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Bioassay—Guided Metabolomic Fingerprinting Analysis of Mediterranean plants using GC-MS and NMR spectroscopy

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Abstract

In our study, the metabolomic fingerprinting analysis of leaves and roots of eight Mediterranean plants was made by an integrated approach of GC-MS and NMR spectroscopic techniques targeted on apolar and polar metabolites respectively, following bioassay test focused on antifungal activity against two phytopathogenic fungi, *Trichoderma harzianum* and *Aspergillus niger*. The eight plant species included two perennial forbs (*Dittrichia viscosa, Acanthus mollis*), two grasses (*Typha latifolia, Festuca drymeia*), one vine (*Hedera helix*), one evergreen tree (*Quercus ilex*), and two deciduous trees (*Fraxinus ornus, Fagus sylvatica*), which have been used as traditional folk remedy. The research aimed at evaluating the chemical compositions of the different species both from a qualitative and a quantitative point of view, to identify the major classes of apolar and polar compounds and to integrate the spectra followed by chemometrics. The highlights of the undertaken work were: i) using an integrated approach of GC-MS and NMR spectroscopic techniques to make an intensive investigation of apolar and polar metabolites of leaves and roots of each species; ii) comparing the variation of metabolite contents in leaves and roots of eight plants simultaneously; iii) correlating internal physiologic properties (chemical profile) with the external bioactivity (antifungal activity) on some degree.

The metabolic fingerprint of the Mediterranean plants showed a complex chemical composition, being specific for each species and plant tissue. Some conclusions were drew as described. Through analyzing the apolar extracts of leaf and root samples of eight species by GC-MS, combined with interpreting method of AMDIS, it was showed that apolar organic extracts were mainly composed of linear saturated fatty acids; 120 apolar metabolites, including fatty acids, n-alkanes, triterpenoids, steroids and oxygenated terpenoids were found. The exceptions were that major apolar metabolites were oxygenated terpenoids in *D. viscosa* leaf and unsaturated fatty acids with the richest component being linoleic acid in *H. helix* root, accounting for the observed antifungal activity. Triterpenoids and steroids were almost exclusively found in

roots. Through analyzing the polar extracts of leaf and root samples of eight species by ^{1}H-NMR, followd by statistical method of Principle Component Analysis (PCA), we found that extracts contained a total of 38 polar metabolites among all samples, including sugars, alkaloids, organic acids, free amino acids and aromatic compounds. *Q. ilex* and *F. ornus* contained large amounts of specific metabolites, quinic acid, quercitol and mannitol. *D. viscosa* and *T. latifolia* were characterized by a high content of aromatic compounds. The separation of *A. mollis* from the other species was due to the presence of betaine and sucrose in leaves and raffinose in roots. Hence, we could conclude that the research developed with the proposed approach possess the advantages of versatility and rapidity, thus making it suitable for a fast comparison among species and plant tissue types.

Keywords: Metabolomics, Fingerprint, GC-MS, AMDIS, NMR, PCA, Mediterranean plants

Chapter 1: General introduction of botanical description of traditional medicinal Mediterranean plants

1.1 Potency of natural products from folk medicinal plants as modern medicine

Higher plants produce a great variety of chemical compounds (Dixon 2001) (Hartmann 2008), traditionally know as secondary metabolites. The name comes from the consideration that plant performances were not directly adversely affected by their absence. Since plant secondary metabolites have been identified to possess important and different functions in the natural environment they are commonly named as “natural products” (Field, Jordán, and Osbourn 2006). Defense response can be induced in most terrestrial plants. The natural products from higher plants show a prominent defensive role and repellence against predators and microbial pathogens, due to their toxic nature.

It is a long history since that mankind used folk plants to help relief from illness. The first written document can be traced back to the period of the early civilization in China, India and the near East, more than five thousand years ago (Mahesh and Satish 2008). A wide range of folk medicinal plant parts was used for extract as raw drugs that were shown to possess a variety of medicinal properties. The different parts used include roots, stems, flowers, fruits, twigs exudates and modified plant organs. In modern medicine, drugs from higher plants continue to play an important role. It is considered that nearly a half of current used drugs are derived from natural products (Kong et al. 2008). In these drugs, all single chemical entities are extracted from higher plants, or modified further synthetically.

There is a hot issue how to exploit the potency of traditional medicinal plants with antimicrobial activity, which has been screened in a number of studies. Studying work on

medicinal plants provides a scientific basis for the popular use against infectious diseases in the modern era. Although hundreds of plant species have been tested for antimicrobial properties, only a small percentage of the estimated plant species has been investigated phytochemically. The majority of them has not been adequately evaluated. Fractions submitted to biological or pharmacological screening are even smaller.

The phytochemical diversity of plants has been previously reviewed by examining their involvement in constitutive (Wittstock and Gershenzon 2002) and inducible chemical defenses (Hammerschmidt 1999), mechanisms of plant resistance (Morrisey and Osbourn 1999) and fitness cost (Heil 2002). The potential exploitation of such molecules plant antimicrobial compounds has also been evaluated. Thousands of diverse natural products, involved in plant defense, have been identified including terpenoids, saponins, phenolics, phenylpropanoids, pterocarpans, stilbenes, alkaloids, glucosinolates, tiosulfinates and indoles (Dixon 2001). This was the reason that prompted us to make a deep research in our study.

Considering the potentiality of plants as sources for antimicrobial drugs with reference to antibacterial and antifungal agents, we selected eight species collected in the Mediterranean area and used as folk plants by analyzing both leaf and root organs for phytochemical researches guided by antifungal studied. The Mediterranean Basin is the region of lands around the Mediterranean Sea, lying between 30° and 45° north and south latitudes that have a Mediterranean climate, with mild, rainy winters and hot, dry summers, which supports characteristic Mediterranean forests, woodlands and scrub vegetation. Special biogeographic environment is able to provide a biodiversity of higher plants and a flourishing folk medicine culture. The species selected were employed in the treatment for their wound healing, anti-inflammatory and disinfectant qualities. All are used to treat general infectious diseases. A scientific inventory of these medicinal plants has been prepared based on a bibliographic review. They were collected in Naples, Campania region, Italy. The antimicrobial activities were established for two extracts

(petroleum ester and water/methanol mixture) from each species. The chemical compositions of the active extracts screened by bioassay test were analyzed by Gas Chromatography (GC-MS) and Nuclear Magnetic Resonance (NMR) Spectroscopy.

The scienfic classification of used eight Mediterranean plants were shown as below.

Tab. 1.1 Scientific classfication of eight Mediterranean plants.

Kingdom	Plantae							
Clade	Angiosperms	Angiosperms	Angiosper	Angiosperms	Angiosperms	Angiosperms	Angiosperms	Angiosperms
Clade	Eudicots	Monocots	Eudicots	Eudicots	Monocots	Eudicots	Eudicots	Eudicots
Clade	Asterids	Commelinids	Asterids	Asterids	Commelinids	Rosids	Asterids	Rosids
Order	Asterales	Poales	Lamiales	Apiales	Poales	Fagales	Lamiales	Fagales
Family	Asteraceae	Typhaceae	Oleaceae	Araliaceae	Poaceae	Fagaceae	Acanthaceae	Fagaceae
Genus	*Dittrichia*	*Typha*	*Fraxinus*	*Hedera*	*Festuca*	*Quercus*	*Acanthus*	*Fagus*
Species	*D. viscosa*	*T. latifolia*	*F. ornus*	*H.helix*	*F. drymeja*	*Q. ilex*	*A. mollis*	*F. sylvatica*

1.2 *Dittrichia viscosa*

Scientific name and etymology: *Dittrichia viscosa* (L.) Greuter is an evergreen perennial weed belonging to the Compositae family (Asteraceae), classfied by Manfred Dittrich. "Viscosa" originated from Latin word means" sticky to touch", referring mainly to the sticky exudate from the glandular hairs of *D. viscosa*. The "viscosa" name covers all the plants with strong typical fragrance. As for the common name "False Yellowhead", it is because the species resembles the related British Yellowhead (*Inula britannica*) (Mifsud 2017).

Morphological and origin description: *D. viscosa* is native to the Mediterranean region, which also widespread distribute in Australia and Asia. The species *D. viscosa* (L.) Greuter consist of *D. viscosa* subsp. *viscosa*, *D. viscosa* subsp. *angustifolia* (Bég.) Greuter, *D. viscosa* subsp. *maritima* (Brullo & De Marco) Greuter , *D. viscosa* subsp. *revoluta* (Hoffmanns. & Link) P. Silva & Tutin. This was formerly included in the genus Inula. Originally, the species was found mainly in dry riverbeds and abandoned fields up to a 1500 m (5000 feet) elevation. Nowadays it is quite common in roadsides and ruderal habitats, and in anthropic altered areas. *D. viscosa* has long and narrow leaves with jagged edges and glandular hairs on the surfaces. The sticky exudate from the glandular hairs diffuses an unpleasant smell. It has been identified that the sticky exudate contains essential oil. When in blossoming period, many yellow flower heads can be produced, each with as many as 16 ray florets and 44 disc florets. The canopy is very dense reaching 150 cm of height and total leaf area per plant comprises 200 cm^2. The species is very resistant to adverse conditions and degraded environments. It is important as food for the caterpillars of certain butterflies and moths.

Uses: *D. viscosa* contains essential oil for which it has been used in traditional medicine since ancient times. Its curative effect was firstly written by Roman scientific encyclopedist and historian Pliny the Elder (AD 23－79)－"the *inula* plant strengthens the teeth, when prepared it is used against cough, boiled juice of its roots evict worms, dried and crushed to powder it is used against

cough and a medicine for stomach craps and against stomach gases, it is useful for healing of poisonous animals bites." (Krispil 1987). M.S. Ali-Shtayeh has summarized the folkloric usage of *D. viscosa* for external use: antispasmodic, sedative, antiseptic, for wounds healing, women infertility, antirheumatic, treat bronchial disorders, expectorant, for haemorrhoids. Used also for internal use as: antipyretic, general tonic, for headache, stomach pain, antispasmodic, antidiabetic, antidiarrheic, antihelmintic (Ali-Shtayeh et al. 1998). So this species has been used as medicine for a long history and targeted on wide symptoms.

Fig. 1.1 *Dittrichia viscosa*

1.3 *Typha latifolia*

Scientific name and etymology: *Typha latifolia* L. is a perennial herbaceous plant, belonging to Typhaceae family, first described by Christiaan Henrik Persoon. The name "*Typha*" comes from ancient Greek word τύφη (túphē) "tufh" which mean "bulrush, cattail", instead "latifolia" is a Latin word meaning "broad leaf" (Rook 2002).

Morpholigical and origin description: This rhizomatous perennial plant was found as a native

plant species in North and South America, Europe, Eurasia, and Africa. They are with long, slender green stalks topped with brown, fluffy, sausage-shaped flowering heads. *T. latifolia* plants are 15–30 dm tall. The spike-like, terminal, cylindric inflorescence has staminate flowers above and pistillate flowers below with a naked axis between the staminate and pistillate flowers. The spike is green when fresh, becoming brown as it matures. The basal leaves are thin with parallel veins running the long, narrow length of the leaf. *T. latifolia* is found at elevations from sea level to 7,500 feet (2,300 m). It is an "obligate wetland" species, meaning that it is always found in or near water (USDA 2018). The species generally grows in flooded areas where the water depth does not exceed 0.8 meters. However, *T. latifolia* has also been reported growing in floating mats in slightly deeper water. It grows mostly in fresh water but also occurs in slightly brackish marshes. Its species is much resistant to the different environment (U.S. Forest Service 2018).

Uses: *T. latifolia* was firstly recorded the medical use “Antihemorrhagic” in ancient Chinese pharmacopeia–shen nong ben cao jing (Anonymous, 25 AD –220 AD) (Kong et al. 2008). It has been also used in traditional Chinese medicine as an anti-inflammatory agent and diuretic (Woo, Choi, and Kang 1983). All parts of the *T. latifolia* are edible when gathered at the appropriate stage of growth due to the high content of nutrients and nutraceuticals in its young shoot. The young shoot is rich in necessary amino acids and vitamins, inorganic salt with potassium and phosphorus. Additionally，the content of each compounds is like that–crude protein 3.16%, crude fiber 4.06%, carbohydrate 80%, crude fat is 1%. The young shoots can be raw or be steamed to eat and also can be made into pickles. The base of the stem and the young flower stalks can be boiled or steamed for eating. The pollen is a fine substitute for flours. The core of rhizome can be ground into flour, too. Hence, it is a potential source of food for the worlds' population (Harrington 1972).

Fig. 1.2 *Typha latifolia*

1.4 *Fraxinus ornus*

Scientific name and etymology: *Fraxinus ornus* L. is a deciduous treet, belonging to Oleaceae family. This name was created by Gabriele D'Annunzio for one of the characters of the tragedy “The Daughter of Jorio”. The name seems to derive from the word “orno or ornello” (Latin: fraxinus ornus, English: flowering ash).

Morphological and origin description: *F. ornus* is native to southern Europe and southwestern Asia. The species is a medium-sized deciduous tree growing to 15–25 m (49–82 ft.) tall with a trunk up to 1 m diameter. The bark is dark grey, remaining smooth even on old trees. The buds are pale pinkish-brown to grey-brown, with a dense covering of short grey hairs. The leaves are in opposite pairs, pinnate, 20–30 cm (7.9–12 in) long, with 5 to 9 leaflets; the leaflets are broad ovoid, 5–10 mm (0.2–0.4 in) long and 2–4 cm (0.8–2 in) broad, with a finely serrated and wavy margin, and short but distinct petiolules 5–15 mm (0.20–0.59 in) long; the autumn color is variable, yellow to purplish. The flowers are produced in dense panicles10–20 cm (3.9–7.9 in)

long after the new leaves appear in late spring, each flower with four slender creamy white petals 5–6 mm (0.20–0.24 in) long; they are pollinated by insects. The fruit is a slender samara 1.5–2.5 cm (0.59–0.98 in) long, the seed 2 mm (0.08 in) broad and the wing 4–5 mm (0.2–0.2 in) broad, green ripening brown.

Uses: A sugary extract from the sap may be obtained by making a cut in the bark, this was compared in late medieval times with the biblical manna. The literary quotation gave rise to the English name of the tree. In fact, the sugar mannose and the sugar alcohol mannitol both derive their names from the extract (Rushforth 1999). The bark and the leaves of *F. ornus* are applied in the Bulgarian and Polish folk medicine against various diseases, including wound healing, diarrhea and dysentery (Kostova and Iossifova 2007).

Fig. 1.3 *Fraxinus ornus*

1.5 *Hedera helix*

Scientific name and etymology: *Hedera helix* L. is an evergreen climbing plant, belonging to Araliaceae family. “Hedera” is the Latin name for ivy, found as early as Publius Vergilius Maro (ancient Roman poet) and Gaius Plinius Secundus (Roman author). It is said to derive from the Greek “hédra” or “haerere”, which mean to “sit”, and which refers to the grasp of its roots. The specific epithet “helix” derives from ancient Greek "twist, turn" (Harrison 2012).

Morphological and origin description: Ivy is native to most of Europe and western Asia.It is a climbing plant, growing to 20–30 m (66–98 ft) high on walls, cliffs even trees. It climbs by means of aerial rootlets with matted pads that cling to the substrate strongly. It generally thrives in a wide range of soil pH with 6.5 being ideal, prefers moist, shady locations and avoids exposure to direct sunlight. (Plants & Flowers—*Hedera helix* Jubilee) The leaves are alternate, 50–100 mm (2–4 in) long, with a 15–20 mm (0.6–0.8 in) petiole. They are divided in two types, with palmately five-lobed juvenile leaves on creeping and climbing stems, and with unlobed cordate adult leaves on fertile flowering stems exposed to full sun. The flowers are produced from late summer to late autumn, individually small, in 3-to-5 cm-diameter (1.2-to-2.0 in) umbels, greenish-yellow. Nectar is rich in flowers, which is an important late autumn food source for bees and other insects. The fruit are purple-black to orange-yellow berries 6–8 mm (0.2–0.3 in) in diameter, ripening in late winter (Brickell 2008). It is an important food for many birds, though somewhat poisonous to humans. From one to five seeds are in each berry, which are dispersed after being eaten by birds.

Uses: The medicinal use of ivy is early well documented. Hippocrate's (Greek physician, 460 BC–370 BC) writings already describe the ivy's root, leaves, and berries as medicines for internal and external use (https://www.avogel.ch/en/plant-encyclopaedia/hedera_helix.php).The leaves and berries were taken orally as an expectorant to treat cough and bronchitis in the past (Deni 1995). In 1597, the British herbalist John Gerard recommended water infused with ivy leaves as a wash for sore or watering eyes (Gerard 1985). Currently, Ivy extracts are part of cough medicines, for example Bronchostad ("BRONCHOSTAD© Hustenlöser-Tropfen-STADA") and Prospan. ("Prospan-With the full power of ivy Prospan ").

Fig. 1.4 *Hedera helix*

1.6 *Festuca drymeja*

Scientific name and etymology: *Festuca drymeja* is a bisexual plant belonging to the grass family Poaceae (subfamily Pooideae), described by Franz Karl Carl Mertens and Wilhelm Daniel Joseph Koch. "Festuca" is a Latin word meaning "stem" or "stalk" first used by Pliny the Elder to describe a weed (Barkworth et al. 2007).

Morphological and origin description: Like other species of this genus, *F. drymeja* is an evergreen and herbaceous perennial tufted grasses with a height range of 10–200 cm (4–79 in) and a cosmopolitan distribution, occurring on every continent except Antarctica.

Uses: Being a kind of highly nutritious forage, *F. drymeja* possess agronomic qualities that make them ideally suited to many livestock farming systems. Some fescues are used as ornamental and turf grasses and as pasture (Stammers et al. 1995).

Fig. 1.5 *Festuca drymeja*

1.7 *Quercus ilex*

Scientific name and etymology: *Quercus ilex* L is a large evergreen oak belonging to family Fagaceae. The species takes its name from holm, an ancient name used for holly, owing to spiny leaves resembling holly'leaves. (https://www.wood landtrust.org.uk/visiting-woods/trees-woods-and-wildlife/british -trees/common-non-native-trees/holm-oak).

Morphological and origin description: They are native to the Mediterranean region (southern Europe and northern Africa), but naturalized in the UK introduced by Thomas Balle. Holm oak can grow to 21–28 m and develop a huge, rounded crown. The bark is black and finely cracked and the young shoots are clothed with a close grey felt. Leaves are very variable in shape, frequently narrowly oval or ovate-lanceolate, dark green to black and concave with a similar coating of pale hairs on the underside. Young leaves and leaves on young plants are toothed, like holly leaves, whereas older leaves and leaves on old plants have smooth edges. The leaves are glossy above and downy below without lobes. Its catkins and yellow male catkins can be seen to hang off the tree in abundance in early spring. After pollination by wind, female flowers develop into acorns and one to three can be produced on a short downy stalk, ripening the first season.

Young acorns are green and mature to a dark red-brown before falling.

Uses: The acorns of *Q. ilex*, like those of the cork oak, are edible (toasted or as a flour) and are an important food for free-range pigs reared for ibérico ham production. *Q. ilex* is used as a folk remedy to treat haemorrhages, chronic diarrhoea and dysentery (Davis 1988) (Baytop 1984). Aacorns boiled in water can also be used as a medicinal treatment for wound disinfections. The holm oak is one of the top three trees used in the establishment of truffle orchards. Truffles grow in an ectomycorrhizal association with the tree's roots. Holm oak timber is incredibly hard and tough. The Romans used the wood for making pillars, the wheels of carts and wagons, as well as agricultural tools. Today it is also used for firewood as it is slow and long lasting.

Fig. 1.6 *Quercus ilex*

1.8 *Acanthus mollis*

Scientific name and etymology: *Acanthus mollis* L., commonly known as bear's breeches or bearsfoot, is an herbaceous perennial plant with an underground rhizome in the genus Acanthus. It belongs to Family Acanthaceae, named by Publius Vergilius Maro. The word "Ac*anthus*" originates from the Greek 'acanthos', meaning and referring to the statuesque flower spikes that last for many weeks, and from the Greek "kantha" meaning thorn and also spine or spike. The sepals are

the only thorn-bearing part of the plant. "*Mollis*" in Latin means soft to touch, referring to leaves. It also has the meaning of swaying, pliant or flexible. (http://www.maltawildplants.com/ACNT/Acanthus_mollis.php)

Morphological and origin description: This plant is native to the Mediterranean region from Portugal and northwest. Africa east to Croatia and it is one of the earliest cultivated species. The species reaches 30–80 cm of height typically, including inflorescence. It has basal clusters of deeply lobed and cut, shiny dark green leaves. The leaves with a long petiole are soft to the touch, up to 40 cm long and 25 cm broad. The inflorescence is a cylindrical spike 30–40 cm long and can produce as many as 120 flowers. The tubular flowers are whitish, and lilac or rose in color.

Uses: *A. mollis* is used as ornamental and folk medicinal plant. It is one of the earliest cultivated species of garden plants (Amenta et al. 2000) (Bremner et al. 2009) (Bader et al. 2015). It is the plant most celebrated in architecture since the Greeks adopted its leaf form for the well-known decoration on the caps of their Corinthian columns. The species of genus *Acanthus* belonging to the family *Acanthaceae* are traditionally used for wound healing, and as disinfectant and diuretic (Llorens 1983). Their extracts exhibit furthermore anti-inflammatory activity.

