Department of Environmental Biology

PhD in Environmental and Evolutionary Biology

XXX cycle - Curriculum Botany

Phytochemical studies on the polar fractions of plants belonging to the Lamiaceae family with aspects of chemotaxonomy, ethno-pharmacology, nutraceutics and phytochemical evolution

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“Nature is not a place to visit. It is home.”

Gary Snyder

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1. Introduction

1.1. Lamiaceae

1.1.1. General

Lamiaceae or Labiatae is a family of plants comprised in the Lamiales order, in the Euasterids I clade of the Asterids class, in the Eudicots type of the Angiosperms group, in the Magnoliophyta division of the Spermatophyta superdivision (Pignatti, 1982).

The first citation of this family is dated back to the Latin writer and naturalist Gaius Pliny Secundus who also indicated the etymology of this word deriving from its most typical genus *Lamium* and descending from the greek term “λάιμος” (Laimos) meaning “jaws” and referring to the fact that the flowers of this plants are able to swallow small insects after they enter the corolla searching for its nectar.

Actually, the scientific name was coined in 1789 by Antoine Laurent de Jussieu in one of his works entitled "*Genera Plantarum - 110*" where he used for the first time the word Labiatae (De Jussieu, 1789).

In 1820, this name was changed into Lamiaceae by the Russian botanist Ivan Ivanovic Martinov in his work "*Tekhno-Botanicheskii Slovar': na latinskom i rossiiskom i-azykakh. Sanktpeterburgie - 355*" and nowadays both names are generally accepted even if Lamiaceae is considered to be more correct (Martinov, 1820).

1.1.2. Botany

From the botanical standpoint, species belonging to this family are characterized by an annual or perennial carriage (Figure 1).

The main biological forms are emicryptophyte and therophyte even though chamaephyte, nanofanerophyte, bushy, arboreal and shrubby species are also present.

The indumentum has a simple pubescence with glandular and non-glandular trichomes.

The stem is principally with a quadrangular section due to the presence of big bundles of collenchyma at its four vertices. No spines are present (Conti et al., 2005; Pignatti, 1982).

Figure 1: Example of the family carriage: *Scutellaria altissima* L.

The leaves are opposite and decussate or verticillated along the stem. They can be sessile or petiolate with a very variable shape ranging from lanceolate to ovate with entire or dentate margins (Figure 2) (Conti et al., 2005; Pignatti, 1982).

Figure 2: Example of the family leaves: *Collinsonia canadensis* L.

The flowers are hermaphrodite and zygomorphous or actinomorphic. They are tetramers or pentamers and generally collected in verticillasters. Each of them is constituted by many flowers (from 2 to 20) and is located circularly on two big leafy bracts which present an alternate disposition. These bracts are subulate or spinose and are densely colored. Bracteoles may be present, too.

The floreal formula is X, K (5), [C (2+3), A 2+2] G (2), (superior), drupe, four nuculae.

The calyx is gamosepalous and more or less actinomorphic. It is constituted by five equal straight lobes which can be lanceolate or triangular. The tube of the calex has about 10 superficial venations.

The corolla is gamopetalous and zygomorphous with the shape of a dilated pipe in the distal part ending with two lips having five lobes. The upper one is short and presents a hood shape and generally an entire margin. The lower one is flattened and is formed by three lobes with the central one bigger then the other two. Sometimes, it is fully pubescent. Its colors are very variable.

The androecium is formed by four fertile didinamous stamens included or protruding form the corolla and located under the lower lip. The anthers are close, paired and pubescent.

The ovary is superior and formed by two sealed carpels. The placentation is axyle. There are four ovules with tegument and kernel. The stylus is inserted into the base of the ovary. The stigma is bifid with equal lobes (Figure 3) (Conti et al., 2005; Pignatti, 1982).

Figure 3: Example of the family inflorescence: *Amasonia campestris* (Aubl.) Moldenke

The fruit is a schizocarp composed by four dry and fleshy nuculae. These are also rounding at the apex. The surface is smooth, glabrous or pubescent or tuberculate. The fruit is able to release semen without endosperm with extreme readiness (Figure 4) (Conti et al., 2005; Pignatti, 1982).

Figure 4: Example of the family fruit: *Callicarpa japonica* Thunb.

In general, Lamiaceae can be found everywhere all over the planet even if some species are endemic (Figure 5). The typical growth habitats of these species are constituted by tropical and temperate climates from 0 to 2500 m a.s.l. (Kadereit, 2004).

Figure 5: Worldwide distribution of Lamiaceae

1.1.3. Phytochemistry

The phytochemistry of Lamiaceae is very complex. Within it, in fact, several different groups and sub-groups co-exist and each presents its own particularities and characteristics from the phytochemical standpoint.

As a matter of fact, species belonging to the Lamiaceae family can be initially distinguished into two major groups. The first one comprises all the species which produce mainly volatile terpenoids, found prevalently in the essential oils. The second one comprises all the species which produce mainly not volatile secondary metabolites, instead.

Some important species belong to the first group such as those of the Mentheae tribe. These species which belong, for example, to the genera *Salvia*, *Rosmarinus*, *Mentha* and *Thymus* are well known for their high and important aromatizing effects which are exactly due to their essential oils. These properties make them very famous in cuisine, perfumery and cosmetics so that many of these species are widely used in these fields as main or only ingredients but also as minor ones due to their great versatility. Moreover, some of these species are able to exert pharmacological and even nutraceutical properties.

The essential oil of these species is especially composed by α-pinene and β-pinene, 1,8-cineole and menthol, germacrene D and cariophyllene, limonene and γ-terpinene plus several fatty acids with a long or short chain (Figure 6).

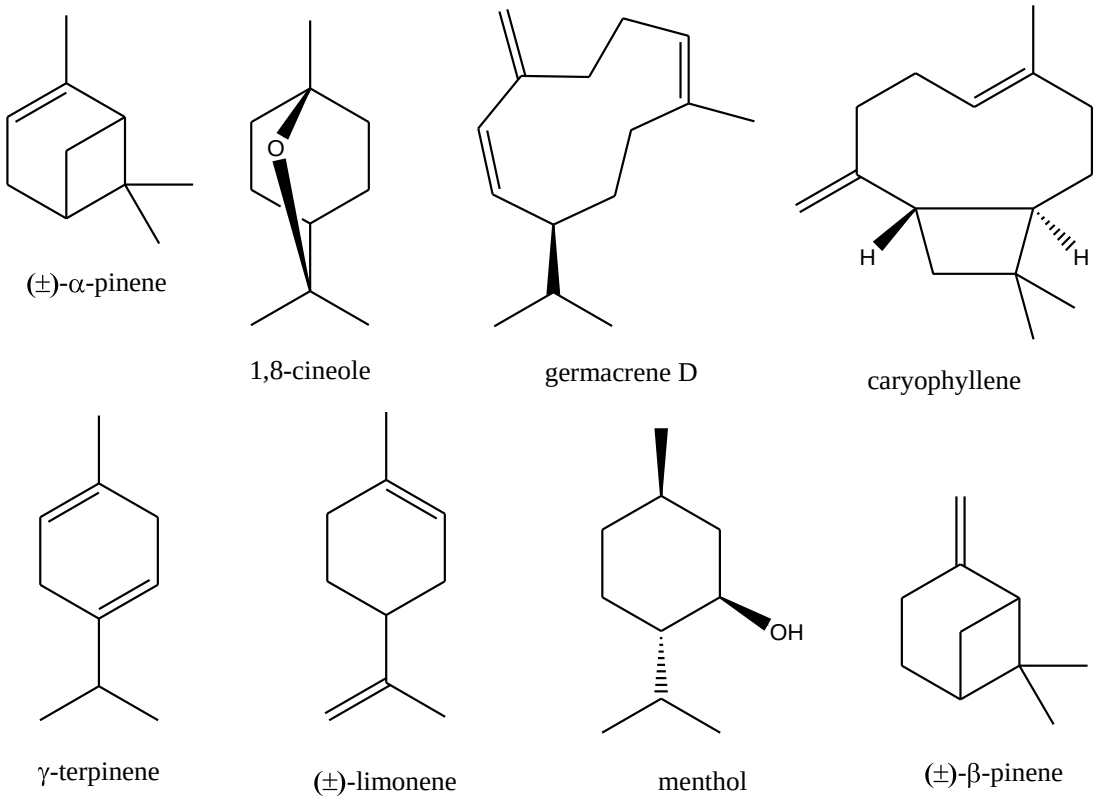


Figure 6: Structures of the main components of the essential oil of Mentheae tribe species

These very volatile compounds belong to the class of natural compounds of terpenoids and the majority of the total constituents of the essential oil of these species are mono, sesqui and di-terpenes meaning that they present a base structure composed by 10, 15 and 20 carbon atoms, respectively.

Anyway, as always, some particular exceptions may also be present but these are mainly due to the growth habitat of the species. In fact, the composition of the essential oil of Lamiaceae is quite stable and similar to each other.

Also, some other important species belong to the group of those producing mainly not volatile secondary metabolites. The secondary metabolites which can be evidenced in these species are also very variable and are represented mainly by terpenoids, flavonoids and iridoids.

Terpenoids are very important compounds which are often biosynthesized by plants. They play an important role as plant protector and attractor especially because of their high aromatic properties. These compounds are constituted by multiples of isoprene (2-methyl-1,3-butadiene) (Figure 7), assembled in a head-tail way, which can be modified in several possible manners. They are built through the mevalonic acid pathway and can have several structures and forms such as linear, cyclic or even a mixture of them simultaneously. At the

moment, more than five thousand different compounds of this kind have been isolated and identified (Degenhardt et al., 2009).

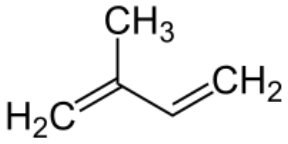


Figure 7: Structure of isoprene

Flavonoids represent, instead, a rich class of compounds which can be evidenced in different plant organs ranging from the roots to the fruits. They have the function to protect the plant from the harmful solar radiations and from pathogens. They are also important for the metabolism of the plant itself and cover an ecological significance, too. In fact, they help in its growth, respiration, physiology and seed germination by attracting insects due to their very bright coloration. At the moment, more than six thousand different compounds of this kind have been isolated and identified. From the chemical point of view, they are polyphenols, constituted by a flavane nucleus of fifteen carbon atoms, according to the biosynthetic scheme C_6C_3-C_6 to form two fused rings in which the first one is a hetero ring containing oxygen and the second one is a benzene ring (Figure 8). To this, a second benzene ring is linked and, according to the bond position (C_2 or C_3), several sub-classes of flavonoids can be formed. Flavonoids can exist both as aglycone and as glycosilated one with the sugar residue that can be linked in several positions of the three rings (Dewick, 2001).

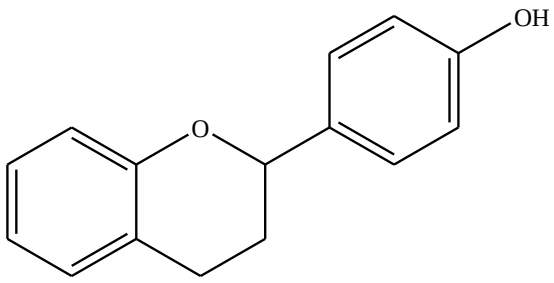


Figure 8: Structure of a generic flavane nucleus

Lastly, iridoids are also a quite widespread class of natural compounds. Their name derives from the Australian ant *Iridomyrmex* which biosynthesizes them as defense agents. From the chemical point of view, they are monoterpenes derived from geraniol and are formed as intermediate products during the synthesis of alkaloids. Iridoids are constituted by only two

isoprene units fused between them to form a cyclopentane ring and a hetero cyclohexane ring containing oxygen (di-hydropyrane) (Figure 9). They are found prevalently in their glycosilated form since this bond provides stability to the whole structure. At the moment about 800 hundred different compounds of this kind have been isolated and identified (Dewick, 2001).

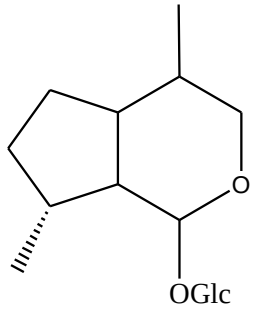


Figure 9: Structure of a generic iridoid

In Lamiaceae, terpenoids, flavonoids and iridoids can present very different structures even according to the species and there are several cases and distinctions to be made. In fact, the composition of the secondary metabolite pattern of Lamiaceae is very unstable and different from one species to the other. In particular, two different sub-classes of terpenoids, three different sub-classes of flavonoids and three other different sub-classes of iridoids are possible in the family and these can be evidenced together in one only species or even not (In this context, paragraph **1.2.5.** at pages 31-35, explores some of these possible distinctions).
Nevertheless, beside these characteristics compounds, other classes of natural secondary metabolites can be found in species belonging to this family such as phenyl-ethanoid glycosides and caffeoyl-quinic acids.
Phenyl-ethanoid glycosides are a very wide class of natural secondary metabolites of the plant kingdom which can be found in different organs. From the chemical point of view, they are constituted by a residue of phenyl-propanoid (caffeic acid) and a residue of phenyl-ethyl (tyrosole) which are both linked to a glucose residue via an ester and a glycosidic bond, respectively. To this glucose residue a second sugar residue constituted by rhamnose, is linked via interglycosidic bond (Figure 10). At the moment more than one hundred different compounds of this kind have been isolated and identified since the two benzene rings may be more or less substituted with different functional groups. Moreover, other sugar residues may be linked to the generic structure (Jiménez and Riguera, 1994).

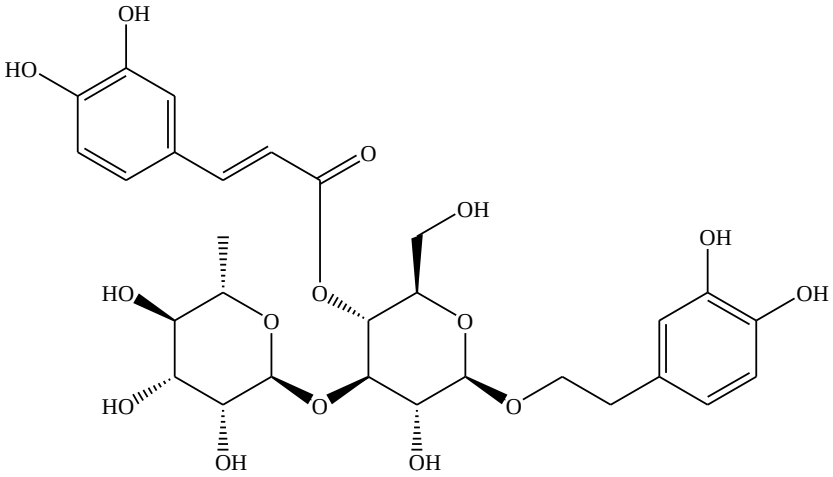


Figure 10: Structure of a generic phenyl-ethanoid glycoside, verbascoside

Lastly, caffeoyl-quinic acids are also quite widespread compounds in the plant kingdom and they can also be evidenced in different organs. From the chemical point of view, they are ester compounds formed by one caffeic acid and one quinic acid. According to the position of this bond (C_3, C_4 or C_5), on the quinic acid residue, several isomers exist (Figure 11). At the moment, only three possible different structures have been isolated and identified. Moreover they also exist as diesters with two caffeoyl-quinic acids that are linked to the same quinic acid but in Lamiaceae this last situation is quite hard to find (Dewick, 2001).

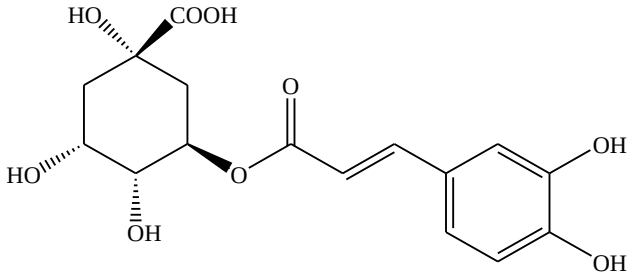


Figure 11: Structure of a generic caffeoyl-quinic acid, chlorogenic acid

1.1.4. Chemotaxonomy

The chemotaxonomy of Lamiaceae is very wide and complex, too.

As described in paragraph **1.1.3.**, several phytochemicals have been reported in the family and, by consequence, Lamiaceae present a wide series of chemotaxonomic markers at every level.

For instance, at a family level, α-pinene, β-pinene, 1,8-cineole, menthol, germacrene D, cariophyllene, limonene and γ-terpinene can be considered to be chemotaxonomic markers of the essential oils of these species since their occurrence is almost ubiquitous in the family. Yet, they do not have absolute valence since these compounds can be also found in other botanical families even if maybe, not all of them in the same time.

For what concerns the chemotaxonomic markers of the polar fractions, they are even more numerous and complex than those of the essential oils since they involve almost all the classes of natural compounds evidenced before in species belonging to this family.

The most important and known chemotaxonomic markers at the family level, are the iridoids, harpagide and 8-*O*-acetyl-harpagide which represent very common compounds in absolutely all the species belonging to this family in spite of their belonging sub-family.

On the other hand, at a more microscopic level including sub-families, genera and species, the chemotaxonomic markers are more specific and different one from the other (for specific details on this last part concerning Ajugoideae and Lamioideae, see Paragraph **1.2.6.** at page 36).

1.1.5. Ethno-pharmacology

Lamiaceae present a very wide and ancient utilization in the ethno-pharmacological field. This is valid for almost all its species with only a few exceptions. Moreover, this concerns both species particularly known for their essential oil and species particularly known for their production of secondary metabolites.

The major examples for the first kind are the essential oils of mint (*Mentha* L. spp.), rosemary (*Rosmarinus* L. spp.), sage (*Salvia* L. spp.), thyme (*Thymus* L. spp.) and marjoram (*Origanum* L. spp.).

Nevertheless, important examples for the second kind are hyssop (*Hyssopus* L. spp.), several *Scutellaria* L. and *Lamium* L. species, some *Marrubium* L. species. In this group, even several species belonging to the Ajugoideae and Lamioideae sub-families are comprised (For further details, see paragraph **1.2.7.** at pages 37-39).

Anyway, the ethno-pharmacological uses of all these species are strictly associated to the presence of several different compounds in them which are able to exert important pharmacological effects.

In general, essential oils are well known to exhibit strong antibacterial properties as well as relaxing and antispasmodic ones. Terpenoids present aromatizing and antiviral capacities. Phenyl-ethanoid glycosides exert antimicrobial and antioxidant effects. Flavonoids are important antioxidant and radical scavenging compounds. Iridoids show very strong antiviral and anti-inflammatory activities, instead. Lastly, caffeoyl-quinic acids are able to display good antioxidant effects (Dewick, 2001).

Yet, it's very important to underline the fact that besides these common properties, a specific compound belonging to one of these natural classes is able to exert specific pharmacological effects. For this, it's always useful to test these compounds for other properties than those commonly known or those which are reported in literature.

In table 8 in paragraph **3.4.** at pages 72-76, the specific pharmacological properties of some compounds are reported.

1.1.6. Nutraceutics

Lamiaceae is also important in the nutraceutical field.

All the species comprised in the Nepetoideae sub-family are used in cuisine especially as flavoring agents because of the impressive and high aromatizing properties of their essential oils.

Some well known and relevant examples of this are rosemary (*Rosmarinus officinalis* L.), sage (*Salvia officinalis* L.), basil (*Ocimum basilicum* L.), savory (*Satureja hortensis* L.), thyme (*Thymus vulgaris* L.), oregano (*Origanum vulgare* L.), marjoram (*Origanum majorana* L.) and mint (*Mentha piperita* L.).

Anyway, some other species, even not producing essential oil, have a similar traditional use because of the high nutraceutical properties brought to the organism by their consumption. This activity, which is actually an antioxidant one, is mainly due to the presence of phenolics i.e. compounds presenting a chemical structure containing phenol groups. These compounds are able to inhibit α-glucosidase preventing cell ageing and stopping degenerative diseases such as Alzheimer and Parkinson diseases since the beginning (Paliyath et al., 2011). Typical example of this part is *Lallemantia royleana* Benth. in Wall. (Ghannadi et al., 2015).

Also a few species of other sub-families present these nutraceutical effects and these belong mainly to the Lamioideae sub-family (for further details see paragraph **1.2.8.** at page 40).

1.1.7. Phylogeny

Lamiaceae is the sixth largest family of Angiosperms comprising 7 sub-families, 16 tribes, 9 sub-tribes, 236 genera and more than 7000 species.

The seven sub-families are: Ajugoideae, Lamioideae, Nepetoideae, Prostantheroideae, Scutellarioideae, Symphorematoideae and Viticoideae.

Eight genera have not been placed in a specific subfamily and are known as incertae sedis: *Acrymia* Prain, *Callicarpa*, *Cymaria* Benth., *Garrettia* Fletch., *Hymenopyramis* Wall. ex Griff., *Peronema* Jack, *Petraeovitex* Oliv. and *Tectona* L.

Generally, this family is considered to be monophyletic both from a morphological and a molecular point of view.

Anyway the monophyly of only five subfamilies (Ajugoideae, Lamioideae, Nepetoideae, Prostantheroideae, Scutellarioideae) has been supported by molecular studies while for what concerns the remaining ones, this has not been satisfactorily confirmed, yet.

As a matter of fact, experimental evidences are demonstrating that Viticoideae may not be monophyletic at all.

Inter-genera relationships have also been illuminated in Ajugoideae, Lamioideae, Nepetoideae, Prostantheroideae and Scutellarioideae.

Moreover, tribal subdivision has been proposed for Lamioideae.

The overall phylogenetic tree inserts Lamiales among one the most derived orders according to molecular data (APG III; Wagstaff et al., 1998) even if it is not the most derived one (Figure 12).

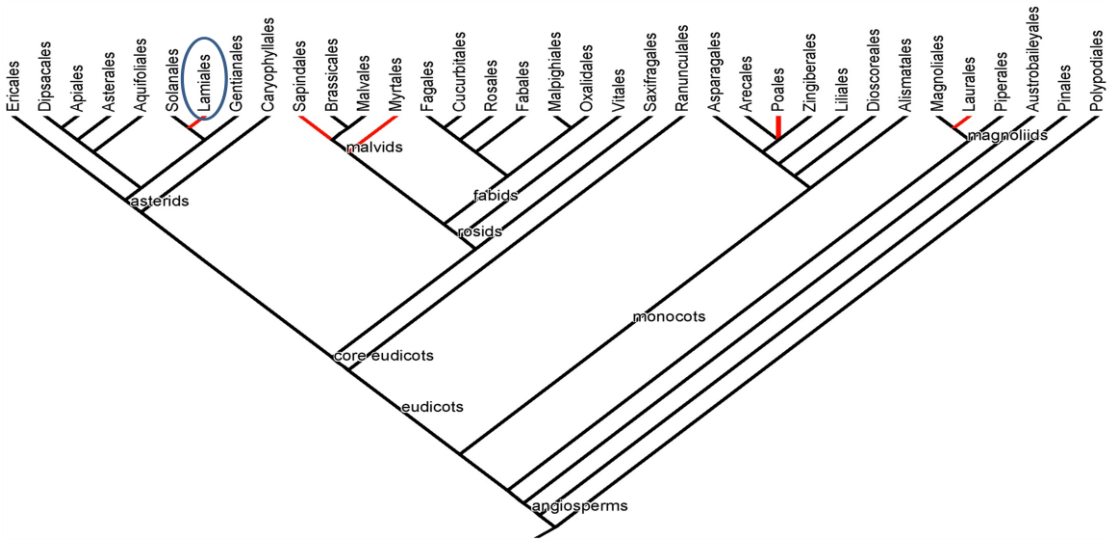


Figure 12: The overall phylogenetic tree of Angiosperms

In the same way, Lamiaceae are considered to be one of the most derived families of the order (Figure 13).

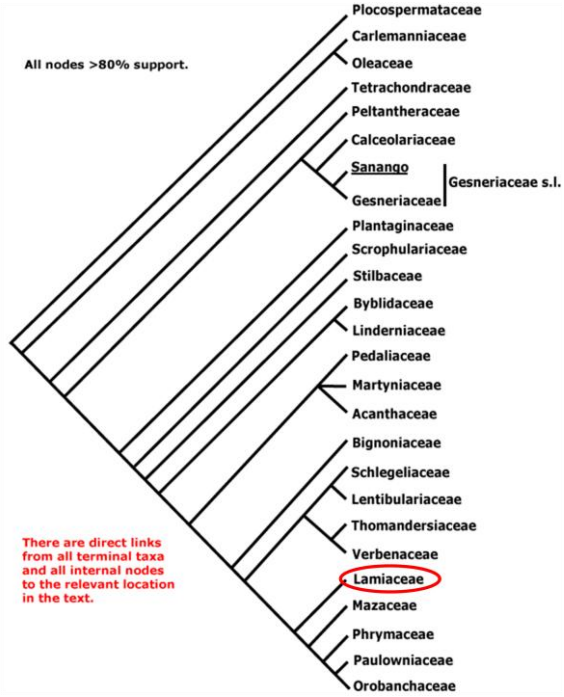


Figure 13: The overall phylogenetic tree of Lamiales

1.2. Studied sub-families and species

1.2.1. General

In this paragraph, the botanical, chemotaxonomic, ethno-pharmacological and nutraceutical features of the subfamilies and species which have been studied during this PhD project are reported.

Actually, only the specific and/or further or different characteristics of these, in every aspect, were added.

1.2.2. Ajugoideae and Lamioideae

Among all the species belonging to this family which could be studied, the attention was focused on two specific subfamilies: Ajugoideae and Lamioideae.

Ajugoideae comprises twenty-eight genera and these genera are all characterized by the fact that they are deprived of the upper lip of the corolla.

The name itself of this family derives from the Latin word “ajugum” with the meaning of yokeless.

The species belonging to this sub-family are perennial and herbaceous with fasciculate roots. The stems are erected and hairy. The leaves are basal and petiolate with an oblong shape. The flowers are collected in verticillasters and have a hairy and gamosepalous calyx. The corolla is zygomorphic and pubescent with a blue-violet color. The flowers are formed by four parallel stamens with yellowish anthers. The ovary is semi-inferior and the stigma is bifid, long and protruding but protected by the leafy bracts of the leaves inserted in the superior verticillum. The flowers bloom from April to July and the pollination takes place through butterflies and bees (Conti et al., 2005; Pignatti, 1982).

Species belonging to this sub-family can be found everywhere all over the planet preferring temperate and tropical climates up to 2500 m a.s.l. (Kadereit, 2004).

Lamioideae, comprises, instead, forty genera and is characterized by the fact that its species are very polymorphic meaning that there are several versions for each species.

Species belonging to this sub-family are also well-known to make interspecific hybrids among each other.

Its name derives from the Greek term “laimos” meaning jaws.

These species are herbaceous, annual or perennial, with tap and rhizomatous roots. The stems are erected and reddish at the base where it’s also branchy. The flowers are petiolate, opposite and alternated without stipels and with a pubescent surface. They are zygomorphic and constituted by a rigid and hairy calyx with five sepals. The corolla has a very variable coloration and is formed by two lips which are very developed. The upper one has a double central lobe to protect the reproduction organs. There are four didinamos stamens which are all fertile. The anthers have rounded woolly or glabrous lobes. The ovary is almost superior with ovate anther cells deriving from two carpels and a bifid stigma. The inflorescence is in several close verticillasters superimposed along the stem, all constituted by sessile and pubescent flowers. The fruit has no endosperm. The pollination takes place through butterflies and bees (Conti et al., 2005; Pignatti, 1982).

Species belonging to this sub-family can be found everywhere all over the planet preferring temperate climates up to 2000 m a.s.l. (Kadereit, 2004).

1.2.3. List of studied species

In the list below, the complete botanical names of the studied species, divided according to the sub-family and the genera, are reported.
In particular, these were:

- *Ajuga chamaepitys* (L.) Schreb. - aerial parts (genus *Ajuga*, sub-family Ajugoideae)
- *Ajuga chamaepitys* (L.) Schreb. - roots (genus *Ajuga*, sub-family Ajugoideae)
- *Ajuga genevensis* L. (genus *Ajuga*, sub-family Ajugoideae)
- *Ajuga reptans* L. (genus *Ajuga*, sub-family Ajugoideae)
- *Ajuga tenorei* C. Presl - leaves (genus *Ajug*a, sub-family Ajugoideae)
- *Teucrium chamaedrys* L. (genus *Teucrium*, sub-family Ajugoideae)
- *Teucrium polium* L. - Northern Iran (genus *Teucrium*, sub-family Ajugoideae)
- *Teucrium polium* L. - Southern Iran (genus *Teucrium*, sub-family Ajugoideae)

- *Galeopsis ladanum* subsp. *angustifolia* (Ehrh. ex Hoffm.) Gaudin (genus *Galeopsis*, sub-family Lamioideae)
- *Melittis melissophyllum* subsp. *albida* (Guss.) P.W.Ball (genus *Melittis*, sub-family Lamioideae)
- *Melittis melissophyllum* L. subsp. *melissophyllum* (genus *Melittis*, sub-family Lamioideae)
- *Sideritis montana* L. subsp. *montana* (genus *Sideritis*, sub-family Lamioideae)
- *Sideritis romana* L. (genus *Sideritis*, sub-family Lamioideae)
- *Stachys affinis* Bunge - rhizomes (genus *Stachys*, sub-family Lamioideae)
- *Stachys alopecuros* (L.) Benth. subsp. *divulsa* (Ten.) Grande (genus *Stachys*, sub-family Lamioideae)
- *Stachys annua* L. (genus *Stachys*, sub-family Lamioideae)
- *Stachys germanica* subsp. *salviifolia* L. (Ten.) Gams. (genus *Stachys*, sub-family Lamioideae)
- *Stachys palustris* L. – Hungary (genus *Stachys*, sub-family Lamioideae)
- *Stachys palustris* L. – France (genus *Stachys*, sub-family Lamioideae)

1.2.4. Botanical characteristics of the studied species

In this sub-paragraph, the distinctive botanical characteristics of the studied species are reported for each one in one own dedicated section. For those about which different organs of the plant or about which different populations of the same species were studied, separately, the botanical characteristics are reported only once.

1.2.4.1. *Ajuga chamaepitys* (L.) Schreb.

Ajuga chamaepitys (L.) Schreb., also known as Ground Pine or Camaepitium, is a grayish-green annual or biannual forb, 5-30 cm tall, with a quadrangular ascending stem. Leaves are opposite, densely appressed, usually tripartite. Flowers, long up to 15 mm, are one or two, brought to leaf axils, with a yellow corolla, more rarely to red-brown, in any case provided with two lips. The fruit contains four small nuts. The leaves are hairy, much divided and three-lobed; the branched stems are often fitted hairy at the base (Figure 14).
It is an Euri-Mediterrranean species. In Italy, it is found in almost all regions, mainly in cultivated fields, urban and ruderal areas from the mountain belt to the sea, behind the dunes, and along the sea coasts of urbanized areas (Conti et al., 2005).
It is also able to establish itself in more consistent groups in fireplaces, after the fire, but only for a few years (Kazanis et al., 2004).

Figure 14: *Ajuga chamaepitys* (L.) Schreb.

1.2.4.2. *Ajuga genevensis* L.

Ajuga genevensis L. is an herbaceous perennial plant presenting purplish-blue flowers in verticillasters. This species is typical of Europe and Asia, while in Italy it is present only in the central and northern regions.

A. genevensis may be distinguished from the congener *A. reptans* L. for the absence of stolons and also for the pubescence which covers all the surface of the stems (Figure 15).
Its favorite habitat is represented by meadows and bushes of hills and mountains where it survives till 1800 m of altitude a.s.l. (Pignatti, 1982; Conti et al. 2005).

Figure 15: *Ajuga genevensis* L.

1.2.4.3. *Ajuga reptans* L.

Ajuga reptans L., also known as Common Bugle or Blue Bugle is a small perennial herbaceous plant.
The botanical description of this species is the following: fasciculate roots; erected stem with opposite and alternated hairs which are present only in the upper part and with many branches; basal leaves which are petiolate, oblong and slightly crenate; cauline leaves which are smaller than the basal ones, sessile, opposite and dentate; inflorescence in a dense raceme with hermaphrodite and zigomorphic flowers which bloom from April to July, are colored in azure-purple and are gathered in four whorls; fruits as rugged schizocarps composed by four nuculae (Figure 16) (Pignatti, 1982).
The distribution area of this species is very wide going from Europe to Japan. In Italy, it can be found everywhere along the national territory growing wild on dump ecosystems such as meadows, hedges and woodlands especially on calcareous substrates with neutral pH till the altitude of 1500 m a.s.l. (Conti et al., 2005).

Figure 16: *Ajuga reptans* L.

1.2.4.4. *Ajuga tenorei* C. Presl - leaves

Ajuga tenorei C. Presl (synonym of *Ajuga acaulis* Brocchi), better known with the common name of Tenore's Bugle, is an herbaceous hemicryptophyte perennial plant.

Its height can reach up to 10 cm and it's characterised by the fact that it has no stem or this is very short for which it can be easily distinguished from the other species of the genus. Its leaves are all collected in a basal rosette and have an oblanceolate shape and are crenate. The flowers are small and blue-violet coloured blooming from May to June (Figure 17) (Pignatti, 1982).

This species is endemic to Italy and grows in stony pastures from 1200 to 2200 m a.s.l. Actually, it can be found in the central and southern regions of Italy with several exceptions: its presence is confirmed with absolute certainty only in Latium, Campania, Abruzzo, Molise, Calabria and Sicily, while there are doubts about its presence in Umbria, Marche and Basilicata and moreover, it has been no longer reported in Apulia (Peruzzi et al., 2014).

Figure 17: *Ajuga tenorei* C. Presl

1.2.4.5. *Galeopsis ladanum* subsp. *angustifolia* (Ehrh. ex Hoffm.) Gaudin

Galeopsis ladanum subsp. *angustifolia* (Ehrh. ex Hoffm.) Gaudin, [syn. *Galeopsis angustifolia* (Ehrh. ex Hoffm.)], is a small annual herbaceous plant.

From the botanical standpoint, the species can reach up to 40 cm height. It is characterized by an ascendant stem with a tetragonal section. The stem is branched only in the upper part and is covered all along with some fuzzy hairs. The leaves are also small, opposite, shortly petiolate, with a full margin. The even small flowers depart from the leaves axillae and are collected in verticillasters four by four. They are also hermaphrodite and zigomorphic blooming from June to October. The fruit is a schizocarp formed by four glabrous nuculae. The roots are tapped (Figure 18).

It is typical of the Northern Mediterranean Basin or even Southern Europe. In Italy, it has a widespread distribution all along the national territory with the only exceptions of Apulia, Calabria and Sardinia whereas its presence is uncertain in Sicily.

The species particularly grows on calcareous rocky cultivated lands and along rivers and railway roads up to 1700 m a.s.l.

Its preferred growth area represents the main reason why this species is classified as critically endangered since it is extremely infesting and its population has dramatically decreased during the last years due to the massive use of fertilizers and herbicides to prevent its seeding (Pignatti, 1982; Conti et al., 2005).

Figure 18: *Galeopsis ladanum* subsp. *angustifolia* (Ehrh. ex Hoffm.) Gaudin

1.2.4.6. *Melittis melissophyllum* subsp. *albida* (Guss.) P.W. Ball

Melittis melissophyllum subsp. *albida* (Guss.) P.W.Ball, or *M. melissophyllum* var. *albida* (Guss.) Nyman [syn. of *Melittis graeca* Klokov] is a perennial herbaceous plant.

Together with the subsp. *melissophyllum*, it represents the only species of the *Melittis* genus that can be found in Italy.

These two subspecies can be distinguished by some morphological factors. In particular, *M. melissophyllum* subsp. *albida* has a taller and a larger stem, with many glandular and short trichomes. Moreover, it has larger leaves and its flowers are white; hence, the origin of its sub-specific name (Figure 19) (Pignatti, 1982; Conti et al., 2005).

This plant is present in Italy only in some southern and central regions and in the islands (Maggi et al., 2011).

Figure 19: *Melittis melissophyllum* subsp. *albida* (Guss.) P.W.Ball

1.2.4.7. *Melittis melissophyllum* L. subsp. *melissophyllum*

Melittis melissophyllum L. subsp. *melissophyllum* is a perennial herb with petiolate to sub-sessile, oblong-ovate, cordate to truncate at the base and coarsely crenate-dentate leaves.

It has big scented flowers with all possible gradations of color, from deep red to purplish to completely white. The fruit is a smooth tetra-achenium, brown at maturity.

The name of the species derives from the fact that its leaves are very similar to those of *Melissa officinalis* L. (Figure 20).

Its habitat is represented by central and southern Europe along tracks and roads covered in half shadow till the altitude of 1400 m a.s.l. In Italy it is quite common along all the territory (Pignatti, 1982; Conti et al., 2005).

Figure 20: *Melittis melissophyllum* L. subsp. *melissophyllum*

1.2.4.8. *Sideritis montana* L. subsp. *montana*

Sideritis montana L. subsp. *montana*, locally known as 'stregonia montana', is a small annual herb characterized by a simple or branched stem and ovate-lanceolate, denticulate leaves. The flowers are grouped in loosely villous-lanate verticillasters appearing from April to July. They have a yellow corolla with brownish-purple margins and green calyx and are endowed with leaf-like bracts (Figure 21).

This species occurs in countries bordering the Mediterranean Sea including North Africa and also in Balkan regions up to Turkey. In Italy it is found in northern and central regions especially in arid pastures on limestones up to 1000 m of altitude (Pignatti, 1982).

Figure 21: *Sideritis montana* L. subsp. *montana*

1.2.4.9. *Sideritis romana* L.

Sideritis romana L., also known as ironwort, is an annual plant.

It is characterized by an erected stem which is simple or branched at the base. It owns elliptic basal leaves, the upper ones with a lanceolate shape. The flowers are inserted in outdistanced verticillasters and the youngest ones fill the inferior part of the inflorescence. The corolla is

yellow with white or pink shades. The fruits are brown trigonal and warty nuculae (Figure 22).

It can be found in the Mediterranean area where it widely grows in arid meadows and grazings till the altitude of 1900 m a.s.l.

In Italy it is present in almost all the territory except for some regions of northern Italy like Veneto, Trentino Alto Adige and Valle d'Aosta and also in Piedmont where its presence is actually unsure (Pignatti, 1982).

It can be mistaken for *Stachys ocymastrum* (L.) Briq. but the latter owns leaves with different shape (oval and crenate along the margins excluding the base) and the youngest flowers fill the superior part of the inflorescence (Conti et al., 2005).

Figure 22: *Sideritis romana* L.

1.2.4.10. *Stachys affinis* Bunge

Stachys affinis Bunge (syn. of *S. sieboldii* Miquel), well known as Chinese artichoke, and chorogi, is an herbaceous plant native to China and Japan, where it has been extensively cultivated for its edible tubers.

Since the nineteenth century this plant has been cultivated in Europe as well (Łuczaj et al., 2011) as, for instance, in Italy, where it is known as 'tuberina'.

In the late twentieth century, this species was gradually forgotten in Europe, but recently Chinese artichoke has come back into fashion following an international trend of rediscovery and revaluation.

Morphologically, it is very similar to *S. palustris* L. (Hegi et al., 1927), although its tubers, also known as 'tubercules', are shorter and thicker (Figure 23).

Figure 23: *Stachys affinis* Bunge

1.2.4.11. *Stachys alopecuros* (L.) Benth. subsp. *divulsa* (Ten.) Grande

Stachys alopecuros (L.) Benth. subsp. *divulsa* (Ten.) Grande is a perennial herbaceous plant with a small, hirsute and sub-cylindrical ascending stem.

The opposite leaves are petiolate and densely hairy on the edge and along the veins. The flowers are gathered in small verticillasters held in a dense spike, yellow-white in color and blooming from June to August-September. The fruits, which are brown colored, are constituted by 4 ovate nuculae (Figure 24).

This plant is characteristic of the mountain habitat of central Italy, being distributed only in a few regions (Umbria, Marche, Abruzzo, Lazio, and Molise), on stony mountain pastures, scrubs, and screes, preferring a calcareous and dry soil up to 2000 m a.s.l. (Pignatti, 1982; Conti et al., 2005).

Figure 24: *Stachys alopecuros* (L.) Benth. subsp. *divulsa* (Ten.) Grande

1.2.4.12. *Stachys annua* L.

Stachys annua L., also known as annual woundwort, is an annual herbaceous plant, characterized by a taproot. Its height can reach up to 40 cm. The leaves are finely dentate and

acute if they bear flowers which have a white-yellowish corolla often spread with red. The upper lip is full and crenulated while the lower one is trilobate with tubes longer than the pubescent calyx. The fruits are dark brown colored (Figure 25).

Its typical growth area is represented by almost all Europe. In Italy, it can be found everywhere except for Apulia and Sardinia and its presence is no longer certain even in Valle D'Aosta, preferring shady places, humid woods and the margins of cultivated lands where it abundantly grows up to 800 m a.s.l. (Pignatti, 1982).

Figure 25: *Stachys annua* L.

1.2.4.13. *Stachys germanica* subsp. *salviifolia* L. (Ten.) Gams.

Stachys germanica subsp. *salviifolia* L. is a perennial emicryptophyta species.

It is characterized by a stem which is densely tomentose or lanate-tomentose. The leaves are very small in length but not in wideness with reticulate veins that resemble those of Sage. The cup has no glandular hairs but presents long teeth of the half pipe. The flowers are collected in pseudo-verticillasters and are distributed at the compact leafy end. They bloom from June to July. The corolla is pink or purple (Figure 26).

It is a species characteristic of the North Mediterranean Area and, in Italy, it is distributed from Liguria to Calabria where it grows on barren limestone and pastures up to 1400 m a.s.l. (Pignatti, 1982; Conti et al., 2005)

Figure 26: *Stachys germanica* subsp. *salviifolia* L. (Ten.) Gams.

1.2.4.14. *Stachys palustris* L.

Stachys palustris L., also known as marsh woundwort, is a typical representative of the mesotrophic hygrophilous Megaforb communities, from lowland to mountain types.

It is a perennial herb, growing up to 1 m, and distributed in humid habitats of Europe. The plant has square stems with opposite leaves, almost stalkless, lanceolate and toothed. The purplish-red flowers are in terminal spikes, with gaps in the lower part of the spike. They are arranged in whorls, each flower consisting of five fused petals. The corolla is two lipped with the upper lip being gently hooded and the lower lip flat and three-lobed. The calyx has five sharply-pointed lobes. The fruit is a dry four-chambered schizocarp (Figure 27).

This species can be very common or very rare following the conservation status and geographical region, with a trend to be rarer in the Mediterranean area. The plant is threatened by habitat destruction such as reclamations, grazing, vegetation cleaning, and cultivations (Pignatti, 1982). *S. palustris* is also considered as a non-indigenous weed aquatic plant introduced accidentally or for gardening purposes in USA as in the freshwater portion of the Hudson River basin (Mills et al., 1996). This species is also an edible plant.

Figure 27: *Stachys palustris* L.

1.2.4.15. *Teucrium chamaedrys* L.

Teucrium chamaedrys L., also known as wall germander, is a small perennial shrubby plant. This species is characterized by an unpleasant smell and presents rhizomatous roots. The stem is ascending, hairy and woody at the base. The leaves are pubescent, opposite and oblong. The upper ones are shortly petiolate while the lower ones are sessile and with a full margin. The flowers are pink-violet, zygomorphic, and collected in small verticillasters blooming from May to July. The fruit is a schizocarp composed by four achenes collected in the persistent calyx (Figure 28).

The species is typical of the Mediterranean Bain but it can be also found in Northern and Eastern Europe. In Italy, its presence is reported along all the national territory where it grows prevalently on meadows and rocky slopes on calcareous soils till the altitude of 1700 m a.s.l. (Pignatti, 1982)

Figure 28: *Teucrium chamaedrys* L.

1.2.4.16. *Teucrium polium* L.

Teucrium polium L. (common name felty germander) is a perennial herbaceous plant with a woody base and a round stalk as well as a pubescent nature. It owns an erected stem, high from 10 to 12 cm, which is fully branched in the upper part. The leaves are 2 cm long and up to 4 mm wide with an intact and folded margin in the lower part while it is crenate and outstretched in the upper one, instead. The flowers are gathered in small verticillasters and their colour range from pink to yellow blooming between April and August (Figure 29) (Negri, 1979).

This species can be found in almost all Mediterranean countries comprising South-western Asia (Feinbrun-Dothan, 1978) and Europe where its typical habitat is represented by dry and

stony hills and deserts till the altitude of about 3000 m a.s.l. (Kovacevic et al., 2001). In Italy it is present almost everywhere along the territory (Pignatti, 1982; Conti et al., 2005).

Figure 29: *Teucrium polium* L.

1.2.5. Phytochemistry of Ajugoideae and Lamioideae and related studied genera and species

Being part of the same family, Ajugoideae and Lamioideae present a quite similar content on secondary metabolites generally constituted by six major classes of natural compounds: terpenoids, phenyl-ethanoid glycosides, flavonoids, organic acids and poly-saccharides.

Yet, some important and interesting differences between these two sub-families for what concerns their exact occurrence exist and they are mainly from a qualitative point of view.

As for terpenoids, two different classes of them can be evidenced in Ajugoideae and Lamioideae: *neo*-clerodanes and *ent*-kauranes diterpenoids.

The difference between these two configurational structures lies in the difference form of connection among all the twenty carbons making part of them. In fact, in the *neo*-clerodanic skeleton, two cyclohexane rings are fused between each other and these can be variously substituted in position and chemical group. The second ring may be furtherly linked to a hetero cyclopentane ring containing oxygen, more or less substituted and, at its own time, this may be linked to a furan ring which does not present substitutions. According to the number, the positions and the kinds of substitutions, several compounds can be identified (Figure 30).

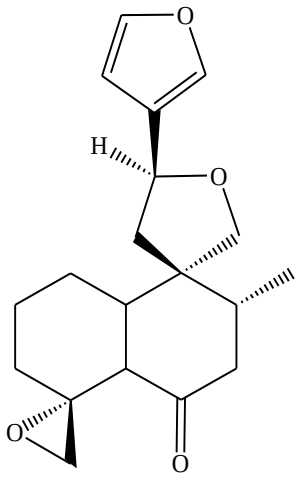


Figure 30: Example of a *neo*-clerodane, teucrasiatin

On the other side, the *ent*-kaurane skeleton is also composed by two fused cyclohexane rings but the second one is, at its own time, fused with another ring which is a cycloheptane one, this time. The number and the kind of substitutions on all these rings lead to the identification of several possible compounds (Figure 31).

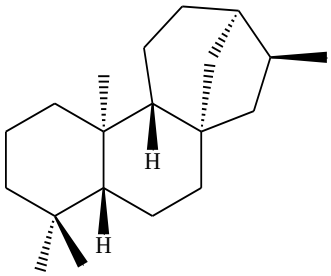


Figure 31: general *ent*-kaurane skeleton

This difference of structure between these two kinds of skeletons, which might seem only a chemical one, is, indeed, very relevant for what concerns the pharmacological field of application of these sub-families and this alone has created their big diversity of employments in ethno-pharmacology and ethno-medicine (see paragraph **1.2.7.** at pages 37-39).

A second important difference is in the belonging sub-class of the flavonoids identified in these sub-families. In fact, in these sub-families, it is very common to find derivatives of apigenin, isoscutellarein and hypolaetin. From the structural standpoint, the difference between these three sub-classes lies, for what concerns isoscutellarein, in the further presence of an hydroxyl group at the position eight of the structure which is totally vacant for the derivatives of apigenin and, for what concerns hypolaetin, in the further presence of another

hydroxyl group at the position 3' in the B ring in respect with isoscutellarein (Figure 32). The presence of several different substituents in the B and C rings, such as methoxy and glycosidic groups, permits the existence of many derivatives.

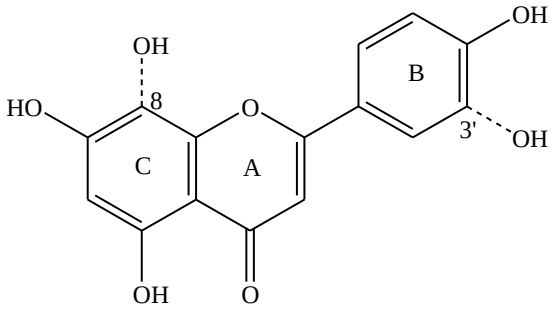


Figure 32: general skeletons of apigenin and isoscutelarrein derivatives

For what concerns the other classes of natural compounds like phenyl-ethanoid glycosides, organic acids and poly-saccharides, their occurrence or absence in both sub-families is mainly due to environmental conditions and to the species and is not likely related to the preference of these compounds to be biosynthesized in a specific sub-family. In fact, particular compounds belonging to these classes are found in both sub-families and other particular compounds have also been found in one only genus or even in one only species.

Indeed, as for iridoids, the speech is much more complex. In fact, even if two particular iridoids (harpagide and 8-*O*-acetyl-harpagide) are very common in both sub-families and thus their presence is almost certain in them, the identification of other specific iridoids depends on the genus, the species and even on their growth habitats. Anyway, the common thing is that all of these present the general structure plus one or two, usually, methyl groups more or less oxidized, linked to this base structure at the positions four and/or eight. Also, here, the presence of several substituents, usually hydroxyl groups, at different positions of the skeleton, makes different compounds. Moreover, it's important the hybridation bond between the carbons seven and eight which also is able to make different compounds (Figure 33).

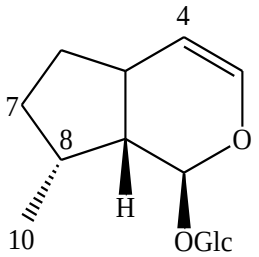


Figure 33: general skeletons of iridoids identified in Ajugoideae and Lamioideae

In the table below (Table 1), the specific phytochemicals identified before in each genus, presented in alphabetical order, are reported.

Genus	Phytochemical compound identified in the species	Reference
Ajuga	- terpenoids such as *neo*-clerodane diterpenoids, triterpenoids, steroidal glucosides - phenyl-ethanoid glycosides - flavonoids, in particular, apigenin derivatives - iridoids such as ajugol, ajugoside, harpagide, 8-*O*-acetyl-harpagide	(Frezza et al., 2017)
Galeopsis	- diterpenoids - flavonoids, isoscutellarein derivatives	(Rodriguez and Savona, 1980; Tomàs-Barberàn et al., 1992).
Melittis	- flavonoids - iridoids, in particular, monomelittoside and melittoside	(Skrzypczak-Pietraszek and Pietraszek, 2014; Tomàs-Barberàn et al., 1991)
Sideritis	- diterpenoids with *ent*-kaurane skeleton - phenylpropanoid glycosides - phenylethanoid glycosides - flavonoids, isoscutellarein derivatives - iridoids	(Kirimer et al., 2004; Giuliani et al., 2011; Venditti et al., 2016a)
Stachys	- terpenoids - phenylethanoid glycosides - flavonoids, isoscutellarein derivatives - iridoids -polysaccharides	(Venditti et al., 2017a)
Teucrium	- terpenoids such as *neo*-clerodane diterpenoids - flavonoids, in particular, apigenin derivatives - iridoids, especially teucardoside	(Essam, 1998; Venditti et al., 2017b)

Table 1: Phytochemicals reported in literature for each studied genus

In the table below (Table 2), instead, the specific phytochemicals identified before in each species, presented in alphabetical order, are reported.

Species	Phytochemical compounds identified in the species	Reference
Ajuga chamaepitys (L.) Schreb	- terpenoids such as *neo*-clerodane diterpenoids, triterpenoids, steroidal glucosides - phenyl-ethanoid glycosides - flavonoids - anthocyanins - iridoids such as ajugol, ajugoside, harpagide	(Venditti et al., 2016b)
Ajuga genevensis L.	- *neo*-clerodane diterpenoids - iridoids such as aucubin	(Malakov et al. 1991; Kostecka-Madalska and Rymkiewicz, 1971)

Ajuga reptans L.	- triglycerides, - phytoecdysteroids - anthocyanins - flavonoids - ionones - iridoids - carbohydrates	(Alekseeva et al., 1999; Breschi et al., 1992; Glyad, 2002; Israili and Lyoussi, 2009; Khan et al., 1999; Manguro et al., 2006; Shoji et al., 1992; Terahara et al., 1996; Tomas et al., 1993).
Ajuga tenorei C. Presl	- none previously reported	-
Galeopsis ladanum subsp. *angustifolia* (Ehrh. ex Hoffm.) Gaudin	- flavonoids - iridoids	(Venditti et al., 2013a; Tomàs-Barberàn et al., 1991)
Melittis melissophyllum subsp. *albida* (Guss.) P.W.Ball	- none previously reported	-
Melittis melissophyllum L. subsp. *melissophyllum*	- coumarins - flavonoids - iridoids such as 8-*O*-acetyl-harpagide, melittoside, monomelittoside	(Maggi et al., 2011a; Skrzypczak-Pietraszek and Pietraszek 2014; Scarpati et al., 1965; Scarpati and Esposito, 1967a, 1967b).
Sideritis montana L. subsp. *montana*	- flavonoid glycosides - phenolic acids	(Venditti et al., 2016a)
Sideritis romana L.	- siderin - flavonoids	(Venturella et al., 1974, 1977
Stachys affinis Bunge	- phenylethanoid glycosides such as acetoside and stachysoside	(Yamahara et al., 1990)
Stachys alopecuros (L.) Benth. subsp. *divulsa* (Ten.) Grande	- phenypropanoids - saponins - iridoids	(Venditti et al., 2013b)
Stachys annua L.	- minor diterpenoids - flavonoids, isoscutellarein derivatives	(Tundis et al., 2014; Venditti et al., 2015a)
Stachys germanica subsp. *salviifolia* L. (Ten.) Gams.	- flavonoids, isoscutellarein derivatives - iridoids -organic acids	(Venditti et al., 2013c; Vundać et al., 2007)
Stachys palustris L.	- sterols - triterpenoids	(Ross and Zinchenko, 1975)
Teucrium chamaedrys L.	- phenylethanoid glycosides - flavonoids - iridoids	(Pacifico et al., 2009; Gross et al., 1988)
Teucrium polium L.	- tannins - saponins - terpenoids among which teucrin is the most important one, - flavonoids especially those derivatives of apigenin - iridoids such as 8-*O*-acetyl-harpagide and teucardoside	(Venditti et al., 2017b)

Table 2: Phytochemicals reported in literature for each studied species

1.2.6. Chemotaxonomy of Ajugoideae and Lamioideae and related studied genera and species

As described in paragraph **1.1.4.** at pages 13-14, the chemotaxonomy of Ajugoideae and Lamioideae is very complex since it involves several phytochemicals belonging to different classes of natural compounds at every level.

At a sub-family level, Ajugoideae and Lamioideae present each two important and absolute chemotaxonomic marker. For both of them, these regard the classes of terpenoids and flavonoids.

For what concerns the former one, the Ajugoideae sub-family is characterized by the presence of *neo*-clerodanes diterpenes whereas the Lamioideae sub-family is characterized by the presence of *ent*-kaurane diterpenes.

Instead, for what concerns flavonoids, derivatives of apigenin are considered to be chemotaxonomic markers for the Ajugoideae sub-family while derivatives of isoscutellarein are chemotaxonomic markers for the Lamioideae sub-family. Actually, derivatives of apigenin cannot be considered as chemotaxonomic markers in absolute since they are very common compounds in several families.

At a genus level, iridoids represent the major chemotaxonomic markers even if they are not the only ones. In particular, in the *Ajuga* genus, ajugol and all of its derivatives can be considered as such while in the *Teucrium* genus, teucardoside is the main chemotaxonomic marker.

On the other hand, in the genera belonging to the Lamioideae sub-family, monomelittoside and melittoside represent the chemotaxonomic markers for excellence in the *Melittis* genus but also for other genera of the sub-family.

In the *Sideritis* genus, siderol, a terpene, is considered to be a chemotaxonomic marker while in the *Stachys* genus, stachysoside, a phenyl-ethanoid glycoside and stachyose, a saccharide, can be deemed as such.

Lastly, *Galeopsis* does not seem to have a chemotaxonomic marker at this level.

Anyway, given the very near proximity of these two sub-families, it’s not rare to see interchanges of chemotaxonomic markers between them at a sub-family and genus level.

Indeed, at the species level, none of these species present a particular chemotaxonomic marker beside *Stachys affinis* Bunge where stachyose is actually that since it has been identified for the first time right in that species.

1.2.7. Ethno-pharmacology of Ajugoideae and Lamioideae and related studied genera and species

The ethno-pharmacology between Ajugoideae and Lamiodeae is very different and is mainly, if not totally, based on phytochemistry.

Species belonging to both sub-families have had an ancient and regular use in the traditional medicine of several countries of all the five continents but, in recent times, something has changed.

In particular, this modification has only concerned the Ajugoideae sub-family which has seen a drastic diminishing of its use in the ethno-pharmacological field because of the presence of particular natural compounds among their phytochemical components.

These compounds are the *neo*-clerodane diterpenoids (see paragraph **1.2.5.** at pages 31-35) and these have been recently proved to exert strong hepatotoxic effects also causing death (Firenzuoli et al., 2002).

On the other side, for what concerns the Lamioideae sub-family, all the ancient medical uses remain and new ones have also been discovered.

As a matter of fact, in ancient times, species belonging to the Ajugoideae sub-family have been used for their powerful astringent, anti-inflammatory, homeopathic and vulnerary properties while species belonging to the Lamioideae sub-family are still used for their powerful vulnerary, antispasmodic, purifying, expectorant, tonic and astringent effects (Pignatti, 1982; Heywood and Richardson, 1972).

As for the genera and species belonging to each sub-family, their ethno-pharmacological uses have followed exactly the same course of those directly concerning the sub-families themselves with the employment of *Ajuga* and *Teucrium* species really decreased and the employment of *Melittis*, *Sideritis* and *Stachys* species which still enjoys great favour or is increased.

In the table below (Table 3), the specific pharmacological properties of each genus together with their ethno-pharmacological employments associated for each country are reported in alphabetical order.

Genus	Pharmacological properties or employments	Places of use	Reference

Ajuga	- antihypertensive, antidiabetic, against intestinal disorders - diuretic - antibacterial, antifungal, insecticide, antimalarial, antimicrobial, anti-inflammatory, antioxidant, antitumor	Africa China World	(Frezza et al., 2017)
Galeopsis	- anti-inflammatory, astringent, antianemic, expectorant, remineralizing. diuretic	World	(Mazza, 2000; Matkowski et al., 2008).
Melittis	- antispasmodic, against flu	World	(Guerrera, 2005)
Sideritis	- tonic, carminative, diuretic, digestive, anti-inflammatory, anti-ulcer, cytostatic, anti-microbial, vulnerary, astringent, flu vaccine and circulatory stimulant	World	(Özcan et al., 2001; Palomino et al., 1996)
Stachys	- against skin disorders, stomachaches, ulcers, asthma, rheumatic pains and cancer, sedative, antispasmodic, diuretic, anti-inflammatory, emmenagogue	World	(Gören, 2014)
Teucrium	- antioxidant, anti-inflammation, analgesic, antiulcer, antimicrobial, hypoglycemic, antispasmodic, antinociceptive, antidiabetic	World	(Hasani-Ranjbar et al., 2010)

Table 3: Pharmacological properties reported in literature for each studied genus

In the table below (Table 4), instead, the specific pharmacological properties of each species, in alphabetical order, together with their ethno-pharmacological employments associated for each country, are reported.

Species	Pharmacological properties or employments	Places of use	Reference
Ajuga chamaepitys (L.) Schreb	- wound healing, diuretic, tonic, emmenagogue, perspirative, menser remover, against scorpion and snake bites, hemorrhoids, stomachaches, jaundice, gout and joint pains, common colds - wound healing, against toothaches, menstrual delays, to cure arthritis, diuretic, depurative - against malaria	Middle East Italy (Abruzzo) Italy (Calabria)	(Venditti et al., 2016b)
Ajuga genevensis L.	- against respiratory tract diseases	Austria	(Vogl et al., 2013)
Ajuga reptans L.	- vasoconstrictor, anti-inflammatory, antirheumatic, antihemorrhagic, antipyretic, antimalarial, antifungal, antibacterial, antitussive, antitumor, tonic, narcotic, astringent, vulnerary, cicatrizing, diuretic, carminative, hypoglycemic and expectorant	World	(Baytop, 1984; Breschi et al., 1992; Chiej et al., 1984; Takasaki et al., 1998; Zargari et al., 1989)
Ajuga tenorei C. Presl	- none reported	-	-
Galeopsis ladanum subsp. *angustifolia* (Ehrh. ex Hoffm.) Gaudin	- antioxidant, sedative, neuroprotective, antiacetylcholinesterasic - against respiratory system infections for their high hematopoietic and fluidizing effects in the bronchi	World Southern Italy	(Czarnecki et al., 1993; Uriarte-Pueyo and Calvo, 2009; Guarino et al., 2008)

Melittis melissophyllum subsp. *albida* (Guss.) P.W.Ball	- none reported	-	-
Melittis melissophyllum L. subsp. *melissophyllum*	- antispasmodic, against insomnia and eye inflammations - as digestive, against cough and sore throat	World (Italy) Abruzzo	(Guarrera, 2005; Idolo et al., 2010)
Sideritis montana L. subsp. *montana*	- against cold, flu and cough - against digestive problems and headaches - spasmolytic, antioxidant, antimicrobial	Turkey Spain World	(Kultur, 2007; De Santayana et al., 2005; Agelet and Vallès, 2001)
Sideritis romana L.	- healing cuts and wounds of the skin, washing the unconcealed parts of the body in case of fright in order to soothe anxiety.	Italy (Madonie area)	(Pignatti, 1982)
Stachys affinis Bunge	- against infections, colds, heart diseases, tuberculosis and pneumonia	China	(Feng et al., 2015; Yamahara et al., 1990)
Stachys alopecuros (L.) Benth. subsp. *divulsa* (Ten.) Grande	- antioxidant, antitumor	World	(Venditti et al., 2013b)
Stachys annua L.	- anticatarrhal, febrifuge, tonic, vulnerary - against evil eye	Italy Italy (Marche)	(Ballelli and Bellomaria, 2005; Bellomaria and Lattanzi, 1982)
Stachys germanica subsp. *salviifolia* L. (Ten.) Gams.	- antimicrobial, myorelaxant, anxiolytic, antioxidant, cytotoxic	World	(Lazarevic et al., 2010; Haznagy-Radnai et al., 2008; Savic et al., 2010)
Stachys palustris L.	- antiseptic, emmenagogue, antispasmodic, emetic, expectorant, haemostatic, nervine, sedative, tonic, vulnerary, against internal and external hemorrhages, gout, cramps and joint pains	World	(Usher et al., 1974; Grieve, 2013)
Teucrium chamaedrys L.	- anti-inflammatory, antirheumatic, astringent, digestive, antispasmodic, diuretic. against coughs, asthma, abscesses, conjunctivitis, cellulite	World	(Özel et al., 2006; Stankovic et al., 2012)
Teucrium polium L.	- anti-inflammatory, antidiabetic, antirheumatic, against bacteria, spasms and anorexia - against indigestions, common colds, urogenital diseases and abdominal pains	Asia Iran	(Venditti et al., 2017b; Abdollahi et al., 2003)

Table 4: Pharmacological properties reported in literature for each studied species

1.2.8. Nutraceutics of Ajugoideae and Lamioideae and related studied genera and species

Also for what concerns the nutraceutical value of species belonging to Ajugoideae and Lamioideae, there is a big difference between the two sub-families.

In fact, if on one side, Ajuoideae is generally known to not have effects of this kind, on the other one, Lamioideae has.

Yet, differences are also present within this same sub-family since not all genera and species belonging to this, possess nutraceutical properties.

Actually, only a few *Sideritis* and *Stachys* species are well-known to exert such activities due to the high content of primary metabolites (mainly proteins, vitamins and mineral salts) whereas *Stachys affinis* Bunge and *Stachys palustris* L. are also known to show these effects because of the presence of particular poly-saccharides, mainly stachyose which is a tetrasaccharide composed by two residues of Galactose, one of Glucose and one of Fructose with important and interesting nutraceutical effects.

In the table below (Table 5), the specific nutraceutical properties reported for each genus, in alphabetical order, are presented.

Genus	Nutraceutical properties	Regional employments	Reference
Ajuga	- none reported	-	-
Galeopsis	- none reported	-	-
Melittis	- none reported	-	-
Sideritis	- flavouring	World	(Pala-Paul et al., 2003)
Stachys	- hypoglycaemic	World	(Łuczaj et al., 2011)
Teucrium	- none reported	-	-

Table 5: Nutraceutical properties reported in literature for each studied genus

In the table below (Table 6), instead, the specific nutraceutical properties reported for each species, in alphabetical order, are presented.

Species	Nutraceutical properties	Places of use	Reference
Ajuga chamaepitys (L.) Schreb	- none reported	-	-
Ajuga genevensis L.	- none reported	-	-
Ajuga reptans L.	- none reported	-	-
Ajuga tenorei C. Presl	- none reported	-	-
Galeopsis ladanum subsp. *angustifolia* (Ehrh. ex Hoffm.) Gaudin	- none reported	-	-

Melittis melissophyllum subsp. *albida* (Guss.) P.W.Ball	- none reported	-	-
Melittis melissophyllum L. subsp. *melissophyllum*	- none reported	-	-
Sideritis montana L. subsp. *montana*	- none reported	-	-
Sideritis romana L.	- none reported	-	-
Stachys affinis Bunge	- antioxidant, antianemic, hypoglycemic	China	(Istituto Botanico Boreali-Occidentali Academiae Sinicae, 1983; Feng et al., 2015; Mercier and Perennes, 1982)
Stachys alopecuros (L.) Benth. subsp. *divulsa* (Ten.) Grande	- none reported	-	-
Stachys annua L.	- none reported	-	-
Stachys germanica subsp. *salviifolia* L. (Ten.) Gams.	- none reported	-	-
Stachys palustris L.	- flavoring	World	(Facciola, 1988; Hedrick, 1972; Łuczaj et al., 2011; Conforti et al., 2009)
Teucrium chamaedrys L.	- none reported	-	-
Teucrium polium L.	- none reported	-	-

Table 6: Nutraceutical properties reported in literature for each studied species

1.2.9. Phylogeny of Ajugoideae and Lamioideae and related studied genera and species

Inside the family, Ajugoideae represents a middle-derived sub-family while Lamioideae represents the most derived one (Wagstaff et al., 1998) (Figure 34).

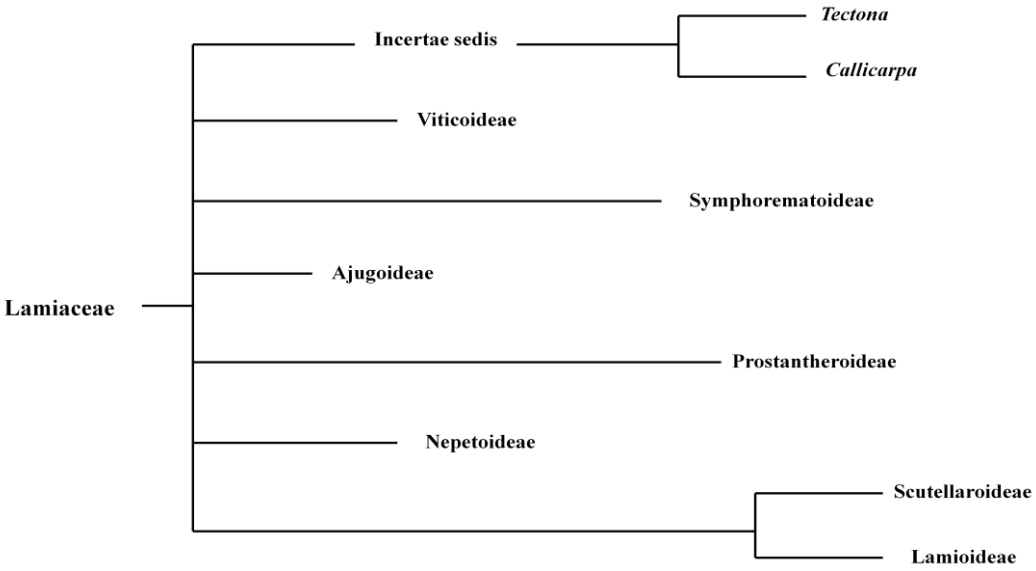


Figure 34: Phylogenetic tree of Lamiaceae

According to genetic data, all the species belonging to the Lamioideae sub-family are also the most derived ones (Wagstaff et al., 1998) (Figure 35).

