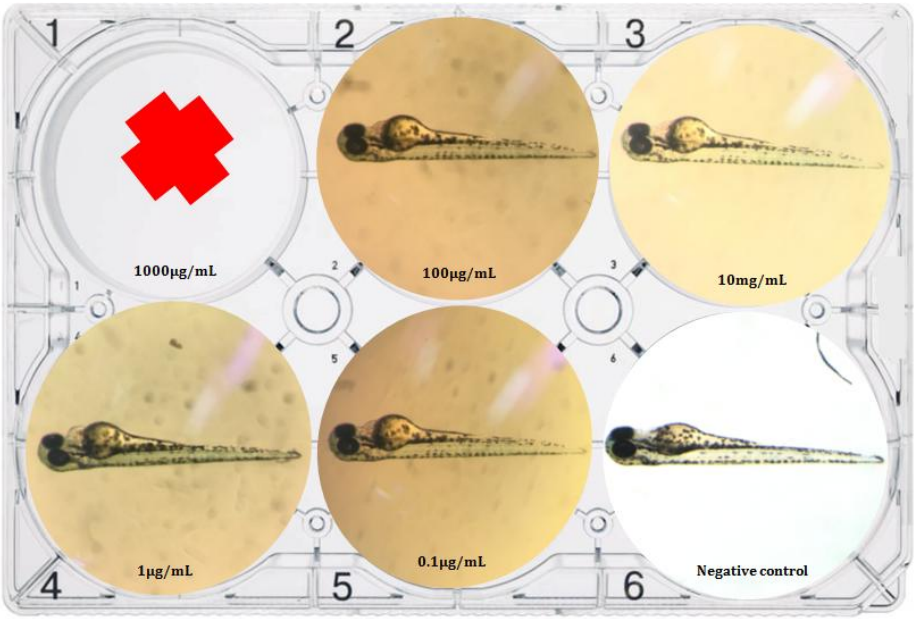


Fig.108. The results obtained for the assessment of the toxicity of roots of *B. alba*

5.5. Discussions

The *in vitro* toxicity at a cellular level of the three species belonging to Cucurbitaceae family was evaluated on two *Plasmodium falciparum* strains (chloroquine resistant and chloroquine sensitive) and on two types of human cellular cell lines, cancerous and healthy. The interest of these tests finds justification in the widely-known cytotoxicity of these species, that is mostly due to their content of cucurbitacins (4). Assessment of the toxicity of these species at a cellular level was though performed in order to justify their possible toxicity. The anti-plasmodial and cytotoxic activities were assessed for total extracts obtained from the main parts of the three species. The results were expressed as the values of IC_{50}, which represent the concentration of the sample needed to obtain 50% inhibition of parasite/cell growth. According to the international guidelines for drug discovery, activities of the extracts can be classified in the following classes, depending on their IC_{50} values: promising activity (IC_{50} ranges between 5µg/ml and 15µg/ml); moderate activity (IC_{50} ranges between 15µg/ml and 50µg/ml); weak activity (IC_{50} is higher than 50 µg/ml). A pure compound is defined as highly active when its IC_{50} is below 1 µg/ml (163).

The anti-plasmodial activity was evaluated for 8 concentrations ranging between 0.8µg/mL and 100µg/mL and values of the IC_{50} were calculated by linear regression of the values obtained for the measured absorbances of each one, after revelation. The IC_{50} for all the tested samples was higher than 50µg/mL, indicating though a weak activity of the samples, at a concentration of 100µg/mL. Same results were obtained for both the 3D7 and the W2 strains. Similarly, **the cytotoxic activity** was assessed for 6 concentrations ranging between 3.2µg/mL and 100µg/mL and values of the IC_{50} were calculated by linear regression of the absorbances obtained after revelation. Values of the IC_{50} were higher than 50µg/mL for most of the samples,

both for cancerous cell lines and for healthy ones. These samples were the ones corresponding to the species *B. alba* and *E. lobata*, that proved weak cytotoxic activity for all their main parts. The only species that proved a cytotoxic activity both on cancerous and on healthy cell lines was *E. elaterium*. It showed moderate cytotoxic activities on cancerous cell lines for mature (IC_{50} of 22.27 ± 10.33μg/mL on HeLa and of 29.59 ± 12.12μg/mL on A549) and young stems (IC_{50} of of 15.28 ± 4.80μg/mL on HeLa and of 22.05 ± 0.88μg/mL on A549) and promising activity for roots (IC_{50} of 11.08 ± 4.94μg/mL on HeLa and of 12.03 ± 1.34μg/mL on A549). It is thus shown that the cytotoxic activity on cancerous cell lines diminishes towards the roots, having its lowest activity in young stems, that are found in the top of the species, and decreasing towards the root, by showing moderate to promising activity for mature stems, that are situated in the close proximity of the roost, which showed the most promising activities. In a similar manner, the cytotoxic activity that was assessed on healthy cell lines produced results in strong correlation with the ones obtained for the cancerous cells. Stems proved moderate decreasing activities, with values from young (IC_{50} of 23.09 ± 2.08μg/mL) to mature (IC_{50} of 20.45 ± 0.57μg/mL) ones and roots proved the most promising activity (IC_{50} of 10.78 ± 0.27μg/mL). If correlating these activities with the composition of compounds that were evaluated in the phytochemical study, it was proven that the species that contained the highest amounts of flavonoids, *B. alba* and *E.lobata*, proved the weakest cytotoxic and anti-plasmodial activities. At the same time, *E .elaterium* was the species that proved the lowest amounts of flavonoids. Moreover, a complementary study of this thesis, that is found in the Annex, proves that this species is the only one that contains in some of its main parts cucurbitacins, which can be the compounds that assign the cytotoxic activity to this species. It is therefore proved that the toxicity at a cellular level for these plants is low, especially for *B. alba* and *E. lobata*. The third species, *E. elaterium* appears to be exhibiting a certain toxicity at a cellular level. Their lack of toxicity or moderate toxicity are in strong correlation with the content of compounds that is evaluated in the studies of the present thesis.

As no toxicity of these species was found at a cellular level and as the main purpose of the present thesis was the study of flavonoids and polyphenols, which are lesser studied in the case of plants belonging to Cucurbitaceae family, the involvement of these species in the events that are related to reduction and oxidation is studied, as it is the main biological activity of which this type of compounds are known (164) .

The antioxidant activity of the three species was though assessed, by several different types of methods. First type of antioxidant assays that were performed were chemical ones, which are aimed to offer preliminary results on the potential involvement of these species in the mechanism of defense towards the aggression of oxidative factors.

Several **chemical assays** were performed in order to assess **the *in vitro* antioxidant activity** of the three species belonging to Cucurbitaceae family. This type of assays is based on the capacity of antioxidants (or mixture of antioxidants, as in the case of extracts) to interact with or neutralize the ROS/RNS. In fact, the oxidative stress is defined as the unbalance between the production of ROS/RNS and the antioxidant defense, which plays an essential role in different pathologies. The *in vitro* chemical assays for the assessment of the antioxidant capacity have been developed to estimate, in a simple experimental way different ways of interacting with the main cause of oxidative stress: the production of ROS/RNS (165). The first of these

strategies concerns the scavenging capacity of antioxidants to neutralize stable free radicals and it is assessed by means of the $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ cations, which are colored, commercially available ($DPPH^{\bullet}$) or which have to be prepared by oxidation of commercially available products ($ABTS^{\bullet+}$) is produced by oxidation of the ABTS with $K_2S_2O_8$) and which usually suffer a decrease of absorbance in the presence of antioxidants. The rate of reaction between antioxidants and $DPPH^{\bullet}$ radical can be monitored not only by spectrophotometry, but also by using normalized double integrated residual EPR signal, which is correlated with the number of paramagnetic species and therefore, the mixture of $DPPH^{\bullet}$ radical and antioxidants decreases the intensity of the signal compared to the $DPPH^{\bullet}$ radical solution. Other strategies concern the reduction of metal ions (FRAP and CUPRAC) and are based on the reduction of Fe^{III} (FRAP method was initially developed to evaluate the ability of human plasma as antioxidant) or Cu^{II} (165,166). The nanoparticles-based assays are based on two stages assessment of the antioxidant capacity: firstly, the reduction of Ag^{+} ions by citrate to form silver seeds and afterwards, the addition of the antioxidant which forms a complex with increased absorbance intensity (166).

All the above described assays (DPPH, CUPRAC, FRAP, TEAC, SNPAC and EPR) were performed in order to establish the interaction of samples with the ROS/RNS, as a single in vitro chemical method is not enough to conclude the antioxidant properties of a sample (165,166). Of all the three species, the one that has proved the most significant antioxidant activity was *E. lobata*. Several samples, collected at different periods of time and at different places were tested. Differences between these samples were noticed. The parts that proved significant antioxidant capacities were the flowers, followed by the leaves and aerial parts. For the flowers, most of the performed methods (DPPH, CUPRAC, FRAP, TEAC and EPR) assigned the most significant antioxidant capacity for the sample EL71, while EL50 was proved the most efficient only in the SNPAC method. The least efficient sample is proved to be EL77 in most of the performed methods (DPPH, FRAP, TEAC, SNPAC and EPR). Results obtained for the assessment of polyphenols in samples of flowers proved that there is a strong correlation between the quantity of polyphenols and the antioxidant activity of these samples. A general trend is observed and concerns samples that are harvested in the stages where the species reaches its highest point of the development, when it seems to accumulate polyphenols. These samples were also the ones that proved to be the most efficient concerning the antioxidant capacity. For the leaves, the most efficient sample was EL47, followed by EL69, in the DPPH, CUPRAC, FRAP and EPR methods. The order is reversed for the TEAC and SNPAC methods. The least efficient proved to be EL75 in all performed methods. Concerning the two samples of aerial parts that were tested, EL76 was proved to be the most efficient than EL48 in all the performed methods. The comparison of different antioxidant efficiency for the flowers, leaves and aerial parts can be found in Table XLIV. Globally, the results of the antioxidant assays correlate to the results of the polyphenol quantification. Samples show a correlation of the accumulation of polyphenols and the increase of the antioxidant capacity, especially for the flowers. If comparing the leaves and aerial parts, it was proved that the leaves have a higher antioxidant capacity than aerial parts, this activity being also correlated with the amounts of polyphenols that are found in their composition. At the same time, compounds seem to have lower concentrations in the aerial parts, due to

the composition of the vegetal product which, besides leaves, may contain other parts of the plant as stems or tendrils.

Table XLIV. The efficiency of the samples of flowers, leaves and aerial parts of *E .lobata*, that proved the most significant antioxidant activity

Method	Flowers	Leaves	Aerial parts
DPPH	EL71 > EL50 > EL77	EL47 > EL69 > EL75	EL76 > EL48
CUPRAC	EL71 > EL77 > EL50	EL47 > EL69 > EL75	EL76 > EL48
FRAP	EL71 > EL50 > EL77	EL47 > EL69 > EL75	EL76 > EL48
TEAC	EL71 > EL50 > EL77	EL69 > EL47 > EL75	EL76 > EL48
SNPAC	EL50 > EL71 > EL77	EL69 > EL47 > EL75	EL76 > EL48
EPR	EL71 > EL50 > EL77	EL47 > EL69 > EL75	EL76 > EL48

On the contrary, *B. alba* showed moderate antioxidant activity and *E.elaterium* showed weak antioxidant activity. All these results correlate with the results obtained in the phytochemical study of flavonoids and polyphenols, which showed moderate content of flavonoids and polyphenols for B.alba and lower concentration of these compounds for *E. elaterium*. For *B. alba*, the most efficient sample was the sample of aerial parts (BA24), especially in the DPPH, CUPRAC, FRAP and TEAC assays. For the SNPAC and EPR the samples that were more efficient were BA2 and BA1. The values that were obtained for *E. elaterium* clearly prove that the species has a weak antioxidant activity. Taken into consideration the results that are obtained in the study of chemical constituents and especially for the study of flavonoids, these results show that there is a strong correlation between the antioxidant capacity of these samples and the content of polyphenols and flavonoids, that is assessed by HPLC-MS method.

In order to support the involvement of these plants in the redox reactions that take place in the biological environment and to support the numerous mechanisms of action by which these species may exhibit their biological activities, anti-catalytic and anti-enzymatic assays were performed. The natural antioxidants may act as unfavourable modulators of the catalytic activity of an enzyme. The natural antioxidants participate at the redox reactions, by maintaining the redox equlibrium in the biological environment, which is favourable for their activity, but also for those of enzymes. When the activity is unfavourable to an enzyme, the activity becomes anti-catalytic. It is for this reason that in the prevention and treatment of pathologies that are related to high ROS production, that the oxidizing enzymes are taken as therapeutic targets in order to control the ROS production.