Fig. 1.7 *Acanthus mollis*

1.9 *Fagus sylvatica*

Scientific name and etymology: *Fagus sylvatica* L., the common beech or European beech, is a deciduous tree belonging to the beech family Fagaceae, explored by Ernest Henry Wilson. Species name comes from the Latin word “fāgus” (beech) and “sylvatica” (forest).

Morphological and origin description: Beech is widely distributed in central and western Europe. In the northern part of its range, beech grows at elevations higher than the southern part. Beech is a large tree, which can grow to heights of normally 25–35 m tall and 1.5 m trunk diameter. It has a typical lifespan of 150–200 years, in rare instances it may live for 250 years. The thin, smooth, silver-grey bark is a typical feature of beech. The leaves are alternate, simple, and entire or with a slightly crenate margins, 5–10 cm long and 3–7 cm broad, with 6–7 veins on each side of the leaf. Beech is a good species for soil conservation because it produces a variety of leaf litter (ca. 900 g/m^2 per year) and has extensive shallow and intermediate roots. Male and female flowers of beech are separated on the same tree. Male flowers are borne in the small

catkins that are a hallmark of the *Fagales* order. There are two nuts produced by female flowers in each cupule, maturing in the autumn 5–6 months after pollination (Wühlisch 2008).

Uses: The leaves of beech can be used to curing fever, diarrhoea, skin liver or respiratory diseases (Cracium 1976). Beech wood is homogeneous with fine pores and conspicuous wood rays. The color varies from nearly white to reddish. The wood has an average density of 700 kg m^{-3} with good stiffness and abrasive resistance but little elasticity. Beech is the most diversely used tree species in Europe, which has 250 known uses for its wood. The wood can produce the Primary Product AM 01-a smoke flavouring used in food (European Food Safety Authority 2010). The nuts are an important food for birds, rodents and in the past also humans. In 19th century England, the nuts were nonetheless pressed to obtain oil that was used for cooking and in lamps. Due to the tannins and alkaloids content, if eaten in large quantities will cause slightly toxicity to humans. After the tannins leached out by soaking, the nuts were also ground to make flour to be eaten (Fergus and Hansen 2005) (Fergus 2002) (Lyle 2006).

Fig. 1.8 *Fagus sylvatica*

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Chapter2: Introduction of metabolomics fingerprinting methodology

2.1 General introduction of metabolomics

In the recent years, many areas of science have seen a great improvement in technologies, equipment, infrastructure, computing capacity, and bioinformatics tools, opening up new opportunities and even generating new fields of scientific research. One of these technologies is metabolomics, where the combined advances in computer hardware, required for reliable and accurate metabolite separation and detection, and its associated software for subsequent data storage, treatment, and analysis, produced progress in the field of the biochemical analysis of biological systems (Hardy and Hall 2012).

The specific concept about "Metabolomics" is the scientific study of large-scale small molecules, commonly known as metabolites, within biological cells, tissues or organisms. A metabolite is a low molecular weight (within a mass range of 50–1500 daltons (Da) organic compound, typically involved in a biological process as substrates, intermediates or products. Some examples of small molecules include: sugars, lipids, amino acids, fatty acids, phenolic compounds, alkaloids and many more. The metabolome represents the complete set of metabolites in a biological cell, tissue, organ or organism, which are the end products of cellular processes (Jordan et al. 2009). Metabolomics is the "systematic study of the unique chemical fingerprints that specific cellular processes leave behind" (Daviss 2005).

Adoption and exploitation of the technology has been rapid both in plant science and beyond–in Fig. 2.1 a research on PubMed shows how the number of publications containing the term "metabolomic" is constantly growing, as well as the numbers of publications containing the terms "metabolomics and plant".

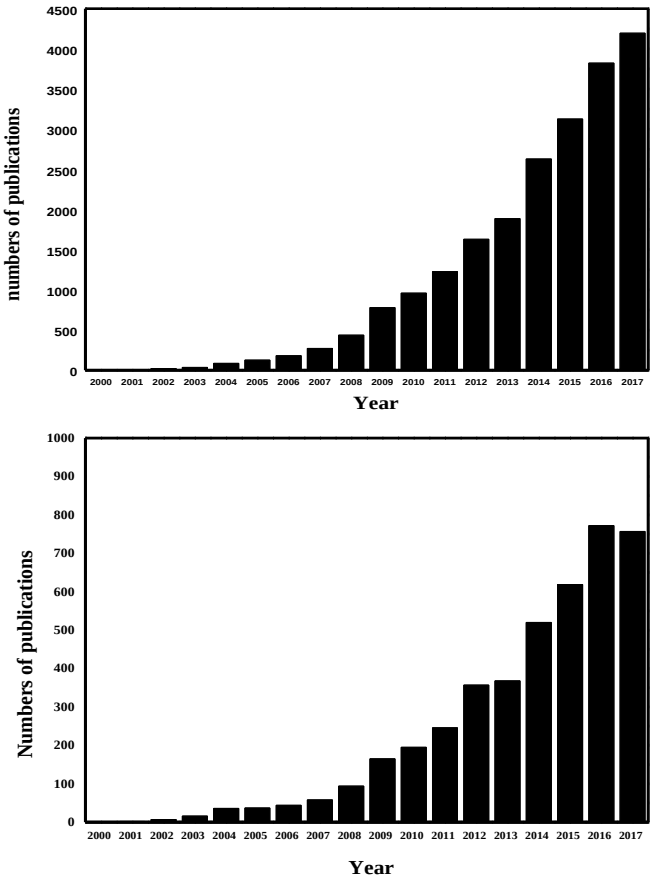

Fig. 2.1 The numbers of publications including the term "*metabolomics* per year referring PubMed (up） and the numbers of publications including the terms "*metabolomics and plant*" per year referring PubMed (down).

As genomics is the study of DNA and genetic information within a cell, and transcriptomics is the study of RNA and differences in mRNA expression; metabolomics is the study of substrates and products of metabolism, which are influenced by both environmental stimuli and genetic perturbation. The relationship between them is like a life paramid genomics, transcriptomics, proteomics and metabolomics, from down to top respectively. DNA and mRNA expression data instruct to transcript proteins and produce metabolites in a cells. Metabolomics is a powerful approach to study metabolites and their concentrations, which is on the top state of the Pyramid. Unlike other "omics" technologies, metabolomics profiling can give an instantaneous snapshot of the physiology of that cell, thus providing a direct "functional readout of the physiological state" of an organism (Hollywood, Brison, and Goodacre 2006). Metabolomics can

best represent the molecular phenotype. The non-invasive nature of metabolomics and its close link to the phenotype make it an ideal tool for the pharmaceutical, preventive healthcare, and agricultural industries, among others. Biomarker discovery and drug safety screens are two examples where metabolomics has already enabled informed decision making.

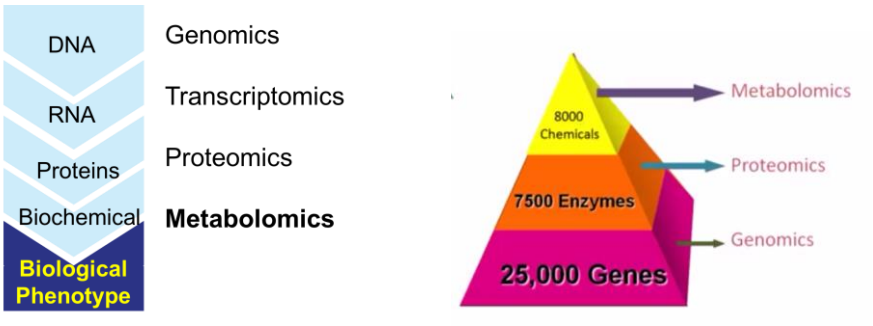

Fig.2.2 A central dogma of biology showing the flow of information from DNA to the phenotype. Associated with each stage is the corresponding systems biology tool, from genomics to metabolomics (left) Constituents of Pyramid of life (D. S. Wishart, 2005) (right)

Because plants are sessile organisms, they cannot escape from changing environmental conditions and plant-attacker interactions that adversely affect their growth and development. Under environmental conditions (abiotic stress) and plant-attacker interactions (biotic stress), plants give responses to produce particular bioactive metabolites for defense. For example, in response to sulfur deficiency, some metabolites will be readjusted (Jorge, Mata, and António 2016).

It is estimated there are around 200,000 metabolites across the plant kingdom, and somewhere between 7,000 and 15,000 within an individual plant species (Fernie 2007). With comparison to many other species, it can be concluded that plants are particularly biochemically rich. Except for those contributing to the color, taste, aroma and scent of fruits and flowers, plant metabolites are associated with many resistance and stress responses in plants. As the end products of cellular regulatory processes, metabolites are the final response of biological systems to genetic alteration or environmental stimuli (Bino et al. 2004). The level of plant metabolites is the essential index that strictly indicates the degree of physiological fluctuations. Therefore, it is

necessary to simultaneously identify and quantify metabolites in plants to understand the dynamics of the metabolites, study fluxes in metabolic pathways and decipher the role of each metabolite in response to.

The studies of the plant metabolomics is composed of analysis of a large number of chemical species with various physical properties, ranges from ionic inorganic compounds to biochemically derived hydrophilic compounds, organic and amino acids, and a series of hydrophobic lipid-related compounds. Plant metabolomics has benefited from a large number of previous methodological approaches and bioanalytical knowledge for the characterization of many chemically diverse classes of metabolites. Because of their highly dynamic in time and space and complexity of plant structures, it is a big challenge for a single analytical technique to separate and characterize all the metabolites in biological sample matrices (https://www.creative-proteomics.com/services/plant-untargeted-metabolomics.htm). The two main approaches used in current plant metabolomics are untargeted and targeted approaches. In contrast to a targeted metabolomics experiment, which measures compounds from known metabolites, an untargeted metabolomics experiment registers all compounds within a certain range, including structurally novel metabolites.

There are typically three steps in an experimental workflow of untargeted approach:

1 Profiling, also known as differential expression, based on finding metabolites with statistically significant variations within control and test sample sets.

2 Compound identification with the determination of the chemical structure of the discovered metabolites.

3 Comprehensive interpretation, the last step, and uncovering biological connections between the metabolites and the biological processes.

In the workflow of discovery metabolomics, analytical reproducibility is critical for expression profiling work; annotation is a tentative identification based on an accurate mass match to a database or a spectral match to a library of spectra; the collected data can be interpreted for biomarker discovery, biological signature/fingerprint selection and pathway mapping. Above are the most important parts in untargeted metabolomic research.

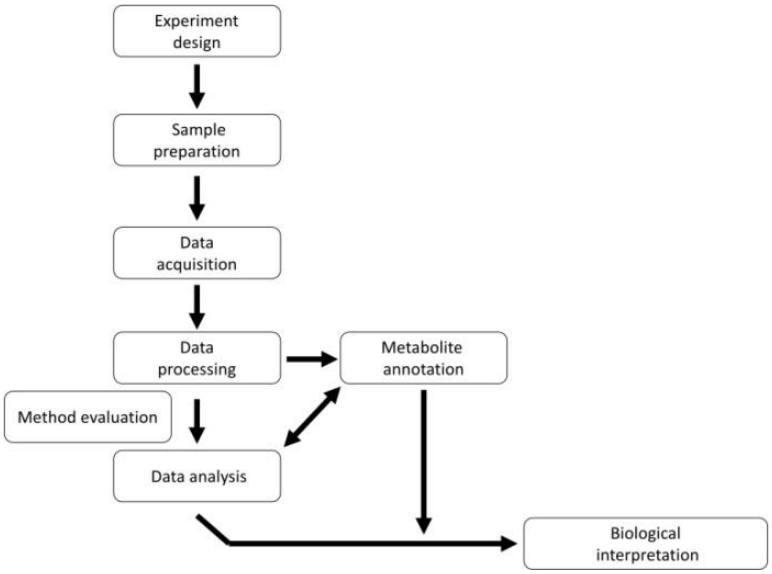

Fig. 3 Typical untargeted metabolomics workflow.

The classical instrumentation useful for the identification and quantification of plant metabolomics includes Mass Spectroscopy (MS), Gas Chromatography (GC), High Performances (Liquid Chromatography (HPLC), Capillary electrophoresis (CE) or hyphenated technologies such as HPLC-MS, GC-MS and CE-MS. The coupling of chromatographic methods such as GC or HPLC with MS can greatly increase coverage of metabolites, which will enhance the biological context by increasing the number of identified metabolites. Other techniques used for plant metabolomics include Fourier Transform Infrared Spectroscopy (FT-IR), Near Infrared Spectroscopy NIR and NMR.

2.2 Typical introduction of Gas Chromatography Mass Spectroscopy (GC-MS)

Mass spectrometry (MS) is an analytical technique that ionizes chemical compounds and sorts the ions based on the mass-to-charge ratio. A mass spectrum is a plot of the ion signal as a function of the mass-to-charge ratio. These spectra are used to determine the elemental or isotopic signature of a sample, the masses of particles and of molecules, and to elucidate the chemical structures of molecules and other chemical compounds.

In a typical MS procedure, a sample, which may be solid, liquid, or gas, is ionized, for example by bombarding it with electrons. This may cause some of the sample's molecules to break into charged fragments. These ions are then separated according to their mass-to-charge ratio, typically by accelerating them and subjecting them to an electric or magnetic field: ions of the same mass-to-charge ratio will undergo the same amount of deflection (Sparkman 2000). The ions are detected by a mechanism capable of detecting charged particles, such as an electron multiplier. Results are displayed as spectra of the relative abundance of detected ions as a function of the mass-to-charge ratio. The atoms or molecules in the sample can be identified by correlating known masses (e.g. an entire molecule) to the identified masses or through a characteristic fragmentation pattern.

Gas chromatography–mass spectrometry (GC-MS) is coupled gas chromatograph with mass spectrometer. The gas chromatograph utilizes a capillary column which depends on the column's dimensions as well as the phase properties. The different molecules of different affinity for the stationary phase will promote separation of the molecules as the sample travels the length of the column.

The molecules in samples are retained by the column and then eluted from the column at different times (the retention time), and this allows the mass spectrometer downstream to capture, ionize, accelerate, deflect, and detect the ionized molecules separately. The mass spectrometer

does this by breaking each molecule into ionized fragments and using their mass-to-charge ratio to detect these fragments further.

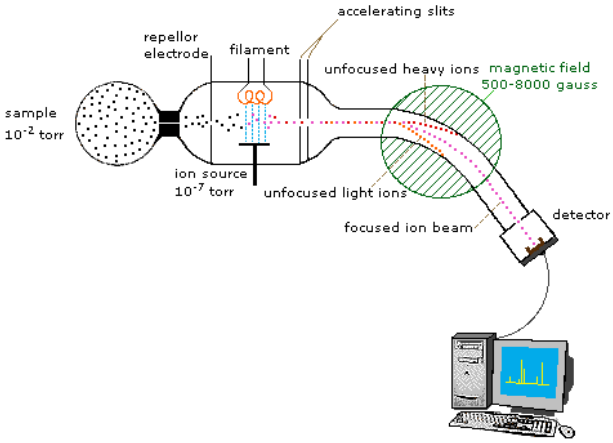

Fig. 2.4 Mechanism of Mass technology

Because of its advantage of high resolution and high reliability, Mass spectrometry is more sensitive for metabolite detection—it can detect analytes routinely in femtomolar to attomolar range. LC-MS is more labile compounds and for compounds hard to derivatize. CE-MS is used for profiling of amino acid in plant cell cultures. Compared to LC-MS and LC-NMR, GC-MS is of analytical reproducibility and lower costs. As one of the widely used analytical techniques in plant metabolomics, GC-MS is utilized to qualitatively and quantitatively analyze a wide range of volatile and derivatized nonvolatile metabolites with high thermal stability （https://www.creative-proteomics.com/services/plant-untargeted-metabolomics.htm）.

However, GC-MS is only capable of analyzing volatile and thermally stable metabolites and requires chemical derivatization to chemically modify non-volatile compounds (e.g. most primary metabolites) to produce volatile derivatives. The derivatization protocol for GC-MS plant metabolomics studies is well established and includes two chemical reactions: methoxyamination and silylation. Nevertheless, some thermolabile metabolites (e.g. sugar phosphates) as well as metabolites that do not become volatile even after derivatization (e.g. large oligosaccharides) are

not amenable to be analyzed with GC-MS, and thereby specific target approaches based on LC-MS are the best choice for their identification and quantification (Jorge et al. 2016).

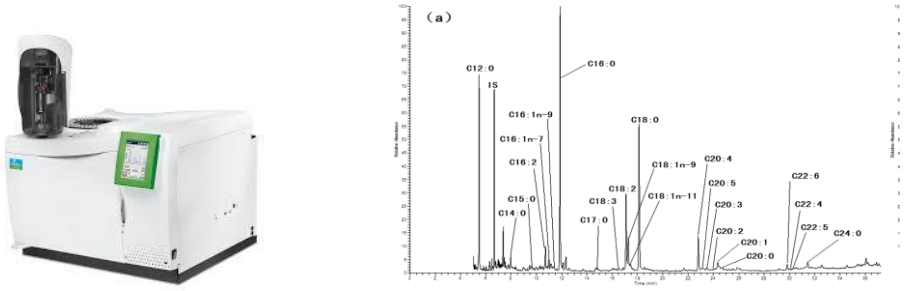

Fig 2.5 GC-MS instrument (left) Spectra of mass data interpretation (right)

2.3 Typical introduction of Nuclear Magnetic Resonance Spectroscopy (NMR)

The technology of nuclear magnetic resonance (NMR) spectroscopy is a spectroscopic technique to apply the presence of anisotropic interactions around atomic nuclei to determine the configuration of organic compounds. When a sample is placed in a magnetic field, the NMR signal is produced by excitation of the nuclei sample with radio waves. The signal is detected with sensitive radio receivers. The intramolecular magnetic field around an atom in a molecule changes the resonance frequency, thus giving access to details of the electronic structure of a molecule and its individual functional groups. Besides identification, NMR spectroscopy provides detailed information about the structure, dynamics, reaction state, and chemical environment of molecules.

The NMR tech can be applied on liquid sample or solid sample. Unlike solid sample, rapid motion of molecules in liquid sample will lead to a variety of NMR line broadening interactions (such as chemical shift anisotropy and dipole-dipole interactions, etc.) averaging out to obtain a spectrum of higher resolution.

The most important nuclei in biomolecular NMR studies are ^{1}H (proton), ^{13}C, ^{15}N, and ^{31}P. Of these, 1H is the most sensitive followed by ^{31}P; both are present at near 100% natural abundance.

In metabolomics, one-dimensional (1D) ^{1}H NMR is the most widely used NMR approach. Signals are either binned and then analyzed or fitted to patterns of signals corresponding to the metabolites expected to be present in the mixture. The latter one can encounter problems in that many 1H signals overlap in ways that offer alternative fitting solutions. It was suggested to overcome the problem by standardizing the analysis in terms of biofluid, solution conditions, data collection protocol, and by employing probabilistic fitting (Ravanbakhsh et al. 2015).

Two dimensional (2D) NMR methods provide improved approaches for unambiguous identification of metabolites in mixtures. These 2D methods include ^{1}H-^{1}H COSY (correlated spectroscopy), ^{1}H-^{1}H TOCSY (total correlation spectroscopy) and ^{1}H-^{13}C HSQC (heteronuclear single-quantum correlation).

In spite of its lower sensitivity for metabolites detection, NMR spectroscopy offers many unparalleled advantages over Mass technology. NMR analysis has distinct advantages—non-destructive requirements for little sample handling and preparation including metabolites in liquid state or intact tissues. It offers benefits for compounds that are difficult to ionize or require derivatization for MS. The tech is easy for the quantification—peak area of compound in NMR spectrum directly related to content of specific nuclei, making precise quantification of compounds in complex mixture by integrating the peak (peak area). NMR plays important role in determining structures of unknown compounds. Through the use of stable isotope labels, NMR can be used to elucidate the dynamics and mechanisms of metabolite transformations and to explore the compartmentalization of metabolic pathways (Markley et al. 2017).

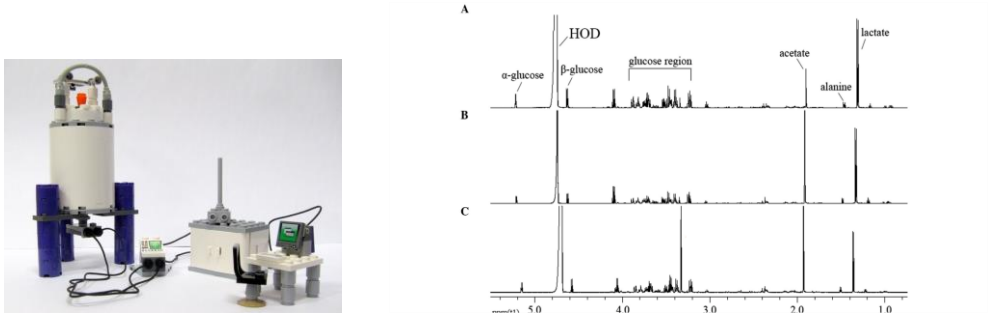

Fig. 2.6 Instrument of NMR (left) Spectra of NMR data interpretation (right)

In our research, the steps needed to perform a gas chromatography mass spectrometry (GC-MS) and nuclear magnetic resonance (NMR) –based untargeted metabolomics experiment guided by *in vitro* bioassay test are detailed. The protocols in this unit describe the conditions necessary for analyzing hydrophilic and hydrophobic metabolites and provide characterization of a metabolite based on novel structure and figure out the correlation between bioassay test and contents of main metabolites.