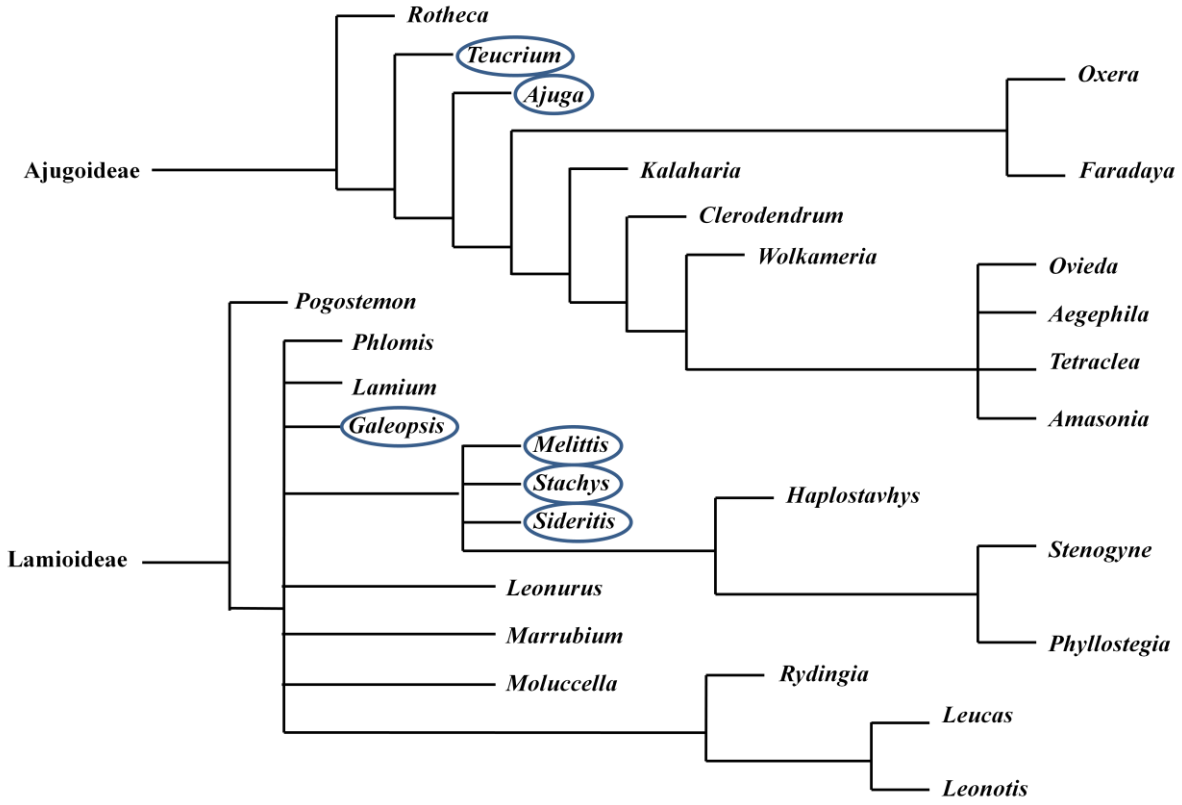


Figure 35: Schematic phylogenetic tree of Ajugoideae and Lamioideae

Among these, the most derived genus is *Sideritis* with *Stachys* right behind it but with several diffractions often with *Sideritis* species themselves. In fact, in recent times, *Sideritis* and *Stachys* genera have been incorporated into an only one as *Stachys*.

Galeopsis genus was recognized as an out-group while *Melittis* genus was recognized as a sister one to the original Stachydeae tribe. Moreover, *Stachys* genus was found to be paraphyletic in respect to all genera of Lamioideae except for *Melittis* (Salmaki et al., 2013).

Inside Ajugoideae, instead, all *Teucrium* species are less evolved than the *Ajuga* species. Actually, the *Ajuga* phylogenetic tree is in common with other genera of the same sub-family and represents the least derived one in this (Bendiksby et al., 2011).

Anyway, both Ajugoideae and Lamioideae are part of a same clade.

2. Aims and objectives

The aims and objectives of this PhD work were multiple.

The first one was to perform a comprehensive phytochemical analysis on several species belonging to the Lamiaceae family, some of which have never been studied before.

The second aim was, for each studied plant, to verify the botanical classification of the species from a phytochemical standpoint.

The third objective was to offer a general overview of the chemotaxonomy of the studied species inside the family also making a strict phytochemical comparison for what concerns their secondary metabolite profiles.

The fourth scope was to provide or not a phytochemical rationale for the possible further uses of each of the studied species in the medicinal field just like many Lamiaceae species or at least to provide a phytochemical rationale for the old and/or current employments of these same species in ethno-pharmacology and ethno-botany.

The fifth one was, finally, to perform an evolutionary study of the species based on phytochemical data in order to draw a Sporne diagram inside the Lamiaceae family which has never been done before at a species level.

All these parallel and complementary research guide lines were carried out by using classical chromatographic techniques such as Column Chromatography and classical spectroscopic techniques such as NMR Spectroscopy and Mass Spectrometry.

Moreover, a detailed literature research on several arguments together with other separation and identification techniques and a careful observation of the biological data were extremely necessary in order to fully accomplish every single point of the objectives of this work.

3. Results and discussion

3.1. General

In this chapter, the results and the relative discussions of the entire work are reported.
These are divided in five sections, phytochemistry, chemotaxonomy, ethno-pharmacology, nutraceutics and phytochemical evolution.
The first section is furtherly sub-divided for each studied plant also according to the studied plant organ while all the remaining ones provide individual and general results together.

3.2. Phytochemistry

3.2.1. *Ajuga chamaepitys* (L.) Schreb - aerial parts

The phytochemical analysis conducted on the iridoidic fraction of *A. chamaepitys* flowering aerial parts led to the isolation and identification of seven compounds: **ajugoside**, **reptoside**, **harpagide**, **8-*O*-acetyl-harpagide**, **5-*O*-β-D-glucopyranosyl-harpagide**, **asperulosidic acid** and **deacetyl-asperulosidic acid** (Figure 36).

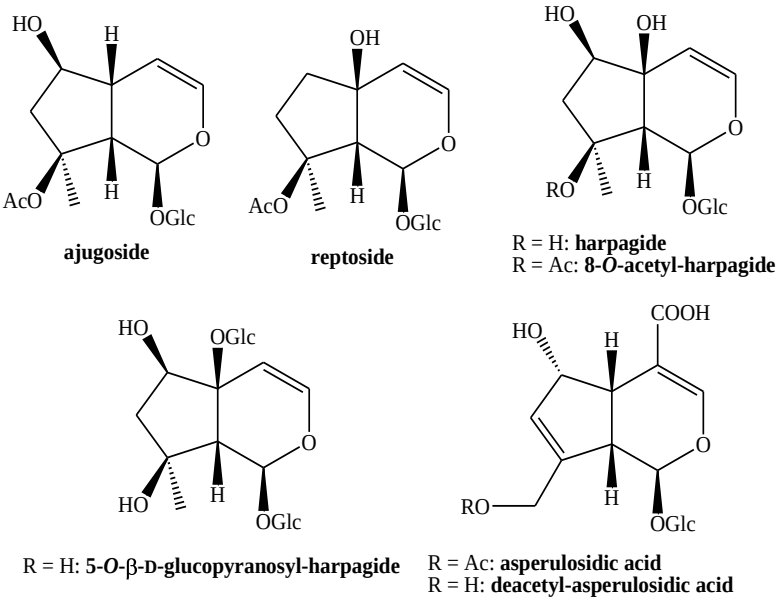


Figure 36: Structures of compounds identified in *A. chamaepitys* - aerial parts

These compounds belong to one only class of natural compounds that is iridoids.

Asperulosidic acid and **deacetyl-asperulosidic acid** represent new compounds for the genus whereas **5-*O*-β-D-glucopyranosyl-harpagide** resulted to be even a new harpagide derivative with an additional glucose residue linked in the position 5 on the iridoid skeleton.

Its structure was established on the basis of spectroscopic evidences by 1D and 2D-NMR analyses.

From the proton NMR experiments, a series of signals consistent with the harpagide iridoidic skeleton were evidenced. These showed a little but visible deshielding effect on H-3, H-4, and H-9 with respect to harpagide. In particular, from the more to the less deshielded signals, these peaks were observed: a doublet at δ 6.51 (J = 6.5 Hz) which may be assigned to H-3; a doublet at 5.76 ppm (J = 1.5 Hz) for H-1; a double doublet at δ 5.24 (J = 6.5/1.2 Hz) assignable to H-4; a broad singlet at 2.92 ppm indicating the H-9; a broad triplet like signal at δ 2.01 integrating for two protons for H-7; a singlet integrating for three protons at 1.33 ppm consistent with a methyl group in geminal with an hydroxyl substituent.

The presence of an additional saccharidic unit was shown by two anomeric protons signals at 4.92 and 4.82 ppm, both doublets with coupling constants of about 8.0 Hz, which indicated a β-configuration for the glycosidic linkage for both sugars.

From ^{13}C-NMR spectra twenty-one peaks were visible, two of which are quaternary carbons, fifteen are methine, three are methylene and one is a methyl.

By means of HSQC experiment, correlations between the proton at δ 4.92 and the carbon at δ 97.4 were evidenced, as well as between δ 4.82 and the carbon at δ 98.0, thus identifying the two anomeric carbons. In addition, the correspondences of H-3/C-3 (6.51/142.8), H-1/C-1 (5.76/92.7), H-4/C-4 (5.26/104.2), H-6/C-6 (4.03/76.0), H-7/C-7 (2.01/45.1), H-9/C-9 (2.92/55.7) and for H-10/C-10 (1.33/24.4) were also shown.

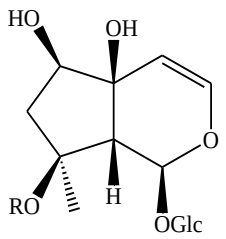
The positions of the glycosidic bonds were evidenced on HMBC experiments since visible correlations between the anomeric proton at δ 4.82 (H-1') and the carbon at δ 92.7 (C-1), indicating the glycosilation in the C-1 position on the iridoidic skeleton, were found, as well as the correlation between the second anomeric proton at δ 4.92 (H-1") and the carbon signal at 78.6 ppm that resulted to be a quaternary carbon by DEPT and APT. This was also confirmed in the HSQC experiment since no correlation with any proton was evidenced. The position of the carbon resonating at 78.6 ppm on the iridoid skeleton was indicated by the correlations in HMBC with H-3 (6.51 ppm) and with H-1 (5.76 ppm). The only quaternary carbon which may have correlation contemporaneously with H-3 and H-1 is C-5, thus the glycosilation occurs on the 5-OH group of harpagide. The two saccharidic units were both

identified as β-D-glucopyranose residues on the basis of their carbon resonances that were consistent with literature data (Breitmaier and Voelter, 1990).

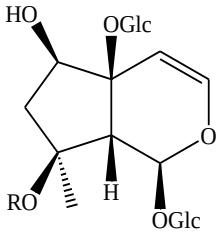
HR-MS experiments confirmed the molecular formula $C_{21}H_{34}O_{15}Na$ for the sodium adduct of this compound.

3.2.2. *Ajuga chamaepitys* (L.) Schreb - roots

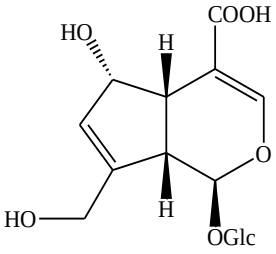
The phytochemical analysis conducted on the iridoidic fraction of *A. chamaepitys* roots led to the isolation and identification of five compounds: **harpagide**, **8-*O*-acetyl-harpagide**, **5-*O*-β-D-glucopyranosyl-harpagide**, **5-*O*-β-D-glucopyranosyl-8-*O*-acetyl-harpagide**, **deacetyl-asperulosidic acid** (Figure 37).



R = H: **harpagide**
R = Ac: **8-*O*-acetyl-harpagide**



R = H: **5-*O*-β-D-glucopyranosyl-harpagide**
R = Ac: **5-*O*-β-D-glucopyranosyl-8-*O*-acetyl-harpagide**



deacetyl-asperulosidic acid

Figure 37: Structures of compounds identified in *A. chamaepitys* - roots

These compounds belong again to one only class of natural compounds that are iridoids.

Again, **deacetyl-asperulosidic acid** represents a new compound for the genus whereas **5-*O*-β-D-glucopyranosyl-harpagide** and **5-*O*-β-D-glucopyranosyl-8-*O*-acetyl-harpagide** are new identified compounds in absolute.

5-*O*-β-D-glucopyranosyl-8-*O*-acetyl-harpagide presents an additional glucose residue linked in the position 5 on the 8-*O*-acetyl-harpagide skeleton.

Also its structure was established on the basis of spectroscopic evidences by 1D and 2D-NMR analyses. In this sense, this compound showed NMR spectra very similar to those of **5-*O*-β-D-glucopyranosyl-harpagide**.

The main differences were visible only in particular areas of the proton and carbon spectra showing further peaks at 2.08 ppm in the proton spectrum and at about 173.5 and 21.6 ppm in the carbon spectrum, all indicating the presence of an acetyl group.

Moreover this group performed a deshielding effect on C-8 resonating now at 87.6 ppm, on H-1 that shifted at lower fields in the proton spectrum (6.12 ppm) and on the methyl in the position 10 with a δ equal to 1.48 ppm. On the contrary, for H-9, a moderate shift to higher fields of 0.3 ppm was observed.

By means of HSQC, the cross peaks relative to this compound were identified In particular, this revealed the presence of correlations between H-1" (4.88 ppm) and C-5 (78.6 ppm) and also between both H-3 (6.48 ppm) and H-4 (5.04 ppm) with C-5 which showed quite the same chemical shift observed for C-5 in **5-*O*-β-D-glucopyranosyl-harpagide**.

High-resolution mass spectrometry experiments confirmed the molecular formula $C_{23}H_{36}O_{16}Na$ for the sodium adduct of **5-*O*-β-D-glucopyranosyl-8-*O*-acetyl-harpagide**.

3.2.3. *Ajuga genevensis* L.

The phytochemical analysis conducted on the polar fraction of this species led to the isolation and identification of three compounds: **rosmarinic acid**, **oleanolic acid** and **maslinic acid** (Figure 38).

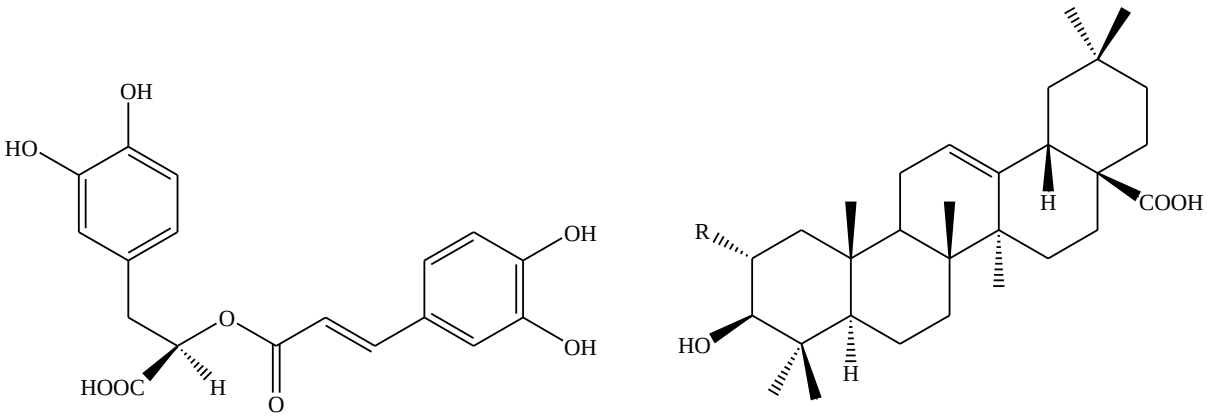


Figure 38: Structures of compounds identified in *A. genevensis*

These compounds belong to two different classes of natural compounds that are phenylpropanoids for **rosmarinic acid** and pentacyclic triterpenes for **oleanolic acid** and **maslinic acid**.

The main constituent of the polar fraction of this species was **rosmarinic acid** while **oleanolic acid** and **maslinic acid** were present only in traces.

Anyway, **rosmarinic acid** and **oleanolic acid** represent new phytochemicals for the species while **maslinic acid** was evidenced for the first time in the genus, during this study.

3.2.4. *Ajuga reptans* L.

The phytochemical analysis conducted on the polar fraction of this species allowed the isolation and identification of thirty-two compounds. In detail, these were: **1-oleoyl-2-linoleoyl-*sn*-glycerol-3-phosphocholine**, **1-miristoyl-2-heptadecenoyl-*sn*-glycerol**, **1,2-di-palmitoyl-*sn*-glycerol**, **martynoside**, **ajugoside**, **harpagide**, **8-*O*-acetyl-harpagide**, **glucose**, **fructose**, **galactose**, **sucrose**, **raffinose**, **stachyose**, **D-mannitol**, **acetic acid**, **caffeic acid**, **formic acid**, **fumaric acid**, **D-lactic acid**, **malic acid**, **pyruvic acid**, **succinic acid**, **α-hydroxy-butyric acid**, **γ-amino-butyric acid (GABA)**, **alanine**, **aspartic acid**, **asparagine**, **lysine**, **glutamine**, **threonine**, **tyrosine** and **valine** (Figure 39).

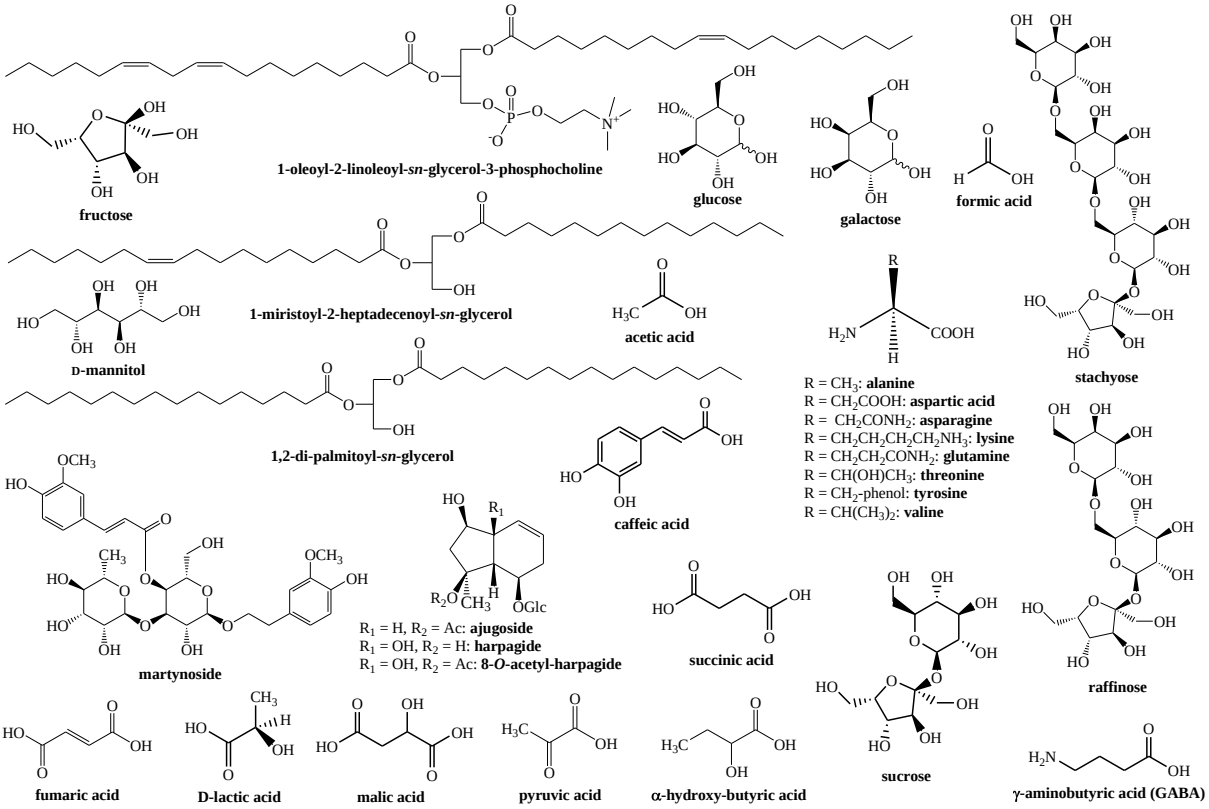


Figure 39: Structures of compounds identified in *A. reptans*

1-oleoyl-2-linoleoyl-*sn*-glycerol-3-phosphocholine is a membrane phospholipid, **1-miristoyl-2-heptadecenoyl-*sn*-glycerol** and **1,2-di-palmitoyl-*sn*-glycerol** are glycerides,

martynoside is one phenyl-ethanoid glycoside, **ajugoside**, **harpagide** and **8-*O*-acetyl-harpagide** are iridoids, **glucose**, **fructose**, **galactose**, **sucrose**, **raffinose** and **stachyose** are saccharides, **D-mannitol** is a linear polyol, **acetic acid**, **caffeic acid**, **formic acid**, **fumaric acid**, **D-lactic acid**, **malic acid**, **pyruvic acid**, **succinic acid**, **α-hydroxy-butyric acid** and **γ-amino-butyric acid (GABA)** are natural organic acids and, lastly, **alanine**, **aspartic acid**, **asparagine**, **lysine**, **glutamine**, **threonine**, **tyrosine** and **valine** are amino acids.

3.2.5. *Ajuga tenorei* C. Presl

The phytochemical analysis conducted on the polar fraction of this species led to the isolation and identification of five compounds: **verbascoside**, **echinacoside**, **ajugoside**, **harpagide** and **8-*O*-acetyl-harpagide** (Figure 40).

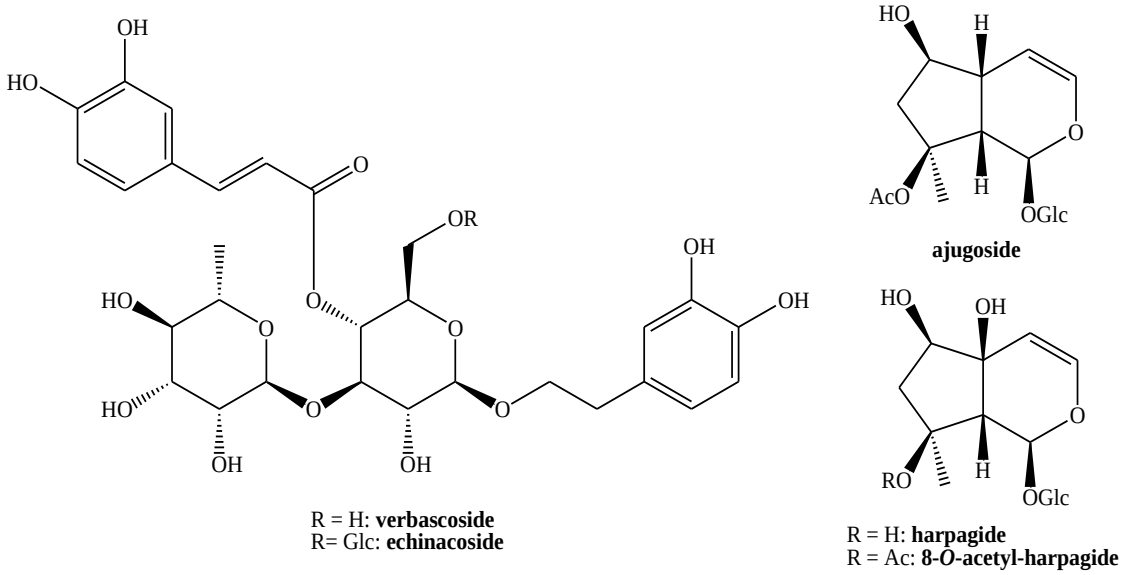


Figure 40: Structures of compounds identified in *A. tenorei*

These compounds belong to two different classes of natural compounds that are phenylethanoid glycosides (PhGs) for **verbascoside** and **echinacoside** and iridoids for **ajugoside**, **harpagide** and **8-*O*-acetyl-harpagide**.

All of them are new phytochemicals for the species.

3.2.6. *Galeopsis ladanum* subsp. *angustifolia* (Ehrh. ex Hoffm.) Gaudin

The phytochemical analysis conducted on the ethanolic extract from the total aerial parts of this species evidenced the presence of nine compounds namely **verbascoside**, **martynoside**, **7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-β-D-glucopyranosyl]oxy}-5,8-dihydroxy-2-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one**, **7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-β-D-glucopyranosyl]oxy}-5,8-dihydroxy-2-(4-methoxyphenyl)-4*H*-1-benzopyran-4-one**, **7-{[2-*O*-β-D-allopyranosyl-(6-*O*-acetyl-β-D-glucopyranosyl)]oxy}-5,8-dihydroxy-2-(3-hydroxy-4-methoxy-phenyl)-4*H*-1-benzopyran-4-one**, **harpagide**, **8-*O*-acetyl-harpagide**, **chlorogenic acid** and **quinic acid** (Figure 41).

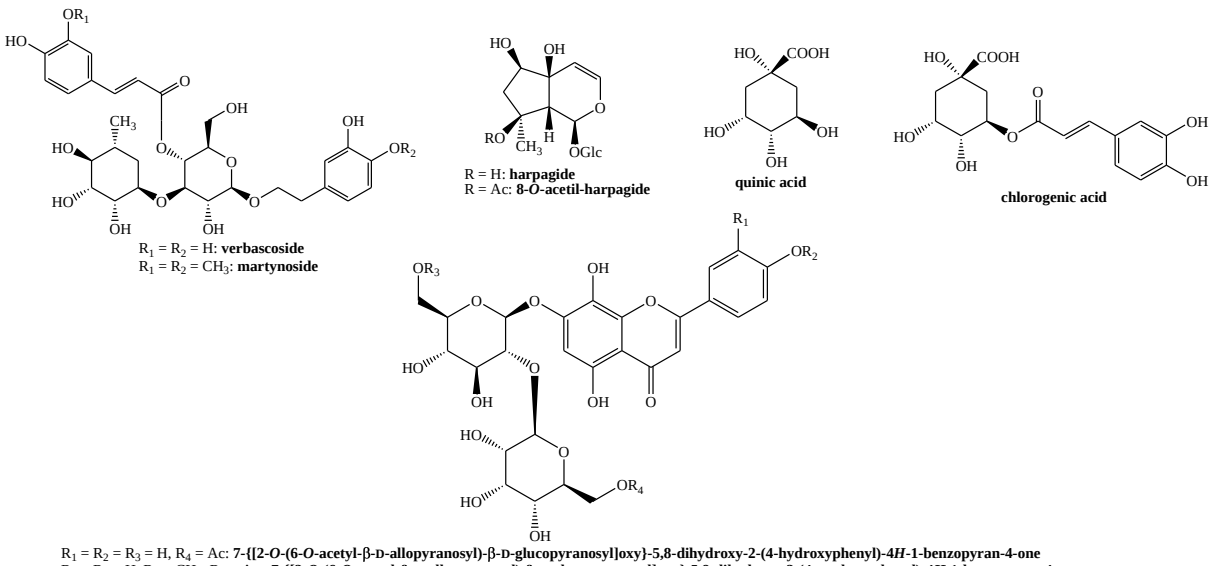


Figure 41: Structures of compounds identified in *G. ladanum* subsp. *angustifolia*

These compounds belong to five different classes of natural products: phenyl-ethanoid glycosides for **verbascoside** and **martynoside**; flavonoids as isoscutellarein derivatives for **7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-β-D-glucopyranosyl]oxy}-5,8-dihydroxy-2-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one** and **7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-β-D-glucopyranosyl]oxy}-5,8-dihydroxy-2-(4-methoxyphenyl)-4*H*-1-benzopyran-4-one** and as hypolaetin derivatives for **7-{[2-*O*-β-D-allopyranosyl-(6-*O*-acetyl-β-D-glucopyranosyl)]oxy}-5,8-dihydroxy-2-(3-hydroxy-4-methoxy-phenyl)-4*H*-1-**

benzopyran-4-one; iridoids for **harpagide** and **8-*O*-acetyl-harpagide**; caffeoyl-quinic acids for **chlorogenic acid**; organic acids for **quinic acid**.

Verbascoside, **7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-β-D-glucopyranosyl]oxy}-5,8-dihydroxy-2-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one**, **7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-β-D-glucopyranosyl]oxy}-5,8-dihydroxy-2-(4-methoxyphenyl)-4*H*-1-benzopyran-4-one**, **7-{[2-*O*-β-D-allopyranosyl-(6-*O*-acetyl-β-D-glucopyranosyl)]oxy}-5,8-dihydroxy-2-(3-hydroxy-4-methoxy-phenyl)-4*H*-1-benzopyran-4-one**, **chlorogenic acid** and **quinic acid** are new phytochemicals for the genus while **martynoside** is a new phytochemical for the species.

3.2.7. *Melittis melissophyllum* subsp. *albida* (Guss.) P.W.Ball

The phytochemical analysis conducted on this species revealed the presence of eight compounds: **verbascoside**, **harpagide**, **8-*O*-acetyl-harpagide**, **melittoside**, **allobetonicoside**, **virginioside**, **geniposidic acid**, **cinnamic acid** (Figure 42).

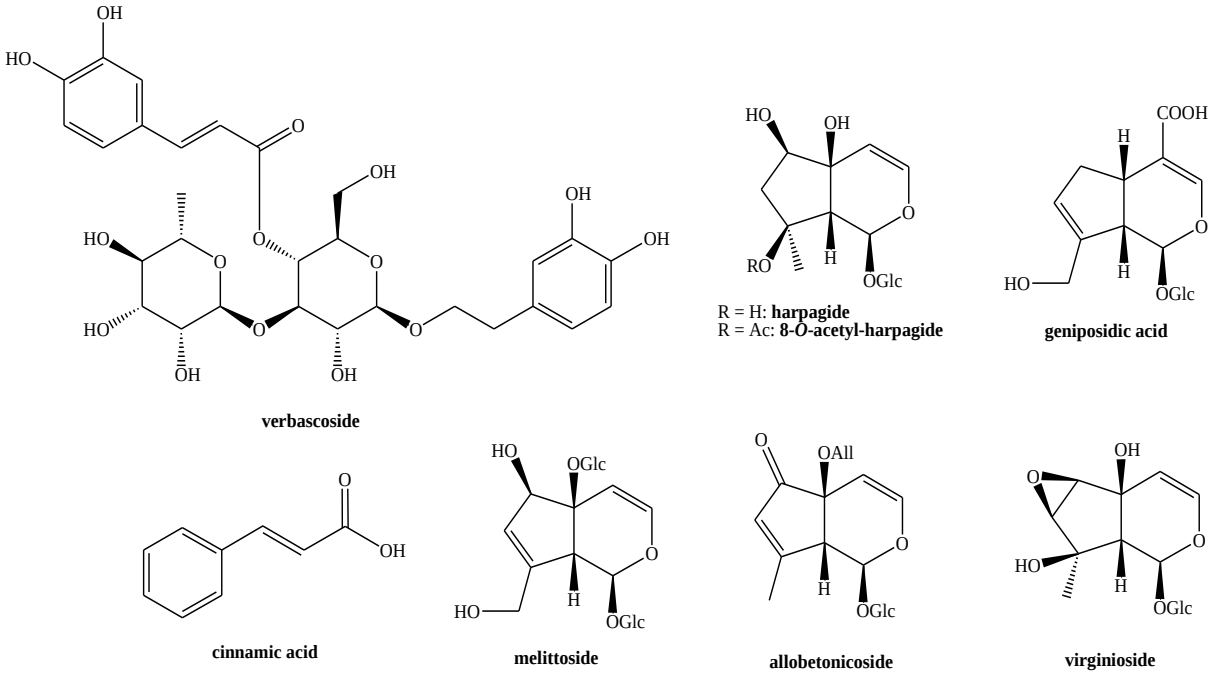


Figure 42: Structures of compounds identified in *M. melissophyllum* subsp. *albida*

These compounds belong to three different classes of natural products namely phenyl-ethanoid glycosides for **verbascoside**, iridoids for **harpagide**, **8-*O*-acetyl-harpagide**,

melittoside, **allobetonicoside**, **virginioside** and **geniposidic acid**, organic acids for **cinnamic acid**.

Harpagide, **8-*O*-acetyl-harpagide**, **melittoside**, **allobetonicoside** and **cinnamic acid** were evidenced for the first time in this species while **virginioside** and **geniposidic acid** are new compounds for the genus.

3.2.8. *Melittis melissophyllum* L. subsp. *melissophyllum*

The phytochemical analysis conducted on the only iridoidic fraction of this species evidenced the presence of six compounds: **harpagide**, **8-*O*-acetyl-harpagide**, **ajugoside**, **monomelittoside**, **melittoside** and **allobetonicoside** (Figure 43).

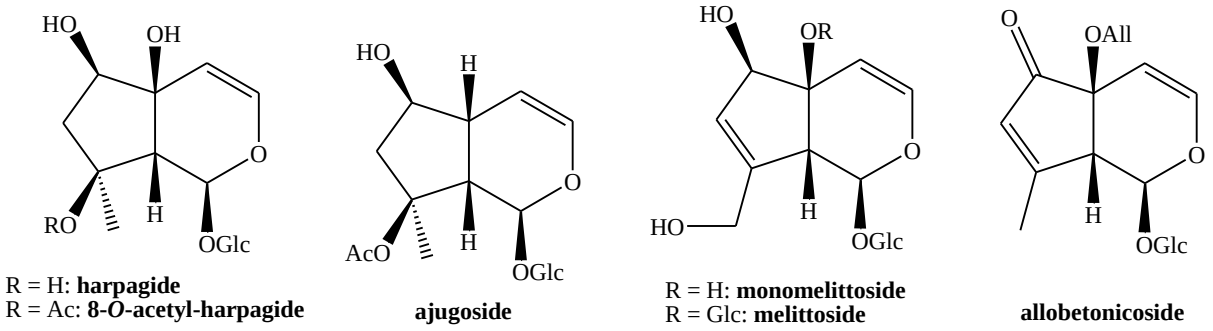


Figure 43: Structures of compounds identified in *M. melissophyllum* subsp. *melissophyllum*

These compounds are all iridoids.

Allobetonicoside represents a new compound for the genus.

3.2.9. *Sideritis montana* L. subsp. *montana*

The phytochemical analysis conducted on the ethanolic extract of this species evidenced the presence of nine compounds i.e. **7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-β-D-glucopyranosyl]oxy}-5,8-dihydroxy-2-(4-methoxyphenyl)-4*H*-1-benzopyran-4-one**, **7-{[2-*O*-β-D-allopyranosyl-(6-*O*-acetyl-β-D-glucopyranosyl)]-oxy}-5,8-dihydroxy-2-(3-hydroxy-4-methoxy-phenyl)-4*H*-1-benzopyran-4-one**, **harpagide**, **8-*O*-acetyl-harpagide**, **melittoside**, **5-*O*-allosyloxy-aucubin**, **8-epiloganic acid**, **chlorogenic acid**, **methyl-arbutin** (Figure 44).

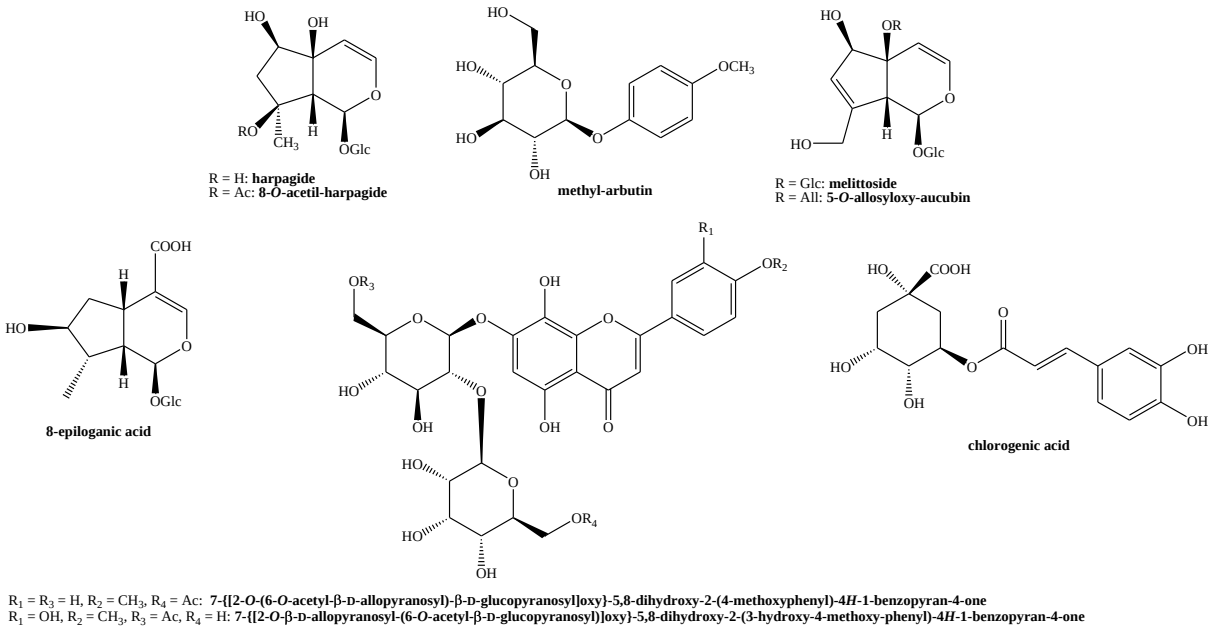


Figure 44: Structures of compounds identified in *S. montana* subsp. *montana*

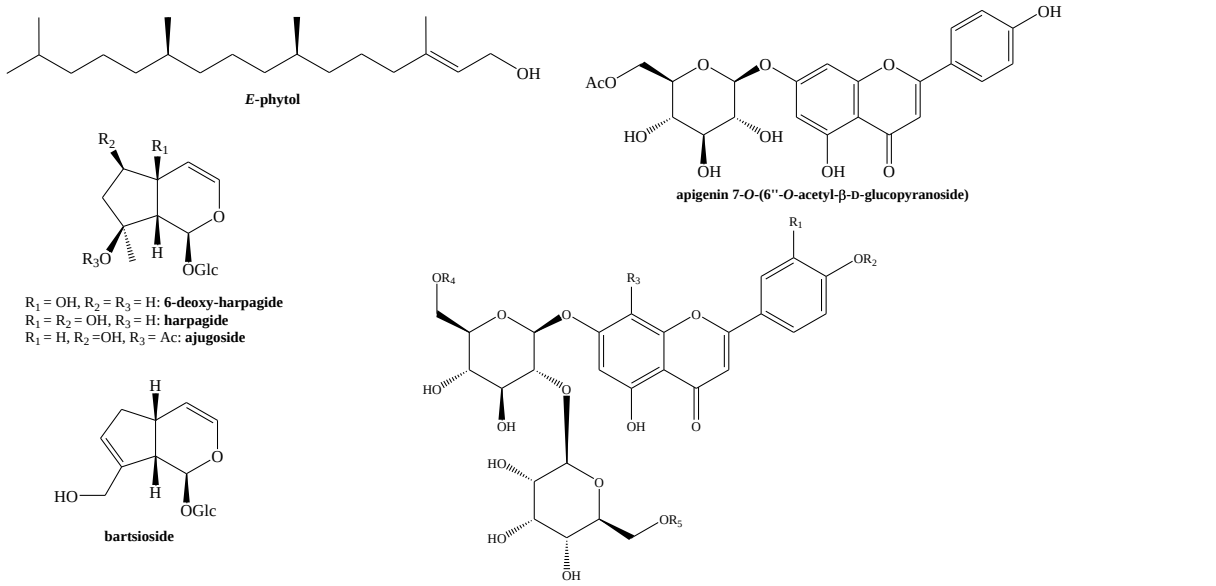
These compounds belong to four different classes of natural products namely flavonoids as isoscutellarein derivatives for **7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-β-D-glucopyranosyl]oxy}-5,8-dihydroxy-2-(4-methoxyphenyl)-4*H*-1-benzopyran-4-one** and **7-{[2-*O*-β-D-allopyranosyl-(6-*O*-acetyl-β-D-glucopyranosyl)]oxy}-5,8-dihydroxy-2-(3-hydroxy-4-methoxy-phenyl)-4*H*-1-benzopyran-4-one**, iridoids for **harpagide**, **8-*O*-acetyl-harpagide**, **melittoside** and **8-epiloganic acid**, caffeoylquinic acids for **chlorogenic acid**; glycosidic hydroquinones for **methyl-arbutin**.

8-epiloganic acid and **methyl-arbutin** were identified for the first time, during this study, as constituents of the genus whereas **7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-β-D-glucopyranosyl]oxy}-5,8-dihydroxy-2-(4-methoxyphenyl)-4*H*-1-benzopyran-4-one**, **7-{[2-*O*-β-D-allopyranosyl-(6-*O*-acetyl-β-D-glucopyranosyl)]oxy}-5,8-dihydroxy-2-(3-hydroxy-4-methoxy-phenyl)-4*H*-1-benzopyran-4-one**, **harpagide**, **8-*O*-acetyl-harpagide** and **chlorogenic acid** are new phytochemicals for the species.

3.2.10. *Sideritis romana* L.

The phytochemical analysis performed on the total fresh aerial parts of this species evidenced the presence of twelve compounds: ***E*-phytol**, **apigenin 7-*O*-(6''-*O*-acetyl-β-D-**

glucopyranoside), **7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-β-D-glucopyranosyl]oxy}-5-hydroxy-2-(3-hydroxy-4-methoxy-phenyl)-4*H*-1-benzopyran-4-one**, **7-{[2-*O*-(6-*O*-acetyl-β-D-glucopyranosyl)-β-D-glucopyranosyl]oxy}-5-hydroxy-2-(3,4-dihydroxyphenyl)-4*H*-1-benzopyran-4-one**, **7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-β-D-glucopyranosyl]oxy}-5-hydroxy-2-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one**, **7-{[2-*O*-β-D-allopyranosyl-β-D-glucopyranosyl]oxy}-5-hydroxy-2-(3-methoxy-4-hydroxy-phenyl)-4*H*-1-benzopyran-4-one**, **7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-β-D-glucopyranosyl]oxy}-5,8-dihydroxy-2-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one**, **7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-6-*O*-acetyl-β-D-glucopyranosyl]oxy}-5,8-dihydroxy-2-(3-hydroxy-4-methoxy-phenyl)-4*H*-1-benzopyran-4-one**, **6-deoxi-harpagide**, **harpagide**, **ajugoside** and **bartsioside** (Figure 45).



R_1 = OH, R_2 = CH_3, R_3 = R_4 = H, R_5 = Ac: **7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-β-D-glucopyranosyl]oxy}-5-hydroxy-2-(3-hydroxy-4-methoxy-phenyl)-4*H*-1-benzopyran-4-one**
R_1 = OH, R_2 = R_3 = R_4 = H, R_5 = Ac: **7-{[2-*O*-(6-*O*-acetyl-β-D-glucopyranosyl)-β-D-glucopyranosyl]oxy}-2-5-hydroxy-(3,4-dihydroxyphenyl)-4*H*-1-benzopyran-4-one**
R_1 = R_2 = R_3 = R_4 = H, R_5 = Ac: **7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-β-D-glucopyranosyl]oxy}-5-hydroxy-2-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one**
R_1 = OCH_3, R_2 = R_3 = R_4 = R_5 = H: **7-[(2-*O*-β-D-allopyranosyl-β-D-glucopyranosyl)oxy]-5-hydroxy-2-(3-methoxy-4-hydroxy-phenyl)-4*H*-1-benzopyran-4-one**
R_1 = R_2 = R_3 = R_4 = H, R_5 = Ac: **7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-β-D-glucopyranosyl]oxy}-5,8-dihydroxy-2-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one**
R_1 = R_3 = OH, R_2 = CH_3, R_4 = R_5 = Ac: **7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-6-*O*-acetyl-β-D-glucopyranosyl]oxy}-5,8-dihydroxy-2-(3-hydroxy-4-methoxy-phenyl)-4*H*-1-benzopyran-4-one**

Figure 45: structures of compounds identified in *S. romana*

These compounds belong to three different classes of natural products: linear diterpenes for ***E*-phytol**, flavonoids as apigenin derivatives for **apigenin 7-*O*-(6''-*O*-acetyl-β-D-glucopyranoside)**, **7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-β-D-glucopyranosyl]oxy}-5-hydroxy-2-(3-hydroxy-4-methoxy-phenyl)-4*H*-1-benzopyran-4-one**, **7-{[2-*O*-(6-*O*-acetyl-β-D-glucopyranosyl)-β-D-glucopyranosyl]oxy}-5-hydroxy-2-(3,4-dihydroxyphenyl)-4*H*-1-benzopyran-4-one**, **7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-β-D-glucopyranosyl]oxy}-5-hydroxy-2-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one** and **7-{[2-*O*-β-D-allopyranosyl-β-D-glucopyranosyl]oxy}-5-hydroxy-2-(3-methoxy-4-hydroxy-phenyl)-4*H*-1-benzopyran-4-**

one and isoscutellarein derivatives for **7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-β-D-glucopyranosyl]oxy}-5,8-dihydroxy-2-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one** and **7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-6-*O*-acetyl-β-D-glucopyranosyl]oxy}-5,8-dihydroxy-2-(3-hydroxy-4-methoxy-phenyl)-4*H*-1-benzopyran-4-one**, iridoids for **harpagide**, **6-deoxy-harpagide**, **ajugoside** and **bartsioside**.

Apigenin 7-*O*-(6''-*O*-acetyl-β-D-glucopyranoside) and **bartsioside** resulted to be new phytochemicals for the family while **6-deoxy-harpagide** was new for the genus.

3.2.11. *Stachys affinis* Bunge - rhizomes

The phytochemical analysis of the rhizomes if this species allowed the identification of nine compounds: **verbascoside**, **leucosceptoside A**, **martynoside**, **harpagide**, **8-*O*-acetyl-harpagide**, **melittoside**, **5-*O*-allosyloxy-aucubin**, **succinic acid** and **stachyose** (Figure 46).

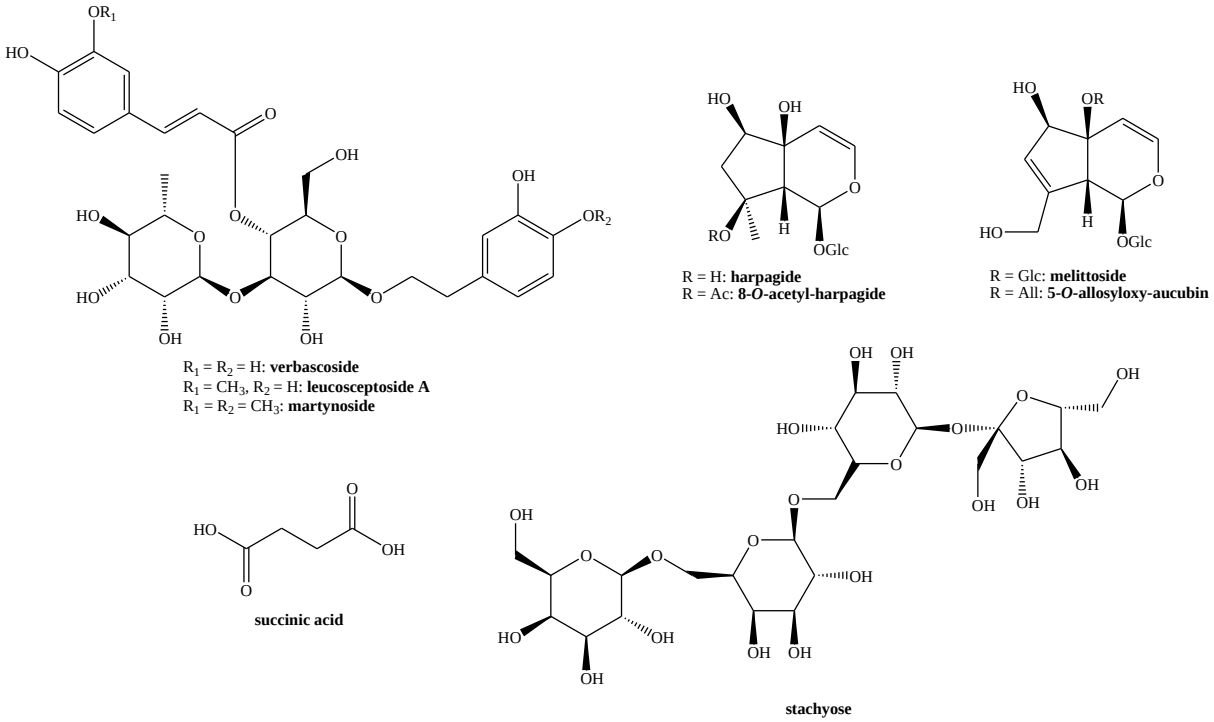


Figure 46: Structures of compounds identified in *S. affinis*

These compounds belong to four different classes of natural metabolites namely phenylethanoid glycosides for **verbascoside**, **leucosceptoside A** and **martynoside**, iridoids for **harpagide**, **8-*O*-acetyl-harpagide**, **melittoside** and **5-*O*-allosyloxy-aucubin**, dicarboxylic acids for **succinic acid** and oligosaccharides for stachyose.

Harpagide, **8-*O*-acetyl-harpagide**, **melittoside** and **5-*O*-allosyloxy-aucubin** represent all new compounds in the species.

3.2.12. *Stachys alopecuros* (L.) Benth. subsp. *divulsa* (Ten.) Grande

The phytochemical analysis of the glycosidic fraction of this species led to the identification of eleven compounds: **verbascoside**, **stachysoside A**, **6-*O*-acetyl-ajugol**, **ajugoside**, **reptoside**, **harpagide**, **8-*O*-acetyl-harpagide**, **allobetonicoside**, **4'-*O*-galactopyranosyl-teuhircoside**, **chlorogenic acid** and **β-arbutin** (Figure 47).

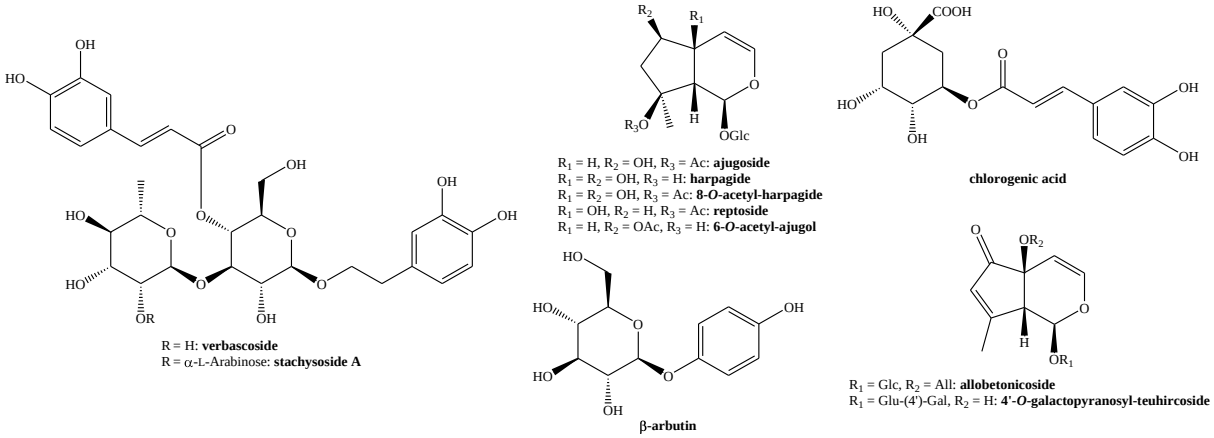


Figure 47: Structures of compounds identified in *S. alopecuros*

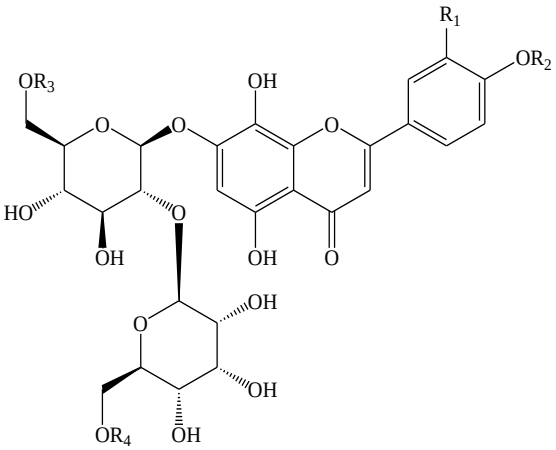
These compounds belong to four different classes of natural metabolites namely phenylethanoid glycosides for **verbascoside** and **stachysoside A**, iridoids for **6-*O*-acetyl-ajugol**, **ajugoside**, **reptoside**, **harpagide**, **8-*O*-acetyl-harpagide**, **allobetonicoside** and **4'-*O*-galactopyranosyl-teuhircoside**, caffeoyl-quinic acids for **chlorogenic acid** and glycosidic hydroquinones for **β-arbutin**.

Ajugoside, **reptoside** and **6-*O*-acetyl-ajugol** resulted to be new iridoids components for this species while **stachysoside A**, **chlorogenic acid** and **β-arbutin** were isolated for the first time from the studied genus.

3.2.13. *Stachys annua* L.

The phytochemical analysis performed on the aerial parts of this species evidenced the presence of two compounds: **7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-β-D-**

glucopyranosyl]oxy}-5,8-dihydroxy-2-(4-methoxyphenyl)-4*H*-1-benzopyran-4-one and **7-{[2-*O*-β-D-allopyranosyl-(6-*O*-acetyl-β-D-glucopyranosyl)]oxy}-5,8-dihydroxy-2-(3-hydroxy-4-methoxy-phenyl)-4*H*-1-benzopyran-4-one** (Figure 48).



$R_1 = R_3 = H$, $R_2 = CH_3$, $R_4 = Ac$: **7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-β-D-glucopyranosyl]oxy}-5,8-dihydroxy-2-(4-methoxyphenyl)-4*H*-1-benzopyran-4-one**
$R_1 = OH$, $R_2 = CH_3$, $R_3 = Ac$, $R_4 = H$: **7-{[2-*O*-β-D-allopyranosyl-(6-*O*-acetyl-β-D-glucopyranosyl)]oxy}-5,8-dihydroxy-2-(3-hydroxy-4-methoxy-phenyl)-4*H*-1-benzopyran-4-one**

Figure 48: Structures of compounds identified in *S. annua*

These compounds are flavonoids as isoscutellarein derivatives.

3.2.14. *Stachys germanica* subsp. *salviifolia* L.

The phytochemical analysis performed on the total aerial parts of this species evidenced the presence of five compounds: **stachysoside A**, **7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-β-D-glucopyranosyl]oxy}-5,8-dihydroxy-2-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one**, **7-{[2-*O*-β-D-allopyranosyl-(6-*O*-acetyl-β-D-glucopyranosyl)]oxy}-5,8-dihydroxy-2-(3-hydroxy-4-methoxy-phenyl)-4*H*-1-benzopyran-4-one**, **harpagide** and **chlorogenic acid** (Figure 49).

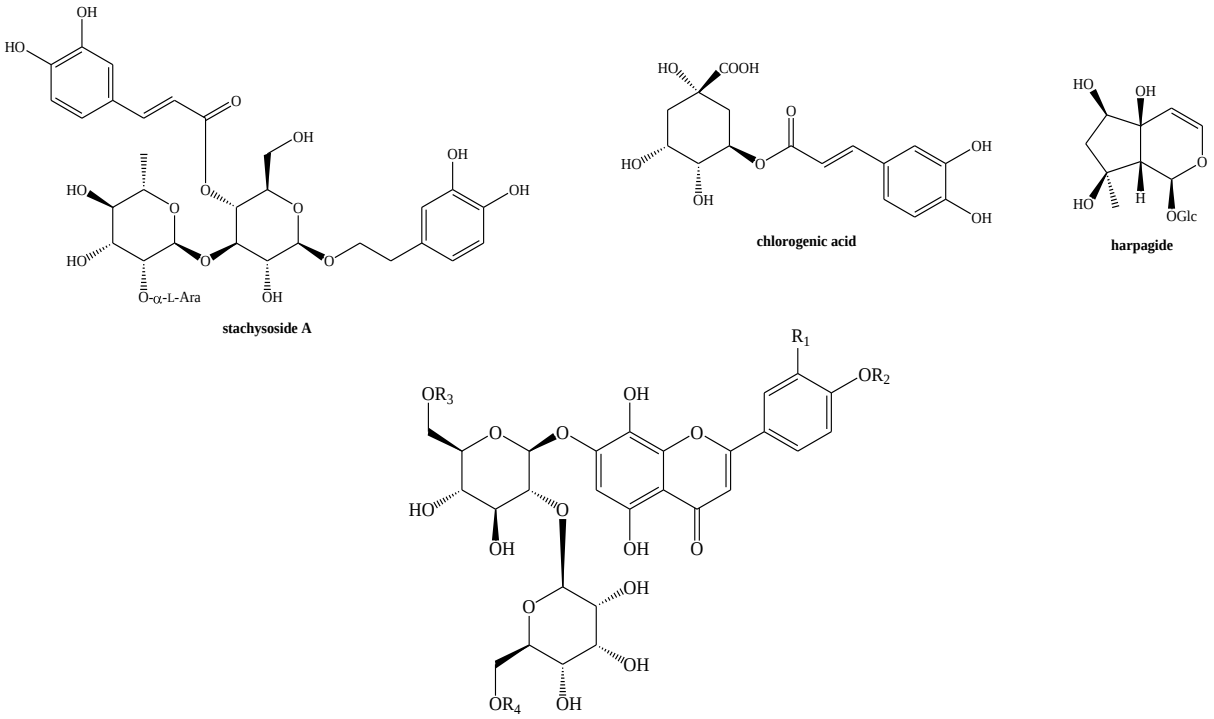


Figure 49: Structures of compounds identified in *S. germanica* subsp. *salviifolia*

These compounds belong to four different classes of natural metabolites which are phenylethanoid glycosides for **stachysoside A**, flavonoids as isoscutellarein derivative for **7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-β-D-glucopyranosyl]oxy}-5,8-dihydroxy-2-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one** and hypolaetin derivative for **7-{[2-*O*-β-D-allopyranosyl-(6-*O*-acetyl-β-D-glucopyranosyl)]oxy}-5,8-dihydroxy-2-(3-hydroxy-4-methoxy-phenyl)-4*H*-1-benzopyran-4-one**, iridoids for **harpagide** and caffeoyl-quinic acids for **chlorogenic acid**.

3.2.15. *Stachys palustris* L. - Hungary

The phytochemical analysis performed on the ethanolic extract of a sample of this species coming from Hungary allowed the identification of eight compounds namely **verbascoside**, **echinacoside**, **7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-β-D-glucopyranosyl]oxy}-5,8-dihydroxy-2-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one**, **7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-β-D-glucopyranosyl]oxy}-5,8-dihydroxy-2-(4-methoxyphenyl)-4*H*-1-benzopyran-4-one**, **harpagide**, **8-*O*-acetyl-harpagide**, **monomelittoside** and **chlorogenic acid** (Figure 50).

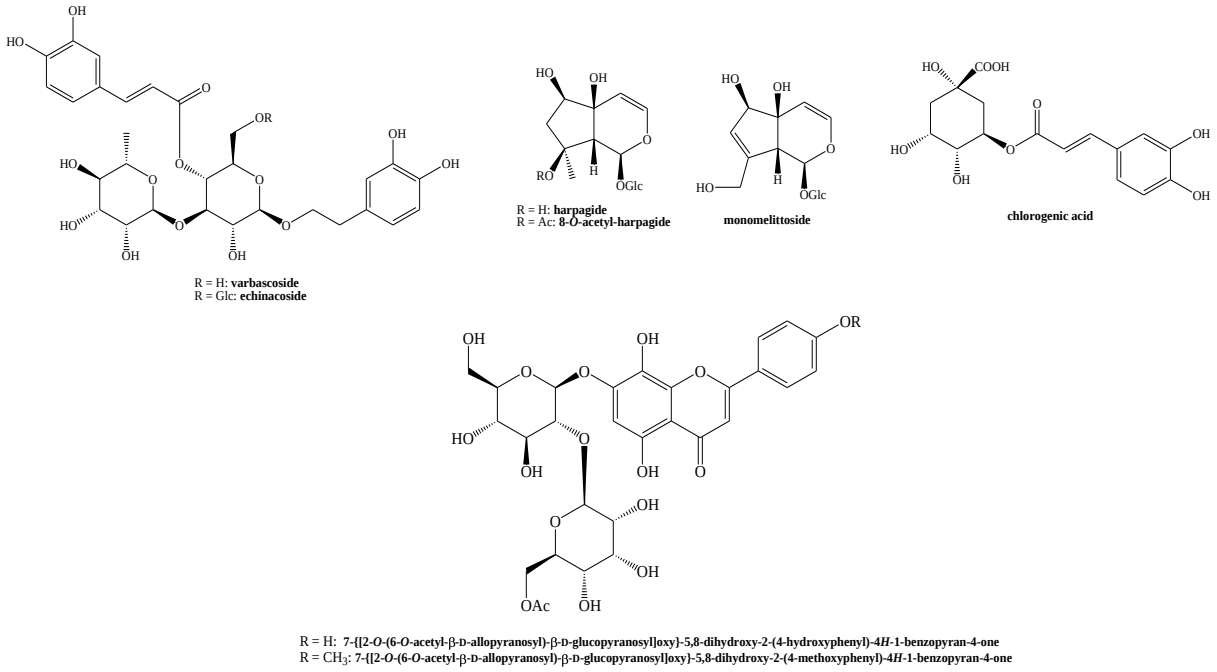


Figure 50: Structures of compounds identified in *S. palustris* collected in Hungary

These compounds belong to four different classes of secondary metabolites such as phenyl-ethanoid glycosides for **verbascoside** and **echinacoside**, flavonoids as isoscutellarein derivatives for **7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-β-D-glucopyranosyl]oxy}-5,8-dihydroxy-2-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one** and **7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-β-D-glucopyranosyl]oxy}-5,8-dihydroxy-2-(4-methoxyphenyl)-4*H*-1-benzopyran-4-one**, iridoids for **harpagide**, **8-*O*-acetyl-harpagide** and **monomelittoside**, caffeoyl-quinic acids for **chlorogenic acid**.

Echinacoside was evidenced, during this study, for the first time in the genus while **verbascoside** and **monomelittoside** were new phytochemicals for what concerns the species.

3.2.16. *Stachys palustris* L. - France

The phytochemical analysis performed on the ethanolic extract of a sample of this species coming from France allowed the identification of eight compounds namely **verbascoside**, **echinacoside**, **7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-β-D-glucopyranosyl]oxy}-5,8-dihydroxy-2-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one**, **7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-β-D-glucopyranosyl]oxy}-5,8-dihydroxy-2-(4-methoxyphenyl)-4*H*-1-benzopyran-4-one**, **harpagide**, **8-*O*-acetyl-harpagide**, **monomelittoside** and **chlorogenic acid** (Figure 51).

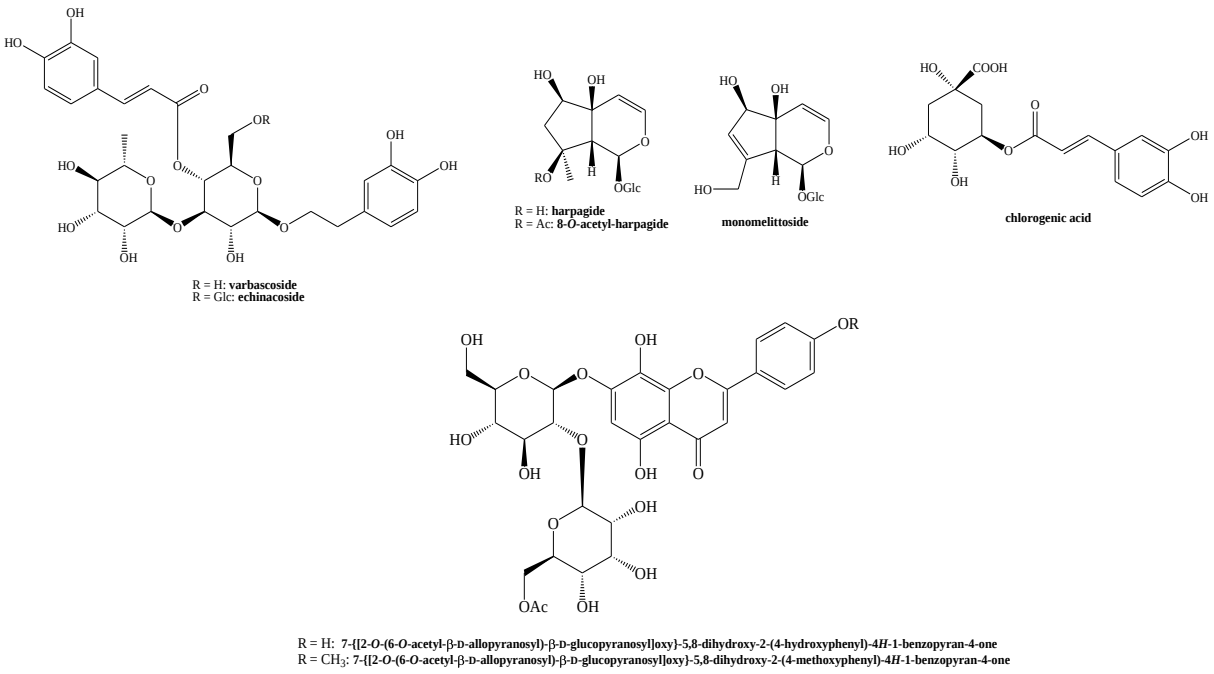


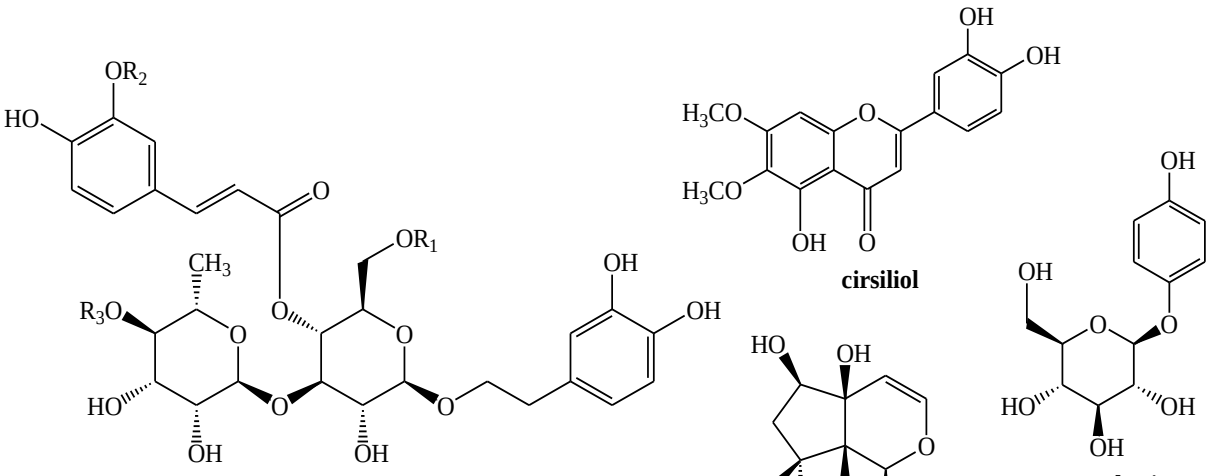
Figure 51: Structures of compounds identified in *S. palustris* collected in France

These compounds belong to four different classes of secondary metabolites such as phenyl-ethanoid glycosides for **verbascoside** and **echinacoside**, flavonoids as isoscutellarein derivatives for **7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-β-D-glucopyranosyl]oxy}-5,8-dihydroxy-2-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one** and **7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-β-D-glucopyranosyl]oxy}-5,8-dihydroxy-2-(4-methoxyphenyl)-4*H*-1-benzopyran-4-one**, iridoids for **harpagide**, **8-*O*-acetyl-harpagide** and **monomelittoside**, caffeoyl-quinic acids for **chlorogenic acid**.