Therefore, **the anti-catalytic activity** of the samples was tested on a model concerning the activity of the horseradish peroxydase (HRP), using hydrogen peroxide as oxidizing agent. In fact, it is an enzyme that catalyses the reductive cleavage of hydrogen peroxide by an electron donor. This enzyme can also catalyze the conversion of the chromogenic substrates and produces light when acting on chemiluminescent substrates (e.g. L012, a luminol-based chemiluminescent (CL) probe). It is a model of oxidizing enzyme that can be similar in its mechanism of action with other enzymes that produce ROS and are involved in pathologies that derive from the increase of the production of ROS. Firstly, a screening of the inhibitory activity on the HRP enzyme was assessed for the total extracts obtained from different parts of the three species. For the *B.alba* species, tests were also performed on the isolated compounds from the leaves of the species. Results were compared to the ones obtained for the compounds

where references were available (saponarin, isoorientin and isovitexin). For total extracts, tested concentrations were ranging between 0.5 and 100 μg/mL and for isolated compounds between 0.01 and 30 μg/mL. Percentages of inhibition where the activity of the enzyme is inhibited at 50% and their corresponding concentrations were established for each tested sample. IC_{50} were calculated for each extract and isolated product, by linear regression of the inhibition percentages.

The samples that proved to be the most efficient were, for the extracts obtained from different parts of the three species, the ones that belong to *B.alba*. Among these samples, the leaves and aerial parts proved to be the most active, having IC_{50} that show promising potential (3.45 ± 0.66 μg/mL and 3.48 ±1.20 μg/mL). The other parts of the species (stems, roots and fruits) show moderate (stems and fruits) and weak activities (roots). All these results are in strong correlation with the results obtained in the study of flavonoids. So, the parts which proved the most important amount of flavonoids are the parts which show the most promising values for the IC_{50}.

The promising activity of the leaves is the main reason for performing the following assays on total extracts of leaves. The leaves of the species were also chosen because of the fact that all the phytochemical assays were performed on these parts and therefore the anti-catalytic assays were also performed on the flavonoids that were isolated from their content. In order to establish the possible connection of the samples with the biological activity and to accurately assign this activity to these products, the isolated compounds were compared to references, where they were available. All products proved promising activities, both for the isolated products and for the references. Differences between references and isolated products appear because of the fact that some of the products contain more than one compound, as shown in the phytochemical studies (the main compound in each fraction is the one that is mentioned as the isolated product). Otherwise, it is clear that the IC_{50} values are close to the ones of the references. For example, saponarin, the main product in the leaves of *B.alba* has the IC_{50} value of the isolated product 0.92 ± 0.55 μg/mL and of the reference 0.79 ± 0.58 μg/mL. It is a compound that also appears alone in the isolated fraction, which reflects in the close values of the IC_{50} for the isolated product and for the reference. The other compounds, isoorientin and isovitexin also show promising inhibitory activity. Lutonarin, a compound whose reference is not commercially available shows an IC_{50} value of 4.35 ± 0.05 μg/mL, also showing important inhibitory potential of the enzyme.

The results of total extracts for *B. alba* are followed by the ones of the extracts of *E. lobata* and *E. elaterium*. The first species shows moderate activity, with values of 10.39 ± 4.80 μg/mL for leaves, 11.68 ± 2.37 μg/mL for flowers and 34.77 ±11.36 μg/mL for aerial parts and weak activity for stems and fruits. Flowers and leaves, the parts that showed significant amounts of flavonoids and polyphenols. The species *E.elaterium* showed the weakest inhibitory activity for the enzyme, with values that exceed 50 μg/mL.

As a general trend, it was noticed that the samples that proved the highest content of flavonoidic and polyphenolic compounds were the ones that proved the most efficient inhibition of the HRP enzyme. Moreover, reference compounds were proved to have similar values of the IC_{50} with the isolated compounds, which allowed to conclude that these compounds appear to be the ones that are responsible for the anti-catalytic activity of the extracts.

The next step after the chemical assessment of the antioxidant activity of the three species is the assessment of their activity on the enzymes that are produced by the main cells that are involved in the inflammatory answer, in order to establish their possible potential as anti-inflammatory agents. The redox reaction that take place *in vivo* are frequent, physiologically normal et also necessary as they maintain the homeostasis. It is by this kind of reactions that the neutrophils and monocytes, two of the most important types of leukocytes that are part of the family of defense cells exhibit their function in the inflammatory answer. The most important enzymes that are involved in this answer are the NADPH oxidase (Nox), the myeloperoxidase, the inducible NO-synthetase. These enzymes form a cascade on which the activation of one determines the activation of the next one by phosphorylation or they provide the necessary substrate for exhibiting their activity. This cascade and mostly these enzymes that are involved will produce the ROS, that will eliminate the harmful agents. If the production by these enzymes of ROS is too high, the excessive quantities of ROS may disturb the redox environment or the homeostasis. Generally, this perturbation occurs at the site of aggression, but it may expand and affect also entire systems, which generates oxidative stress, which produces the change in the equilibrium of the redox environment, by favouring the reactive species. Thus, the ROS produce deleterious effects on the organism, by oxidizing important biological molecules (e.g. proteins, phospholipids in the composition of membranes etc.), which can destroy their structure and change their functionality. The high levels of ROS represent the basis of numerous pathologies that have inflammatory components as for example cardio-vascular ones. This is the main reason for which the modulation of enzymatic production of ROS in order to maintain the most the physiological level that is needed for homeostasis and to avoid the excesses, is an adequate approach in the prevention and in the struggle against pathologies that are related to inflammation. This is why Nox, MPO but also NADPH are considered very much lately as therapeutical targets. Moreover, some well known no-steroidal anti-inflammatory that are used in the tretment of pathologies associated to inflammation may develop secondary reactions, that are not favourable.

The study of **the global antioxidant activity** of the extracts obtained from the three plants belonging to Cucurbitaceae family was aimed therefore to support the involvement of these plants in the biological response specific to numerous disorders which is inflammation. It assesses the global antioxidant activity on equine neutrophils isolated *ex vivo*, that are activated with PMA and in vitro on a model of HL-60 human monocytes transformed in macrophages that are also activated by the PMA. The study of the anti-catalytic activity of the extracts allowed to perform an adequate screening of the products and concentrations that were involved in the biological activity. Based on these results, it could be possible to calculate the values of the IC_{50} for the inhibition of the enzyme. Same ranges of concentrations were also tested for the assessment of the global antioxidant activity.

The model of *equine neutrophils isolated ex vivo* showed that the total extract has potent inhibitory activity (compared to DMSO, the solvent) on concentrations ranging between 25 and 100 µg/mL. The main compound isolated from the leaves of the species, saponarin showed almost similar active range of concentrations (10 and 100 µg/mL). The most inhibitory activity was even though showed for lutonarin, isoorientin and isovitexin, which have potent inhibitory at all the tested concentration

(ranging between 1 and 100 µg/mL). Moreover, the range of concentrations that shows potent inhibitory activity on the isolated neutrophils show clear dose-dependent activity.

On the other side, the model of *HL-60 human monocytes transformed in macrophages* shows that, compared to DMSO, the inhibitory activity of the samples is too close, so that it couldn't be possible to establish whether the inhibitoy activity of the enzyme is due to the solvent or the sample in itself. The only compound that could show clear inhibitory activity is isoorientin, with a dose-dependent inhibitory answer in the range of concentrations of 10 and 100 µg/mL. As no conclusion could be extracted from these assays, but as values for the inhibition of neutrophils show promising activity, the change of solvent with one that can show a lesser inhibitory activity could help to establish the inhibitory activities of the samples. The DMSO is used, in this model, as a conversion factor for the monocytes in macrophages (48h incubation of the monocytes with 1.25% DMSO) and as a solvent for the samples (incubation over night with the cells). Its inhibitory activity is though significant so, in order to properly assign the activity to the samples, the use of another solvent for the samples is necessary (e.g. ethanol, water).

The **zebrafish larvae model** is a method that assesses the *in vivo* toxicity of extracts or drugs, used by researchers worldwide as an alternative to animal testing (167), since zebrafish larvae are early life-stages of animals that are not protected, as suggested by the directives of EU (2010/63/EU) until 120h after fertilization, when they can be regarded as independently feeding (168). It is a method of assessement of special toxicity which has proven excellent results in the medical field of research of different pathologies (167,169). Latest studies have proven that they are excellent study models for the research in numerous fields, as cancer (170–172), brain disorders (173), motor neuron diseases or other neuroscience-related disorders (174,175).

As species belonging to Cucurbitaceae family are largely known for their toxicity, the *in vivo* assessment of their toxicity was necessary in order to justify the possibility of their future use in therapy for their antioxidant or anti-inflammatory activities. The species that proved the most significant results for the phytochemical and antioxidant assays, *B. alba*, is the one that was tested by this model. Samples that were tested are extracts of leaves, stems, aerial parts and roots. The results obtained hereby complete the ones that were obtained *in vitro* for the assessment of the cellular toxicity, antioxidant and anti-inflammatory activities, so that the study of these plants becomes complete and may be able to bring important data on the potential of these plants as possible medicinal products.

The observation of the zebrafish, from the early stages of development (embryos of 24h) until 72h of their development, after 3 different treatments with different doses of extracts, shows that no special toxicity may be assigned to the extracts of this species. Leaves, aerial parts and roots showed similar results concerning the different concentrations of extract. Highest doses of extract that were tested (1 and 10 mg/mL) were the only ones that killed the zebrafish from their early stages of the development (embryos). A possible explanation for this fact is the osmolarity of the medium, which determines the death of the zebrafish. The only sample where the concentration of 1 mg/mL showed lagging in the process of the development of the zebrafish was the stem extract. 10 out of 20 embryos showed important slowing in the process of leaving the chorion, which shows evident sign of

weakness. Zebrafish that were able to get out of their chorion show obvious signs of lagging in different levels: slow heart rate, slow breathing, motility problems and even morphological changes. At concentrations of 10^{-1} mg/mL and below, all the zebrafish showed no special toxicity on different levels. Cardiovascular, respiratory, dermatological and morphological changes of the zebrafish were monitored before and after the treatment with the different doses of extracts in the 3 days of treatment. No special toxicity was observed for none of the tested samples. All the zebrafish show the same parameters that concern heart rate, respiratory rate, but also same development rhytm. No other problems were noticed for none of the tested samples: e.g. morphological changes, motility problems or dermatological issues. Even the root extract did not determine special toxicity, despite the fact that scientific literature cites an important toxicity of these parts of the species (28,29). All parameters that are observed for each of these samples are compared with the ones of the control zebrafish, that are not treated with the samples.

5.6. Conclusions

Assessment of the biological activity of the three species belonging to Cucurbitaceae family is the last step in the study of these plants, which completes data that are brought by the present thesis. Biological activities that are tested hereby are aimed to bring data that concern the toxicity of these species, but also the potential use in the treatment of some disorders.

Anti-plasmodial and cytotoxicity assays show that there is no toxicity at a cellular level for the total extracts obtained from different parts of the three species at a concentration of 100 μg/mL.

Study of the antioxidant activity of these species is performed by different methods. Chemical assays for the antioxidant activity are performed and confirm the potential of these samples to act on different levels such as neutralizing stable free radicals or reduction of metal ions.

The global antioxidant activity is assessed in order to confirm the involvement of these species in the processes that are also involved in inflammation. The study of the inhibitory activity on different paths that are directly connected to the production of ROS/RNS is performed after the assessment of the anti-catalytic activity of these samples, which is aimed not only to perform a screening of the most active concentrations for each sample, but also to bring data on the inhibitory activity for the catalytic activity of a peroxydase. The models that are used are the activated equine neutrophils isolated *ex vivo* and the *in vitro* model of monocytes transformed into macrophages. On the first model, there is a clear inhibitory activity which is shown especially by the isolated compounds from the leaves of *B. alba*. The compounds that show the most significant activity are lutonarin, isoorientin and isovitexin. The model of the HL-60 human monocytes transformed into macrophages shows an activity of the samples which is close to the one of the solvent (DMSO), which is also the conversion factor of monocytes to macrophages. Further studies are needed in order to confirm the activity of these samples on this model. Thus, the potential of these plants as antioxidant and anti-inflammatory agents could be established. The attempt to establish the inhibitory activity on the last model, using another solvent for the samples may help to confirm the involvement of the samples in this process.

The toxicity of the samples was assessed on an *in vivo* model of zebrafish larvae, which are treated with different concentration of extract in 72h. After the treatment, no special toxicity was noticed at any level (cardiovascular, respiratory, dermatological) and no special involvement of the extracts in the motility or in the development of the embryos was observed. Tested samples included stems, leaves, aerial parts and roots of *B.alba*. None of these proved special toxicity, even though scientific literature indicates certain toxicity. The fact that no toxicity is shown at a starting concentration of 100 μg/mL is in a strong correlation with the assays of cellular toxicity, that showed no such toxicity at the same dose.

All these assays bring important scientific evidence on the fact that the three studied species have an important potential as medicinal species.