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Chapter 3: Materials and methods

3.1 Chemicals and devices

The information on chemicals and instruments used in experiments were presented in Table 3.1 and Table 3.2 respectively. The reagents used for the extraction procedure were analytical grade. Reagents used for analytical analysis (GC-MS and NMR) were gradient grade.

Tab. 3.1 Information on chemicals used in experiments

Agent name	Abbreviation	Molecule formula	Manufacturer
Petroleum ether (99.8%)	PET	C_6H_{14}	Carlo Erba
Dichloromethane (99.8%)	DCM	CH_2Cl_2	Carlo Erba
Methyl Alcohol	MeOH	CH_4O	Carlo Erba
Methyl Alcohol	MeOH	CH_4O	Romil
Hydrochloric Acid	HCl	HCl	
Deionized Water	DI	H_2O	
Potato+Dextrose+Agar	PDA		VWR CHEMICALS
Lysogeny Broth	LB		VWR CHEMICALS
Deuterium Oxide (99.9 atom % D)	D_2O	D_2O	Aldrich
4,4-Dimethyl-4-Silapentane Sulfonate Acid (99.90 atom % D)	DSS	$C_6H_{16}NaO_3SSi$	Aldrich
Standard of Fatty Acid Methyl Esters			

Tab. 3.2 Information on instruments used in experiments

Instrumental name	Instrument model	Manufacturer
Blender	DPA141	Moulinex, France
Rotor-Evaporator	R-114	Büchi, Switzerland
Rotor-Evaporator	VV2000	Heidolph, Germany
Microscope	HM-LUX	Leitz Wetzlar, Germany
Microscope	INV. N. 181	Wild Heerbrugg, Switzerland
Autoclave		Pbi international, America
Water Bath		Kottermann, Germany
Sterile Bench	Steril-VBH	Angelantoni Life Science, Italy
Laminar Flow	Vertical 700	Asal Srl, Italy

3.2 Plant materials

A total pool of leaves and roots for each species were selected from Mediterranean and

temperate ecosystems (Southern Italy). The species pool includes two grasses (*Festuca drymeia*, *Typha latifolia*), two perennial forbs (*Acanthus mollis*, *Dittrichia viscosa*), one vine (*Hedera helix*), one evergreen tree (*Quercus ilex*), and two deciduous trees (*Fagus sylvatica*, *Fraxinus ornus*). Voucher specimens were prepared under the direction of Prof. Giuliano Bonanomi.

For each species, a number >10 of individuals from natural communities were randomly selected at the sampling sites. Fresh leaves were collected and dried (40°C until constant weight was reached) and then stored in sealed plastic bags at room temperature. For the same 10 individuals for each species, root samples were collected. Briefly, roots were gently washed to remove soil particles and dried by means of laboratory paper, subsequently dried at 40°C until constant weight was reached. Roots were deposited in plastic bag at dark and dry conditions. When chemical extraction started, dry leaves and roots were transported to the Laboratory of Organic Chemistry, Department of Agricultural and Food Sciences, University of Naples Federico II for preprocessing.

3.3 Plant materials extraction and isolation

Preprocessing: the tissues of leaves and roots of eight Mediterranean folk medicinal plants were ground into small pieces by a blender. Then they were stored and sorted in boxes at cool and dry spot at room temperature.

Apolar phase extraction：Each sample of four grams was dissolved in 20 mL petroleum ether and then was stirred uniformly for 1 h at room temperature. If the solvent turned too concentrated, it was added more petroleum ether. After stirring, the mixture was filtrated with paper, then the transparent filtrate was transferred into a vial. Afterwards, the vial was air-dried at room temperature as well as the residues of the sample. Finally, the dried organic samples were stored at +4 ℃ until analysis. The polarity of petroleum ether is 0.01, which can extract lipids, wax, essential oils, isolated steroids, triterpenes and so on. (Song Xiaokai 2017)

Polar phase extraction：The residues of sample were weighted and then dissolved in 20 mL

methanol: water=60:40. Mixed by vortex (9000 rpm 1 min). After centrifugation (3000 rpm, 5 min), the aqueous and solid fractions were separated, with particular attention to collect the transparent polar extract and discard the interphase and precipitate fraction. The polar extraction was rotor-evaporated to dryness under vacuum at 35°C. Finally, dried samples were stored at +4 °C until analysis. The polarity of methanolwater mixture allowed to extract alkaloid salts, glycosides, tannins, and amino acid, sugars, salts and so on.

3.4 Protocols of Bioassay Test

Antifungal activity of the apolar and polar phases of each plant species was tested against two fungal species: a widespread air-borne pathogen, causal agent of the black mold disease on certain fruits and vegetables (*Aspergillus niger*), and an antagonistic fungus that is also used as a fungicide. (*Trichoderma harzianum*). Microbes were obtained from the Laboratory of Plant Pathology, Department of Agricultural Sciences, University of Naples Federico II.

Shortly, fungal inoculum was obtained by adding 10 ml of sterile water to seven-day-old cultures, grown in Petri dishes containing Potato Dextrose Agar (PDA), and scraping the culture surface to obtain conidia. The suspension was filtered, centrifuged, washed twice with sterile water and adjusted to a concentration of 1×10^6 mL^{-1} by hemocytometer. 1 mL Spore suspension was prepared in distilled water. Extracts were applied at three concentrations (1000, 100 and 50 ppm) in a 96-well Elisa plate and incubated at room temperature. Fungal hyphal length of germinating spore was measured usually after 7 days of incubation. Biological activity by *in vitro* was described in details test as below.

3.4.1. Substrate preparing and spore collection quantitatively

All the experimental items used should be sterilized, including distill water, PDA, LB, Petri dishes, vials tips, pipette, etc. The regular cycle of Autoclave sterilizing procedure is 120°C × 40 min under vacuum.

Preparation of media for culturing fungus—Agar 39 gram/L (PDA: potato+dextrose+agar

media (solid); LB: Lysogeny broth (liquid). The edge of culture surface are scraped to obtain conidia of the new growing fungus (*A. niger*, *T. harzianum*) and transferred onto the center of PDA. The sealed Elisa plate are stored at cool and airy place at room temperature and observed their growth for successive days. Usually the LB control will grow fully of the well of the plate from 3-7 days depending on different room temperature from 25°C to 10°C.

Preparation of spore suspension—the surface of fungus cultures is scraped and it is added distilled water to make raw spore solution, filtrated and centrifuged at 3000 rmp for 5 min twice. Then slide are made to observe under the microscope with eyepieces of 20 or 40 times amplification. The numbers of spores of ten grids (or five grids, both on the top and below of hemocytometer) counted and got the average number to calculate the concentration of the spore. Spore concentration is targeted on nearly 1×10^6 mL^{-1}, it means nearly each grid contain 5-6 spores. When necessary, the procedure was repeated to obtain the ideal concentration of spore solution.

Preparation of stock solution of plant extracts—empty vials are weighted and then the stool used to scrape nearly 2 mg extract into the vial. 20 μL ethanol are used to solubilize and vortexed for a few minutes. The extracts are dissolved and when necessary a water bath to heat at nearly 45~50°C is used. Then 180μL distill water is added to prepare 10^4 ppm stock solution (Ethanol: water=1:9). The precise weight is recorded and stock solution is stored in the fridge.

3.4.2. Design of bioassay

Preparation of Elisa Plate: The solvents is added step by step, using different types of pipette and tips, paying attention to add everything into the well and shake a little bit to mix. The sequence is water, Lb, substance, spore suspension. All the bioassay experiments were performed in triplicate to assure their reproducibility. The bioassay design of Elisa plate on *Typha latifolia* (Ty) is taken as an example as showed below.

Tab. 3.3 The bioassay design of Elisa plate on *Typha latifolia (Ty)*. **“**1000, 100, 50” mean concentration of substance. **“**r1, r2, r3” refer numbers of replication “L, R” “P, A” represent “leaf, root” “polar, apolar” respectly. **“**Lb” is short

of" inoculation control with Lb". "H_2O" means "inoculation control with water". "no inoc" means "no inoculation with only water"

	1	2	3	4	5	6
A	1000 Ty LP-r1	1000 Ty LP-r2	1000 Ty LP-r3	1000 Ty RP-r1	1000 Ty RP-r2	1000 Ty RP-r3
B	100 Ty LP-r1	100 Ty LP-r2	100 Ty LP-r3	100 Ty RP-r1	100 Ty RP-r2	100 Ty RP-r3
C	50 Ty LP-r1	50 Ty LP-r2	50 Ty LP-r3	50 Ty RP-r1	50 Ty RP-r2	50 Ty RP-r3
D	1000 Ty LA-r1	1000 Ty LA-r2	1000 Ty LA-r3	1000 Ty RA-r1	1000 Ty RA-r2	1000 Ty RA-r3
E	100 Ty LA-r1	100 Ty LA-r2	100 Ty LA-r3	100 Ty RA-r1	100 Ty RA-r2	100 Ty RA-r3
F	50 Ty LA-r1	50 Ty LA-r2	50 Ty LA-r3	50 Ty RA-r1	50 Ty RA-r2	50 Ty RA-r3
G	Lb-r1	Lb-r2	Lb-r3	H_2O r1	H_2O r2	H_2O r3
H	no inoc-r1	no inoc-r2	no inoc-r3			

Specific volumes of constituents were showed as below.

Tab. 3.4 Specific volume of Lb, substance, spore solution and water in each well in Elisa plate.

	Lb/µl	Substance/µl	Spore/µl	Water/µl	Total/µl
1000 ppm	10	10	10	70.0	100
100 ppm	10	1	10	79.0	100
10 ppm	10	0.5	10	79.5	100
0 ppm	0	0	10	90	100
Lb control	10	0	10	80	100

3.4.3. Measurement of hyphal growth

After inoculating the fungus for 7 days, the fungal hyphal growth were observed under microscopes. Firstly, it was setted the LB control as "10" (the maximal) and empty control (no inoculated) as "0" (the minimal), to measure all the fungal hyphal growth of germination spores from 0 to 10. Fungal growth was statistically analyzed by one-way ANOVA and Duncan test by using concentrations of plant extracts as a factor for each fungus. Significance was evaluated in all cases at $P < 0.05$.

3.5 GC-MS analysis

Gas Chromatography Mass Spectroscopy: In order to obtain volatile compounds, nonpolar extracts were derivatized before analysis by GC-MS. For GC-MS: use 1mL 1N MeOH: HCl =97:3 solvent to solubilize the dry samples, then the vials were left at 50 °C overnight. This methanolysis action was to make fatty acid derivatized. The solvents were rotor-evaporated to dryness, then 1 mL DCM was added to resolubilize the dry samples. 1 μL of each derivatized sample was injected in a pulsed splitless mode into an Agilent-6850 GC system with 5977E MSD operating in EI mode at 70 eV. The system was equipped with a 30 m × 0.25 mm id fused-silica capillary column with 0.25 μm HP-5MS stationary phase (Agilent technologies, UK). The injection temperature was set at 270 °C. Helium was used as carrier gas at a constant flow rate of 0.8 mL /min. Separation of the non-polar extract was achieved using a temperature program of 80 °C for 1 min, then ramped at 10 °C/min to 320 °C and held for 1 min. (de Falco et al. 2018)

3.6 NMR analysis

Nuclear magnetic resonance spectroscopy: ^{1}H NMR spectroscopy were acquired for the polar extracts, recurring to deuterium oxide as solvent: 600 μl of D_2O (purity of 99.8%) were used to dissolve the dry extracts, which were then transferred into a 5 mm NMR tube, 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS) was used as an internal standard. The pH was adjusted to 6.0 by using KH_2PO_4 and 1 N NaOD as buffering agents. All spectra were acquired at 298 K with Varian Unity Inova spectrometer operating at a ^{1}H frequency of 400.422 MHz. The recycle time was set to 5 s and a 45° pulse angle was used. Chemical shifts were referred to DSS signal (δ 0.00 ppm). All spectra were processed using Mestranova program, phased and baseline corrected manually (de Falco and Lanzotti 2018).

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Chapter 4: Bioassay test results and discussion

4.1 Yield of Hydrophilic and lipophilic extracts

We can see from the table that apolar leaf extract yield was very low, less than 1%, ranging from 0.16% to 0.99%. While the polar leaf extract yield was high ranging from 0.53% to 12.44%. Apolar root extracts, with relative even lower yield, possessed the yield fluctuating from 0.03% to 0.59% while the polar root extract had the yield from 0.95% to 9.05%. The yields of apolar and polar extract among different plants were related to the solubility of major metabolites of each species in the petroleum ether and in the mixture of water and methanol we used.

Tab. 4.1 the weight and the percent yield of plant apolar and polar extracts

Species	AL /mg	Yield /%	PL /mg	Yield /%	AR /mg	Yield /%	PR /mg	Yield /%
D. viscosa (Dry)	39.6	0.99%	468	12.44%	5.9	0.15%	285.7	7.3%
D. viscosa (Fresh)	14.3	0.36%	109.2	6.85%	23.9	0.59%	180.4	5.53%
T. latifolia	5.4	0.19%	150	5.24%	1.7	0.04%	54.5	1.36%
F. ornus	32.4	0.81%	348.7	8.99%	2.8	0.07%	95.4	2.46%
H. helix	5.2	0.13%	278.5	7.11%	19.8	0.49%	151.7	3.87%
F. drymeja	9.4	0.24%	41.6	1.05%	1.8	0.04%	38.4	0.95%
Q. ilex (Dry)	6.7	0.17%	165.4	4.12%	18.3	0.45%	340	9.05%
Q. ilex (Fresh)	17.9	0.45%	143.9	4.55%				
F. sylvatica	6.2	0.155%	20.1	0.53%	1.5	0.0370%	60	1.5%
A. mollis	33.7	0.84%	255	6.6%	1.4	0.0346%	58.5	1.5%

4.2 The discussion of antifungal activity of extracts on bioassay test results

Percentage growth of mycelium for tested microorganisms was calculated using the following formula:

Formula 4.1: $$\frac{Experimental\ well\ growth\text{-}Blank\ well\ growth\ \left(no\ Inoculation\ control\right)\times 100\%}{Negative\ control\ growth\ \left(Lb\ control\right)}$$

The activity of the extracts of eight plants against fungus were divided into three groups on the base of strong, moderate and none activity and described as below Fig 4.1-4.3. The original data and general leaner model results were at Tab. 4.2 and Tab. 4.3.

Tab. 4.2 Growth values and relative standard deviation of *T.harzianum* in LB media expressed as percentage respect control in presence of polar and apolar extract from leaf and roots at three different concentrations from eight plant species.

		Leaf		Root		Leaf		Root	
		Apolar	Polar	Apolar	Polar	Apolar	Polar	apolar	polar
	50	103.96±0	103.96±0	103.96±0	103.96±0	101.45±0	111.59±0	101.45±0	101.45±0
D. viscosa	100	103.96±0	103.96±0	103.96±0	103.96±0	101.45±0	121.74±10.14	101.45±0	101.45±0
	1000	41.58±0	93.56±0	69.3±60	97.03±12	33.82±5.86	131.89±0	101.45±0	101.45±0
	50	103.96±0	103.96±0	103.96±0	93.56±10.39	101.45±0	101.45±0	101.45±0	101.45±0
T. latifolia	100	100.49±6	93.56±0	103.96±0	93.56±0	98.07±5.86	101.45±0	101.45±0	101.45±0
	1000	103.96±0	93.56±0	103.96±0	17.32±6	71.01±52.71	101.45±0	101.45±0	101.45±0
	50	103.96±0	103.96±0	69.3±6	103.96±0	101.45±0	101.45±0	101.45±0	101.45±0
H. helix	100	103.96±0	103.96±0	83.17±0	103.96±0	101.45±0	101.45±0	101.45±0	101.45±0
	1000	90.01±6	10.4±0	10.4±0	103.96±0	101.45±0	101.45±0	60.86±0.17	101.45±0
	50	103.96±0	41.58±0	103.96±0	103.96±0	101.45±0	67.63±58.57	101.45±0	101.45±0
F. ornus	100	103.96±0	31.18±0	100.49±6	103.96±0	101.45±0	101.45±0	71.01±52.71	101.45±0
	1000	31.19±10.39	20.79±0	24.25±24	114.36±0	101.45±0	101.45±0	101.45±0	101.45±0
	50	103.96±0	34.65±60.02	103.96±0	103.96±0	101.45±0	0±0	101.45±0	67.63±58.56
F. drymeja	100	103.96±0	0±0	103.96±0	103.96±0	101.45±0	0±0	101.45±0	101.45±0
	1000	93.56±0	103.96±0	79.7±12	93.56±0	101.45±0	0±0	101.45±0	0±0
	50	100.45±6	103.96±0	103.96±0	103.96±0	101.45±0	111.59±0	101.45±0	101.45±0
A. mollis	100	103.96±0	103.96±0	107.42±6	83.17±20.79	101.45±0	111.59±0	101.45±0	101.45±0
	1000	103.96±0	114.36±0	114.36±0	86.63±30.01	101.45±0	121.74±0	101.45±0	101.45±0
	50	114.36±0	114.36±0	103.96±0	100.49±6	101.45±0	101.45±0	101.45±0	101.45±0
F. sylvatica	100	114.36±0	114.36±0	100.49±6	110.89±6	101.45±0	101.45±0	101.45±0	101.45±0
	1000	124.75±0	124.75±0	117.82±6	103.96±0	101.45±0	111.59±0	101.45±0	101.45±0
	50	103.96±0	103.96±0	103.96±0	103.96±0	104.83±5.86	104.83±5.86	101.45±0	101.45±0
Q. ilex	100	103.96±0	103.96±0	103.96±0	103.96±0	104.83±5.86	108.21±5.86	101.45±0	101.45±0
	1000	103.96±0	103.96±0	103.96±0	103.96±0	111.59±0	131.89±0	101.45±0	101.45±0

Tab. 4.3 Degree of freedom (F) and p.value results of GLMs models on growth of *T.harzianum* and *A.niger* (see tab S 4.1) with repect to different categorical predictor. Significant p-values in bold.

Categorical predictors	*T. harzianum*		*A. niger*	
	F	p value	F	p value
Plant Species	54.67	**< .001**	64.39	**< .001**
Polarity of extract	14.73	**< .001**	4.23	**< .001**
Plant portion	8.99	**0.003**	30.75	**0.041**
Concentration	56.89	**< .001**	3.80	**< .001**
Species*Polarity	16.08	**< .001**	86.22	**0.024**
Species*Plant portion	27.90	**< .001**	22.65	**< .001**
Polarity*Plant portion	62.67	**< .001**	16.05	**< .001**
Species*Concentration	18.71	**< .001**	3.56	**< .001**
Polarity*Concentration	15.13	**< .001**	4.98	**0.007**
Plant portion*Concentration	4.14	**0.017**	2.50	0.084
Species*Polarity*Plant portion	43.69	**< .001**	29.44	**< .001**
Species*Polarity*Concentration	14.65	**< .001**	4.83	**< .001**
Species*Plant portion*Concentration	7.09	**< .001**	4.29	**< .001**
Polarity*Plant portion*Concentration	0.78	0.456	11.84	**< .001**
Whole combinations	12.56	**< .001**	3.31	**< .001**

4.2.1. *Dittrichia viscosa* and *Typha latifolia*

Dittrichia viscosa

Fig. 4.1 A showed that the antifungal effect of the dry and fresh leaf apolar extract of *D. viscosa* towards the two fungal species *Trichoderma harzianum* and *Aspergillus niger* was always dependent on their concentration. The growth of mycelia decreased, followed parallel by the extract concentration increasing. On the greatest concentration (1000 ppm), the inhibition effect was 60% comparing with control. This performance was superior to the previous finding it was elucidated that leaf hexane extracts of *D. viscosa* at higher concentration (6000 ppm) reduced mycelial growth of *T. harzianum* (inhibited by 62%) by Faten Omezzine et al (Omezzine et al. 2011). While the leaf extract, using mixture of methanol and water, didn't showed effect against the two tested fungi, in comparison to apolar samples possessing antifungal activity. In our present

research, dry samples extracts appeared to show similar antifungal activity of the fresh ones, maybe because the active metabolites were stable not easily volatizing with the artificial dehydration process. Additionally, it was indicated that the sensitivity of the phytopathogen *A. niger* to apolar leaf extracts was equal to the antagonist fungus *T. harzianum* (Fig. 4.1A). It was the first time that the inhibition effect of *D. viscosa* leaf organic extract was discovered on *A. niger* although there were a plenty of studies demonstrating high activity on colony growth of various tested fungi, except *Aspergillus spp.*

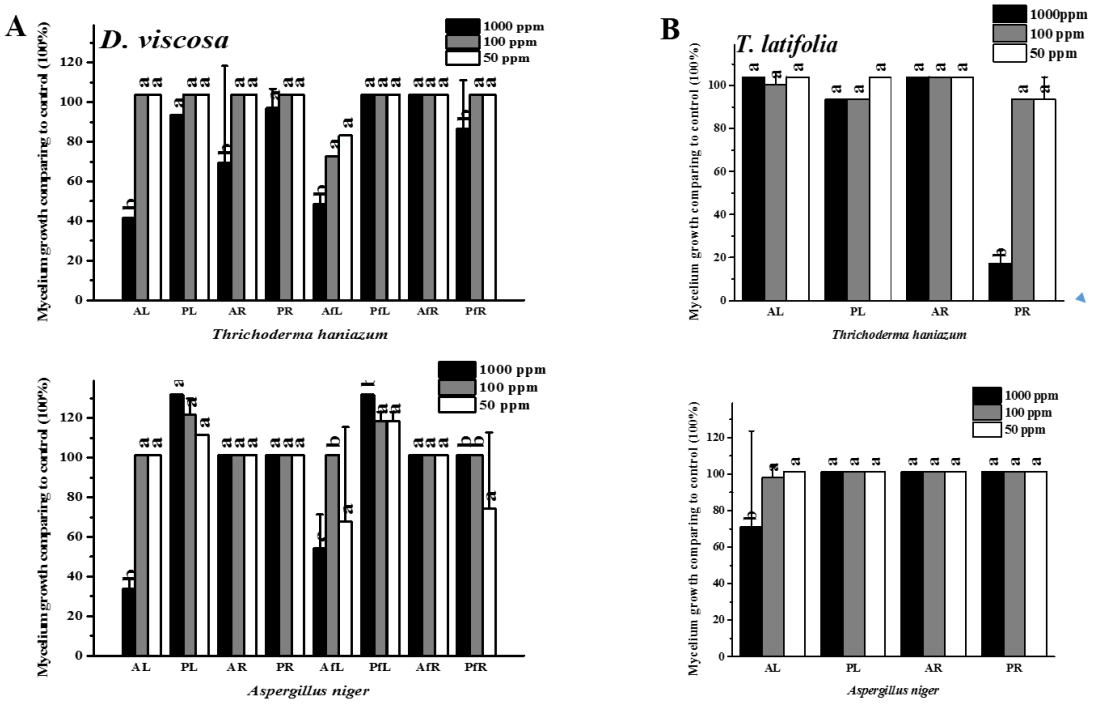

Fig. 4.1 Antifungal activity of polar and apolar mixtures, isolated from *D. viscosa* and *T. latifolia* at three concentrations (1000, 100 and 50 ppm.). Data represent fungal growth expressed in percentage compared to control (=100). Values are average of three replicates ± standard deviation. For each kind of extract, values with different letters indicate significant differences for $P < 0.05$ according to Duncan test. Fungal species were *T. harzianum* (upper chart) and *A. niger* (lower chart).