Echinacoside was evidenced, during this study, for the first time in the genus while **verbascoside** and **monomelittoside** were for what concerns the species.

3.2.17. *Teucrium chamaedrys* L.

The phytochemical analysis performed on the ethanolic extract of this species evidenced the presence of eight compounds: **verbascoside**, **forsythoside b**, **samioside**, **alyssonoside**, **harpagide**, **8-*O*-acetyl-harpagide**, **cirsiliol** and **β-arbutin** (Figure 52).



$R_1 = R_2 = R_3 = H$: **verbascoside**
$R_1 = \beta$-D-Apiose, $R_2 = R_3 = H$: **forsythoside b**
$R_1 = R_2 = H$, $R_3 = \beta$–D-Apiose: **samioside**
$R_1 = \beta$–D-Apiose, $R_2 = CH_3$, $R_3 = H$: **allysonoside**

R = H: **harpagide**
R = Ac: **8-*O*-acetyl-harpagide**

Figure 52: Structures of compounds identified in *T. chamaedrys*

These phytochemicals belong to four different classes of natural compounds i.e. phenyl-ethanoid glycosides (**verbascoside**, **forsythoside b**, **samioside** and **alyssonoside**), iridoids (**harpagide** and **8-*O*-acetyl-harpagide**), flavonoids (**cirsiliol**) and glycosidic hydroquinones (**β-arbutin**).

B-arbutin resulted to be a new compound for the genus.

3.2.18. *Teucrium polium* L. - Northern Iran

The phytochemical analysis conducted on the polar fraction of *T. polium* from Northern Iran led to the isolation and identification of twelve compounds: ***E*-phytol**, **pheophorbide a**, **teucrasiatin**, **20-*O*-acetyl-teucrasiatin**, **oleanolic acid**, **maslinic acid**, **verbascoside**, **apigenin**, **luteolin**, **acacetin**, **apigenin 7-*O*-β-glucoside**, **cirsimaritin** (Figure 53).

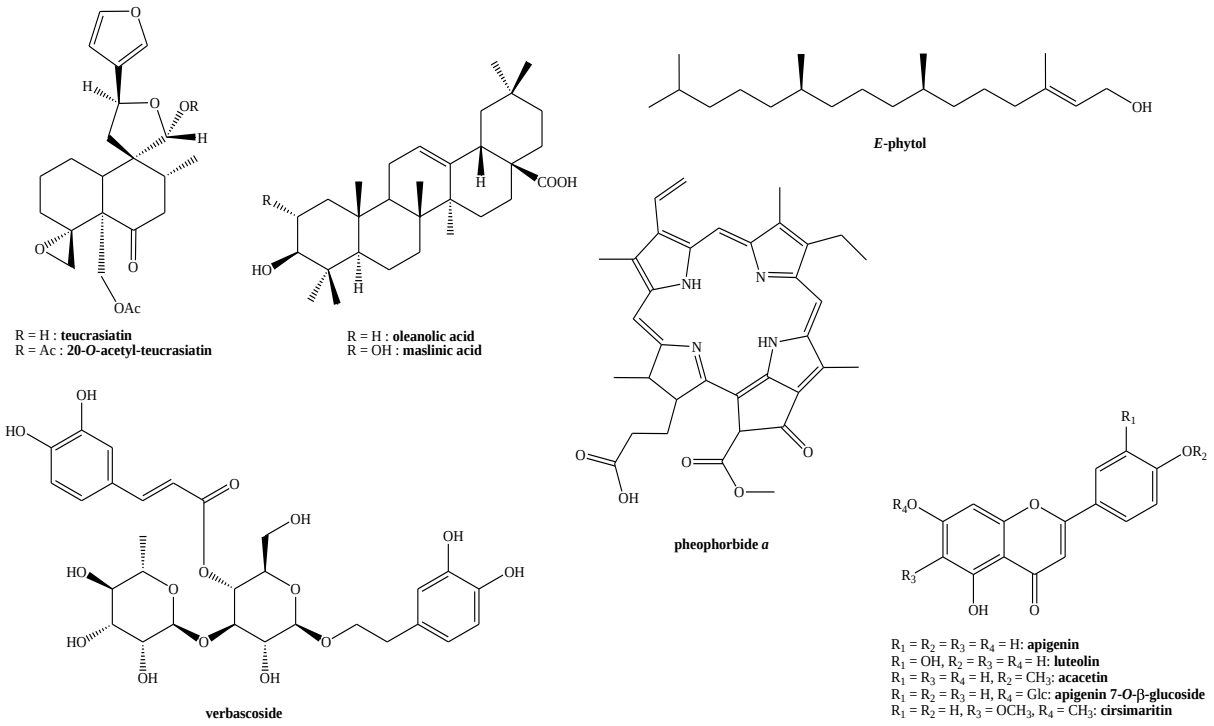


Figure 53: Structures of compounds identified in *T. polium* collected in Northern Iran

These compounds belong to five different classes of natural products which are: linear diterpenes for ***E*-phytol**, tetrapyrroles for **pheophorbide a**, *neo*-clerodane diterpenes for **teucrasiatin** and **20-*O*-acetyl-teucrasiatin**, pentacyclic triterpenic acids for **oleanolic acid** and **maslinic acid**, phenyl-ethanoid glycosides for **verbascoside** and flavonoids for **apigenin**, **luteolin**, **acacetin**, **apigenin 7-*O*-β-glucoside**, **cirsimaritin**.

***E*-phytol**, **teucrasiatin**, **oleanolic acid** and **maslinic acid** represent new phytochemicals for the species whereas **20-*O*-acetyl-teucrasiatin** was even isolated for the first time as a natural compound from *Teucrium* even if it was already known as a synthetic derivative of **teucrasiatin** (Rodriguez et al., 1996).

Its structure was hypothesized on the basis of extensive NMR data and then confirmed by Mass Spectrometry experiments.

In the proton NMR several signals were visible: at 6.48, 7.48 ppm and 7.53 ppm which are in accordance with the presence of a β-substituted furan ring; a doublet at 1.06 ppm with a coupling constant of 6.8 Hz indicating the presence of a secondary methyl group at C-17; a 4,18-oxirane function was recognized by the presence of an AB system for geminal protons on C-18 at 3.00 (J = 5.0, 2.0 Hz) and 2.42 (J = 5.0 Hz); a spiro-20,12-hemiacetal function forming a five member ring with carbons C-9, C-11, C-12 and C-20.

This is a structural motif already observed in several neo-clerodane isolated from *Teucrium* species (Bruno et al., 1995a, 1995b).

The two acetyl functions were revealed by the presence of two singulets integrating each for three protons at 2.18 and 2.00 ppm.

The ^{13}C-NMR revealed the presence of 24 peaks which confirmed the β-substituted furan ring moiety (144.8, 140.9, 126.2 and 109.8 ppm), the two acetyl substituents (172.3, 171.5, 21.6 and 20.7 ppm), a carbonyl function (208.4 ppm) and the three quaternary carbons at 62.5, 55.4 and 54.0 ppm for C-4, C-5 and C-9, respectively.

In the HSQC experiment no correlations were shown by these quaternary carbons, of course. The last diagnostic signal at 99.9 ppm revealed the presence of a hemiacetalic carbon at C-20. This last signal resulted to be more deshielded in respect of that observed in **teucrasiatin** (93.8 ppm), giving a first evidence of an acetylation in this position.

The acetylation site was then confirmed by HMBC correlations between H-20 (6.32 ppm) and the acetyl moiety (171.5 ppm) and also between H-20 and C-9 (54.0 ppm). The reverse correlation between the methyl group of acetyl moiety (2.18 ppm) and C-20 (99.9 ppm) was also observed.

The second acetylation site appeared to be on C-19, as well as in **teucrasiatin** and was confirmed by correlations between H-19 (AB system, 4.75 and 4.29 ppm) and the carboxylic carbon of this second acetyl moiety (172.3 ppm) and also between its methyl protons at 2.00 ppm and the carbon resonating at 64.9 ppm (C-19).

The keto-function resulted to be in the position 6 since correlations between the secondary methyl (C-17) protons and the protons linked to C-8 and C-7 (TOCSY), and long range correlations (HMBC) between H-17 (1.06 ppm) and C-8 (43.8 ppm), C-7 (47.4 ppm), C-9 (55.4 ppm) and C-6 (208.4 ppm) were observed.

NOESY experiments confirmed the configuration at C-20 as (20R)-20-*O*-acetyl-20,12-hemiacetal.

The HR-ESI–MS spectrum in positive ion mode confirmed the molecular formula $C_{24}H_{30}O_8Na$ for the sodium adduct of this compound.

In addition, the ESI-MS^2 experiments confirmed the presence of the two acetyl moieties which were lost in the fragmentation: *m/z* 409.15 $[M+Na-AcO]^+$ and *m/z* 315.11 $[M+Na-2AcO-OH]^+$.

3.2.19. *Teucrium polium* L. - Southern Iran

The phytochemical analysis of *T. polium* from Southern Iran evidenced the presence of ten compounds: **10-β-hydroxy-teucjaponin B**, **picropolin**, **teupolin I**, **poliumoside**, **cirsilineol**, **cirsimaritin**, **4'-*O*-methyl-luteolin** (**diosmetin)**, **cirsiliol**, **apigenin**, **apigenin 7-*O*-rutinoside (isorhoifolin)** (Figure 54).

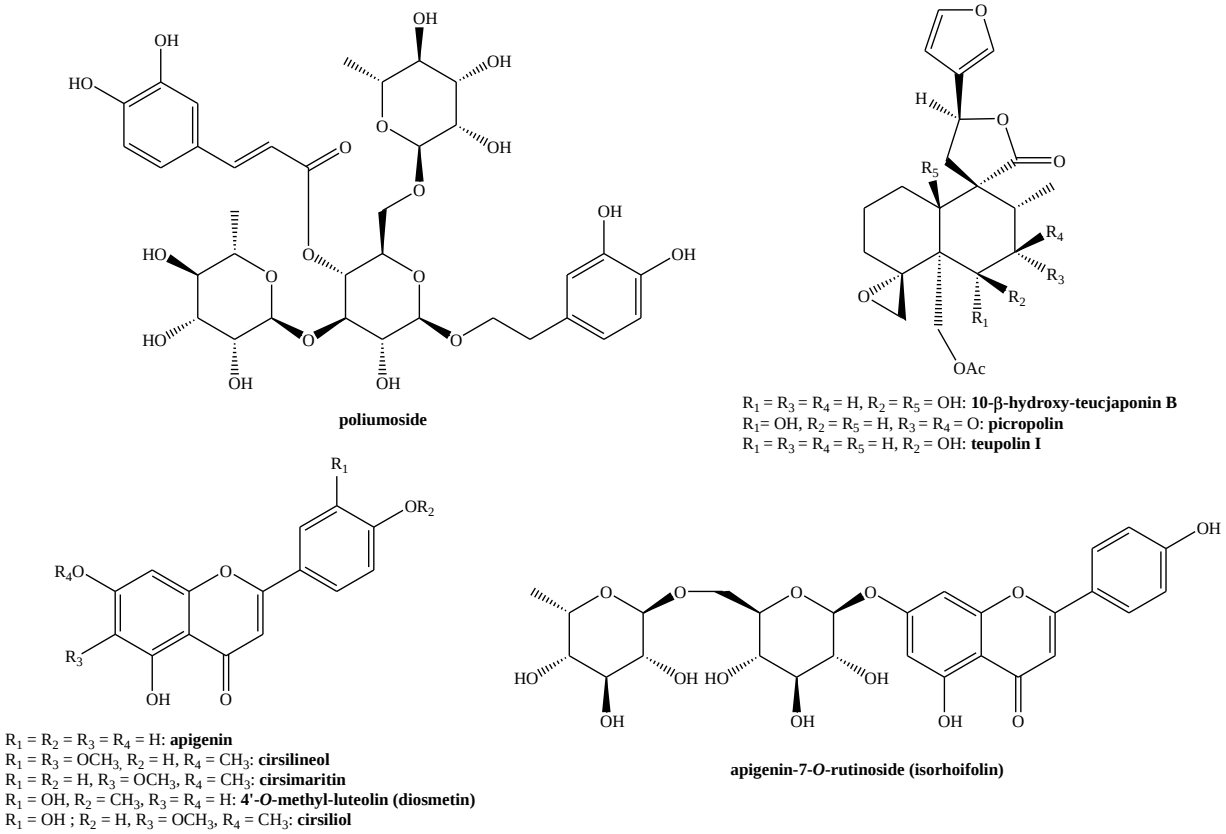


Figure 54: Structures of compounds identified in *T. polium* collected in Southern Iran

These compounds belong to three different classes of natural products which are *neo*-clerodane diterpenes for **10-β-hydroxyteucjaponin B**, **picropolin** and **teupolin I**, phenyl-ethanoid glycosides for **poliumoside**, flavonoids for **cirsilineol**, **cirsimaritin**, **diosmetin**, **cirsiliol**, **apigenin**, **apigenin 7-*O*-rutinoside** (**isorhoifolin**).

10-β-hydroxyteucjaponin B was evidenced for the first time, during this study, as constituent of this species.

3.3. Chemotaxonomy

During this work, several phytochemicals belonging to a total of thirteen different major classes of natural compounds were evidenced.

In particular, these classes were: membrane phospholipids, glycerides, tetrapyrroles, terpenoids, phenyl-ethanoid glycosides (PhGs), flavonoids, iridoids, caffeoyl-quinic acids, glycosidic hydroquinones, polyols, saccharides, organic acids and amino acids.

Anyway, some classes were in common among several studied species while others were peculiar of only few species or even of an only one.

During this work, a total of one hundred and sixty-nine phytochemical compounds were evidenced for all the studied species, too.

In particular, these compounds were: **1-oleoyl-2-linoleoyl-*sn*-glycerol-3-phosphocholine**, **1-miristoyl-2-heptadecenoyl-*sn*-glycerol**, **1,2-di-palmitoyl-*sn*-glycerol**, **pheophorbide a**, ***E*-phytol**, **10-β-hydroxy-teucjaponin B**, **picropolin**, **teupolin I**, **teucrasiatin**, **20-*O*-acetyl-teucrasiatin**, **rosmarinic acid**, **oleanolic acid**, **maslinic acid**, **verbascoside**, **martynoside**, **forsythoside b**, **samioside**, **alyssonoside**, **leucosceptoside A**, **echinacoside**, **poliumoside**, **stachysoside A**, **apigenin**, **luteolin**, **acacetin**, **cirsiliol**, **cirsimaritin**, **cirsilineol**, **4'-*O*-methyl-luteolin (diosmetin)**, **apigenin 7-*O*-β-glucoside**, **apigenin 7-*O*-rutinoside (isorhoifolin)**, **apigenin 7-*O*-(6''-*O*-acetyl-β-D-glucopyranoside)**, **7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-β-D-glucopyranosyl]oxy}-5-hydroxy-2-(3-hydroxy-4-methoxyphenyl)-4*H*-1-benzopyran-4-one**, **7-{[2-*O*-(6-*O*-acetyl-β-D-glucopyranosyl)-β-D-glucopyranosyl]oxy}-5-hydroxy-2-(3,4-dihydroxyphenyl)-4*H*-1-benzopyran-4-one**, **7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-β-D-glucopyranosyl]oxy}-5-hydroxy-2-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one**, **7-{[2-*O*-β-D-allopyranosyl-β-D-glucopyranosyl]oxy}-5-hydroxy-2-(3-methoxy-4-hydroxy-phenyl)-4*H*-1-benzopyran-4-one**, **7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-β-D-glucopyranosyl]oxy}-5,8-dihydroxy-2-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one**, **7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-β-D-glucopyranosyl]oxy}-5,8-dihydroxy-2-(4-methoxyphenyl)-4*H*-1-benzopyran-4-one**, **7-{[2-*O*-β-D-allopyranosyl-(6-*O*-acetyl-β-D-glucopyranosyl)]oxy}-5,8-dihydroxy-2-(3-hydroxy-4-methoxy-phenyl)-4*H*-1-benzopyran-4-one**, **7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-6-*O*-acetyl-β-D-glucopyranosyl]oxy}-5,8-dihydroxy-2-(3-hydroxy-4-methoxy-phenyl)-4*H*-1-benzopyran-4-one**, **8-epiloganic acid**, **ajugoside**, **reptoside**, **harpagide**, **8-*O*-acetyl-harpagide**, **monomelittoside**, **melittoside**, **allobetonicoside**, **virginioside**, **geniposidic acid**, **6-deoxy-**

harpagide, **bartsioside**, **6-*O*-acetyl-ajugol**, **5-*O*-allosyloxy-aucubin**, **asperulosidic acid**, **deacetyl-asperulosidic acid**, **5-*O*-β-D-glucopyranosyl-harpagide**, **5-*O*-β-D-glucopyranosyl-8-*O*-acetyl-harpagide**, **4'-*O*-galactopyranosyl-teuhircoside**, **chlorogenic acid**, **β-arbutin**, **methyl-arbutin**, **D-mannitol**, **glucose**, **fructose**, **galactose**, **sucrose**, **raffinose**, **stachyose**, **acetic acid**, **caffeic acid**, **cinnamic acid**, **formic acid**, **fumaric acid**, **D-lactic acid**, **malic acid**, **pyruvic acid**, **quinic acid**, **succinic acid**, **α-hydroxy-butyric acid**, **γ-amino-butyric acid (GABA)**, **alanine**, **aspartic acid**, **asparagine**, **lysine**, **glutamine**, **threonine**, **tyrosine** and **valine**.

Also, in this case, some compounds were in common among several studied species while others were peculiar of only few species or even of an only one.

In the next table (Table 7), the specific identified compounds in alphabetical order are compared for their occurrence in all the studied species which are also specified.

Compound	Species in which the compound was evidenced
1-oleoyl-2-linoleoyl-*sn*-glycerol-3-phosphocholine	- *Ajuga reptans* L.
1-miristoyl-2-heptadecenoyl-*sn*-glycerol	- *Ajuga reptans* L.
1,2-di-palmitoyl-*sn*-glycerol	- *Ajuga reptans* L.
pheophorbide a	- *Teucrium polium* L. - Northern Iran
***E*-phytol**	- *Sideritis romana* L. - *Teucrium polium* L. - Northern Iran
10-β-hydroxy-teucjaponin B	- *Teucrium polium* L. - Southern Iran
picropolin	- *Teucrium polium* L. - Southern Iran
teupolin I	- *Teucrium polium* L. - Southern Iran
teucrasiatin	- *Teucrium polium* L. - Northern Iran
20-*O*-acetyl-teucrasiatin	- *Teucrium polium* L. - Northern Iran
rosmarinic acid	- *Ajuga genevensis* L.
oleanolic acid	- *Ajuga genevensis* L. - *Teucrium polium* L. - Northern Iran
maslinic acid	- *Ajuga genevensis* L. - *Teucrium polium* L. - Northern Iran
verbascoside	- *Ajuga tenorei* C. Presl - *Galeopsis ladanum* subsp. *angustifolia* (Ehrh. ex Hoffm.) Gaudin - *Melittis melissophyllum* L. subsp. *albida* (Guss.) P.W.Ball - *Stachys affinis* Bunge - rhizomes - *Stachys alopecuros* (L.) Benth. subsp. *divulsa* (Ten.) Grande - *Stachys palustris* L. - Hungary - *Stachys palustris* L. - France - *Teucrium chamaedrys* L. - *Teucrium polium* L. - Northern Iran
martynoside	- *Ajuga reptans* L. - *Galeopsis ladanum* subsp. *angustifolia* (Ehrh. ex Hoffm.) Gaudin - *Stachys affinis* Bunge - rhizomes
forsythoside b	- *Teucrium chamaedrys* L.
leucosceptoside A	- *Stachys affinis* Bunge - rhizomes
samioside	- *Teucrium chamaedrys* L.
alyssonoside	- *Teucrium chamaedrys* L.

echinacoside	- *Ajuga tenorei* C. Presl - leaves - *Stachys palustris* L. - Hungary - *Stachys palustris* L. - France
poliumoside	- *Teucrium polium* L. - Southern Iran
stachysoside A	- *Stachys alopecuros* (L.) Benth. subsp. *divulsa* (Ten.) Grande - *Stachys germanica* subsp. *salviifolia* L.
apigenin	- *Teucrium polium* L. - Northern Iran - *Teucrium polium* L. - Southern Iran
luteolin	- *Teucrium polium* L. - Northern Iran
acacetin	- *Teucrium polium* L. - Northern Iran
cirsiliol	- *Teucrium chamaedrys* L. - *Teucrium polium* L. - Southern Iran
cirsimaritin	- *Teucrium polium* L. - Northern Iran - *Teucrium polium* L. - Southern Iran
cirsilineol	- *Teucrium polium* L. - Southern Iran
4'-*O*-methyl-luteolin (diosmetin)	- *Teucrium polium* L. - Southern Iran
apigenin 7-*O*-β-glucoside	- *Teucrium polium* L. - Northern Iran
apigenin 7-*O*-rutinoside (isorhoifolin)	- *Teucrium polium* L. - Southern Iran
apigenin 7-*O*-(6''-*O*-acetyl-β-D-glucopyranoside)	- *Sideritis romana* L.
7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-β-D-glucopyranosyl]oxy}-5-hydroxy-2-(3-hydroxy-4-methoxyphenyl)-4*H*-1-benzopyran-4-one	- *Sideritis romana* L.
7-{[2-*O*-(6-*O*-acetyl-β-D-glucopyranosyl)-β-D-glucopyranosyl]oxy}-5-hydroxy-2-(3,4-dihydroxyphenyl)-4*H*-1-benzopyran-4-one	- *Sideritis romana* L.
7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-β-D-glucopyranosyl]oxy}-5-hydroxy-2-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one	- *Sideritis romana* L.
7-{[2-*O*-β-D-allopyranosyl-β-D-glucopyranosyl]oxy}-5-hydroxy-2-(3-methoxy-4-hydroxy-phenyl)-4*H*-1-benzopyran-4-one	- *Sideritis romana* L.
7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-β-D-glucopyranosyl]oxy}-5,8-dihydroxy-2-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one	- *Galeopsis ladanum* subsp. *angustifolia* (Ehrh. ex Hoffm.) Gaudin - *Sideritis romana* L. - *Stachys germanica* subsp. *salviifolia* L.
7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-β-D-glucopyranosyl]oxy}-5,8-dihydroxy-2-(4-methoxyphenyl)-4*H*-1-benzopyran-4-one	- *Galeopsis ladanum* subsp. *angustifolia* (Ehrh. ex Hoffm.) Gaudin - *Sideritis montana* L. subsp. *montana* - *Stachys annua* L.
7-{[2-*O*-β-D-allopyranosyl-(6-*O*-acetyl-β-D-glucopyranosyl)]oxy}-5,8-dihydroxy-2-(3-hydroxy-4-methoxy-phenyl)-4*H*-1-benzopyran-4-one	- *Galeopsis ladanum* subsp. *angustifolia* (Ehrh. ex Hoffm.) Gaudin - *Sideritis montana* L. subsp. *montana* - *Stachys annua* L. - *Stachys germanica* subsp. *salviifolia* L.
7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-6-*O*-acetyl-β-D-glucopyranosyl]oxy}-5,8-dihydroxy-2-(3-hydroxy-4-methoxy-phenyl)-4*H*-1-benzopyran-4-one	- *Sideritis romana* L.
8-epiloganic acid	- *Sideritis montana* L. subsp. *montana*
ajugoside	- *Ajuga chamaepitys* (L.) Schreb. - aerial parts - *Ajuga reptans* L. - *Ajuga tenorei* C. Presl - leaves - *Melittis melissophyllum* L. subsp. *melissophyllum* - *Sideritis romana* L. - *Stachys alopecuros* (L.) Benth. subsp. *divulsa* (Ten.) Grande

reptoside	- *Ajuga chamaepitys* (L.) Schreb. - aerial parts - *Stachys alopecuros* (L.) Benth. subsp. *divulsa* (Ten.) Grande
harpagide	- *Ajuga chamaepitys* (L.) Schreb. - aerial parts - *Ajuga chamaepitys* (L.) Schreb. - roots - *Ajuga reptans* L. - *Ajuga tenorei* C. Presl - leaves - *Galeopsis ladanum* subsp. *angustifolia* (Ehrh. ex Hoffm.) Gaudin - *Melittis melissophyllum* L. subsp. *albida* (Guss.) P.W.Ball - *Melittis melissophyllum* L. subsp. *melissophyllum* - *Sideritis montana* L. subsp. *montana* - *Sideritis romana* L. - *Stachys affinis* Bunge - rhizomes - *Stachys alopecuros* (L.) Benth. subsp. *divulsa* (Ten.) Grande - *Stachys germanica* subsp. *salviifolia* L. - *Stachys palustris* L. - Hungary - *Stachys palustris* L. - France - *Teucrium chamaedrys* L.
6-deoxy-harpagide	- *Sideritis romana* L.
8-*O*-acetyl-harpagide	- *Ajuga chamaepitys* (L.) Schreb. - aerial parts - *Ajuga chamaepitys* (L.) Schreb. - roots - *Ajuga reptans* L. - *Ajuga tenorei* C. Presl - leaves - *Galeopsis ladanum* subsp. *angustifolia* (Ehrh. ex Hoffm.) Gaudin - *Melittis melissophyllum* L. subsp. *albida* (Guss.) P.W.Ball - *Melittis melissophyllum* L. subsp. *melissophyllum* - *Sideritis montana* L. subsp. *montana* - *Stachys affinis* Bunge - rhizomes - *Stachys alopecuros* (L.) Benth. subsp. *divulsa* (Ten.) Grande - *Stachys palustris* L. - Hungary - *Stachys palustris* L. - France - *Teucrium chamaedrys* L.
monomelittoside	- *Melittis melissophyllum* L. subsp. *melissophyllum* - *Stachys palustris* L. - Hungary - *Stachys palustris* L. - France
melittoside	- *Melittis melissophyllum* L. subsp. *albida* (Guss.) P.W.Ball - *Melittis melissophyllum* L. subsp. *melissophyllum* - *Sideritis montana* L. subsp. *montana* - *Stachys affinis* Bunge - rhizomes
allobetonicoside	- *Melittis melissophyllum* L. subsp. *albida* (Guss.) P.W.Ball - *Melittis melissophyllum* L. subsp. *melissophyllum* - *Stachys alopecuros* (L.) Benth. subsp. *divulsa* (Ten.) Grande
virginioside	- *Melittis melissophyllum* L. subsp. *albida* (Guss.) P.W.Ball
geniposidic acid	- *Melittis melissophyllum* L. subsp. *albida* (Guss.) P.W.Ball
bartsioside	- *Sideritis romana* L.
6-*O*-acetyl-ajugol	- *Stachys alopecuros* (L.) Benth. subsp. *divulsa* (Ten.) Grande
5-*O*-allosyloxy-aucubin	- *Sideritis montana* L. subsp. *montana* - *Stachys affinis* Bunge - rhizomes
asperulosidic acid	- *Ajuga chamaepitys* (L.) Schreb. - aerial parts
deacetyl-asperulosidic acid	- *Ajuga chamaepitys* (L.) Schreb. - aerial parts - *Ajuga chamaepitys* (L.) Schreb. - roots
5-*O*-β-D-glucopyranosyl-harpagide	- *Ajuga chamaepitys* (L.) Schreb. - aerial parts - *Ajuga chamaepitys* (L.) Schreb. - roots
5-*O*-β-D-glucopyranosyl-8-*O*-acetyl-harpagide	- *Ajuga chamaepitys* (L.) Schreb. - roots
4'-*O*-galactopyranosyl-teuhircoside	- *Stachys alopecuros* (L.) Benth. subsp. *divulsa* (Ten.) Grande
	- *Galeopsis ladanum* subsp. *angustifolia* (Ehrh. ex Hoffm.) Gaudin - *Sideritis montana* L. subsp. *montana*

chlorogenic acid	- *Stachys alopecuros* (L.) Benth. subsp. *divulsa* (Ten.) Grande - *Stachys germanica* subsp. *salviifolia* L. - *Stachys palustris* L. - Hungary - *Stachys palustris* L. - France
β-arbutin	- *Stachys alopecuros* (L.) Benth. subsp. *divulsa* (Ten.) Grande - *Teucrium chamaedrys* L.
methyl-arbutin	- *Sideritis montana* L. subsp. *montana*
D-mannitol	- *Ajuga reptans* L.
glucose	- *Ajuga reptans* L.
fructose	- *Ajuga reptans* L.
galactose	- *Ajuga reptans* L.
sucrose	- *Ajuga reptans* L.
raffinose	- *Ajuga reptans* L.
stachyose	- *Ajuga reptans* L. - *Stachys affinis* Bunge - rhizomes
acetic acid	- *Ajuga reptans* L.
caffeic acid	- *Ajuga reptans* L.
cinnamic acid	- *Melittis melissophyllum* L. subsp. *albida* (Guss.) P.W.Ball
formic acid	- *Ajuga reptans* L.
fumaric acid	- *Ajuga reptans* L.
lactic acid	- *Ajuga reptans* L.
malic acid	- *Ajuga reptans* L.
pyruvic acid	- *Ajuga reptans* L.
quinic acid	- *Galeopsis ladanum* subsp. *angustifolia* (Ehrh. ex Hoffm.) Gaudin
succinic acid	- *Ajuga reptans* L. - *Stachys affinis* Bunge - rhizomes
α-hydroxy-butyric acid	- *Ajuga reptans* L.
γ-amino-butyric acid (GABA)	- *Ajuga reptans* L.
alanine	- *Ajuga reptans* L.
aspartic acid	- *Ajuga reptans* L.
asparagine	- *Ajuga reptans* L.
lysine	- *Ajuga reptans* L.
glutamine	- *Ajuga reptans* L.
threonine	- *Ajuga reptans* L.
tyrosine	- *Ajuga reptans* L.
valine	- *Ajuga reptans* L.

Table 7: Correlation between evidenced compounds and their presence in the studied species

As it can be seen from this table, **harpagide** and **8-*O*-acetyl-harpagide** are the phytochemicals which were evidenced in more species (fifteen for **harpagide** and thirteen for **8-*O*-acetyl-harpagide**, respectively). This fact is not uncommon and unusual since these two compounds are considered to be the main chemotaxonomic markers of the entire Lamiaceae family and for this their occurrence is wide in both Ajugoideae and Lamioideae sub-families.

As expected, *neo*-clerodane terpenoids were evidenced only in the Ajugoideae sub-family even if with an important peculiarity. In fact, their presence (**10-β-hydroxy-teucjaponin B**, **picropolin**, **teupolin I**, **teucrasiatin**, **20-*O*-acetyl-teucrasiatin**) was reported only in the *Teucrium* genus and only in one species (*T. polium* L.). This might mean that within the

Ajugoideae sub-family there might be the co-existence of two different trends. In the first one, *Teucrium polium* L. is inserted and this corresponds to those species which bio-synthesize *neo*-clerodane diterpenes while in the second one, the other studied species of the *Teucrium* genus (*T. chamaedrys* L.) and all the studied species of the *Ajuga* genus (*A. chamaepitys* (L.) Schreb, *A. genevensis* L., *Ajuga reptans* L. and *Ajuga tenorei* C. Presl) are inserted and these do not bio-synthesize *neo*-clerodane diterpenes. Yet, further studies are necessary in order to confirm the validity of this supposition and the botanical and/or ecological reasons of this different behaviour. Moreover, A. *chamaepitys* (L.) Schreb. was studied only for its iridoid content and so no information was collected about other compounds.

In addition, *A. genevensis* L. also proved to be a very strange species from the chemotaxonomic point of view. As a matter of fact, this species did not bio-synthesize not only *neo*-clerodane compounds but also iridoidic compounds, even its chemotaxonomic markers. This is quite unusual since, at least, the occurrence of **ajugoside** and, by extension, of its derivatives, were supposed to be sure since that compound is considered to be the chemotaxonomic marker for excellence of all the *Ajuga* genus (Venditti et al., 2016c) but this did not take place in this case. Instead, in other *Ajuga* species (*A. chamaepitys* (L.) Schreb, *A. reptans* L., *A. tenorei* C. Presl) and also in some Lamioideae species, this compound was reported. Indeed, the latter fact is not surprising since Lamioideae species are, anyway, taxonomically near to the Ajugoideae ones and the mutual occurrence of some compounds is not rare.

In this same context, it's also important to underline the complete absence of iridoids in *Teucrium polium* L., even teucardoside, the chemotaxonomic marker of the entire *Teucrium* genus (Venditti et al., 2017b). Yet, at least, this can be explained by environmental hypothesis. In fact, both the exemplars of this species were collected at quite high altitudes and the absence of iridoids may be due to the non-necessity of these exemplars to bio-synthesize compounds useful for their self-defense against insects and other animals like iridoids are able to do. This hypothesis is corroborated by the fact that *T. chamaedrys* L. which was collected at about 180 m a.s.l., did bio-synthesize iridoids since at that altitude the survival of insects and other animals is very favored.

Nevertheless, many species of both sub-families have also seen the occurrence of particular compounds known as phenyl-ethanoid glycosides such as **verbascoside** and all its derivatives. Actually, these were more abundant in species belonging to the Lamioideae sub-family but this does not have any chemotaxonomic meaning. In fact, phenyl-ethanoid glycosides do not have a chemotaxonomic relevance by themselves since they are well spread phytochemicals.

Yet, these compounds gain chemotaxonomic importance when they are evidenced together with iridoids (Jensen, 1992) because this seems to be limited to particular genera of the Lamiaceae family. In this sense, the presence of **verbascoside** and **poliumoside** in *Teucrium polium* L. is irrelevant since no iridoids were found in that species but the presence of **verbascoside**, **forsythoside b**, **samioside** and **alyssonoside** in *Teucrium chamaedrys* L. is important, since several iridoids were found together with them. It's fundamental to highlight the fact that these phenyl-ethanoid glycosides were found prevalently in species of the Lamioideae sub-family which, in the end, have demonstrated to be richer in iridoids. This is, in fact, one peculiarity of this sub-family and is perfectly in accordance with the modern general chemotaxonomic theory which sees their presence more suitable to this sub-family.

Several flavonoids were evidenced as well but, from this point of view, some expectations were satisfied. In fact, in spite of the fact that no *Ajuga* species showed any presence of any kind of flavonoid, flavonoids, derivatives of apigenin, were evidenced mainly in Ajugoideae species (with the only exception of **apigenin 7-*O*-(6''-*O*-acetyl-β-D-glucopyranoside)** in *Sideritis romana* L.) while flavonoids, derivatives of isoscutellarein and hypolaetin, were evidenced only in Lamioideae species. This different distribution of flavonoids is typical. Moreover, it's also typical the fact that the isoscutellarein and hypolaetin derivatives found in Lamioideae species, owned a particular structure constituted by the typical flavonoidic skeleton to which a dimer of glucose and allose with a further acetylic group on one or both residues, is linked via a glycosidic bond. In fact, compounds with this specific structure have chemotaxonomic relevance because they are considered to be further chemotaxonomic markers of the Lamioideae sub-family given that their occurrence seems to be limited to only few genera such as *Galeopsis*, *Sideritis* and *Stachys* (Venditti et al., 2015a) which is exactly where they were evidenced.

For what concerns the other iridoids, only a couple of differences from what expected were evidenced. The expectations were to find **melittoside** and **monomelittoside** mainly in *Melittis* species and however in Lamioideae species because they are considered to be the specific chemotaxonomic markers of the *Melittis* genus and, in some way, of the Lamioideae sub-family, too (Venditti et al., 2016d). Another expectation was to find **5-*O*-allosyloxy-aucubin** in *Sideritis* species where it plays the role of specific chemotaxonomic marker and this also occurred. Also the presence of diglycosidic iridoids was expected, even if maybe not all the evidenced ones such as **5-*O*-β-D-glucopyranosyl-harpagide** and **5-*O*-β-D-glucopyranosyl-8-*O*-acetyl-harpagide** which, actually, resulted to be newly identified phytochemicals. In fact, these compounds may be considered as additional chemotaxonomic markers of the Lamiaceae

family where they have been evidenced several times before. Lastly, **reptoside** is the chemotaxonomic marker of several genera belonging to the Lamiaceae family.

On the other side, the presence of **asperulosidic acid**, **deacetyl-asperulosidic acid**, **geniposidic acid**, **bartsioside** and **6-deoxy-harpagide** was not expected, at all. All of them have been rarely reported before in Lamiaceae being more characteristics and distinctive of other families. For example, **asperulosidic acid**, **deacetyl-asperulosidic acid** represent the chemotaxonomic markers of Rubiaceae species. **Bartsioside** is the chemotaxonomic marker of Scrophulariaceae and Plantaginaceae. Instead, **geniposidic acid** and **6-deoxy-harpagide** are just rare compounds in the Lamiaceae family itself.

Lastly, all the other compounds are primary metabolites which means that they are essential for the life cycle of the species and for this they are ubiquitous and have no chemotaxonomic relevance.

3.4. Ethno-pharmacology

In the table below (Table 8), all the pharmacological properties associated to every compound are reported. The compounds are listed according to their belonging natural classes and for each pharmacological property a reference is provided.

Compound	Pharmacological properties	References
1-oleoyl-2-linoleoyl-*sn*-glycerol-3-phosphocholine	- hypotensive - antioxidant	(Teres et al., 2008)
1-miristoyl-2-heptadecenoyl-*sn*-glycerol	- antifungal	(Avia and Bélanger, 2001)
1,2-di-palmitoyl-*sn*-glycerol	- antioxidant - selective cytotoxic	(Harada et al., 2002)
pheophorbide a	- photocytotoxic - antiviral - inhibitory on uterine contractions	(Venditti et al., 2017b)
***E*-phytol**	- antiradical - antimicrobial - cytotoxic	(Venditti et al., 2016e)
10-β-hydroxy-teucjaponin B	- none specific reported in literature	-
picropolin	- none specific reported in literature	-
teupolin I	- none specific reported in literature	-
teucrasiatin	- none specific reported in literature	-
20-*O*-acetyl-teucrasiatin	- none specific reported in literature	-
rosmarinic acid	- antioxidant - neuroprotective	(Popov et al., 2013; Coelho et al., 2015; Hasanein and Mahtaj, 2015)
oleanolic acid	- antiproliferative - anti-inflammatory	(Leal et al., 2013; Galipalli et al., 2014; Martìn et al., 2014)
maslinic acid	- antiproliferative	(Leal et al., 2013; Galipalli

	- anti-inflammatory	et al., 2014; Martín et al., 2014)
verbascoside	- antimicrobial, especially against *Staphylococcus aureus*, - anti-inflammatory	(Avila et al., 1999; Speranza et al., 2010)
martynoside	- antiestrogenic - cytotoxic - antioxidant	(Papoutsi et al., 2006; Saracoğlu et al., 1995; Wang et al., 1996)
forsythoside b	- neuroprotective - antiseptic	(Jiang et al., 2010; Jiang et al., 2012)
leucosceptoside A	- antiestrogenic - antioxidant	(Papoutsi et al., 2006; Wang et al., 1996)
samioside	- antioxidant - radical scavenging - antimicrobial	(Kyriakopoulou et al., 2001)
alyssonoside	- radical scavenging	(Heilmann et al., 2000)
echinacoside	- antimicrobial, especially against *Staphylococcus aureus* and *Streptococci* - antioxidant - anti-inflammatory	(Stoll et al., 1950; Wu et al., 2007; Caufin et al., 2014)
poliumoside	- antioxidant - antityrosinase - neuroprotective - antibacterial - DNA polymerases inhibitory	(Venditti et al., 2017c)
stachysoside A	- none specific reported in literature	-
apigenin	- anti-inflammatory - antioxidant - antiallergic - anticancer - antidiabetic - pancreatic-protective - antihyperglycemic	(Lyang et al., 1999; Sarkarizi et al., 2015; Esmaeili and Sadeghi, 2009; Stefkov et al., 2011)
luteolin	- anti-inflammatory - antioxidant - antiallergic - anticancer	(Lyang et al., 1999)
acacetin	- anti-inflammatory - antioxidant - antiallergic - anticancer	(Lyang et al., 1999)
cirsiliol	- relaxant - sedative - hypnotic - pancreatic-protective - antihyperglycemic	(Mustafa et al., 1992; Esmaeili and Sadeghi, 2009; Stefkov et al., 2011; Viola et al., 1997)
cirsimaritin	- anti-inflammatory - antioxidant - antiallergic - anticancer - pancreatic-protective - antihyperglycemic	(Lyang et al., 1999; Esmaeili and Sadeghi, 2009; Stefkov et al., 2011)
cirsilineol	- antioxidant - antibacterial - pancreatic-protective - antihyperglycemic - antidiabetic	(Esmaeili and Sadeghi, 2009; Stefkov et al., 2011; Sarkarizi et al., 2015)
	- antioxidant - antibacterial	(Esmaeili and Sadeghi,

4'-*O*-methyl-luteolin (diosmetin)	- pancreatic-protective - antihyperglycemic - antidiabetic	2009; Stefkov et al., 2011; Sarkarizi et al., 2015)
apigenin 7-*O*-β-glucoside	- anti-inflammatory - antioxidant - antiallergic - anticancer	(Lyang et al., 1999)
apigenin 7-*O*-rutinoside (isorhoifolin)	- antioxidant - antibacterial - pancreatic-protective - antihyperglycemic - antidiabetic	(Esmaeili and Sadeghi, 2009; Stefkov et al., 2011; Sarkarizi et al., 2015)
apigenin 7-*O*-(6''-*O*-acetyl-β-D-glucopyranoside)	- none specific reported in literature	-
7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-β-D-glucopyranosyl]oxy}-5-hydroxy-2-(3-hydroxy-4-methoxy-phenyl)-4*H*-1-benzopyran-4-one	- none specific reported in literature	-
7-{[2-*O*-(6-*O*-acetyl-β-D-glucopyranosyl)-β-D-glucopyranosyl]oxy}-5-hydroxy-2-(3,4-dihydroxyphenyl)-4*H*-1-benzopyran-4-one	- none specific reported in literature	-
7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-β-D-glucopyranosyl]oxy}-5-hydroxy-2-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one	- none specific reported in literature	-
7-{[2-*O*-β-D-allopyranosyl-β-D-glucopyranosyl]oxy}-5-hydroxy-2-(3-methoxy-4-hydroxy-phenyl)-4*H*-1-benzopyran-4-one	- none specific reported in literature	-
7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-β-D-glucopyranosyl]oxy}-5,8-dihydroxy-2-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one	- none specific reported in literature	-
7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-β-D-glucopyranosyl]oxy}-5,8-dihydroxy-2-(4-methoxyphenyl)-4*H*-1-benzopyran-4-one	- none specific reported in literature	-
7-{[2-*O*-β-D-allopyranosyl-(6-*O*-acetyl-β-D-glucopyranosyl)]oxy}-5,8-dihydroxy-2-(3-hydroxy-4-methoxy-phenyl)-4*H*-1-benzopyran-4-one	- none specific reported in literature	-
7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-6-*O*-acetyl-β-D-glucopyranosyl]oxy}-5,8-dihydroxy-2-(3-hydroxy-4-methoxy-phenyl)-4*H*-1-benzopyran-4-one	- none specific reported in literature	-
8-epiloganic acid	- none specific reported in literature	-
ajugoside	- antioxidant	(Erukainure et al., 2014)
reptoside	- antioxidant - anti-inflammatory - antiarthritic	(Venditti et al., 2016b; Gautam et al., 2011; Kaithwas et al., 2012)
	- antioxidant	(Binyu et al., 2013;

harpagide	- antitumor - anti-inflammatory - antibacterial - against osteoporosis	Guarrera, 2005; Konoshima et al., 2000; Chung et al., 2016; Venditti et al., 2016b)
6-deoxy-harpagide	- anti-inflammatory - antiarthritic	(Gautam et al., 2011; Kaithwas et al., 2012)
8-*O*-acetyl-harpagide	- antioxidant - antitumor - anti-inflammatory - antibacterial - antiviral - analgesic - antiarthritis	(Binyu et al., 2013; Guarrera, 2005; Konoshima et al., 2000; Xie et al., 2005; Venditti et al., 2016b)
monomelittoside	- antioxidant - neuroprotective	(Tundis et al., 2014)
melittoside	- antioxidant - anti-inflammatory - neuroprotective	(Samuelsen, 2000; Tundis et al., 2014)
allobetonicoside	- none specific reported in literature	-
virginioside	- none specific reported in literature	-
geniposidic acid	- lowers blood pressure - repairs soft tissue injury - anti-inflammatory - cancer preventive - against osteoporosis - antiatherosclerotic - promotes collagen synthesis	(Gao et al., 2015; Li et al., 1998)
bartsioside	- anti-inflammatory	(Venditti et al., 2016e)
6-*O*-acetyl-ajugol	- none specific reported in literature	-
5-*O*-allosyloxy-aucubin	- antioxidant - anti-inflammatory	(Samuelsen, 2000)
asperulosidic acid	- inhibitory - antimicrobial	(Ling et al., 2003; West et al., 2012)
deacetyl-asperulosidic acid	- antimicrobial	(West et al., 2012)
5-*O*-β-D-glucopyranosyl-harpagide	- antioxidant	(Venditti et al., 2016b)
5-*O*-β-D-glucopyranosyl-8-*O*-acetyl-harpagide	- antioxidant	(Venditti et al., 2016b)
4'-*O*-galactopyranosyl-teuhircoside	- none specific reported in literature	-
chlorogenic acid	- antioxidant - topical wound healing - antiviral - antiproliferative - inhibits carcinogenesis - reduces the risk of cardiovascular diseases	(Venditti et al., 2017a; Cho et al., 2010)
β-arbutin	- antibacterial - tyrosinase-inhibiting	(Yarnell et al., 2002; Sugimoto et al., 2005)
methyl-arbutin	- antibacterial - anti-inflammatory	(Schindler et al., 2002; Dos Santos et al., 2006)
D-mannitol	- reduces intracranial and intraocular pressure - against specific kidney failures - preserves renal function - laxative for children	(AHFS Drug Information, 2005; Baxter Healthcare, 2005)
glucose	- favors brain activity	-
fructose	- none reported in literature	-
galactose	- none reported in literature	-

sucrose	- antioxidant	(Balbani et al., 2006)
raffinose	- none reported in literature	-
stachyose	- antibacterial	(Smith et al., 2002)
acetic acid	- antibacterial, especially against skin infections	(Nagoba et al., 2013)
caffeic acid	- none reported in literature	-
cinnamic acid	- antifungal - anticancer - cancer-preventive - antileukaemic	(Shi et al., 2005; Niero and Machado-Santelli, 2013; Yao et al., 2012; Zhang et al., 2014)
formic acid	- antibacterial - cures warts	(Bhat et al., 2001; Reutemann and Kieczka, 2002)
fumaric acid	- antipsoriasis	(Balak et al., 2016)
D-lactic acid	- none reported in literature	-
malic acid	- painkilling - exfoliating	(Kornhauser et al., 2010)
pyruvic acid	- increases cardiac function	(Hermann et al., 1961)
quinic acid	- antioxidant - astringent	(Pero et al., 2009)
succinic acid	- none reported in literature	-
α-hydroxy-butyric acid	- none reported in literature	-
γ-amino-butyric acid (GABA)	- none reported in literature	-
alanine	- none reported in literature	-
aspartic acid	- none reported in literature	-
asparagine	- none reported in literature	-
lysine	- none reported in literature	-
glutamine	- none reported in literature	-
threonine	- none reported in literature	-
tyrosine	- none reported in literature	-
valine	- none reported in literature	-

Table 8: Pharmacological properties associated to every evidenced phytochemical

As it can be seen from this table, almost all the compounds evidenced during the phytochemical studies of each plant, are endowed with pharmacological properties.

The only exceptions are represented by **raffinose**, **succinic acid**, **α-hydroxy-butyric acid**, **γ-amino-butyric acid (GABA)**, **alanine**, **aspartic acid**, **asparagine**, **lysine**, **glutamine**, **threonine**, **tyrosine** and **valine** for which no pharmacological property is reported in literature.

Nevertheless, for some compounds such as **10-β-hydroxyteucjaponin B**, **picropolin**, **teupolin I**, **teucrasiatin**, **20-*O*-acetyl-teucrasiatin**, **stachysoside A**, **7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-β-D-glucopyranosyl]oxy}-5-hydroxy-2-(3-hydroxy-4-methoxyphenyl)-4*H*-1-benzopyran-4-one**, **7-{[2-*O*-(6-*O*-acetyl-β-D-glucopyranosyl)-β-D-glucopyranosyl]oxy}-5-hydroxy-2-(3,4-dihydroxyphenyl)-4*H*-1-benzopyran-4-one**, **7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-β-D-glucopyranosyl]oxy}-5-hydroxy-2-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one**, **7-{[2-*O*-β-D-allopyranosyl-β-D-**

glucopyranosyl]oxy}-5-hydroxy-2-(3-methoxy-4-hydroxy-phenyl)-4*H*-1-benzopyran-4-one, **7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-β-D-glucopyranosyl]oxy}-5,8-dihydroxy-2-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one**, **7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-β-D-glucopyranosyl]oxy}-5,8-dihydroxy-2-(4-methoxyphenyl)-4*H*-1-benzopyran-4-one**, **7-{[2-*O*-β-D-allopyranosyl-(6-*O*-acetyl-β-D-glucopyranosyl)]oxy}-5,8-dihydroxy-2-(3-hydroxy-4-methoxy-phenyl)-4*H*-1-benzopyran-4-one**, **7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-6-*O*-acetyl-β-D-glucopyranosyl]oxy}-5,8-dihydroxy-2-(3-hydroxy-4-methoxy-phenyl)-4*H*-1-benzopyran-4-one**, **8-epiloganic acid**, **allobetonicoside**, **virginioside**, **6-*O*-acetyl-ajugol** and **4'-*O*-galactopyranosyl-teuhircoside** no specific pharmacological properties are reported in literature. Yet, some considerations can be made about this fact according to their structure. For what concerns **10-β-hydroxy-teucjaponin B**, **picropolin**, **teupolin I**, **teucrasiatin**, **20-*O*-acetyl-teucrasiatin**, these are *neo*-clerodane diterpene compounds and literature data report that all *neo*-clerodane diterpenoids have antifeedant as well as hepatotoxic properties (Bruno et al., 2002; Pacifico et al., 2012). As for **stachysoside A** which is a phenyl-ethanoid glycosides, these compounds are well known to exhibit antioxidant properties (Venditti et al., 2017a). **7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-β-D-glucopyranosyl]oxy}-5-hydroxy-2-(3-hydroxy-4-methoxy-phenyl)-4*H*-1-benzopyran-4-one**, **7-{[2-*O*-(6-*O*-acetyl-β-D-glucopyranosyl)-β-D-glucopyranosyl]oxy}-5-hydroxy-2-(3,4-dihydroxyphenyl)-4*H*-1-benzopyran-4-one**, **7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-β-D-glucopyranosyl]oxy}-5-hydroxy-2-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one**, **7-{[2-*O*-β-D-allopyranosyl-β-D-glucopyranosyl]oxy}-5-hydroxy-2-(3-methoxy-4-hydroxy-phenyl)-4*H*-1-benzopyran-4-one**, **7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-β-D-glucopyranosyl]oxy}-5,8-dihydroxy-2-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one**, **7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-β-D-glucopyranosyl]oxy}-5,8-dihydroxy-2-(4-methoxyphenyl)-4*H*-1-benzopyran-4-one**, **7-{[2-*O*-β-D-allopyranosyl-(6-*O*-acetyl-β-D-glucopyranosyl)]oxy}-5,8-dihydroxy-2-(3-hydroxy-4-methoxy-phenyl)-4*H*-1-benzopyran-4-one** and **7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-6-*O*-acetyl-β-D-glucopyranosyl]oxy}-5,8-dihydroxy-2-(3-hydroxy-4-methoxy-phenyl)-4*H*-1-benzopyran-4-one** are hypolaetin and isoscutellarein derivatives which are reported to exert strong antioxidant and anticholinesterase activities (Venditti et al., 2016e). Lastly, **8-epiloganic acid**, **allobetonicoside**, **virginioside**, **6-*O*-acetyl-ajugol** and **4'-*O*-galactopyranosyl-teuhircoside** are iridoids and these are known to possess anti-inflammatory effects (Bianco et al., 1990). According to these premises, it's not awkward to think that all these compounds may indeed present, at least, these same properties associated

to each natural class of compounds even if further pharmacological studies are necessary to verify this and also if they present other specific properties.

From the ethno-pharmacological standpoint, the presence of all these compounds is very important and some of them may justify and explain the ethno-pharmacological uses of the species in which they have been evidenced.

This is true for *Ajuga chamaepitys* (L.) Schreb., *Ajuga reptans* L., *Galeopsis ladanum* subsp. *angustifolia* (Ehrh. ex Hoffm.) Gaudin, *Melittis melissophyllum* L. subsp. *melissophyllum*, *Sideritis montana* L. subsp. *montana*, *Sideritis romana* L., *Stachys affinis* Bunge, *Stachys alopecuros* (L.) Benth. subsp. *divulsa* (Ten.) Grande, *Stachys annua* L., *Stachys germanica* subsp. *salviifolia* L. (Ten.) Gams., *Stachys palustris* L.

On the other hand, a particular case is given by *Teucrium polium* L. In fact, if on one side, the presence of some compounds has the same value as described before, on the other one, the further presence of other compounds justifies and explains why this species is starting to be abandoned as ethno-pharmacological remedy. All the matter lies in which of these two opposite effects is the most important but several deeper studies in this sense are necessary to answer this even if some conclusions have already been made.

Another strange case is represented by *Teucrium chamaedrys* L. Just like many other species belonging to the Ajugoideae sub-family, this species is no longer used in the ethno-pharmacological fields due to the possible presence of particular compounds, the *neo*-clerodane diterpenes with strong anti-beneficial effects. Yet, during the phytochemical study performed by us on this species, none of these compounds was evidenced and nothing explains and justifies its non current employment in this sense. It is possible that this condition is only a particular case of the studied specimen maybe due to the growth habitat or also due to the period of collection of the plant itself and for this further phytochemical studies on the entire species are absolutely necessary. Anyway, this work suggests that no judgement based on preliminary results, reported literature and/or personal thoughts should be made in order to avoid the loss of potential good effects deriving from natural sources.

It is also important the highlight the situations of *Ajuga genevensis* L. and of *Ajuga tenorei* (C.) Presl and *Melittis melissophyllum* L. subsp. *albida* (Guss.) P.W.Ball. For what concerns the former one, the compounds evidenced by the phytochemical study on the species do not justify and explain its current ethno-pharmacological employments but rather may suggest new ones such as anti-inflammatory and antioxidant because of the fact that the evidenced compounds exert these pharmacological properties. Yet, it is possible, also in this case, that this condition is only temporary for the same previous explanations and for this, other

pharmacological studies are necessary to verify this hypothesis of employment also because the total phytocomplex may show different results from the single compounds composing it.
Instead, for *Ajuga tenorei* (C.) Presl and *Melittis melissophyllum* L. subsp. *albida* (Guss.) P.W.Ball, no ethno-pharmacological employments are reported in literature and this might be the right occasion to eventually suggest some, especially as antioxidant, anti-inflammatory and anticancer but the same speech presented for the possible technical problems of use of *Ajuga genevensis* L. is also valid for these species. For this case, the suggestion is to never stop at the actual knowledge because there are many things, especially in nature, which have not been discovered and studied, yet.

3.5. Nutraceutics

On the other hand, in the table below (Table 9), all the nutraceutical properties associated to every compound are reported. The compounds are listed according to their belonging natural classes and for each nutraceutical property a reference is provided.
Actually a pure nutraceutical survey on all the studied species was not performed except for *Ajuga reptans* L. since, in all the other cases, this was not the primary aim of the study.

Compound	Nutraceutical properties	References
1-oleoyl-2-linoleoyl-*sn*-glycerol-3-phosphocholine	- physiologic	(Teres et al., 2008)
1-miristoyl-2-heptadecenoyl-*sn*-glycerol	- reduces the total level of cholesterol in blood	(Avia and Bélanger, 2001)
1,2-di-palmitoyl-*sn*-glycerol	- none reported in literature	-
pheophorbide a	- none reported in literature	-
***E*-phytol**	- none reported in literature	-
10-β-hydroxy-teucjaponin B	- none reported in literature	-
picropolin	- none reported in literature	-
teupolin I	- none reported in literature	-
teucrasiatin	- none reported in literature	-
20-*O*-acetyl-teucrasiatin	- none reported in literature	-
rosmarinic acid	- none reported in literature	-
oleanolic acid	- none reported in literature	-
maslinic acid	- none reported in literature	-
verbascoside	- none reported in literature	-
martynoside	- none reported in literature	-
forsythoside b	- none reported in literature	-
leucosceptoside A	- none reported in literature	-
samioside	- none reported in literature	-
alyssonoside	- none reported in literature	-
echinacoside	- none reported in literature	-
poliumoside	- none reported in literature	-
stachysoside A	- none reported in literature	-
apigenin	- none reported in literature	-

luteolin	- none reported in literature	-
acacetin	- none reported in literature	-
cirsiliol	- none reported in literature	-
cirsimaritin	- none reported in literature	-
cirsilineol	- none reported in literature	-
4'-*O*-methyl-luteolin (diosmetin)	- none reported in literature	-
apigenin 7-*O*-β-glucoside	- none reported in literature	-
apigenin 7-*O*-rutinoside (isorhoifolin)	- none reported in literature	-
apigenin 7-*O*-(6''-*O*-acetyl-β-D-glucopyranoside)	- none reported in literature	-
7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-β-D-glucopyranosyl]oxy}-5-hydroxy-2-(3-hydroxy-4-methoxyphenyl)-4*H*-1-benzopyran-4-one	- none reported in literature	-
7-{[2-*O*-(6-*O*-acetyl-β-D-glucopyranosyl)-β-D-glucopyranosyl]oxy}-5-hydroxy-2-(3,4-dihydroxyphenyl)-4*H*-1-benzopyran-4-one	- none reported in literature	-
7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-β-D-glucopyranosyl]oxy}-5-hydroxy-2-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one	- none reported in literature	-
7-{[2-*O*-β-D-allopyranosyl-β-D-glucopyranosyl]oxy}-5-hydroxy-2-(3-methoxy-4-hydroxy-phenyl)-4*H*-1-benzopyran-4-one	- none reported in literature	-
7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-β-D-glucopyranosyl]oxy}-5,8-dihydroxy-2-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one	- none reported in literature	-
7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-β-D-glucopyranosyl]oxy}-5,8-dihydroxy-2-(4-methoxyphenyl)-4*H*-1-benzopyran-4-one	- none reported in literature	-
7-{[2-*O*-β-D-allopyranosyl-(6-*O*-acetyl-β-D-glucopyranosyl)]oxy}-5,8-dihydroxy-2-(3-hydroxy-4-methoxy-phenyl)-4*H*-1-benzopyran-4-one	- none reported in literature	-
7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-6-*O*-acetyl-β-D-glucopyranosyl]oxy}-5,8-dihydroxy-2-(3-hydroxy-4-methoxy-phenyl)-4*H*-1-benzopyran-4-one	- none reported in literature	-
8-epiloganic acid	- none reported in literature	-
ajugoside	- none reported in literature	-
reptoside	- none reported in literature	-
harpagide	- none reported in literature	-
6-deoxy-harpagide	- none reported in literature	-
8-*O*-acetyl-harpagide	- none reported in literature	-
monomelittoside	- none reported in literature	-
melittoside	- none reported in literature	-

allobetonicoside	- none reported in literature	-
virginioside	- none reported in literature	-
geniposidic acid	- none reported in literature	-
bartsioside	- none reported in literature	-
6-*O*-acetyl-ajugol	- none reported in literature	-
5-*O*-allosyloxy-aucubin	- none reported in literature	-
asperulosidic acid	- none reported in literature	-
deacetyl-asperulosidic acid	- none reported in literature	-
5-*O*-β-D-glucopyranosyl-harpagide	- none reported in literature	-
5-*O*-β-D-glucopyranosyl-8-*O*-acetyl-harpagide	- none reported in literature	-
4′-*O*-galactopyranosyl-teuhircoside	- none reported in literature	-
chlorogenic acid	- improves the metabolism of glucose - antiobesity	(Venditti et al., 2017a; Cho et al., 2010)
β-arbutin	- none reported in literature	-
methyl-arbutin	- none reported in literature	-
D-mannitol	- increases toxin elimination	(AHFS Drug Information, 2005; Baxter Healthcare, 2005)
glucose	- against hypoglycemia	(Caballero et al., 2016)
fructose	- contrasts diabetes mellitus and insulin resistance	(Caballero et al., 2016)
galactose	- reduces glucose concentration in blood	(De Smet et al., 2009)
sucrose	- sweetening	(Caballero et al., 2016)
raffinose	- anticaloric	(Caballero et al., 2016)
stachyose	- lowers plasma glucose levels	(Zhang et al., 2004)
acetic acid	- additive	(Caballero et al., 2016)
caffeic acid	- antioxidant - anti-inflammatory - α-glucosidase inhibitory - cytotoxic	(Matsuno, 1992; Olthof et al., 2001; Venditti et al., 2015b)
cinnamic acid	- none reported in literature	-
formic acid	- none reported in literature	-
fumaric acid	- antioxidant	(Kornhauser et al., 2010)
D-lactic acid	- flavoring	(Kornhauser et al., 2010)
malic acid	- energy boost	(Kornhauser et al., 2010)
pyruvic acid	- improves metabolism	(Jaimes, 2015)
quinic acid	- none reported in literature	-
succinic acid	- excipient	(Zeikus et al., 1999)
α-hydroxy-butyric acid	- none reported in literature	-
γ-amino-butyric acid (GABA)	- relaxing - antianxiety - anticonvulsive	(Chapouthier and Venault, 2001; Foster and Kemp, 2006)
alanine	- provides energy - synthesizes proteins	(Brennan et al., 2002)
aspartic acid	- none specific reported in literature	-
asparagine	- none specific reported in literature	-
lysine	- none specific reported in literature	-
glutamine	- none specific reported in literature	-
threonine	- none specific reported in literature	-
tyrosine	- none specific reported in literature	-
valine	- none specific reported in literature	-

Table 9: Nutraceutical properties associated to every evidenced phytochemical

As it can be seen from the table above, only some compounds present nutraceutical properties. In particular, these compounds are the glycerides, the saccharides and the organic acids with the further adding of chlorogenic acid.

The nutraceutical effects that these compounds are able to exert are very diverse and cover very different aspects ranging from mere food additives to real pharmacological activities among which the cytotoxic one (for **caffeic acid**) is probably the most important.

Anyway, for other compounds, it's not less interesting to note that the real pharmacological activities cover the antioxidant (**caffeic acid** and **fumaric acid**), the anticonvulsive (**γ-amino-butyric acid (GABA)**) and the antitoxin accumulation (**D-mannitol**) ones, thus making them quite relevant and fundamental from this point of view.

Nevertheless, a particular case is represented by the amino acids. Nothing specific about the nutraceutical properties of these compounds is reported in literature but they do exert them only according to their nature. In fact, they all possess at least physiological properties since they are very important for providing energy and for the production of proteins which are both extremely necessary for the correct development and functioning of animal and vegetal organisms.

All these compounds may explain some of the ethno-pharmacological properties associated to every studied species maybe playing a synergic role with the other identified phytochemicals in the total effect of the phytocomplex of the plant. Yet, further and deeper studies are necessary to establish their real function and mechanism of action in this.

3.5. Phytochemical evolution

The basilar concept of phytochemical evolution is that the more oxidized is the compound, the more evolved is that biogenetic pathway and, by consequence, the more evolved is that plant species.

For this reason, through the study of the secondary metabolites pattern of a plant species, it's possible to have an idea of the evolution of the plant itself.

In this context, this concept was applied to iridoids and to the particular sub-group of flavonoids identified in Lamiaceae (flavones, 2-phenylchromen-4-one).

Both of these compounds represent the most common compounds of the family and then they can provide more generic informations.

In order to make this particular study, the parameters known as mean oxidation value (mOV) and mean Sporne index (mSI) must be taken into account.

For what concerns the first one, it is calculated basing on a simple math formula which is the following, elaborated by Kaplant and Gottlieb (1982):

$$(mOV) = [\Sigma\alpha(OV\alpha \cdot N\alpha)]/Ntot$$

with: $\alpha = 0$; $\alpha > 0$,

where: $OV\alpha$ is the oxidation value of a compound of the same class having the value α;

$N\alpha$ is the number of compounds of the same class in the genus having OV with the same value α;

Ntot is the number of total compounds of the same class present in the genus

This is valid for both classes of compounds studied.

For what concerns iridoids, the oxidation value (OV) is calculated by assigning additive point values for each functional group present in a certain iridoid, according to their increased oxidation.

In particular, the additive point values for each functional group are the following:

- 1 point for each ring, double bond, oxy-function
- 2 points for an oxo-function
- 3 points for each carboxyl
- 4 point for the lack of either C-10 or C-11
- 6 points for the lack of both C-10 and C-11

In this calculation, one important assumption is done:

- the presence of OGly and OAc is equal to the presence of OH

The count of the point values for each iridoidic structure is made starting from a base one which corresponds to the iridotrial skeleton (Figure 55).

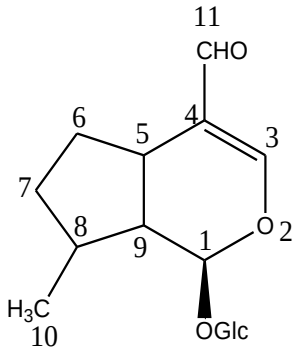


Figure 55: Structure of the iridotrial skeleton

To this base compound, a total oxidation value of 0 is assigned.

At this point, it was possible to calculate the oxidation value of each iridoid identified in this study which were: **ajugoside**, **6-*O*-acetyl-ajugol**, **reptoside**, **harpagide**, **8-*O*-acetyl-harpagide**, **6-deoxy-harpagide**, **5-*O*-β-D-glucopyranosyl-harpagide**, **5-*O*-β-D-glucopyranosyl-8-*O*-acetyl-harpagide**, **asperulosidic acid**, **deacetyl-asperulosidic acid**, **geniposidic acid**, **monomelittoside**, **melittoside**, **5-*O*-allosyloxy-aucubin**, **4'-*O*-galactopyranosyl-teuhircoside**, **bartsioside**, **allobetonicoside**, **virginioside** and **8-epiloganic acid**.

In the table below (Table 10), the oxidation values of these iridoids, calculated as previously described in this paragraph, are reported.

Compound	OV
8-epiloganic acid	5
ajugoside	7
reptoside	7
6-deoxy-harpagide	7
bartsioside	7
6-*O*-acetyl-ajugol	7
harpagide	8
8-*O*-acetyl-harpagide	8
5-*O*-β-D-glucopyranosyl-harpagide	8
5-*O*-β-D-glucopyranosyl-8-*O*-acetyl-harpagide	8
5-*O*-allosyloxy-aucubin	8

monomelittoside	9
melittoside	9
allobetonicoside	9
virginioside	9
4'-*O*-galactopyranosyl-teuhircoside	9
geniposidic acid	10
asperulosidic acid	11
deacetyl-asperulosidic acid	11

Table 10: Calculation of the Oxidation Values (OV) for every evidenced iridoid

The validity and exactness of this method of calculation modified by us starting from an idea of Kaplant and Gottlieb (1982) and widely modified by us, is incontrovertible since **8-epiloganic acid** presented the lowest oxidation value whereas **asperulosidic acid** and **deacetyl-asperulosidic acid** presented the highest ones.
In fact, **8-epiloganic acid** is considered to be the precursor of all the kind of iridoids found in the Lamiaceae family and so it must have a low oxidation value. On the other hand, **asperulosidic acid** and **deacetyl-asperulosidic acid**, which present in their structure several oxidized functional groups, must have a high oxidation value.

Also for the flavonoids of the flavones sub-group, the oxidation value is calculated by assigning additive point values as suggested by Gottlieb et al., (1982) and slightly modified by us.
Yet, these additive point values regard, this time, only the further presence, in certain positions of their structure, of hydroxyl groups which have 1 point value.