6. General discussions

B. alba, *E. lobata* and *E. elaterium* are three species belonging to Cucurbitaceae family that are known for their allopathic and homeopathic use, in traditional or modern medicine. Scientific literature brings data on these species, but information is not enough to establish the potential of these species as medicinal plants. This is the main reason for directing the studies in this thesis towards different approaches, that may finally offer important information in order to complete existing data and confirm their importance in this field. The three approaches that are studied hereby refer to the botanical, phytochemical and biological study of these species.

Differences and similarities between the three species are found at all levels. Most of the similarities that are found between these species are related to *B. alba* and *E. elaterium*. In fact, these species have an important connection, that starts on the phylogenetic line. The different approaches that are performed show that there is clearly a connection between the two species. Botanic studies show that there is a clear connection between the two species, that concerns the macroscopic and microscopic features of the main organs. For example, roots present the same macroscopic and microscopic features, that concern primary and secondary structures. Other parts of the species present similar features. Microscopically, the feature that is found as a general similarity are the bicollateral vascular bundles, that are found in almost all of the organs of these species. Differences between the two related species concern the presence of the tendrils for *B. alba* and their absence for *E. elaterium*. Otherwise, all of the other features are specific for the organ they belong to. Relationship that exists between these two species stops at the phylogenetic connection, which is in direct correlation with the botanic characters. The phytochemical and pharmacological approaches of the two related species show that there are significant differences between these species. Even though the most important compounds that are found in the composition of the two species are cucurbitacins and flavonoids, it seems that not the same compounds are responsible for the biological activities. Phytochemical studies performed hereby, that concern especially the study of flavonoids, show that flavonoids are present in the composition of *B. alba* in important amounts, while in the composition of *E. elaterium* they are present in lower amounts. Cucurbitacins were only detected in *E. elaterium.* As these compounds are the ones that are responsible for the biological activities, it is clear that these activities are also different. The anti-plasmodial and cytotoxic effect are not present at a concentration of 100 µg/mL for *B. alba* and are present with weak values for *E. elaterium*, while the antioxidant assays have shown promising activity for *B.alba* and weak activity for *E. elaterium*. As the present thesis is aimed to study the flavonoids in the composition of these species, the biological activities that are determined by these compounds are developed for *B.alba*.

Overall, regarding the three species that are studied hereby, there are also similarities and differences. From the botanic point of view, in fact, the three species that are studied in the present thesis present the same important microscopic feature that concerns bicollateral vascular bundles. It is a feature that is characteristic for plants that belong to Cucurbitaceae family and it is found, with some rare exceptions, in most of the microscopic structures of the organs belonging to the three species.

Exceptions are found especially in the anatomical structures of stems, which, for example, for *E. lobata* contain both collateral and bicollateral vascular bundles. Phytochemical studies show that the greatest amount of flavonoids and polyphenols are also present for this species, which is followed by *B. alba* and *E. elaterium*, the latter being the one that contains the lowest amounts of these compounds. Concerning the biological activities of these species, there is a clear lack of toxicity at a cellular level for the species *B. alba* and *E. lobata*, while *E. elaterium* shows a certain cytotoxicity. Other bio-activity that is proved by the studies in this thesis and have shown significant results is the antioxidant one. There is a clear evidence that there is a connection between the compounds that are assessed in the composition of these species and their biological activities. Thus, the species that have proven important amounts of flavonoids and polyphenols are the ones that have proven to have the most significant results for the antioxidant activities. Therefore, is was shown by the HPLC-MS study of polyphenols and by the assessment of the antioxidant activity by chemical assays that *E. lobata* is the species that is the richest in flavonoids and polyphenols and has therefore the most significant results that concern the antioxidant activities. It is followed by *B. alba* and *E. elaterium* has proved to be the poorest in flavonoids and showing the lowest antioxidant activity.

On the other side, the complete phytochemical profile is elucidated only for *B.alba*. This is the most important reason for choosing to further study the antioxidant mechanisms that are involved in the bio-activity that is shown by this species. Thus, it could be proved that this species is involved in the redox processes that accompany the inflammatory process. After a screening of the inhibitory activity on a peroxidase, it was proved that this species is the one that shows the most significant values of the inhibitory activity, which was a supplementary reason for further studying its global antioxidant activity, which was assessed by two different models, that use freshly isolated equine neutrophils *ex vivo* activated by PMA and human monocytes transformed into macrophages activated by PMA. Results show that there is a significant inhibitory activity for the total extract, but especially for the isolated compounds in its composition. Moreover, this species is the one that is tested for in vivo toxicity on the cellular model of zebrafish larvae. The tested samples (extracts of stems, leaves, aerial parts and roots) showed no special toxicity on the early stages of development of the zebrafish embryos. There is a strong and important correlation between the doses that are assessed for the *in vitro* assays of cellular toxicity and the doses that are assessed for the *in vivo* model of zebrafish larvae.

All the similarities between the three species are derived from the fact that these species belong to the same family and are related to different aspects that concern the three major direction that the present thesis is following: botanical, phytochemical and assessment of the biological activity. Differences that appear between these species are the ones that differentiate and individualize each species.

7. General conclusions

The comparative study of the three species belonging to Cucurbitaceae family allows to bring important data on each of them and, at the same time, on the similarities and differences between them.

Botanical studies allowed to establish the microscopic and macroscopic features of each species that is studied. Firstly, macroscopic analysis could be performed by the observation of the botanical features, but also by using adequate methods such as stereomicroscopy. Microscopic analysis allowed to identify the anatomical structures of each studied organ. For the three species, both vegetative and reproductive organs were studied, by different methods that allowed obtaining the most adequate sections through these organs. By the examination of the photos that are obtained for each of these organs belonging to each species, the anatomical structure could be established. Thus, by the correlation of all these, not only the botanical features of these plants could be established, but also a complete description of each one could be provided. This represents the first step towards a complete description of these species, offering, at the same time, the necessary data in order to correctly establish the identity of each species.

Regarding the phytochemical study, the present thesis is aimed to provide information on the flavonoids, which are compounds that were not frequently studied in the composition of these species. Actually, plants belonging to Cucurbitaceae family are widely known for the presence in their composition in cucurbitacins. The thesis is aimed to bring into attention that, besides the presence of cucurbitacins, in the composition of these species, there are also other compounds, lesser studied, but which may prove significant involvement in the known biological activities, or may even assign new biological activities. Therefore, the study of flavonoids was performed by spectrophotometric, chromatographic and spectroscopic methods. Firstly, the study of flavonoids started with the quantification of the total flavonoids, polyphenols and hydroxycinnamic acids. Afterwards, flavonoids and polyphenols were identified and quantified by HPLC-DAD and HPLC-MS methods. Identified flavonoids were isolated by preparative HPLC-DAD and identification of their chemical structure was performed by MS and NMR techniques. Thus, in the composition of the leaves of *B. alba* (the plant part containing most flavonoids) lutonarin, saponarin, isoorientin and isovitexin could be identified, isolated and quantified. The only compound that has not been cited before in the composition of the species is isoorientin. It is the first study of the kind which evaluated different parts of the species and performs the analysis of flavonoids on the richest parts. The species proved therefore to contain mostly flavone-C-glucosides. In the composition of *E. lobata*, O-heterosides proved to be the main flavonoidic compounds. Parts that proved to be the richest were flowers and leaves. The analysis were mostly performed on flowers. The complete identification of the flavonoids in the composition of flowers is not completely finished. Despite this fact, isorhamnetin and quercetin derivatives were found. Astragalin, isoquercitrin and rutin were identified, after the comparison with references. On the contrary, *E. elaterium* proved to be the poorest in flavonoids, and in the composition of aerial parts only rutin could be identified.

The assessment of the biological activity for this species followed to establish new perspectives on the use of these plants for biological activities they are not frequently cited for. The first step was the assessment of their toxicity at a cellular level, as these plants are known for their toxicity. No cellular toxicity was found for *B. alba* and *E. lobata* and a low cytotoxicity was noticed for *E.elaterium*. Antioxidant activity was subsequently assessed by chemical assays. The most active species was *E. lobata*, followed by *B. alba* and *E. elaterium.* Next, the study of the global antioxidant activity was studied in order to justify the involvement of these plants in the inhibition of redox processes that are also involved in inflammation. The study of global antioxidant activity is based on a screening that aims to assess the capacity of these plants to act as unfavourable modulators of the activity of an enzyme (the HRP enzyme). In this assay, the most active species was *B. alba*, followed by *E. lobata* and *E. elaterium.* Global antioxidant activity on neutrophils and macrophages was only assessed for *B.alba*, as it proved the most important potential for the involvement in the redox processes, that may accompany inflammation. Significant inhibitory activity of the leaves extract and of its isolated compounds was found on two models, the equine isolated neutrophils *ex vivo* and activated with PMA and the human HL-60 monocytes transformed in macrophages and activated with PMA. The most active compounds proved to be lutonarin, isoorientin and isovitexin. Not least, for this species, the *in vivo* toxicity was assessed on a zebrafish larvae model. No special toxicity was found for the stems, leaves, aerial parts and roots. The concentrations correlate with the ones tested for cellular toxicity, which may indicate the fact that the species may have low toxicity. Moreover, it was found that all these results of biological assays are in strong correlation with their composition in bio-active compounds.

Therefore, the study of these plants, that is performed by three different approaches, brings new and important data, both on the composition of these species in flavonoidic compounds, but also on the biological activities that they are responsible for. The present study is the first of a kind that treats these plants in this manner.

8. Originality and innovative contributions of the thesis

The present thesis is following three major directions that are aimed to provide a complete study of three species belonging to Cucurbitacee family, namely *Bryonia alba* L., *Echinocystis lobata (Michx*.) Torr. et A. Gray and *Ecballium elaterium* (L.) A. Rich.

The first approach that is offered by this thesis is the botanical study of the three species. This is the first approach of that kind that is done for each species. Even though scientific literature offers some data on the botanical description of some of the features of these species, no complete and conclusive data on these species could be found and therefore the information that is brought hereby offer a new approach towards the characterization of the microscopic and macroscopic description of these species. The methods that are used for the microscopic study allow to bring data that are presented for the first time in this manner. It is thus the first description of the anatomical structure of reproductive organs for *B. alba* and the first description of all the vegetative and reproductive organs for *E. obata* and *E. elaterium*. For the trichomes of the species, the ones belonging to *B. alba* and *E. lobata* are described for the first time (the ones of *B. alba* are also described by SEM). Therefore, the results obtained hereby in the botanical study are original and may complete the existing data in scientific literature and help to perform the complete and correct identification of these species.

The second section of this thesis is in itself the most innovative realisation as it describes the study of the composition in flavonoids of these species. Even if the plants belonging to Cucurbitaceae family are largely known for their content of cucurbitacins, the flavonoids are lesser known and studied. They are nevertheless important compounds and the present studies were aimed to show that they might be responsible for biological activities that were never described before in scientific literature. The novelty of this study concerns therefore the HPLC-DAD and HPLC-MS study of flavonoids and polyphenols, followed by their isolation by preparative HPLC-DAD. Isolated products by these techniques were subsequently analyzed in order to properly identify their structure by means of MS and NMR techniques. In the last stage, these products were used for the biological assays and were tested in order to establish their involvement in the biological activities that the total extracts exhibited.

The biological activities that are assessed in this study are also novel and innovative. These plants are generally known for their cytotoxicity, as their content of cucurbitacins is largely cited. The present study showed no significant cytotoxicity for the extracts belonging to the three plants. These findings are important, as the plants that proved weak cytotoxicity (*B. alba* and *E. lobata*) are the ones that prove to contain the highest amounts of flavonoids. The only species that has proved moderate cytotoxicity is *E. elaterium*, the species which contains the lowest amounts of flavonoids. Another important novelty is the assessment of the anti-plasmodial activity of these plants. Even though the anti-plasmodial tests showed no activity, the results correlate with the ones obtained for the assessment of the cytotoxicity, showing that there is no cellular toxicity for these samples. It is especially the extracts of the species that are richer in flavonoids that have shown no toxicity. Subsequently, the species were studied for the antioxidant properties, as the flavonoids are well known to

exhibit such properties. Our work demonstrated antioxidant properties for these species. Classical and chemical assays were used. The capacity to inhibit the activity of an enzyme was assessed on the HRP model and it was found that the most potent extracts were the ones of *B. alba*. Subsequently, this extract and its isolated products were tested for the global antioxidant activity on two different models, the equine isolated neutrophils *ex vivo* and activated with PMA and the human HL-60 monocytes transformed in macrophages and activated with PMA. All these assays are performed for the first time for the species *B. alba*. By justifying the involvement of these products in the redox processes, it could be justified that these products can be involved in the processes that accompany inflammation.

By the different approaches that were treated in the present thesis, new and important data could be added to scientific literature regarding the three species belonging to Cucurbitaceae family. Originality and innovative contributions are found not only in the different ways of describing these species, but also in the obtained results, that can help to justify the importance of these plants as medicinal species. Therefore, *Bryonia alba* L., *Echinocystis lobata (Michx*.) Torr. et A. Gray and *Ecballium elaterium* (L.) A. Rich. have proved to be important sources of bioactive compounds and may have an important potential in the treatment of different disorders. Nevertheless, their absence of toxicity should be confirmed with more samples and *in vivo* studies.