Since *D. viscosa* belongs to the family Compositae and has been applied as traditional herbaceous perennial medicine for its therapeutic effects, different chemical investigations and antimicrobial activities tests (phytopathogenic fungi, dermatophytic fungi, yeasts, and bacteria) have been reported. According to previous studies, extracts made from the aerial part of this plant exhibited a strong fungicidal activity *in vitro* and *in vivo*. Most fungicidal compounds isolated

were lipophilic.

Maoz et.al (Maoz, Kashman, and Neeman 1999) investigated that a sesquiterpene (tayunin), isolated from the leaves of *D. viscose*, inhibited the growth of *Microsporum canis* at a concentration of 10 μg/mI and *Trichophyton rubrum* at 50 μg/mI (MIC). Cafarchia's paper (Cafarchia et al. 2002) reported that the high concentration of the sesquiterpene (carboxyeudesmadiene) in essential oil of fresh *D. viscosa* leaf may contribute to great antifungal acitivity *in vitro* against Dermatophyte and *Candida spp.* even at low concentration (0.01mg/L). Wang and Cohen's group (Wang, Ben-Daniel, and Cohen 2004) (Cohen et al. 2006) investigated that the oily pastes of *D. viscosa* Leaves, obtained by extraction with a mixture of acetone and n-hexane, were used for the controlling plant downy mildew caused by *Pseudoperonospora cubensis*, *Phytophthora infestans*, *Blumeria graminis* f. sp. *tritici*, *Puccinia helianthi* and *Plasmopara viticola*. Chemical analyses conducted on the paste samples showed the presence of tomentosin, inuviscolide, costic acid and iso-costic acid. In addition, Caboni (Caboni et al. 2011) demonstrated that the high activity *D. viscosa* extract on colony growth of *Botryotinia fuckeliana, Monilinia laxa, Monilinia fructigena* and *Penicillium digitatum* was based on the chemical constituents on two sesquiterpene lactones (inuviscolide, tomentosin) and three sesquiterpene acids (costic acid, hydroxycostic acid, ilicic acid).

Thus, lipophilic extract of *D. viscosa* can be used as a much potential alternative for the control for fungus. For the acting mechanism, M. Maoz and Neeman (Maoz and Neeman 2000) discovered that *D. viscosa* leaf extract caused a decline in chitin content, a very important constituent of fungal cell wall, probably resulting in the antimycotic activity against dermatophytes and *Candida albicans*. The same group (Berdicevsky et al. 2001) also found that the extract containing tayunin caused dramatic changes in the hyphae and spore morphology due to severe damage in the fungal cell coat. Theses points provided good evidence to explain the strong antifungal activity of *D. viscosa* leaf extract.

Tab.4.4 List of references of the species *D. viscosa* with related antifungal and antimycotic activities

Plant organ	Antimicrobial compounds	Activity (**Antifungal**)	References
Leaves	Carboxyeudesmadiene	*Microsporum canis, M. gypseum, Trichophyton mentagrophytes, T. terrestre* and *Candida albicans, C. parapsilosis*	(Cafarchia et al. 2002)
Aerial parts		*Trichophyton mentagrophytes, T. violaceum*	(Ali‐Shtayeh and Abu Ghdeib 1999)
Whole plant	Methylated quercetins	*Candida albicans*	(Talib, Abu Zarga, and Mahasneh 2012)
Young shoots	Sesquiterpene lactone and acids	*Botryotinia fuckeliana, Monilinia laxa, M. fructigena* and *Penicillium digitatum*	(Mamoci et al. 2011)
Leaves	Tomentosin, inuviscolide, costic acid and iso-costic acid.	*Pseudoperonospora cubensis, Phytophthora infestans, Blumeria graminis f. sp. tritici, Puccinia helianthi.*	(Wang et al. 2004)
Leaves		*Microsporum canis* and *Trichophyton rubrum*	(Maoz and Neeman 1998)
Fresh and dry roots and shoots	Volatiles	*Helminthosporium sativumx*; *Fusarium oxysporum* f. sp. *lycopersici*	(Qasem, Al-Abed, and Abu-Blan 1995)
Leaves	Tayunin	*Microsporum canis* and *Trichophyton rubrum*	(Maoz et al. 1999)
Leaves		*Candida spp., Malassezia pachydermatis, M. furfur, Microsporum canis* and *Aspergillus fumigatus strains*	(Cafarchia et al. 2017)
Shoots (stem and leaves)	Costic acid and iso-costic acid	*Oomycetes, Ascomycetes* and *Basidiomycetes*	(Cohen et al. 2002)
Leaves		*Botrytis cinerea, Alternaria solani, Cladosporium sp., Fusarium oxysporum f. sp. melonis, Rhizoctonia solani, Sphaerotheca cucurbitae*	(Abou-Jawdah et al. 2004)
Leaves	Tomentosin and costic acid.	*Plasmopara viticola*	(Cohen et al. 2006)
Leaves and flower		*Trichoderma harzianum* and *T. viride; Fusarium oxysporum* f. sp. *melonis, F. oxysporum* f. sp. *lycopersici* and *F. oxysporum* f. sp. *tuberosi*	(Omezzine et al. 2011)

Leaves	Thymol and carvacrol in essential oil	*Fusarium moniliforme* and *Phytophthora capsici*	(Müller-Riebau, Berger, and Yegen 1995)
Leaves	Compounds to synthesize chitin	*Microsporum canis*, *Trichophyton rubrum* and *Candida albicans*	(Maoz and Neeman 2000)
Fresh aerial part		*Botrytis cinerea*, *Alternaria solani*, *Penicillium* sp., *Cladosporium* sp.; *Fusarium oxysporum* f. sp. *melonis* and *Verticillium dahlia*	(Abou-Jawdah et al. 2004)
Leaves with stems		*Geotrichum candidum*	(Talibi et al. 2012)
Leaves with stems		*Penicillium italicum*	(Askarne et al. 2012)
Leaves	Mono- and dicaffeoyl quinic acids	*Fusarium polyphialidicum*, *F. oxysporum*, *F. equiseti*, *F. accuminatum*, *F. scirpi*, *Septoria nodorum* and *Sclerotinia sclerotiorum*	(Mahmoudi et al. 2016)
Leaves	Phtalide compounds	*Fusarium culmorum* and *F. graminearum*	(Haoui et al. 2016)

Typha latifolia

Observing the mycelia growth condition in the apolar and polar extract of *T. latifolia* tissues, it was shown (Fig. 4.1B) that only polar root extract possessed significant activity against *T. harzianum* at the highest concentration (1000 ppm), in the presence of 30% mycelia growth compared with control (70% inhibition). Besides, all the other extracts showed little or no ability against both assayed phytopathogens.

In the previous researches, not so many data on the antimicrobial activities of *T. latifolia* extracts were reported. The ether extract of *T. latifolia* had partial inhibition against gram-positive gram bacterial *Staphylococcus aureus* by Agar dilution method (Carlson, Douglas, and Robertson 1947). In addition, the dichloromethane extracts of roots of *T. latifolia* were active against *Bacillus subtilis*, another gram-positive bacteria, using the diffusion method in solid media (Eduardo et al. 2006). While on antialgal activity, ethyl extracts of the whole *T. latifolia* was observed to show inhibition effect on the blue-green algae (particularly on T 625 *Synechococcus leopoliensis* and on T 1444 *Anabaenaflosaquae)* (Aliotta et al. 1990).

T. latifolia L. is one kind of helophytes with high allelopathic interactions (Szczepanska 1987). Several studies have focused on characterizing their biologically active metabolites to better understand the invasive properties. Ozawa and Imagawa (Ozawa and Imagawa 1988) found eleven phenolic compounds in female flowers of *T. latifoglia.* Ishida and co-workers (Ishida et al., 1988) identified a new flavonol glucoside isorhamnetin 3-rutinoside-7-rhamnoside from the dried pollen of *T. latifolia* L. extract, which showed antihemorrhagic activity. The research group of M. D. Greca isolated several free and acyl glucosylated stigmasterols (Della Greca et al. 1990) (M. D. Greca, Monaco, and Previtera 1990) and two carotenoid-like compounds, Blumenol A and (3R,5R,65,9ε)5,6-epoxy-3-hydroxy-β-ionol from extracts of *T. latifolia* (M. Della Greca et al. 1990). He et al. (He et al. 2015) proved that the root samples had higher concentrations of several

n-alkyl coumarates and ferulates in root than in leaf of *T. latifolia* by GC-MS spectroscopy Other species of *Typha*, like *T. domingensis*, also were tested in effective biological activity. For instance, aqueous extracts of leaves, stems, and roots of *T. domingensis* inhibited the growth of the water fern *Salvinia minima*, in bioassays(Gallardo, Martin, and Martin 1998). The most active phenolic compounds were 2-chlorophenol and salicylaldehyde, especially when extracted from roots. All these mentioned compounds might be proven to have phytotoxic properties, or to explain the antifungal activity of polar root extract in our present test as well.

Tab. 4.5 List of organic compounds from *T. latifolia* with related antibacterial and antialgal activities

Plant organ	Antimicrobial compounds	Activity	References
		Antibacterial	
Whole plant, Root		*Staphylccoccus aureus* *Bacillus subtilis*	(Carlson et al. 1947) (Eduardo et al. 2006)
		Antialgal	
	Steroids and unsaturated fatty acids	Blue-green algae mainly on T 625 *Synechococcus leopoliensis* and T 1444 *Anabaena flosaquae*	(Aliotta et al. 1990)

4.2.2. *Hedera helix, Fraxinus ornus* and *Festuca drymeja*

Hedera helix

Antifungal screening (Fig 4.2A) by adding apolar and polar extract of *Hedera helix* to the medium of the fungal pathogens showed that polar leave and apolar root extracts both exhibited an appreciable inhibitory activity, particularly at the highest dosage (90% inhibition at 1000 ppm). The effect was more distinct on *T. harzianum* than *A. niger*. At the same concentration of 1000ppm of apolar root extract, the mycelia growth was inhibited by 40% (Fig 4.2A). Besides, apolar leaf and polar root leachates showed weak or no ability against the two assayed phytopathogens.

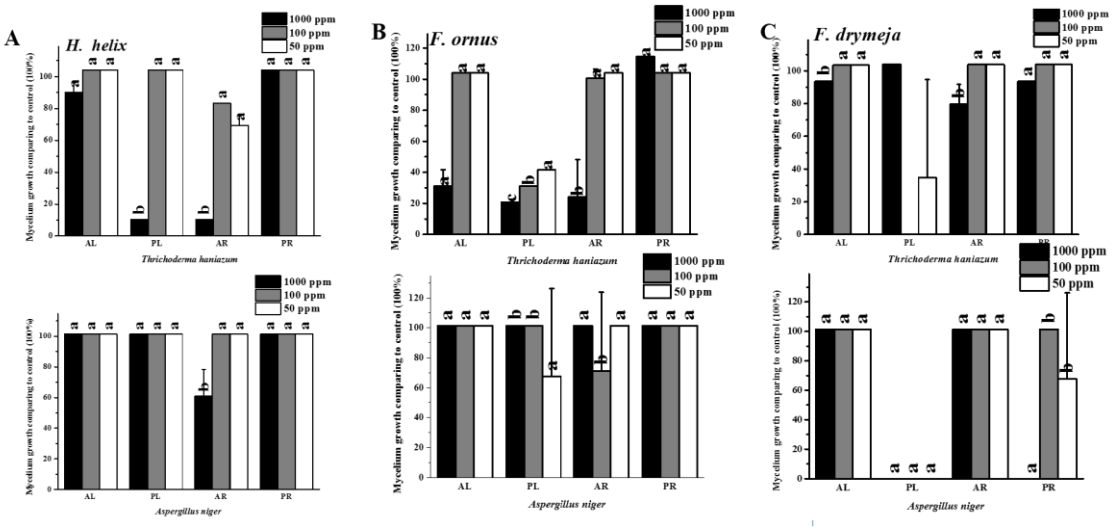

Fig. 4.2 Antifungal activity of polar and apolar mixtures, isolated from *H. helix, F. ornus* and *F. drymeja* at three concentrations (1000, 100 and 50 ppm.). Data represent fungal growth expressed in percentage compared to control (=100). Values are average of three replicates ± standard deviation. For each kind of extract, values with different letters indicate significant differences for $P < 0.05$ according to Duncan test. Fungal species were *T. harzianum* (upper chart) and *A. niger* (lower chart).

Hedera helix L. is a plant characterized by having abundant saponins. Hederacoside B and C, α-hederin and hederasaponin were the four major saponins found in *H. helix* (Pasich, Terminska, and Demczuk 1983). The pharmacological effects of this plant, including antifungal activity, were studied in deep in many scientific researches. As early as 1947, the strong effect of *H. helix* water extracts on the germination of the conidia of *Venturia inaequalis* was observed by Gilliver (Gilliver 1947). In 1979, Margaretha Leven (Leven et al. 1979) identified the inhibition effect against *Trichophyton rubrum, T. mentagrophytes, Microsporum canis* and *Candida albicans* comparing with the growth zone of standard. Oana ROşca-Casian et al. (Roşca-Casian et al. 2017) assessed *in vitro* antifungal activity of the 50% ethanol extract obtained from *H. helix* leaves against phytopathogenic fungi (*Aspergillus niger, Botrytis cinerea, B. tulipae, Fusarium oxysporum* f. sp. *tulipae, Penicillium gladioli,* and *Sclerotinia sclerotiorum*) using an agar dilution assay (MIC = 10–14%). The antibacterial activity of the saponins of *H. helix* was studied by Cioaca et al. (Cioaca, Margineanu, and Cucu 1978). The saponins of this plant presented antimicrobial activity against all 23 strains tested, representing 22 different bacteria and one yeast strain. Referring to these literatures, it was reasonable the high antifungal activity possessed by

leaf polar extract in our present study.

Tab. 4.6 List of organic compounds from the species *H. helix* with related antifungal and antibacterial activities

Plant organs	Antimicrobial compounds	Activity	References
		Antifungal	
		Venturia inaequalis	(Gilliver 1947)
		Trichophyton rubrum and *T. mentagrophytes Microsporum canis, Candida albicans*	(Leven et al. 1979)
Leaf		*Fusarium oxysporum*	(Bibi et al. 2016)
Leaf	Rutin, quercetin, kaempferol, stigmasterol and saponins	*Aspergillus niger, Botrytis cinerea, B. tulipae, Fusarium oxysporum* f. sp. *tulipae, Penicillium gladioli,* and *Sclerotinia sclerotiorum*	(Roşca-Casian et al. 2017)
		Antibcacterial	
	Saponins	22 different bacterias	(Cioaca et al. 1978)
		Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa	(Leven et al. 1979)

Fraxinus ornus

Concerning on *Fraxinus ornus*, each of apolar and polar leaf and apolar root eluates possessed an evident inhibition effect of polar leaf toward *T. harzianum* (Fig 4.2B). Particularly on polar leaf leachate, a dramatic inhibition effect has been detected, even at low concentration (inhibiting 60% at 50 ppm). The controlling effect was considered in correlation with the concentration of the extracts added in mycelial media, the antifungal activity level increasing in parallel to the rise of content of extracts. On the apolar leaf and root extracts, the controlling impact on the hyphae growth were presented on the highest concentration. While *A. niger* grew uneffectively in contrast to control.

The stem bark *F. ornus* L.is commonly used in the traditional medicine for wound healing. Lambrev and co-workers (Lambrev et al. 1961) revealed a clear antibacterial activity of the ethanolic extract and decoctions from the bark of *F. ornus* against *Staphylococcus aureus, Bacillus*

subtilis and *Leptospira ponoma*. Jurd et al. (Jurd et al. 1971) that the inhibitory effects of daphnetin and aesculetin (occurring free in *Fraxinus* species) on the growth of 22 species of bacteria, yeast and molds were measured. Grujic-Vacic's group (Grujic-Vaciæ et al. 1989) carried out that the aqueous extracts of the leaves of *F. ornus* showed strong inhibition on the growth of *Candida albicans* with zones of inhibition of 25 and 22 mm, while the extracts of the barks expressed inhibitory activity against *Staphylococcus aureus* (zones of inhibition 13 and 15 mm). Kostova's group (Iossifova et al. 1994) investigated that the antimicrobial activity of different groups of bark constituents of *F. ornus*. In the group of the coumarins Esculetin, Esculin, Soscopoletin, 7-Methylesculin, Scoparon, Fraxetin, Fraxin and 6,7,8-Trimethoxycoumarin, a clear correlation between structure and antimicrobial activity against *S. aureus* and *E. coli* was observed. While Ligstroside Insularoside, Ornosol inhibited the growth of *S. aureus* and *Cladosporum cucumerinum*. In another study the same group (Iossifova T. 2000) found the caffeoyl esters of phenylethanoid glycosides verbascoside and isoacteoside as inhibitors of *B. subtilis* at 2.5 μg/spot.

Referring to the papers of Kostova's group focusing on the active chemical constituents of *F. ornus* extract, the activity of the extracts against *S. aureus* was dependent on their hydroxycoumarin contents and there was a clear correlation between structure and antimicrobial activity (Iossifova et al. 1994) (Kostova and Iossifova 2002). It was confirmed that not only the major constituents like Esculetin and Fraxetin, but also but also their glucosides Esculin and Fraxin, may have antimicrobial activity, consistent with our bioassay results, that is the polar and polar leaf extract simultaneous presented antifungal activity. The principle may be explained considering that the major active metabolites had the basic moiety skeleton of hydroxycoumarin.

Tab. 4.7 List of organic compounds from the species *F. ornus* with related antifungal and antibacterial activities

Plant organ	Antimicrobialcompounds	Activity	References
		Antifungal	
Leaf		*Candida albicans*	(Grujic-Vaciæ et al. 1989)
Bark	Fraxin, esculetin, fraxetin	*Candida sp.*	(Kostova and Iossifova 2002)
		Antibacterial	
Bark	Esculin and fraxin	*Staphylococcus aureus, Bacillus subtilis* and *Leptospira ponoma.*	(Lambrev et al. 1961)
	Daphnetin, aesculetin	22 species of bacterias	(Jurd et al. 1971)
Bark	Esculetin, Esculin, soscopoletin, 7-Methylesculin, Scoparon, Fraxetin, Fraxi 6, 7,8- Tri methoxy coumarin; Ligstroside Insularoside, ornosol	*Staphylococcus aureus Escherichia coli Cladosporum cucumerinum*	(Iossifova et al. 1994)
Bark	Verbascoside and isoacteoside	*Bacillus subtilis*	(Iossifova T. 2000)

Festuca drymeja

Concerning the bioactivity of *Festuca drymeja* extracts, it was displayed unstably that polar leaf metabolites have the strong inhibition effect on tested microorganisms (Fig. 4.2 C). On the contrary, apolar and polar root tissue extracts showed weak or no ability focused on the mycelia growth condition of both *T. harzianum* and *A. niger*. On the details of statistical data of mycelia growth, it was abnormal that there was no symptom suggesting that antifungal activity was concentration-related with the leachates. No biological activity has been reported for *F. drymeja*. However, the presence of alkaloids in the extracts of *Festuca protensis* with antioxidant effect have been reported by Robbins et al. (Robbins et al. 1972).