Also in this case one important assumption is done:
- the presence of OGly, OAc and OMe is equal to the presence of OH

The base structure for flavonoids is the following:

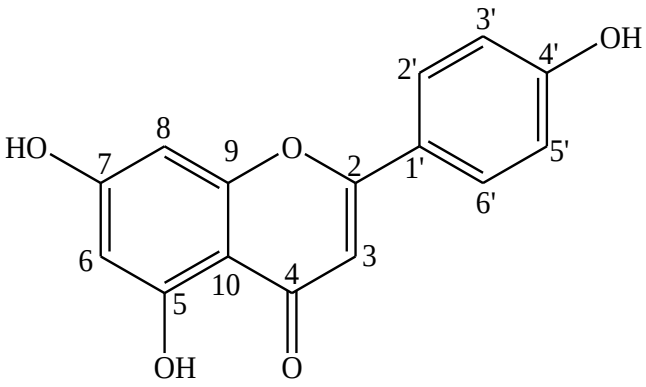


Figure 56: Structure of the base flavonoidic compound

To this base compound a total oxidation value of 0 is assigned.

At this point, it was possible to calculate the oxidation value of each flavonoid identified in this study which were: **apigenin**, **luteolin**, **acacetin**, **cirsiliol**, **cirsimaritin**, **cirsilineol**, **4'-*O*-methyl-luteolin (diosmetin)**, **apigenin 7-*O*-β-glucoside**, **apigenin 7-*O*-rutinoside (isorhoifolin)**, **apigenin 7-*O*-(6''-*O*-acetyl-β-D-glucopyranoside)**, **7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-β-D-glucopyranosyl]oxy}-5-hydroxy-2-(3-hydroxy-4-methoxy-phenyl)-4*H*-1-benzopyran-4-one**, **7-{[2-*O*-(6-*O*-acetyl-β-D-glucopyranosyl)-β-D-glucopyranosyl]oxy}-5-hydroxy-2-(3,4-dihydroxyphenyl)-4*H*-1-benzopyran-4-one**, **7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-β-D-glucopyranosyl]oxy}-5-hydroxy-2-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one**, **7-{[2-*O*-β-D-allopyranosyl-β-D-glucopyranosyl]oxy}-5-hydroxy-2-(3-methoxy-4-hydroxy-phenyl)-4*H*-1-benzopyran-4-one**, **7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-β-D-glucopyranosyl]oxy}-5,8-dihydroxy-2-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one**, **7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-β-D-glucopyranosyl]oxy}-5,8-dihydroxy-2-(4-methoxyphenyl)-4*H*-1-benzopyran-4-one**, **7-{[2-*O*-β-D-allopyranosyl-(6-*O*-acetyl-β-D-glucopyranosyl)]oxy}-5,8-dihydroxy-2-(3-hydroxy-4-methoxy-phenyl)-4*H*-1-benzopyran-4-one**, and **7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-6-*O*-acetyl-β-D-glucopyranosyl]oxy}-5,8-dihydroxy-2-(3-hydroxy-4-methoxy-phenyl)-4*H*-1-benzopyran-4-one**

In the table below (Table 11), the oxidation values of these flavonoids, calculated as previously described in this paragraph, are reported.

Compound	OV
apigenin	1
luteolin	2
acacetin	1
cirsiliol	3
cirsimaritin	2
cirsilineol	3
4'-*O*-methyl-luteolin (diosmetin)	2
apigenin 7-*O*-β-glucoside	1
apigenin 7-*O*-rutinoside (isorhoifolin)	1
apigenin 7-*O*-(6''-*O*-acetyl-β-D-glucopyranoside)	1
7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-β-D-glucopyranosyl]oxy}-5-hydroxy-2-(3-hydroxy-4-methoxy-phenyl)-4*H*-1-benzopyran-4-one	2
7-{[2-*O*-(6-*O*-acetyl-β-D-glucopyranosyl)-β-D-glucopyranosyl]oxy}-5-hydroxy-2-(3,4-dihydroxyphenyl)-4*H*-1-benzopyran-4-one	2
7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-β-D-glucopyranosyl]oxy}-5-hydroxy-2-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one	1
7-{[2-*O*-β-D-allopyranosyl-β-D-glucopyranosyl]oxy}-5-hydroxy-2-(3-methoxy-4-hydroxy-phenyl)-4*H*-1-benzopyran-4-one	2
7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-β-D-glucopyranosyl]oxy}-5,8-dihydroxy-2-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one	2
7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-β-D-glucopyranosyl]oxy}-5,8-dihydroxy-2-(4-methoxyphenyl)-4*H*-1-benzopyran-4-one	2
7-{[2-*O*-β-D-allopyranosyl-(6-*O*-acetyl-β-D-glucopyranosyl)]oxy}-5,8-dihydroxy-2-(3-hydroxy-4-methoxy-phenyl)-4*H*-1-benzopyran-4-one	3
7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-6-*O*-acetyl-β-D-glucopyranosyl]oxy}-5,8-dihydroxy-2-(3-hydroxy-4-methoxy-phenyl)-4*H*-1-benzopyran-4-one	3

Table 11: Calculation of the Oxidation Values (OV) for every evidenced flavonoid

Also in this case, the validity of the results is incontrovertible since apigenin and its derivatives presented the lowest oxidation values while hypolaetin and its derivatives had

medium oxidation values and isoscutellarein and its derivatives had the highest oxidation values.

On the other side, the mean Sporne index (mSI) is a parameter which gives informations about the evolution of families by a pure morphological standpoint and was elaborated by Sporne (1980).

It is valid for all species and is calculated according to this math formula:

$$mSI=(SI_{\alpha}/M)\cdot 100$$

where: SI_{α} is the Sporne Index calculated for each species;
M is the maximum value of the Sporne Index possible for that same species

The Sporne index (SI) is based on 30 morphological characters which are listed in the table below (Table 12):

1) Woody habit	16) Stamens numerous
2) Vessel ends scalariform	17) Anther-tapetum parietal
3) Vessel sides scalariform	18) Pollen binucleate
4) Parenchyma apotracheal	19) Pollen pauci-aperturate
5) Rays heterogeneous	20) Carpels numerous
6) Spiral phyllotaxy	21) More than one seed per carpel
7) Leaf margin toothed	22) Ovules anatropous
8) Stipules present	23) Two integuments
9) Leaves glandular	24) Integument bundles
10) Flowers actinomorphic	25) Aril present
11) Flowers unisexual	26) Ovules crassinucellate
12) Perianth hypogynous	27) Endosperm nuclear
13) Petals or tepals imbricate	28) Seeds albuminous
14) Petals or tepals numerous	29) Leucanthocyanins present
15) Petals free	30) Ellagitannins present

Table 12: List of the morphological characters for the calculation of the Sporne Index (SI)

For each character, it is given:

- the symbol (+) if the character is present
- the symbol (–) if the character is absent

In the tables below (Tables 13, 14, 15) the specific presence and/or absence of the thirty morphological characters for each studied plant, is reported.

Three tables are present. The first one contains species belonging to Ajugoideae sub-family while the other two contain species belonging to the Lamioideae sub-family.

Morphological characters	Ajugoideae					
	Species					
	Ajuga chamaepitys (L.) Schreb	*Ajuga genevensis* L.	*Ajuga reptans* L.	*Ajuga tenorei* C. Presl	*Teucrium chamaedrys* L.	*Teucrium polium* L.
Woody habit	-	-	-	-	+	+
Vessel ends scalariform	-	-	-	-	-	-
Vessel sides scalariform	-	-	-	-	-	-
Parenchyma apotracheal	-	-	-	-	-	-
Rays heterogeneous	+	+	+	+	+	+
Spiral phyllotaxy	-	-	-	-	-	-
Leaf margin toothed	+	+	+	+	+	+
Stipules present	-	-	-	-	-	-
Leaves glandular	+	+	+	+	+	+
Flowers actinomorphic	-	-	-	-	-	-
Flowers unisexual	-	-	-	-	-	-
Perianth hypogynous	+	+	+	+	+	+
Petals or tepals imbricate	-	-	-	-	-	-
Petals or tepals numerous	-	-	-	-	-	-
Petals free	-	-	-	-	-	-
Stamens numerous	-	-	-	-	-	-
Anther-tapetum parietal	+	+	+	+	+	+
Pollen binucleate	+	+	+	+	+	+
Pollen pauci-aperturate	+	+	+	+	+	+
Carpels numerous	-	-	-	-	-	-
More than one seed per carpel	-	-	-	-	-	-
Ovules anatropous	+	+	+	+	+	+
Two integuments	-	-	-	-	-	-
Integument bundles	-	-	-	-	-	-
Aril present	-	-	-	-	-	-
Ovules crassinucellate	-	-	-	-	-	-
Endosperm nuclear	-	-	-	-	-	-
Seeds albuminous	+	+	+	+	+	+
Leucanthocyanins present	-	-	-	-	-	-
Ellagitannins present	-	-	-	-	-	-
Legend: + = present; - = absent						

Table 13: Presence or absence of the Sporne morphological characters in the studied Ajugoideae species

Morphological characters	Lamioideae (1)				
	Species				
	Galeopsis ladanum subsp. *angustifolia* (Ehrh. ex Hoffm.) Gaudin	*Melittis melissophyllum* subsp. *albida* (Guss.) P.W.Ball	*Melittis melissophyllum* L. subsp. *melissophyllum*	*Sideritis montana* L. subsp. *montana*	*Sideritis romana* L.
Woody habit	-	-	-	-	-
Vessel ends scalariform	-	-	-	-	-
Vessel sides scalariform	-	-	-	-	-
Parenchyma apotracheal	-	-	-	-	-
Rays heterogeneous	+	+	+	+	+
Spiral phyllotaxy	-	-	-	-	-
Leaf margin toothed	+	+	+	+	+
Stipules present	-	-	-	-	-
Leaves glandular	+	+	+	+	+
Flowers actinomorphic	-	-	-	-	-
Flowers unisexual	-	-	-	-	-
Perianth hypogynous	+	+	+	+	+
Petals or tepals imbricate	-	-	-	-	-
Petals or tepals numerous	-	-	-	-	-
Petals free	-	-	-	-	-
Stamens numerous	-	-	-	-	-
Anther-tapetum parietal	+	+	+	+	+
Pollen binucleate	+	+	+	+	+
Pollen pauci-aperturate	+	+	+	-	-
Carpels numerous	-	-	-	-	-
More than one seed per carpel	-	-	-	-	-
Ovules anatropous	+	+	+	+	+
Two integuments	-	-	-	-	-
Integument bundles	-	-	-	-	-
Aril present	-	-	-	-	-
Ovules crassinucellate	-	-	-	-	-
Endosperm nuclear	-	-	-	-	-
Seeds albuminous	+	+	+	+	+
Leucanthocyanins present	-	-	-	-	-
Ellagitannins present	-	-	-	-	-

Table 14: Presence or absence of the Sporne morphological characters in the studied Lamioideae species - part one

Morphological characters	Lamioideae (2)				
	Species				
	Stachys affinis Bunge	*Stachys alopecuros* (L.) Benth. subsp. *divulsa* (Ten.) Grande	*Stachys annua* L.	*Stachys germanica* subsp. *salviifolia* L. (Ten.) Gams.	*Stachys palustris* L.
Woody habit	-	-	-	-	-
Vessel ends scalariform	-	-	-	-	-
Vessel sides scalariform	-	-	-	-	-
Parenchyma apotracheal	-	-	-	-	-
Rays heterogeneous	+	+	+	+	+
Spiral phyllotaxy	-	-	-	-	-
Leaf margin toothed	+	+	+	+	+
Stipules present	-	-	-	-	-
Leaves glandular	+	+	+	+	+
Flowers actinomorphic	-	-	-	-	-
Flowers unisexual	-	-	-	-	-
Perianth hypogynous	+	+	+	+	+
Petals or tepals imbricate	-	-	-	-	-
Petals or tepals numerous	-	-	-	-	-
Petals free	-	-	-	-	-
Stamens numerous	-	-	-	-	-
Anther-tapetum parietal	+	+	+	+	+
Pollen binucleate	+	+	+	+	+
Pollen pauci-aperturate	+	+	+	+	+
Carpels numerous	-	-	-	-	-
More than one seed per carpel	-	-	-	-	-
Ovules anatropous	+	+	+	+	+
Two integuments	-	-	-	-	-
Integument bundles	-	-	-	-	-
Aril present	-	-	-	-	-
Ovules crassinucellate	-	-	-	-	-
Endosperm nuclear	-	-	-	-	-
Seeds albuminous	+	+	+	+	+
Leucanthocyanins present	-	-	-	-	-
Ellagitannins present	-	-	-	-	-

Table 15: Presence or absence of the Sporne morphological characters in the studied Lamioideae species - part two

At this point, it was possible to calculate the Sporne index (SI) for each studied plant which is calculated according to this math formula:

$$SI=\Sigma_X(\text{symbol (-)})$$

where: Σ_X(symbol (-)) is the summatory of the (-) values relative to each species; each symbol (-) corresponds to one mark

The SI values for each studied species are reported in the table below (Table 16):

Species	Sporne Index (SI)
Ajuga chamaepitys (L.) Schreb	21
Ajuga genevensis L.	21
Ajuga reptans L.	21
Ajuga tenorei C. Presl	21
Galeopsis ladanum subsp. *angustifolia* (Ehrh. ex Hoffm.) Gaudin	21
Melittis melissophyllum subsp. *albida* (Guss.) P.W.Ball	21
Melittis melissophyllum L. subsp. *melissophyllum*	21
Sideritis montana L. subsp. *montana*	22
Sideritis romana L.	22
Stachys affinis Bunge	21
Stachys alopecuros (L.) Benth. subsp. *divulsa* (Ten.) Grande	21
Stachys annua L.	21
Stachys germanica subsp. *salviifolia* L. (Ten.) Gams.	21
Stachys palustris L. - France	21
Stachys palustris L. - Hungary	21
Teucrium chamaedrys L.	20
Teucrium polium L. - Northern Iran	20
Teucrium polium L. - Southern Iran	20

Table 16: Sporne Index (SI) values for each studied species

In the table below (Table 17), it is reported the mean Sporne index for each studied species. The M value for each studied species is equal to 30.

Species	Mean Sporne Index (mSI)
Ajuga chamaepitys (L.) Schreb	70
Ajuga genevensis L.	70
Ajuga reptans L.	70
Ajuga tenorei C. Presl	70
Galeopsis ladanum subsp. *angustifolia* (Ehrh. ex Hoffm.) Gaudin	70
Melittis melissophyllum subsp. *albida* (Guss.) P.W.Ball	70
Melittis melissophyllum L. subsp. *melissophyllum*	70
Sideritis montana L. subsp. *montana*	73
Sideritis romana L.	73
Stachys affinis Bunge	70
Stachys alopecuros (L.) Benth. subsp. *divulsa* (Ten.) Grande	70
Stachys annua L.	70
Stachys germanica subsp. *salviifolia* L. (Ten.) Gams.	70

Stachys palustris L. - France	70
Stachys palustris L. - Hungary	70
Teucrium chamaedrys L.	67
Teucrium polium L. - Northern Iran	67
Teucrium polium L. - Southern Iran	67

Table 17: Mean Sporne Index (mSI) values for each studied species

Lastly, in the tables below (Tables 18, 19), the mean Oxidation Values for the iridoids (mOV_i) and flavonoids (mOV_f) evidenced in each studied species are reported:

Species	Mean Oxidation Value for iridoids (mOV_i)
Ajuga chamaepitys (L.) Schreb	68/8 = **8.5**
Ajuga genevensis L.	**0**
Ajuga reptans L.	23/3 = **7.7**
Ajuga tenorei C. Presl	23/3 = **7.7**
Galeopsis ladanum subsp. *angustifolia* (Ehrh. ex Hoffm.) Gaudin	16/2 = **8.0**
Melittis melissophyllum subsp. *albida* (Guss.) P.W.Ball	53/6 = **8.8**
Melittis melissophyllum L. subsp. *melissophyllum*	50/6 = **8.3**
Sideritis montana L. subsp. *montana*	38/5 = **7.6**
Sideritis romana L.	29/4 = **7.3**
Stachys affinis Bunge	33/4 = **8.3**
Stachys alopecuros (L.) Benth. subsp. *divulsa* (Ten.) Grande	55/7 = **7.9**
Stachys annua L.	**0**
Stachys germanica subsp. *salviifolia* L. (Ten.) Gams.	8/1 = **8.0**
Stachys palustris L. - France	25/3 = **8.3**
Stachys palustris L. - Hungary	25/3 = **8.3**
Teucrium chamaedrys L.	16/2 = **8.0**
Teucrium polium L. - Northern Iran	**0**
Teucrium polium L. - Southern Iran	**0**

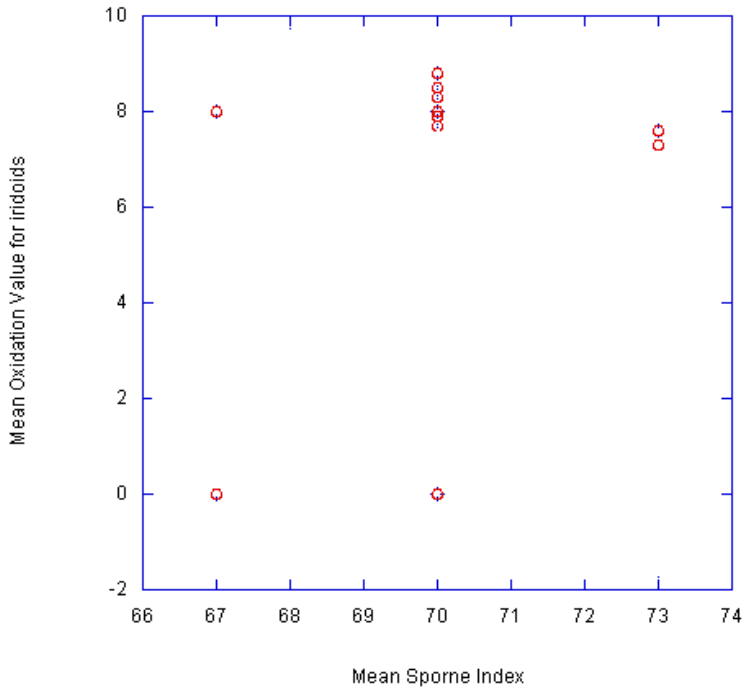
Table 18: Mean Oxidation Values (mOV_i) for iridoids evidenced in each studied species

Species	Mean Oxidation Value for flavonoids (mOV_f)
Ajuga chamaepitys (L.) Schreb	**0**
Ajuga genevensis L.	**0**
Ajuga reptans L.	**0**
Ajuga tenorei C. Presl	**0**
Galeopsis ladanum subsp. *angustifolia* (Ehrh. ex Hoffm.) Gaudin	7/3 = **2.3**
Melittis melissophyllum subsp. *albida* (Guss.) P.W.Ball	**0**
Melittis melissophyllum L. subsp. *melissophyllum*	**0**
Sideritis montana L. subsp. *montana*	5/2 = **2.5**
Sideritis romana L.	13/7 = **1.9**
Stachys affinis Bunge	**0**
Stachys alopecuros (L.) Benth. subsp. *divulsa* (Ten.) Grande	**0**
Stachys annua L.	5/2 = **2.5**
Stachys germanica subsp. *salviifolia* L. (Ten.) Gams.	5/2 = **2.5**
Stachys palustris L. - France	**0**

Stachys palustris L. - Hungary	**0**
Teucrium chamaedrys L.	3/1 = **3.0**
Teucrium polium L. - Northern Iran	7/5 = **1.4**
Teucrium polium L. - Southern Iran	12/6 = **2.0**

Table 19: Mean Oxidation Values (mOV_i) for flavonoids evidenced in each studied species

With these two values, it was then possible to draw two graphs of phytochemical evolution between the mean Oxidation Value of iridoids and the mean Sporne Index and between the mean Oxidation Value of flavonoids and the mean Sporne Index again (Graph 1, Graph 2). Graph 1 is reported below.



Graph 1: Evolution diagram for iridoids in studied Lamiaceae species

In Graph 1 concerning iridoids, there are two spots at 0 for what concerns the Mean Oxidation value. The first one presenting a Mean Sporne Index of 67 corresponds to the species *Ajuga genevensis* L. while the second one at a value of Mean Sporne Index of 70 corresponds to the two studied species of *Teucrium polium* L. from Northern Iran and from Southern Iran. For all of them this result is due to the fact that no iridoids were reported from these species during the phytochemical study but this does not represent an absolute value since the absence of iridoids may have been caused by many factors, both environmental and of collection.

Going upward along the y axis from a Mean Sporne Index value of 67, another spot can be seen and this corresponds to *Teucrium chamaedrys* L.

Instead, going upward along the y axis from a Mean Sporne Index value of 70, several spots are present and these are very near to each other. Twelve species correspond to these spots namely *Ajuga chamaepitys* (L.) Schreb, *Ajuga reptans* L., *Ajuga tenorei* C. Presl, *Galeopsis ladanum* subsp. *angustifolia* (Ehrh. ex Hoffm.) Gaudin, *Melittis melissophyllum* subsp. *albida* (Guss.) P.W.Ball, *Melittis melissophyllum* L. subsp. *melissophyllum*, *Stachys affinis* Bunge, *Stachys alopecuros* (L.) Benth. subsp. *divulsa* (Ten.) Grande, *Stachys annua* L., *Stachys germanica* subsp. *salviifolia* L. (Ten.) Gams., *Stachys palustris* L. - France and *Stachys palustris* L. - Hungary.

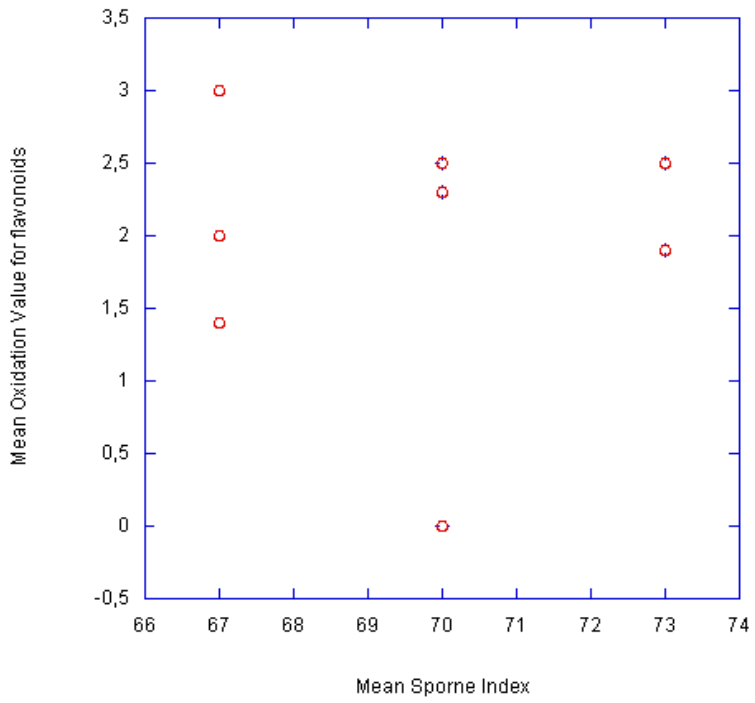
In this context, it's very important to underline the fact that the *Melittis* species spots are those which are very next to the *Stachys* species ones and this is extremely relevant from the phylogenetic point of view. In fact, in recent times, *Melittis* genus has been inserted into the *Stachys* one basing upon genetic data. These results seem to confirm this also from the phytochemical evolution standpoint.

Lastly, the two spots going up upward along the y axis from a Mean Sporne Index value of 73, correspond to the two studied *Sideritis* species, *Sideritis montana* L. subsp. *montana* and *Sideritis romana* L. It's not strange to find them so very close but the spots should be reversed. In fact, according to genetic data, *Sideritis montana* L. subsp. *montana* should be less evolved than *Sideritis romana* L. Thus, the phytochemical evolution displayed an opposite result.

Actually, this happened also for the other species present in the second group discussed but only for a few of them. In fact, *Galeopsis ladanum* subsp. *angustifolia* (Ehrh. ex Hoffm.) Gaudin should be less evolved than all the others but this is not and in this case, in fact, it is placed in the middle of them (Salmaki et al., 2013).

Anyway, this is only a first approach to this argument and further studies will be necessary to fully verify this aspect but, at least, this phytochemical evolution study started to raise this fact.

Indeed Graph 2 is reported below.



Graph 2: Evolution diagram for flavonoids in studied Lamiaceae species

Also, in Graph 2 concerning flavonoids, things are very similar. Yet, the spot having a Mean Oxidation value of 0 corresponds to all the studied *Ajuga* and *Melittis* species and to most of the studied *Stachys* species, *Stachys affinis* Bunge, *Stachys alopecuros* (L.) Benth. subsp. *divulsa* (Ten.) Grande, *Stachys palustris* L. - France and *Stachys palustris* L. - Hungary. Anyway, for some of them, *Ajuga chamaepitys* (L.) Schreb., *Melittis melissophyllum* L. subsp. *melissophyllum* and *Stachys alopecuros* (L.) Benth. subsp. *divulsa* (Ten.) Grande, different compounds from iridoids were not sought.

For what concerns *Teucrium* species, everything is in accordance with the phylogenetic evolution while for *Sideritis* species, the spots should be again reversed (Salmaki et al., 2013; Li et al., 2016).

4. Conclusions

One comprehensive phytochemical analysis was then performed on nineteen plants belonging to the Ajugoideae and Lamioideae sub-families of the Lamiaceae family.

In particular, these plants were: *Ajuga chamaepitys* (L.) Schreb. - aerial parts, *Ajuga chamaepitys* (L.) Schreb. - roots, *Ajuga genevensis* L., *Ajuga reptans* L., *Ajuga tenorei* C. Presl - leaves, *Teucrium chamaedrys* L., *Teucrium polium* L. from Northern Iran, *Teucrium polium* L. from Southern Iran, *Galeopsis ladanum* subsp. *angustifolia* (Ehrh. ex Hoffm.) Gaudin, *Melittis melissophyllum* subsp. *albida* (Guss.) P.W.Ball, *Melittis melissophyllum* L. subsp. *melissophyllum*, *Sideritis montana* L. subsp. *montana*, *Sideritis romana* L., *Stachys affinis* Bunge - rhizomes, *Stachys alopecuros* (L.) Benth. subsp. *divulsa* (Ten.) Grande, *Stachys annua* L., *Stachys germanica* subsp. *salviifolia* L. (Ten.) Gams., *Stachys palustris* L. from Hungary and St*achys palustris* L. from France.

One hundred and sixty-nine phytochemical compounds were evidenced for all the studied species. In particular, these compounds were: **1-oleoyl-2-linoleoyl-*sn*-glycerol-3-phosphocholine**, **1-miristoyl-2-heptadecenoyl-*sn*-glycerol**, **1,2-di-palmitoyl-*sn*-glycerol**, ***E*-phytol**, **pheophorbide a**, **10-β-hydroxy-teucjaponin B**, **picropolin**, **teupolin I**, **teucrasiatin**, **20-*O*-acetyl-teucrasiatin**, **rosmarinic acid**, **oleanolic acid**, **maslinic acid**, **verbascoside**, **martynoside**, **forsythoside b**, **samioside**, **alyssonoside**, **leucosceptoside A**, **echinacoside**, **poliumoside**, **stachysoside A**, **apigenin**, **luteolin**, **acacetin**, **cirsiliol**, **cirsimaritin**, **cirsilineol**, **4'-*O*-methyl-luteolin (diosmetin)**, **apigenin 7-*O*-β-glucoside**, **apigenin 7-*O*-rutinoside (isorhoifolin)**, **apigenin 7-*O*-(6''-*O*-acetyl-β-D-glucopyranoside)**, **7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-β-D-glucopyranosyl]oxy}-5-hydroxy-2-(3-hydroxy-4-methoxy-phenyl)-4*H*-1-benzopyran-4-one**, **7-{[2-*O*-(6-*O*-acetyl-β-D-glucopyranosyl)-β-D-glucopyranosyl]oxy}-5-hydroxy-2-(3,4-dihydroxyphenyl)-4*H*-1-benzopyran-4-one**, **7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-β-D-glucopyranosyl]oxy}-5-hydroxy-2-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one**, **7-{[2-*O*-β-D-allopyranosyl-β-D-glucopyranosyl]oxy}-5-hydroxy-2-(3-methoxy-4-hydroxy-phenyl)-4*H*-1-benzopyran-4-one**, **7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-β-D-glucopyranosyl]oxy}-5,8-dihydroxy-2-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one**, **7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-β-D-glucopyranosyl]oxy}-5,8-dihydroxy-2-(4-methoxyphenyl)-4*H*-1-benzopyran-4-one**, **7-{[2-*O*-β-D-allopyranosyl-(6-*O*-acetyl-β-D-glucopyranosyl)]oxy}-5,8-dihydroxy-2-(3-hydroxy-

4-methoxy-phenyl)-4*H*-1-benzopyran-4-one, **7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-6-*O*-acetyl-β-D-glucopyranosyl]oxy}-5,8-dihydroxy-2-(3-hydroxy-4-methoxy-phenyl)-4*H*-1-benzopyran-4-one**, **8-epiloganic acid**, **ajugoside**, **reptoside**, **harpagide**, **8-*O*-acetyl-harpagide**, **monomelittoside**, **melittoside**, **allobetonicoside**, **virginioside**, **geniposidic acid**, **6-deoxy-harpagide**, **bartsioside**, **6-*O*-acetyl-ajugol**, **5-*O*-allosyloxy-aucubin**, **asperulosidic acid**, **deacetyl-asperulosidic acid**, **5-*O*-β-D-glucopyranosyl-harpagide**, **5-*O*-β-D-glucopyranosyl-8-*O*-acetyl-harpagide**, **4′-*O*-galactopyranosyl-teuhircoside**, **chlorogenic acid**, **quinic acid**, **β-arbutin**, **methyl-arbutin**, **cinnamic acid**, **D-mannitol**, **glucose**, **fructose**, **galactose**, **sucrose**, **raffinose**, **stachyose**, **acetic acid**, **caffeic acid**, **formic acid**, **fumaric acid**, **lactic acid**, **malic acid**, **pyruvic acid**, **succinic acid**, **α-hydroxy-butyric acid**, **γ-amino-butyric acid (GABA)**, **alanine**, **aspartic acid**, **asparagine**, **lysine**, **glutamine**, **threonine**, **tyrosine** and **valine**.

Anyway, most of these compounds were found in common among several studied species while others were peculiar of only a few species or even of an only one.

Among them, it's very important to underline **20-*O*-acetyl-teucrasiatin**, **5-*O*-β-D-glucopyranosyl-harpagide** and **5-*O*-β-D-glucopyranosyl-8-*O*-acetyl-harpagide** which represent brand new compounds recognized for the first time as natural constituents of plants.

From the chemotaxonomic point of view, the presence of specific markers of different kind in several studied species was observed thus confirming their current botanical classifications. Nevertheless, in some cases, their absence was also evidenced suggesting the possibility to use phytochemistry for the botanical classification of the species together with genetic data. Moreover, in several cases, different markers belonging to different families from Lamiaceae were found, demonstrating chemotaxonomic correlations among families but also strange molecular patterns for Lamiaceae species in some cases.

From the pharmacological point of view, most of the isolated compounds possess important pharmacological properties both specific and general. In many cases, these allowed to provide a phytochemical rationale for the ethno-pharmacological employments or not of the species where they were evidenced since they are totally responsible of these effects. Nevertheless, in some cases, they suggested other potential pharmacological uses for the species and/or the possibility to use them for the first time. Yet, further pharmacological studies in this sense are necessary for confirmation of this hypothesis. In one case, *Ajuga reptans* L., a nutraceutical general analysis was also performed and the possibility to use this species in the nutraceutical field was suggested.

Lastly, two preliminary diagrams concerning the phytochemical evolution were drawn and these showed concordance with the phylogenetic evolution for some species and genera but also some discordance for others, in particular, for the exact position in the evolutionary tree of some species and this might be the starting base for further studies in this sense even if they did not absolutely want to be reliable in an absolute way.

5. Experimental

5.1. Plant materials

In the table below (Table 20), all the collection areas of the studied plants are reported. Moreover, the specific studied plant organs, the date of collection, the geographic coordinates and the altitudes of collection are added.

As it can be seen, most of them were collected along the national territory while two of them came from the European Continent and other two even from Asia.

Sub-family Ajugoideae			
Genus *Ajuga*			
Species name	**Studied Organ**	**Place of collection with geographical coordinates and altitude**	**Date of collection**
Ajuga chamaepitys (L.) Schreb.	aerial parts	Roccamattea (Pievebovigliana, MC); 43°05'53" N, 13°07'37" E; 370 m a.s.l.	May 2014
Ajuga chamaepitys (L.) Schreb.	roots	Roccamattea (Pievebovigliana, MC); 43°05'53" N, 13°07'37" E; 370 m a.s.l.	May 2014
Ajuga genevensis L.	aerial parts	Dolomitis Mountains, lake of Braies; 46°41'39" N, 12°05'08" E; 1496 m a.s.l.	August 2013
Ajuga reptans L.	aerial parts	Badia Morronese swamps area (Sulmona, (AQ); 42°54'26" N, 13°55'17" E; 320 m. a.s.l.	April 2015
Ajuga tenorei C. Presl	leaves	Rivisondoli (AQ); 41°86'13" N; 13°98'99" E; 1750 m a.s.l.	June 2015
Genus *Teucrium*			
Species name	**Studied Organ**	**Place of collection with geographical coordinates and altitude**	**Date of collection**
Teucrium chamaedrys L.	aerial parts	Pre-Apennines area of Latium region (Italy); 41°61'51" N, 13°54'78" E; about 180 m a.s.l	July 2015
Teucrium polium L.	aerial parts	Alborz mountain (Northern Iran); 36°06'37" N, 51°79'16" E; 2000 m a.s.l.	May 2015
Teucrium polium L.	aerial parts	Koohpayeh mountains (South Western Iran); 30°31'57" N, 57°11'42" E; about 2200 m a.s.l.	June 2014

Sub-family Lamioideae			
Genus *Galeopsis*			
Species name	**Studied Organ**	**Place of collection with geographical coordinates and altitude**	**Date of collection**
Galeopsis ladanum subsp. *angustifolia* (Ehrh. ex Hoffm.) Gaudin	aerial parts	Civita di Oricola (Abruzzo region); 42º02'58" N, 13º02'21" E; about 600 m a.s.l.	August 2015
Genus *Melittis*			
Species name	**Studied Organ**	**Place of collection with geographical coordinates and altitude**	**Date of collection**
Melittis melissophyllum subsp. *albida* (Guss.) P.W.Ball	aerial parts	Pre-park Area of Abruzzo, Lazio and Molise National Park (Molise region); 41°67'15" N, 14°03'41" E; 750 m a.s.l.	June 2015
Melittis melissophyllum L. subsp. *melissophyllum*	aerial parts	Piedilapiaggia (MC); 43°09'10"N, 13°07'18"E; 600 m a.s.l.	May 2012
Genus *Sideritis*			
Species	**Studied Organ**	**Place of collection with geographical coordinates and altitude**	**Date of collection**
Sideritis montana L. subsp. *montana*	aerial parts	Monte Capolapiaggia (MC); 43°08'45" N, 13°07'05" E; 700 m a.s.l.	June 2014
Sideritis romana L.	aerial parts	Latium hills, in the province of Frosinone; 41°61'54" N, 13°54'81" E; 150 m a.s.l.	June 2014
Genus *Stachys*			
Species	**Studied Organ**	**Place of collection with geographical coordinates and altitude**	**Date of collection**
Stachys affinis Bunge	rhizomes	Gazoldo degli Ippoliti (MN); 45°11'02" N, 10°34'04" E; 35 m a.s.l.	December 2014
Stachys alopecuros (L.) Benth. subsp. *divulsa* (Ten.) Grande	aerial parts	Pizzo Tre Vescovi (MC); 42°58'07" N, 13°14'10" E; 1520 m a.s.l.	August 2015
Stachys annua L.	aerial parts	Capolapiaggia (MC); 43°80'86" N, 13°80'65" E; 627 m a.s.l.	August 2013
Stachys germanica subsp. *salvifolia* L.	aerial parts	Majella National Park; 41°68'11" N, 14°05'42" E; 715 m a.s.l.	June 2013
Stachys palustris L.	aerial parts	Pellérd (Hungary); 46°03'02" N, 18°08'02" E; 109 m a.s.l.	August 2015
Stachys palustris L.	aerial parts	Inzinzac-Lochrist (Morbihan, France); 47°49'04" N, 03°14'04" O; 8 m a.s.l.	September 2015

Table 20: Collection details for each studied species

5.2. Chemicals

During all this study, the following reagents and solvents were used: ethanol 96% and distilled water for the extraction procedure; *n*-butanol, distilled water, chloroform, methanol, ethyl-acetate and *n*-hexane, as pure compounds or in mixtures at different concentrations among them all for the separation procedure by Column Chromatography; silica gel or acidified silica gel both with particle sizes of 40-63 μm as stationary phases for the Chromatography Columns; H_2SO_4 2N as spray reagent for the development of TLCs; aqueous solution of $FeCl_3$ as spray reagent for the development of chromatographic papers in order to verify the possible presence of phenol compounds; methanolic solution of vanillin/HCl (3% w/v) for the development of chromatographic papers in order to verify the possible presence of iridoid compounds; $CDCl_3$, CD_3OD and D_2O as deuterated solvents for the sample preparation for NMR Spectroscopy; RS methanol with the aim to dissolve samples before analysis in Mass Spectrometry.

All the natural solvents having RPE purity grade if not differently specified, were purchased from Sigma-Aldrich together with all the deuterated solvent and the RS methanol.

Silica gel was, instead, purchased from Fluka Analyticals and the pre-coated silica gel TLCs "60 F254" were purchased from Merck.

5.3. Instrumentation

The following instruments were used: Rotavapor RII by Büchi for the solvent evaporation at reduced pressure; vacuum pump "Jet Standard" by "General Scientific Instrument"; lyophilizer belonging to the "Analitica De Mori" company.

NMR spectra were recorded on a Varian (now Agilent Technologies) Mercury 300 MHz instrument and/or on a Bruker Avance II 400 MHz instrument with the chemical shifts expressed in ppm.

The signal of TMS (s) at 0 ppm was used as reference for spectra in $CDCl_3$; the internal solvent signal corresponding to CD_2HOD (p) at 3.31 ppm was the reference for spectra in CD_3OD; the signal at 4.79 (s) related to HDO was the reference for spectra in D_2O, instead.

MS spectra were performed on a Q-TOF MICRO spectrometer (Micromass, now Waters, Manchester, UK) equipped with an ESI source that operated in the negative and/or positive

ion mode. The flow rate of sample infusion was 20 L/min. with 100 acquisitions per spectrum. Data were analyzed by using the MassLynx software developed by Waters.
HPLC experiments were carried out on a Waters (Now Agilent) 2487 HPLC instrument equipped with a Merck 50983 LiChrospher100 RP-18 column (5 μm, 250 × 4 mm) and a binary pump Waters 1525, managed with Breeze software and coupled with a Waters 2487 UV-Vis detector double absorbance.

5.4. Methodology

5.4.1. Extraction of metabolites

The complete extraction of metabolites from every fresh or dry plant material was achieved by following a specific methodology.
The collected plant material, consisting on the total aerial parts or on a particular aerial or sub-aerial part, was inserted in a conical flask and immediately filled with an extraction solution. In particular, this was generally ethanol 96% or a solution at different concentrations of ethanol 96% and distilled water.
The choice of using one solution or the other depended on the target of the study: if the target was only secondary metabolites, only ethanol 96% was used whereas if the target was together primary and secondary metabolites, both ethanol 96% alone and the solutions of ethanol 96% and distilled water were used.
The utilized amount of these solutions was different according to the total amount of the plant material. In general, the plant material was covered up with these solutions in order to have a more functional extraction.
The whole flask containing plant material and the extraction solution was left closed for at least 48 hours in order to let metabolites come into solution.
The occurred passage of metabolites into the solution is clearly visible since the solution itself changed its coloration passing from transparent to a darker color, typically dark green.
At this point, the solution was filtered on a normal funnel in a round-bottom flask and concentrated under reduced pressure at 50° C.
Throughout this, pH was checked on litmus paper. This check was necessary to verify that pH was not too acid or too basic (the optimal range is 5.5-8.5) because one too acid or too basic

environment might have caused unwanted secondary reactions on the metabolites such as the hydrolysis of the ester and sugar groups. T

The procedure of concentration was repeated till the end of the mother solution.

In addition to this, the extraction procedure was repeated for every plant material for three times in order to have an exhaustive extraction of the studied species.

Once concentration was over, the total extract was freeze-dried for as much time as necessary and lyophilized in order to preserve also temperature-sensitive compounds present in the extract.

After this passage, solid extracts with different colorations, generally dark green again, were obtained and this were scratched from the round-bottom flask walls, collected in big vials, weighed, well closed and stored in the fridge for the following analysis steps.

5.4.2. Separation of metabolites

The separation of metabolites was achieved by means of classical column chromatography on silica gel or by HPLC.

5.4.2.1. Column Chromatography

Column Chromatography (CC), also known as liquid-solid chromatography, is an analytical technique whose aim is to separate the components of one homogeneous solution.

The structure of a chromatographic column is very simple. This is constituted by a glass tube whose forms and dimensions are very variable and depend on the substance which must be examined but also on the quantity of adsorbing material to be used for the separation. Lastly, at the lower point of the column, a small plug is present and this is in order to regulate the flow of the eluate.

From the theory standpoint, CC is based on the different distribution of its components between two phases, a stationary and a mobile one, along a well defined direction through a direct interaction between the active sites of the adsorbent solid and the functional groups present in the molecules of the mixture to separate.

The adsorbent solid is usually silica gel but other materials can be used such as cellulose, polyamide and allumine.

The solutes are bound to the stationary phase by dipole-dipole interactions or dipole-induced dipole interactions in a reversible way. This kind of interactions is the result of a competitive complex phenomenon which occurs between the molecules of the mobile phase and those of the solute for the linkage to the active sites of the fixed phase.

The mobile phases often correspond to mixtures of solvents at different polarity such as *n*-butanol-water, methanol-chloroform, diethyl ether-dichloromethane and *n*-hexane-ethyl acetate.

By this process, it is, then, possible to separate the components of a mixture from each other since different times are necessary for each component to run the entire column and elute out. The eluents which were able to catch, during the passage in the column, single components of the studied complex mixture, can be collected in single fractions and so they are separated mechanically (Figure 57).

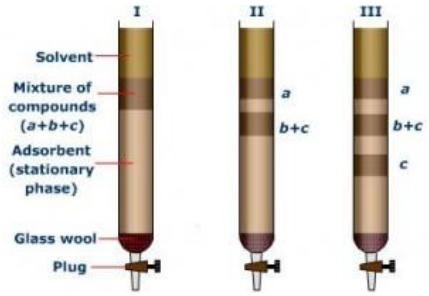


Figure 57: Theoric separation of compounds present in mixtures by column chromatography

According to the type of stationary phase use, the components elute by their major or minor polarity. In the case of silica gel, the most polar compounds elute at the end while the least polar compounds elute first. In the case of cellulose, it is exactly the opposite.

For these reasons, Column Chromatography represented one of the most used separative techniques in analytical chemistry. In fact, in recent times, its employment has drastically decreased mainly because of three factors: the excessive sample preparation, the long lengths of time necessary to totally perform a single analysis and the little resolving power (the ability to distinguish among similar compounds) of the technique.

At its place, new methods have been taking more and more space in the separation of substances since they are able to overcome all the problems associated to classical Column Chromatography (Harwood and Moody, 1989).

5.4.2.2. Preparation of acidic silica gel

Acidified silica gel was prepared according to a standard protocol.
100 g of normal silica gel were weighed and inserted in a flask in which 250 mL of a solution of HCl 12N and distilled water in ratio 1:5 (v/v) were then poured.
The whole was left so for at least 24 hours.
Then, the solution was vacuum-filtered and the solid was washed more times with distilled water until its neutralization. This passage was necessary in order to eliminate every trace of chlorides. Their absence was checked by the AgNO3 method.
Then the silica gel was dried in oven at 120 °C for 48 hours.
At this point, it was ready for use.

5.4.2.3. HPLC

HPLC (High Performance Liquid Chromatography or High Pressure Liquid Chromatography) is a powerful analytical technique which is used in order to separate compounds in a complex mixture.
The HPLC instrument is constituted by: a series of pumps through which the eluting mixtures pass at a pre-fixed flow time; a chromatographic column which can be long from 3 to 30 cm and is packed with different fixed phases according to the nature of the compounds to be separated (for example, polymers with different numbers of carbon atoms present, phenyl-hexyl, cellulose); a sampler which carries the sample into the flow of the mobile phase; a detector which can be an UV lamp, a fluorescence lamp, a photodiode array or a Mass Spectrometer.
This detector is formed also by a calculator which permits a continuous analysis of the column exit and generates a signal that is proportional to the quantity of that constituent of the mixture present in the column which is why HPLC is also used for quantitative analysis. Actually, the most common detectors are UV lamps which are lamps emitting a light comprised in wavelengths between 200 and 450 nm (Figure 58).

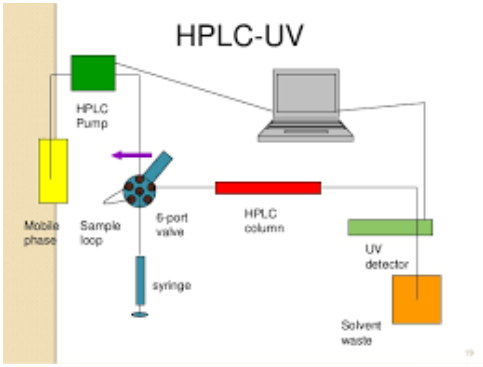


Figure 58: Operative scheme of a typical HPLC-UV instrument

From the strictly technical point of view, HPLC is based on the ability of a certain fixed phase to adsorb a certain kind of compound and on a certain mobile phase (usually a mixture of solvents having a different polarity) to desorb it and carry it away along the former phase.

In practice, each constituent of the mixture sample interacts with the fixed phase in a different way according to its chemical structure which causes different elution times for that specific constituent in respect with the others. In fact, the bigger is the affinity of a compound to the stationary phase, the bigger is the time requested for that compound to exit the chromatographic column. This time is known as retention time (Rt) and is calculated in seconds.

There are several important differences between this technique and the other liquid chromatographic ones. The first one is that HPLC works in reversed phase, meaning that the most polar compound elutes first from the column. Another one is that high pressures are used in order to perform the separation which leads to the employment of columns having quite small dimensions and to minor times to perform the chromatographic run. Moreover, the resolving power of HPLC is much higher than the other classical chromatographic techniques which allow the possibility to distinguish better among similar compounds.

For all these reasons HPLC has now become the most important analytical method in separating substances in spite of the usual expensive costs for its installation and maintenance (Skoog, 1995).

5.4.3. Active charcoal treatment

The method of the active charcoal treatment is based on a recent patent of Ballero and co-workers (Ballero et al., 2009) and consists in suspending the crude extract in water to favor its

adsorption on a charcoal/celite/polyamide mixture in ratio 10:1:1 (w/w/w) until no positive reaction with vanillin/HCl spray reagent.

This is a rapid, highly sensitive and specific test for the screening of the presence of iridoid glucosides in a crude extract as well as in a chromatographic fraction.

From the experimental standpoint, it is performed on a filter paper where the sample solution is seeded and then treated with the spray reagent.

The majority of iridoid glucosides, after heating at 120°C, gives characteristic colored spots. In many cases, it is possible to identify the compound on the basis of the obtained color shade.

If the aqueous suspension gives positive reaction to the vanillin/HCl test, the iridoids were not completely adsorbed on the charcoal/celite/polyamide mixture.

This step is conducted on an empirical basis and the adsorbing mixture is added in aliquots until any reaction occurs.

The resulting suspension is then stratified on a Gooch funnel and the water is filtered.

The charcoal/celite/polyamide cake is then eluted with distilled water to eliminate the very polar compounds (mainly mono- and disaccharides) not adsorbed but present in the residual moisture.

To verify the absence of saccharides, a drop of the sample solution is placed on a TLC plate, treated with H_2SO_4 2N spray reagent and then heated revealing or not a corresponding spot.

To eliminate the saccharidic compounds, different volumes of distilled water might be necessary according to the amount of the starting material and to the wanted purpose.

At this point, the desorption procedure is started with mixtures of ethanol 96% and distilled water at different concentration ratios between them according to the kind of iridoid compounds which have to be eluted from the charcoal/celite/polyamide cake.

Anyway, these mixtures head toward an increasing amount of ethanol 96% percentages.

The mixtures which are usually used for this contain 30, 60 and 96% of ethanol in order to elute different classes of iridoids.

The elution of compounds is monitored by reaction with vanillin/HCl reagent often accompanied by H_2SO_4 2N and heating.

Solvent polarity is changed when the spot intensity of the drop of the sample solution sensibly decrease.

All the elutions are conducted by avoiding the complete drying of the charcoal mixture.

In this manner, different fractions at different polarity are collected and after elimination of the solvent under reduced pressure and freeze drying, dried solids are recovered.

Chromatographic separation on silica gel columns are then carried out on one of more of these solids and their choice is due to preliminary TLC analysis and the vanillin/HCl test favoring those having more spots on both.

5.4.4. Identification of metabolites

The identification of metabolites was conducted through two spectroscopic techniques: NMR Spectroscopy and MS Spectrometry.

5.4.4.1. NMR Spectroscopy

NMR (Nuclear Magnetic Resonance) Spectroscopy, is a spectroscopic technique widely used in pure organic chemistry, but also in diagnostics, in order to identify the structure of unknown compounds present as pure or in complex mixtures.
The instrument is constituted by a gap where the sample is inserted. This is surrounded by a circular coil where an electric current is in continuous motion to generate a magnetic field. This whole is immerged in a vacuum environment generated by N_2 which is also useful to hold in the heat created by the irradiation with the magnetic field. Another gas, He, is also present in the instrument to create a more pronounced vacuum and to avoid chemical and/or physical interactions between the sample and the outer environment thus performing the most accurate analysis possible (Figure 59).

Figure 59: Typical NMR instrument

NMR is based on the fact all the nuclei of atoms rotate around themselves due to their electric charge generating an intrinsic magnetic field. This can be oriented if these nuclei undergo a magnetization by an external uniform magnetic field.

Actually not all nuclei can do this but only those having a magnetic dipole momentum different from 0. In practice, all the stable isotopic nuclei satisfy this condition and so only these can be used in NMR Spectroscopy.

After this magnetization, these nuclei are now able to adsorb energy at specific radiofrequencies, inverting so their rotation axis.

This energy adsorption causes the promotion of an electron from an internal orbital and thus in a relaxed state, to an outer orbital and thus to an excited state. This promotion occurs in resonance with the frequency of the applied magnetic field.

After a certain length of time, this electron decays returning to its original orbital and during this process, the signal is recorded by the detector.

Yet, this recording gives a graphic where absorbance is reported on the y axis while the decay times of the electrons are reported on the x axis. From this graphic, no useful information could ever be collected.

For this reason the detector is linked to an elaborator, usually a computer, which is set on a mathematical function called “The Fourier Transform” which permits the conversion of this graphic to another one where absorbance is reported on the y axis and the resonance frequencies of the atoms are reported on the x axis.

This resonance frequency depends on the potency of the external magnetic field and on the chemical characteristics of the studied nucleus. The latter dependence is useful whereas the former is not because this may cause differences on the results if the analysis is performed with different instruments.

Just in order to remedy this, on the x axis, the frequency of resonance of the atoms is not conventionally reported as such but one of its mathematical derivatives. This derivative is called chemical shift (δ) which is defined as the difference between the resonance frequency of the studied atom minus the resonance frequency of a standard compound, all divided for the potency of the instrument.

The standard compound is tetramethyl-silane ($(CH_3)_4Si$) which has a resonance frequency equal to 0.

This technique is very useful because every atom or better every functional group has its own peculiar resonance frequency.

By consequence, studying the δ values present in a spectrum, it is possible to identify with absolute sureness, the functional groups present in the studied molecule.

These δ values can be obtained by performing NMR spectra of several nuclei (^{1}H, ^{13}C, ^{19}F are The most commonly used) which provide similar and/or further informations on the chemical structure of the studied molecule.

Yet, the final molecular structure of the compounds is decoded also by studying other parameters that are well visible in specific spectra such as coupling constants (vicinity of hydrogen atoms both baring a double or triple bond), integrals (only for ^{1}H spectra which indicate the number of equivalent protons present on the same functional group), signal splitting (vicinity of other functional groups in respect with the one taken into account).

Lastly, by performing bidimensional experiments i.e. performing in the same time the promotion of electrons belonging to different nuclei of the same molecule, it is possible to have other informations such as the direct connection between a proton and its carbon (HSQC: contemporaneous promotions of the electrons of one ^{1}H nucleus and of one ^{13}C nucleus), the vicinity of a certain proton with a certain carbon (HMBC: contemporaneous promotions of the electrons of one ^{1}H nucleus and of one ^{13}C nucleus), the correlation of a certain proton with another proton (COSY: contemporaneous promotions of the electrons of one ^{1}H nucleus and of one other ^{1}H nucleus), the total spin system present in a molecule (TOCSY: contemporaneous promotions of electrons of one ^{1}H nucleus and one other ^{1}H nucleus).

The structure obtained by this technique is always the right one which makes NMR very reliable. Moreover, it's quite cheap, easy to use and little invasive since the molecules present in the sample are never destroyed and can be recovered if necessary.

For this NMR is the top technique for this kind of analysis (Silverstein, 2006).

5.4.4.2. Mass Spectrometry

Mass Spectrometry (MS) is an analytical technique which is used for the identification of unknown compounds, present as pure or in mixtures even in traces, providing their molecular weight.

It is based on the separation of the components of a mixture, which travel all along the instrument as ions, according to their mass/charge ratio (*m/z*).

These ions of the components are achieved by irradiating the initial liquid matrix with an electron beam having a known energy.

The typical instrument is constituted by an injector system for the sample, an ion source, an analyzer and a detector (Figure 60).

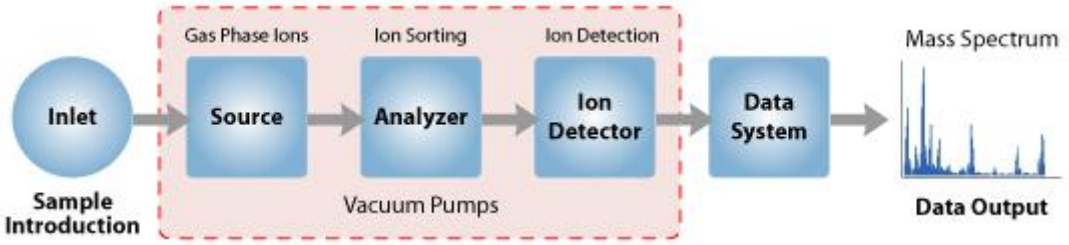


Figure 60: Operative scheme of a typical HPLC-UV instrument

The injector system may be constituted by an auto-sampler or only by a simple valve through which a manual direct injection can be done.

The type of ion source used may be very variable such as ESI (Electron Spray Ionization), EI (Electron Ionization), CI (Chemical Ionization), API (Atmospheric Pressure Ionization), FAB (Fast Atom Bombardment), MALDI (Matrix Assisted Laser Desorption Ionization).

For this study, the ESI source was used.

ESI is a soft ionization method which occurs in liquid phase and at atmospheric pressure and allows the analysis of molecules having a medium polarity and high molecular weight.

This method does not destroy the sample and for this it is called as soft ionization method. This means that every result obtained correspond to the molecular weight of a molecular present in the sample.

From the technical standpoint, the ESI works as such: the sample constituted by a very volatile solvent and the analyte, is pushed in a capillary at whose extremity a voltage of about 20 Volts is applied. This voltage is able to ionize and in the meantime, evaporate the entire sample. The presence of a very volatile solvent is important to dilute the sample and avoid an extreme concentration of the analyte which may cause obstructions and false results and for the next passage in which the solvent must be eliminated. This solvent is usually methanol but also acetonitrile can be employed. Indeed, the ionization and the evaporation of the sample, is absolutely necessary for this technique since the MS instrument is only able to detect ion and gaseous compounds.

The elimination of the solvent occurs this way. The presence of this initial capillary allows the formation of small drops attached to it and these grow wider and wider as soon as more

amount of sample passes. At one point, these drops are too big to be kept by the capillary also because of the presence of charges which place themselves all along the surface of the drop following a physical principle. There is a Coulombian explosion and the drop is pushed away from the capillary also because, at the other extremity, an opposite charge voltage is present which attracts the drop. During this process of attraction, the drops pass through a space where N_2 is free to wander and this gas is able to create vacuum but also to catch the ion molecules of the solvent which are taken away. Actually, this is possible only for small and extremely volatile solvent molecules. So, at the other extremity which corresponds to the entrance of the analyzer, only the ion molecules of the analyte arrive.

There are several kinds of analyzers such as Quadrupole (Q), Time of Flight (TOF), Ion Trap (IT) and Orbitrap.

For this study, the analyzer was a combined Quadrupole-Time of Flight (Q-TOF) one.

The Quadrupole analyzer is constituted by four metal bars which are parallel among them two by two. The horizontal bars undergo a continuous voltage having the same charge of the ions of the analyte while the vertical bars undergo an alternated charge voltage.

All of this generates an electric field which forces the ions of the analyte to go an oscillating trajectory which is different for each *m/z* ratio. In particular, ions which have an *m/z* value too different form the set one between specific ranges are taken away and don't reach for the detector because they are forced to crash with the metal bars which dissipate their charge and are then expelled.

Anyway, different ions are affected by the electric field in different way. In fact, small ions are affected by this in a minor way in respect to the big ones which are affected in a big way. This fact causes that all the ions of the analyte present in the sample exit the Quadrupole in different moments and this goes against the base principle of the technique which requires an only measurement and not a scan.

For this reason, several expedients have been taken over the years in order to avoid this such as the use of multiple Quadruples in series and with the passing of time even newer and more sophisticated ones are replacing the old.

On the other hand, the TOF analyzer is a quite long tube which is run all along by a voltage. This voltage permits the ions to pass but, also in this case, ions having different *m/z* values will need different times to go through it.

At the end of the tube, the detector is placed and again we would have a scan and not a measurement.

To avoid this, two modifications have been created: the reflectron TOF and the orthogonal TOF.

For this study, the reflectron TOF was used. This modification consists, in practice, of the fact that at the end of the tube the detector is no longer present but rather an ion trap is.

This ion trap which presents a voltage having an opposite charge to that of the analyte ions has the task to catch and keep the analyte ions and the smaller is the analyte ion which enters the trap the bigger is the force of attraction that the trap employs to keep it.

In this view, the use of this ion trap is fantastic because one small ion will need little time to go through the tube but its permanence in the ion trap is longer and stronger than a big ion. In fact, while the small ion is kept in the trap, the big ion has all the time to reach for it in the same trap and is kept with a minor force.

When all the ions enter the trap, its voltage is changed coming to be of the same charge sign of the analyte ions. This causes all the analyte ions to be now expelled form the trap and to be forced to go through another tube which is parallel to the first one. The ions go through this tube because the original one is closed and because at the end of this second tube there is an opposite voltage which attracts them.

Yet, things are now changed since the analyte ions that first exit the trap are the big ones which were kept with a minor force. So the big ions advance but this fact doesn't change their speed which is slow as usual.

After some time, also the small analyte ions exit the traps and their speed is high as usual. Yet, differently from before, these are now behind the big ones and so, at the end of this second tube, they will arrive simultaneously having an only measurement.

Also the detector may be of different kind but it is usually a thermocouple which has the task to multiply the signal and let it arrive to the elaborator which is a computer.

The elaborator provides a chromatogram or mass spectrum which reports on the y axis, the relative abundance of the peak and on the x axis, the *m/z* value for each molecule present in the sample.

This *m/z* value is correlated to the chemical structure of the molecule and to its ionization conditions. Instead, the relative abundance of the peak is correlated to the amount of that molecule present in the sample but also to the easiness of that same molecule to ionize in that positive or negative mode.

For this reason, no quantitative analysis can be performed by MS analysis.

Actually the *m/z* value which is reported on the mass spectrum is not the real value for what concerns the molecular weight. In many occasions, it corresponds to the formation of particular adducts during the passage of the ion through the instrument.
In our operative conditions, with an ESI source and a Q-TOF analyzer, the *z* value is 1 since ESI is not able to produce multi-charged compounds, and so the *m/z* value is the *m* value formed by the analyte plus Proton, Natrium or Potassium, in positive ion mode or minus one Proton in negative ion mode.
For this reason, MS experiments are very important because they can give informations at least on the classes of compounds the studied molecule belongs to.
Moreover, they are very useful to confirm the structure hypothesis derived from the direct study of the results of other spectroscopic techniques (Cotter, 1994; Kenneth and Rubinson, 2002).

5.4.5. Preparation of the samples

5.4.5.1. For Column Chromatography

The standard procedure of preparation of the samples for the column chromatography separation method in silica gel which corresponds to the only kind of Column Chromatography employed during this study, is the following:

- the sample is inserted in a flask of proper dimensions according to the amount of it to be used
- this is solubilized in the minimal amount of solvent which is usually chloroform, methanol or distilled water
- at this point, silica gel is added until no liquid is still visible
- the mixture is shaken and the solvent is evaporated at reduced pressure by Rotavapor in order to avoid problems of interference with the eluting system of the chromatographic column which might cause little or no separation
- a very thin powder-like solid is obtained
- a proper column is chosen according to the amount of silica gel to insert in and a piece of cotton is put in it at the plug level. This is to avoid the total simultaneous elution of everything from the column

- silica gel, previously solubilized in the minimal amount of eluting system, chosen after preliminary tests (usually TLC on the total extract), is inserted in this column and is packed in it in the most homogenous way possible. This is important in order to avoid the formation of bubbles which may cause the formation of preferential paths within the column itself and the non-correct separation of the compounds in the analyte mixture. For this, it is usually advised to pour all the solubulized silica gel in one only time and to shake it through the column with the aid of a gum tube.
- once silica gel is well packed in the chromatographic column (which can be seen by touching the upper part of the sedimentation with a glass bar and try its resistance which should be very strong if the packing is complete), the whole is left so for at least five minutes in order to let silica gel really stabilize itself in it. During this passage, particular attention must be put to always leave a little amount of eluting system above the deposited silica gel. In fact, if this should ever finish, the air entering from the free upper extremity of the chromatographic column, would surely cause fractures in the deposited silica gel which would lead to the formation of air bubbles in it and, by consequence, of preferential paths as previously described
- after this period, the mixture composed by the sample and silica gel can be poured in the chromatographic column and this is also packed with the same methodology already described.
- when this is also packed, the final surface of the sedimentation is covered by a piece of cotton which protects it from air and from the pressure caused by the continuous adding of the eluting system for more days. Also in this case, a little amount of liquid must be always present over this point for the same reasons of before.
- at this point, the chromatographic column is ready and at the proper time, fractions can be collected

5.4.5.2. For HPLC analysis

The standard procedure of preparation of the samples for HPLC analysis is the following:

- the sample to analyze is inserted in a flask with proper dimensions and previously weighed
- the sample is solubilized in the minimal amount of solvent

- at this point, this is concentrated at reduced pressure by Rotavapor and by a high vacuum pump in order to eliminate all the solvent traces
- the flask is re-weighed in order to have the weight of the sample obtained by difference between the current weight and the initial one
- a solution of the sample is then prepared by pouring the wanted volume of solvent in its HPLC purity grade
- the solution is left so for at least ten minutes in order to have the total solubilization of the material
- at this point, a small portion of it (usually a few μLs) is taken and this is directly injected in the HPLC instrument

5.4.5.3. For NMR analysis

The standard procedure of preparation of the samples for NMR analysis is the following:

- the liquid sample containing the interest metabolites is inserted in a calibrated flask
- this is concentrated at reduced pressure by Rotavapor until a dried extract is obtained. For the only fractions deriving from separations in which *n*-butanol was used, this passage is not enough itself. In fact, after the dried extract is obtained, a triple wash with distilled water is necessary in order to eliminate every trace of *n*-butanol which not only causes problems for the next passage, but is also able to cause problems for the final analysis since this solvent present NMR peaks in areas of the spectrum where other interest signals are present, thus creating confusion of assignment
- at this point, solubility tests are performed in order to verify the best solvent which perfectly or at least better solubilizes the sample. Usually the solvents used for this are chloroform, methanol and distilled water.
- after choosing the best solvent, the sample is properly washed with 0.5 mL of it for three times in order to make it more soluble in the corresponding deuterated solvent in the next passage
- at every wash, the sample is concentrated at reduced pressure while, after the last time, the sample is also concentrated at the high vacuum pump in order to eliminate every trace of the solvent. In fact, if this should be also present during the NMR experiment, there would be problems in setting the most proper parameters for it and the analysis might be affected

- then, the flask is weighed and the real weight of the sample is taken as described in the previous section. For better experiments, this weight should be comprised between the range 20-50 mg
- at this point, the sample is solubilized in 0.5 mL of the chosen deuterated solvent and is poured in an NMR tube which is later closed, labeled and analyzed

5.4.5.4. For MS analysis

The standard procedure of preparation of the samples for MS analysis is the following:

- a small amount of the liquid sample consisting in one or two μLs, is taken directly from the NMR tube or from the flask containing it by a capillary tube and poured in a test tube
- in this, a quantity of about 2 mL of methanol having HPLC purity grade, is also poured
- the test tube is shaken in order to provide the best solubilization possible of the two different components
- then, the test tube is closed, labeled and analyzed

5.5. Supplementary Material

In this chapter, all the specific details of the work conducted on each plant material are reported. These concern both the chromatographic and spectrometric sections.
For what concerns the procedures, these are the same as described throughout chapter **5.4**.
As for the identification of metabolites, this was achieved by direct comparison of our spectroscopic data with those reported in literature and/or with spectroscopic data of standards present in our laboratory.
In this chapter, the NMR and MS data of all the identified compounds are also reported. Anyway, due to their frequent occurrence, for each compound, these data are given but not for every plant meaning that the NMR and MS data of same compounds are reported only once in the chapter referring to those data every time that the compound was evidenced again in other species.

5.5.1. *Ajuga chamaepitys* (L.) Schreb - aerial parts

A portion of 10.0 g of the crude extract from the aerial parts obtained as described in paragraph **5.4.1.**, was treated with a mixture of active charcoal/celite/polyamide in ratio 10:1:1 for the total weight of 15.1 g according to the method described in the paragraph **5.4.3.**
The desorption of the constituents was performed by mixtures of EtOH/H_2O at the concentration of EtOH equal to 30, 60 and 95 %.
Three fractions at different polarity were then collected and, always according to the procedure described in the paragraph **5.4.3.**, 3.80, 0.90 and 0.18 g of the total dried crude extracts were obtained.
After a careful analysis by spectrometric and chromatographic means (TLC, Paper Chromatography), it was decided to fractionate the sample deriving from the desorption by the mixture water-ethanol in ratio 70:30 (v/v).

On a portion of this, for the weight of 2.0 g, a column chromatography on silica gel was conducted.
The weight of silica gel was 60.0 g (ratio 1:30 w/w) and *n*-butanol saturated with water (82:18, v/v) was the eluting system.
From this chromatographic step, one hundred and thirty fractions were collected.
After TLC analysis, these were collected putting together those having the same Rf values, as follows (Table 21):

Fractions	Weight (mg)
1-7	26.9
8-9	37.0
10-19	52.3
20	20.0
21-25	49.9
26	28.6
27-50	43.0
51-60	16.0
61-64	6.0
65-80	19.5
81-99	12.2
100-130	300.0

Table 21: Fraction assemblies and corresponding weights for chromatographic column 1

From fraction 20, the presence of a mixture of **ajugoside** and **reptoside** (Guiso et al., 1974) (Figures 102, 125), in ratio 1:1, was evidenced.

Ajugoside: NMR and MS data are reported in paragraph **5.5.8.** at page 158.