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ANNEX I. Study of the cucurbitacins in the composition of different parts belonging to the three species

1. TLC studies

Conditions:

- **Test solution**: extracts of the samples described in Table below (concentration 1mg/mL) in methanol;

Species	Sample name	Part of the species
Bryonia alba L.	BA27	Stems
	BA40	Fruits
	BA25	Root
	BA6	Leaves
	BA7	Aerial part
Echinocystis lobata (Michx.) Torr. et A. Gray	EL73	Stems
	EL74	Leaves
	EL75	Aerial parts
	EL76	♂ flowers
	EL77	Fruits
Ecballium elaterium (L.) A .Rich.	EE82	Mature stems
	EE83	Young stems
	EE84	Leaves
	EE85	Aerial part
	EE86	Roots

- **Plate:** Silica gel 60 F_{254} – precoated TLC plates (Merck, Germany);
- **Mobile phase:** Dichloromethane: Methanol (95:10 V/V);
- **Application:** 10µL as bands;
- **Development:** over a path of 10cm;
- **Drying:** in air;
- **Detection:** spray with vanilin phosphoric acid reagent; examination in VIS.

Results:

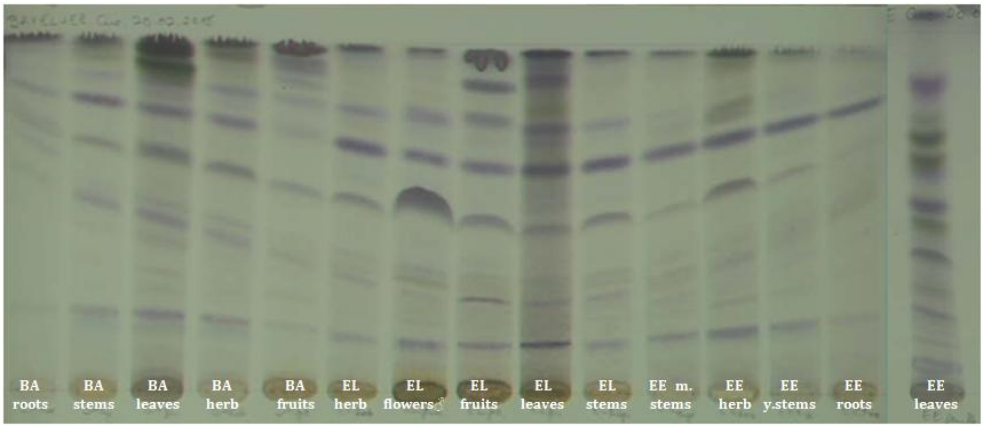

Fig.1. TLC analysis of different samples belonging to the three species

2. HPLC-DAD

Conditions:

- **Apparatus**: Hewlett Packard Agilent 1100 system, equipped with an Agilent 1100 quaternary pump, an Agilent 1100 degaser and an Agilent 1100 Automatic Liquid Sampler;
- **Test solution**: extracts of the samples described in Table below (concentration 0.1mg/mL) in ethyl acetate, that are evaporated under reduced pressure and dissolved in methanol;

Species	Sample name	Part of the species
Bryonia alba L.	BA27	Stems
	BA40	Fruits
	BA25	Root
	BA2	Leaves
	BA3	Aerial part
Echinocystis lobata (Michx.) Torr. et A. Gray	EL73	Stems
	EL74	Leaves
	EL75	Aerial parts
	EL76	♂ flowers
	EL77	Fruits
Ecballium elaterium (L.) A. Rich.	EE82	Mature stems
	EE83	Young stems
	EE84	Leaves
	EE85	Aerial part
	EE86	Roots

- **Reference solutions:** Cucurbitacin B, D, E, I (Sigma-Aldrich, Germany);
- **Column:** Hypersil ODS C18 column (250 mm x 4.6, i.d.; particle size 5 μm; Thermo Scientific);
- **Temperature:** room temperature (25°C);
- **Flow rate:** 1mL/min;
- **Injection volume:** 10μL;
- **Mobile phase:** water (A) and acetonitrile (B), with the following gradient :

Minute	% A	%B
0-20	20	80
20-22	60	40
22-40	60	40
40-41	90	10
41-46	90	100
46-47	20	80
47-65	20	80

- **Detection:** UV, at 235nm.

Results:

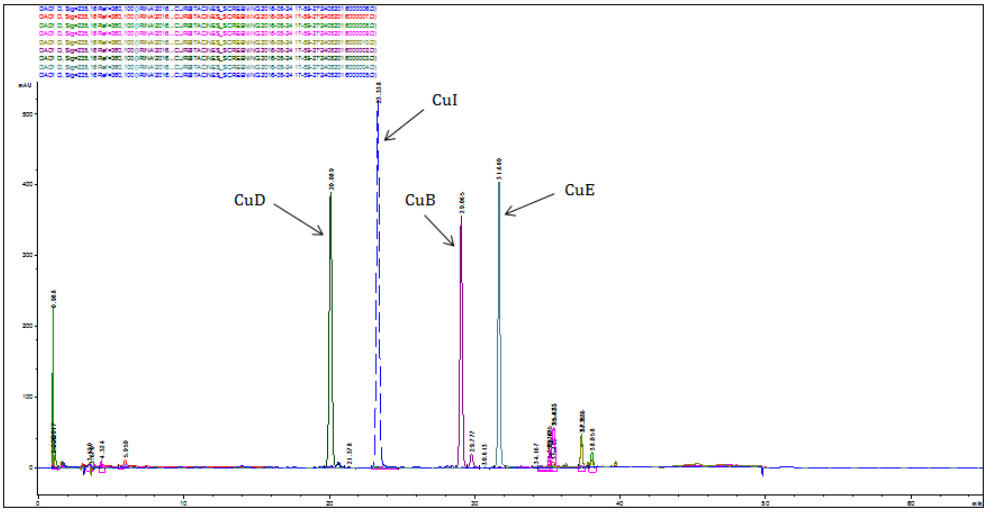

Fig.2. HPLC-DAD chromatogram showing the profile of cucurbitacins in different samples of *B. alba* (blue - BA leaves; red – BA stems; dark green – BA fruits; rose – BA aerial parts; light green – BA roots)

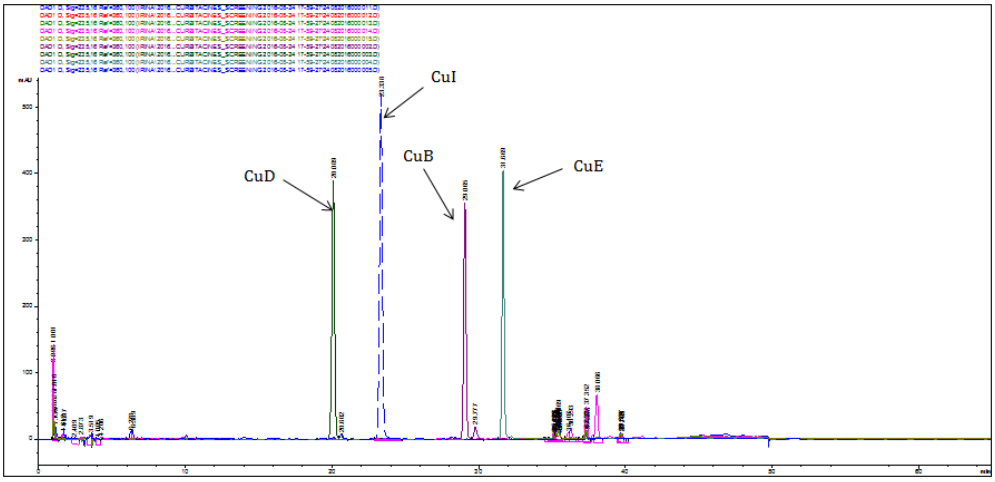

Fig.3. HPLC-DAD chromatogram showing the profile of cucurbitacins in different samples of *E. lobata* (blue - EL leaves; red – EL flowers; dark green – EL stems; rose – EL fruits; light green – EL aerial parts)

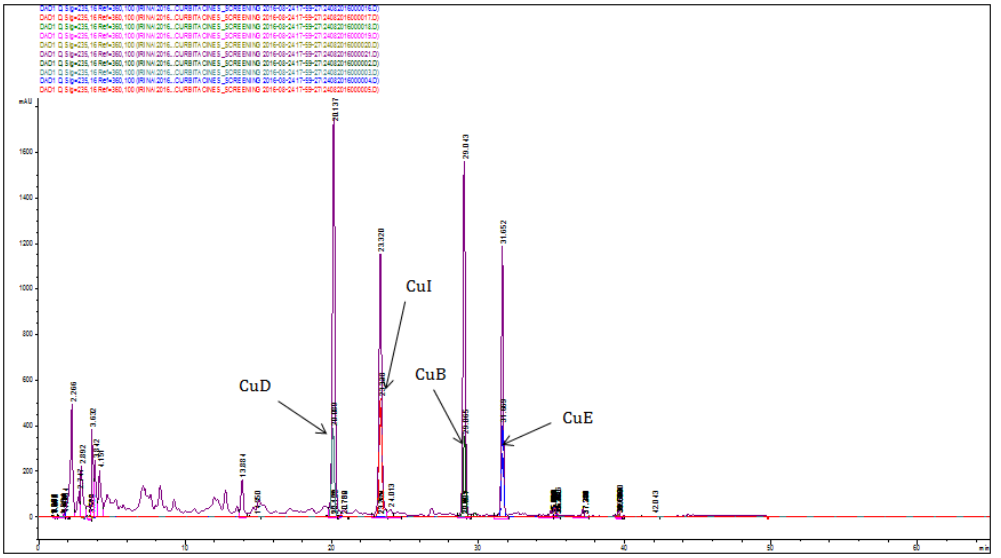

Fig.4. HPLC-DAD chromatogram showing the profile of cucurbitacins in different samples of *E. elaterium* (blue –EE leaves; red – EE young stem; dark green – EE mature stem; rose – EE aerial parts; light green – EE roots; violet – EE fruits)

General discussions:

The TLC analysis shows the presence of numerous spots in the composition of the three species, that are violet in the VIS light, after revelation with the vanilin phosphoric reagent. Scientific literature cites the compounds as possible cucurbitacin genins or cucurbitacin heterosides (176), but as the revelation reagent is not a specific one for these compounds, the TLC analysis is not conclusive. The HPLC-DAD analysis of cucurbitacins shows that these compounds are not present in the extracts belonging to different parts of the three species. References that are tested in the present study are genins that are named Cucurbitacin B, D, E and I. The only species that shows the presence of all the four genins is *E. elaterium*, in its fruits. All of the other species and their parts do not contain these compounds.

Conclusions:

Despite the fact that scientific literature cites the presence of cucurbitacins in the composition of the three species, studies performed hereby prove that there low amounts or no cucurbitacins (among the tested genins: cucurbitacin B, D, E and I) are found in the compostion of the main parts belonging to *Bryonia alba* L., *Echinocystis lobata (Michx.*) Torr. et A. Gray and *Ecballium elaterium* (L.) A. Rich.