4.2.3. *Acanthus mollis, Fagus sylvatica* and *Qeurcus ilex*

It was clear to see from the bar chart that the extracts of *A. mollis*, *F. sylvatica* and *Q. ilex* showed nearly no ability to inhibit mycelial growth. (Fig. 4.3) In all the extracts incorporated with fungus, it appeared the much similar mycelia growth situation, nearly 100% in comparison to

control. There were limited valuable literatures that refer to the fungistatic activity of *A. mollis* metabolites. For *F. sylvatica* and *Q. ilex,* the targets of most tested antimicrobial activity were bacteria. The results obtained by Jara (Jara et al. 2017) showed that ethyl acetate and ethanol extracts of *A. mollis* leaf and flower had the highest antifungal activity measured on various strains of Candida, which may be related to the high antioxidant activity. In the article of Brav (Brav 1997), natural benzoxazolinone (BOA) and derivatives, isolated from *A. mollis* seed extract, inhibited the growth of *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans*. There are references to the use of the antibacterial effect of *F. sylvatica* leaves against *Helicobacter pylori* (Frédérich et al. 2009), *Burkholderia coagulans* and *Alcaligenes xylosoxydans* (Lindberg, Willför, and Holmbom 2004). The *F. sylvatica* L. leaves were proven to be rich in polyphenols and to have antimicrobial activity against *Escherichia coli ATCC 8739* and *Staphylococcus epidermidis* (Nicu et al. 2016) (Nicu et al. 2018).Tănase et al. (Tănase et al. 2018) isolated the polyphenol compounds, including vanilic acid, catechin, taxifolin and syringing, and tested the antibacterial activity against *Staphylococcus aureus* and Methicillin-resistant *S. aureus*. Concerning *Q. ilex*, (Leven et al. 1979) tested the water extract of the whole *Q. ilex* plant against 5 fungus, *Aspergillus flavus, A. fumigatus, Trichophyton rubrum, T. Mentagrophytes, Microsporum canis,* and *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Proteus vulgaris.* Berahou and co-workers (Berahou et al. 1979) investigated the ethyl acetate, n-butanol and aqueous layer of *Q. ilex* bark that resulted effective against all bacterial strains tested at MICs ranging from 128 to 512 μg/mL. Güllüce et al. (Güllüce et al. 2004) identified inhibition effects of methanol extract of *Q. ilex* leaf towards the growth of all *Candida albicans* isolates and 35 bacterial strains of 7 bacteria genera. In the paper of Anastasia et al. (Karioti, Bilia, and Skaltsa 2010), the isolated compounds from *Q. ilex* leaves extracts containing flavonoids, proanthocyanidins and phenolic acids, showed generally higher activity against 14 fungal species than bifonazole and ketoconazole.

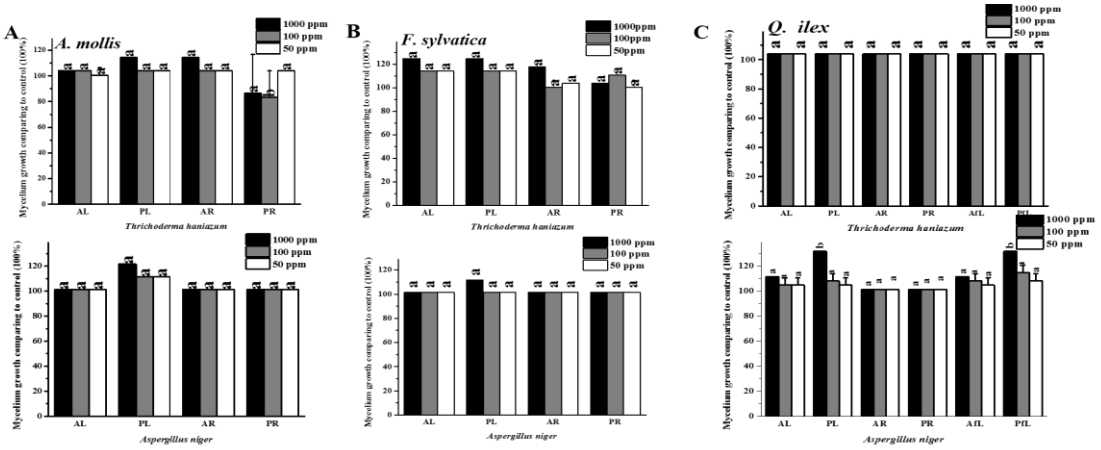

Fig. 4.3 Antifungal activity of polar and apolar mixtures, isolated from *A. mollis, F. sylvatica* and *Q. ilex* at three concentrations (1000, 100 and 50 ppm.). Data represent fungal growth expressed in percentage compared to control (=100). Values are average of three replicates ± standard deviation. For each kind of extract, values with different letters indicate significant differences for $P < 0.05$ according to Duncan test. Fungal species were *T. harzianum* (upper chart) and *A. niger* (lower chart).

Tab. 4.8 List of organic compounds from *A. mollis*, *F. sylvatica* and *Q. ilex* with related antifungal and antibacterial activities

Species and organs	Antimicrobial compounds	Activity	References
A. mollis		**Antifungal**	
Leaf and flowers	Phenols	*Candida glabrata, C. parasilopsis, C. tropicalis, C. lusitaniae, C. albicans, C. albicans spp. C. guillermondii, C. dublinensis*	(Jara et al. 2017)
Seed	Benzoxazolinone (BOA) and the derivatives	*Candida albicans*	(Brav 1997)
		Antibacterial	
Seed	Benzoxazolinone (BOA) and the derivatives	*Staphylococcus aureus*, *Escherichia coli*	(Brav 1997)
F.sylvatica		**Antibacterial**	
Wood knot	Catechin	*Bacillus coagulans, Alcaligenes xylosoxydans*	(Lindberg et al. 2004)
Leaf		*Helicobacter pylori*	(Frédérich et al. 2009)
Leaf	Polyphenolic compounds	*Escherichia coli, Staphylococcus epidermidis*	(Nicu et al. 2016) (Nicu et al. 2018)
Bark	Vanilic acid, catechin, taxifolin and syringin	*Staphylococcus aureus, Methicillin-resistant S. aureus*	(Tănase et al. 2018)
Q.ilex		**Antifungal**	

Whole plant	polyphenols and tannins	*A. flavus, A. fumigatus, Trichophyton rubrum, T. mentagrophytes, Microsporum canis*	(Leven et al. 1979)
Leaves		*Candida albicans*	(Güllüce et al. 2004)
Leaves	Quercetin-3-O-glucoside Procyanidin Catechin	*Altenaria alternata fries, Aspergillus flavus, A. fumigatus, A. niger, A. ochraceus, A. versicolor; Aureobasidium pullulans , Cladosporium cladosporioides, Fusarium trincintum Corda, F. sporotrichioides, Fulvia fulvum, Penicillium funiculosum, P. ochrochloron, and Trichoderma viride C. albicans*	(Karioti et al. 2010)
		Antibacterial	
Whole plant	Polyphenols and tannins	*Staphylococcus aureus. Escherichia coli, Pseudomonas aeruginosa, Proteus vulgaris*	(Leven et al. 1979)
Bark		*Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus* and *S.epidermidis Proteus mirabilis, Klebsiella pneumonia, Bacillus subtillis, Salmonella typhimurium, Vibrio colerae, Streptococcus pyogenes, S. agalactiae*	(Berahou et al. 1979)
Leaves		35 bacterial strains of 7 bacteria genera including *Brucella, Bacillus, Enterobacter, Neisseria, Pseudomonas* and *Escherichia*	(Güllüce et al. 2004)
Leaves	Quercetin-3-O-glucoside Procyanidin Catechin	*Escherichia coli, Pseudomonas aeruginosa, Salmonella typhimurium and Proteus mirabilis. Listeria monocytogenes, Bacillus cereus, Micrococcus flavus and Staphylococcus aureus.*	(Karioti et al. 2010)

4.3 Conclusion

We discussed the results of the bioassay test conducted by measuring the mycelia growth of two fungus *Trichoderma hazianum* and *Aspergillus niger* in the apoar and polar extracts of each sample of 8 plants. The inhibition effect against *T. hazianum* were observed on the apolar leaf extracts of *D. viscosa*, polar root extract of *T. latifolia*, polar leaf and apolar root extracts of *H. helix*, apolar leaf, polar leaf and apolar root extracts of *F. ornus*, polar leaf extracts of *F. drymeja*. As for *A.niger*, a resistant phtytopathogen, it was only susceptible to the apolar extract of *D. viscosa* leaf. It was found that the results of bioassay test consistent with the previous studies in most cases.

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Chapter 5: Metabolite profiling of apolar extracts of plants by GC-MS spectroscopy

5.1 Data analysis method

AMDIS

Data was processed with the Automated Mass Spectrometry Deconvolution and Identification System Software (AMDIS), provided by the National Institute of Standards and Technology (NIST). AMDIS was applied automatically and manually to identify target and untarget compounds from GC/MS data file. The relative amounts of separated metabolites were calculated from Total Ion Chromatography (TIC) by the computerized integrator.

AMDIS has been a powerful tool for the detection of trace compounds. Each component was compared to the library of target compounds and with spectra in database of library NIST 11. When default value was exceeded, the matching factor of the target map and the map of the deconvoluted component was reported.

Kovats Index:

In our present study, Kovats Index, the Gas Chromatogram index of component was used to help identify compounds. The retention index or Kovats Index (RI or KI) concept was proposed by Kovats in 1958 and the retention of the component was calibrated with two adjacent normal alkanes. The retention index of the normal alkanes is specified to be equal to 100 times the number of carbon atoms in the alkane molecule. The RI of the normal alkane is independent of column temperature and other operating conditions. In 1963, Van Den Dool et al. (Van den Dool 1963) introduced a concept of linear temperature-programmed retention index after estimating.

Linear programming

Formula 5.1 $$RI = 100Z + 100\left[TR(x) - TR(z)\right] / \left[TR(z+1) - TR(z)\right]$$

Note: *TR(x), TR(z), TR(z+1)* represent the retention time of the component and the carbon number *Z, Z+1* n-alkane, respectively. And *TR(z)< TR(x)<TR(z+1)*.

On a non-polar column, the linear saturated fatty acid methyl ester has a retention index plus 200 (FAME corrected retention index, N=2), which is very close to the retention index of n-alkane of the same carbon number. Cross-references or even substitutes can be considered in practice. Our standard of a sequence of saturated fatty acid methyl ester were shown below, which helped us to qualify the metabolites more properly when combined with similar MS interpretation result. (Tab.5.1)

Tab. 5.1 Definition Retention Index and Corrected Definition Retention Index of a sequence of Linear Saturated Fatty Acid Methyl Ester standard.

Linear Saturated Fatty Acid Methyl Ester（FAME)					
FEMA	Carbon number of FAME	Retention Time	Definition Retention Index	Corrected Definition Retention Index	Referred Retention Index
C13:0	14	12.6407	1400	1600	1608
C15:0	16	14.8570	1600	1800	1807
C16:0	17	15.8697	1700	1900	1909
C17:0	18	16.8662	1800	2000	2012
C18:0	19	17.7880	1900	2100	2111
C19:0	20	18.7025	2000	2200	2210
C20:0	21	19.5446	2100	2300	2311
C21:0	22	20.3895	2200	2400	2410
C22:0	23	21.1649	2300	2500	2502
C23:0	24	21.9139	2400	2600	2612
C24:0	25	22.6659	2500	2700	2712

5.2Metabolite profiling of apolar extracts

We used gas chromatography coupled to mass spectrometry (GC-MS) with increasing separation capability, allowing determine the quali-quantitative profile of the studied Mediterranean plants. In this way, it was possible to identify single fatty acids on the basis of their

molecular weight. The Fig.5.1 showed the total ions chromatogram of GC-MS data of all the leaves and roots samples of Mediterranean species, which were cleaned up by AMDIS in advance.

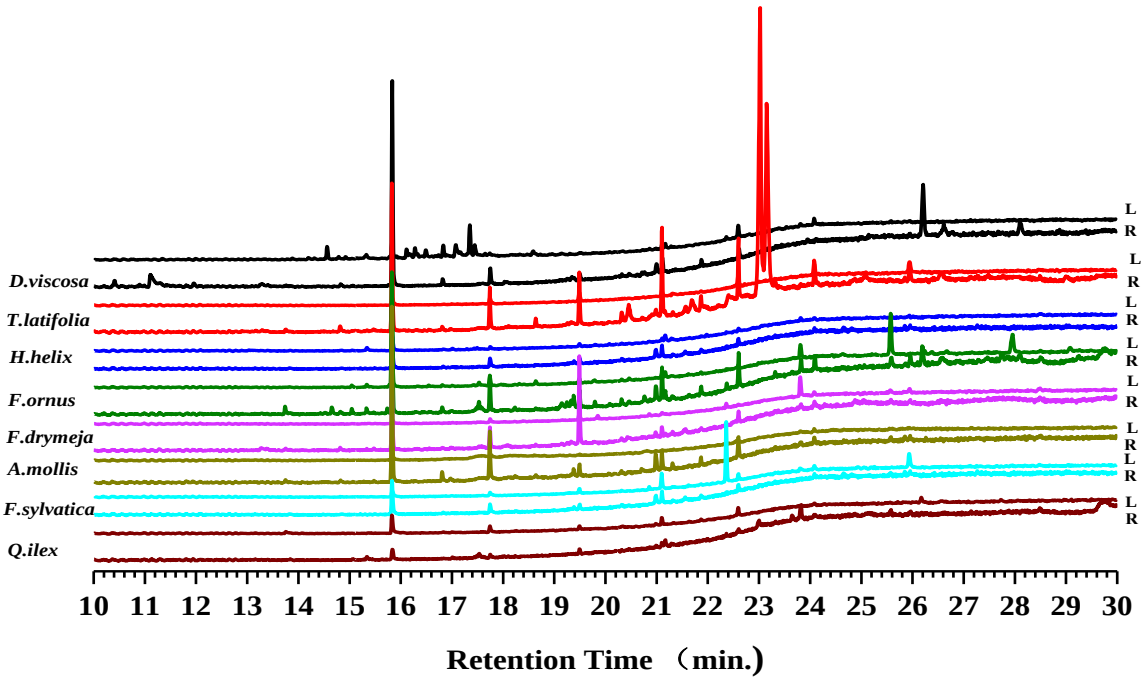

Fig. 5.1. TIC (total ions chromatogram) of GC-MS of leaves (L) and roots (R) of Mediterranean species.

NIST and AMDIS was used in library search with full scan-mode in the GC-MS analysis. Automatic and manual intepreting mothod were applied when identifying metabolites. The matching probability with NIST library of the presence of confirmation by most automatic analysis was higer (60%-99%) than by manual analysis (40%-70%), which based on the purity of the compounds. The full description of the full scan including retention time and typical fragment ions of compounds characterization were present in the Tab. 5.2 at the end of this chapter.

The data of all the species under investigation allowed to characterize 120 metabolites, belonging to several classes of organic compounds-saturated and unsaturated fatty acids, n-alkanes, steroids, triterpenoids, oxygenated terpenoids, apolar phenols and others. The count of metabolites extracted was usually higher in the root extracts. Especially *F. ornus* root contained 47 organic compounds, reaching the maximum value (Fig. 5.2).

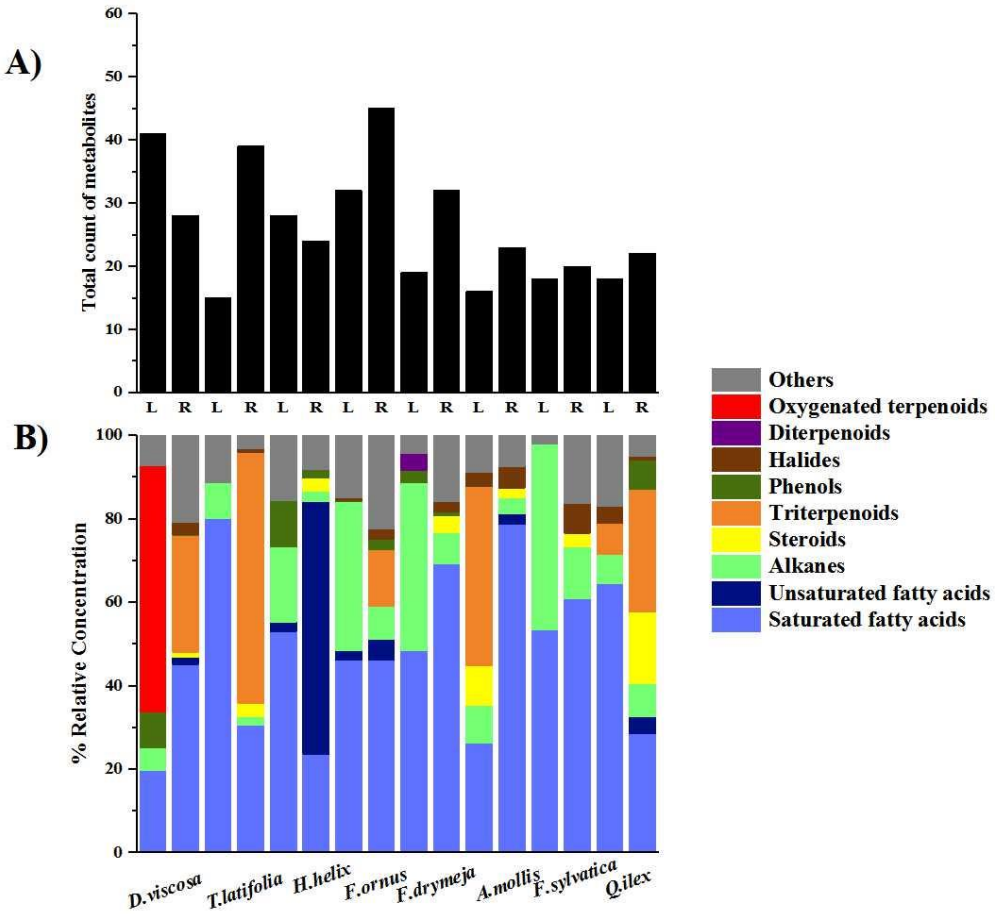

Fig. 5.2 A) Total count of organic compounds in apolar leaf (L) and root (R) extracts; b) Relative concentration (%) for class of organic compounds in apolar leaf and root extracts of each species.

Fatty acids

Among all the metabolites identified, fatty acids were ubiquitous metabolites in all analyzed samples. The main fragment ions of saturated fatty acids were 143 m/z,87m/z,74m/z,69m/z,55m/z and molecular ions. The carbon atom number of saturated fatty acid were odd or even from 14 to 30. However, the contents of fatty acids with even numbers (for e.g. Palmitic acid (16:0), Stearic acid (18:0), Arachidic acid (20:0) Behenic acid (22:0)) were much higher than the ones with odd numbers. It was considered to be related with biosynthetic pathway of fatty acids, the biosynthetic pathway of even fatty acids is much more energy-saving to form Acetyl coenzyme A (Acetyl-CoA) (Herbert 1989). The most abundant saturated fatty acid was palmitic acid (16:0) among nearly all the samples, which also was the most common saturated fatty acid distributed in organisms. The

relative percent of saturated fatty acid ranged from a maximal value 78.6% in *A. mollis* root to minimal value 19.75% in *D. viscosa* leaf. In addition, 9,10-dihydroxystearic acid at 20.76 min, a derivatives from stearic acids was detected. Except linear chain fatty acids, a traces of branched chain fatty acid also were discovered in some samples, like for example 14-methyl-, Hexadecanoic acid and 10-methyl- Hexadecanoic acid (Tab. 5.1).

In contrast to saturated fatty acids, unsaturated fatty acids were determined at little concentration. Nevertheless, what attracted our attention was that the concentration of linoleic acid (C18:2) in *H. helix* root reaching at 60.37%. This seemed could explain the good antifungal activity of the apolar root extract of this plant species.

n-Alkanes

Beside fatty acids, n-alkanes were wide-spread in the extracts. As we know, n-alkanes are important constituents of plant lipids to keep moisture balance of leaf surface (Eglinton and Hamilton 1963). n-alkanes had the characteristic ions of a sequence of 57m/z,71m/z,85m/z,99m/z, while the molecular ion was not always visible. The n-alkanes were detected in all the samples except *D. viscosa* root extract, from tricosane (23C) to tritriacosane (33C). For n-alkanes, one with the odd number of carbon atom were more abundant than ones with even number. *F. sylvatica* leaf extract had the richest heptacosane (C27) in leaf extract at 34.63% (Fig.5.2).

Triterpenoids

Triterpenoids were present at higher concentrations in all studied roots extracts than leaf extracts, with an exception of *A. mollis*. From the figure 5.1, it was shown obviously that there were two large peaks at 23.01 min and 23.15 min. on the TIC of *T. latifolia* roots extracts, identified as Friedelan-3-one and D:A-Friedelan-2-one occupying 29.82% and 22.94 % respectively of the total root apolar extract (Figure 5.3). And 2,6,6,9,2',6',6',9'-Octamethyl-[8,8']

bi[tricyclo[5.4.0.0(2,9)]undecyl], Ursane-3,16-dione, alpha and beta Amyrin were representative triterpenoids always found among the root samples (Tab.5.1).