Reptoside: NMR and MS data are reported in paragraph **5.5.12** at page 183.

From fraction 26, the presence of a mixture of **harpagide** and **8-*O*-acetyl-harpagide** (Scarpati et al., 1965) (Figures 104, 105), in ratio 1:1, was evidenced.

Harpagide: NMR and MS data are reported in paragraph **5.5.8.** at page 160.

8-*O*-acetyl-harpagide: NMR and MS data are reported in paragraph **5.5.8.** at pages 161-161.

Since not all the present compounds could be clearly identified from this separation step, a second column chromatography was performed on an assembly of fractions deriving from the first one, 100-130, for the total weight of 300.0 mg.
The correspective amount of silica gel was 7.5 g (ratio 1:25 w/w), this time, and the eluting system consisted of a mixture of *n*-butanol/methanol/water 70:10:30 (v/v/v).
From this chromatographic step, thirty-nine fractions were collected.
After TLC analysis, these were collected putting together those having the same Rf values, as follows (Table 22):

Fractions	Weight (mg)
1-8	7.8
9-11	20.0
12-30	87.9
31-39	10.3

Table 22: Fraction assemblies and corresponding weights for chromatographic column 2

From the assembly of fractions 9-11, **8-*O*-acetyl-harpagide** (Scarpati et al., 1965) (Figure 105), was detected as almost pure compound

8-*O*-acetyl-harpagide: NMR and MS data are reported in paragraph **5.5.8.** at pages 160-161.

Since not all the present compounds could be clearly identified from this separation step, again, a third column chromatography was performed on an assembly of fractions deriving from the second one, 12-30, for the total weight of 87.9 mg.

The correspective amount of silica gel was 5.0 g (ratio 1:57 w/w), this time, and the eluting system consisted of a mixture of *n*-butanol/methanol/water 70:10:25 (v/v/v).

From this chromatographic step, sixty-eight fractions were collected.

After TLC analysis, these were collected putting together those having the same Rf values, as follows (Table 23):

Fractions	Weight (mg)
1-8	1.4
9-12	1.7
13-15	5.4
16-20	4.9
21-23	18.7
24-29	16.6
30-32	13.3
33-41	12.1
42-44	4.0
45-68	21.2

Table 23: Fraction assemblies and corresponding weights for chromatographic column 3

From the assembly of fractions 13-15, **asperulosidic acid** (Bianco et al., 1978) (Figure 61) (Spectrum 1) was evidenced as almost pure compound

Asperulosidic acid: ^{1}H-NMR (300MHz, D_2O) δ: 7.58 (1H, s, H-3), 6.10 (1H, s, H-7), 5.22 (1H, s, H-1), 4.90 (1H, d, *J* = 7.8 Hz, H-1'), 4.23 (2H, dd, *J* = 15.6/7.9 Hz, H-10), 3.12 (1H, t, *J* = 7.1 Hz, H-5), 2.66 (1H, br. t, *J* = 9.5 Hz, H-9), 2.15 (3H, s, OAc).

ESI-MS: *m/z* 455.10 $[M+Na]^+$; *m/z* 431.11 $[M-H]^-$.

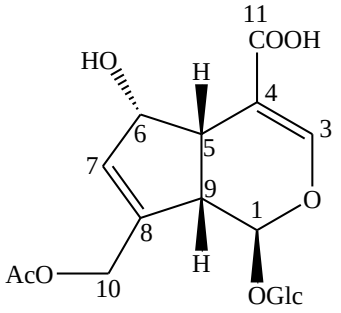
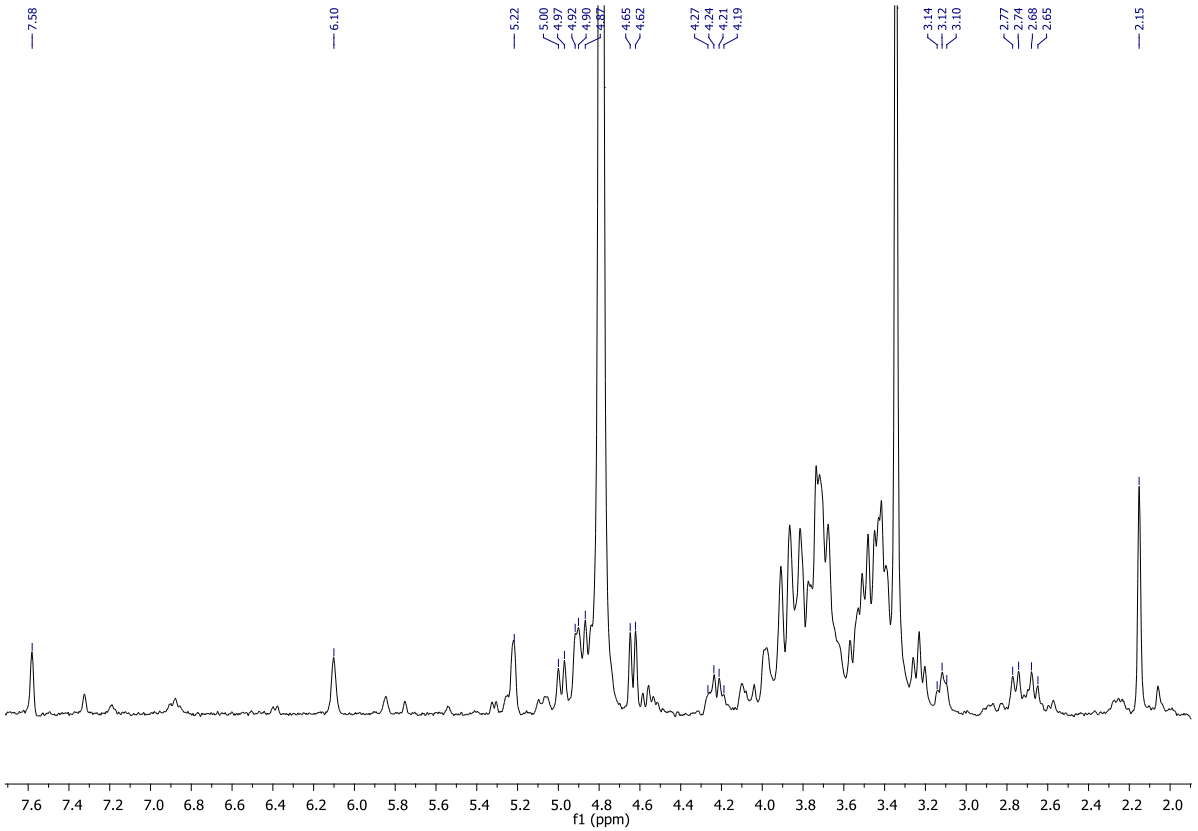


Figure 61: Structure of **asperulosidic acid**



Spectrum 1: ^{1}H-NMR of **asperulosidic acid**

From the assembly of fractions 21-23 (Spectra 2, 3), a mixture of **deacetyl-asperulosidic acid** (Bianco et al., 1978) (Figure 64) and of **5-*O*-β-D-glucopyranosyl-harpagide** (Figure 62), in ratio 1:4, was evidenced.

Deacetyl-asperulosidic acid: NMR and MS data are reported in paragraph **5.5.2.** at page 127.

5-*O*-β-D-glucopyranosyl-harpagide: ^{1}H-NMR (400 MHz, D_2O) δ: 6.51 (1H, d, *J* = 6.5 Hz, H-3), 5.76 (1H, d, *J* =1.5 Hz, H-1), 5.26 (1H, dd, *J* = 6.5/1.2 Hz, H-4), 4.92 (1H, d, *J* = 8.0 Hz, H-1), 4.82 (1H, d, *J* = 8.1 Hz, H-1), 4.03 (1H, m, H-6), 2.92 (1H, br. s, H-9), 2.01 (2H, br. t, *J* = 5.0 Hz, H-7), 1.33 (3H, s, H-10).
^{13}C-NMR (100 MHz, D_2O) δ: 142.8 (C-3), 104.2 (C-4), 97.4 (C-1), 98.0 (C-1), 92.7 (C-1), 78.6 (C-5), 76.9 (C-8), 76.3 (C-5), 76.1 (C-5), 75.9 (C-3), 75.8 (C-6), 75.4 (C-3), 73.3 (C-2), 72.9 (C-2), 69.5 (C-4), 69.2 (C-4), 60.7 (C-6), 60.4 (C-6), 55.7 (C-9), 45.1 (C-7), 24.4 (C-10).
ESI-MS: *m/z* 549.15 $[M+Na]^+$; *m/z* 525.19 $[M-H]^-$.
HR-ESI-MS: *m/z* 549.1804 $[M+Na]^+$ ($C_{21}H_{34}O_{15}Na$).

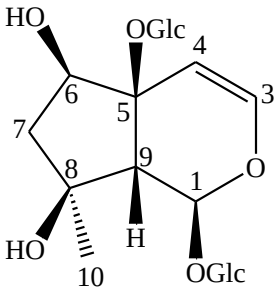
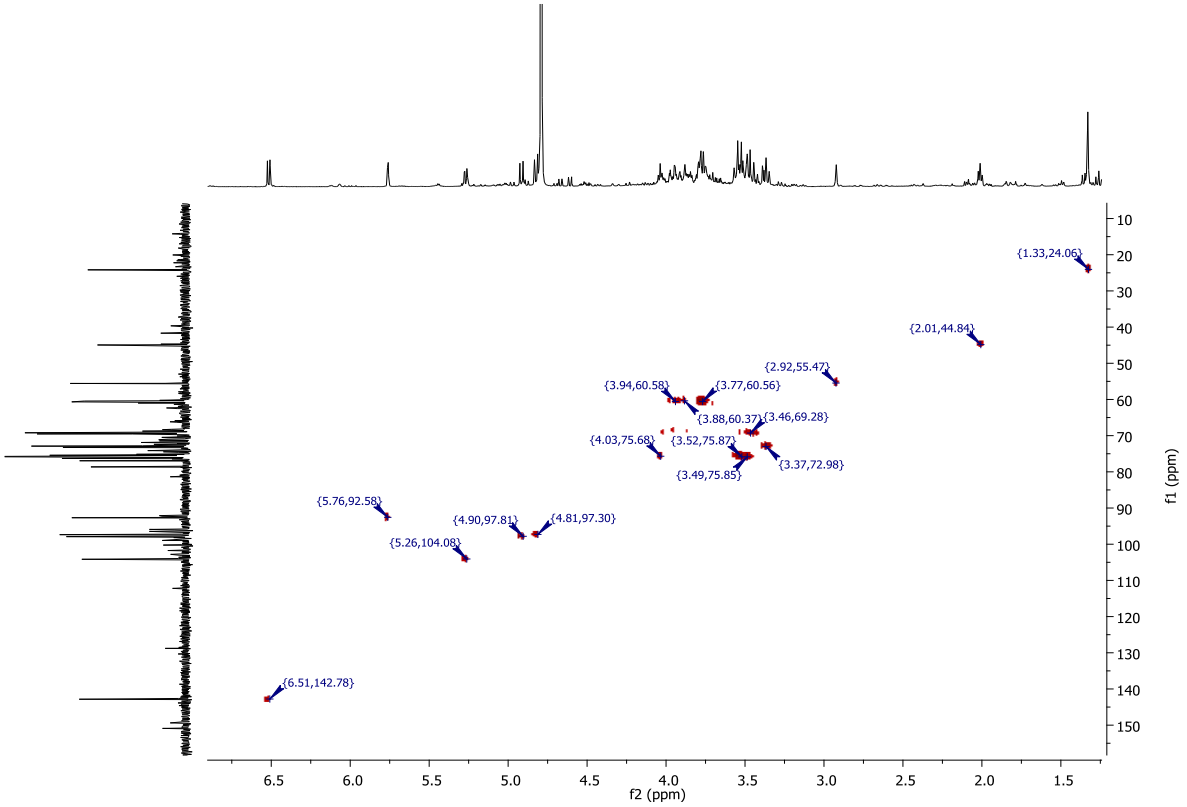
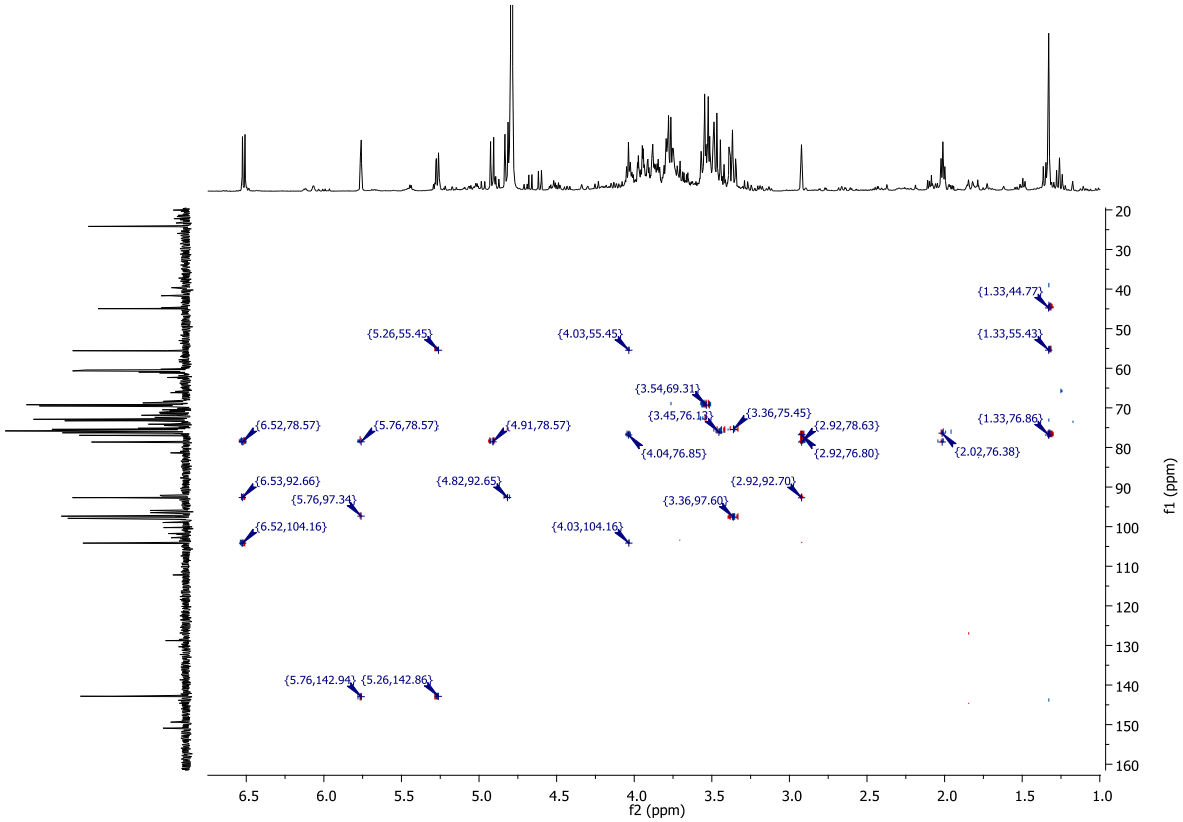


Figure 62: Structure of **5-*O*-β-D-glucopyranosyl-harpagide**



Spectrum 2: HSQC spectrum of the assembly of fractions 21-23



Spectrum 3: HMBC spectrum of the assembly of fractions 21-23

5.5.2. *Ajuga chamaepitys* (L.) Schreb - roots

An aliquot of 4.0 g of the crude extract obtained from the roots as described in paragraph **5.4.1.**, was treated with the adsorbing mixture composed by active charcoal/celite/polyamide for the total weight of 6.1 g according to the method reported in the paragraph **5.4.3**.

The desorption of the constituents was performed by an only mixture of EtOH/H_2O at the concentration of EtOH equal to 30 %.

One fraction at different polarity was then collected and, always according to the procedure described in the paragraph **5.4.3.**, 0.80 g of the total dried cool extract was obtained.

On the total amount of this, a column chromatography on silica gel for the weight of 16.0 g (ratio 1:20 w/w) and using *n*-butanol saturated with water (82:18, v/v) as eluting system, was conducted

From this chromatographic step, one-hundred and forty fractions were collected.

After TLC analysis, these were collected putting together those having the same Rf values, as follows (Table 24):

Fractions	Weight (mg)
1-8	16.4
9-15	22.8
16-29	31.1
30-42	22.9
43-68	39.9
69-93	41.0
94-104	34.6
105-128	36.6
129-131	8.0
132-140	7.7

Table 24: Fraction assemblies and corresponding weights for chromatographic column 1

From the assembly of fractions 9-15, **8-*O*-acetyl-harpagide** (Guiso et al., 1974) (Figure 105), was evidenced as almost pure compound

8-*O*-acetyl-harpagide: NMR and MS data are reported in paragraph **5.5.8.** at pages 160-161.

From the assembly of fractions 30-42, a mixture of **harpagide** and **8-*O*-acetyl-harpagide** (Guiso et al., 1974) (Figures 104, 105), in ratio 2:1, was evidenced.

Harpagide: NMR and MS data are reported in paragraph **5.5.8.** at page 160.

8-*O*-acetyl-harpagide: NMR and MS data are reported in paragraph **5.5.8.** at pages 160-161.

From the assembly of fractions 94-104 (Spectra 4, 5), a mixture of **5-*O*-β-D-glucopyranosyl-harpagide** (Figure 62) and of **5-*O*-β-D-glucopyranosyl-8-*O*-acetyl-harpagide** (Figure 63), in ratio 1.5:1, was evidenced.

5-*O*-β-D-glucopyranosyl-harpagide: NMR and MS data are reported in paragraph **5.5.1.** at page 122.

5-*O*-β-D-glucopyranosyl-8-*O*-acetyl-harpagide: ^{1}H-NMR (400 MHz, D_2O) δ: 6.48 (1H, d, *J* = 6.4 Hz, H-3), 6.12 (1H, br. s, H-1), 5.04 (1H, dd, *J* = 6.4/1.5 Hz, H-4), 4.88 (1H, d, *J* = 7.9 Hz, H-1), 4.81 (partially overlapped with HDO, H-1), 4.04-3.36 (overlapped sugars signals), 3.87 (1H, m, H-6), 2.89 (1H, br. s, H-9), 2.24-2.17 (2H, m, H-7a, H-7b), 2.08 (3H, s, CH_3CO), 1.48 (3H, s, H-10).

^{13}C-NMR (100 MHz, D_2O) δ : 173.5 (OAc), 142.6 (C-3), 105.0 (C-4), 98.9 (C-1), 98.6 (C-1), 93.7 (C-1), 87.8 (C-8), 78.6 (C-5), 76.9 (C-5), 76.2 (C-6), 75.8 (C-5), 75.6 (C-3), 75.4 (C-3), 73.2 (C-2), 72.9 (C-2), 69.5 (C-4), 69.2 (C-4), 61.2 (C-6), 61.1 (C-6), 53.0 (C-9), 44.3 (C-7), 21.6 (OAc), 21.3 (C-10).

HR-ESI-MS: *m/z* 591.1901 $[M+Na]^+$ ($C_{23}H_{36}O_{16}Na$).

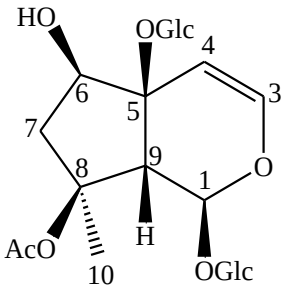
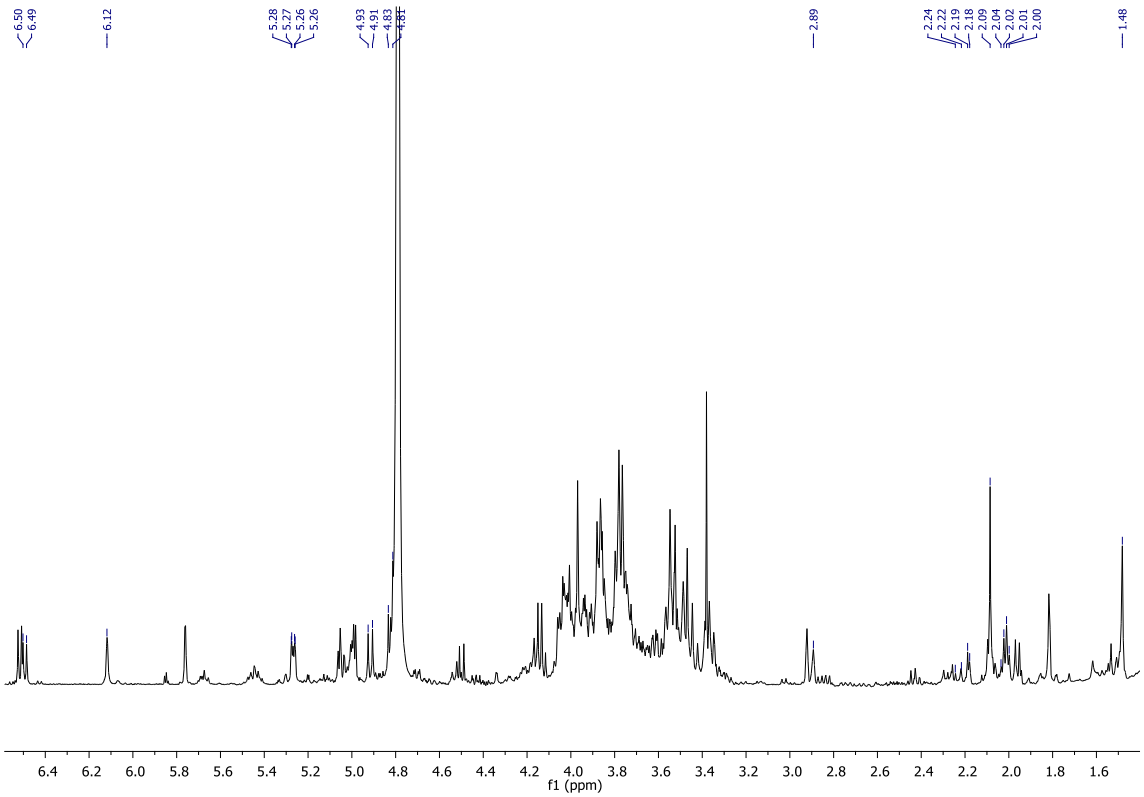
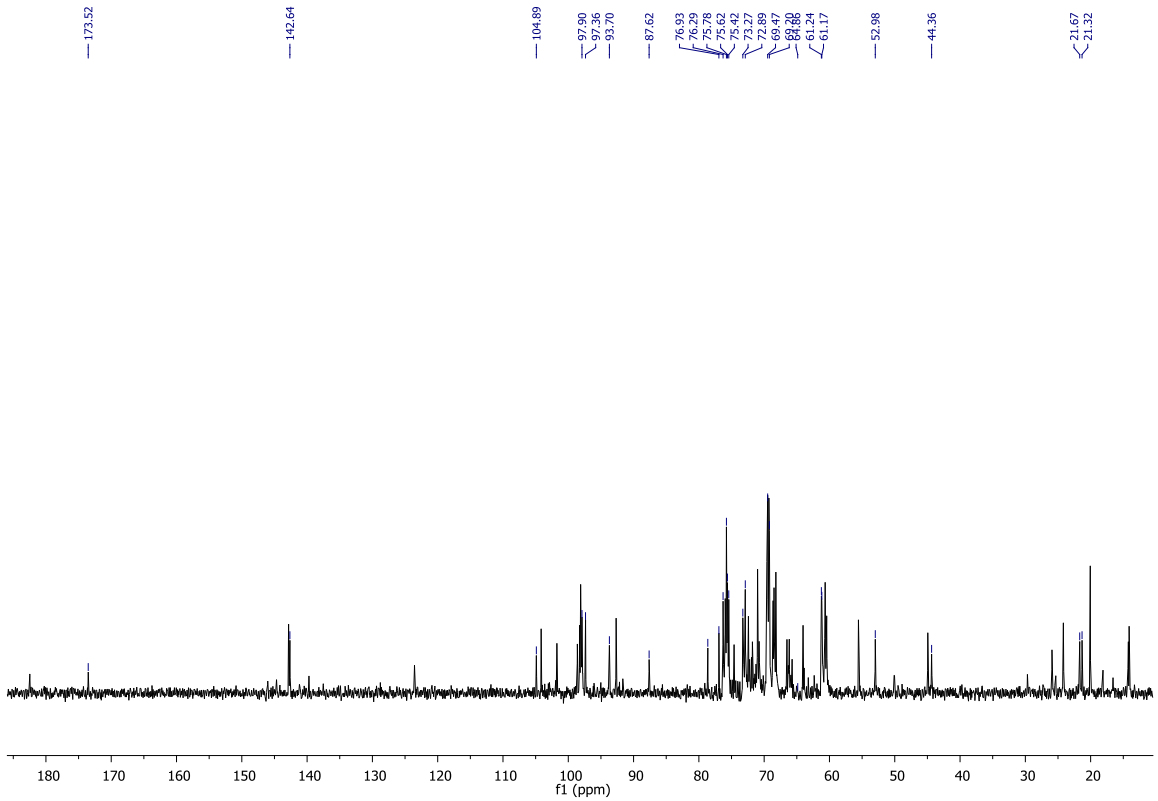


Figure 63: Structure of **5-*O*-β-D-glucopyranosyl-8-*O*-acetyl-harpagide**



Spectrum 4: ^{1}H-NMR of the assembly of fractions 94-104



Spectrum 5: ^{13}C-NMR of the assembly of fractions 94-104

From the assembly of fractions 129-131, **deacetyl-asperulosidic acid** (Bianco et al., 1978) (Figure 64) (Spectrum 6) was evidenced as almost pure compound.

Deacetyl-asperulosidic acid: ^{1}H-NMR (300 MHz, D_2O) δ: 7.48 (1H, s, H-3), 6.04 (1H, s, H-7), 5.24 (1H, d, *J* = 4.0 Hz, H-1), 4.96 (1H, d, *J* = 8.0 Hz, H-1'), 4.29 (2H, d, *J* = 14.5 Hz, H-10), 3.11 (1H, br. t, *J* = 7.3 Hz, H-5), 2.64 (1H, br. t, *J* = 7.8 Hz, H-9).
ESI-MS: *m/z* 549.15 $[M+Na]^+$; *m/z* 525.19 $[M-H]^-$.

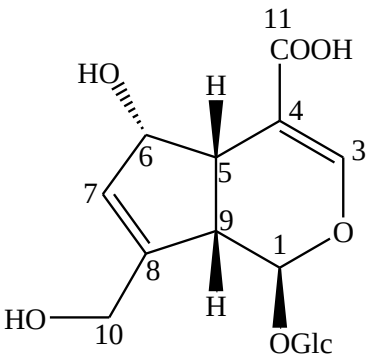
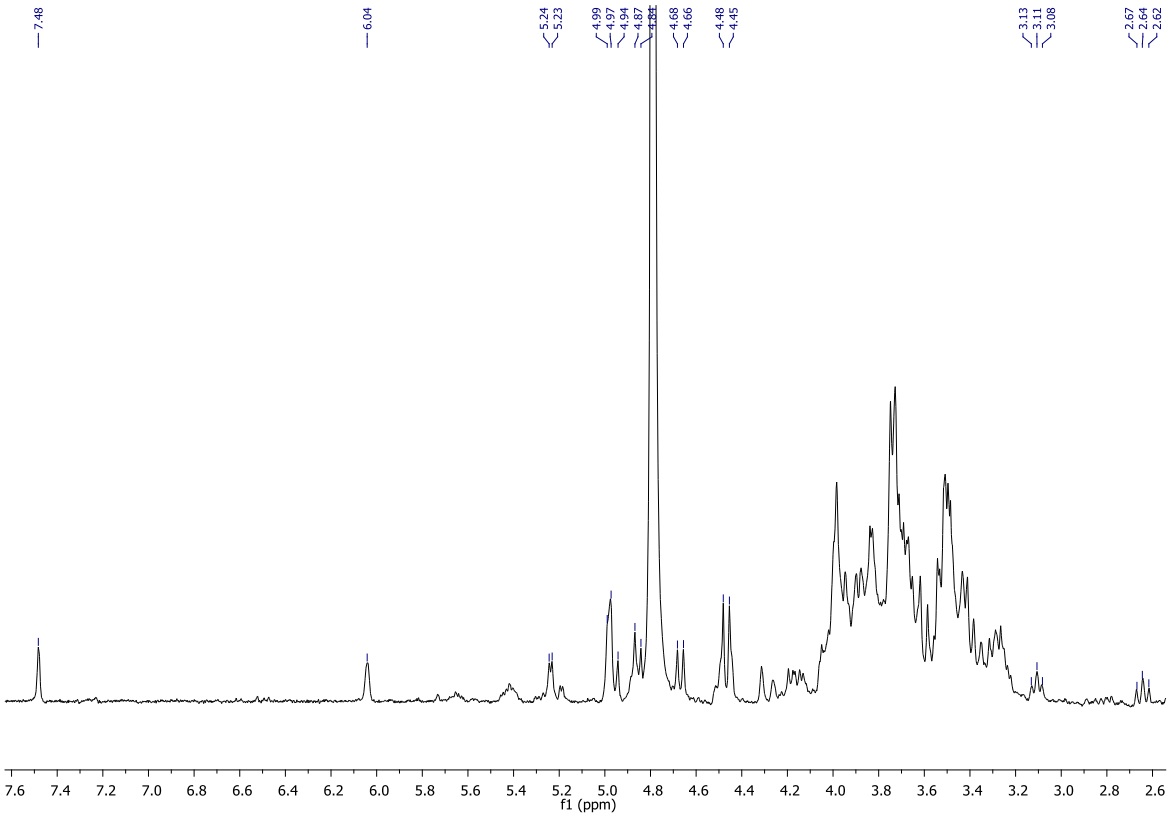


Figure 64: Structure of **deacetyl-asperulosidic acid**



Spectrum 6: ^{1}H-NMR of **deacetyl-asperulosidic acid**

5.5.3. *Ajuga genevensis* L.

The total dried crude extract obtained as described in paragraph **5.4.1.** for the weight of 0.64 g was subjected to a first chromatographic separation using a correspective amount of silica gel of 15.2 g (ratio 1:25) and a mixture of *n*-butanol and distilled water in concentration ratio 82:18 (v/v) as eluting system.

From this chromatographic step, eighty-two fractions were collected.

After TLC analysis, these were collected putting together those having the same Rf values, as follows (Table 25):

Fractions	Weight (mg)
1-2	15.7
3-14	49.4
15-16	10.1
17-24	14.6
25-28	14.2
29-32	12.3
33-38	6.9
39-56	9.5
57-82	22.7

Table 25: Fraction assemblies and corresponding weights for chromatographic column 1

From the assembly of fractions 17-24, **rosmarinic acid** (Ticli et al., 2005) (Figure 65) (Spectra 7, 8) was evidenced as almost pure compound.

Rosmarinic acid: ^{1}H-NMR (300 MHz, D_2O) δ: 7.32 (1H, d, *J* = 15.9 Hz, H-β), 6.93 (1H, d, *J* = 1.8 Hz, H-2''), 6.81 (1H, dd, *J* = 8.2, 1.8 Hz, H-6''), 6.76 (1H, d, *J* = 1.6 Hz, H-2'), 6.75 (1H, d, *J* = 8.0 Hz, partially overlapped with H-2', H-5''), 6.68 (1H, d, *J* = 8.0 Hz, H-5'), 6.62 (1H, dd, *J* = 8.0, 1.6 Hz, H-6'), 6.11 (1H, d, *J* = 15.9 Hz, H-α), 4.99 (1H, dd, *J* = 9.4/3.3 Hz, H-2), 3.06 (1H, dd, *J* = 14.1/3.3 Hz, H-3a), 2.95 (1H, dd, *J* = 14.1/9.4 Hz, H-3b).
^{13}C-NMR (75 MHz, CD_3OD), δ: 176.9 (C-1), 168.9 (C-4), 147.0 (C-4'), 146.2 (C-3'), 144.2 (C-β), 143.8 (C-4''), 142.7 (C-3''), 130.1 (C-1'), 127.0 (C-1''), 122.7 (C-6'), 121.9 (C6''), 117.3 (C-2'), 116.2 (C2''), 116.1 (C5''), 115.2 (C-5'), 114.2 (C-α), 76.2 (C-2), 36.83 (C-3).
ESI-MS: *m/z* 383.05 $[M+Na]^+$; *m/z* 358.98 $[M-H]^-$.

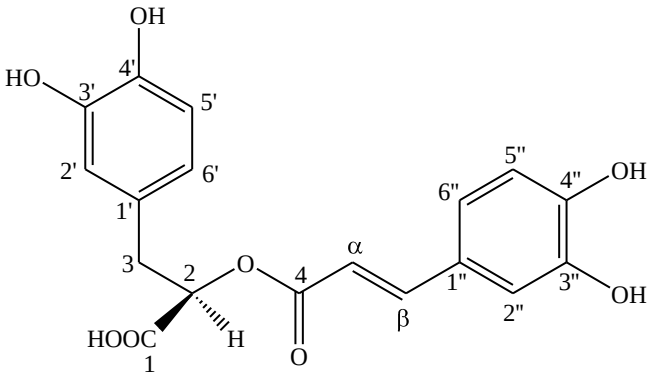
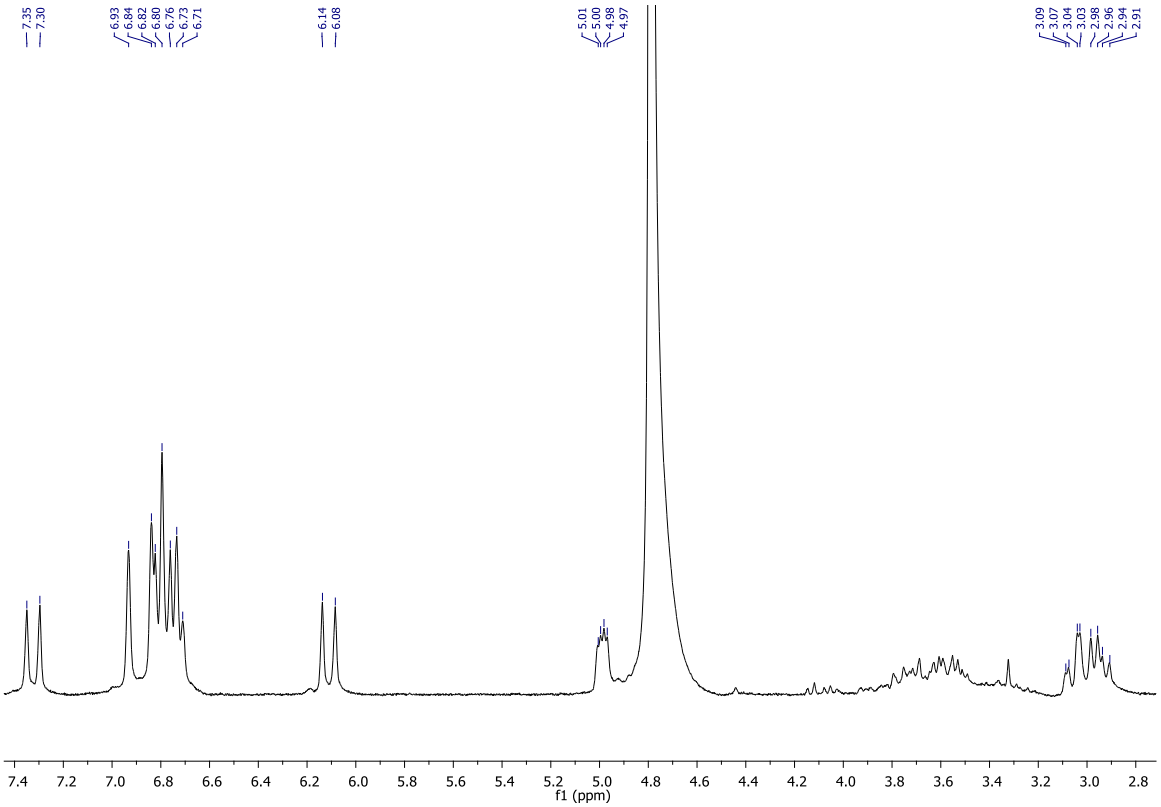
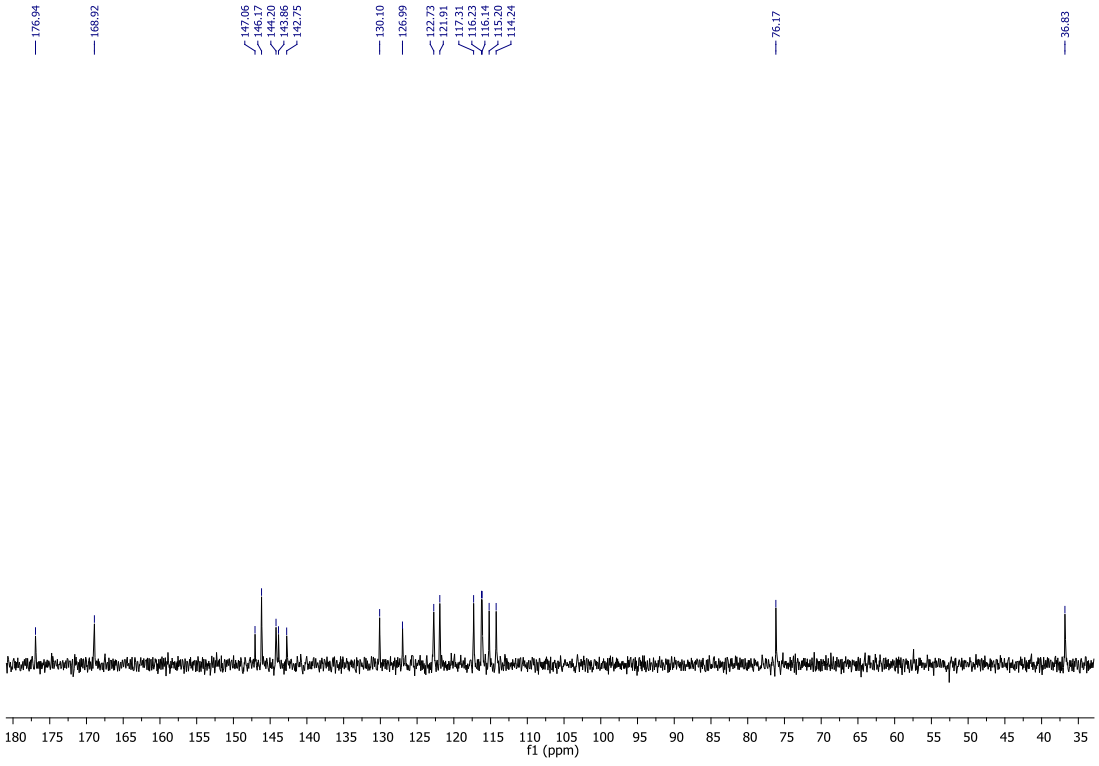


Figure 65: Structure of **rosmarinic acid**



Spectrum 7: ^{1}H-NMR spectrum of **rosmarinic acid**



Spectrum 8: ^{13}C-NMR spectrum of **rosmarinic acid**

Since not all the present compounds could be clearly identified from this separation step, a second column chromatography was performed on an assembly of fractions deriving from the first one, 3-14, for the total weight of 49.4 mg.

The correspective amount of silica gel was 1.45 g (ratio 1:30 w/w), this time, and the eluting system consisted of a mixture of chloroform/methanol at different concentrations.

The initial one was 99:1 (v/v) but during the chromatographic run, this was changed in order to raise the polarity of the solution and let the elution of the most polar compounds, to 95:5 (v/v), 9:1 (v/v) and lastly 8:2 (v/v).

From this chromatographic step, seventy fractions were collected.

After TLC analysis, these were collected putting together those having the same Rf values, as follows (Table 26):

Fractions	Weight (mg)
1-8	1.7
9-15	1.5
16-21	3.1
22	1.1
23-29	3.9
30-33	7.5
36-51	8.9
52-53	1.5
54-70	10.1

Table 26: Fraction assemblies and corresponding weights for chromatographic column 1

From the assembly of fractions 30-33, **oleanolic acid** (Seebacher et al., 2003) (Figure 66) (Spectrum 9) was evidenced as almost pure compound.

Oleanolic acid: ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 5.27 (1H, br. t, *J* = 3.2 Hz, H-12), 3.22 (1H, dd, *J* = 9.6/4.3 Hz, H-3), 2.82 (1H, dd, *J* = 13.2/6.5 Hz, H-18), 1.19 (3H, s, H-27), 0.98 (3H, s, H-25), 0.95 (3H, s, H-30), 0.93 (3H, s, H-29), 0.85 (3H, s, H-26), 0.77 (3H, s, H-24).

ESI-MS: *m/z* 479.61$[M+Na]^+$; *m/z* 455.55 $[M-H]^-$.

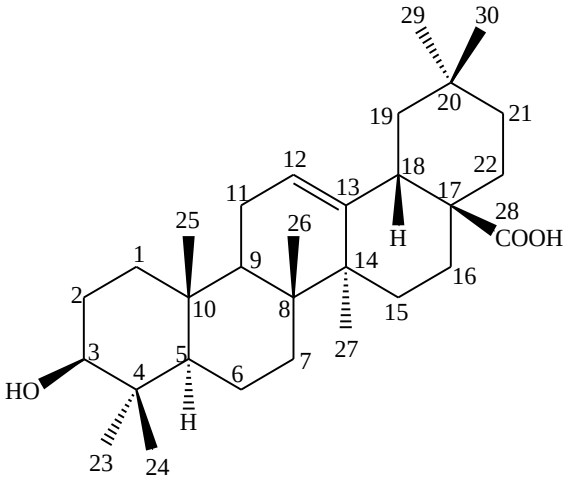
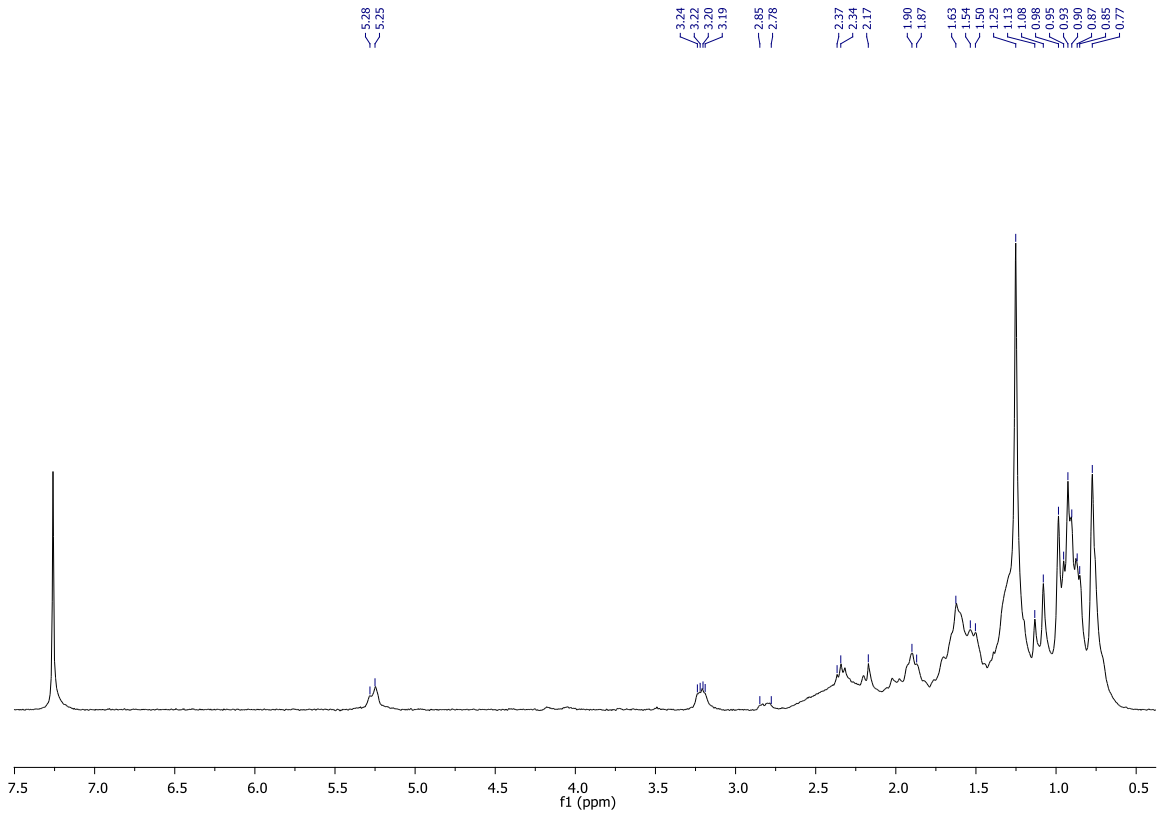


Figure 66: Structure of **oleanolic acid**



Spectrum 9: ^{1}H-NMR spectrum of **oleanolic acid**

From the assembly of fractions 52-53, **maslinic acid** (Taniguchi et al., 2002) (Figure 67) (Spectrum 10) was evidenced as almost pure compound, instead.

Maslinic acid: ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 5.26 (1H, br. t, *J* = 3.0 Hz, H-12), 3.65 (1H, m, H-2), 1.10 (3H, s, H-27), 1.02 (3H, s, H-23), 0.95 (3H, s, H-25), 0.88 (3H, s, H-30), 0.85 (3H, s, H-29), 0.79 (3H, s, H-24).

ESI-MS: *m/z* 495.30 $[M+Na]^+$; *m/z* 471.33 $[M-H]^-$.

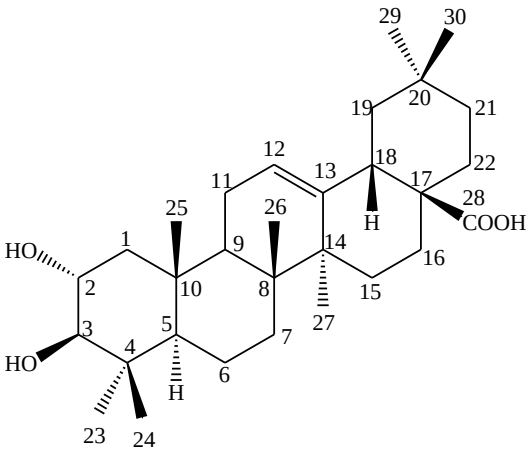
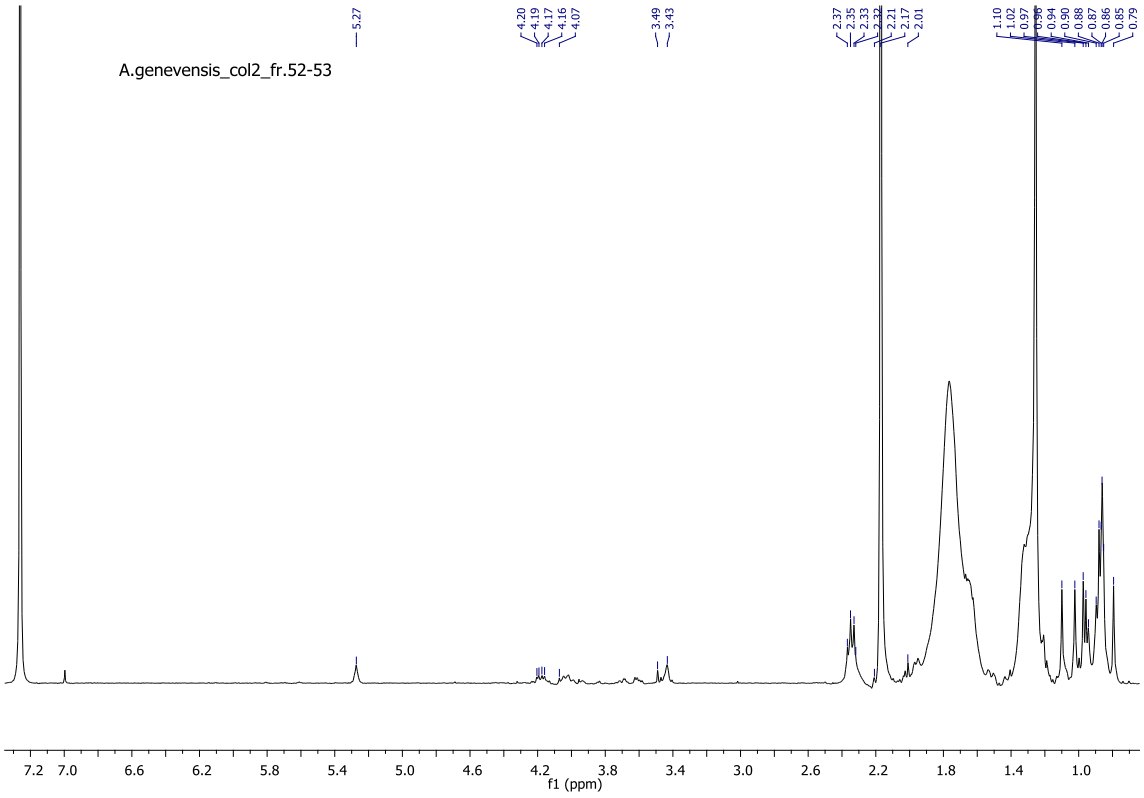


Figure 67: Structure of **maslinic acid**



Spectrum 10: ^{1}H-NMR spectrum of **maslinic acid**

5.5.4. *Ajuga reptans* L.

An aliquot of the total dried crude extract obtained as described in paragraph **5.4.1.**, for the weight of 2.4 g was subjected to a first chromatographic separation using a corresponding amount of silica gel of 96.2 g (ratio 1:40 w/w) and, as eluting system, a solution of *n*-butanol and distilled water at concentration 82:18 (v/v).

During the chromatographic run, the polarity of the eluting solution was raised in order to let the elution of the most polar compounds and so this became a solution of *n*-butanol, methanol and distilled water at concentration 70:10:30 (v/v/v).

From this chromatographic step, one hundred and fifty one fractions were collected.

After TLC analysis, these were collected putting together those having the same Rf values, as follows (Table 27):

Fractions	Weight (mg)
1-2	6.6
3-31	298.2
32-41	7.9
42-46	5.7
47-70	23.7
71-93	24.0
94-100	4.9
101-144	21.1
145-151	8.8
Methanol column wash	57.9

Table 27: Fraction assemblies and corresponding weights for chromatographic column 1

From the assembly of fractions 42-46, **ajugoside** (Venditti et al., 2016d) (Figure 102), was evidenced as almost pure compound.

Ajugoside: NMR and MS data are reported in paragraph **5.5.8.** at page 158.

From the assembly of fractions 42-46, **harpagide** and **8-*O*-acetyl-harpagide** (Venditti et al., 2016d) (Figures 104, 105), were evidenced in mixture in ratio 1:5.

Harpagide: NMR and MS data are reported in paragraph **5.5.8.** at page 160.

8-*O*-acetyl-harpagide: NMR and MS data are reported in paragraph **5.5.8.** at pages 160-161.

From the assembly of fractions 145-151, **caffeic acid** (Venditti et al., 2015b) (Figure 68), **harpagide** and **8-*O*-acetyl-harpagide** (**7**) (Venditti et al., 2016d) (Figures 104, 105), were evidenced in an only mixture in ratio 1:3:4.

Caffeic acid: ^{1}H-NMR (300 MHz, CD_3OD) δ: 7.60 (1H, d, *J* = 16.1 Hz, H-β), 7.07 (1H, br. s, H-2), 6.96 (1H, br. d, *J* = 8.1 Hz, H-6), 6.79 (1H, d, *J* = 8.1 Hz, H-5), 6.31 (1H, d, *J* = 16.1 Hz, H-α).
ESI-MS: *m/z* 203.10 $[M+Na]^+$.

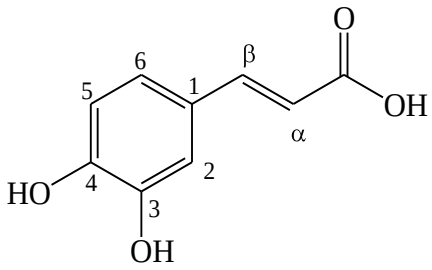


Figure 68: Structure of **caffeic acid**

Harpagide: NMR and MS data are reported in paragraph **5.5.8.** at page 160.

8-*O*-acetyl-harpagide: NMR and MS data are reported in paragraph **5.5.8.** at pages 160-161.

From the methanol column wash, an only mixture of **glucose** (Figure 69), **fructose** (Figure 70), **galactose** (Figure 71) **sucrose** (Figure 72), **raffinose** (Figure 73), **stachyose** (Figure 122), **D-mannitol** (Figure 74), **acetic acid** (Figure 75), **formic acid** (Figure 76), **fumaric acid** (Figure 77), **D-lactic acid** (Figure 78), **malic acid** (Figure 79), **pyruvic acid** (Figure 80), **succinic acid** (Figure 121), **α-hydroxy-butyric acid** (Figure 81), **γ-amino-butyric acid (GABA)** (Figure 82), **alanine** (Figure 83), **aspartic acid** (Figure 84), **asparagine** (Figure 85), **glutamine** (Figure 86), **lysine** (Figure 87), **threonine** (Figure 88), **tyrosine** (Figure 89) and **valine** (Figure 90) (McIntyre and Vogel, 1989; Sciubba et al., 2014a, 2014b; Venditti et al., 2017d), in ratio not detectable, was evidenced.

α-glucose: ^{1}H-NMR (400 MHz, D_2O) δ: 5.24 (1H, d, *J* = 3.7 Hz, H-1), 3.98 (1H, m, Ha-6), 3.87 (1H, m, H-5), 3.75 (1H, m, Hb-6), 3.70 (1H, m, H-3), 3.56 (1H, m, H-2), 3.41 (1H, m, H-4).

ESI-MS: *m/z* = 203.09 $[M+Na]^+$; *m/z* = 179.16 $[M-H]^-$.

β-glucose: ^{1}H-NMR (400 MHz, D_2O) δ: 4.63 (1H, d, *J* = 8.0 Hz, H-1), 3.90 (1H, m, Ha-6), 3.73 (1H, m, Hb-6), 3.51 (1H, m, H-3), 3.48 (1H, m, H-5), 3.42 (1H, m, H-4), 3.25 (1H, m, H-2).
ESI-MS: *m/z* = 203.09 $[M+Na]^+$; *m/z* = 179.16 $[M-H]^-$.

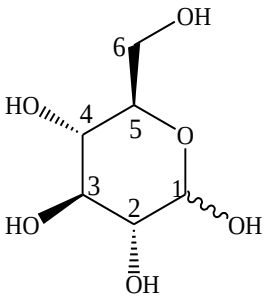


Figure 69: Structure of **glucose**

Fructose: ^{1}H-NMR (400 MHz, D_2O) δ: 4.17 (1H, d, *J* = 8.8 Hz, H-2), 4.03 (1H, m, Ha-5), 5.00 (1H, m, H-4), 3.91 (1H, m, H-3), 3.82 (1H, m, Hb-5), 3.57 (2H, m, H-6).
ESI-MS: *m/z* = 203.09 $[M+Na]^+$; *m/z* = 179.16 $[M-H]^-$.

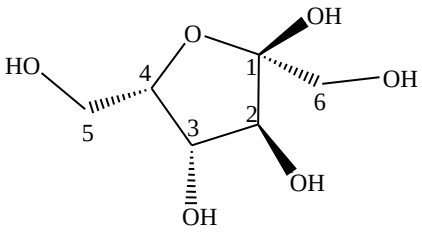


Figure 70: Structure of **fructose**

α-Galactose: ^{1}H-NMR (400 MHz, D_2O) δ: 5.25 (1H, d, *J* = 3.7 Hz, H-1), 4.07 (1H, m, H-5), 3.95 (2H, m, H-4), 3.84 (1H, m, H-3), 3.70 (2H, m, H-6), 3.48 (1H, m, H-2).
ESI-MS: *m/z* = 203.09 $[M+Na]^+$; *m/z* = 179.16 $[M-H]^-$.

β-Galactose: ^{1}H-NMR (400 MHz, D_2O) δ: 4.57 (1H, d, *J* = 8.0 Hz, H-1), 3.92 (1H, m, H-4), 3.74 (2H, m, H-6), 3.70 (1H, m, H-5), 3.64 (1H, m, H-3), 3.48 (1H, m, H-2).
ESI-MS: *m/z* = 203.09 $[M+Na]^+$; *m/z* = 179.16 $[M-H]^-$.

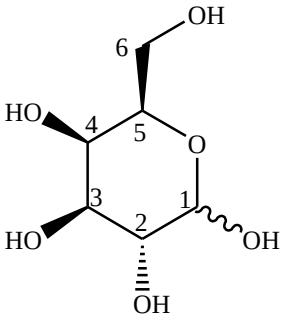


Figure 71: Structure of **galactose**

Sucrose: ^{1}H-NMR (400 MHz, D_2O) δ: 5.40 (1H, d, *J* = 3.9 Hz, H-1), 4.20 (1H, d, *J* = 8.8 Hz, H-2'), 4.07 (1H, m, H-3'), 3.90 (1H, m, H-4'), 3.85 (1H, m, H-5), 3.82 (4H, m, H-6 and H-5'), 3.79 (1H, m, H-3), 3.69 (2H, s, H-6'), 3.59 (1H, m, H-2), 3.48 (1H, m, H-4).
ESI-MS: *m/z* = 365.17 $[M+Na]^+$; *m/z* = 342.09 $[M-H]^-$.

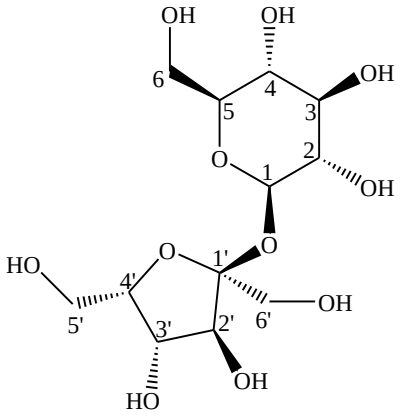


Figure 72: Structure of **sucrose**

Raffinose: ^{1}H-NMR (400 MHz, D_2O) δ: 5.44 (1H, d, *J* = 3.8 Hz, H-1'), 5.00 (1H, d, *J* = 3.7 Hz, H-1), 4.25 (1H, d, *J* = 8.7 Hz, H-2''), 4.06 (1H, m, H-3''), 4.04 (3H, m, Ha-5', Ha-6'), 3.94 (1H, m, H-5), 3.90 (2H, m, H-4'' and H-2), 3.85 (1H, m, H-3), 3.82 (2H, m, H-4 and H-5''), 3.80 (3H, m, H-6 and H-3'), 3.70 (1H, m, Hb-6'), 3.69 (2H, s, H-6''), 3.59 (1H, m, H-2).
ESI-MS: *m/z* = 527.24 $[M+Na]^+$; *m/z* = 503.29 $[M-H]^-$.

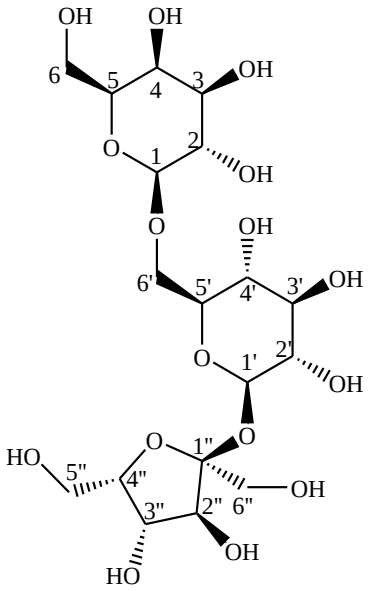


Figure 73: Structure of **raffinose**

Stachyose: NMR and MS data are reported in paragraph at page **5.5.11.** at page 180.

D-mannitol: ^{1}H-NMR (400 MHz, D_2O) δ: 3.85 (4H, m, H-1 and H-6), 3.78 (2H, m, H-2 and H-5), 3.73 (2H, m, H-3 and H-4).
ESI-MS: *m/z* = 205.20 $[M+Na]^+$; *m/z* = 181.17 $[M-H]^-$.

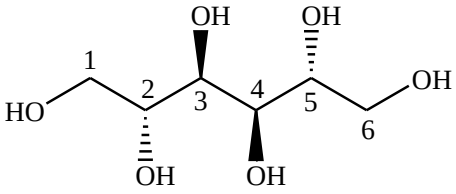


Figure 74: Structure of **D-mannitol**

Acetic acid: ^{1}H-NMR (400 MHz, D_2O) δ: 1.91 (3H, s, CH_3)
ESI-MS: *m/z* = 59.13 $[M-H]^-$.

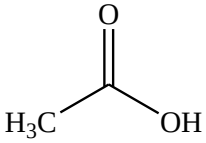


Figure 75: Structure of **acetic acid**

Formic acid: ^{1}H-NMR (400 MHz, D_2O) δ: 8.44 (1H, s, $HCOO^-$).

ESI-MS: *m/z* = 45.23 $[M-H]^-$.

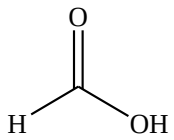


Figure 76: Structure of **formic acid**

Fumaric acid: ^{1}H-NMR (400 MHz, D_2O) δ: 6.48 (2H, s, H-α' and H-α").

ESI-MS: *m/z* = 115.09 $[M-H]^-$.

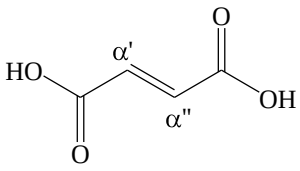


Figure 77: Structure of **fumaric acid**

D-Lactic acid: ^{1}H-NMR (400 MHz, D_2O) δ: 4.12 (1H, m, H-α), 1.30 (3H, d, *J* = 4.4 Hz, CH_3).

ESI-MS: *m/z* = 89.04 $[M-H]^-$.

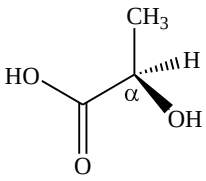


Figure 78: Structure of **D-lactic acid**

Malic acid: ^{1}H-NMR (400 MHz, D_2O) δ: 4.32 (1H, dd, *J* = 10.0/3.0 Hz, H-α), 2.70 (1H, dd, *J* = 15.4/3.0 Hz, H-β), 2.37 (1H, dd, *J* = 15.4/10.0 Hz, H-β').

ESI-MS: *m/z* = 133.07 $[M-H]^-$.

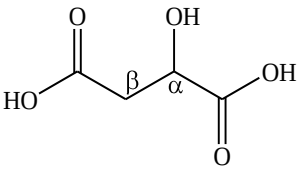


Figure 79: Structure of **malic acid**

Pyruvic acid: ^{1}H-NMR (400 MHz, D_2O) δ: 2.39 (3H, s, CH_3)
ESI-MS: *m/z* = 87.12 $[M-H]^-$.

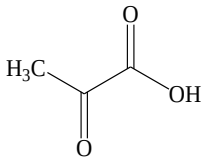


Figure 80: Structure of **pyruvic acid**

Succinic acid: NMR and MS data are reported in paragraph **5.5.11.** at page 179.

α-hydroxy-butyric acid: ^{1}H-NMR (400 MHz, D_2O) δ: 4.00 (1H, m, H-α), 1.70 (2H, m, H-β), 0.89 (3H, m, CH_3).
ESI-MS: *m/z* = 103.02 $[M-H]^-$.

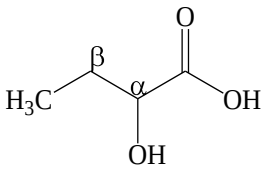


Figure 81: Structure of **α-hydroxy-butyric acid**

γ-amino-butyric acid (GABA): ^{1}H-NMR (400 MHz, D_2O) δ: 3.00 (2H, m, H-γ), 2.30 (2H, m, H-α), 1.91 (2H, m, H-β).
ESI-MS: *m/z* = 102.22 $[M-H]^-$.

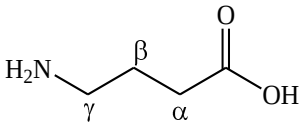


Figure 82: Structure of **γ-amino-butyric acid (GABA)**

Alanine: ^{1}H-NMR (400 MHz, D_2O) δ: 3.79 (1H, m, H-α), 1.48 (3H, d, *J* = 7.3 Hz, CH_3)
ESI-MS: *m/z* = 88.05 $[M-H]^-$.

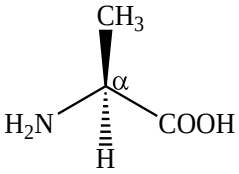


Figure 83: Structure of **alanine**

Aspartic acid: ^{1}H-NMR (400 MHz, D_2O) δ: 3.91 (1H, m, H-α), 2.79 (1H, m, H-β'), 2.70 (1H, m, H-β)

ESI-MS: *m/z* = 132.13 $[M-H]^-$.

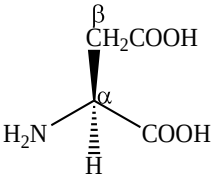


Figure 84: Structure of **aspartic acid**

Asparagine: ^{1}H-NMR (400 MHz, D_2O) δ: 4.01(1H, m H-α), 2.98 (1H, m, H-β'), 2.86 (1H, m, H-β).

ESI-MS: *m/z* = 132.13 $[M-H]^-$.

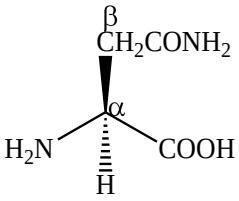


Figure 85: Structure of **asparagine**

Glutamine: ^{1}H-NMR (400 MHz, D_2O) δ: 3.78 (1H, m, H-α), 2.44 (2H, m, CH_2-γ), 2.13 (2H, m, CH_2-β).

ESI-MS: *m/z* = 145.22 $[M-H]^-$.

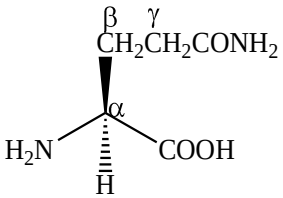


Figure 86: Structure of **glutamine**

Lysine: ^{1}H-NMR (400 MHz, D_2O) δ: 3.71 (1H, m, H-α), 3.01 (2H, m, CH_2-ε), 1.91 (2H, m, CH_2-β), 1.71 (2H, m, CH_2-δ), 1.42 (2H, m, CH_2-γ).
ESI-MS: *m/z* = 145.22 $[M-H]^-$.

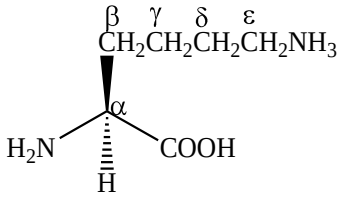


Figure 87: Structure of **lysine**

Threonine: ^{1}H-NMR (400 MHz, D_2O) δ: 4.25 (1H, m, H-β), 3.60 (1H, m H-α), 1.33 (3H, d, *J* = 6.9 Hz, CH_3-γ).
ESI-MS: *m/z* = 118.10 $[M-H]^-$.

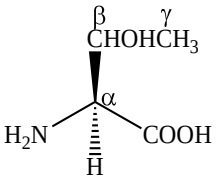


Figure 88: Structure of **threonine**

Tyrosine: ^{1}H-NMR (400 MHz, D_2O) δ: 7.19 (2H, m, H-2 and H-6), 6.86 (1H, m, H-3 and H-5), other signals overlapped with carbohydrate resonances.
ESI-MS: *m/z* = 180.19 $[M-H]^-$.

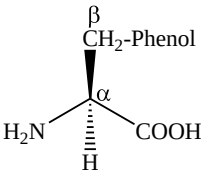


Figure 89: Structure of **tyrosine**

Valine: ^{1}H-NMR (400 MHz, D_2O) δ: 2.13 (1H, m, H-β), 1.03 (3H, d, *J* = 8.3 Hz, γ-CH_3), 0.98 (3H, d, *J* = 8.3 Hz, δ-CH_3), H-α (1H) is overlapped with carbohydrate resonances.
ESI-MS: *m/z* = 116.09 $[M-H]^-$.

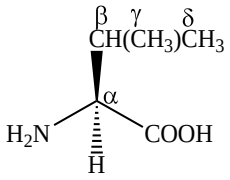


Figure 90: Structure of **valine**

Since not all compounds could be clearly detected from this chromatographic step, another chromatographic separation was performed on the assembly of fractions 3-31 deriving from the first one for the total weight of 298.2 mg.