ANNEX II. List of reagents and solvents

Reagents	Solvents	References
Alum carmine Alfa Aesar®	**Disinfectol ® (Ethanol)** ChemLab	**Saponarin** Extrasynthese
Malachite green Merck	**Methanol** HiPerSolv Chromanorm VWR Chemicals	**Isovitexin** Sigma Aldrich
Toluidin blue Sigma Aldrich	**Methanol** LiChrosolv ® Merck	**Vitexin** Sigma Aldrich
Iodine green International Laboratory	**Ethyl acetate** Anala R NORMAPUR VWR Chemicals	**Rutin** Extrasynthese
Lactic acid 90% Merck	**Acetonitrile** LiChrosolv® Merck	**Isoquercitrin** Extrasynthese
Soudan G Red	**Acetone** AnalaR NORMAPUR VWR Chemicals	**Astragalin (Kaempferol-3-O-glucoside)** Extrasynthese
Anilin sulphate UCB	**CD_3OD** EurisoTop Gif-sur-Yvette France	**Apigenin** Extrasynthese
Iodine	**Xylene** Lachner Chemicals	**Luteolin** Extrasynthese
Potassium iodide		**Chlorogenic acid,** Sigma Aldrich
Paraffin		***p*-Coumaric acid** Sigma Aldrich
Chloralhydrate hydroxide Sigma Aldrich		**Caffeic acid** Sigma Aldrich
Hydrochloric acid Merck		**Rutin** Sigma Aldrich
Potassium hydroxide Sigma Aldrich		**Apigenin** Sigma Aldrich
Formic acid 98% GPR Rectapur VWR Chemicals		**Quercetin** Sigma Aldrich
Glacial acetic acid 99-100% ChemLab		**Isoquercitrin** Sigma Aldrich
Diphenyl-borate-amino-ethanol Sigma Aldrich		**Quercitrin** Sigma Aldrich
PEG 400 VWR Chemicals		**Hyperoside** Sigma Aldrich
Boric acid Merck		**Kaempferol** Sigma Aldrich
Oxalic acid Merck		**Myricetol** Sigma Aldrich
Trifluoroacetic acid Uvasol® Merck		**Fisetin** Sigma Aldrich
Sodium acetate Sigma Aldrich		**Cichoric acid** Dalton
Aluminium chloride Sigma Aldrich		**Caftaric acid** Dalton
Folin Ciocâlteu reagent Sigma Aldrich		**Ferulic acid** Roth
Hydrochloric acid Sigma Aldrich		**Sinapic acid** Roth
Sodium nitrite Sigma Aldrich		**Gentisic acid** Roth
Sodium molibdate Sigma Aldrich		**Gallic acid** Roth
Sodium hydroxide Sigma Aldrich		**Patuletin** Roth
Lithium Lactate Sigma Aldrich		**Luteolin** Roth
DMEM Lonza		**Hyperoside** Extrasynthese
RPMI 1640 (1x) Medium Gibco		**Orientin** Extrasynthese
PBS Lonza		**Quercitrin** Extrasynthese
DMSO Merck		**Isoorientin** Extrasynthese
Grunwald Merck		**Caffeic acid** Sigma Aldrich
Giemsa Merck		**Cucurbitacin B** Sigma Aldrich
Saponin Alfa Aesar		**Cucurbitacin D** ChromaDex
FBS Sigma Aldrich		**Cucurbitacin E** Sigma Aldrich
PenStrep Lonza		**Cucurbitacin I** ChromaDEx

L-glutamine Lonza		
Gentamicin Lonza		
Glucose-hypoxanthine Sigma Aldrich		
APAD Sigma Aldrich		
Triton X100 Sigma Aldrich		
PES Sigma Aldrich		
NBT Sigma Aldrich		
WST1 Roche		
DPPH Sigma Aldrich		
ABTS$^{\bullet+}$ Sigma Aldrich		
Trolox Sigma Aldrich		
2,4,6-tripyridyl-s-triazine Sigma Aldrich		
Neocupreine Sigma Aldrich		
Silver nitrate Sigma Aldrich		
Trisodium citrate Sigma Aldrich		
HRP Sigma Aldrich		
H_2O_2 Sigma Aldrich		
Percoll Sigma Aldrich		
HBSS Sigma Aldrich		
PMA Sigma Aldrich		
Calcium chloride Merck		
L012 Wako Chemicals GBH		

ANNEX III. Vegetal material. Tested samples

Table I. Collected samples of the three species belonging to Cucurbitaceae family, with collection date and place

Nr.	Species	Harvesting date	Harvesting place	Part of the species
1.	*Bryonia alba* L.	06.05.2014	Cluj, Parcul Feroviarilor	Stems
2.				Leaves
3.				Aerial part
4.				Roots
5.	*Bryonia alba* L.	20.05.2014	Cluj, Parcul Feroviarilor	Stems
6.				Leaves
7.				Aerial part
8.				Roots
10.	*Bryonia alba* L.	14.06.2014	Cluj, Gheorgheni	Stems
11.				Leaves
12.				Aerial part
13.				Roots
14.	*Echinocystis lobata* (Michx.) Torr. & A. Gray	09.06.2014	Hunedoara, Lăpuşnic	Stems
15.				Leaves
16.				Aerial part
17.				Roots
18.	*Echinocystis lobata* (Michx.) Torr. & A. Gray	21.06.2014	Cluj, Apahida	Stems
19.				Leaves
20.				Aerial part
21.				Roots
22.	*Bryonia alba* L.	10.07.2014	Cluj, Parcul Feroviarilor	Stems
23.				Leaves
24.				Aerial part
25.				Roots
26.				Fruits
27.	*Bryonia alba* L.	14.07.2014	Cluj, Mănăştur	Stems
28.				Leaves
29.				Aerial part
30.				Fruits
31.	*Echinocystis lobata* (Michx.) Torr. & A. Gray	16.07.2014	Cluj, Apahida	Stems

32.				Leaves
33.				Aerial part
33*				Flowered aerial part
34.				Flowers
35.	*Bryonia alba* L.	31.07.2014	Cluj, Parc Iulius Mall	Stems
36.				Leaves
37.				Aerial part
38.				Roots
39.				Fruits
40.	*Bryonia alba* L.	31.07.2014	Cluj, Parc Iulius Mall	Ripe fruits
41.	*Echinocystis lobata* (Michx.) Torr. & A. Gray	05.08.2014	Hunedoara, Simeria	Stems
42.				Leaves
43.				Aerial part
44.				Roots
45.				Flower buds
46.	*Echinocystis lobata* (Michx.) Torr. & A. Gray	05.08.2014	Hunedoara, Simeria	Stems
47.				Leaves
48.				Aerial part
49.				Roots
50.				♂ flowers
51.	*Bryonia alba* L.	18.08.2014	Nefrologie, Cluj-Napoca	Fresh fruits, not ripe
52.	*Echinocystis lobata* (Michx.) Torr. & A .Gray	19.08.2014	Aiud-Teiuș, Alba	Mature stems
53.				Young stems
54.				Leaves
55.				Aerial part
56.				♂ flowers
57.				Fruits, not ripe
58.				Fresh fruits, not ripe
59.	*Echinocystis lobata* (Michx.) Torr. & A. Gray	19.08.2014	Aiud-Teiuș, Alba	Mature stems
60.				Young stems
61.				Leaves
62.				Aerial part
63.				♂ flowers
64.				Fruits, not ripe
65.				Fresh fruits, not ripe

66.	*Echinocystis lobata* (Michx.) Torr. & A. Gray	23.08.2014	Deva, Hunedoara	Aerial part + fruits
67.	*Echinocystis lobata* (Michx.) Torr. & A. Gray	23.08.2014	Deva, Hunedoara	Mature stems
68.				Young stems
69.				Leaves
70.				Aerial part
71.				♂ flowers
72.				Fruits
73.	*Echinocystis lobata* (Michx.) Torr. & A. Gray	23.08.2014	Deva, Hunedoara	Mature stems
74.				Young stems
75.				Leaves
76.				Aerial part
77.				♂ flowers
78.	*Echinocystis lobata* (Michx.) Torr. & A. Gray	23.08.2014	Deva, Hunedoara	Fresh ♂ flowers
79.				Fresh fruits
82.	*Ecballium elaterium* (L.) A. Rich.	05.09.2014	Constanța	Mature stems
83.				Young stems
84.				Leaves
85.				Aerial part
86.				Roots
87.				Fresh fruits

ANNEX IV. RMN spectra of isolated compounds from the leaves of *B.alba*

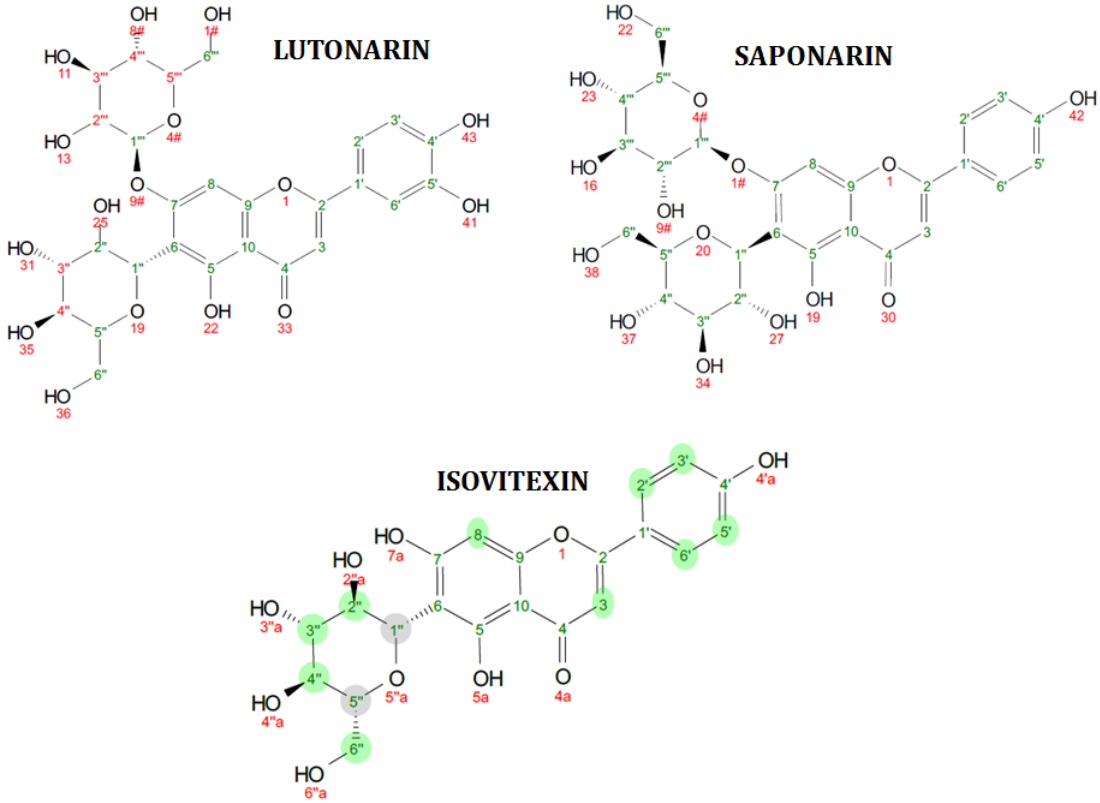

Fig.1. Structure of lutonarin, saponarin and isovitexin isolated from the leaves of *B. alba*, with corresponding signals annotated on the structure