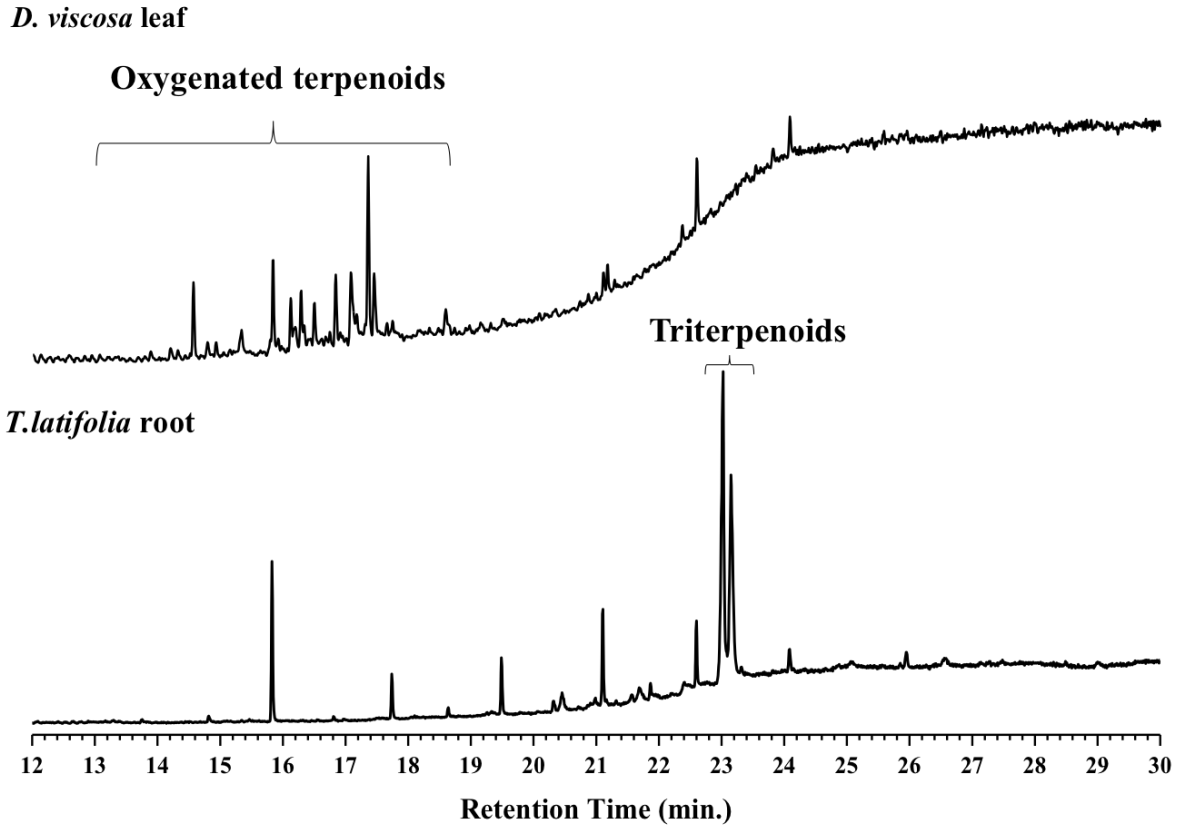

Fig. 5.3. TIC (total ions chromatogram) of GC-MS of *D. viscosa* leaves and *T. latifolia* roots.

Steroids

Steroids have the similar metabolomic rout with triterpenoids (Xu, Fazio, and Matsuda 2004) and the relative percent calculated ran from 1.3%（*D. viscosa* root）to 9.59% (*A. mollis* root) in a near range. 7-Dehydrodiosgenin was the important steroid occurring in three root samples, in *T. latifolia* root (0.7%) *A. mollis* root (2.3%) *F. sylvatica* root (3.2%) at 25.58 min.

Oxygenated terpenoids

Oxygenated terpenoids were unique metabolites in *D. viscosa* leaf, analyzed at retention time from 13.88 min to 18.58 min (Figure 5.3).Based on the Retention Index and Ms spectrum, the most compounds in theses range were identified as oxygenated sesquiterpenoids, such 2-Methyl-2-[2-(2,6,6-trimethyl-3-methylene-cyclohex-1-enyl) -vinyl]-[1,3] dioxolane at 14.56min with the molecular formula $C_{16}H_{24}O_2$. This class of metabolites represented 59.05% of all the metabolites in *D. viscosa* leaf. So it was the possible reason why the apolar extract of *D. viscosa* leaf was so active against assayed fungus.

Phenols

Lastly, it was interesting to find four apolar phenols in trace amounts in the extracts---Phenol, 2-(1,1-dimethylethyl)-4-(1-methyl-1-phenylethyl)- (268 m/z, 253 m/z; 17.013min), phenol2,6-bis(1,1-dimethylethyl)-4 (1-methyl-1-phenylethyl) (309 m/z 324 m/z, 17.438 min), phenol,2,4-bis[1-methyl-1-phenylethyl] (330 m/z,315 m/z; 21.17 min.)2,4-Bis(dimethylbenzyl)-6-t-butylphenol (386 m/z, 371 m/z;21.289 min.)

5.3 Conclusion

Through analyzing the apolar phase of leaf and root samples of each species by GC-MS, we found that fatty acids, n-alkanes, terpenoids and steroids were ubiquitous among the samples.The most abundant metabolites among these species were always saturated fatty acids with the relative content from 19.75% (*D. viscosa* leaf) to 79.6% (*A. mollis* root), with four exceptions. In *D. viscosa* leaf, oxygenated terpenoids were most abundant than fatty acids. In *H. helix* root, the major compounds were unsaturated fatty acids, particularly linoleic acid. These can be counted for the reason why the two apolar extracts have antifungal activity in the bioassy and the concentration of triterpenoids, exceeding the one of fatty acids, was the richest in *A. mollis* leaf and *Q. ilex* root extracts. For apolar extracts in leaf and root samples of *F. ornus* and root samples of *F. drymeja*, the metabolite amount were identified as many as 32, 47 and 36, suggesting a

relative wild range of organic molecules. All chemical properties of the apolar metabolites presented could contribute to the phenomenon of antifungal activity at some degree.

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Tab. 5.2. Relative concentration (%) of main metabolites of *Acanthus mollis*, *Dittrichia viscosa*, *Festuca drymeja*, *Fraxinus ornus*, *Fagus sylvatica*, *Hedera helix*, *Quercus ilex*, *Typha latifolia* apolar leaf and root extracts. Quantification was determined by integrating peak areas in GC-MS analysis.

		A.mollis		*D. viscosa*		*F. sylavtica*		*F.ornus*		*H. helix*		*Q. ilex*		*T.latifolia*		*F.drymeja*	
Compounds	Rt (min)	leaf	root	leaf	root	leaf	root	leaf	root	leaf	root	leaf	root	leaf	root	leaf	root
4-ter-butylcatechol,dimethyl ether 180,165,135,105,91,77	10.41	n.d.	n.d.	n.d.	2.2± 0.2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Ethanone,1(3-bromophenyl)- 198,183,117	10.90	n.d.	n.d.	n.d.	0.7± 0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
2',4'-dimethoxy acetophenone 180,165,135,105,91,77	11.11	n.d.	n.d.	n.d.	6.±0. 4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
4',6'-Dimethoxy-2',3'-dimethylacetophenone, 208,193,91	11.95	n.d.	n.d.	n.d.	1.3± 0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
S-Indacene,1,2,3,5,6,7-hexahydro -1,1,7,7-tetramethyl-, 214,199,201,103	13.24	n.d.	n.d.	n.d.	1.2± 0.4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Isobutyl methyl phthalate 163,149,181	13.29	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.2± 0	n.d.	n.d.
Tetradecanoic acid (C14:0), 242,143,87,74,55	13.74	n.d.	1.0± 0.1	n.d.	0.5± 0.1	n.d.	n.d.	n.d.	1.2± 0.1	n.d.	n.d.	3.1± 0.1	n.d.	n.d.	0.4± 0.1	n.d.	0.8± 0.1
4,4,5,8-Tetramethyl-chroman-2-One 204,189,91	13.88	n.d.	n.d.	0.8± 0.1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Oxygenated terpenoid 246, 246,220,204,189,91	14.19	n.d.	n.d.	1.1± 0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

Oxygenated terpenoid 248, 248,109,91	14.31	n.d.	n.d.	0.9± 0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
9-methyltetradecanoic acid 213,157,143,74,55	14.43	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.3± 0.1	n.d.	n.d.	n.d.	n.d.	n.d.	0.1± 0	n.d.	0.4± 0.1
Tetradecanoic acid, 12-methyl- 87,74,69,55	14.52	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.4± 0.1	n.d.	n.d.	n.d.	n.d.	n.d.	0.1± 0	n.d.	0.3± 0.1
2-Methyl-2-[2-(2,6,6-trimethyl-3-methylene-cyclohex-1-enyl)-vinyl]-[1,3]dioxolane 248,233,201,173,91,79,67	14.56	n.d.	n.d.	4.9± 0.1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
8aH-2,4a-methanonapen-8a-ol octahydro-1,1,5,5'tertramethyl- 222,180,74,67,55	14.66	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.2± 0.1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Ethanone,1-(7-hydroxy-5-methoxy -2,2-dimethyl-2H-1-benzopyran-8-yl)- 248, 233,173,91	14.79	n.d.	n.d.	1.3± 0.1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Pentadecanoic acid (C15:0) 256,143,87,74,55	14.82	n.d.	1.0± 0.1	n.d.	0.8± 0	n.d.	n.d.	n.d.	0.5± 0	n.d.	0.8± 0.1	n.d.	n.d.	n.d.	0.5± 0	n.d.	1.2± 0.1
4,7-Methanofuro[3,2-c]oxacycloundecin-6(4H)-one, 7,8,9,12 -tetra hydro - 3,11-dimethyl- 246, 178, 91	14.91	n.d.	n.d.	1.1± 0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
2-Pentadecanone, 6,10,14- 3- trimethyl - 250,123,109,95,85,71,58	15.04	n.d.	n.d.	n.d.	0.3± 0.1	n.d.	n.d.	0.9± 0.01	0.8± 01	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
3-Quinoxalinepropanoic acid, 3-methoxy- 246,187,159,115	15.21	n.d.	n.d.	0.4± 0.1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
3-Quinoxalinepropanoic acid deriv 246,187,159	15.3	n.d.	n.d.	1±0. 1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

1,2-Benzenedicarboxylic acid bis(2-methylpropyl) ester 223,149,57	15.36	n.d.	0.1± 0	1.8± 0.1	0.2± 0.1	n.d.	n.d.	1.0± 0.1	0.9± 0.1	4±0. 6	0.4± 0.02	0.8± 0.1	2.1± 0.1	n.d.	0.2± 0	0.6± 0.1	0.5± 0.1
Pentadecanoic acid, 14-methyl- 143,87,74	15.47	n.d.	0.5± 0.2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.2± 0	n.d.	0.8± 0.2
Pentadecanoic acid, 13-methyl- 270,87,74,55	15.47	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.3± 0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Palmitoleic acid(C 16:1) 236,152,141,110,97,74,55	15.74	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.8± 0	n.d.	n.d.	1.9± 0.1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
10-Methyl hexadecenoic acid 236,74,69,55	15.74	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.8± 0.1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
3,4,6,7-Tetrahydrobenzo[1,2-b:5,4-b']dipyran-2, 8-dione 218,175	15.79	n.d.	n.d.	1.0± 0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Palmitic acid(C 16:0) 270,143,87,74,55	15.86	4.8± 1.2	37±0 .7	5.9± 0.1	28.5 ±1.7	5.1± 1.1	21± 1.2	26.9 ±3	15.4 ±0.5	20.4 ±0.4	9.9± 0.7	18.1 ±2.4	6.8± 0.6	1±0. 2	7.7± 0.2	3.7± 0.4	17.5 ±1.6
2-Naphthaleneacetic acid, 6-methoxy-, alpha,-methyl-, 244,185,169,157,141	16.12	n.d.	n.d.	3.2± 0.2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
5,8-Dimethyl-1,2,3,4-tetrahydro-1-naphthol, 246,158,143,128,91	16.19	n.d.	n.d.	2.6± 0.1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
1-Methoxy-3,7-dimethyl-5-oxo-5,6,7,8-tetrahyd ronaphthalene-2-carboxylic acid 230,202,187,159, 91,77	16.28	n.d.	n.d.	4.0± 0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
1,2-Benzenedicarboxylic acid, butyl 2-methylpropyl ester 279,167,149	16.31	n.d.	n.d.	n.d.	0.2± 0.1	n.d.	n.d.	n.d.	0.3± 0.1	n.d.	n.d.	n.d.	n.d.	n.d.	0.1± 0	n.d.	0.6± 0.1

5,8-Dimethyl-1,2,3,4-tetrahydro-1-naphthol deriv 158,143,128,91	16.33	n.d.	n.d.	1.6± 0.2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
5,19-Cyclo-5beta-androst-6-ene-3,17-dione, 282,223,117	16.40	n.d.	n.d.	0.7± 0.3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Hexadecanoic acid,14-methyl 284, 87,77,55	16.46	n.d.	0.4± 0.1	n.d.	n.d.	n.d.	n.d.	0.7± 0.1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.1± 0	n.d.	0.5± 0.1
1,2,3,4,5,6,7,8-Octahydrophenanthrene-9-carboxylic acid 244,212,183,153,132,117,91	16.49	n.d.	n.d.	3.1± 0.1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Hexadecanoic acid,10-methyl 284,241,185,143,87,74	16.55	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.6± 0.1
5,8,11-Heptadecatriynoic acid 271,155,141,129,91,55	16.57	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	2.1± 0.2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Benzo[e](1H)indene, 1,2,3a,4,5, 9b-hexahydro-7-methoxy-3-oxo-3a,9b-dimethyl- 244,229,115	16.61	n.d.	n.d.	0.8± 0.1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Oxygenated terpenoid 232 (1) 232,105,91	16.67	n.d.	n.d.	0.6± 0.1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Oxygenated terpenoid 232 (2) 233,217,152,105,93	16.74	n.d.	n.d.	0.9± 0.1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Heptadecanoic acid (C 17:0) 284,143,87,74,69	16.81	n.d.	2.4± 0.1	n.d.	1.3± 0.1	n.d.	1.5± 0.5	0.8± 0.1	0.8± 0	1.6± 0.5	0.7± 0	1.3± 0.6	n.d.	n.d.	0.3± 0.1	n.d.	0.8± 01

Ambros-2-en-12-oic acid, 6 beta,8alpha -dihydroxy-4-oxo-, 12,8-lactone, acetate, (11R)-, 264,246,232,177,91,79,67,55	16.83	n.d.	n.d.	4.3± 0.1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Methyl octan-2-yl phthalate 181,163,149	17.01	n.d.	1.1± 0.2	n.d.	3.1± 0.3	0.7± 0.3	0.9 ±0. 2	n.d.	0.5± 0.1	n.d.	n.d.	1±0. 1	n.d.	1.3± 0.3	0.2± 0.03	n.d.	0.8± 0.1
Phenol, 2-(1,1-dimethylethyl)-4 -(1-methyl-1-phenylethyl)- 268,253,57	17.01	n.d.	n.d.	0.7± 0.1	n.d.	n.d.	n.d.	0.8± 0.2	0.4± 0.2	2.5± 0.5	0.4± 0	n.d.	1.1± 0.1	n.d.	n.d.	n.d.	n.d.
Benzo[e]isobenzofuran-1,4-dione,1,3,4,5,5a,6,7, 8,9,9a-decahydro-6,6,9a-trimethyl, 220,159,119,105	17.06	n.d.	n.d.	8.0± 0.1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
5-Isopropenyl-4,8a-dimethyl-1,2,3,5,6,7,8,8a-oc tahydro-naphthalen-2-ol 159,121,91,79,53	17.17	n.d.	n.d.	2.1± 0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Thiophene-2-carbonitrile,5-tert-butyl-3-(4-chlor obenzylidenamino)- 302,287	17.30	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.2± 0.2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
1-Methoxy-3,7-dimethyl-5-oxo-5,6,7,8-tetrahyd ronaphthalene-2-carboxylic acid 262,230,187,145,91,77,67,55	17.35	n.d.	n.d.	11.8 ±0.4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Heptadecanoic acid,16-methyl- 298,143,87,74	17.40	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.4± 0.05	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Phenol,2,6-bis(1,1-dimethylethyl)-4-(1-methyl-1 -phenylethy)-l 324,309,119,57	17.43	n.d.	n.d.	4.5± 0.2	0.12 ±0.1	n.d.	n.d.	0.5± 0	0.5± 0.2	1.5± 0.1	0.2± 0.1	n.d.	0.7± 0.2	n.d.	n.d.	0.6± 0.1	n.d.

Linoleic acid（C18:2） 280,110,95,81,67,55	17.49	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.0± 0.2	2.2± 0.2	44.5 ±3.9	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
11,14-Octadecadienoic acid 294,81,67,55	17.49	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.4± 0.3	n.d.	n.d.	n.d.	n.d.
Triterpenoid 426 (1) 426,218,203,189	17.52	17.8 ±2.2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Steroid 410 (1) 410,395,57	17.53	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.2± 0.3
6-Octadecenoic acid,(E)- 296,264,97,83,69,55	17.53	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	2.7± 0.7	n.d.	n.d.	n.d.	n.d.
Oleic acid (C18:1) 264,97,83,69,55	17.55	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.2± 0.2	1.6± 0.2	n.d.	8±3. 9	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
C18:1 296,264,83,69,55	17.58	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.0± 0.3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Stearic acid (C 18:0) 298,143,87,74,69	17.77	7.6± 2.1	10.4 ±0.5	1.3± 0.2	3.1± 0.3	3.5± 0.4	6.5± 0.4	2.9± 0.2	4.4± 0.2	3.5± 0.1	1.9± 0.1	7.5± 0.9	2.6± 0.2	7.6± 0.5	2.3± 0.02	3.8± 0.2	5.3± 0.4
Steroid 410 (2) 410,174,57	18.09	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.5± 0.5
Triterpenoids 426 (2) 426,218	18.16	20.2 ±8.5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
5,8,11-Eicosatriynoic acid, 173,155,141,128,115,91,77,55	18.25	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	3.9± 0.1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Unsaturated fatty acid 294 294, 96, 55	18.31	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.0± 0.2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Oxygenated terpenoid 210 210,175	18.51	n.d.	n.d.	0.6± 0.03	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

Oxygenated terpenoid 233 233,210,201,173,121,59	18.59	n.d.	n.d.	2.4± 0.2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Triterpenoid 426 (3) 426,218	18.63	5.1± 1.7	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Nonadecanoic acid (C19:0) 312,143,87,74,69	18.64	n.d.	1.3± 0.3	n.d.	0.4± 0.1	n.d.	n.d.	n.d.	0.5± 0.1	n.d.	n.d.	n.d.	n.d.	n.d.	0.6± 0.01	n.d.	0.8± 0.1
Tricosane (23C) 113,99,85,71,57	19.25	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.8± 0.2	n.d.	n.d.	n.d.	n.d.	0.2± 0	n.d.	n.d.
Eicosenoic,13-methyl- 199,74,69,55	19.34	n.d.	n.d.	n.d.	1.0± 0.1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Eicosenoic acid,11-methyl- 234,199,74,69,55	19.38	n.d.	n.d.	n.d.	0.7± 0.1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
8,11,14-Eicosatrienoic acid 320,150,87,74,55	19.38	n.d.	2.4± 0.2	n.d.	n.d.	n.d.	n.d.	n.d.	1.4± 0.2	n.d.	n.d.	n.d.	n.d.	n.d.	0.1± 0	n.d.	n.d.
Arachidic acid (C 20:0) 326,143,87,74,69	19.52	1.8± 0.4	3.1± 0.2	0.7± 0.3	1.4± 0.03	3.7± 0.5	4.0 ±0.2	1.9± 0.1	2.1± 0.1	3.7± 0.2	1.9± 0.3	4.6± 0.4	3.6± 0.2	9.9± 0.2	2.9± 0.1	7.0± 0.7	20.1 ±1.7
Thiophene,3-nitro-2-(2-thienylsulfonyl)- 127,99,71,55	19.80	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.6± 0.1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Dehydroabietic acid 314,299,239,165,141,117	19.85	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	4.1± 0.2	n.d.
Tetracosane(24C) 113,99,85,71,57	20.08	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.6± 0	1.6± 0.2	n.d.	n.d.	n.d.	n.d.	0.2± 0	n.d.	n.d.
Heneicosylic acid (C21:0) 340,143,87,74,69	20.32	n.d.	1.5± 0.2	n.d.	1.1± 0.3	1.2± 0.3	2.5± 0.2	0.8± 0.2	1±0. 1	1.4± 0.1	0.8± 0.2	1.6± 0.3	n.d.	n.d.	1±0. 1	n.d.	1.3± 0

Stigmast-4-en-3-one 412,397,370,229,124,91,55	20.43	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	2.0± 0.2	n.d.	2.0± 0.3
Dodecanoic acid, tetradecyl ester 396, 201,97,83,57	20.46	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.0± 0.2	0.9± 0.1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Octadecanoic acid,9,10-dihydroxy- 187,155,138,87,69,55	20.76	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.2± 0.1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
a-Homocholest-4a-en-3-one 398,136,123,107	20.81	9.5± 0.4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Pentacosane (25C) 113,99,85,71,57	20.86	n.d.	n.d.	0.9± 0.5	n.d.	1.9± 0.3	0.8± 0.4	0.5± 0.3	0.6± 0.1	2.7± 0.1	0.6± 0.2	n.d.	1.1± 0.4	1.31 ±0.3	0.2± 0.1	3.6± 0.3	0.8± 0.1
9,10-dichloro-,Octadecanoic acid 294,263,87,74,69,55	20.98	3.3± 0.9	5.1± 0.3	n.d.	2.2± 0.3	n.d.	6.9± 0.2	n.d.	2.6± 0.2	n.d.	n.d.	4.0± 0.8	0.9± 0.2	n.d.	0.7± 0	n.d.	2.2± 0.4
Behenic acid (C22:0) 354,143,87,74,69	21.10	2.9± 0.9	4.8± 0.4	1.9± 0.1	2.4± 0.2	9.9± 0.9	7.0± 0.2	1.4± 0.1	3.8± 0.2	3.1± 0.3	2.1± 0.1	7.2± 0.3	3.1± 0.4	6.6± 0.3	4.8± 0.1	3.2± 0.3	3.0± 0.2
Phenol,2,4-bis{1-methyl-1-phenylethyl}- 330,315,150,103,91	21.17	n.d.	n.d.	2.3± 0.3	n.d.	n.d.	n.d.	1.7± 0.1	1.2± 0	7.2± 0.1	1.4± 0.1	n.d.	3.8± 0.2	n.d.	n.d.	2.3± 0.6	0.9± 0.1
2,4-Bis(dimethylbenzyl)-6-t-butylphenol 386,371,119,91	21.29	n.d.	n.d.	0.9± 0.2	n.d.	n.d.	n.d.	n.d.	0.5± 0.3	2.8± 0.2	0.5± 0.2	n.d.	1.5± 0.2	n.d.	n.d.	n.d.	n.d.
Phthalic acid, di(oct-3-yl) ester 279,167,149	21.32	n.d.	1.9± 0.3	n.d.	0.3± 0.1	1.3± 0.3	1.3± 0.2	n.d.	0.6± 0.3	2.1± 0.2	n.d.	1.3± 0.3	n.d.	2.9± 0.9	0.4± 0	1±0	1.7± 0.4
1(10),9(11)-B-Homolanistadiene 410,395,119,107,95	21.57	n.d.	n.d.	n.d.	n.d.	n.d.	3.1± 0.6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.8± 0	n.d.	n.d.
Hexacosane (26C) 113,99,85,71,57	21.63	n.d.	n.d.	n.d.	n.d.	1.5± 0.2	n.d.	0.8± 0.2	0.6± 0.2	1.9± 0.7	n.d.	2.2± 0.9	n.d.	n.d.	n.d.	2.2± 0.4	n.d.
Friedelan derivates 426 426,163,95,81,55	21.69	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.8± 0.3	n.d.	n.d.