The correspective amount of silica gel was 12.1 g (ratio 1:40 w/w) and the eluting system was a solution of dichloromethane and methanol at different concentrations.

The first one was 95:5 (v/v) but during the chromatographic run this was changed in order to let the elution of the most polar compounds passing to 9:1 (v/v), 8:2 (v/v), 7:3 (v/v) and 6:4 (v/v).

From this chromatographic step, one hundred and sixty-three fractions were collected.

After TLC analysis, these were collected putting together those having the same Rf values, as follows (Table 28):

Fractions	Weight (mg)
1-7	18.9
8-10	7.3
11-15	9.1
16-19	4.8
20-57	36.1
58-90	31.2
91-163	24.7

Table 28: Fraction assemblies and corresponding weights for chromatographic column 2

From the assembly of fractions 8-10, **1-miristoyl-2-heptadecenoyl-*sn*-glycerol** (Sciubba et al., 2014a; Venditti et al., 2017d) (Figure 91) was evidenced as almost pure compound.

1-miristoyl-2-heptadecenoyl-*sn*-glycerol: ^{1}H-NMR (300 MHz, CD_3OD) δ: 5.31 (2H, m, H-10' and H-11'), 5.12 (1H, m, H-2), 4.26 (1H, dd, *J* = 11.8/4.2 Hz, Hb-1), 4.12 (1H, dd, *J* = 11.8/4.2 Hz, Ha-1), 3.80 (1H, m, Ha-3), 3.70 (1H, m, Hb-3), 2.35 (4H, m, $\underline{CH_2}$-CO_2^- (heptadecenoyl plus miristoyl)), 2.02 (4H, m, H-9' and H-12'), 1.58 (4H, m, $\underline{CH_2}$-CH_2-CO_2^-

(heptadecenoyl plus miristoyl)), 1.29 (36H, m, n-CH_2 (heptadecenoyl plus miristoyl), 0.91-0.88 (6H, m, CH_3 (heptadecenoyl) and CH_3 (miristoyl)).

ESI-MS: *m/z* = 575.47 $[M+Na]^+$.

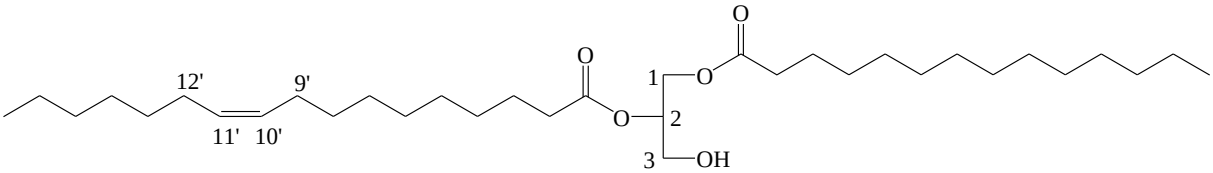


Figure 91: Structure of **1-miristoyl-2-heptadecenoyl-*sn*-glycerol**

From the assembly of fractions 16-19, **1,2-di-palmitoyl-*sn*-glycerol** (Sciubba et al., 2014a; Venditti et al., 2017d) (Figure 92) was evidenced as almost pure compound.

1,2-di-palmitoyl-*sn*-glycerol: ^{1}H-NMR (300 MHz, CD_3OD) δ: 5.12 (1H, m, H-2), 4.25 (1H, dd, *J* = 11.9/4.3 Hz, Hb-1), 4.12 (1H, dd, J = 11.9/4.3 Hz, Ha-1), 3.81 (1H, m, Ha-3), 3.68 (1H, m, Hb-3), 2.36 (4H, m, $\underline{CH_2}$-CO_2^-), 1.56 (4H, m, $\underline{CH_2}$-CH_2-CO_2^-), 1.29 (50H, m, n-CH_2), 0.89 (6H, m, CH_3).

ESI-MS: *m/z* = 591.51 $[M+Na]^+$.

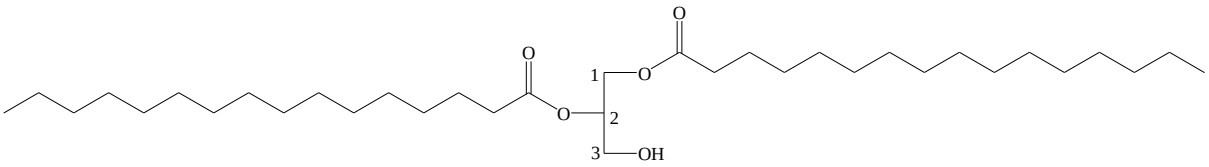


Figure 92: Structure of **1,2-di-palmitoyl-*sn*-glycerol**

From the assembly of fractions 58-90, **martynoside** (Yalcin et al., 2003) (Figure 98) was evidenced again as almost pure compound.

Martynoside: NMR and MS data are reported in paragraph **5.5.6.** at page 152.

From the assembly of fractions 91-163, **1-oleoyl-2-linoleoyl-*sn*-glycerol-3-phosphocholine** (Sciubba et al., 2014b) (Figure 93) was evidenced as almost pure compound.

1-oleoyl-2-linoleoyl-*sn*-glycerol-3-phosphocholine: ^{1}H-NMR (300 MHz, CD_3OD) δ: 5.35 (6H, m, H-9', H-10', H-12', H-13', H-9'' and H-10''), 5.10 (1H, m, H-2), 4.26 (1H, dd, *J* =

11.8/4.2 Hz, Hb-1), 4.12 (1H, dd, *J* = 11.8/4.2 Hz, Ha-1), 4.07 (2H, m, CH_2-2), 3.80 (1H, m, Ha-3), 3.70 (1H, m, Hb-3), 3.51 (2H, m, CH_2-1), 3.38 (9H, s, $N^+(CH_3)_3$), 2.79 (2H, m, H-11'), 2.35 (4H, m, $\underline{CH_2}$-CO_2^- (oleoyl plus linoleoyl)), 2.02 (8H, m, H-8', H-14', H-8'' and H-11''), 1.58 (4H, m, $\underline{CH_2}$-CH_2-CO_2^- (oleoyl plus linoleoyl)), 1.29 (40H, m, n-CH_2 (oleoyl plus linoleoyl), 0.91-0.88 (6H, m, CH_3)

ESI-MS: *m/z* = 807.25 $[M+Na]^+$.

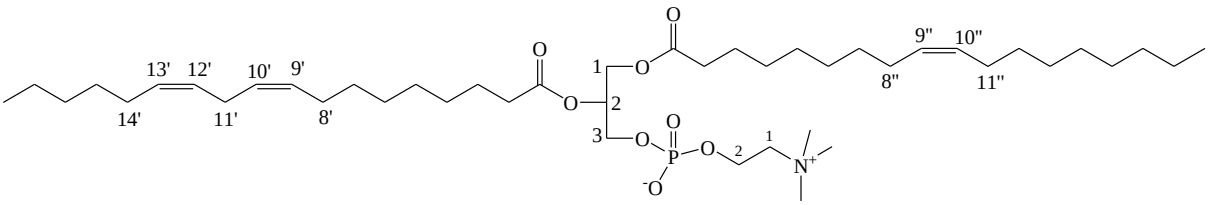


Figure 93: Structure of **1-oleoyl-2-linoleoyl-*sn*-glycerol-3-phosphocholine**

5.5.5. *Ajuga tenorei* C. Presl - leaves

An aliquot of the total dried crude extract obtained as described in paragraph **5.4.1.** for the weight of 2.6 g, underwent a first chromatographic separation using a correspective amount of silica gel of 78.0 g (ratio 1:30) and a mixture of *n*-butanol and distilled water in concentration ratio 82:18 (v/v) as eluting system.

From this chromatographic step, eighty-four fractions were collected.

After TLC analysis, these were collected putting together those having the same Rf values, as follows (Table 29):

Fractions	Weight (mg)
1-9	54.7
10-29	120.1
30-37	50.2
38-48	11.9
49-67	9.4
68-84	102.5

Table 29: Fraction assemblies and corresponding weights for chromatographic column 1

From the assembly of fractions 15-16, **verbascoside** (Venditti et al., 2016f) (Figure 94) (Spectrum 11) was evidenced as almost pure compound.

Verbascoside: ^{1}H NMR (CD_3OD, 300 MHz) δ: 7.67 (1H, d, *J* = 15.8 Hz, H-β), 7.21 (1H, br. s, H-2'), 7.07 (1H, br. d, *J* = 8.1 Hz, H-4'), 6.94 (1H, d, *J* = 8.1 Hz, H-5'), 6.80 (1H, d, *J* = 2.1 Hz, H-2''), 6.69 (1H, br. d, *J* = 7.9 Hz, H-6''), 6.57 (1H, d, *J* = 7.9 Hz, H-5''), 6.38 (1H, d, *J* = 15.8 Hz, H-α), 5.19 (1H, br. s, H-1'''), 4.36 (1H, d, *J* = 7.6 Hz, H-1), 3.74-3.49 (overlapped saccharidic signals), 2.80 (2H, m, H-β'), 1.06 (3H, d, *J* = 6.0 Hz, H-6''')

ESI-MS: *m/z* 647.25 $[M+Na]^+$; *m/z* 622.90 $[M-H]^-$.

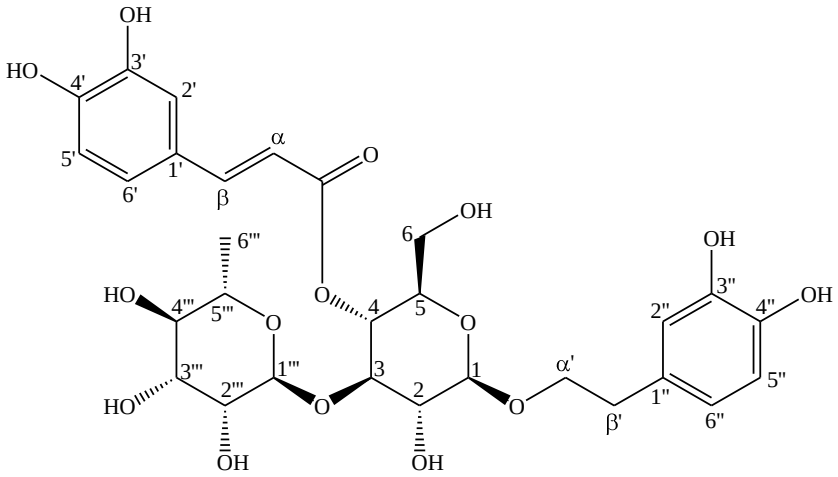
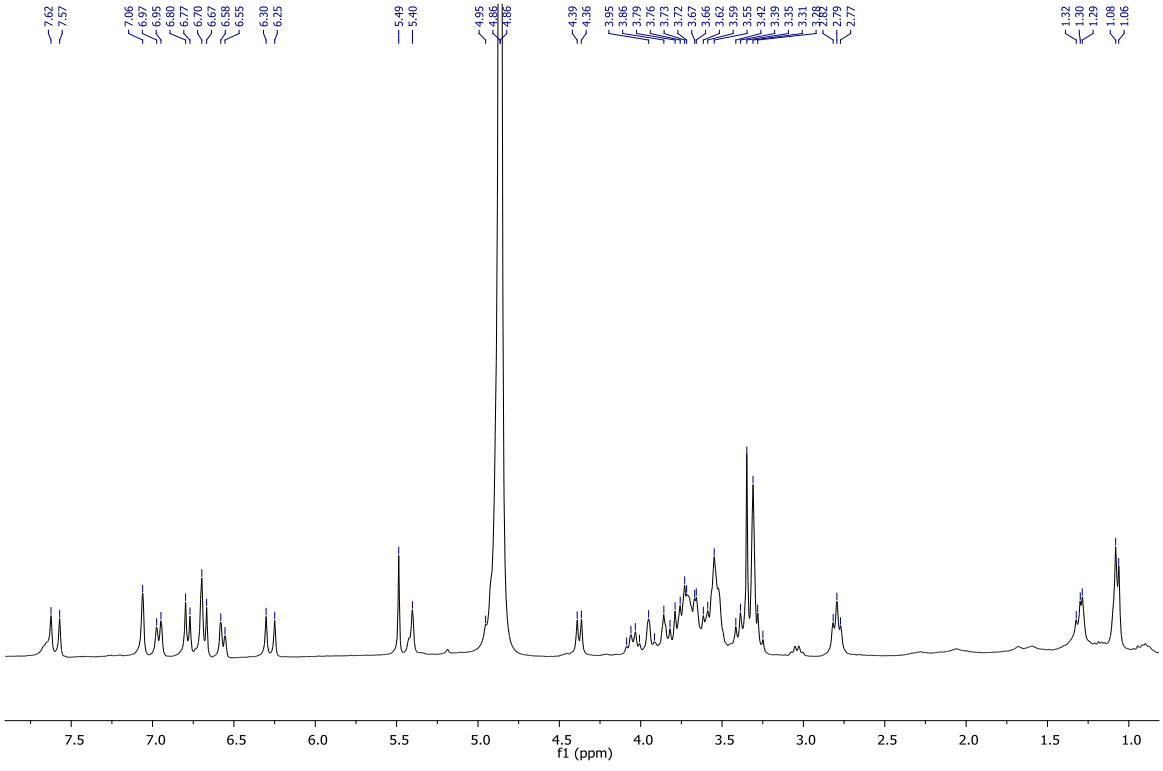


Figure 94: Structure of **verbascoside**



Spectrum 11: ^{1}H-NMR spectrum of **verbascoside**

From the assembly of fractions 49-67, a mixture of **harpagide** and **8-*O*-acetyl-harpagide** (Guiso et al., 1974) (Figures 104, 105), in ratio 1:1, was evidenced.

Harpagide: NMR and MS data are reported in paragraph **5.5.8.** at page 160.

8-*O*-acetyl-harpagide: NMR and MS data are reported in paragraph **5.5.8.** at pages 160-161.

Since not all the present compounds could be clearly identified from this separation step, a second column chromatography was performed on an assembly of fractions deriving from the first one, 10-29, for the total weight of 120.1 mg.
The correspective amount of silica gel was 3.1 g (ratio 1:25 w/w), this time, and the eluting system consisted of a mixture of chloroform/methanol at different concentrations.
The initial one was 95:5 (v/v) but during the chromatographic run, this was changed in order to raise the polarity of the solution and let the elution of the most polar compounds, to 9:1 (v/v), 8:2 (v/v), and lastly 7:3 (v/v).
From this chromatographic step, seventy fractions were collected.
After TLC analysis, these were collected putting together those having the same Rf values, as follows (Table 30):

Fractions	Weight (mg)
1-5	8.2
6-13	5.5
14-19	4.3
20-35	17.8
36-42	14.4
43-53	13.2
54	2.7
55-69	37.5

Table 30: Fraction assemblies and corresponding weights for chromatographic column 2

From the assembly of fractions 14-19, **ajugoside** (Guiso et al., 1974) (Figure 102), was evidenced as almost pure compound.

Ajugoside: NMR and MS data are reported in paragraph **5.5.8.** at page 158.

From fraction 54, **echinacoside** (Kobayashi et al., 1984) (Figure 95) was evidenced as almost pure compound, instead.

Echinacoside: ^{1}H-NMR (300 MHz, CD_3OD) δ: 7.60 (1H, d, *J* = 16.2 Hz, H-β), 7.21 (1H, br. s, H-2'), 7.09 (1H, br. d, *J* = 8.3 Hz, H-6'), 6.94 (1H, d, *J* = 8.3 Hz, H-5'), 6.83 (1H, br. s, H-2''), 6.78 (1H, br. d, *J* = 8.2 Hz, H-6''), 6.56 (1H, d, *J* = 8.2 Hz, H-5''), 6.38 (1H, d, *J* = 16.2 Hz, H-α), 5.22 (1H, br. s, H-1'''), 4.35 (1H, d, *J* = 7.4 Hz, H-1), 4.03 (1H, m, H-α'), 3.97-3.52 (overlapped signals of saccharides), 2.79 (2H, m, H-β'), 1.06 (3H, d, *J* = 6.0 Hz, H-6''').
ESI-MS: *m/z* 809.47 $[M+Na]^+$; *m/z* 785.04 $[M-H]^-$.

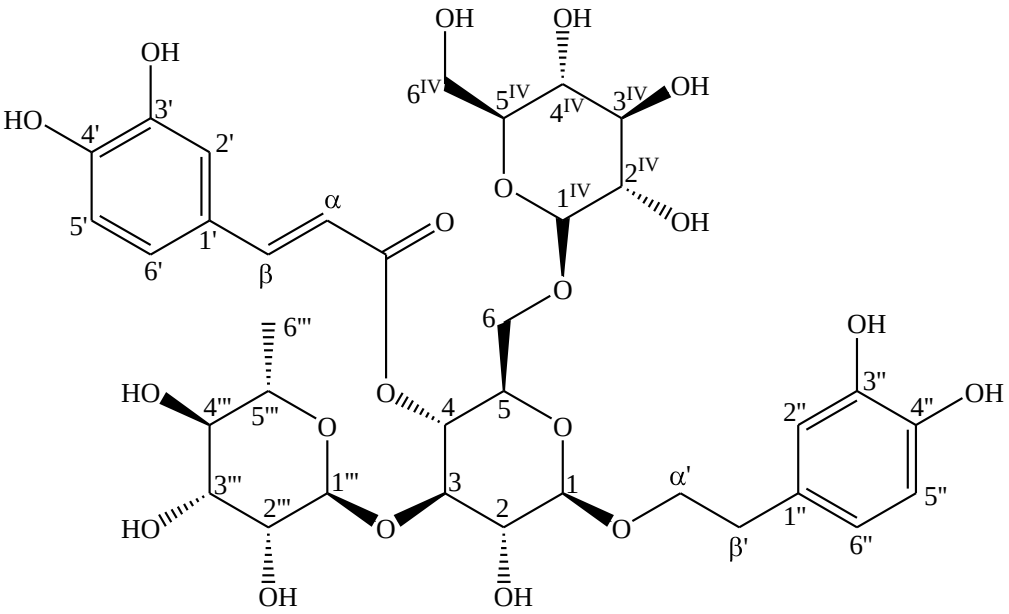


Figure 95: Structure of **echinacoside**

5.5.6. *Galeopsis ladanum* subsp. *angustifolia* (Ehrh. ex Hoffm.) Gaudin

An aliquot of the total dried crude extract obtained as described in paragraph **5.4.1.** for the weight of 1.8 g was subjected to a first chromatographic separation using a correspective amount of silica gel of 80.0 g (ratio 1:45 w/w) and a mixture of *n*-butanol and distilled water (82:18 v/v) as eluting system.

During the chromatographic run, the polarity of the eluting solution was raised in order to let the elution of the most polar compounds and so this became a solution of *n*-butanol, methanol and distilled water at concentration 70:10:30 (v/v/v).

From this chromatographic step, one-hundred and ninety-six fractions were collected.

After TLC analysis, these were collected putting together those having the same Rf values, as follows (Table 41):

Fractions	Weight (mg)
2-19	981.2
20-25	31.4
26-30	24.8
31-46	15.9
47-63	22.2
64-93	39.9
78-82	76.7
94-178	39.8
179-196	16.5

Table 41: Fraction assemblies and corresponding weights for chromatographic column 1

From the assembly of fractions 46-56, **verbascoside** (Venditti et al., 2016a) (Figure 94), **7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-β-D-glucopyranosyl]oxy}-5,8-dihydroxy-2-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one** (Lecher and Mabry, 1987) (Figure 129), **7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-β-D-glucopyranosyl]oxy}-5,8-dihydroxy-2-(4-methoxyphenyl)-4*H*-1-benzopyran-4-one** (Venditti et al., 2014) (Figure 96), **7-{[2-*O*-β-D-allopyranosyl-(6-*O*-acetyl-β-D-glucopyranosyl)]oxy}-5,8-dihydroxy-2-(3-hydroxy-4-methoxy-phenyl)-4*H*-1-benzopyran-4-one** (Venditti et al., 2014) (Figure 128), were evidenced as an only mixture in ratio 1:3:2:1.

Verbascoside: NMR and MS data are reported in paragraph **5.5.5.** at page 146.

7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-β-D-glucopyranosyl]oxy}-5,8-dihydroxy-2-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one: NMR and MS data are reported in paragraph **5.5.14.** at page 189.

7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-β-D-glucopyranosyl]oxy}-5,8-dihydroxy-2-(4-methoxyphenyl)-4*H*-1-benzopyran-4-one: ^{1}H-NMR (300 MHz, CD_3OD): 8.02 (2H, d, *J* = 8.1 Hz, H-2', H-6'), 7.12 (2H, d, *J* = 8.1 Hz, H-3', H-5'), 6.77 (1H, s, H-3), 6.68 (1H, s, H-6), 5.09 (1H, d, *J* = 8.2 Hz, H-1'''), 4.95 (overlapped with solvent signal, H-1''), 3.89 (3H, s, CH_3O), 3.85-3.42 (overlapped signals of carbohydrates), 2.01 (3H, s, CH_3CO).
ESI-MS: *m/z* 689.18 $[M+Na]^+$.

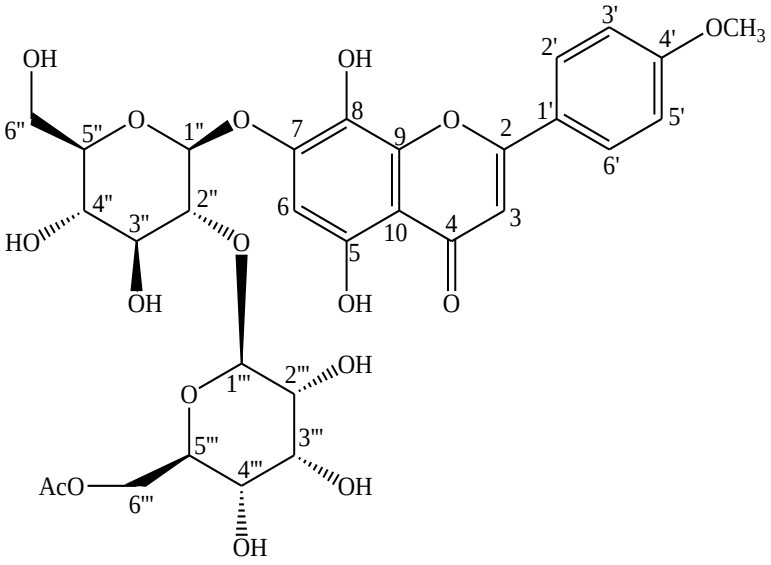


Figure 96: Structure of **7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-β-D-glucopyranosyl]oxy}-5,8-dihydroxy-2-(4-methoxyphenyl)-4*H*-1-benzopyran-4-one**

7-{[2-*O*-β-D-allopyranosyl-(6-*O*-acetyl-β-D-glucopyranosyl)]oxy}-5,8-dihydroxy-2-(3-hydroxy-4-methoxy-phenyl)-4*H*-1-benzopyran-4-one: NMR and MS data are reported in paragraph **5.5.13.** at page 187.

From the assembly of fractions 78-82, **harpagide** and **8-*O*-acetyl-harpagide** (Guiso et al., 1974) (Figures 104, 105), were evidenced as an only mixture in ratio 1:3.

Harpagide: NMR and MS data are reported in paragraph **5.5.8.** at page 160.

8-*O*-acetyl-harpagide: NMR and MS data are reported in paragraph **5.5.8.** at pages 160-161.

From the assembly of fractions 94-178, **chlorogenic acid** (Caprioli et al., 2016) (Figure 108), and **quinic acid** (Pauli et al., 1988) (Figure 97) were evidenced in mixture in ratio 1:2.

Chlorogenic acid: NMR and MS data are reported in paragraph **5.5.9.** at page 165.

Quinic acid: ^{1}H-NMR (300MHz, D_2O) δ: 4.12 (1H, m, H-4), 3.99 (1H, overlapped signal with carbohydrates, H-5), 3.52 (1H, overlapped signal with carbohydrates, H-3), 2.11 (4H, m, Ha-2, Hb-2, Ha-6, Hb-6).
ESI-MS: *m/z* 215.19 $[M+Na]^+$; *m/z* 191.09 $[M-H]^-$.

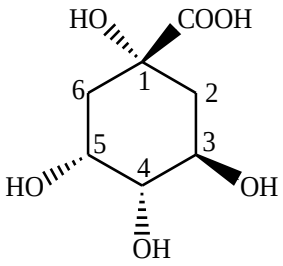


Figure 97: Structure of **quinic acid**

Since not all compounds could be clearly identified in this chromatographic step, another chromatographic separation was performed on an assembly of fractions deriving from the first one, in particular, 2-19, with a total weight of 981.2 mg.

This time, the correspective amount of silica gel was 40.0 g (ratio 1:40 w/w) and the eluting system consisted of a solution of dichloromethane and methanol at different concentrations.

The first one was 95:5 (v/v) but during the chromatographic run this was changed in order to let the elution of the most polar compounds passing to 9:1 (v/v), 8:2 (v/v), 7:3 (v/v) and 6:4 (v/v).

From this chromatographic step, one-hundred and twenty-seven fractions were collected. After TLC analysis, these were collected putting together those having the same Rf values, as follows (Table 32):

Fractions	**Weight (mg)**
1-9	23.4
10-14	7.9
15-17	6.8
18-34	54.7
35-39	41.6
40-43	9.8
44-47	7.7
48-53	6.6
54-75	12.9
82-92	19.0
93-127	17.4

Table 32: Fraction assemblies and corresponding weights for chromatographic column 2

From the assembly of fractions 54-75, **martynoside** (Yalcin et al., 2003) (Figure 98) was evidenced as almost pure compound.

Martynoside: ^{1}H-NMR (300 MHz, CD_3OD): δ 7.66 (1H, d, *J* = 15.9 Hz, H-β), 7.20 (1H, br. s, H-2'), 7.08 (1H, br. d, *J* = 8.5 Hz, H-6'), 6.94 (1H, d, *J* = 8.5 Hz, H-5'), 6.87 (1H, br. d, *J* = 8.2 Hz, H-6''), 6.84 (1H, br. s, H-2''), 6.73 (1H, d, *J* = 8.2 Hz, H-5''), 6.37 (1H, d, *J* = 15.9 Hz, H-α), 4.44 (1H, d, *J* = 8.2 Hz, H-1'), 3.88 (3H, s, O-CH_3(caff.)), 3.81 (3H, s, O-CH_3(tyr.)), 2.81 (2H, overlapped signal, H-β'), 1.05 (3H, d, *J* = 6.2 Hz, H-6''').
ESI-MS: *m/z* 675.19 $[M+Na]^+$.

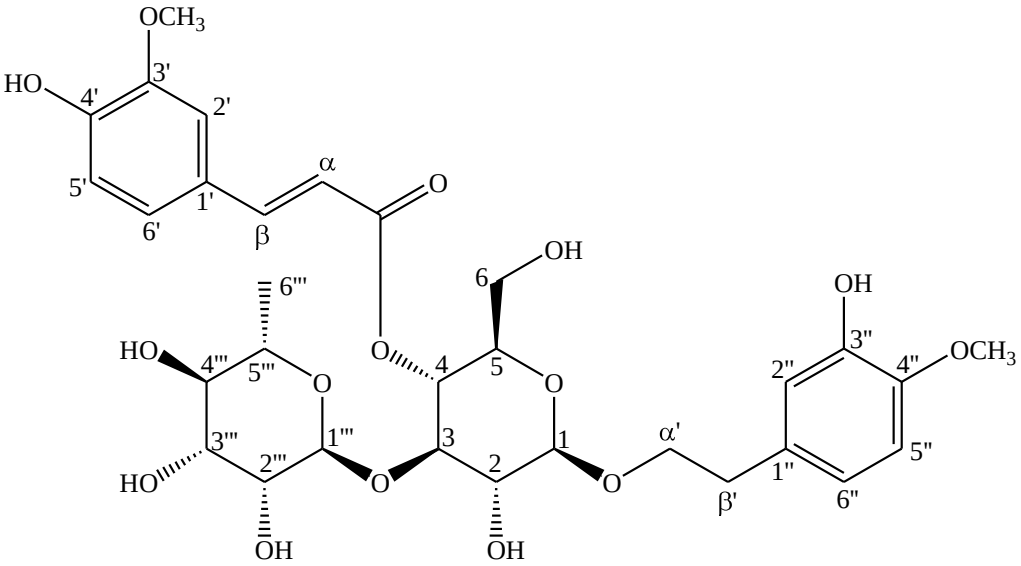


Figure 98: Structure of **martynoside**

5.5.7. *Melittis melissophyllum* subsp. *albida* (Guss.) P.W.Ball

An aliquot of the total dried crude extract obtained as described in paragraph **5.4.1.** for the weight of 10.1 g was subjected to an active charcoal treatment as described in paragraph **5.4.3.**.
From this procedure, four fractions of different polarity were collected recovering 1.18 g, 2.45 g, 0.170 g and 0.048 g of solids, by extracting respectively with distilled water and the 30, 60 and 96% ethanolic solutions.
Only the 30% fraction gave a positive reaction for the presence of iridoids and so only this was used for chromatographic separation.

The whole 2.45 g of it was chromatographed on silica gel for the amount of 90 g (ratio 1:30 w/w) using *n*-butanol saturated with distilled water (82:18, v/v) as eluting system.
From this chromatographic step, one-hundred and fifteen fractions were collected.

After TLC analysis, these were collected putting together those having the same Rf values, as follows (Table 33):

Fractions	Weight (mg)
1-13	21.4
14-17	14.5
18-24	12.7
25-39	89.2
40-41	9.9
42-55	74.1
56-64	35.8
65-115	222.3

Table 33: Fraction assemblies and corresponding weights for chromatographic column 1

From the assembly of fractions 14-17, **8-*O*-acetyl-harpagide** (Venditti et al., 2016d) (Figure 105), was evidenced as almost pure compound.

8-*O*-acetyl-harpagide: NMR and MS data are reported in paragraph **5.5.8.** at pages 160-161.

From the assembly of fractions 25-39, **harpagide**, **8-*O*-acetyl-harpagide** and **verbascoside** (Venditti et al., 2016d, 2016f) (Figures 104, 105, 94), were evidenced in mixture in ratio 3:3:1.

Harpagide: NMR and MS data are reported in paragraph **5.5.8.** at page 160.

8-*O*-acetyl-harpagide: NMR and MS data are reported in paragraph **5.5.8.** at pages 160-161.

Verbascoside: NMR and MS data are reported in paragraph **5.5.5.** at page 146.

From the assembly of fractions 42-55, **harpagide** and **virginioside** (Venditti et al., 2016d; Jensen et al., 1989) (Figures 104, 99), were evidenced in mixture in ratio 4:1.

Harpagide: NMR and MS data are reported in paragraph **5.5.8.** at page 160.

Virginioside: NMR and MS data are reported in paragraph **5.5.7.** at pages 154-155.

From the assembly of fractions 65-115, **melittoside** and **allobetonicoside** (Venditti et al., 2016d) (Figures 107, 103), were evidenced in mixture in ratio 9:1.

Melittoside: NMR and MS data are reported in paragraph **5.5.8.** at page 162.

Allobetonicoside: NMR and MS data are reported in paragraph **5.5.8.** at page 159.

Since not all compounds could be clearly identified from this separation step, a second chromatographic separation was performed on the assembly of fractions 42-55 deriving from the first chromatographic separation for the total weight of 74.1 mg.
The correspective amount of silica gel was 2.6 g (ratio 1:40 w/w) and the eluting system consisted of a mixture of chloroform/methanol at different concentrations.
The initial one was 85:15 (v/v) but during the chromatographic run, this was changed to increase the polarity and let the elution of the most polar compounds, passing to 8:2 (v/v), 7:3 (v/v) and, lastly, 6:4 (v/v).
From this chromatographic step, sixty fractions were collected.
After TLC analysis, these were collected putting together those having the same Rf values, as follows (Table 34):

Fractions	Weight (mg)
1-21	9.4
22-26	8.0
27-36	3.6
37-39	10.1
40-45	1.8
46	2.5
47-60	5.4

Table 34: Fraction assemblies and corresponding weights for chromatographic column 2

From the assembly of fractions 22-26, **virginioside** (Jensen et al., 1989) (Figure 99) was evidenced as pure compound.

Virginioside: ^{1}H-NMR (300 MHz, CD_3OD) δ: 6.25 (1H, d, *J* = 6.4 Hz, H-3), 5.67 (1H, d, *J* = 2.5 Hz, H-1), 4.94 (1H, d, *J* = 6.5 Hz, H-4), 4.56 (1H, d, *J* = 7.8 Hz, H-1'), 3.89 (1H, d, *J* = 10.3 Hz, H-6'a), 3.48 (1H, d, *J* = 3.6 Hz, H-6), 2.01 (1H, m, H-9), 1.24 (3H, s, H-10).

^{13}C-NMR (75 MHz, CD_3OD) δ: 141.5 (C-3), 103.6 (C-4), 98.7 (C-1'), 93.5 (C-1), 77.3 (C-8), 76.7 (C-5'), 75.8 (C-3'), 72.9 (C-2'), 70.0 (C-4'), 69.8 (C-5), 61.3 (C-7), 61.2 (C-6), 61.1 (C-6'), 52.3 (C-9), 24.3 (C-10).

HR-ESI-MS: *m/z* 385.1111 $[M+Na]^+$ ($C_{15}H_{22}O_{10}Na$)

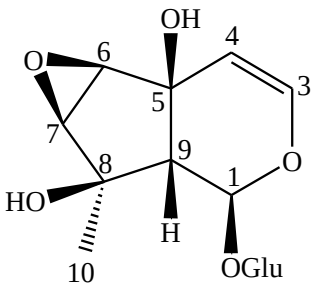


Figure 99: Structure of **virginioside**

From the assembly of fractions 37-39, **harpagide** and **geniposidic acid** (Venditti et al., 2016d; Guarnaccia et al., 1972) (Figure 104, 100) were evidenced in mixture in ratio 3:1.

Harpagide: NMR and MS data are reported in paragraph **5.5.8.** at page 160.

Geniposidic acid: ^{1}H-NMR (300 MHz, CD_3OD) δ: 7.50 (1H, br. s, H-3), 5.80 (1H, br. s, H-7), 5.16 (1H, d, *J* = 7.6 Hz, H-1), 4.72 (1H, d, *J* = 7.8 Hz, H-1'), 4.32 (1H, br. d, *J* = 12.8 Hz, H-10a), 4.20 (1H, br. d, *J* = 12.8 Hz, H-10b), 3.12 (1H, br. s, partially overlapped with sugar signals, H-9), 2.88-2.80 (H, m, H-6a), 2.76-2.66 (1H, br. t, *J* = 7.4 Hz, H-5), 2.17-2.04 (1H, m, H-6b).

^{13}C-NMR (75 MHz, CD_3OD) δ: 170.7 (C-11), 153.0 (C-3), 144.9 (C-8), 128.5 (C-7), 108.7 (C-4), 100.4 (C-1'), 98.3 (C-1), 78.4 (C-3'), 78.2 (C-5'), 74.8 (C-2'), 71.8 (C-4'), 62.8 (C-10), 61.5 (C-6'), 47.1 (C-9), 39.8 (C-6), 36.8 (C-5).

ESI-MS: *m/z* 397.20 $[M+Na]^+$; *m/z* 373.11 $[M-H]^-$.

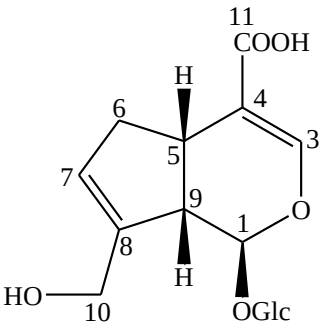


Figure 100: Structure of **geniposidic acid**

Since, again, not all compounds could be clearly identified from the first separation step, a third chromatographic separation was performed on the assembly of fractions 65-115 deriving from the first chromatographic separation for the total weight of 222.3 mg.

The correspective amount of silica gel was 7.7 g (ratio 1:35 w/w) and the eluting system consisted of a mixture of *n*-butanol-methanol-distilled water 70:10:30 (v/v/v).

From this chromatographic step, fifty-seven fractions were collected.

After TLC analysis, these were collected putting together those having the same Rf values, as follows (Table 45):

Fractions	Weight (mg)
1-10	21.1
11-12	13.6
13-18	14.4
19-28	31.4
29-30	6.5
31-41	35.5
42-51	49.8
52-57	16.6

Table 35: Fraction assemblies and corresponding weights for chromatographic column 3

From the assembly of fractions 11-12, **cinnamic acid** (Tran et al., 2009) (Figure 101) was evidenced as almost pure compound.

Cinnamic acid: ^{1}H-NMR (300 MHz, D_2O) δ: 7.42 (3H, m, H-3, H-4 and H-5), 7.37 (1H, d, *J* = 15.9 Hz, H-β), 6.58 (1H, d, *J* = 15.9 Hz, H-α).

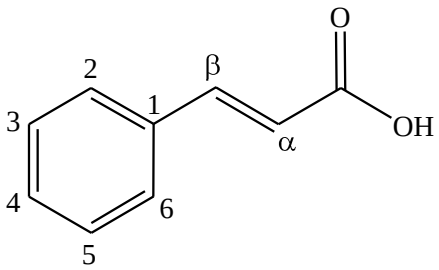


Figure 101: Structure of **cinnamic acid**

From the assembly of fractions 29-30, **allobetonicoside** (Venditti et al., 2016d) (Figure 103), was evidenced as almost pure compound.

Allobetonicoside: NMR and MS data are reported in paragraph **5.5.8.** at page 159.

From the assembly of fractions 42-51, **melittoside** (Venditti et al., 2016d) (Figure 107), was evidenced as almost pure compound.

Melittoside: NMR and MS data are reported in paragraph **5.5.8.** at page 162.

5.5.8. *Melittis melissophyllum* L. subsp. *melissophyllum*

The total dried crude extract obtained as described in paragraph **5.4.1.** for the weight of 31.7 g was subjected to an active charcoal treatment as described in paragraph **5.4.3**.
From this procedure, four fractions of different polarity were collected recovering 0.3 g, 3.6 g, 0.16 g and 0.12 g of solids, by extracting respectively with distilled water and the 10, 30, 60 and 90% ethanolic solutions.
The 30% fraction gave the highest positive reaction for the presence of iridoids and so this was used for the chromatographic separation.

The whole 3.6 g of it was chromatographed on silica gel for the amount of 90 g (ratio 1:30 w/w) using *n*-butanol saturated with distilled water (82:18, v/v) as eluting system.
During the chromatographic run, the polarity of the eluting system was raised in order to let the elution of the most polar compounds and this became a solution of *n*-butanol/methanol/distilled water 70:10:30 (v/v/v).
From this chromatographic step, one-hundred and eighty fractions were collected.

After TLC analysis, these were collected putting together those having the same Rf values, as follows (Table 36):

Fractions	Weight (mg)
1-13	41.3
14-19	36.4
20-33	25.9
34-50	29.3
51-89	122.6
90-107	26.6
108-110	11.5
111-133	31.1
134-135	105.7
136-159	99.7
160-161	50.4
162-170	44.5
171-173	22.2
174-180	11.3

Table 36: Fraction assemblies and corresponding weights for chromatographic column 1

From the assembly of fractions 20-33, **ajugoside** (Venditti et al., 2016d) (Figure 102) was evidenced as almost pure compound.

Ajugoside: ^{1}H-NMR (300 MHz, D_2O) δ: 6.28 (1H, br. d, *J* = 6.2 Hz, H-3), 5.90 (1H, br. s, H-1), 5.00-4.60 (1H, m, H-4), 2.90 (2H, m, H-9, H-5), 2.35 (2H, m, H-7), 2.01 (3H, s, OAc), 1.52 (3H, s, H-C10).
ESI-MS: *m/z* 412.98 $[M+Na]^+$.

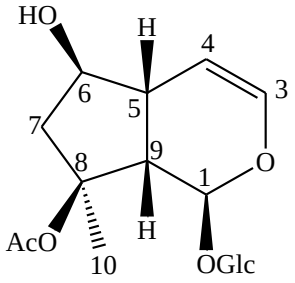


Figure 102: Structure of **ajugoside**

From the assembly of fractions 34-50, a mixture of **harpagide**, **8-*O*-acetyl-harpagide** and **monomelittoside** (Venditti et al., 2016d) (Figures 104, 105, 107), in ratio 3:2:1, was evidenced.

Harpagide: NMR and MS data are reported in paragraph **5.5.8.** at page 160.

8-*O*-acetyl-harpagide: NMR and MS data are reported in paragraph **5.5.8.** at page 160-161.

Monomelittoside: NMR and MS data are reported in paragraph **5.5.8.** at page 162.

From the assembly of fractions 134-135, a mixture of **allobetonicoside** and **melittoside** (Venditti et al., 2016d) (Figures 103, 107) in ratio 3:1, was evidenced.

Allobetonicoside: ^{1}H-NMR (300 MHz, D_2O) δ: 6.52 (1H, d, *J* = 5.8 Hz, H-3), 5.87 (1H, br. s, H-1), 5.18 (1H, d, *J* = 5.8 Hz, H-4), 4.95 (d, partially overlapped with HDO, H-C1"), δ 2.49 (1H, s, H-10).
^{13}C-NMR (75 MHz, D_2O) δ: 206.3 (C-6), 178.4 (C-8), 127.8 (C-7), 144.9 (C-3), 104.9 (C-4), 98.43 (C-1'), 96.1 (C1"), 92.3 (C-1), 77.5 (C-5), 76.8 (C-5'), 75.7 (C-3'), 72.9 (C-2'), 72.4 (C5"), 72.1 (C-2"), 70.2(C-3"), 69.8 (C4'), 66.5 (C4"), 62.5 (C6'), 60.9 (C-6").
ESI-MS: *m/z* 544.80 $[M+Na]^+$.

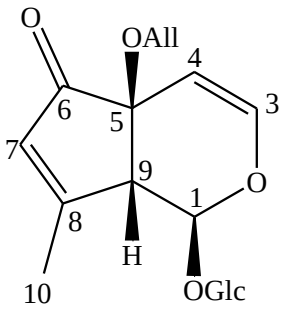


Figure 103: Structure of **allobetonicoside**

Melittoside: NMR and MS data are reported in paragraph **5.5.8.** at page 162.

Since not all compounds could be clearly identified from this separation step, a second chromatographic separation was performed on the assembly of fractions 51-89 deriving from the first chromatographic separation for the total weight of 311.4 mg.
The correspective amount of silica gel was 10.0 g (ratio 1:90 w/w) and the eluting system consisted of a mixture of *n*-butanol/methanol/distilled water 70:10:30 (v/v/v).
From this chromatographic step, sixty-four fractions were collected.

After TLC analysis, these were collected putting together those having the same Rf values, as follows (Table 37):

Fractions	Weight (mg)
1-11	9.9
12-15	5.4
16-17	14.3
18-20	15.9
21-25	11.2
26-27	8.7
28-33	15.1
34-60	21.7

Table 37: Fraction assemblies and corresponding weights for chromatographic column 2

From the assembly of fractions 16-17, a mixture of **harpagide** and **8-*O*-acetyl-harpagide** (Venditti et al., 2016d) (Figures 104, 105), in ratio 1:4, was evidenced.

Harpagide: ^{1}H-NMR (400 MHz, D_2O) δ: 6.42 (1H, d, *J* = 6.4 Hz, H-3), 5.78 (1H, d, *J* = 0.9 Hz, H-1), 5.09 (1H, dd, *J* = 6.4/1.5 Hz, H-4), 4.76 (1H, d, *J* = 8.2 Hz, H-1'), 2.60 (1H, s, H-9), 1.92 (1H, dd, *J* = 14.9/5.8, H-7a), 1.79 (1H, dd, *J* = 14.9/5.8, H-7b), 1.30 (3H, s, H-10).
^{13}C-NMR (100 MHz, D_2O) δ: 141.2 (C-3), 107.4 (C-4), 96.7 (C-1'), 94.2 (C-1), 76.2 (C8), 78.8 (C5'), 72.6 (C2'), 72.3 (C3'), 71.3 (C4'), 62.5 (C6'), 44.5 (C 7), 21.6 (C10).
ESI-MS: *m/z* 386.91 $[M+Na]^+$; *m/z* 362.95 $[M-H]^-$.

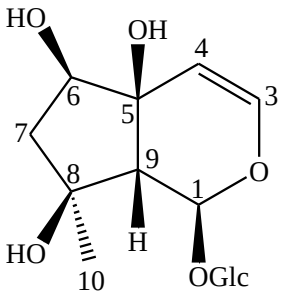


Figure 104: Structure of **harpagide**

8-*O*-acetyl-harpagide: ^{1}H-NMR (400 MHz, D_2O) δ: 6.37 (1H, br. d, *J* = 6.4 Hz, H-3), 6.12 (1H, d, *J* = 1.2 Hz, H-1), 5.05 (1H, dd, *J* = 6.4/1.3 Hz, H-4), 2.95 (1H, s, H-9), 2.10 (1H, br. d, *J* = 15.7 Hz, H-7a), 2.09 (3H, s, OAc), 1.95 (1H, dd, *J* = 15.7/4.4 Hz, H-7b), 1.49 (3H, s, H-10).

^{13}C-NMR (100 MHz, D_2O) δ: 174.33 (OAc), 146.5 (C-3), 106.6 (C-4), 98.5 (C-1'), 88.0 (C-8), 77.2 (C-6), 76.4 (C-3'), 74.6 (C-2'), 71.6 (C-4'), 62.8 (C-6'), 53.2 (C-9), 45.6 (C-7), 21.9 (C-10). 21.4 (OAc).

ESI-MS: *m/z* 428.91 $[M+Na]^+$.

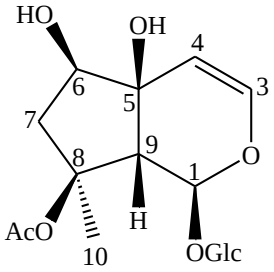


Figure 105: Structure of **8-*O*-acetyl-harpagide**

Since not all compounds could be clearly identified from this separation step, a second chromatographic separation was performed on the assembly of fractions 108-170 deriving from the first chromatographic separation for the total weight of 469.6 mg.

The correspective amount of silica gel was 10.0 g (ratio 1:30 w/w) and the eluting system consisted again of a mixture of *n*-butanol/methanol/distilled water 70:10:30 (v/v/v).

From this chromatographic step, eighty-seven fractions were collected.

After TLC analysis, these were collected putting together those having the same Rf values, as follows (Table 38):

Fractions	Weight (mg)
1-6	8.8
7-8	6.9
9-11	30.9
12-17	24.6
18-22	11.0
23-28	10.3
29-32	6.7
33-41	38.9
42-64	45.2
65-87	60.7

Table 38: Fraction assemblies and corresponding weights for chromatographic column 2

From the assembly of fractions 7-8, **monomelittoside** (Venditti et al., 2016d) (Figure 106) was evidenced as almost pure compound.

Monomelittoside: ^{1}H-NMR (300 MHz, D_2O) δ: 6.50 (1H, d, *J* = 6.4 Hz, H-3), 5.73 (1H, br. s, H-7), 5.25 (1H, d, *J* = 3.7 Hz, H-1), 5.07 (1H, dd, *J* = 6.4/1.5 Hz, H-4), 4.40 (1H, d, *J* = 8.0 Hz, H-6), 4.25 (2H, m, H-10), 2.95 (1H, br s, H-9).
^{13}C-NMR (75 MHz, D_2O) δ: 151.2 (C-8), 142.9 (C-3), 127.0 (C-7), 105.0 (C-4), 98.2 (C-1'), 80.8 (C-5), 74.3 (C-2'), 71.6 (C-4'), 63.5 (C-6'), 60.9 (C-10), 52.0 (C-9).
ESI-MS: *m/z* 384.90 $[M+Na]^+$; *m/z* 360.94 $[M-H]^-$.

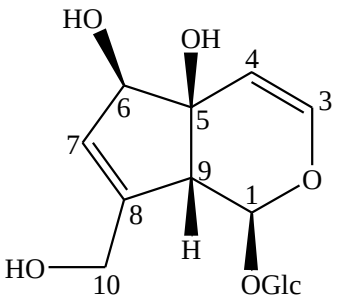


Figure 106: Structure of **monomelittoside**

From the assembly of fractions 9-11, a mixture of **monomelittoside** and **melittoside** (Venditti et al., 2016d) (Figures 106, 107), was evidenced in ratio 1:1.

Monomelittoside: NMR and MS data are reported in paragraph **5.5.8.** at pages 162.

Melittoside: NMR and MS data are reported in paragraph 5.5.8. at page 162.

From the assembly of fractions 12-17, **melittoside** (Venditti et al., 2016d) (Figure 107) was evidenced as almost pure compound.

Melittoside: ^{1}H-NMR (400 MHz, D_2O) δ: 6.55 (1H, d, *J* = 6.3 Hz, H-3), 5.90 (1H, br. s, H-7), 5.48 (1H, d, *J* = 5.4 Hz, H-1), 5.21 (1H, d, *J* = 6.3 Hz, H-4), 4.94 (1H, s, H-C1'), 4.89 (1H, s, H-1''), 4.64 (1H, br. s, H-6), 4.30 (2H, q like, *J* = 7.8 Hz, H-10), 3.39 (1H, d, *J* = 5.9 Hz, H-9).
^{13}C-NMR (100 MHz, D_2O) δ: 144.6 (C-8), 143.4 (C-3), 127.6 (C-7), 104.7 (C-4), 98.0 (C-1'), 97.8 (C-1''), 95.2 (C-1), 80.7 (C-5), 79.9 (C-6), 62.3 (C-6'), 63.4 (C6''), 60.7 (C-10), 50.6 (C-9).
ESI-MS: *m/z* 546.93 $[M+Na]^+$.

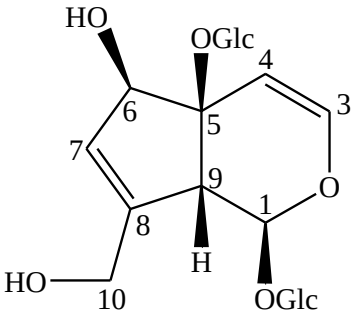


Figure 107: Structure of **melittoside**

From the assembly of fractions 23-28, **allobetonicoside** (Venditti et al., 2016d) (Figure 103), was evidenced again in mixture with several saccharides.

Allobetonicoside: NMR and MS data are reported in paragraph **5.5.8.** at page 159.

5.5.9. *Sideritis montana* L. subsp. *montana*

An aliquot of the total dried crude extract obtained as described in paragraph **5.4.1.** for the weight of 3.0 g was subjected to a first chromatographic separation using a correspective amount of silica gel of 75.0 g (ratio 1:25 w/w) and a mixture of *n*-butanol and distilled water (82:18 v/v) as eluting system.

During the chromatographic run, the polarity of the eluting solution was raised in order to let the elution of the most polar compounds and so this became a solution of *n*-butanol, methanol and distilled water at concentration 70:10:30 (v/v/v).

From this chromatographic step, one-hundred and ninety-five fractions were collected.

After TLC analysis, these were collected putting together those having the same Rf values, as follows (Table 39):

Fractions	Weight (mg)
1-23	600.0
24-31	85.1
32-39	110.4
40-51	77.4
52-56	47.2
57-61	44.4
62-71	45.0
72-82	68.6
83-92	50.2
93-110	92.0
111-117	37.7
118-134	73.4
135-149	45.1
150-195	129.6
Methanol column wash	56.7

Table 39: Fraction assemblies and corresponding weights for chromatographic column 1

From the assembly of fractions 32-39, a mixture of **7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-β-D-glucopyranosyl]oxy}-5,8-dihydroxy-2-(4-methoxyphenyl)-4*H*-1-benzopyran-4-one** and **7-{[2-*O*-β-D-allopyranosyl-(6-*O*-acetyl-β-D-glucopyranosyl)]oxy}-5,8-dihydroxy-2-(3-hydroxy-4-methoxy-phenyl)-4*H*-1-benzopyran-4-one** (Lenherr and Mabry, 1987; Venditti et al., 2014) (Figures 96, 128), in ratio 2:1, was evidenced.

7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-β-D-glucopyranosyl]oxy}-5,8-dihydroxy-2-(4-methoxyphenyl)-4*H*-1-benzopyran-4-one: NMR and MS data are reported in paragraph **5.5.6.** at page 149.

7-{[2-*O*-β-D-allopyranosyl-(6-*O*-acetyl-β-D-glucopyranosyl)]oxy}-5,8-dihydroxy-2-(3-hydroxy-4-methoxy-phenyl)-4*H*-1-benzopyran-4-one: NMR and MS data are reported in paragraph **5.5.13.** at page 187.

From the assembly of fractions 57-61, **8-*O*-acetyl-harpagide** (Venditti et al., 2016d) (Figure 105), was evidenced as almost pure compound.

8-*O*-acetyl-harpagide: NMR and MS data are reported in paragraph **5.5.8.** at pages 160-161.

From the assembly of fractions 83-92, **harpagide** (Venditti et al., 2016d) (Figure 104), was evidenced as almost pure compound.

Harpagide: NMR and MS data are reported in paragraph **5.5.8.** at page 160.

From the assembly of fractions 118-134, **chlorogenic acid** (Caprioli et al., 2016) (Figure 108) (Spectrum 12) was evidenced as almost pure compound.

Chlorogenic acid: ^{1}H-NMR (300 MHz, D_2O) δ: 7.63 (1H, d, *J* = 15.8 Hz, H-β), 7.16 (1H, br. s, H-2'), 7.09 (1H, br. d, *J* = 8.1 Hz, H-6'), 6.88 (1H, d, *J* = 8.1 Hz, H-5'), 6.35 (1H, d, *J* = 15.8 Hz, H-α), 5.31 (1H, m, H-3), 4.17 (1H, m, H-4), 2.20-1.99 (4H, m, H-2a, H-2b, H-6a, H-6b). ESI-MS: *m/z* 377.21 $[M+Na]^+$; *m/z* 353.27 $[M-H]^-$.

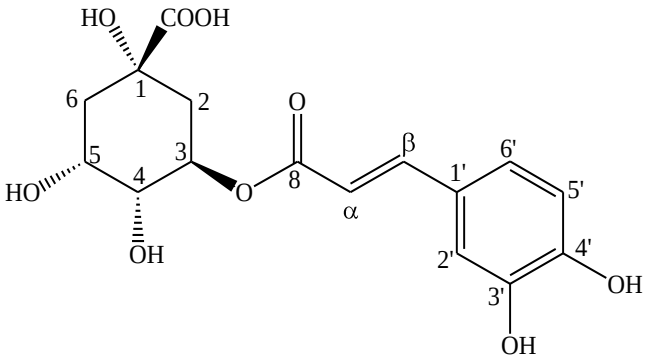
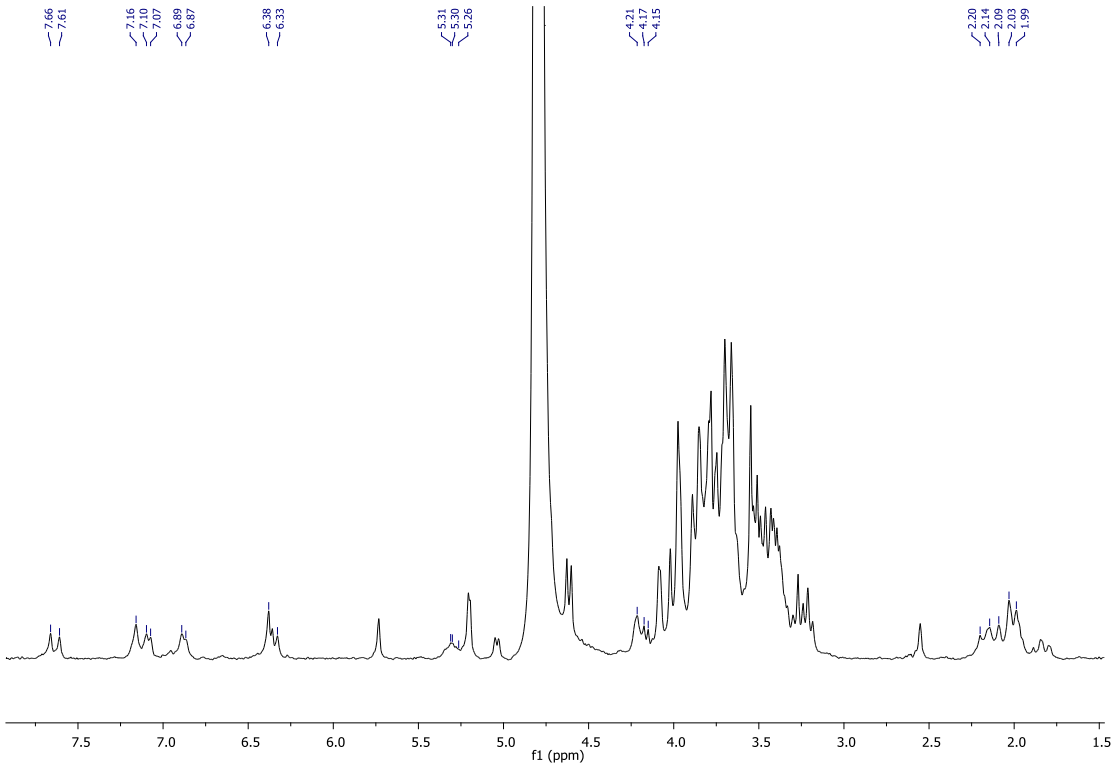


Figure 108: Structure of **chlorogenic acid**



Spectrum 12: ^{1}H-NMR spectrum of **chlorogenic acid**

From the methanol column wash (Spectrum 13), a mixture of **5-*O*-allosyloxy-aucubin** and **melittoside** (Serrilli et al., 2006; Venditti et al., 2016d) (Figures 109, 107), in ratio 1:1.5, was evidenced

5-*O*-allosyloxy-aucubin: ^{1}H-NMR (300 MHz, D_2O) δ: 6.46 (1H, d, *J* = 6.6 Hz, H-3), 5.84 (1H, br. s, H-7), 5.38 (1H, d, *J* = 3.4 Hz, H-1), 5.06 (1H, d, *J* = 6.6 Hz, H-4), 4.61 (1H, d, *J* = 8.4 Hz, H-1'), 4.53 (1H, d, *J* = 0.6 Hz, H-6), 3.24 (1H, d, *J* = 3.3 Hz, H-9).
ESI-MS: *m/z* $[M+Na]^+$ = 547.05.

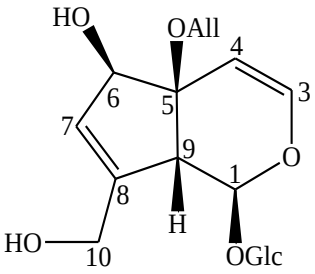
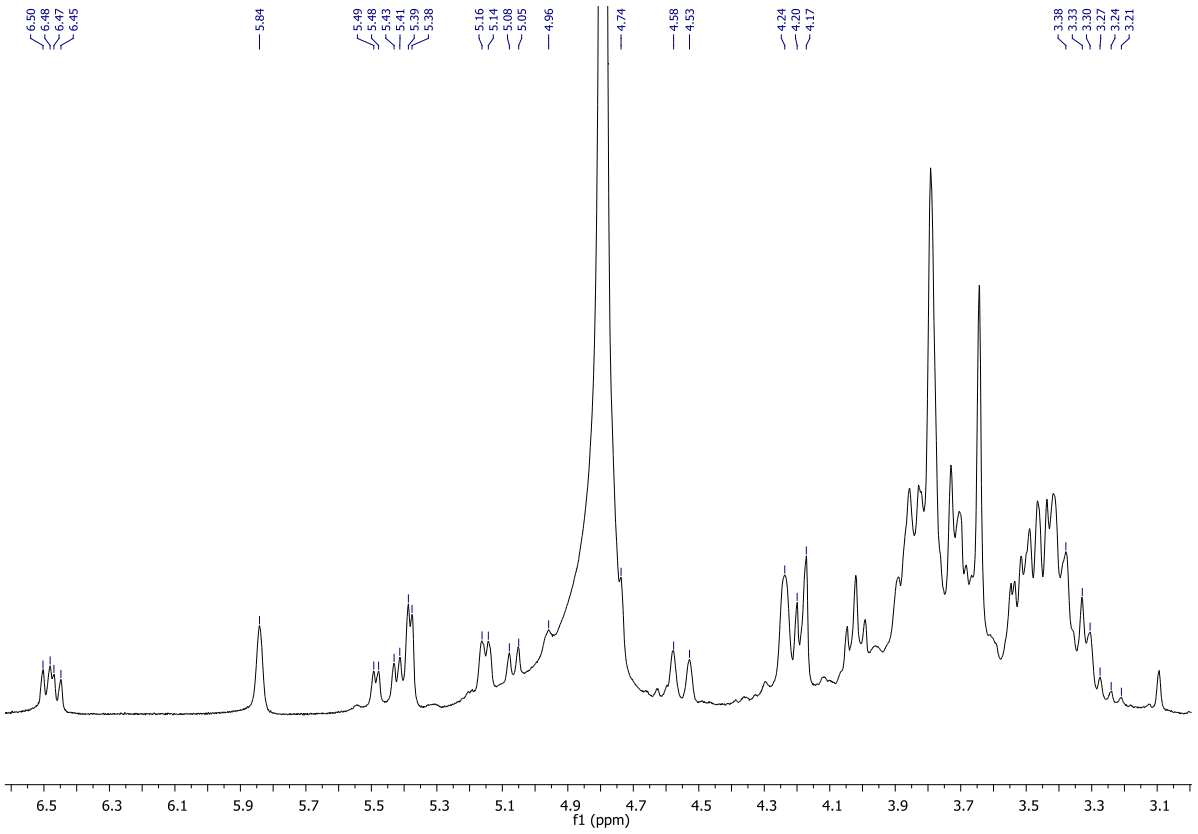


Figure 109: Structure of **5-*O*-allosyloxy-aucubin**



Spectrum 13: ^{1}H-NMR spectrum of the assembly of the methanol column wash

Melittoside: NMR and MS data are reported in paragraph **5.5.8.** at page 162.

Since not all compounds could be clearly identified from this separation step, the total dried crude extract itself for the weight of 5.0 g underwent an active charcoal treatment as described in paragraph **5.4.3**.
From this procedure, four fractions of different polarity were collected recovering 2.35 g, 0.21 g, 0.17 g and 0.30 g of solids, by extracting respectively with distilled water and the 10, 30, 60 and 96% ethanolic solutions.

The 30% fraction gave the highest positive reaction for the presence of iridoids and so this all was used for the further chromatographic separation with a correspective amount of silica gel of 75.0 g (ratio 1:35 w/w) and again a mixture of *n*-butanol and distilled water (82:18 v/v) as eluting system.
From this chromatographic step, one-hundred and ninety-eight fractions were collected.
After TLC analysis, these were collected putting together those having the same Rf values, as follows (Table 40):

Fractions	Weight (mg)
1-8	13.2
9-18	56.5
19-27	51.4
28-38	34.9
39-167	224.8
168-198	200.1

Table 40: Fraction assemblies and corresponding weights for chromatographic column 2

From the assembly of fractions 19-27, **methyl-arbutin** (Lutterbach and Stöckigt, 1995) (Figure 110) was evidenced as almost pure compound.

Methyl-arbutin: ^{1}H-NMR (400 MHz, D_2O) δ: 6.98 (2H, d, *J* = 9.0 Hz, H-2, H-6), 6.79 (2H, d, *J* = 9.0 Hz, H-3, H-5), 4.91 (1H, d, *J* = 7.7 Hz, H-1'), 4.04-3.04 (overlapped signals of saccharides), 3.68 (3H, s, $\underline{CH_3}O$).
ESI-MS: *m/z* $[M+Na]^+$ = 309.27.

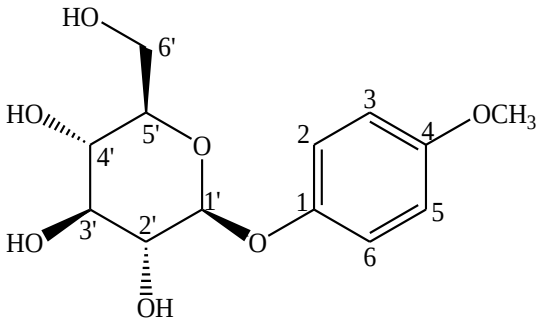


Figure 110: Structure of **methyl-arbutin**

From the assembly of fractions 39-167, a mixture of **harpagide**, **8-*O*-acetyl-harpagide and 8-epiloganic acid** (Venditti et al., 2016d, 2016f) (Figures 104, 105, 111), was evidenced in ratio 3:2:1.

Harpagide: NMR and MS data are reported in paragraph **5.5.8.** at page 160.

8-*O*-acetyl-harpagide: NMR and MS data are reported in paragraph **5.5.8.** at pages 160-161.

8-epiloganic acid: ^{1}H-NMR (300 MHz, D_2O) δ: 7.07 (1H, s, H-3), 5.55 (1H, br. s, H-1), 4.68 (1H, d, *J* = 7.9 Hz, H-1'), 3.06 (1H, m, H-5), 2.73 (1H, m, H-9), 2.21 (1H, m, H-8), 1.93 (2H, m, H-6), 1.05 (3H, d, *J* = 7.1 Hz, H-10).
ESI-MS: *m/z* 399.19 $[M+Na]^+$; *m/z* $[M-H]^-$ = 375.28.

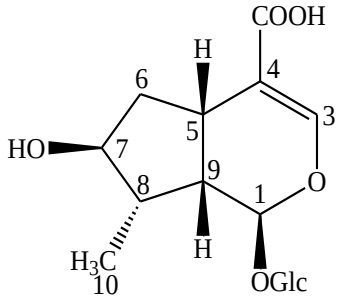


Figure 111: Structure of **8-epiloganic acid**

From the assembly of fractions 168-198, **5-*O*-allosyloxy-aucubin** and **melittoside** (Serrilli et al., 2006; Venditti et al., 2016b) (Figures 109, 107), in ratio 3:2, was evidenced, again.

5-*O*-allosyloxy-aucubin: NMR and MS data are reported in paragraph **5.5.9.** at page 166.

Melittoside: NMR and MS data are reported in paragraph **5.5.8.** at page 162.

5.5.10. *Sideritis romana* L.

An aliquot of the total dried crude extract obtained as described in paragraph **5.4.1.** for the weight of 5.15 g was subjected to a first chromatographic separation on silica gel column, using a correspective amount of silica gel of 100.0 g (ratio 1:20 w/w) and a mixture of *n*-butanol and distilled water (82:18 v/v) as eluting system.

During the chromatographic run, the polarity of the eluting solution was raised in order to let the elution of the most polar compounds and so this became a solution of *n*-butanol, methanol and distilled water at concentration 70:10:30 (v/v/v).

From this chromatographic step, one-hundred and seventy-nine fractions were collected.

After TLC analysis, these were collected putting together those having the same Rf values, as follows (Table 51):

Fractions	Weight (mg)
1-23	47.7
24-25	14.9
26-51	41.3
52-57	15.6
58-70	25.5
71-94	83.3
95-105	21.2
106-117	19.9
118-131	44.4
132-160	58.8
161-163	16.2
164-168	21.4
169-179	31.0

Table 41: Fraction assemblies and corresponding weights for chromatographic column 1

From the assembly of fractions 24-25, **(*E*)-phytol** (Venditti et al., 2016g) (Figure 135), was evidenced as almost pure compound.

(*E*)-phytol: NMR and MS data are reported in paragraph **5.5.18.** at page 204.

From the assembly of fractions 52-57, **apigenin 7-*O*-(6''-*O*-acetyl-β-D-glucopyranoside)** (Svelhiková et al., 2004) (Figure 112) (Spectra 14, 15) was evidenced as almost pure compound.