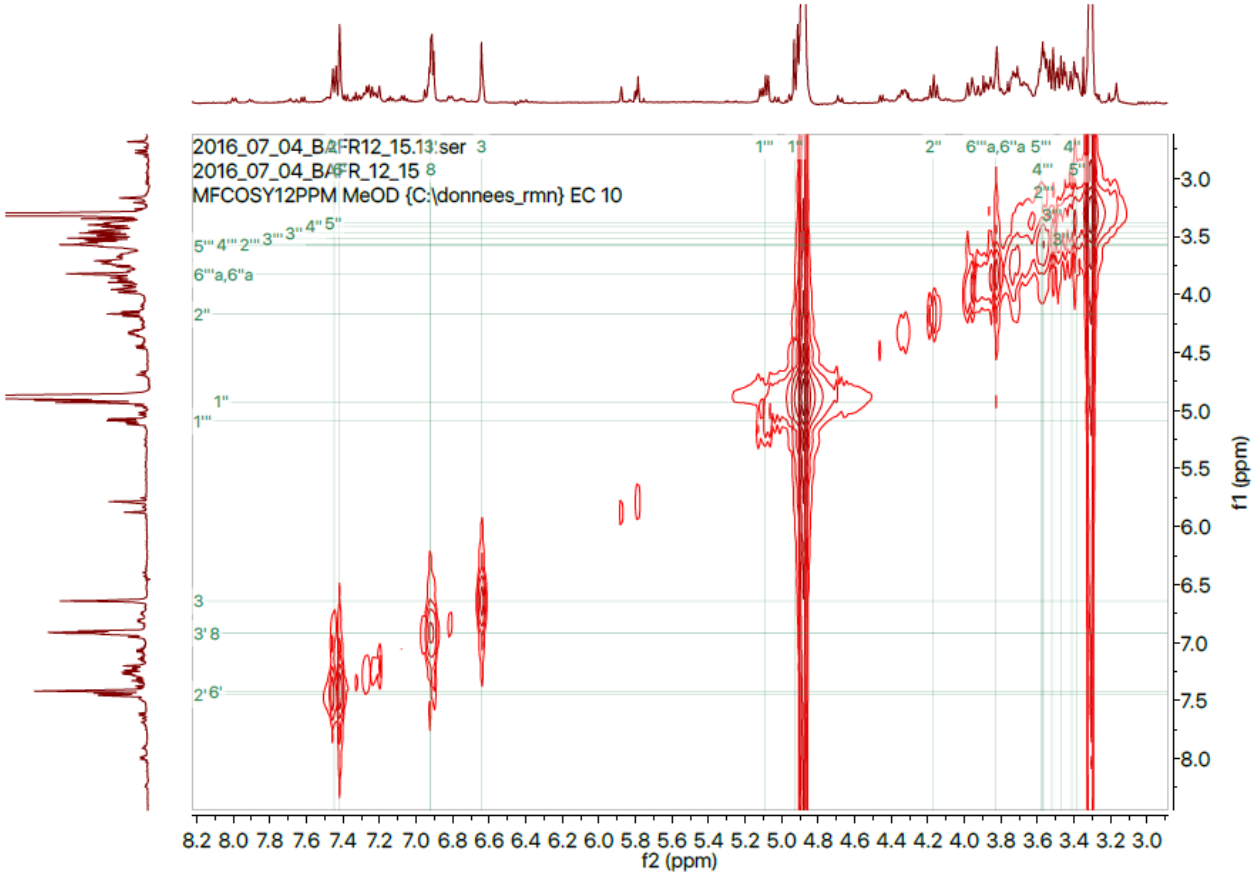

Fig.2. The COSY spectrum of lutonarin isolated from the leaves of *B. alba*

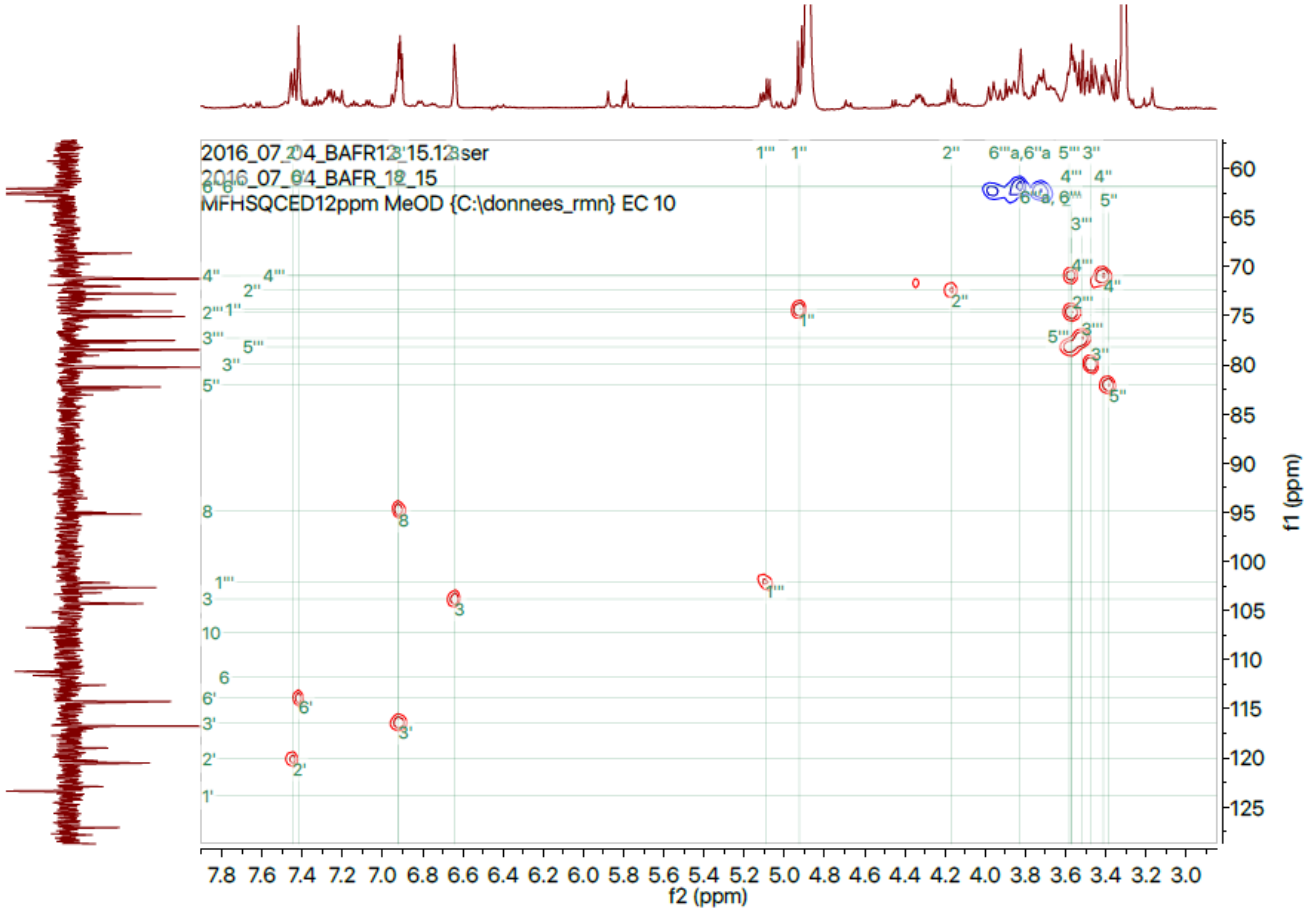

Fig.3. The HSQC spectrum of lutonarin isolated from the leaves of *B. alba*

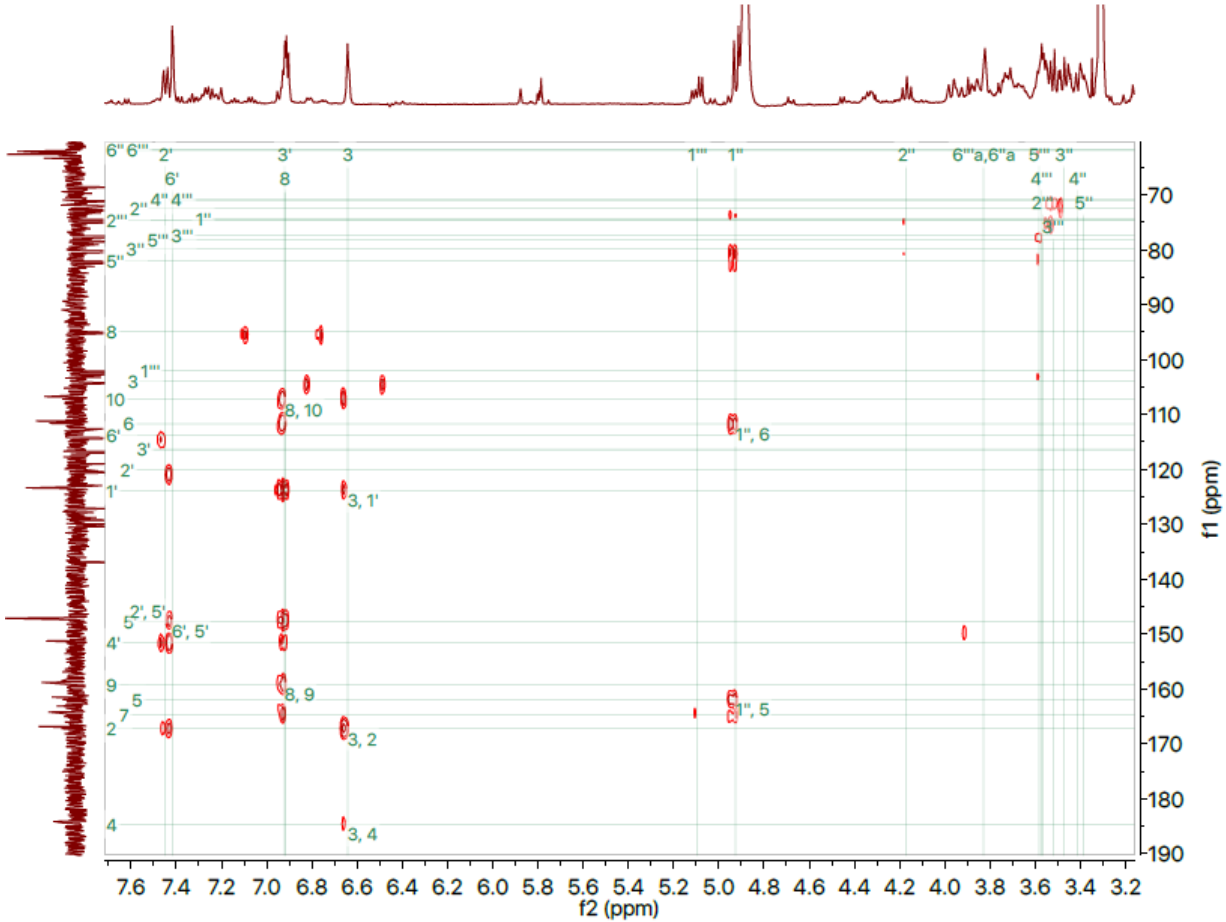

Fig.4. The HMBC spectrum of lutonarin isolated from the leaves of *B. alba*

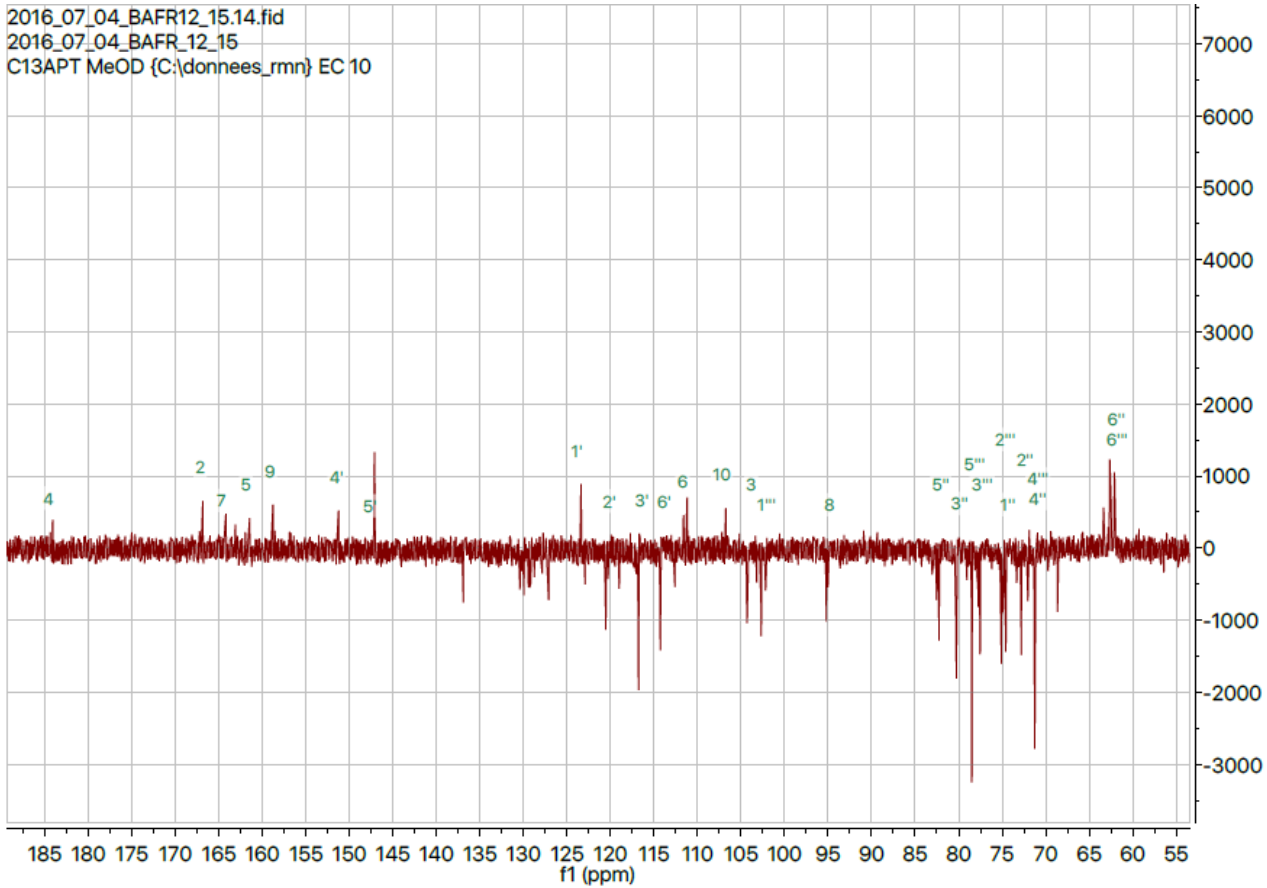

Fig.5. The ^{13}C-APT spectrum of lutonarin isolated from the leaves of *B. Alba*