Tricosylic acid (C23:0) 368,143,87,74,69	21.88	n.d.	2.8± 0.5	0.8± 0.4	1.5± 0.1	1.6± 0.4	2.9± 0.2	1.0± 0.1	1.5± 0.2	2.1± 0.7	1.3± 0.1	3.1± 0.1	n.d.	3.5± 0.7	1.1± 0.1	n.d.	1.5± 0.2
2,2'-Isopropylidenebis(6-methoxy-3-methylbenzofuran) 364,351,349	21.98	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	3.7± 0.4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Heptacosane (27C) 113,99,85,71,57	22.35	2.3± 0.7	0.5± 0.2	1.8± 0.2	n.d.	34.7 ±2.8	2.3± 0.4	0.9± 0.2	0.9± 0.1	1.9± 0.3	0.9± 0.3	n.d.	1.8± 0.4	2±0. 8	n.d.	5.1± 0.3	1.2± 0.4
Ursa-9(11),12-dien-3-yl acetate 466,407,255	22.40	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.7± 0.2	n.d.	n.d.
Lignoceric acid (C24:0) 382,143,87,74,69	22.64	2.8± 0.5	5.2± 0.2	4.9± 0.3	1.8± 0.2	4.9± 0.3	4.2± 0.5	1.4± 0.6	4.1± 0.2	3.7± 0.4	2.6± 0.4	7.9± 0.4	3.4± 0.5	11.2 ±0.2	3.6± 0.4	2.7± 0.6	2.7± 0.5
Friedelan-3-one 426,125,109,95,69,55	23.01	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	3.1± 0.2	n.d.	4.0± 0.7	n.d.	29.8 ±0.7	n.d.	n.d.
Octacosane(28C) 113,99,85,71,57	23.06	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.9± 0.3	0.13 ±0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
D:A-Friedooleanan-2-one 426,302,163,123,109,95,81,69,55	23.15	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	22.9 ±0.6	n.d.	n.d.
Pentacosylic acid (C25:0) 396,143,87,74,69	23.32	n.d.	1.±0. 3	0.6± 0.1	0.8± 0	1.6± 0.3	1.8± 0.2	0.9± 0.3	1.1± 0	1.6± 0.3	0.8± 0.3	3±0. 6	n.d.	1.5± 0.3	0.9± 0.2	2±0. 9	1.1± 0.2
Cholestane-3,5,6-triol,(3.beta.,5.alpha.,6.beta)- 419,402,137,95,69,55	23.65	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	4.9± 1	n.d.	n.d.	n.d.	n.d.
Nonacosane (29C) 113,99,85,71,57	23.81	3.3± 1.7	1.8± 0.5	1.8± 0.2	n.d.	4.3± 1.1	1.4± 0.6	4.8± 0.4	1.7± 0.1	6.0± 0.5	1.1± 0.5	2.9± 0.8	4.1± 1.2	0.9± 0.4	0.4± 0.1	18.2 ±0.9	1.9± 0.2
Cholestane-3,6,7-triol, (3.beta.,5.alpha.,6.beta.,7.beta.)- 419,402,137,85,71,57	23.82	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	8.1± 1.2	n.d.	n.d.	n.d.	n.d.

Cholesta-8,14-dien-3-ol, (3beta)- 384,369	23.95	n.d.	n.d.	n.d.	1.3± 0.3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Cerotic acid(C26:0) 410,143,87,74,55	24.14	3.4± 0. 7	2.7± 0.5	3.1± 0.2	1.1± 0.2	5.1± 2	2.7± 0.6	1.4± 0.6	2.2± 0.1	3.9± 0.7	0.8± 0.3	3.7± 0.7	2.6± 0.9	13.7 ±0.6	0.7± 0.1	5.4± 0.8	3.3± 0.5
Triacontane(30C) 113,99,85,71,57	24.62	n.d.	n.d.	n.d.	n.d.	n.d.	3.2± 1.1	1.3± 0.1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Heptacosanoic acid (C27:0) 424,143,87,74,55	24.95	n.d.	n.d.	n.d.	n.d.	1.6± 0.2	n.d.	n.d.	1±0. 1	1±0. 1	n.d.	n.d.	n.d.	1±0. 04	0.4± 0.1	3.8± 0.9	1.8± 0.3
Triterpenoid 426 426,384,369	25.06	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.8± 0.1	n.d.	n.d.
Hentriacontane (31C) 113,99,85,71,57	25.57	2.7± 0.5	1.7± 0.4	0.8± 0.2	n.d.	0.8± 0.1	3±0. 4	15.4 ±2.2	2.2± 0.2	3.1± 0.6	n.d.	2.1± 0.4	1.9± 0.3	0.8± 0.3	0.8± 0.3	7.3± 1.5	2.2± 0.3
7-Dehydro diosgenin 394,143,69,55	25.85	n.d.	2.3± 0.3	n.d.	n.d.	n.d.	3.2± 0.3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.7± 0.1	n.d.	n.d.
Montanic acid (C28:0) 438,143,87,74,55	26.04	2.1± 0.5	1.9± 0.4	0.7± 0.1	n.d.	12.8 ±0.6	3.3± 0.3	6.6± 0.1	1.8± 0	2.6± 0.3	n.d.	1.2± 0.1	2.9± 0.7	16.8 ±1	1.6± 0.1	9.5± 0.3	2.1± 0.7
2,6,6,9,2',6',6',9'-Octamethyl-[8,8'] bi[tricyclo[5.4.0.0(2,9)]undecyl] 410,395,205,189,95,81,69,55	26.21	n.d.	n.d.	n.d.	14.2 ±0.2	n.d.	n.d.	n.d.	3.9± 0.03	n.d.	n.d.	7.3± 0.7	n.d.	n.d.	n.d.	n.d.	n.d.
Ursane-3,16-dione 440,299,190	26.54	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.7± 0.1	n.d.	n.d.
Ursane-3,16-dione deriv 440,299,190	26.58	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.9± 0.9	n.d.	n.d.
C(14a)-Homo-27-nor-14beta-gammaceran-3alpha-ol 410,395,274,95,81,55	26.61	n.d.	n.d.	n.d.	5.1± 0.4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

Dotriacontane (32C) 113,99,85,71,57	26.66	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.0± 0.3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Nonacosanoic acid (C29:0) 452,143,87,74,55	27.11	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.6± 0.1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.0± 0.3
Tritriacontane (33C) 113,99,85,71,57	27.96	0.8± 0.7	n.d.	n.d.	n.d.	1.5± 0.3	1.4± 0.4	9.3± 1	1.2± 0.1	n.d.	n.d.	n.d.	n.d.	n.d.	0.2± 0.1	3.8± 0.5	1.4± 0.7
2,2,4a,6a,8a,9,12b,14a-Octamethyl- 410, 395,218,95,81,55	28.10	n.d.	n.d.	n.d.	5.8± 0.9	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Melissic acid (C30:0) 466,143,87,74,55	28.49	0.8± 0.2	1.6± 0.3	n.d.	n.d.	2.3± 1.3	1.3± 0.3	0.9± 0.4	1.7± 0.1	4.2± 0.5	n.d.	2.2± 0.3	3.4± 0.4	2.2± 0.8	0.5± 0.2	7.3± 0.9	2.0± 0.4
Triterpenoid 428 428,396,359	28.99	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.9± 0.3	n.d.	n.d.
beta-Amyrin 426,218,109.95,69,55	29.25	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	2.3± 0.7	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
alpha amyrin 426,218,109.95,69,55	29.78	n.d.	n.d.	n.d.	2.8± 0.2	n.d.	n.d.	n.d.	7.2± 0.7	n.d.	n.d.	n.d.	29.4 ±5.3	n.d.	n.d.	n.d.	n.d.

Note: Data refers to mean ± standard deviation of triplicate spectra. n.d.: not dete

Chapter 6: Metabolite profiling of polar extracts of plants by NMR spectroscopy

6.1 Multivariate data analysis method

Resulting dataset from ^{1}H-NMR was examined through Multivariate approach in order to obtain information of its underlying structure and the effect of multiple variables on the chemical differentiations between the plant species and plant tissues object of the study. Previous to apply multivariate approach, each dataset was normalized to minimize small differences and subsequently mean-centered.

For ^{1}H-NMR, the description of statistical analyses refers to range scaled data, in order to preserve experimental biological information. Total dataset was plotted according to PCA, in order to explain main chemical species producing differentiations among plant and root extracts. Given the high number of resonance regions and the unbalanced presence of chemical classes that are constitutively more produced with respect to other, we perform three additional PCA on different resonance regions. Resonance regions were clustered according to common chemical classes as described following: i) Aromatic/ phenolic compounds regions (from δ 10.5 to 5.5); ii) Carbohydrates regions (from δ 5.5 to 3.0) iii) Aliphatic regions (from δ 3.0 to 0.5).

Data ordination and normalization was performed by means of Excel software, while Multivariate analysis and plotting was performed in Statistica 10 software (StatSoft, Inc., Tulsa, OK).

6.2 Results and discussion

6.2.1. Metabolite profiling of polar extracts

An integrate spectroscopic approach combined with multivariate data analysis was applied on

eight Mediterranean plants. The metabolic profile of leaves and roots was obtained to comprehensively evaluate the metabolome of each species and how its chemical composition was distributed in two compartments of each plant species. On the basis of our previous experience(de Falco et al. 2016) (de Falco et al. 2017), the polar extracts were analyzed by NMR analysis, while the apolar extracts were investigated through GC-MS, because of the strong overlapping of the methylene signals in the ^{1}H-NMR spectra.

Each polar extract showed a very intricate profile, with free aliphatic and aromatic amino acids, carbohydrates, organic acids and aromatic compounds; the qualitative and quantitative metabolite profile was peculiar of each analyzed species (Fig. 6.1).

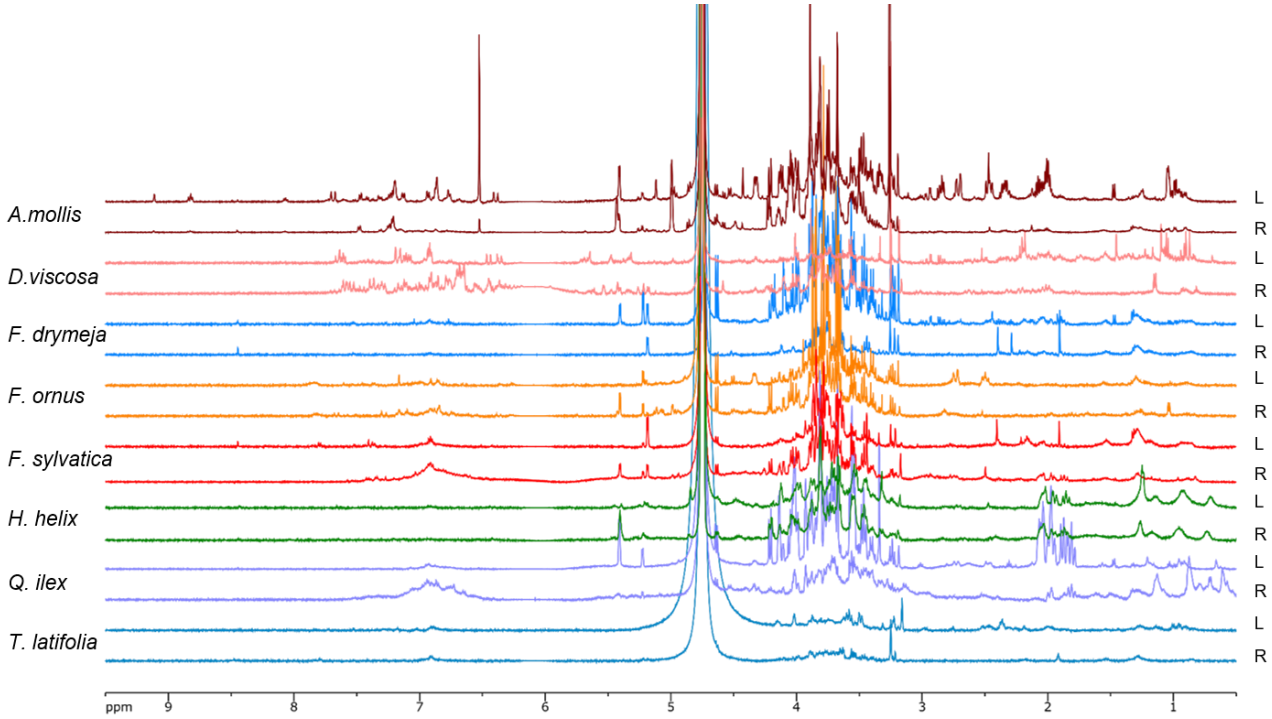

Fig. 6.1. ^{1}H-NMR at 500 MHz in D_2O of leaves (L) and roots (R) of Mediterranean species.

For more convenient data interpretation, the 1H-NMR spectra were divided in three regions: the aliphatic region between 0.5-3.10 ppm, the sugar region between 3.10-5.50 ppm and the aromatic region ranging from 5.50 to 8.5 ppm (Fig. 6.2).

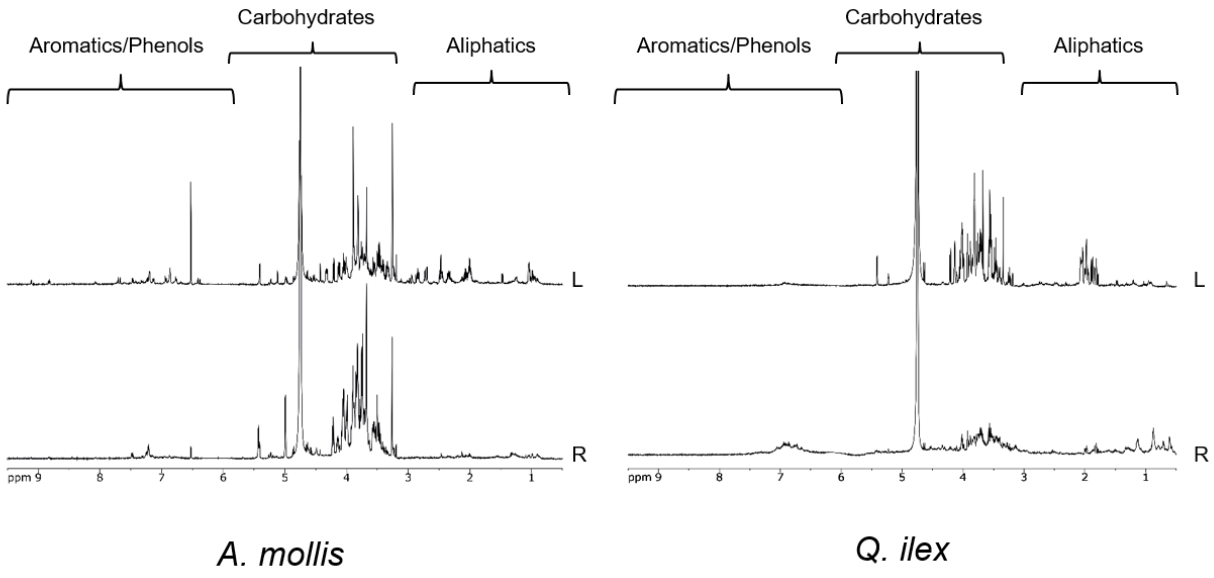

Fig. 6.2. 1H-NMR spectra at 500 MHz in D_2O of A. mollis and Q. ilex leaves (L) and roots (R).

The aliphatic region contained signal related to amino acids and organic acids. Diagnostic methyl doublets typical of isoleucine (Ile) and valine (Val), resonated at 0.91 ppm and 1.01 ppm, respectively, and the methyl triplet of leucine (Leu) at 0.95 ppm allowed their qualitative and a quantitative assignment. Moreover, doublets at δ 1.46 (J 7.0 Hz) and δ 1.32 were associated to alanine (Ala) and threonine (Thr), respectively (Tab. 6.1). The typical region of methylene groups closes to a carbonyl group in 1H-NMR spectra showed a triplet at 2.98 ppm attributed to the γ-methylene protons of γ-amino butyric acid (GABA), as well as two double doublets at 2.84 and 2.94 ppm, corresponding to the diastereotopic hydrogens of asparagine (Asn). A mention is due to proline (Pro), whose recognized has been obtained by three multiplets at δ 1.99, 2.06 and 2.34, and to glutamic acid (Glu) with the typical multiplet signals at δ 2.05, 2.10 and 2.36. Pro and Glu were not always present in the studied species, but when they occurred in the plant were present at reasonable amounts, although it was not easy to distinguish between them (Tab.6.1). All monosaccharides and alditols were quantified by integrating the signals indicated in Tab.6.1. Finally, the aromatic region was selected from 5.51 to 8.50, excluding three multiplet signals at 7.32, 7.36 and 7.40 ppm corresponding to phenylalanine (Phe), and two doublets at 6.80 and 7.12 ppm, corresponding to tyrosine (Tyr). Some aromatic signals were determined, as chlorogenic

acid (CA) (Tab. 6.1)

The results showed that all analyzed samples have carbohydrates as major metabolites. In detail, the analysis of the leaves indicated *F. ornus* and in *Q. ilex* to contain a rather high content of monosaccharides, due to the presence of additional alditols. In the ^{1}H-NMR spectra of *F. ornus* leaves, the signals of mannitol were easily recognized by the presence of two coupled double doublets at δ 3.66 and 3.85, a double triplet at δ 3.75 and a doublet at δ 3.79. Mannitol was the major component of manna, which is produced from *Fraxinus* sp. especially under stress conditions.(Stoop, Williamson, and Pharr 1996) In our study mannitol alone represented 45.1% in weight of the total metabolome of *F. ornus*. The holm oak (*Q. ilex)* contained two metabolites quercitol and quinic acid (QA) deriving from the shikimic acid pathway (Wilson et al. 1998), whose signals resonated mostly in the sugar region. This is probably the reason for the high sugar content found for this species. It has been reported that QA and quercitol are the most abundant metabolites in Q. ilex and in other species of Quercus (Sardans et al. 2014) (Passarinho et al. 2006); their production is a reaction to biotic and osmotic stress. Quantitative determination of QA and quercitol was not easy due to their nearness in the ^{1}H-NMR spectra; to avoid any kind of overlapping, we choose to integrate the signal at δ 1.81 for quercitol and the signal at δ 1.87 for QA. In this way, we were able to quantitate quercitol and QA which represented 18.9% and 13.9% of the all holm oak leaves polar extract, respectively. On the contrary, *D. viscosa* had the lowest amount of carbohydrates (10.4%) (Fig. 6.3, Tab. 6.1 and 6.2).