Apigenin 7-*O*-(6''-*O*-acetyl-β-D-glucopyranoside): ^{1}H-NMR (400 MHz, CD_3OD) δ: 7.90 (2H, d, *J* = 8.8 Hz, H-2', H-6'), 6.96 (2H, d, *J* = 8.8 Hz, H-3', H-5'), 6.79 (1H, d, *J* = 2.1 Hz, H-8), 6.68 (1H, s, H-3), 6.52 (1H, d, *J* = 2.1 Hz, H-6), 5.08 (1H, d, *J* = 7.3 Hz, H-1''), 4.47 (1H, dd, *J* = 12.0/2.2 Hz, H-6''a), 4.26 (1H, dd, *J* = 12.0/7.1 Hz, H-6''b), 2.09 (3H, s, OAc).
^{13}C-NMR (100 MHz, CD_3OD) δ: 184.1 (C-4), 172.7 (OAc), 166.7 (C-2), 164.6 (C-7), 162.9 (C-5), 158.9 (C-9), 129.6 (C-2', C-6'), 123.1 (C-1'), 117.1 (C-3', C-5'), 104.9 (C-10), 104.2 (C-3), 101.5 (C-1''), 101.1 (C-8), 96.2 (C-6), 77.8 (C-3''), 75.6 (C-5''), 74.7 (C-2''), 71.6 (C-4''), 64.8 (C-6''), 20.8 (OAc).
ESI-MS: *m/z* 496.97 $[M+Na]^+$; *m/z* 472.92 $[M-H]^-$.

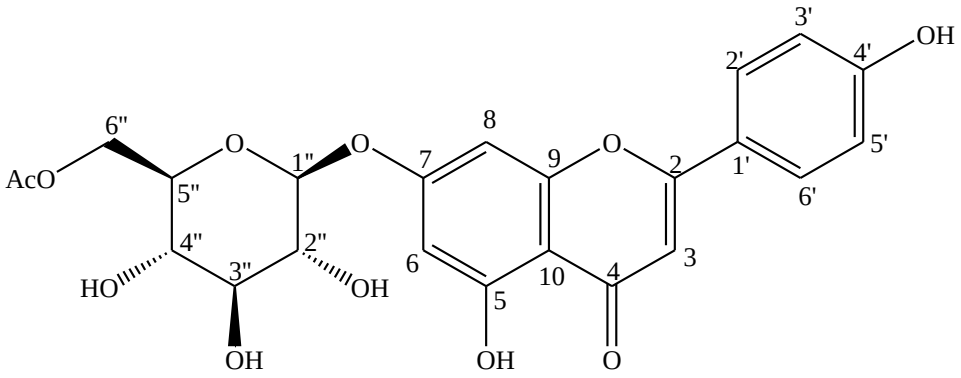
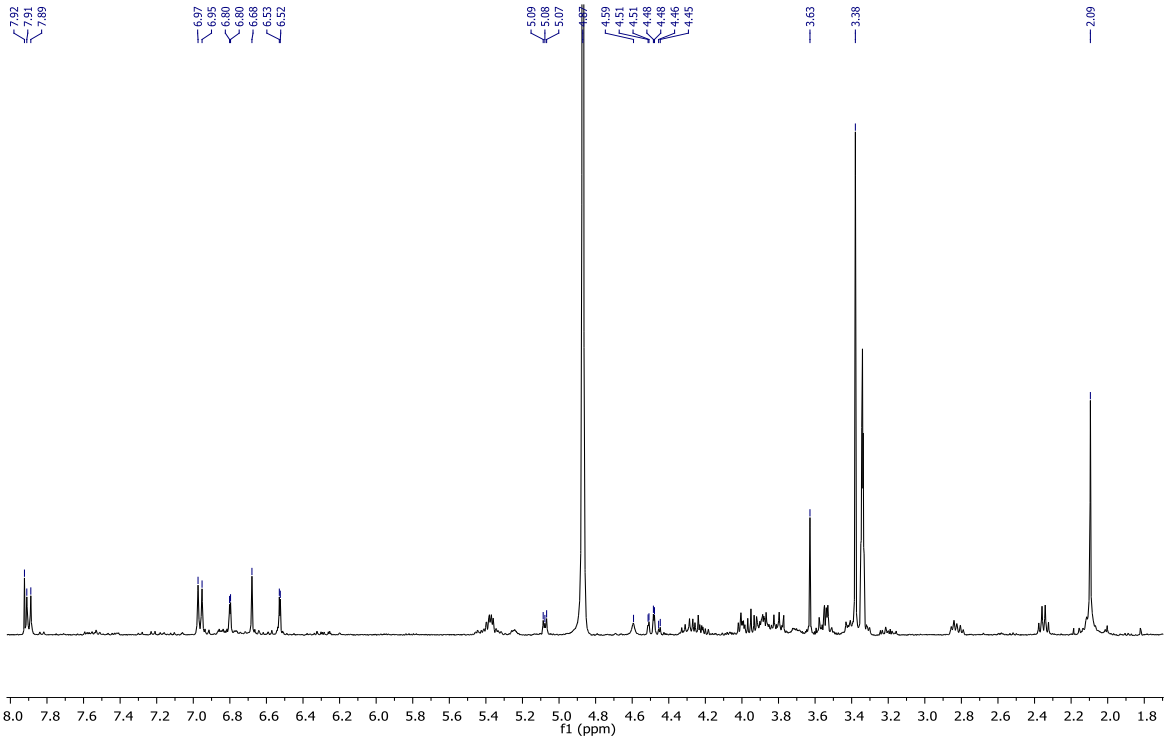
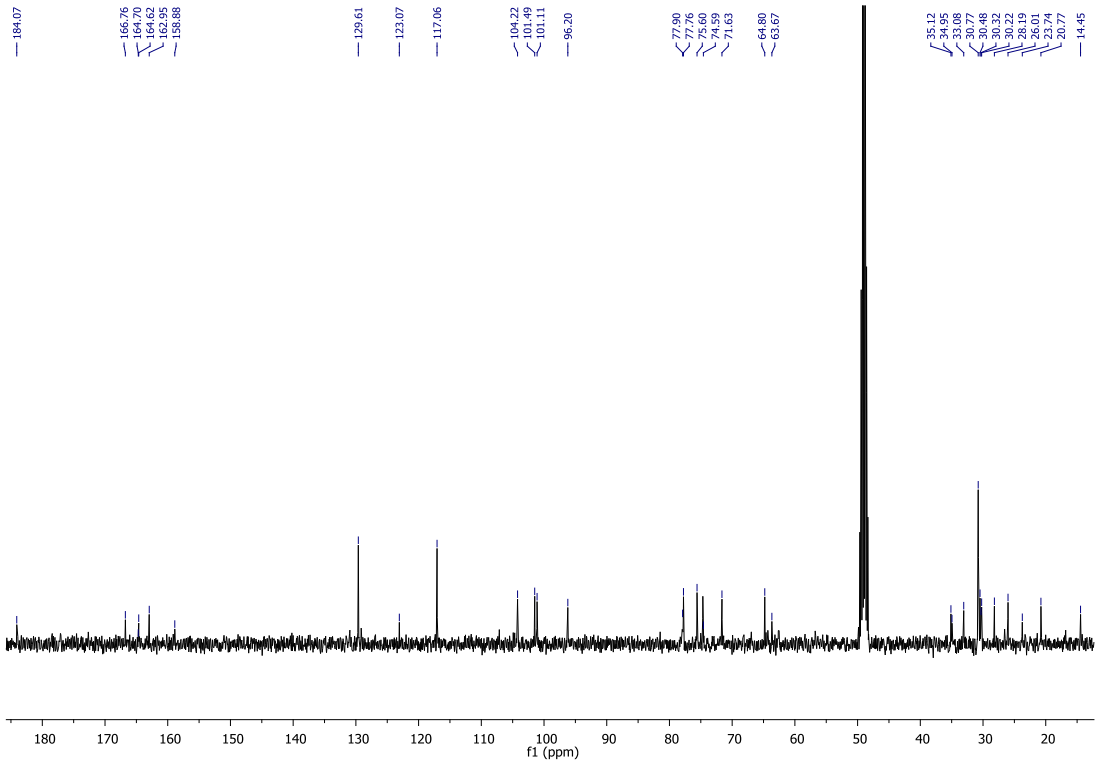


Figure 112: Structure of **apigenin 7-*O*-(6''-*O*-acetyl-β-D-glucopyranoside)**



Spectrum 14: ^{1}H-NMR spectrum of **apigenin 7-*O*-(6''-*O*-acetyl-β-D-glucopyranoside)**



Spectrum 15: ^{13}C-NMR spectrum of **apigenin 7-*O*-(6''-*O*-acetyl-β-D-glucopyranoside)**

From the assembly of fractions 71-94, **7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-β-D-glucopyranosyl]oxy}-5-hydroxy-2-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one** (Figure 113), **7-{[2-*O*-(6-*O*-acetyl-β-D-glucopyranosyl)-β-D-glucopyranosyl]oxy}-5-hydroxy-2-(3,4-dihydroxyphenyl)-4*H*-1-benzopyran-4-one** (Figure 114), **7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-β-D-glucopyranosyl]oxy}-5-hydroxy-2-(3-hydroxy-4-methoxy-phenyl)-4*H*-1-benzopyran-4-one** (Figure 115) and **7-{[2-*O*-β-D-allopyranosyl-β-D-glucopyranosyl]oxy}-5-hydroxy-2-(4-hydroxy-3-methoxy-phenyl)-4*H*-1-benzopyran-4-one** (Figure 116) (El-Ansari et al., 1991; Rabanal et al., 1982; Venditti et al., 2016e) were evidenced as an only mixture in ratio 5:4:2:1.

7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-β-D-glucopyranosyl]oxy}-5-hydroxy-2-(4-hydroxyphenyl)-4*H*-benzopyran-4-one: ^{1}H-NMR (400 MHz, DMSO) δ: 7.98 (2H, d, *J* = 8.2 Hz, H-2', H-6'), 6.94 (1H, s, H-3), 6.92 (2H, d, *J* = 8.2 Hz, H-3', H-5'), 6.73 (1H, d, *J* = 2.2 Hz, H-6), 6.42 (1H, d, *J* = 2.1 Hz, H-8), 5.18 (1H, d, *J* = 7.5 Hz, H-1''), 4.80 (1H, d, *J* = 7.9 Hz, H-1'''), 4.12 (1H, dd, *J* = 11.8/1.9 Hz, H-6'''a), 4.00 (1H, dd, *J* = 11.8/5.7 Hz, H-6'''b), 1.89 (3H, s, OAc).

ESI-MS: *m/z* 659.12 $[M+Na]^+$; *m/z* 635.02 $[M-H]^-$.

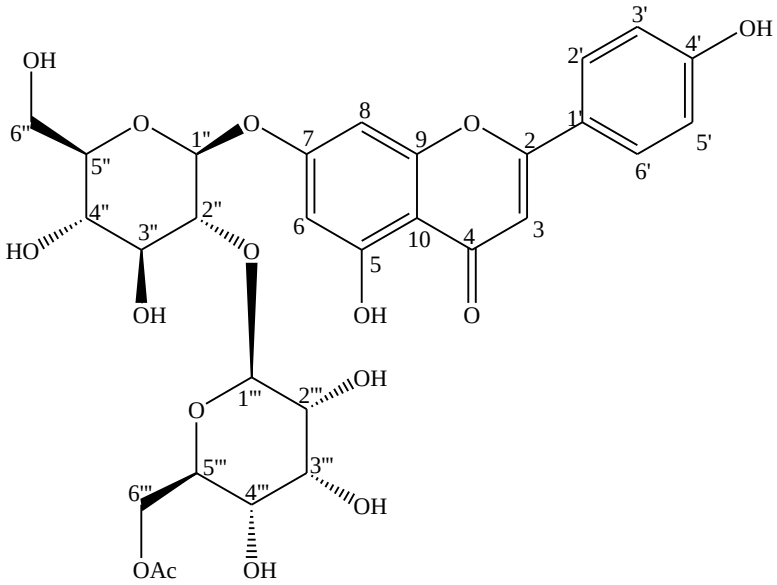


Figure 113: Structure of **7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-β-D-glucopyranosyl]oxy}-5-hydroxy-2-(4-hydroxyphenyl)-4*H*-benzopyran-4-one**

7-{[2-*O*-(6-*O*-acetyl-β-D-glucopyranosyl)-β-D-glucopyranosyl]oxy}-5-hydroxy-2-(3,4-dihydroxyphenyl)-4*H*-1-benzopyran-4-one: ^{1}H-NMR (400 MHz, DMSO) δ: 7.65-7.62 (1H, br. s, H-2'), 7.52 (1H, br. d, *J* = 8.5 Hz, H-6'), 6.90 (1H, d, *J* = 8.4 Hz, H-5'), 6.83 (1H, d, *J* = 1.9 Hz, H-6), 6.70 (1H, s, H-3), 6.51 (1H, d, *J* = 1.9 Hz, H-8), 5.24 (1H, d, *J* = 6.7 Hz, H-1"), 4.98 (1H, d, *J* = 8.0 Hz, H-1'"), 4.16 (1H, dd, *J* = 12.2/1.7 Hz, H-6'"a), 2.06 (3H, s, OAc).
ESI-MS: *m/z* 675.10 $[M+Na]^+$; *m/z* 650.99 $[M-H]^-$.

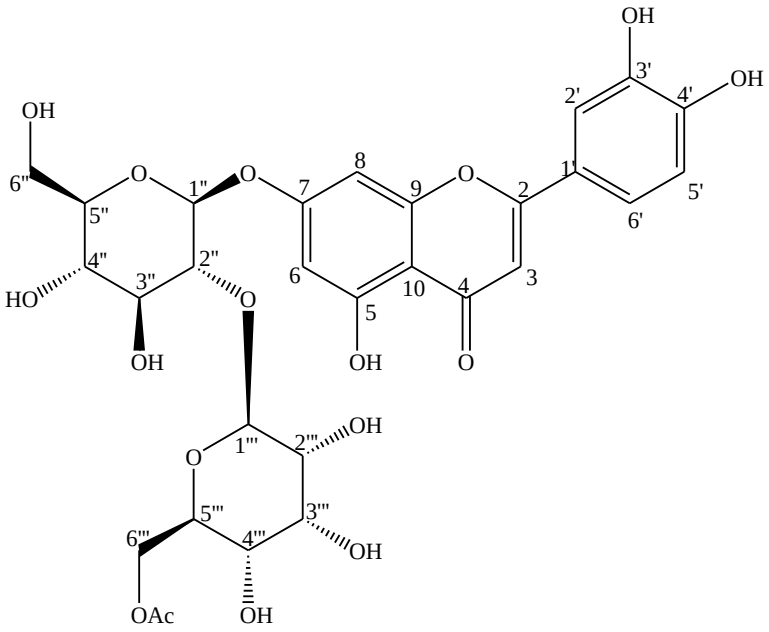


Figure 114: Structure of **7-{[2-*O*-(6-*O*-acetyl-β-D-glucopyranosyl)-β-D-glucopyranosyl]oxy}-5-hydroxy-2-(3,4-dihydroxyphenyl)-4*H*-1-benzopyran-4-one**

7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-β-D-glucopyranosyl]oxy}-5-hydroxy-2-(3-hydroxy-4-methoxy-phenyl)-4*H*-1-benzopyran-4-one: ^{1}H-NMR (400 MHz, DMSO) δ: 7.60 (2H, m, H-2', H-6'), 6.98 (1H, s, H-3), 6.94 (1H, d, *J* = 8.9 Hz, H-5'), 6.80 (1H, d, *J* = 1.8 Hz, H-6), 6.45 (1H, d, *J* = 1.8 Hz, H-8), 5.21 (1H, d, *J* = 7.5 Hz, H-1"), 4.79 (1H, d, *J* = 7.9 Hz, H-1'"), 4.12 (1H, dd, *J* = 11.7/1.8 Hz, H-6'"a), 4.00 (1H, dd, *J* = 11.7/5.4 Hz, H-6'"b), 3.89 (3H, s, OCH_3), 1.94 (3H, s, OAc).

ESI-MS: *m/z* 689.11 $[M+Na]^+$; *m/z* 665.02 $[M-H]^-$.

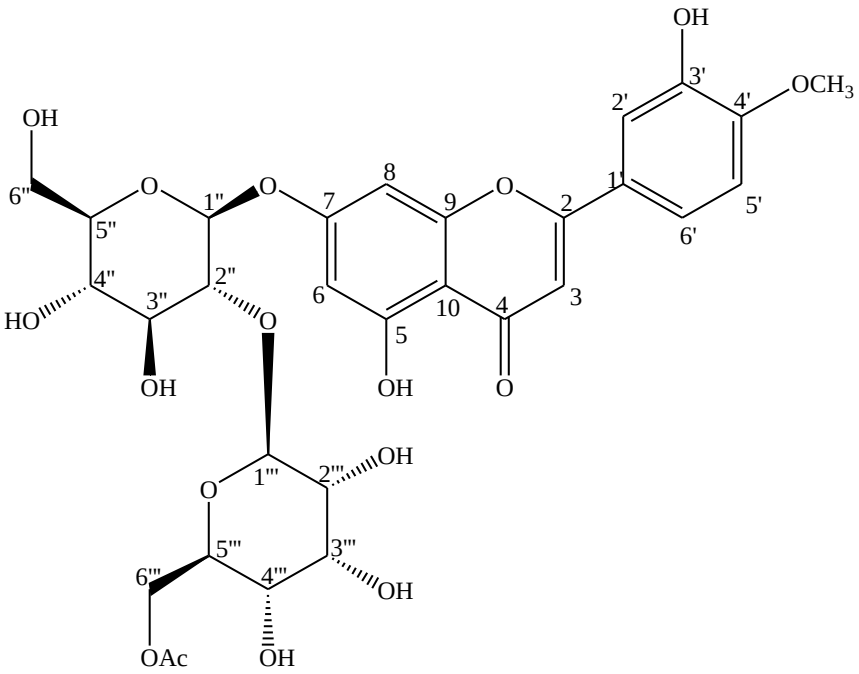


Figure 115: Structure of **7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-β-D-glucopyranosyl]oxy}-5-hydroxy-2-(3-hydroxy-4-methoxy-phenyl)-4*H*-1-benzopyran-4-one**

7-{[2-*O*-β-D-allopyranosyl-β-D-glucopyranosyl]oxy}-5-hydroxy-2-(4-hydroxy-3-methoxy-phenyl)-4*H*-1-benzopyran-4-one: ^{1}H-NMR (400 MHz, DMSO) δ: 7.56 (1H, dd, *J* = 8.5/2.1 Hz, H-6'), 7.54 (1H, d, *J* = 2.1 Hz, H-2'), 6.95 (1H, d, *J* = 8.5 Hz, H-5'), 6.89 (1H, d, *J* = 2.2 Hz, H-6), 6.71 (1H, s, H-3), 6.53 (1H, d, *J* = 2.2 Hz, H-8), 5.27 (1H, d, *J* = 7.2 Hz, H-1''), 5.01 (1H, d, *J* = 7.8 Hz, H-1'''), 3.98 (3H, s, 3'-OCH_3).

ESI-MS: *m/z* 646.67 $[M+Na]^+$; *m/z* 622.85 $[M-H]^-$.

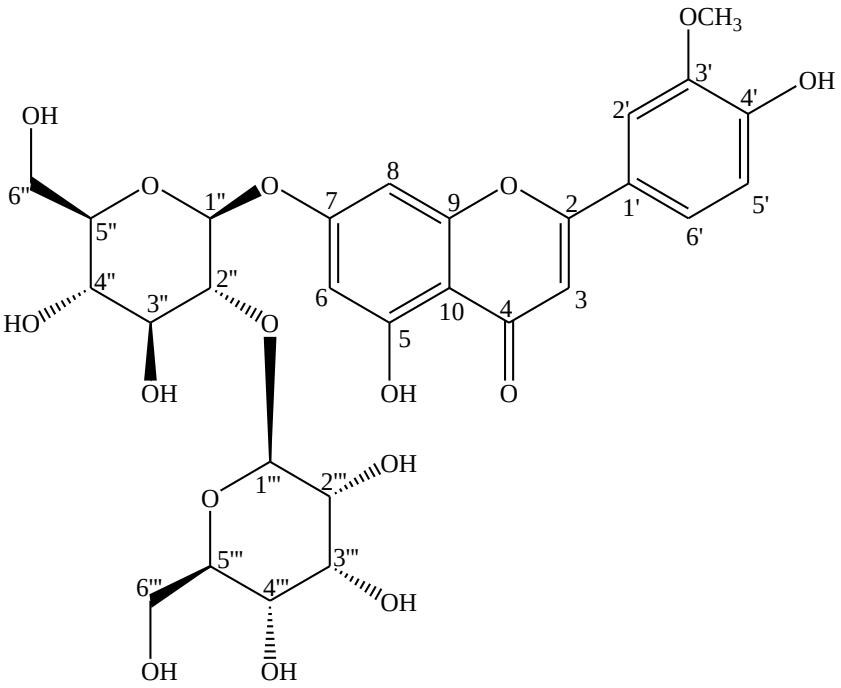


Figure 116: Structure of **7-{[2-*O*-β-D-allopyranosyl-β-D-glucopyranosyl]oxy}-5-hydroxy-2-(4-hydroxy-3-methoxy-phenyl)-4*H*-1-benzopyran-4-one**

From the assembly of fractions 118-131, **7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-β-D-glucopyranosyl]oxy}-5,8-dihydroxy-2-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one** and **7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-6-*O*-acetyl-β-D-glucopyranosyl]oxy}-5,8-dihydroxy-2-(3-hydroxy-4-methoxy-phenyl)-4*H*-1-benzopyran-4-one** (Figures 129, 117) (Lehnerr and Mabry, 1987) were evidenced as an only mixture in ratio 3:1.

7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-β-D-glucopyranosyl]oxy}-5,8-dihydroxy-2-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one: NMR and MS data are reported in paragraph **5.5.14.** at page 189.

7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-6-*O*-acetyl-β-D-glucopyranosyl]oxy}-5,8-dihydroxy-2-(3-hydroxy-4-methoxy-phenyl)-4*H*-1-benzopyran-4-one: ^{1}H-NMR (400 MHz, CD_3OD) δ: 7.56 (1H, br. d, *J* = 8.9 Hz, H-6'), 7.55 (1H, br. s, H-2'), 6.96 (1H, d, *J* = 8.6 Hz, H-5), 6.84 (1H, br. s, H-6), 6.53 (1H, s, H-3), 5.25 (1H, d, *J* = 7.8 Hz, H-1"), 5.00 (1H, d, *J* = 8.0 Hz, H-1'"), 3.99 (3H, s, 4'-OCH_3), 2.01 (3H, s, OAc (Glc)), 1.99 (3H, s, OAc (All)). ESI-MS: *m/z* 746.97 $[M+Na]^+$; *m/z* 723.58 $[M-H]^-$.

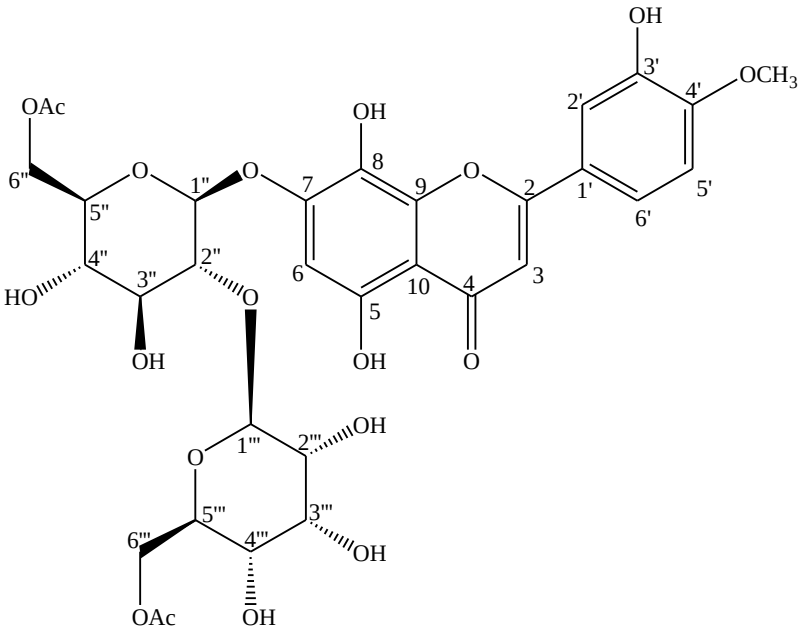


Figure 117: Structure of **7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-6-*O*-acetyl-β-D-glucopyranosyl]oxy}-5,8-dihydroxy-2-(3-hydroxy-4-methoxy-phenyl)-4*H*-1-benzopyran-4-one**

From the assembly of fractions 161-163, **6-deoxy-harpagide** and **harpagide** (Sticher et al., 1975; Venditti et al., 2016d) (Figures 118, 104), were evidenced as an only mixture in ratio 1:1.

6-deoxy-harpagide: 1H-NMR (300 MHz, D_2O) δ: 6.20 (1H, m, H-3), 5.75 (1H, br. s, H-4), 5.44 (1H, d, *J* = 7.7 Hz, H-1), 4.46 (1H, d, *J* = 7.8 Hz, H-1'), 3.92-3.37 (overlapped signals of saccharides), 2.91 (1H, br. s, H-9), 2.79-2.69 (2H, m, H-6), 2.32-2.29 (2H, m, H-7), 1.32 (3H, s, H-10).

ESI-MS: *m/z* 371.31 $[M+Na]^+$.

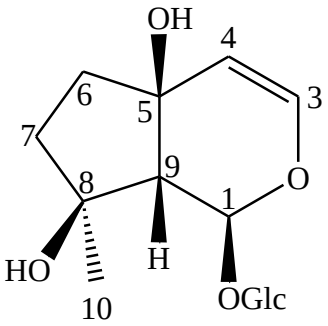


Figure 118: Structure of **6-deoxy-harpagide**

Harpagide: NMR and MS data are reported in paragraph **5.5.8.** at page 160.

From the assembly of fractions 169-179, **ajugoside** and **bartsioside** (Figure 102, 119) (Venditti et al., 2016d; Bianco et al., 1976) were evidenced as an only mixture in ratio 2:1.

Ajugoside: NMR and MS data are reported in paragraph **5.5.8.** at page 158.

Bartsioside: ^{1}H-NMR (400 MHz, CD_3OD) δ: 6.20 (1H, dd, *J* = 6.3/2.3 Hz, H-3), 5.85 (1H, d, *J* = 1.7 Hz, H-7), 5.40 (1H, d, *J* = 5.2 Hz, H-1), 4.98 (1H, d, *J* = 6.2 Hz, H-4), 4.24 (2H, br. d, *J* = 10.0 Hz, H-10), 3.16 (2H, m, H-5, H-9), 2.84-2.70 (1H, m, H-6a), 2.24-2.14 (1H, m, H-6b).
ESI-MS: *m/z* 353.14 $[M+Na]^+$.

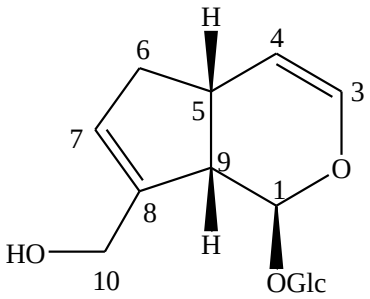


Figure 119: Structure of **bartsioside**

5.5.11. *Stachys affinis* Bunge - rhizomes

An aliquot of the total dried crude extract obtained as described in paragraph **5.4.1.** for the weight of 1.5 g was subjected to a first chromatographic separation on silica gel column, using a correspective amount of silica gel of 100.0 g (ratio 1:20 w/w) and a mixture of *n*-butanol and distilled water (82:18 v/v) as eluting system.
During the chromatographic run, the polarity of the eluting solution was raised in order to let the elution of the most polar compounds and so this became a solution of *n*-butanol, methanol and distilled water at concentration 70:10:30 (v/v/v).
From this chromatographic step, one-hundred and eighteen fractions were collected.
After TLC analysis, these were collected putting together those having the same Rf values, as follows (Table 42):

Fractions	Weight (mg)
1-2	14.7
3-5	23.4
6-17	19.2
18-41	72.1
42-59	35.5
60-66	19.7
67-80	16.6
81-98	15.4
99-108	13.7
109-118	11.1
Methanol column wash	87.9

Table 42: Fraction assemblies and corresponding weights for chromatographic column 1

From the assembly of fractions 3-5, **verbascoside**, **leucosceptoside A** and **martynoside** (Venditti et al., 2016g) (Figures 94, 120, 98), was evidenced as an only mixture in ratio 5:2:1.

Verbascoside: NMR and MS data are reported in paragraph **5.5.5.** at page 146.

Leucosceptoside A: ^{1}H-NMR (300 MHz, CD_3OD) δ: 7.56 (1H, d, *J* = 15.6 Hz, H-β), 7.08 (1H, d, *J* = 1.8 Hz, H-2'), 6.97 (2H, m, overlapped signals, H-6, H6''), 6.80 (1H, d, *J* = 8.2 Hz, H-5'), 6.72 (1H, d, *J* = 2.1 Hz, H-2'), 6.56 (1H, d, *J* = 8.0 Hz, H-5''), 6.38 (1H, d, *J* = 15.6 Hz, H-α), 5.19 (1H, br s, H-1), 4.37 (1H, d, *J* = 7.9 Hz, H-1'''), 3.89 (3H, s, OCH_3), 2.87-2.70 (2H, br. t, *J* = 7.0 Hz, H-β'), 1.09 (3H, d, *J* = 6.1 Hz, H-6''').
ESI-MS: *m/z* 660.84 $[M+Na]^+$.

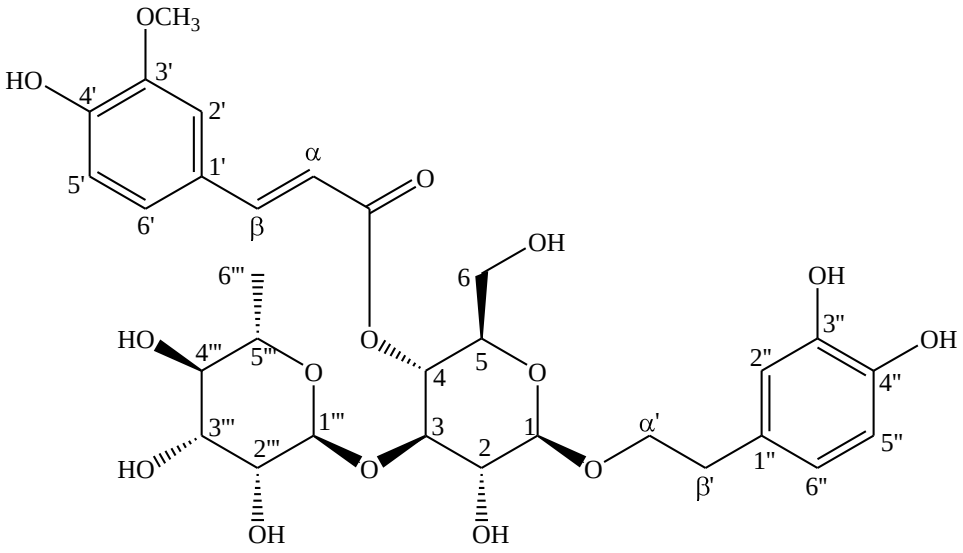


Figure 120: Structure of **leucosceptoside A**

Martynoside: NMR and MS data are reported in paragraph **5.5.6.** at page 152.

From the assembly of fractions 18-41, **harpagide** and **8-*O*-acetyl-harpagide** (Venditti et al., 2016d) (Figures 104, 105), were evidenced as a mixture in ratio 6:1.

Harpagide: NMR and MS data are reported in paragraph **5.5.8.** at page 160.

8-*O*-acetyl-harpagide: NMR and MS data are reported in paragraph **5.5.8.** at pages 160-161.

From the assembly of fractions 60-66, **melittoside** and **5-*O*-allosyloxy-aucubin** (Venditti et al., 2016d; Serrilli et al., 2006) (Figures 107, 109), were evidenced as a mixture in ratio 2:1.

Melittoside: NMR and MS data are reported in paragraph **5.5.8.** at page 162.

5-*O*-allosyloxy-aucubin: NMR and MS data are reported in paragraph **5.5.9.** at page 166.

From the assembly of fractions 109-118, **succinic acid** and **stachyose** (Sciubba et al., 2014a; McIntyre and Vogel, 1989) (Figure 121, 122) were evidenced as a mixture in ratio 1:5.

Succinic acid: ^{1}H-NMR (400 MHz, D_2O) δ: 2.47 (4H, s, α-CH_2 and β- CH_2)
ESI-MS: *m/z* = 117.31 $[M-H]^-$.

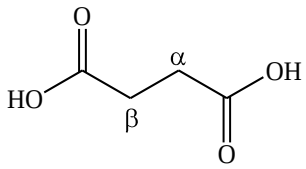


Figure 121: Structure of **succinic acid**

Stachyose: NMR and MS data are reported in paragraph **5.5.11.** at page 122.

From the methanol column wash, **stachyose** (McIntyre and Vogel, 1989) (Figure 122) was evidenced as almost pure compound.

Stachyose: ^{1}H-NMR (400 MHz, D_2O) δ: 5.41 (1H, d, *J* = 4.0 Hz, H-1''), 4.98 (2H, m, H-1, H-1'), 4.27 (1H, d, *J* = 8.9 Hz, H-2'''), 4.22 (1H, m, H-5'), 4.13 (1H, m, H-5''), 4.07 (4H, m, H-4, H-4' and H-6''), 4.00 (2H, m, H-2' and H-5'''), 3.85-3.74 (14H, m, H-3, H-4, H-5, CH_2-6, H-3', CH_2-6', H-3'', H-4''', CH_2-5''', CH_2-6'''), 3.58 (1H, m, H-2''), 3.52 (1H, m, H-4'').
ESI-MS: *m/z* = 689.30 $[M+Na]^+$; *m/z* = 665.28 $[M-H]^-$.

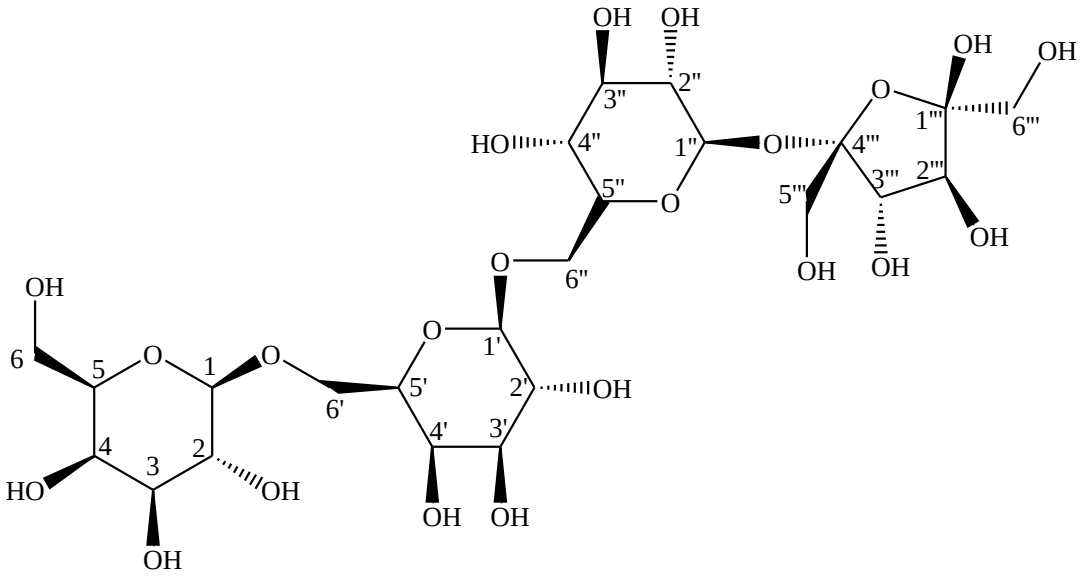


Figure 122: Structure of **stachyose**

5.5.12. *Stachys alopecuros* (L.) Benth. subsp. *divulsa* (Ten.) Grande

The total dried crude extract obtained as described in paragraph **5.4.1.** for the weight of 86.7 g was subjected to an active charcoal treatment as described in paragraph **5.4.3.**.
From this procedure, four fractions of different polarity were collected recovering 23.9 g, 4.70 g and 0.82 g of solids, by extracting respectively with distilled water and the 30, 60 and 96% ethanolic solutions.
The 60% fractions gave the highest positive reaction for the presence of iridoids and so this was used for the chromatographic separation.

The first chromatographic separation was performed on an aliquot of the 60% ethanolic elution for the weight of 4.0 g using 120.0 g of silica gel (ratio 1:30 w/w). The eluting system was a mixture of *n*-butanol/distilled water (82:18 v/v).
From this chromatographic step, one-hundred and sixty-nine fractions were collected.

After TLC analysis, these were collected putting together those having the same Rf values, as follows (Table 43):

Fractions	Weight (mg)
1-7	12.1
8-15	12.4
16-24	11.2
25-29	7.8
30-32	3.2
33-42	9.1
43-45	25.0
46-52	48.8
53-54	113.7
55-69	71.1
70-88	981.2
89-94	91.7
95-112	109.4
113-117	39.7
118-122	48.3
123-132	68.5
133-135	29.1
136-143	49.1
144-163	100.7
164-165	13.3
166-169	24.7

Table 43: Fraction assemblies and corresponding weights for chromatographic column 1

From the assembly of fractions 8-15, **verbascoside** (Venditti et al., 2016f) (Figure 94) was evidenced as almost pure compound.

Verbascoside: NMR and MS data are reported in paragraph **5.5.5.** at page 146.

From the assembly of fractions 17-24, a mixture of **verbascoside** and **stachysoside A** (Venditti et al., 2016f; Nishimura et al., 1991) (Figure 94, 123), in ratio 5:1, was evidenced.

Verbascoside: NMR and MS data are reported in paragraph **5.5.5.** at page 146.

Stachysoside A: ^{1}H-NMR (300 MHz, CD_3OD) δ: 7.60 (1H, d, *J* = 15.6 Hz, H-β''), 7.06 (1H, br. s, H-2''), 6.98 (1H, br. d, *J* = 8.5 Hz, H-6''), 6.78 (1H, d, *J* = 8.5 Hz, H-5''), 6.70 (1H, br. s, H-2'), 6.68 (1H, d, *J* = 8.0 Hz, H-5'), 6.57 (1H, br. d, *J* = 8.1 Hz, H-6'), 6.28 (1H, d, *J* = 15.6 Hz, H-α''), 5.42 (1H, br. s, H-1'''), 4.43 (1H, d, *J* = 7.9 Hz, H-1), 4.31 (1H, d, *J* = 7.2 Hz, H-1'''), 2.81 (2H, d, *J* = 7.1 Hz, H-β'), 1.16 (3H, d, *J* = 6.0 Hz, H-6''').

ESI-MS: *m/z* 779.16 $[M+Na]^+$; *m/z* 755.20 $[M-H]^-$.

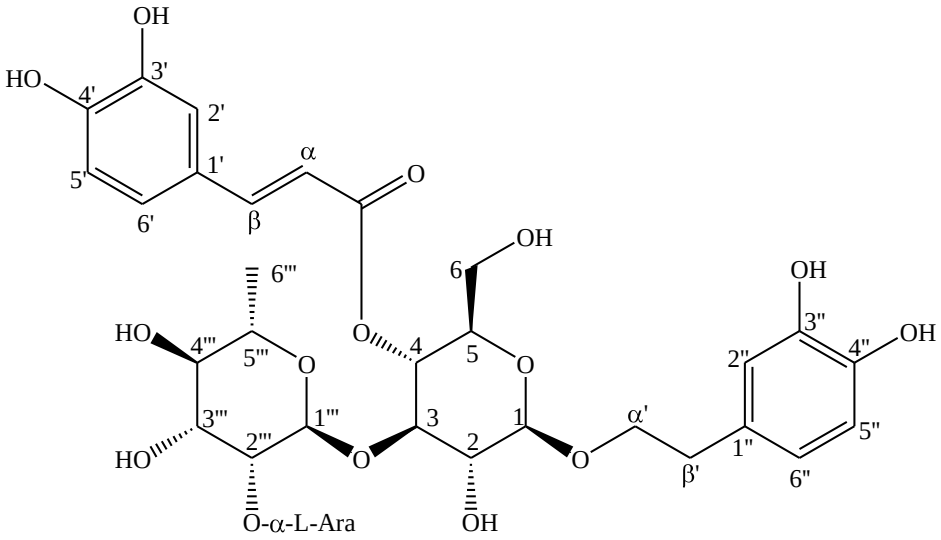


Figure 123: Structure of **stachysoside A**

From the assembly of fractions 30-32, **β-arbutin** (Thies and Sulc, 1950) (Figure 124) was evidenced as almost pure compound.

β-arbutin: ^{1}H-NMR (300 MHz, CD_3OD) δ: 7.07 (2H, d, *J* = 8.6 Hz, H-3, H-5), 6.69 (2H, d, *J* = 8.6 Hz, H-2, H-6), 4.95 (1H, partially obscured by HDO signal, H-1'), 3.96-3.79 (m, overlapped glucose protons), 3.77-3.54 (m, overlapped glucose protons), 3.18 (1H, br. t, *J* = 7.9 Hz, H-2').
ESI-MS: *m/z* 311.22 $[M+K]^+$.

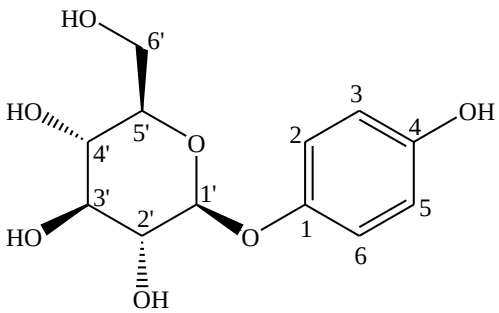


Figure 124: Structure of **β-arbutin**

From the assembly of fractions 43-45, a mixture of **reptoside** and **6-*O*-acetyl-ajugol** (Guiso et al., 1974; Tasdemir et al., 1999) (Figures 125, 126), in ratio 1:1.5, was evidenced.

Reptoside: ^{1}H-NMR (300 MHz, D_2O) δ: 6.40 (1H, d, *J* = 6.5 Hz, H-3), 5.90 (1H, br s, H-1), 5.02 (1H, dd, *J* = 6.4/1.3 Hz, H-4), 4.75 (1H, d, partially obscured by the solvent signal, H-1'), 3.92 (1H, dd, *J* = 12.4/1.9 Hz, H-6'a), 3.71 (1H, br. d, *J* = 12.4 Hz, H-6'b), 3.26 (1H, dd, *J* = 8.3/8.2 Hz, H-2'), 2.65 (1H, br. s, H-9), 2.04 (3H, s, OAc), 1.93 (2H, m, H-6a, H-7a), 1.71 (2H, m, H-6b, H-7b), 1.43 (3H, s, H-10).
ESI-MS: *m/z* 413.04 $[M+Na]^+$.

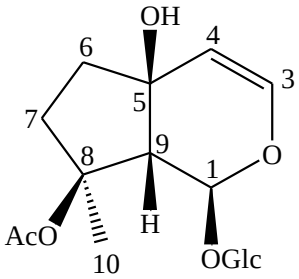


Figure 125: Structure of **reptoside**

6-*O*-acetyl-ajugol: ^{1}H-NMR (300 MHz, D_2O) δ: 6.34 (1H, dd, *J* = 6.4/1.8 Hz, H-3), 5.50 (1H, d, *J* = 2.8 Hz, H-1), 4.89 (1H, dd, *J* = 6.4/2.8 Hz, H-4), 4.81 (1H, obscured by the solvent signal, H-6), 4.70 (1H, d, *J* = 8.0 Hz, H-1'), 3.90 (1H, dd, *J* = 12.1/1.6 Hz, H-6'), 3.68 (1H, br. d, *J* = 12.2 Hz, H-6'b), 3.24 (1H, m, H-2'), 2.79 (1H, br. t, *J* = 6.5 Hz, H-5), 2.34 (1H, dd, *J* = 7.9/2.7 Hz, H-9), 2.12 (1H, br. dd, *J* = 14.0/6.5 Hz, H-7a), 2.08 (3H, s, OAc), 1.93 (1H, dd, *J* = 13.8/8.6 Hz, H-7b), 1.38 (3H, s, H-10).
ESI-MS: *m/z* 413.04 $[M+Na]^+$.

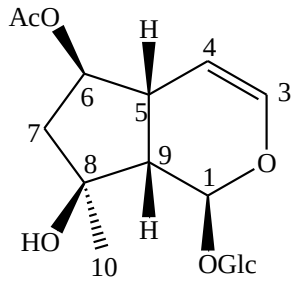


Figure 126: Structure of **6-*O*-acetyl-ajugol**

From the assembly of fractions 53-54, **ajugoside** (Venditti et al.,2016d) (Figure 102), was evidenced as pure compound.

Ajugoside: NMR and MS data are reported in paragraph **5.5.8.** at page 158.

From the assembly of fractions 70-88, **8-*O*-acetyl-harpagide** (Venditti et al., 2016d) (Figure 105), was evidenced as almost pure compound.

8-*O*-acetyl-harpagide: NMR and MS data are reported in paragraph **5.5.8.** at pages 160-161.

From the assembly of fractions 95-112, a mixture of **8-*O*-acetyl-harpagide** and **chlorogenic acid** (Venditti et al., 2016d; Caprioli et al., 2016) (Figures 105, 108), in ratio 1:2, was evidenced.

8-*O*-acetyl-harpagide: NMR and MS data are reported in paragraph **5.5.8.** at pages 160-161.

Chlorogenic acid: NMR and MS data are reported in paragraph **5.5.9.** at page 165.

From the assembly of fractions 118-122, a mixture of **harpagide** and **chlorogenic acid** (Venditti et al., 2016d; Caprioli et al., 2016) (Figures 104, 108), in ratio 3:1 was evidenced.

Harpagide: NMR and MS data are reported in paragraph **5.5.8.** at page 160.

Chlorogenic acid: NMR and MS data are reported in paragraph **5.5.9.** at page 165.

From the assembly of fractions 133-135, a mixture of **chlorogenic acid** and **allobetonicoside** (Caprioli et al., 2016; Venditti et al., 2016d) (Figures 108, 103), in ratio 10:1, was evidenced.

Chlorogenic acid: NMR and MS data are reported in paragraph **5.5.9.** at page 165.

Allobetonicoside: NMR and MS data are reported in paragraph **5.5.8.** at page 159.

From the assembly of fractions 144-163, **allobetonicoside** (Venditti et al., 2016d) (Figure 103), was evidenced as pure compound.

Allobetonicoside: NMR and MS data are reported in paragraph **5.5.8.** at page 159.

From the assembly of fractions 166-169, a mixture of **allobetonicoside** (Venditti et al., 2016d) and **4'-*O*-galactopyranosyl-teuhircoside** (Figure 103, 127), in ratio 1:3, was evidenced.

Allobetonicoside: NMR and MS data are reported in paragraph **5.5.8.** at page 159.

4'-*O*-galactopyranosyl-teuhircoside: ^{1}H-NMR (300 MHz, D_2O) δ: 6.43 (1H, d, *J* = 6.4 Hz, H-3), 6.13 (1H, br. s, H-7), 5.94 (1H, br. s, H-1), 4.98 (1H, d, *J* = 6.4 Hz, H-4), 4.80 (1H, partially overlapped with solvent signal, H-1"), 4.73 (1H, d, *J* = 8.1 Hz, H-1'), 2.29 (3H, s, H-10).
ESI-MS: *m/z* 529.12 $[M+Na]^+$.

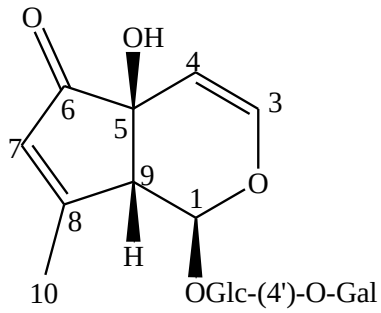


Figure 142: Structure of **4'-*O*-galactopyranosyl-teuhircoside**

5.5.13. *Stachys annua* L.

An aliquot of the total dried crude extract obtained as described in paragraph **5.4.1.** for the weight of 2.0 g out of 8.5 g was subjected to a first chromatographic separation on silica gel column, using a correspective amount of silica gel of 60.0 g (ratio 1:30 w/w) and a mixture of *n*-butanol and distilled water (82:18 v/v) as eluting system.
During the chromatographic run, the polarity of the eluting solution was raised in order to let the elution of the most polar compounds and so this became a solution of *n*-butanol, methanol and distilled water at concentration 70:10:30 (v/v/v).
From this chromatographic step, forty-nine fractions were collected.
After TLC analysis, these were collected putting together those having the same Rf values, as follows (Table 44):

Fractions	Weight (mg)
1	318.8
2-11	266.0
12-22	222.3
23-46	110.4

Table 44: Fraction assemblies and corresponding weights for chromatographic column 1

No compound could be clearly identified from this chromatographic step and for this a second chromatographic separation was carried out on a specific assembly deriving from the first one, 2-11 for the total weight of 266.0 g using a correspective amount of silica gel of 8.0 g (ratio 1:35 w/w) and a chloroform as eluting system.

From this chromatographic step, thirty-three fractions were collected.

After TLC analysis, these were collected putting together those having the same Rf values, as follows (Table 45):

Fractions	Weight (mg)
1-4	6.0
5-13	13.7
14-39	65.2

Table 45: Fraction assemblies and corresponding weights for chromatographic column 2

No compound could be clearly identified even from this chromatographic step and for this a third chromatographic separation was carried out on another specific assembly deriving from the first one, 12-22 for the total weight of 222.3 g using a correspective amount of silica gel of 8.0 g (ratio 1:35 w/w) and a mixture of chloroform/methanol acidified with gaseous CO_2 at different concentrations as eluting system.

The initial one was 8:2 (v/v) but during the chromatographic run, this was changed to increase the polarity and let the elution of the most polar compounds, passing to 7:3 (v/v) and 6:4 (v/v).

From this chromatographic step, seventy-two fractions were collected.

After TLC analysis, these were collected putting together those having the same Rf values, as follows (Table 46):

Fractions	Weight (mg)
1-7	10.9
8-9	5.0
10-16	6.7
17-25	31.5
26-31	28.0
32-44	85.4
45-50	22.3
51-57	12.3
58-72	69.2

Table 46: Fraction assemblies and corresponding weights for chromatographic column 3

From the assembly of fractions 8-9, a mixture of **7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-β-D-glucopyranosyl]oxy}-5,8-dihydroxy-2-(4-methoxyphenyl)-4*H*-1-benzopyran-4-one** and **7-{[2-*O*-β-D-allopyranosyl-(6-*O*-acetyl-β-D-glucopyranosyl)]oxy}-5,8-dihydroxy-2-(3-hydroxy-4-methoxy-phenyl)-4*H*-1-benzopyran-4-one** (Lehnerr & Mabry, 1987; Venditti et al., 2014) (Figures 96, 128) was evidenced in ratio 3:1.

7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-β-D-glucopyranosyl]oxy}-5,8-dihydroxy-2-(4-methoxyphenyl)-4*H*-1-benzopyran-4-one: NMR and MS data are reported in paragraph **5.5.6.** at page 149.

7-{[2-*O*-β-D-allopyranosyl-(6-*O*-acetyl-β-D-glucopyranosyl)]oxy}-5,8-dihydroxy-2-(3-hydroxy-4-methoxy-phenyl)-4*H*-1-benzopyran-4-one: ^{1}H-NMR (300 MHz, CD_3OD) δ: 7.79 (1H, br. d, *J* = 8.0 Hz, H-6'), 7.53 (1H, br. s, H-2'), 7.25 (1H, d, *J* = 8.0 Hz, H-5'), 6.84 (1H, s, H-6), 6.74 (1H, s, H-3), 5.12 (1H, d, *J* = 8.0 Hz, H-1"), 5.04 (1H, d, *J* = 7.9 Hz, H-1'"), 4.46 (1H, *J* = 10.8 Hz, Ha-6"'), 3.95 (3H, s, 4'-OCH_3), 2.10 (3H, s, OAc).
ESI-MS: *m/z* 705.16 $[M+Na]^+$.

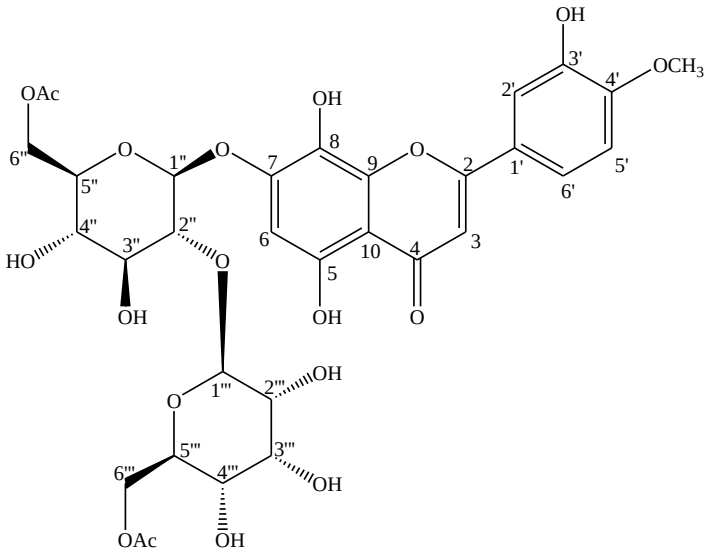


Figure 128: Structure of **7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-β-D-glucopyranosyl]oxy}-5,8-dihydroxy-2-(3-hydroxy-4-methoxy-phenyl)-4*H*-1-benzopyran-4-one**

5.5.14. *Stachys germanica* subsp. *salviifolia* L. (Ten.) Gams.

An aliquot of the total dried crude extract obtained as described in paragraph **5.4.1.** for the weight of 4.2 g was subjected to a first chromatographic separation on silica gel column, using a correspective amount of silica gel of 60.0 g (ratio 1:30 w/w) and a mixture of *n*-butanol and distilled water (82:18 v/v) acidified with gaseous CO_2 as eluting system.

During the chromatographic run, the polarity of the eluting solution was raised in order to let the elution of the most polar compounds and so this became a solution of *n*-butanol, methanol and distilled water at concentration 70:10:30 (v/v/v).

From this chromatographic step, one hundred and thirty-eight fractions were collected.

After TLC analysis, these were collected putting together those having the same Rf values, as follows (Table 47):

Fractions	Weight (mg)
1-10	32.2
11-27	585.5
28-38	569.3
39-51	45.2
52-59	33.6
60-66	30.2
67-72	56.8
73-84	52.1
85-92	14.2
93-97	34.8
98-106	48.7
107-118	60.7
119-123	30.3
124-138	85.6

Table 47: Fraction assemblies and corresponding weights for chromatographic column 1

From the assembly of fractions 39-51, **7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-β-D-glucopyranosyl]oxy}-5,8-dihydroxy-2-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one** (Lehnerr & Mabry, 1987) (Figure 129) (Spectrum 16) was evidenced as almost pure compound.

7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-β-D-glucopyranosyl]oxy}-5,8-dihydroxy-2-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one: ^{1}H- NMR (300 MHz, CD_3OD) δ: 7.92 (2H, d, *J* = 8.9 Hz, H-2', H-6'), 6.95 (2H, d, *J* = 8.9 Hz, H-3', H-5'), 6.79 (1H, br. s, H-3), 6.63 (1H, br. s, H-6), 5.08 (1H, d, *J* = 8.1 Hz, H-1'''), 4.95 (partially overlapped with solvent signal, H-1''), 4.08-3.45 (overlapped signals of saccharides), 2.00 (3H, s, OAc).
^{13}C NMR: δ 183.0 (C-4), 171.6 (OAc), 164.9 (C-2), 161.5 (C-4'), 143.7 (C-9), 130.1 (C-2', C-6'), 121.8 (C-1'), 115.3 (C-3', C-5'), 102.5 (C-6, C-3), 101.5 (C-1''), 82.6 (C-2''), 67.0 (C-4'''), 63.6 (C-6'''), 19.4 (OAc).
ESI-MS: *m/z* 675.05 $[M+Na]^+$.

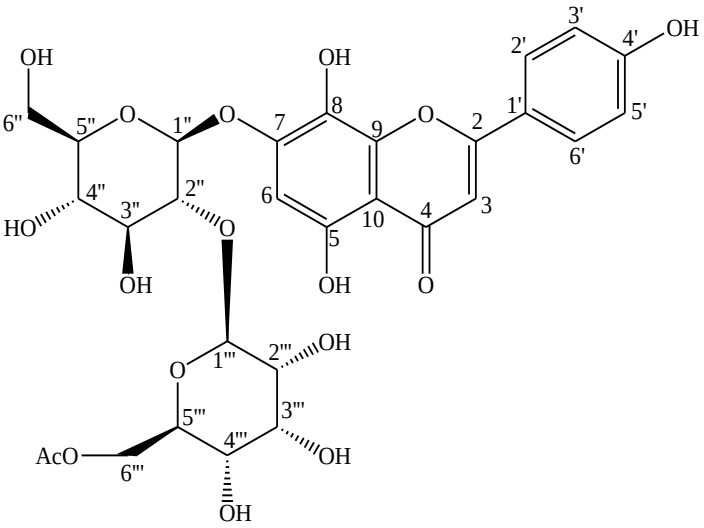
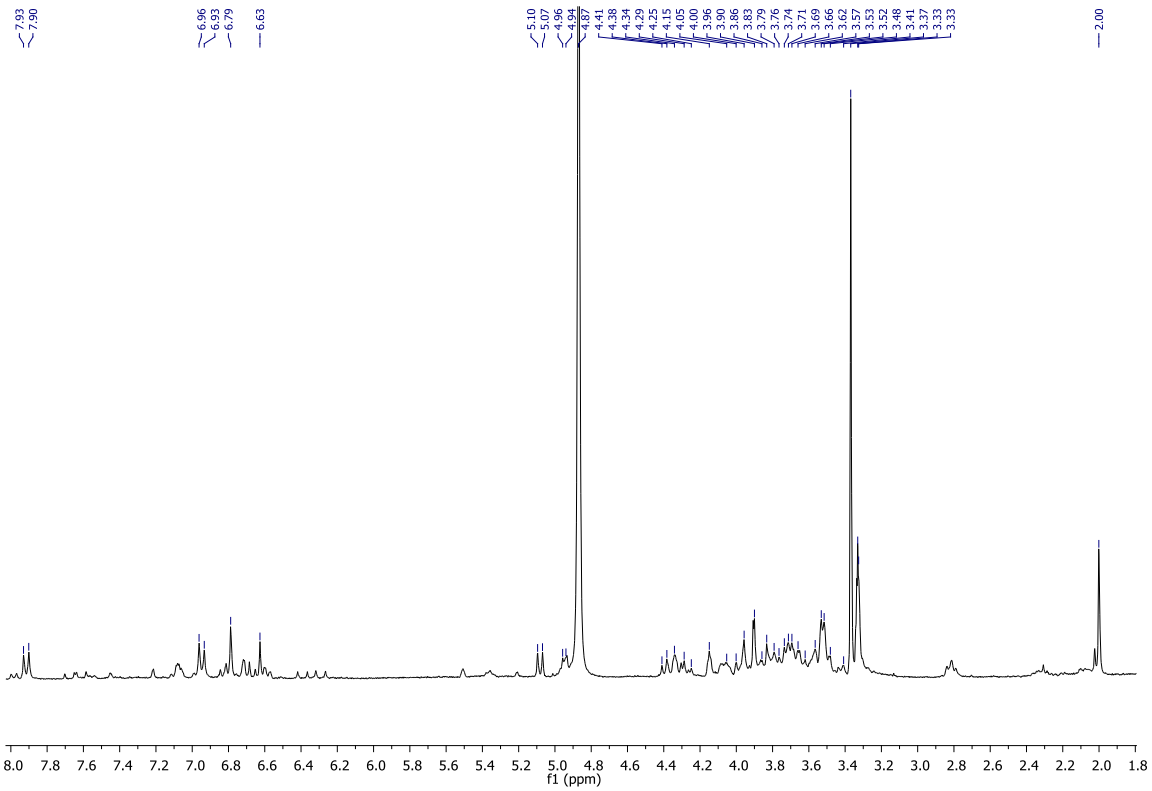


Figure 129: Structure of **7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-β-D-glucopyranosyl]oxy}-5,8-dihydroxy-2-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one**



Spectrum 16: ^{1}H-NMR spectrum of **7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-β-D-glucopyranosyl]oxy}-5,8-dihydroxy-2-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one**

From the assembly of fractions 52-59, **7-{[2-*O*-β-D-allopyranosyl-(6-*O*-acetyl-β-D-glucopyranosyl)]oxy}-5,8-dihydroxy-2-(3-hydroxy-4-methoxy-phenyl)-4*H*-1-**

benzopyran-4-one and **stachysoside** (Venditti et al., 2014) (Figure 128, 123), were evidenced in an only mixture in ratio 3:1.

7-{[2-*O*-β-D-allopyranosyl-(6-*O*-acetyl-β-D-glucopyranosyl)]oxy}-5,8-dihydroxy-2-(3-hydroxy-4-methoxy-phenyl)-4*H*-1-benzopyran-4-one: NMR and MS data are reported in paragraph **5.5.13.** at page 187.

Stachysoside: NMR and MS data are reported in paragraph **5.5.12.** at page 181.

From the assembly of fractions 85-92, **chlorogenic acid** and **harpagide** (Caprioli et al., 2016; Venditti et al., 2016d) (Figures 108, 104), were evidenced in mixture in ratio 1:1.

Chlorogenic acid: NMR and MS data are reported in paragraph **5.5.9.** at page 165.

Harpagide: NMR and MS data are reported in paragraph **5.5.8.** at page 160.

5.5.15. *Stachys palustris* L. - Hungary

An aliquot of the total dried crude extract obtained as described in paragraph **5.4.1.** for the weight of 4.0 g was subjected to a first chromatographic separation on silica gel column, using a correspective amount of silica gel of 60.0 g (ratio 1:30 w/w) and a mixture of *n*-butanol and distilled water (82:18 v/v) as eluting system.
During the chromatographic run, the polarity of the eluting solution was raised in order to let the elution of the most polar compounds and so this became a solution of *n*-butanol, methanol and distilled water at concentration 70:10:30 (v/v/v).
From this chromatographic step, one hundred and thirty-eight fractions were collected.
After TLC analysis, these were collected putting together those having the same Rf values, as follows (Table 48):

Fractions	Weight (mg)
1-12	121.3
13-19	36.9
20-24	25.5
25-40	64.1
41-49	32.2
50-54	16.9
55-71	36.7
72-80	13.3
81-89	25.2
90-94	21.1
95-105	48.8
106-110	51.1
111-145	119.5

Table 48: Fraction assemblies and corresponding weights for chromatographic column 1

From the assembly of fractions 20-24, **verbascoside** (Venditti et al., 2016f) (Figure 94), was evidenced as almost pure compound.

Verbascoside: NMR and MS data are reported in paragraph **5.5.5.** at page 146.

From the assembly of fractions 50-54, **echinacoside** (Kobayashi et al., 1984) (Figure 95), was evidenced as almost pure compound.

Echinacoside: NMR and MS data are reported in paragraph **5.5.5.** at page 148.

From the assembly of fractions 72-80, **7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-β-D-glucopyranosyl]oxy}-5,8-dihydroxy-2-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one** and **7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-β-D-glucopyranosyl]oxy}-5,8-dihydroxy-2-(4-methoxyphenyl)-4*H*-1-benzopyran-4-one** (Lenherr and Mabry, 1987; Venditti et al., 2014) (Figures 129, 96), were evidenced in mixture in ratio 3:1.

7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-β-D-glucopyranosyl]oxy}-5,8-dihydroxy-2-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one: NMR and MS data are reported in paragraph **5.5.14.** at page 189.

7-{[2-*O*-(6-*O*-acetyl-β-D-allopyranosyl)-β-D-glucopyranosyl]oxy}-5,8-dihydroxy-2-(4-methoxyphenyl)-4*H*-1-benzopyran-4-one: NMR and MS data are reported in paragraph **5.5.6.** at page 149.

From the assembly of fractions 90-94, **chlorogenic acid** and **harpagide** (Caprioli et al., 2016; Venditti et al., 2016d) (Figures 108, 104) were evidenced in mixture in ratio 5:1.

Chlorogenic acid: NMR and MS data are reported in paragraph **5.5.9.** at page 165.

Harpagide: NMR and MS data are reported in paragraph **5.5.8.** at page 160.

From the assembly of fractions 106-110, **8-*O*-acetyl-harpagide** and **monomelittoside** (Venditti et al., 2016d) (Figure 105, 106) were evidenced in mixture in ratio 3:2.

8-*O*-acetyl-harpagide: NMR and MS data are reported in paragraph **5.5.8.** at pages 160-161.

Monomelittoside: NMR and MS data are reported in paragraph **5.5.8.** at page 162.

5.5.16. *Stachys palustris* L. - France

The total dried crude extract was obtained as described in paragraph **5.4.1.** for the weight of 16.6 g.
The phytochemical analysis of this sample was conducted only by means of TLC directly comparing a portion of its total crude dried with the concentration of 20 mg/mL with a portion of the total crude dried extract of the sample from Hungary with the same concentration after run in *n*-butanol saturated with distilled water in ratio 82:18 (v/v).
After this, a direct comparison on a semi-preparative TLC between the two plant samples and the compound standards present in our laboratory was performed evidencing the presence of the same compounds as found in *Stachys palustris* L. from Hungary.

5.5.17. *Teucrium chamaedrys* L.

An aliquot of the total dried crude extract obtained as described in paragraph **5.4.1.** for the weight of 3.0 g, underwent a first chromatographic separation using a correspective amount of silica gel of 120.0 g (ratio 1:30) and a mixture of *n*-butanol and distilled water in concentration ratio 82:18 (v/v) as eluting system.

During the chromatographic run, the polarity of the eluting solution was raised in order to let the elution of the most polar compounds and so this became a solution of *n*-butanol, methanol and distilled water at concentration 70:10:30 (v/v/v).
From this chromatographic step, one hundred and forty-five fractions were collected.
After TLC analysis, these were collected putting together those having the same Rf values, as follows (Table 49):

Fractions	Weight (mg)
1-12	375,0
13-14	62,0
15-35	71,1
36-37	22,4
38-43	27.6
44-45	16,5
46-59	21,3
60-81	12,2 g
82-86	16,9 g
87-100	42,0 g
101-145	97,3 g

Table 49: Fraction assemblies and corresponding weights for chromatographic column 1

From the assembly of fractions 13-14, **verbascoside** (Venditti et al., 2016f) (Figure 94), was evidenced as almost pure compound.

Verbascoside: NMR and MS data are reported in paragraph **5.5.5.** at page 146.

From the assembly of fractions 15-35 (Spectra 17, 18), **forsythoside b**, **samioside** and **alyssonoside** (Saracoğlu et al., 2002; Yalçin et al., 2003) (Figures 130, 131, 132), were evidenced in an only mixture in ratio 5:1:1.

Forsythoside b: ^{1}H-NMR (400 MHz, CD_3OD): δ 7.60 (1H, d, *J* = 15.9 Hz, H-β), 7.06 (1H, d, *J* = 2.0 Hz, H-2'), 6.96 (1H, dd, *J* = 8.2/2.0 Hz, H-6'), 6.78 (1H, d, *J* = 8.2 Hz, H-5'), 6.70 (1H, d, *J* = 2.0 Hz, H-2''), 6.68 (1H, d, *J* = 8.0 Hz, H-5''), 6.57 (1H, dd, *J* = 8.0/2.0 Hz, H-6''), 6.27 (1H, d, *J* = 15.9 Hz, H-α), 5.12 (1H, d, *J* = 1.6 Hz, H-1'''), 4.89 (1H, d, *J* = 2.4 Hz, H-1^{IV}), 4.37 (1H, d, *J* = 7.9 Hz, H-1), 4.02-3.98 (1H, m, H-α'), 3.95-3.35 (overlapped signals of saccharides), 2.93-2.68 (1H, m, H-β'), 1.07 (3H, d, *J* = 6.2 Hz, H-6''').
^{13}C-NMR (100 MHz, CD_3OD): δ 168.25 (COO(caff.)), 149.6 (C-4'), 148.0 (C-β), 146.8 (C-3'), 146.1 (C-3''), 144.7 (C-4''), 131.4 (C-1), 127.7 (C-1'), 123.2 (C-5'), 121.3 (C-5''), 117.3 (C-

2''), 116.3 (C-6''), 115.2 (C-2'), 114.7 (C-α), 109.1 (C-1^{IV}), 104.2 (C-1), 103.0 (C-1'''), 81.9 (C-3), 80.4 (C-3^{IV}), 76.1 (C-2^{IV}), 76.0 (C-2), 75.1 (C-4^{IV}), 74.1 (C-4'''), 74.0 (C-5), 72.6 (C-α'), 72.3 (C-2'''), 71.9 (C-3'''), 70.4 (C-5'''), 68.9 (C-6), 65.0 (C-5^{IV}), 36.6 (C-β'), 18.5 (C-6'''). ESI-MS: *m/z* 779.37 $[M+Na]^+$.