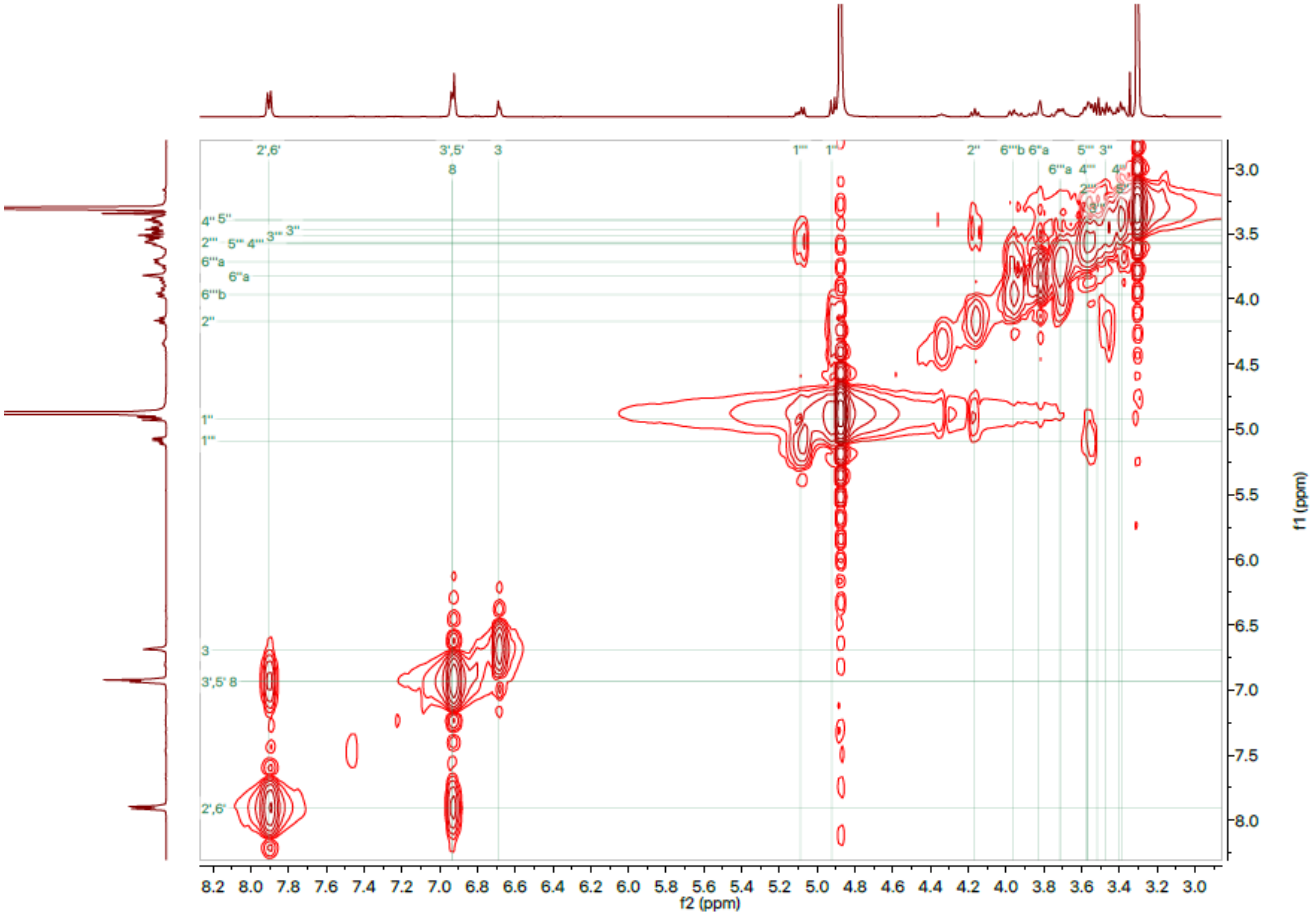

Fig.6. The COSY spectrum of saponarin isolated from the leaves of *B. alba*

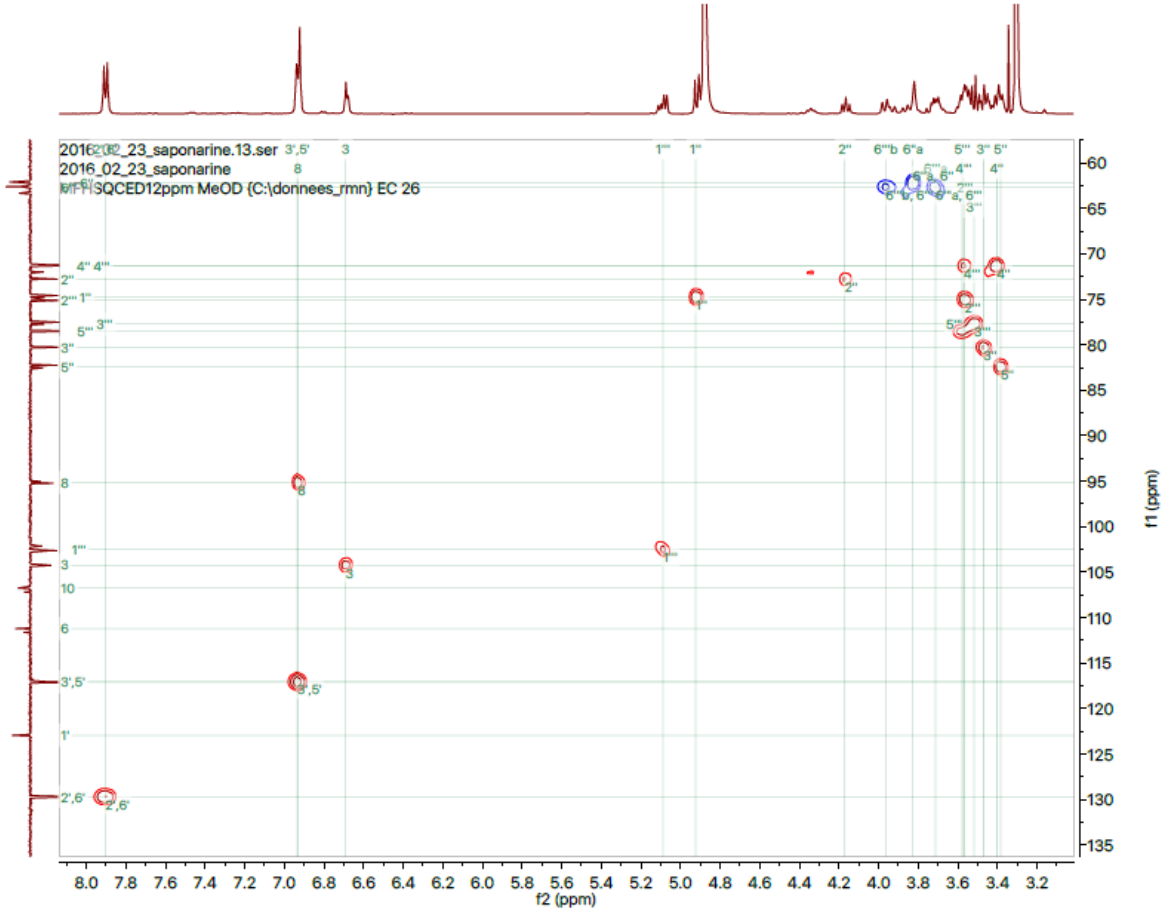

Fig.7. The HSQC spectrum of saponarin isolated from the leaves of *B. Alba*

Fig.8. The HMBC spectrum of saponarin isolated from the leaves of *B. Alba*

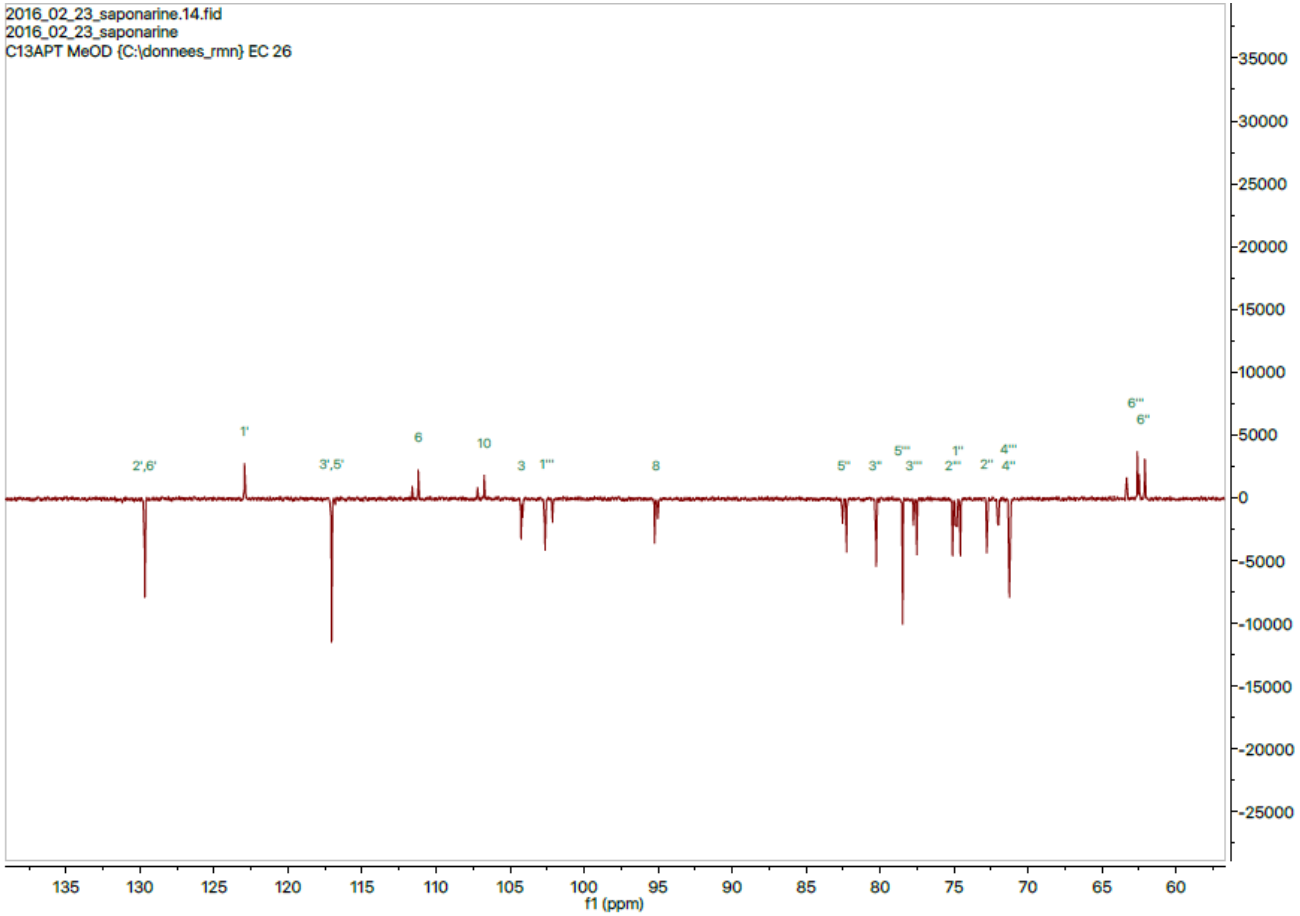

Fig.9. The ^{13}C-APT spectrum of saponarin isolated from the leaves of *B. alba*

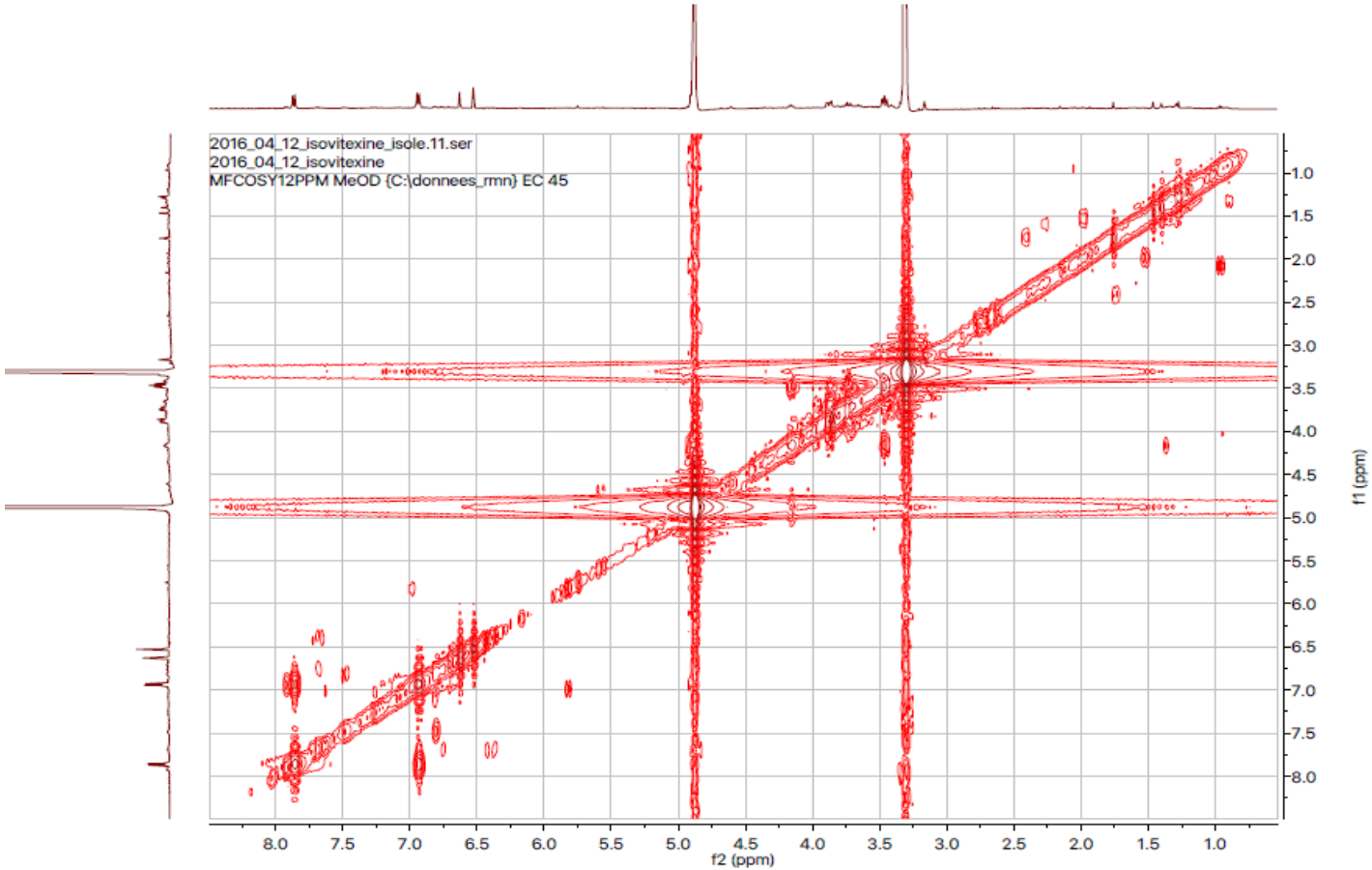

Fig.10. The COSY spectrum of isovitexin isolated from the leaves of *B. alba*

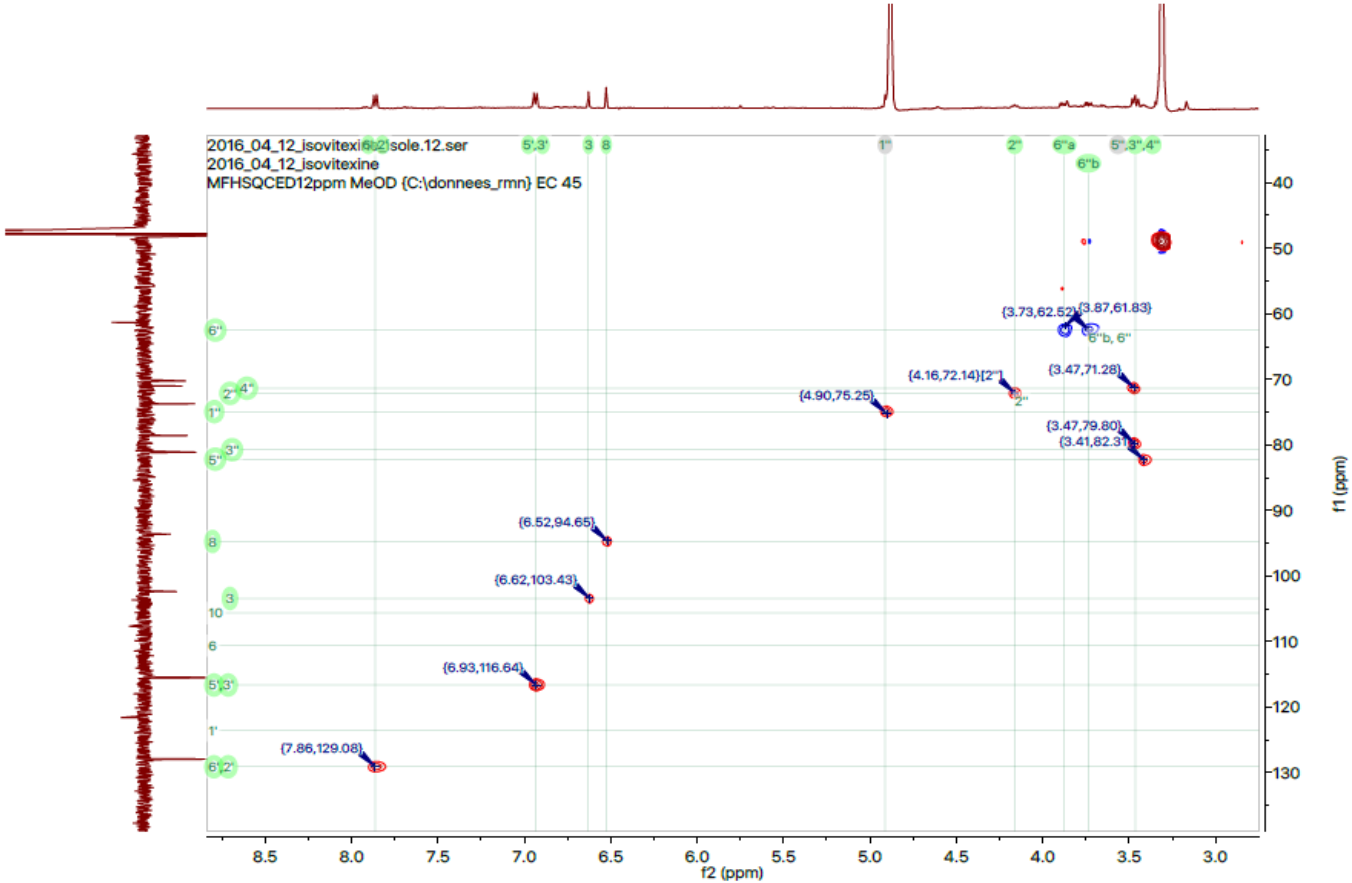

Fig.11. The HSQC spectrum of isovitexin isolated from the leaves of *B. alba*

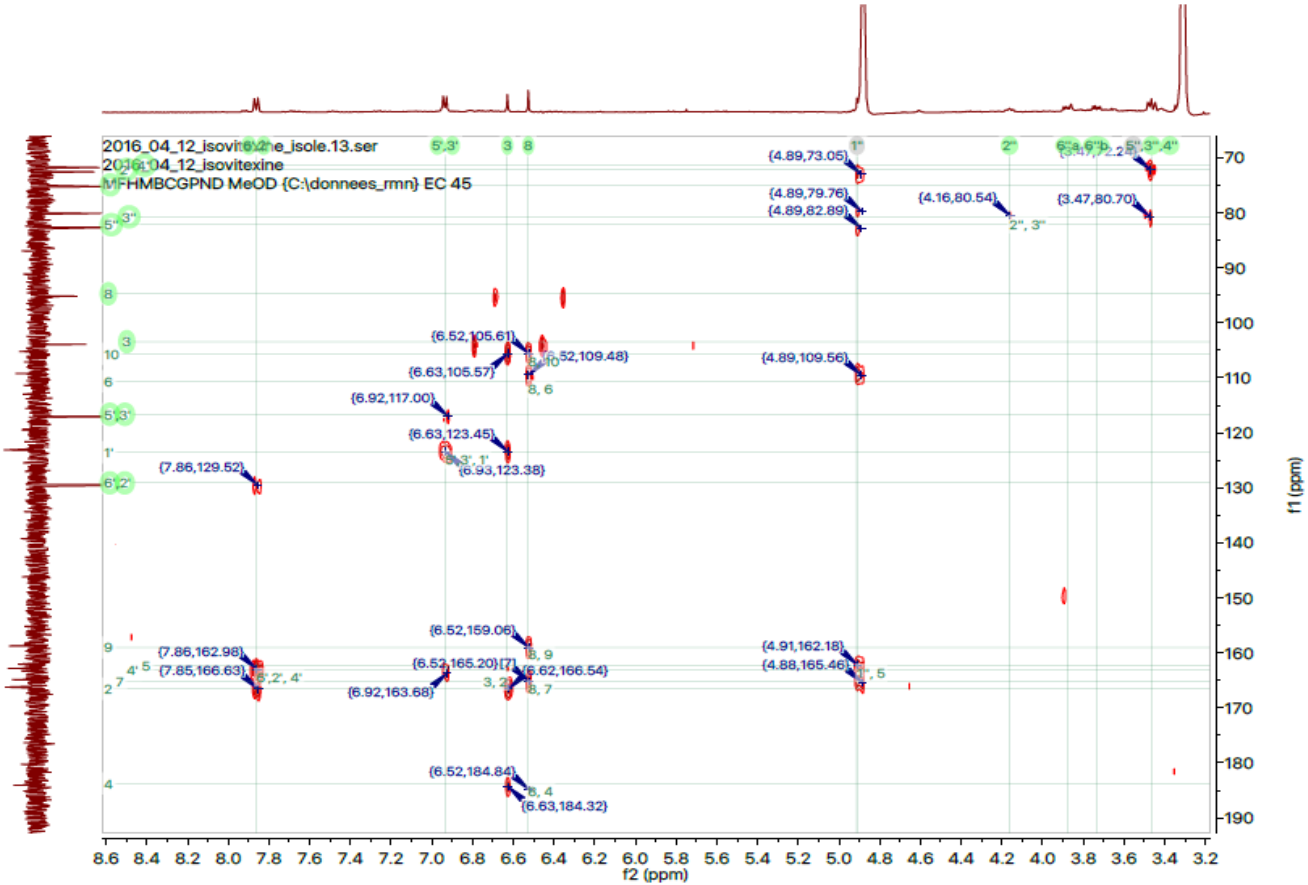

Fig.12. The HMBC spectrum of isovitexin isolated from the leaves of *B. alba*

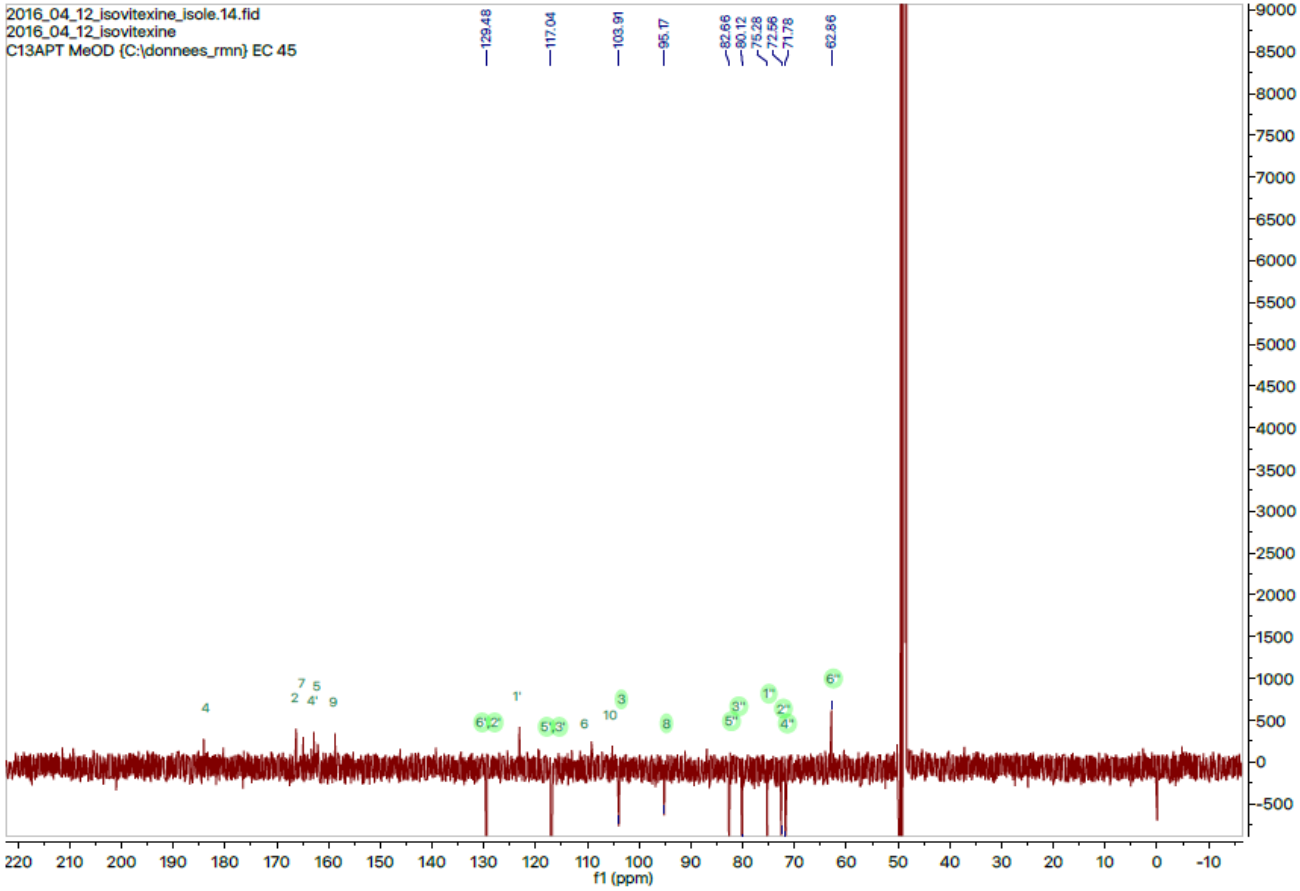

Fig.13. The ^{13}C-APT spectrum of isovitexin isolated from the leaves of *B. alba*