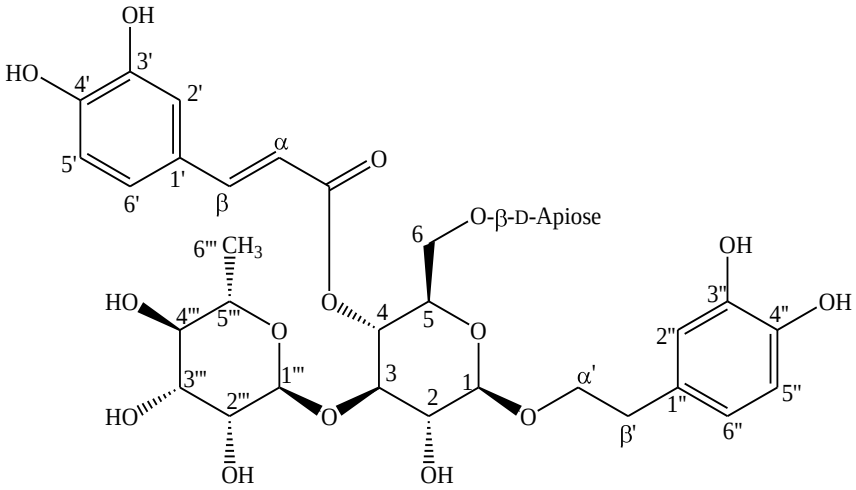


Figure 130: Structure of **forsythoside b**

Samioside: ^{1}H-NMR (400 MHz, CD_3OD): δ 7.56 (1H, d, *J* = 15.9 Hz, H-β), 7.10 (1H, br. s, H-2'), 7.01 (1H, br. d, *J* = 8.3 Hz, H-6'), 6.78 (1H, d, *J* = 8.3 Hz, H-5'), 6.70 (1H, d, *J* = 2.0 Hz, H-2''), 6.68 (1H, d, *J* = 8.0 Hz, H-5''), 6.57 (1H, dd, *J* = 8.0/2.0 Hz, H-6''), 6.27 (1H, d, *J* = 15.9 Hz, H-α), 5.28 (1H, d, *J* = 1.5 Hz, H-1'''), 5.24 (1H, d, *J* = 3.0 Hz, H-1^{IV}), 4.37 (1H, d, *J* = 7.9 Hz, H-1), 4.02-3.98 (1H, m, H-α'), 3.95-3.35 (overlapped signals of saccharides), 2.93-2.68 (1H, m, H-β'), 1.10 (3H, d, *J* = 5.9 Hz, H-6'''). ESI-MS: *m/z* 675.48 $[M+Na]^+$.

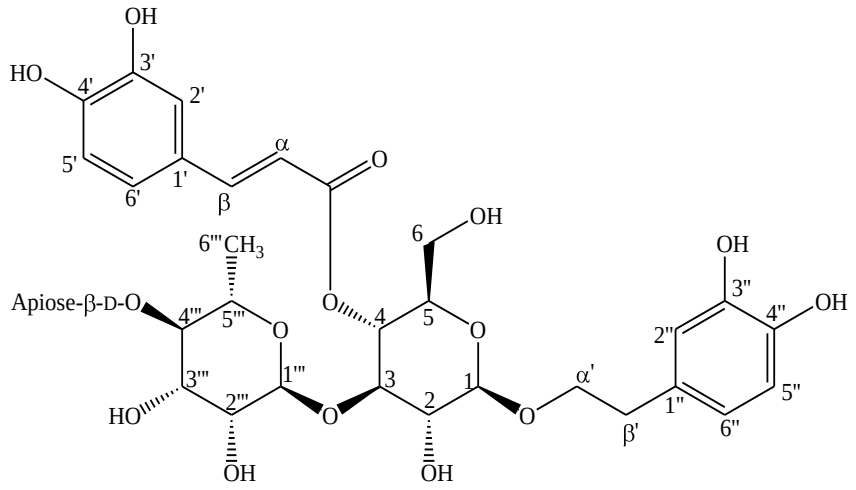


Figure 131: Structure of **samioside**

Alyssonoside: ^{1}H-NMR (400 MHz, CD_3OD) δ: 7.66 (1H, d, *J* = 16.0 Hz, H-β), 7.20 (1H, br. s, H-2'), 7.10 (1H, br. d, *J* = 8.1 Hz, H-6'), 6.90 (1H, d, *J* = 8.1 Hz, H-5'), 6.73 (1H, br. s, H-2''), 6.64 (1H, d, *J* = 8.2 Hz, H-5''), 6.55 (1H, br. d, *J* = 8.2 Hz, H-6''), 6.29 (1H, d, *J* = 16.0 Hz, H-α), 5.19 (1H, d, *J* = 1.7 Hz, H-1'''), 4.87 (1H, d, *J* = 2.1 Hz, H-1^{IV}), 4.33 (1H, d, *J* = 8.0 Hz, H-1), 4.02-3.98 (1H, m, H-α'), 3.81 (3H, s, O-CH_3), 3.80-3.35 (overlapped signals of saccharides), 2.93-2.68 (1H, m, H-β'), 1.05 (3H, d, *J* = 6.2 Hz, H-6''').

ESI-MS: *m/z* 793.44 $[M+Na]^+$.

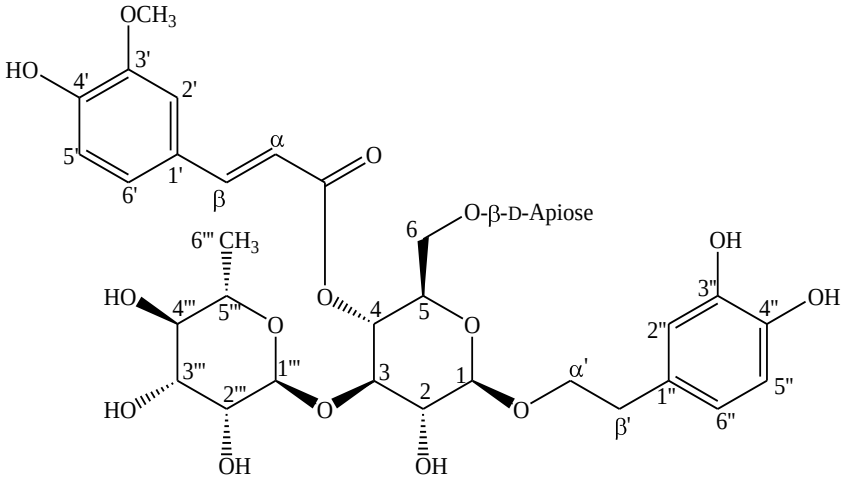
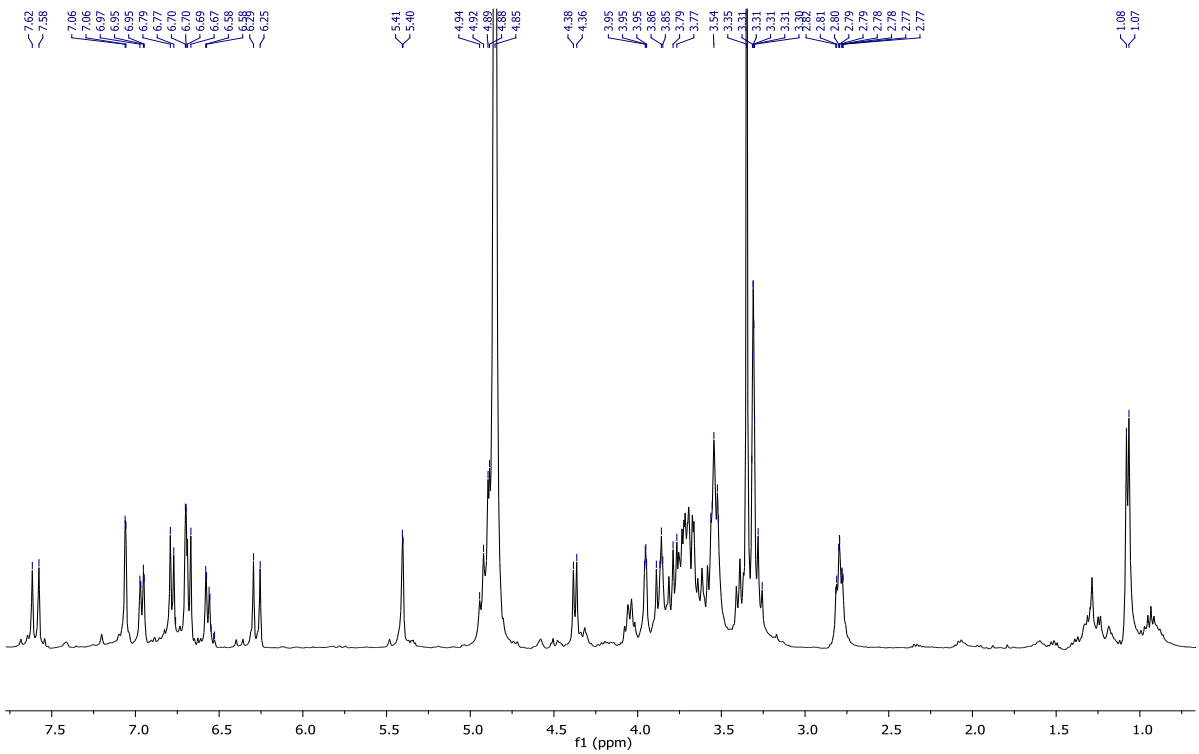
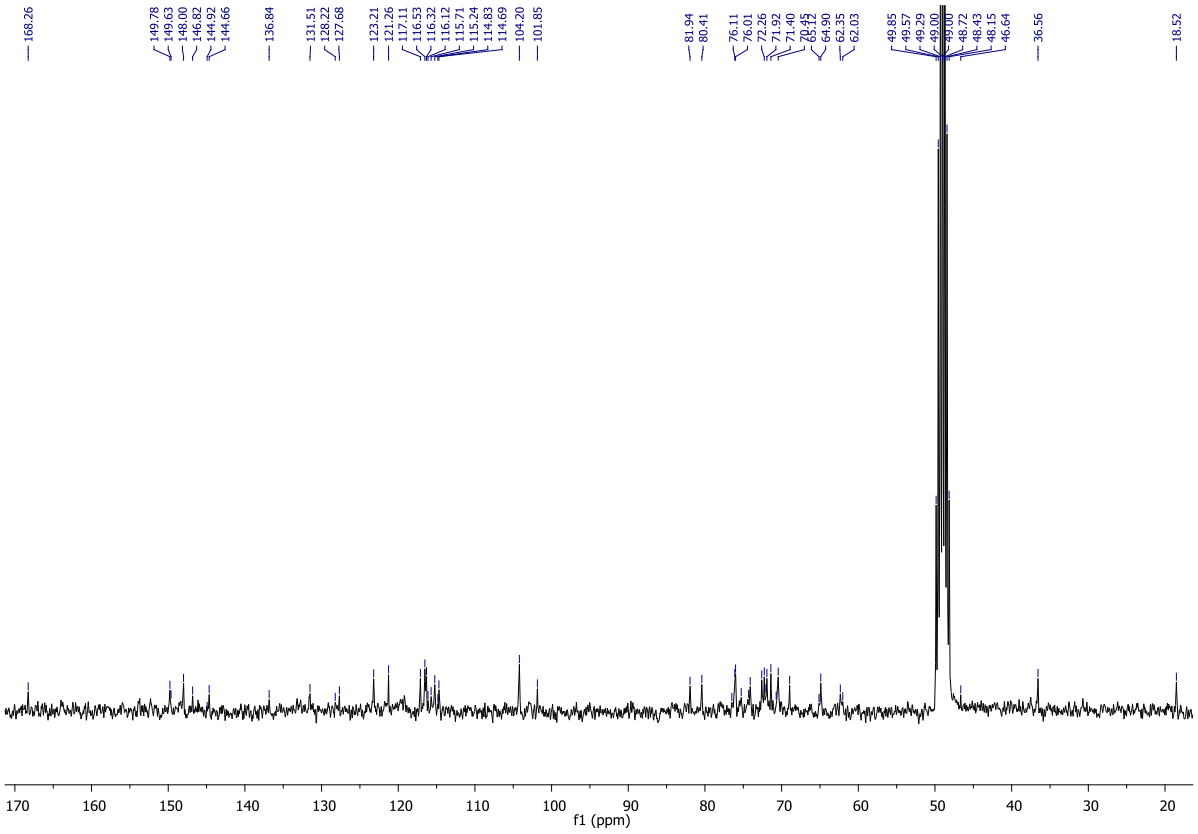


Figure 132: Structure of **alyssonoside**



Spectrum 17: ^{1}H-NMR of the assembly of fractions 15-35



Spectrum 18: ^{13}C-NMR of the assembly of fractions 15-35

From the assembly of fractions 82-86, **forsythoside b** and **8-*O*-acetil-harpagide** (Figures 130, 105), were evidenced in mixture in ratio 1:3 (Saracoğlu et al., 2002; Venditti et al., 2016d).

Forsythoside b: NMR and MS data are reported in paragraph **5.5.17.** at pages 194-195.

8-*O*-acetil-harpagide: NMR and MS data are reported in paragraph **5.5.8.** at pages 160-161.

From the assembly of fractions 101-145, **forsythoside b** and **harpagide** (Figures 130, 104), were evidenced in mixture in ratio 1:2 (Saracoğlu et al., 2002; Venditti et al., 2016d).

Forsythoside b: NMR and MS data are reported in paragraph **5.5.17.** at at pages 194-195.

Harpagide: NMR and MS data are reported in paragraph **5.5.8.** at page 160.

Since not all the present compounds could be clearly identified from this separation step, a second column chromatography was performed on an assembly of fractions deriving from the first one, 2-12, for the total weight of 375.0 mg.
The correspective amount of silica gel was 16.0 g (ratio 1:40 w/w), this time, and the eluting system consisted of a mixture of chloroform/methanol at different concentrations.
The initial one was 95:5 (v/v) but during the chromatographic run, this was changed in order to raise the polarity of the solution and let the elution of the most polar compounds, to 9:1 (v/v), 8:2 (v/v), and lastly 7:3 (v/v).
From this chromatographic step, fifty-six fractions were collected.
After TLC analysis, these were collected putting together those having the same Rf values, as follows (Table 50):

Fractions	Weight (mg)
1-5	16.6
9-10	20.1
11-14	36.9
15-31	61.2
32-33	2,2
34-43	35,5
44-45	16,5
44-56	24.4

Table 50: Fraction assemblies and corresponding weights for chromatographic column 2

From the assembly of fractions 44-56, **cirsiliol** and **β-arbutin** (**8**) (Alwahsh et al., 2015; Venditti et al, 2017b) (Figures 145, 124), were evidenced in mixture in ratio 3:1.

Cirsiliol: NMR and MS data are reported in paragraph **5.5.19.** at page 216.

β-arbutin: NMR and MS data are reported in paragraph **5.5.12.** at page 182.

5.5.18. *Teucrium polium* L. - Northern Iran

An aliquot of the total dried crude extract obtained as described in paragraph **5.4.1.** for the weight of 3.0 g was subjected to a first chromatographic separation using a correspective amount of silica gel of 75.0 g (ratio 1:25 w/w) and a mixture of chloroform/methanol at different concentrations as eluting system.

The initial one was 95:5 (v/v) but during the chromatographic run, this was changed in order to raise the polarity of the solution and let the elution of the most polar compounds, to 9:1 (v/v), 8:2 (v/v), 7:3 (v/v) and lastly 6:4 (v/v).

From this chromatographic step, one hundred and eighty-four fractions were collected.

After TLC analysis, these were collected putting together those having the same Rf values, as follows (Table 51):

Fractions	Weight (mg)
3-47	28.7
54-58	33.5
59-60	33.8
61-86	663.7
87-95	10.7
96-106	7.8
161-163	15.7
164-184	34.6
Wash	30.8

Table 51: Fraction assemblies and corresponding weights for chromatographic column 1

From the assembly of fractions 59-60, **apigenin** (Faidi et al., 2014) (Figure 133) was evidenced as almost pure compound.

Apigenin: ^{1}H-NMR (CD_3OD, 300 MHz) δ: 7.86 (2H, d, *J* = 8.6 Hz, H-2', H-6'), 6.94 (1H, d, *J* = 8.6 Hz, H-3', H-5'), 6.60 (1H, s, H-3), 6.46 (1H, s, H-8), 6.20 (1H, s, H-6).

ESI-MS: *m/z* 293.18 $[M+Na]^+$.

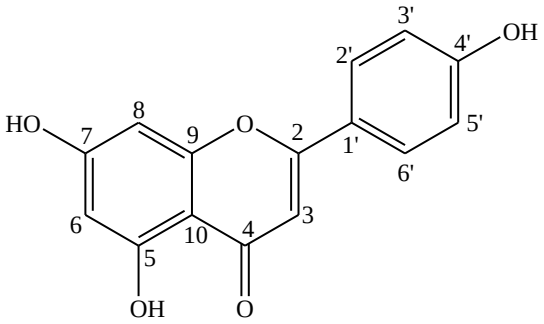


Figure 133: Structure of **apigenin**

From the assembly of fractions 185-200, **verbascoside** (Venditti et al., 2016f) (Figure 94), was evidenced as almost pure compound.

Verbascoside: NMR and MS data are reported in paragraph **5.5.5.** at page 146.

Since not all the present compounds could be clearly identified from this separation step, a second column chromatography was performed on an assembly of fractions deriving from the first one, 61-86, for the total weight of 663.7 mg.

The correspective amount of silica gel was 26.5 g (ratio 1:40 w/w), this time, and the eluting system consisted of a mixture of *n*-hexane/ethyl acetate at different concentrations.

The initial one was 8:2 (v/v) but during the chromatographic run, this was changed in order to raise the polarity of the solution and let the elution of the most polar compounds, to 7:3 (v/v) and 6:4 (v/v).

From this chromatographic step, one hundred and eighty-two fractions were collected.

After TLC analysis, these were collected putting together those having the same Rf values, as follows (Table 52):

Fractions	Weight (mg)
1-2	6.5
5	1.8
6-14	3.9
15-16	9.4
17-40	19.2
41-44	7.8
46-79	9.9
80-82	3.6
83-95	19.8
96-139	21.1
140-144	8.0
145-159	7.9
160-162	1.9
163-184	11.5

Table 52: Fraction assemblies and corresponding weights for chromatographic column 2

From the assembly of fractions 140-144, **20-*O*-acetyl-teucrasiatin** (**teupoliumin**) (Rodriguez et al., 1996) (Figure 134) (Spectra 19, 20) was evidenced as almost pure compound.

20-*O*-acetyl-teucrasiatin (**teupoliumin**): ^{1}H-NMR (400 MHz, CD_3OD) δ: 7.55-7.51 (1H, m, H-16), 7.48 (1H, t, *J* = 1.7 Hz, H-15), 6.49-6.46 (1H, m, H-14), 6.32 (1H, s, H-20), 5.17 (1H, dd, *J* = 10.1/7.7 Hz, H-12), 4.75 (1H, d, *J* = 11.5 Hz, Ha-19), 4.29 (1H, d, *J* = 11.5 Hz, Hb-19), 3.00 (1H, dd, *J* = 5.0/2.0 Hz, Ha-18), 2.59 (1H, dd, *J* = 13.4/8.7 Hz, Ha-11), 2.58 (1H, d, *J* = 13.2 Hz, Ha-7), 2.42 (1H, d, *J* = 5.0 Hz, Hb-18), 2.39 (1H, overlapped, Hb-7), 2.18 (3H, s,

$\underline{CH_3}CO$), 2.03-1.98 (1H, partially overlapped, Hb-11), 2.03 (1H, br. d, *J* = 6.9 Hz, H-8), 2.00 (3H, s, $\underline{CH_3}CO$), 1.06 (3H, d, *J* = 6.9 Hz, H-17).

^{13}C-NMR (100 MHz, CD_3OD) δ: 208.4 (C-6), 172.3 (AcO), 171.5 (AcO), 144.8 (C-15), 140.9 (C-16), 126.2 (C-13), 109.8 (C-14), 99.9 (C-20), 73.8 (C-12), 64.9 (C-18), 62.5 (C-4), 55.4 (C-5), 54.0 (C-9), 53.1 (C-10), 51.5 (C-19), 47.4 (C-7), 45.0 (C-11), 43.3 (C-8), 33.8 (C-3), 26.1 (C-2), 25.0 (C-1), 21.6 (C-24), 20.7 (C-22), 17.9 (C-17).

HR-ESI-MS: *m/z* 469.1838 $[M+Na]^+$ for $C_{24}H_{30}O_8Na$.

ESI-MS-MS: *m/z* 469.17 $[M+Na]^+$ (253), *m/z* 409.15 $[M+Na\text{-}AcO]^+$ (2141), *m/z* 315.11 $[M+Na\text{-}2AcO\text{-}OH]^+$ (4052).

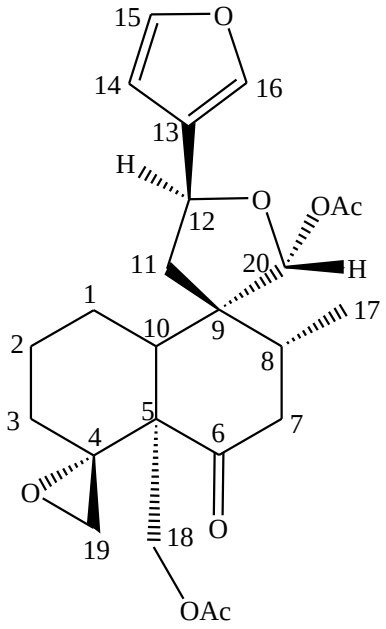
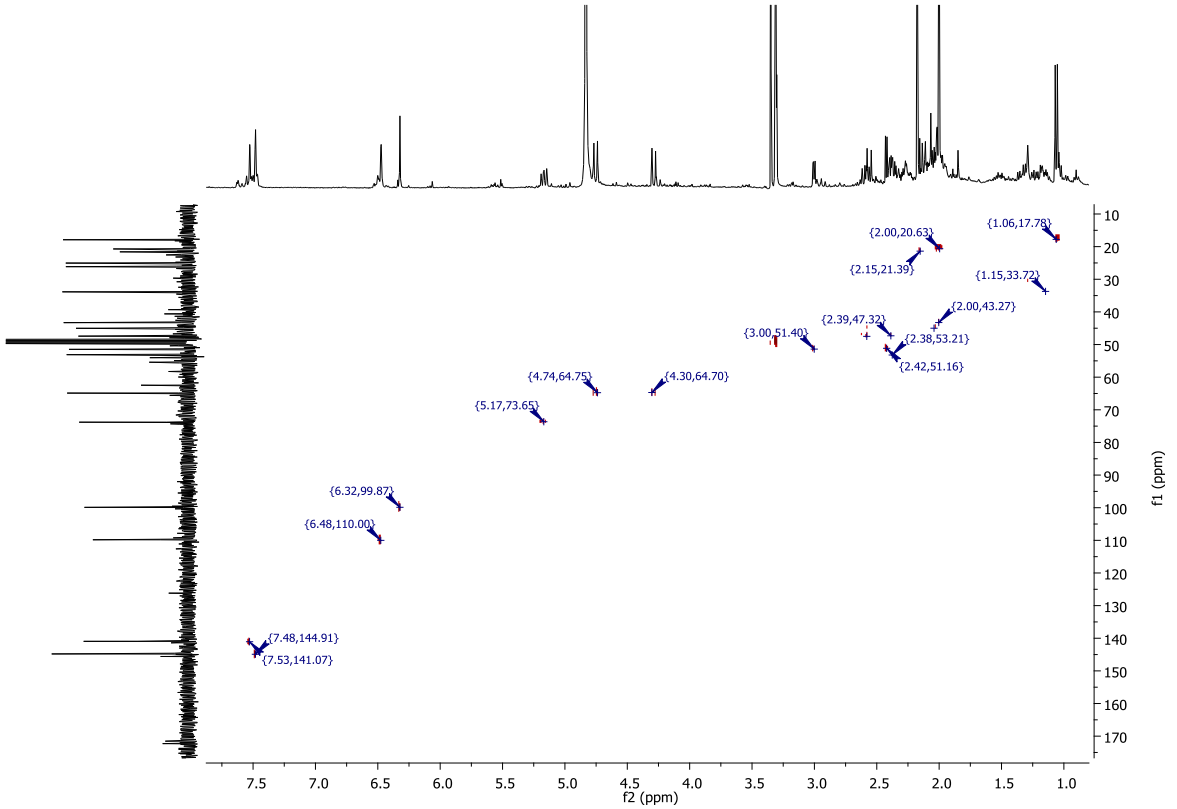
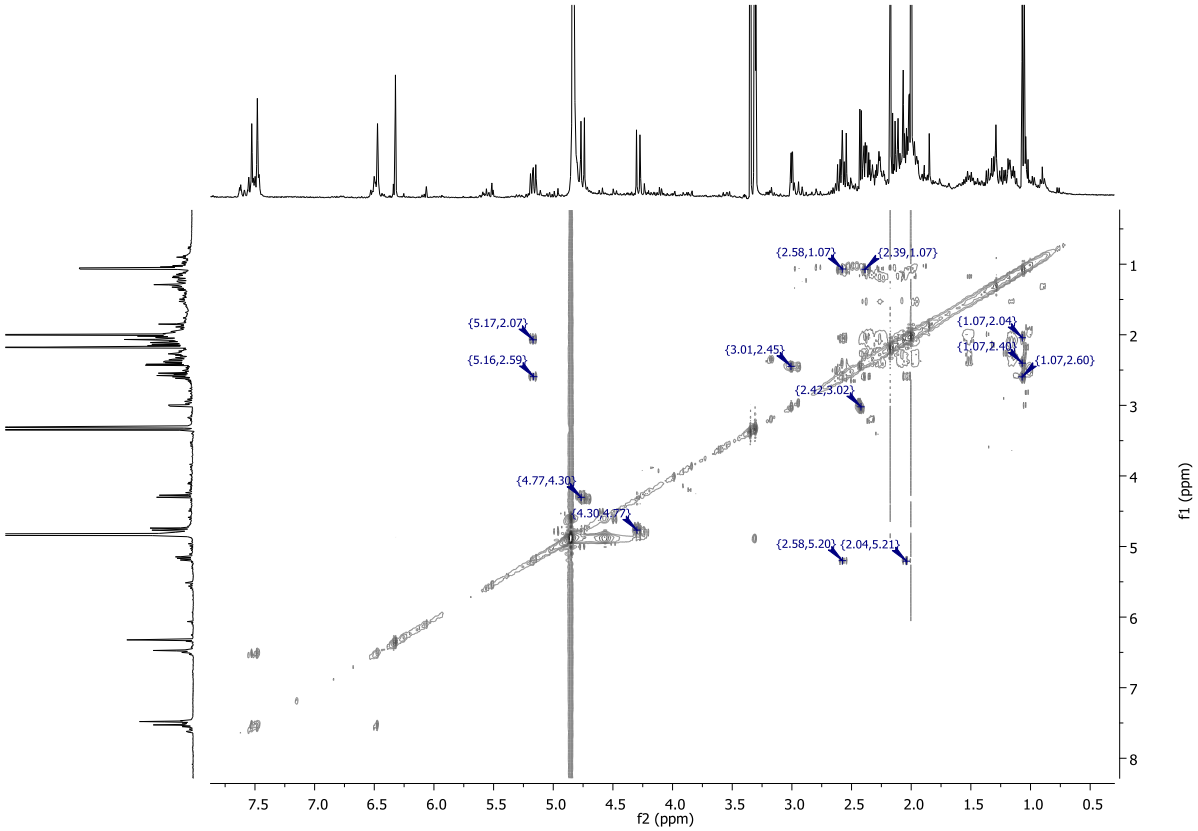


Figure 134: Structure of **20-*O*-acetyl-teucrasiatin**



Spectrum 19: HSQC spectrum of **20-*O*-acetyl-teucrasiatin** (**teupoliumin**)



Spectrum 20: TOCSY spectrum of **20-*O*-acetyl-teucrasiatin** (**teupoliumin**)

A third chromatographic separation was conducted on the assembly of fractions (96-184) obtained from the first separation step for the total weight of 40.5 mg because of the possible presence of several flavonoids which was supposed on the basis of the preliminary screening.

In this case, a qualitative HPLC approach was applied to identify these compounds working with a solution of 5 mg of the assembly solubilized in 1 mL of methanol (solution concentration = 5 mg/mL).
The method used was originally developed by Svelìkovà and co-workers (Svelìkovà et al., 2004) but slight modifications were introduced in order to optimize the separation of the components.
The applied HPLC parameters were the following: solvent A = 81% ultra-pure water containing 19% (HPLC grade) acetonitrile (MeCN) and 0.1% trifluoroacetic acid (TFA); solvent B = 100% MeCN.
The gradient program was: 0 min (95% A, 5% B), 5 min (80% A, 20% B), 10 min (70% A, 30% B), 15 min (65% A, 35% B), 20 min (40% A, 60% B), 22.5 min (100% B), 25 min (100% B), 27.5 min (95% A, 5% B), 35 min (95% A, 5% B).
The flow rate was 1.0 mL/min and the injection volume was 10 μL.
The UV–VIS detector was set at 220 nm and 335 nm.

From this step three further compounds were identified by co-injection of standard compounds: **apigenin 7-*O*-glucoside** (R.t. 7.60), **acacetin** (R.t. 13.40) and **luteolin** (R.t. 28.27).

For a more accurate screening of the less polar fraction, a portion of the crude extract (3.5 g) was suspended in water (150 mL) and partitioned with dichloromethane (3 × 100 mL).
After evaporation of the organic solvent, 1.7 g of crude material was recovered.
This was chromatographed on silica gel CC (68.0 g) using *n*-hexane/ethyl acetate (98:2 v/v) as eluting mixture.
During the chromatographic run, the concentration ratio of the constituents of the eluting system was changed to 95:5 (v/v), 85:15 (v/v), 7:3 (v/v) and 6:4 (v/v), in order to raise the polarity of the solution and let the elution of the most polar compounds.
From this chromatographic step, two hundred and thirty-nine fractions were collected.
After TLC analysis, these were collected putting together those having the same Rf values, as follows (Table 53):

Fractions	Weight (mg)
1-10	16.6
11-39	39.7
40-67	51.1
68-78	12.1
79-110	48.8
111-128	19.3
129-133	27.2
134-144	27.1
145-156	56.9
162-239	400.0

Table 53: Fraction assemblies and corresponding weights for chromatographic column 3

From the assembly of fractions 68-78, **(*E*)-phytol** (Venditti et al., 2016g) (Figure 135), was evidenced as almost pure compound.

(*E*)-phytol: ^{1}H-NMR ($CDCl_3$, 300 MHz) δ: 5.41 (1H, br. t, *J* = 6.9 Hz, H-2), 4.15 (2H, d, *J* = 6.9 Hz, H-2), 2.01 (2H, t, *J* = 7.4 Hz, H-4), 1.67 (3H, s, H-20), 1.56-1.48 (3H, overlapped signals, H-7, H-11, H-15), 1.39-1.32 (2H, m, H-5), 1.32-1.20 (14H, overlapped signals, H-6, H-8, H-9, H-10, H-12, H-13, H-14), 0.87-0.84 (12H, overlapped signals, H-16, H-17, H-18, H-19).
ESI-MS: *m/z* 319.18 $[M+Na]^+$; *m/z* 615.24 $[2M+Na]^+$; *m/z* 295.23 $[M-H]^-$.

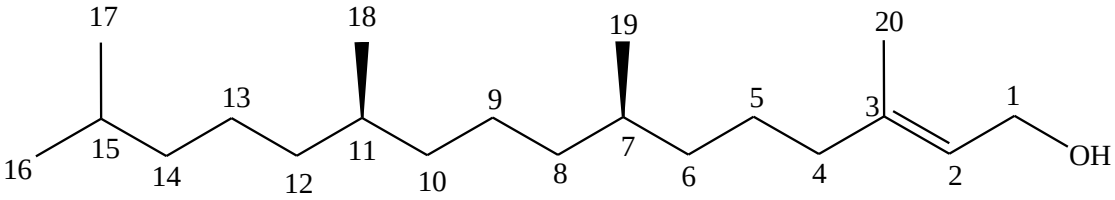


Figure 135: Structure of **(*E*)-phytol**

From the assembly of fractions 129-133, **oleanolic acid** (Seebacher et al., 2003) (Figure 66), was evidenced as almost pure compound.

Oleanolic acid: NMR and MS data are reported in paragraph **5.5.3.** at page 131.

From the assembly of fractions 134-144, **pheophorbide a** (Chee et al., 2005) (Figure 136) was evidenced as almost pure compound.

Pheophorbide a: ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 9.52 (1H, s, H-1'), 9.39 (1H, s, H-5), 8.56 (1H, s, H-20), 7.99 (1H, dd, *J* = 17.8/11.5 Hz, H-3'), 6.32-6.26 (2H, overlapped signals, Ha-32, H-13''), 6.20 (1H, m, Hb-3''), 4.51 (1H, m, H-18), 4.21 (1H, m, H-17), 3.87 (3H, s, H-13^{IV}), 3.68 (3H, s, H-12'), 3.40 (3H, s, H-2'), 3.24 (3H, s, H-7'), 2.46 (2H, m, H-17'), 2.33 (2H, m, H-17''), 1.82 (3H, m, H-18'), 1.74 (3H, overlapped, H-8'').
ESI-MS: *m/z* 615.27 $[M+Na]^+$.

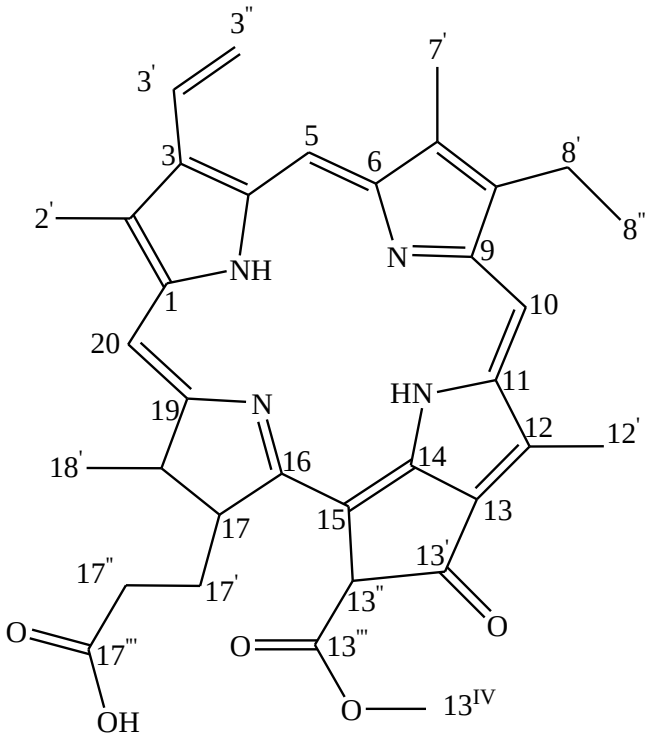


Figure 102: Structure of **pheophorbide a**

From the assembly of fractions 145-156, **maslinic acid** (Taniguchi et al., 2002) (Figure 67), was evidenced as almost pure compound.

Maslinic acid: NMR and MS data are reported in paragraph **5.5.3.** at page 133.

The assembly of fractions (162-239), for a weight of 400 mg, was further purified on silica gel column chromatography (16.0 g) (ratio 1:40 w/w) with chloroform/methanol (9:1)..
From this chromatographic step, thirty-six fractions were collected.
After TLC analysis, these were collected putting together those having the same Rf values, as follows (Table 54):

Fractions	Weight (mg)
1-3	78.8
4-7	129.4
8-19	41.1
20-36	31.2

Table 54: Fraction assemblies and corresponding weights for chromatographic column 4

No further compound was evidenced from this chromatographic step.

The assembly of fractions 4-7 for the weight of 129.4 mg was re-subjected to a chromatographic step on silica gel for the weight of 5.0 g (ratio 1:40) and eluted with chloroform/methanol starting with a mixture 99:1 and gradually passing to 98:2 (v/v), 97:3 (v/v), 95:5 (v/v) and 9:1 (v/v).
From this chromatographic step, eighty-eight fractions were collected.
After TLC analysis, these were collected putting together those having the same Rf values, as follows (Table 55):

Fractions	Weight (mg)
1-21	11.8
22-44	16.7
45-56	9.8
57-60	3.6
61-86	21.0

Table 55: Fraction assemblies and corresponding weights for chromatographic column 5

From the assembly of fractions 57-60, **cirsimaritin** (Alwahsh et al., 2015) (Figure 137) was evidenced as almost pure compound.

Cirsimaritin: ^{1}H-NMR (300 MHz, CD_3OD) δ: 7.90 (2H, d, *J* = 8.8 Hz, H-2', H-6'), 6.95 (2H, d, *J* = 8.8 Hz, H-3', H-5'), 6.83 (1H, s, H-8), 6.67 (1H, s, H-3), 3.99 (3H, s, 7-$\underline{CH_3}O$), 3.84 (3H, s, 6-$\underline{CH_3}O$).
ESI-MS: *m/z* 314.27 $[M+Na]^+$.

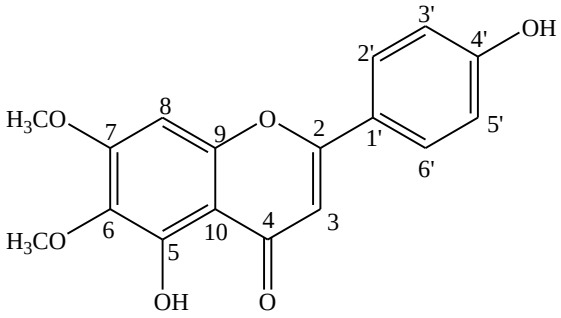


Figure 137: Structure of **cirsimaritin**

5.5.19. *Teucrium polium* L. - Southern Iran

An aliquot of the total dried crude extract obtained as described in paragraph **5.4.1.** for the weight of 3.0 g was subjected to a first chromatographic separation using a correspective amount of silica gel of 90.0 g (ratio 1:30 w/w) and a mixture of *n*-butanol and distilled water (82:18 v/v) as eluting system.

During the chromatographic run, the polarity was increased passing to a solution of *n*-BuOH/MeOH/H_2O at the concentration ratio of 70:10:30 (v/v/v) in order to favor the elution of the more polar components.

From this chromatographic step, seventy-nine fractions were collected.

After TLC analysis, these were collected putting together those having the same Rf values, as follows (Table 56):

Fractions	Weight (mg)
1-4	44.2
5-20	498.7
21-50	511.1
51-79	322.9

Table 56: Fraction assemblies and corresponding weights for chromatographic column 1

No clear compound could be identified from this chromatographic passage.

For this reason, a second chromatographic separation was performed on the assembly of fractions 5-20 deriving from the first one.

The correspective amount of silica gel was 25.0 g (ratio 1:50 w/w) and the eluting system was a mixture of chloroform/methanol at different concentrations. The initial one was 97:3 (v/v)

but during the chromatographic run, this was gradually changed by rising the polarity to 9:1 (v/v), 8:2 (v/v), 7:3 (v/v) and, lastly, 6:4 (v/v).

From this chromatographic step, one-hundred and fifty fractions were collected.

After TLC analysis, these were collected putting together those having the same Rf values, as follows (Table 57):

Fractions	Weight (mg)
1-12	9.9
13	6.0
14-46	325.9
47-64	33.2
65-102	111.1
103-108	19.4
109-118	20.7
119-144	45.8
145-150	4.9

Table 57: Fraction assemblies and corresponding weights for chromatographic column 2

From the assembly of fractions 13, **cirsilineol** (Alwahsh et al., 2015) (Figure 138), **10-β-hydroxy-teucjaponin B** (Coll and Tandrón, 2005) (Figure 139), **picropolin** (Brieskorn and Pfeuffer, 1967) (Figure 140) and **teupolin I** (Malakov et al., 1979) (Figure 141) were evidenced in an only mixture in ratio 1:1:1:1.

Cirsilineol: ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 7.41 (1H, d, *J* = 2.0 Hz, H-2'), 7.34 (1H, br. d, *J* = 8.6 Hz, H-6'), 7.03 (1H, d, *J* = 8.6 Hz, H-5'), 6.58 (1H, s, H-8), 6.55 (1H, s, H-3), 4.00 (3H, s, 3'-$\underline{CH_3}O$), 3.97 (3H, s, 7-$\underline{CH_3}O$), 3.92 (3H, s, 6-$\underline{CH_3}O$).

ESI-MS: *m/z* 344.93 $[M+H]^+$.

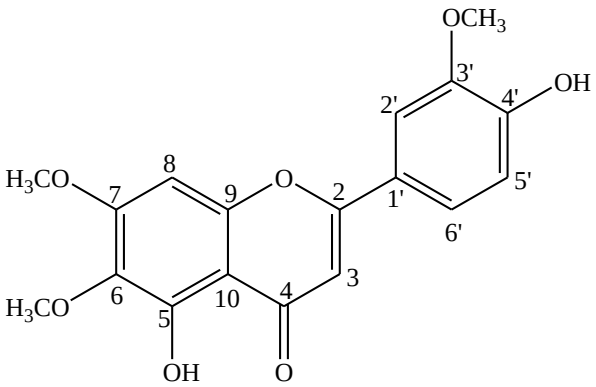


Figure 138: Structure of **cirsilineol**

10-β-hydroxy-teucjaponin B: ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 7.46 (1H, br. s, H-16), 7.45 (1H, br. s, H-15), 6.39 (1H, br. s, H-14), 5.11 (1H, m, H-19a), 4.70 (1H, m, H-19b), 4.16 (1H, br. d, *J* = 11.0 Hz,, H-6), 2.12 (3H, s, AcO), 0.99 (3H, d, *J* = 7.0 Hz, H-17).
ESI-MS: *m/z* 442.93 $[M+Na]^+$.

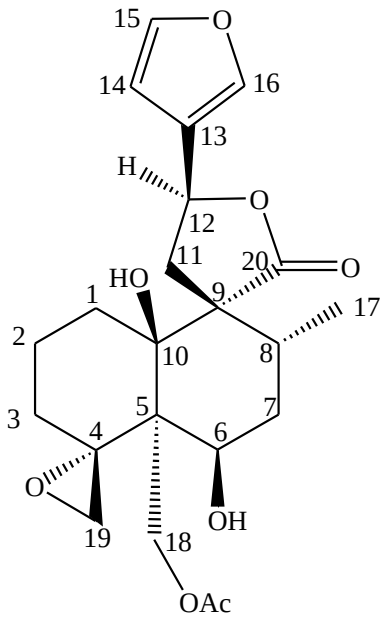


Figure 139: Structure of **10-β-hydroxy-teucjaponin B**

Picropolin: ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 7.46 (1H, br s, H-16), 7.42 (1H, br s, H-15), 6.38 (1H, br s, H-14), 5.52 (1H, m, H-12), 4.99 (1H, br. d, *J* = 12.8 Hz, H-19a), 4.79-4.64 (1H, m, H-19b), 3.23 (1H, overlapped with other signals, H-18a), 2.55 (1H, d, *J* = 3.5 Hz, partially overlapped, H-18b), 2.16 (3H, s, AcO), 0.85 (3H, d, *J* = 7.1 Hz, H-17).
ESI-MS: *m/z* 440.93 $[M+Na]^+$.

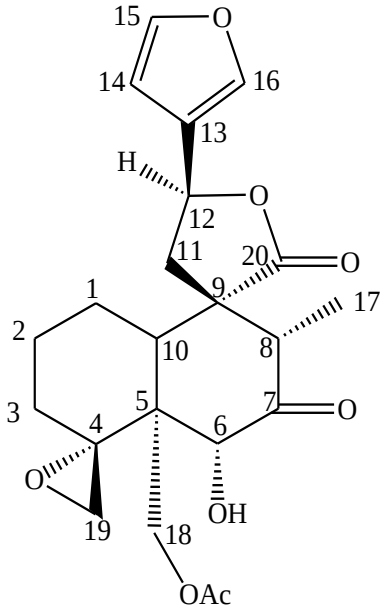


Figure 140: Structure of **picropolin**

Teupolin I: ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 7.41 (1H, d, *J* = 3.4 Hz, H-16), 7.40 (1H, br. s, H-15), 6.35 (1H, br. s, H-14), 5.31-5.22 (1H, m, H-12), 4.99 (1H, d, *J* = 12.4 Hz, H-19a), 4.57 (1H, d, *J* = 12.4 Hz, H-19b), 3.62-3.47 (1H, m, H-6), 3.23-3.13 (1H, m, H-18a), 2.06 (3H, s, AcO), 0.96 (3H, d, *J* = 6.5 Hz, 1H).

ESI-MS: *m/z* 426.94 $[M+Na]^+$.

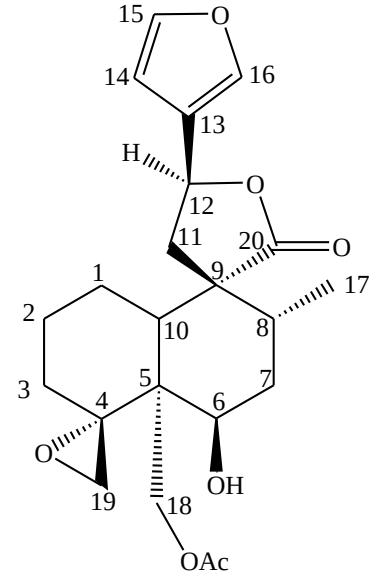


Figure 141: Structure of **teupolin I**

From the assembly of fractions 103-108, **apigenin-7-*O*-rutinoside** (**isorhoifolin**) (Boghrati et al., 2016) (Figure 142) (Spectrum 21) was evidenced as almost pure compound.

Apigenin-7-*O*-rutinoside (**isorhoifolin**): ^{1}H-NMR (300 MHz, CD_3OD) δ: 7.87 (2H, d, *J* = 8.8 Hz, H-2', H-6'), 6.94 (2H, d, *J* = 8.8 Hz, H-3', H-5'), 6.75 (1H, d, *J* = 2.0 Hz, H-8), 6.64 (1H, s, H-3), 6.51 (1H, d, *J* = 2.0 Hz, H-6), 5.04 (1H, d, *J* = 7.0 Hz, H-1"), 4.72 (1H, br. s,H-1'"), 1.17 (3H, d, *J* = 6.2 Hz, H-6'").

^{13}C-NMR (75 MHz, CD_3OD) δ: 184.0 (C-4), 166.8 (C-7), 164.7 (C-2), 163.0 (C-4'), 162.8 (C-9), 158.9 (C-5), 129.6 (C-2', C-6'), 123.1 (C-1'), 117.1 (C-3', C-5'), 107.1 (C-10), 104.1 (C-3), 102.1 (C-1'"), 101.5 (C-1"), 101.1 (C-8), 96.2 (C-6), 77.8 (C-3"), 77.1 (C-5"), 74.7 (C-2"), 74.1 (C-4'"), 72.4 (C-3'"), 72.1 (C-2'"), 71.3 (C-4"), 69.9 (C-5'"), 67.4 (C-6"), 17.9 (C-6'").

ESI-MS: *m/z* 600.87 $[M+Na]^+$; *m/z* 577.09 $[M-H]^-$.

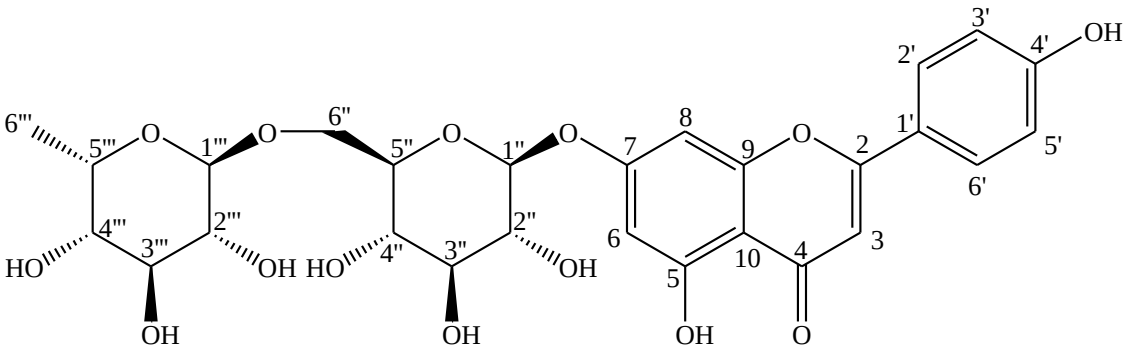
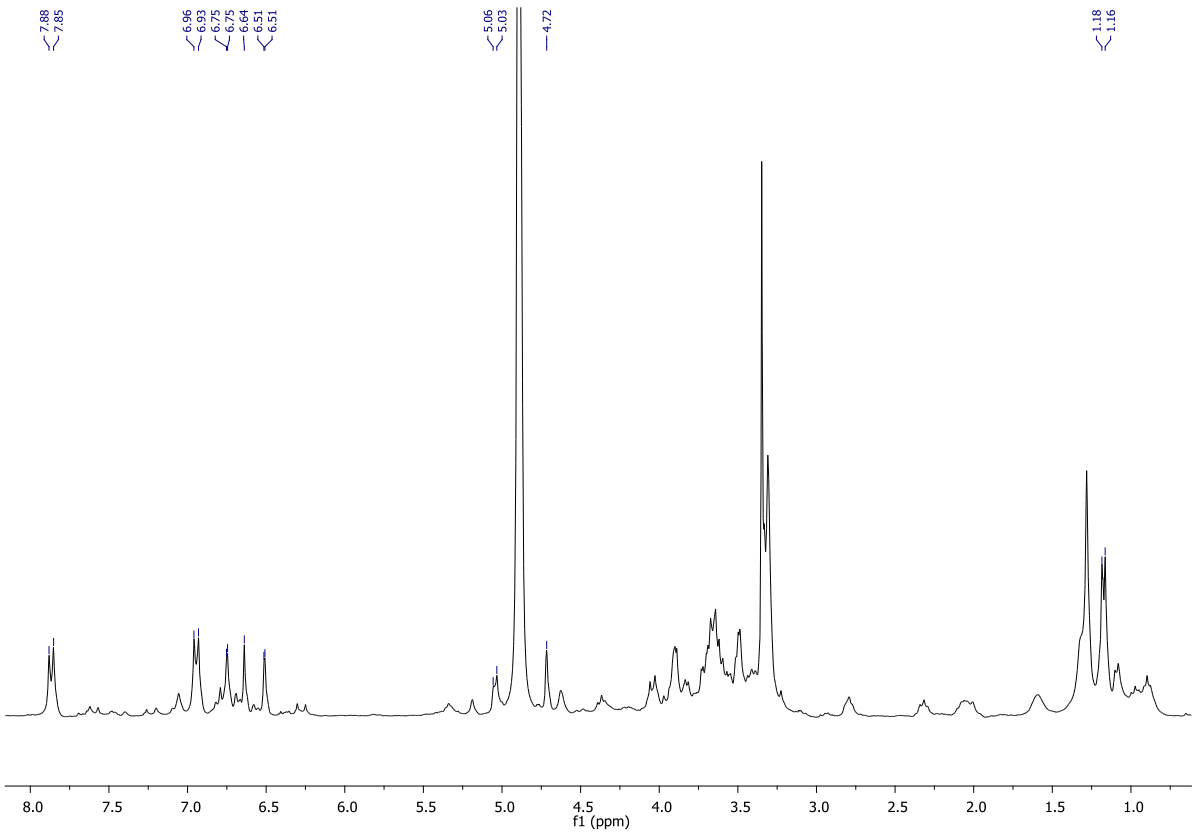


Figure 108: Structure of **apigenin-7-*O*-rutinoside** (**isorhoifolin**)



Spectrum 21: ^{1}H-NMR spectrum of **apigenin-7-*O*-rutinoside** (**isorhoifolin**)

From the assembly of fractions 119-144, **poliumoside** (Andary et al, 1985) (Figure 143) (Spectra 22, 23) was evidenced as almost pure compound.

Poliumoside: ^{1}H-NMR (300 MHz, CD_3OD) δ: 7.60 (1H, d, *J* = 15.9 Hz, H-β), 7.07 (1H, br. s, H-2"), 6.96 (1H dd, *J* = 8.2/1.9 Hz, H-6"), 6.78 (1H, d, *J* = 8.2 Hz, H-5"), 6.70 (1H, d, *J* = 2.1 Hz, H-2'), 6.69 (1H, d, *J* = 8.0 Hz, H-5'), 6.57 (1H, dd, *J* = 8.0/2.1 Hz, H-6'), 6.28 (1H, d, *J* = 15.9 Hz, H-α), 5.19 (1H, br. s, H-1^{IV}), 4.63 (1H, br. s, H-1'"), 4.38 (1H, d, *J* = 7.9 Hz, H-1(Glc)), 4.03-3.26 (overlapped carbohydrate signals), 2.80 (2H, br. t, *J* = 7.1 Hz, H-β'), 1.20 (3H, d, *J* = 6.2 Hz, H-6^{IV}), 1.08 (3H, d, *J* = 6.1 Hz, H-6'").

^{13}C-NMR (75 MHz, CD_3OD) δ: 168.0 (COOH caff), 149.8 (C-4"), 148.0 (C-β), 146.8 (C-3"), 146.1 (C-3'), 144.7 (C-4'), 131.4 (C-1'), 127.6 (C-1"), 123.2 (C-6"), 121.3 (C-6'), 117.1 (C-2'), 116.5 (C-5"), 116.4 (C-5'), 115.2 (C-2"), 114.7 (C-α), 104.3 (C-1), 103.0 (C-1^{IV}), 102.2 (C-1'"), 81.6 (C-3), 76.1 (C-2), 74.7 (C-5), 73.9 (C-4'"), 73.7 (C-4^{IV}), 72.4 (C-2'"), 72.3 (C-α'), 72.2 (C-2^{IV}), 72.1 (C-3'"), 72.0 (C-3^{IV}), 70.4 (C-5'"), 70.3 (C-4), 69.9 (C-5^{IV}), 67.5 (C-6), 30.8 (C-β'), 18.4 (C-6'"), 18.0 (C-6^{IV}).

ESI-MS: *m/z* 792.97 $[M+Na]^+$; *m/z* 768.85 $[M-H]^-$.

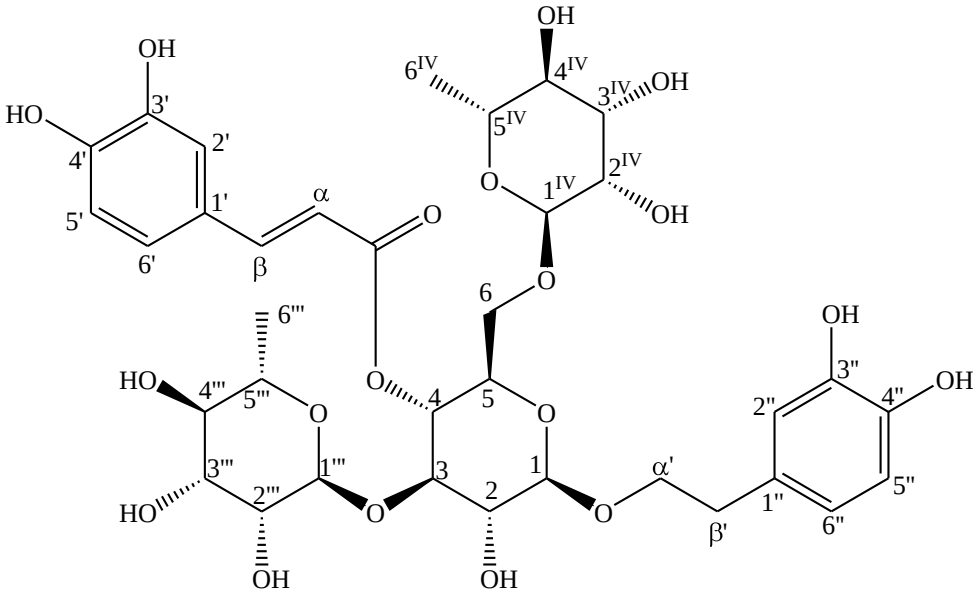
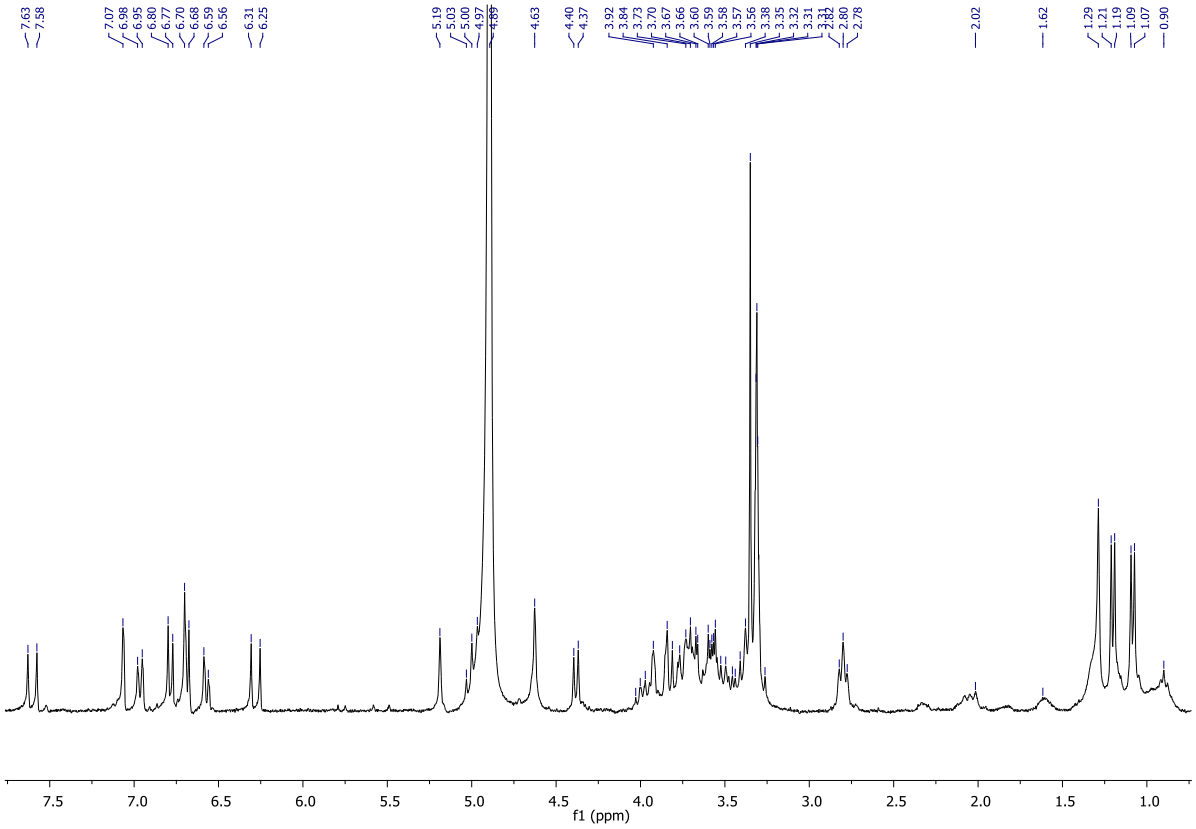
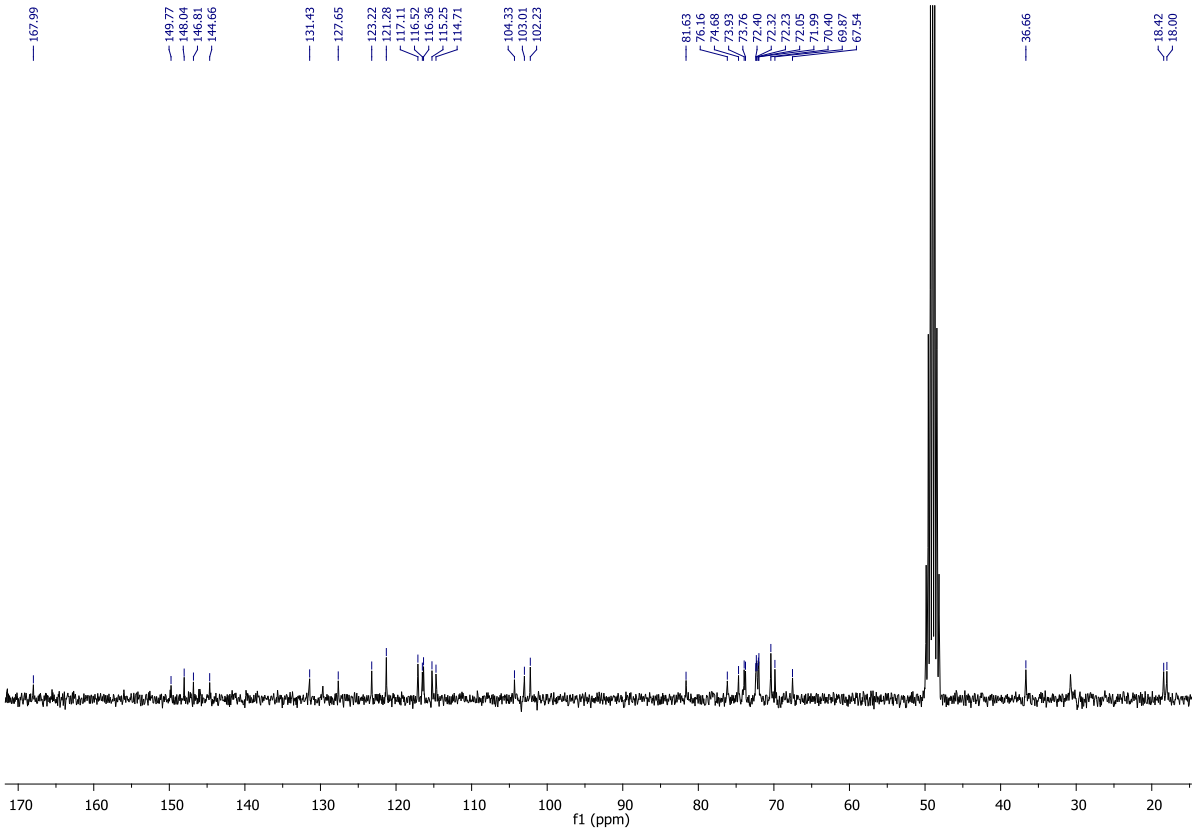


Figure 143: Structure of **poliumoside**



Spectrum 22: ^{1}H-NMR spectrum of **poliumoside**



Spectrum 23: ^{13}C-NMR spectrum of **poliumoside**

Since not all the present compounds could be clearly identified, a third separation step was conducted on the assembly of fractions 14-46 deriving from the second step for a total weight of 325.9 mg.

The correspective amount of acidic silica gel was 10.0 g (ratio 1:30 w/w) and a mixture of *n*-hexane/ethyl acetate at different concentrations, previously saturated with CO_2, was used as eluting system. The initial concentration between the components was 85:15 (v/v) but then during the chromatographic run, it was changed to increase the polarity to 70:30 (v/v), 60:40 (v/v) and lastly, 50:50 (v/v).

From this chromatographic step, two-hundred and fifteen fractions were collected.

After TLC analysis, these were collected putting together those having the same Rf values, as follows (Table 58):

Fractions	Weight (mg)
1-24	16.6
25-45	21.1
46-56	7.4
57-86	17.5
87-90	1.9
91-95	1.2
96-108	5.3
109-158	30.0
159-215	28.8

Table 58: Fraction assemblies and corresponding weights for chromatographic column 3

From the assembly of fractions 46-56, **cirsimaritin** (Alwahsh et al., 2015), **4'-*O*-methyl-luteolin (diosmetin**) (Park et al., 2007) and **apigenin** (Faidi et al., 2014) (Figure 137, 144, 133) were evidenced in an only mixture in ratio 10:2:1.

Cirsimaritin: NMR and MS data are reported in paragraph **5.5.18.** at page 206.

4'-*O*-methyl-luteolin (diosmetin): ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 7.69 (1H, dd, *J* = 8.5/1.8 Hz, H-6'), 7.57 (1H, d, *J* = 1.8 Hz, H-2'), 6.99 (1H, d, *J* = 8.5 Hz, H-5'), 6.56 (1H, s, H-3), 6.45 (1H, d, *J* = 2.1 Hz, H-8), 6.29 (1H, d, *J* = 2.1 Hz, H-6), 3.96 (3H, s, 4'-O$\underline{CH_3}$).
ESI-MS: *m/z* 301.34 $[M+H]^+$.

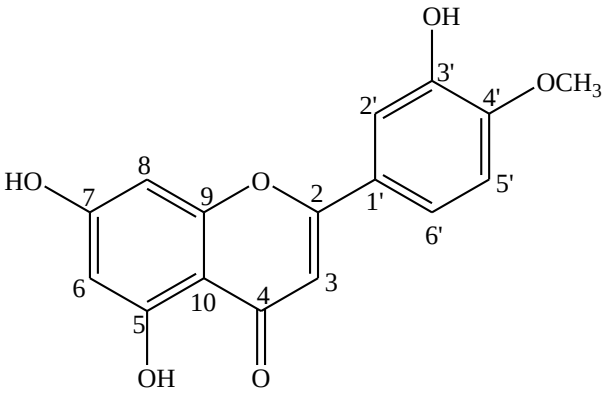


Figure 144: Structure of **4'-*O*-methyl luteolin (diosmetin**)

Apigenin: NMR and MS data are reported in paragraph **5.5.18.** at page 199.

From the assembly of fractions 57-86, **cirsimaritin** (Alwahsh et al., 2015) (Figure 137), was evidenced as almost pure compound.

Cirsimaritin: NMR and MS data are reported in paragraph **5.5.18.** at page 206.

From the assembly of fractions 87-90, **cirsimaritin** (Alwahsh et al., 2015) and **cirsiliol** (Alwahsh et al. 2015) (Figures 137, 145), were evidenced in mixture in ratio 2:1.

Cirsimaritin: NMR and MS data are reported in paragraph **5.5.18.** at page 206.

Cirsiliol: NMR and MS data are reported in paragraph **5.5.18.** at page 216.

From the assembly of fractions 96-108, **cirsiliol** (Alwahsh et al. 2015) (Figure 145), was evidenced as almost pure compound.

Cirsiliol: ^{1}H-NMR (400 MHz, CD_3OD) δ: 7.44 (1H, dd, *J* = 8.3/2.2 Hz, H-6'), 7.41 (1H, d, *J* = 2.2 Hz, H-2'), 6.92 (1H, d, *J* = 8.3 Hz, H-5'), 6.80 (1H, s, H-8), 6.61 (1H, s, H-3), 3.99 (3H, s, 7-O$\underline{CH_3}$), 3.84 (3H, s, 6-O$\underline{CH_3}$).
ESI-MS: *m/z* 331.18 $[M+H]^+$.

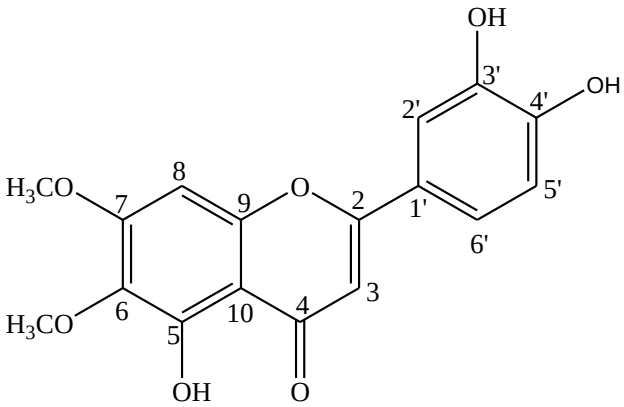


Figure 145: Structure of **cirsiliol**

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