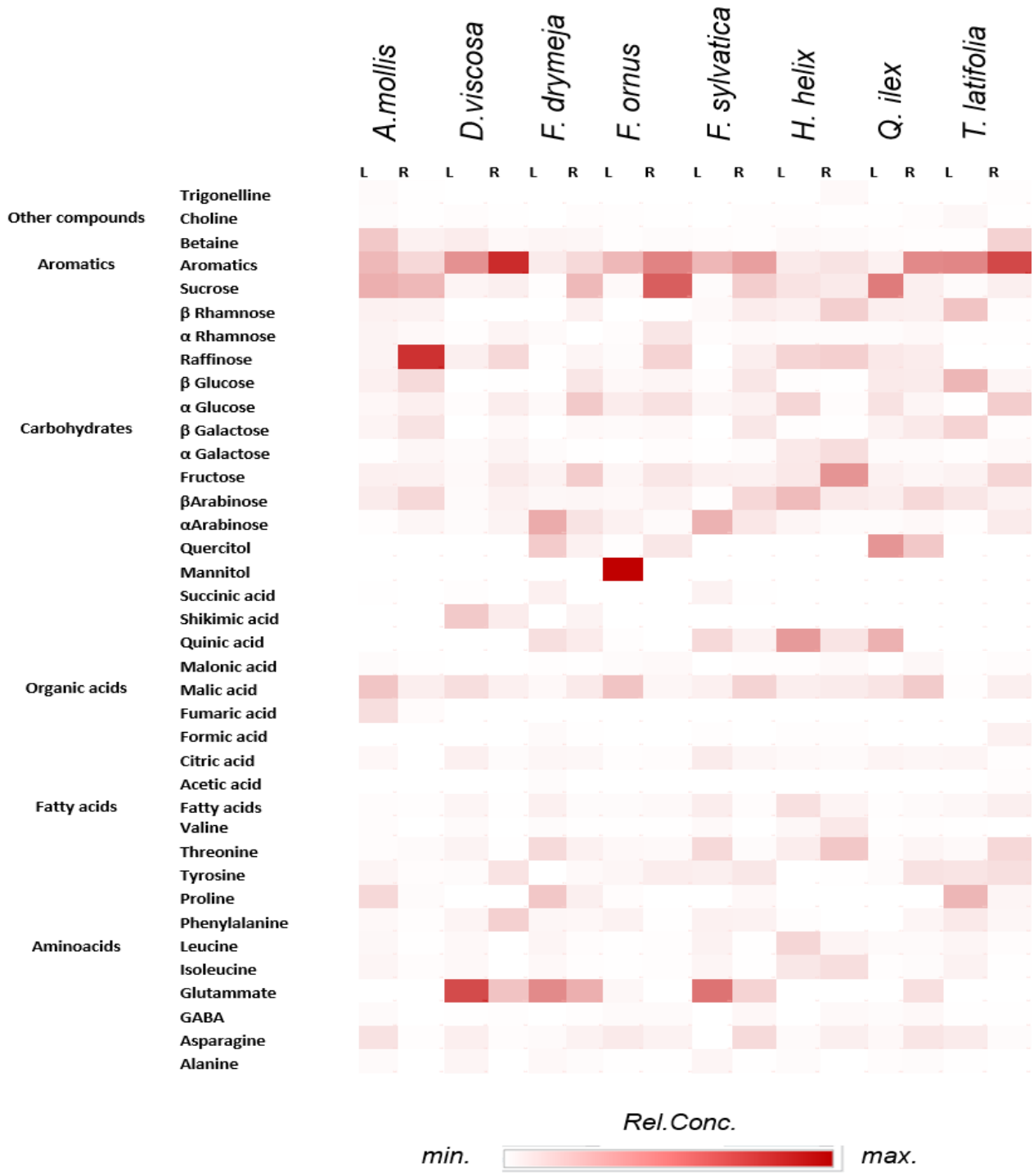

Fig. 6.3. Heat-map of the relative concentration (%) of metabolites in leave and root polar extract of each species

The organic acid total content was almost the same in all analyzed leaves (~10%) with the exception of *T. latifolia, H. helix,* and *Q. ilex.* The former contained the lowest content of organic acid (2.6%) among the analyzed leaves. The latter present the highest content, reaching respectively 23.2% and 21.0%, due to the presence of QA, absent in the other analyzed plants (Fig. 5.3, Tab. 5.2). Moreover, the leaves of *A. mollis* showed a high amount of betaine, recognizable from a singlet at 3.25 ppm (Tab. 6.1).

The leaves of *D. viscosa*, *F. sylvatica*, and *F. drymeja* showed a high percentage of amino acids, which was partially due to the high values of glutamic acid (31.9%, 24.8% and 20.9%, respectively) (Fig.6.3). Aromatic compounds were particularly abundant in *D. viscosa* and *T. latifolia* leaves, followed by *F. sylvatica*, *F. ornus* and *A. mollis*. The lowest content of aromatic compounds was found in *Q. ilex* leaves (Fig. 6.3).

Concerning root tissue, the carbohydrate content of the analyzed species was generally around 50% of the comprehensive metabolite content of polar extract, with some notable exceptions. *A. mollis* extract had 80.6% of sugar content, due to the presence of several sugar residues, from which raffinose (Raff) was predominant with 36.5% of total extract. *F. ornus* contained 62.7% of sugars, the most abundant being sucrose (Sucr). Moreover, particularly low is the content of amino acids and organic acids in these species. On the contrary, *D. viscosa* and *T. latifolia* had the lowest percentage of carbohydrate content and the highest percentage of aromatic compounds (Fig. 6.3).

6.2.2. Multivariate Data Analysis

Coupled with metabolic profiling, the multivariate approach ordinated plant species according to their respective metabolic characteristics. Through PCA we obtained a general view of the underling structure of the data. The principal components were displayed as a set of scores (PC), which highlights clustering or outliers, and a set of loadings (p), which emphasizes the influence of input variables on PC. The multivariate methodology was applied both for data originated from

NMR and tested for the ordination of plant according to chemical characteristics present in three different regions putatively assigned to aromatic/ phenolic compounds (from δ 10.5 to 5.5), carbohydrates regions (from δ 5.5 to 3.0), and aliphatic compounds (from δ 3.0 to 0.5).

In PCA performed for the totality of the regions from ^{1}H-NMR spectra, the first 2 components explained the 71.4% of the variance among the samples (PC1 65.6 and PC2 5.8%). Results are showed in figure 6.4 A and 6.5 B for loadings and score plots, respectively. In a general view, we observed a marked ordination of loadings values according to the respective plant species. Inversely, metabolic profile of the plants does not discriminate among plant portion from which metabolite was extracted. The general variation among plant species was triggered by carbohydrates, while aliphatic and aromatic/phenolics regions has a decreased discriminant power. This is likely explained by the normal attitude to accumulate carbohydrates as nutrient source from photosynthetic pathways (Heldt, Piechulla, and Heldt 2011). Given this, carbohydrates mediate the unidirectional disposition of the samples in its correspondent area showing a generalized positive association of all the samples with carbohydrate signals. However, peculiar number of specific metabolites generate distinctive disposition among plant species. For instance, *D. viscosa* leaves extracts is characterized by the presence of betaine, that also appears to be responsible of the separation of *A. mollis* leaves extracts from the other plant extracts. So far, mannitol signals are majorly associated to the well-known manna producer species *F. ornus* and the quercitol to the oak *Q. ilex*. In both the cases, the metabolite disposition appears to be few distinctive by the point of view of the plant organs in which the metabolite was extracted. More generally, *H. helix, F. ornus, F. drymeja, F. sylvatica, D. viscosa* for roots and *F. ornus, F. drymeja, F. sylvatica* for leaves associate with the aforementioned mannitol and glutamic acid, sucrose, fructose, shikimic acid and raffinose.

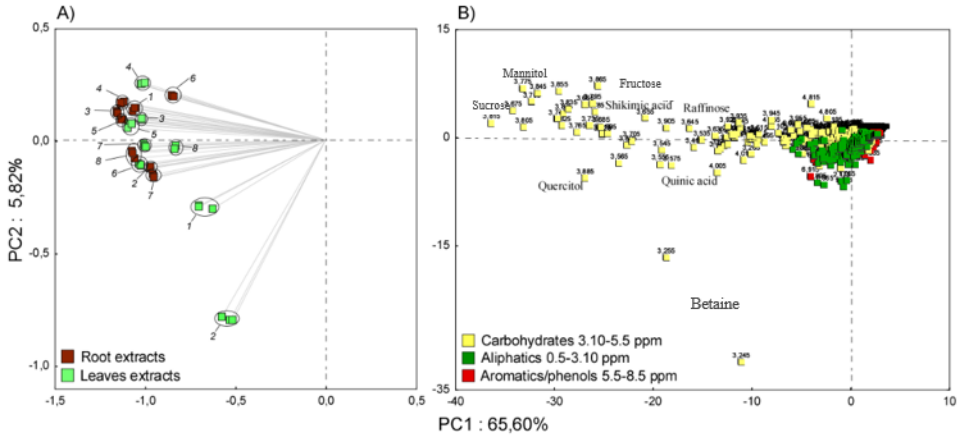

Fig. 6.4 Principal component analysis (PCA) ordination of eight Mediterranean plant leaves and roots based on ^{1}H-NMR resonance spectra from polar (A and B) fractions. A: variable loadings;B: factorial scores of resonance intervals of 0.01 ppm and retention time value. Explained variance of principal components is reported on the axis labels. Plants in loading plots are numbered as: 1. *A. mollis*, 2. *D. viscosa*, 3. *F. drimeja*, 4. *F. ornus*, 5. *F. sylvatica*, 6. *H. helix*,7. *Q. ilex*, 8. *T. latifolia*.

To avoid the hiding action of carbohydrates on the other spectral regions we analyzed these in separate way with the same multivariate approach. Fig. 6.5A and 6.5C showed the PCA ordinationof different extracts according to their chemical composition. The PCA explained totally the 62.0% of the variance in the sample (PC1 51.7% and PC2 10.3%). In this case, we observed a marked differentiation of *A. mollis* and *H. helix* root and leaf extracts with respect to other species mainly operated by the higher content of fumaric acid. Intermediate position was instead acquired by *F. drymeja* leaves extract associated by higher content of tyrosine. The other species aggregated in same directional ordination that was given by the similarity of the spectral regions between 6.96 and 6.83 ppm. For those regions, we unassigned the signals given the high level of uncertainty. In addition, residuals signals from carbohydrates and amino acidic compounds interfere in the interpretation of the spectra limiting our multivariate approach. For the PCA of carbohydrate region (Fig. 6.5 C and 6.5 D), is observed a specular disposition of the data to those of the comprehensive PCA (Fig. 6.5 A and 6.5C). Indeed, the PCA of carbohydrate region explain 72.6% of the variance with respect to the 71.4% explained by the PCA of the overall dataset. For the PCA of the aliphatic region, lower level of explained variance was reported (Fig. 6.5E and

6.5F). Nonetheless, *A. mollis* and *Q. ilex* leaves and *D. viscosa* roots differentiate for quinic acid andproline contents rather than other species that differentiate for the contents of threonine and signals of rhamnose, acetic acid/GABA and residual signals from polar portion of fatty acids.

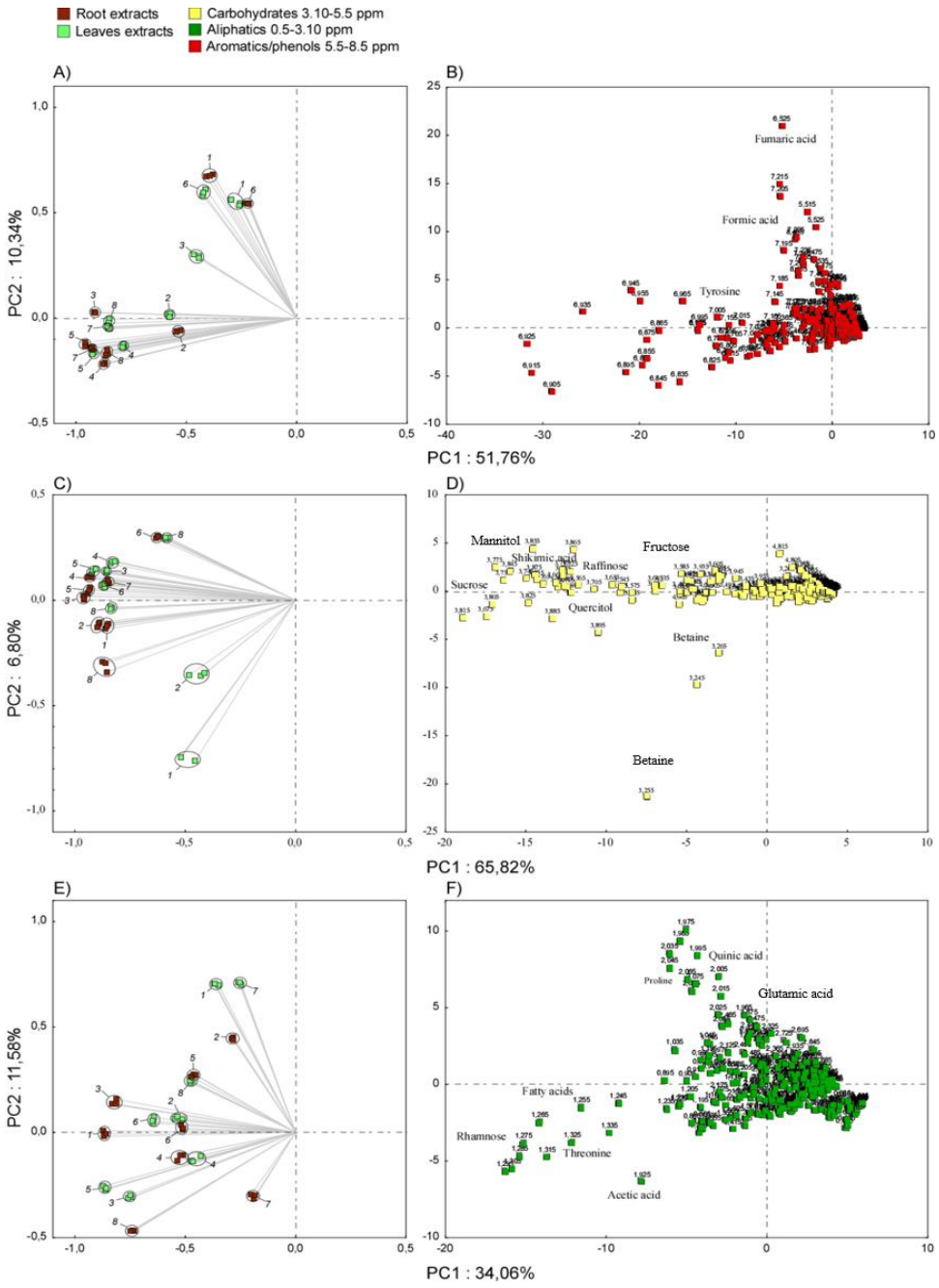

Fig. 6.5. Principal component analysis (PCA) ordination of ^{1}H-NMR resonance intervals: (A and B) from δ 10.5 to 5.5; (C and D) from δ 5.5 to 3.0; (E and F) from δ 3.0 to 0.5. Left: variable loadings; right: factorial scores of resonance intervals of 0.01 ppm. Explained variance of principal components is reported on the axis labels. Plants in loading plots are numbered as: 1. *A. mollis*, 2. *D. viscosa*, 3. *F. drimeja*, 4. *F. ornus*, 5. *F. sylvatica*, 6. *H. helix*, 7. *Q. ilex*, 8. *T. latifolia*.

6.3 Conclusion

Q. ilex and *F. ornus* contained large amounts of specific metabolites, quinic acid, quercitol and mannitol, usually produced from plants during stress conditions. Besides being involved in osmotic stress, quercitol has been recently used as a building block in the synthetic strategy for antidiabetic compounds. *D. viscosa* was characterized by a high content of aromatic compounds at the expense of carbohydrate production in the polar fraction. The separation of *A. mollis* from the other species was due to the presence of betaine and sucrose in leaves and raffinose in roots.All chemical properties of the polar metabolites presented could contribute to the phenomenon of antifungal activity at some degree.

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Tab. 6.1. ^{1}H-NMR chemical shifts, assignment and multiplicity at 500 MHz in D_2O of organic compounds detected in the polar extracts of all plants (leaves and roots).

Compound	Assignment	^{1}H (ppm)	Multiplicity [J (Hz)]
Acetic acid (AC)	CH_3	1.90*	s
Citric acid (CI)	α,γ-CH	2.56*	t [15.0]
	α',γ'-CH	2.67	t [15.0]
Formic acid (FO)	HCOOH	8.45*	s
Fumaric acid (FU)	α,β-CH=CH	6.51*	s
Malic acid (MA)	β'-CH	2.47	dd [15.7, 8.9]
	β-CH	2.70	dd [15.7, 3.7]
	α-CH	4.31*	dd [8.9, 3.7]
Malonic acid (MO)	CH_2	3.10*	s
Quinic acid (QU)	CH_2- 1,1'	1.87*, 2.09	
	CH_2- 5,5'	2.05, 2.00	
	CH-3	3.55	
	CH-2	4.02	
	CH-4	4.15	
Shikimic acid (SH)	CH_2-7	2.22*, 2.76	
	CH-5	3.75	
	CH-6	4.02	
	CH-4	4.43	
	CH-3	6.69	
Succinic acid (SU)	CH_2-2,3	2.43	s
Amino acids			
Alanine (Ala)	β-CH_3	1.46	d [7.0]
Asparagine (Asn)		2.84	dd [4.0, 16.0]
		2.94*	dd [4.0, 16.0]
		4.01	
γ-aminobutyrate (GABA)	CH_2	1.90*	
	-$COCH_3$	2.33	
	γ-CH_2	2.98	t [7.0]
Glutamic acid (Glu)	β,β'-CH	2.05, 2.10*	m
	γ-CH_2	2.36	m
	α-CH	3.77	
Isoleucine (Ile)	δ-CH_3	0.91	t [7.0]
	γ'-CH_3	1.01*	d [7.0]
Leucine (Leu)	δ-CH_3	0.95*	d [7.0]
Phenylalanine (Phe)	CH-2,6	7.32	m
	CH-4	7.36*	m
	CH-3,5	7.40	m
Proline (Pro)	γ-CH_2	2.06, 2.34*	m
	γ-CH_2	1.99	m
	CH_2	3.33, 3,41	
	α-CH	4.12	
Threonine (Thr)	CH_3	1.32	d [7.0]
Tyrosine (Tyr)	CH-5,9	6.78*	m
	CH-6,8	7.21	m
Valine (Val)	γ'-CH_3	0.99	d [7.0]
	γ-CH_3	1.01*	d [7.0]

Carbohydrates			
α-Arabinose (α-Ara)	CH-1	5.19*	
β-Arabinose (β-Ara)	CH-1	4.49*	
α-Fructofuranose (α-Fruf)	CH-3	4.10*	
	CH-5	4.05	
β -Fructofuranose (β-Fruf)	CH-3	4.10*	
	CH-4	4.10*	
	CH_2-6,6’	3.81, 3.65	
β -Fructopyranose (β-Frup)	CH_2-1,1’	3.56, 3.70	
	CH-3	3.79	
	CH-4	3.88	
	CH_2-1,1’	3.70, 4.03	
α-Galactose (α-Gal)	CH-1	5.21*	
β-Galactose (β-Gal)	CH-2	3.50	
	CH-3	3.66	
	CH-4	3.94	
	CH-1	4.54*	d [8.0]
α-Glucose (α-Glu)	CH-4	3.25	
	CH-2	3.53	
	CH-5	3.83	
	CH-3	3.70	
	CH-1	5.22*	d [4.0]
β-Glucose (β-Glu)	CH-4	3.25	
	CH-2	3.53	
	CH-5	3.83	
	CH-3	3.70	
	CH-1	4.62*	d [4.0]
Raffinose (Raff)	GLC-C_2H	3.55	
	GLC-C_3H	3.78	
	GLC-C_5H	4.08	
	GLC-C_1H	5.42*	d [4.0]
α-Rhamnose (α-Rha)	CH_3	1.28	d [6.0]
	CH-1	5.10*	d [1.0]
β- Rhamnose (β-Rha)	CH_3	1.26	d [6.0]
	CH-1	4.85*	bs
Sucrose (Sucr)	GLC CH-1	5.41*	d [4.0]
	CH-4	3.46	
	CH-2	3.55	
	CH-3	3.75	
	CH_2-6	3.81	
	CH-5	3.83	
	FRU CH_2-1’	3.67	
	CH_2-6’	3.81	
	CH-5’	3.89	
	CH-4’	4.04	
	CH-3’	4.21	
Mannitol	CH_2	3.66	dd [5.3, 10.0]
	CH_2	3.75	dt [5.3, 8.0]
	CH_2	3.79	d [8.0]
	CH_2	3.85*	dd [2.0, 10.0]

Quercitol	CH-1	4.13	
	CH-2	3.92	
	CH-3	3.70	dd
	CH-4	3.55	
	CH-5	3.74	m
	CH_2-6	1.81*, 1.99	
Aromatic compounds			
Aromatics (Arom)**	CH-		
Fatty acids			
Fatty acids (FA)	β-CH_2	1.25*	
Other compounds			
Betaine (Bet)	$N(CH_3)^{3+}$	3.26*	s
Choline (Cho)	$N(CH_3)^{3+}$	3.19*	s
Trigonelline (Tri)	CH_3	4.43	s
	CH-4	8.07*	
	CH-3,5	8.80	
	CH-1	9.08	

* Signal selected for quantitation.
** Referring to caffeic acid.

Tab.6.2. Relative concentration (%) of main metabolites of *Acanthus mollis*, *Dittrichia viscosa*, *Festuca drymeja*, *Fraxinus ornus*, *Fagus sylvatica*, *Hedera helix*, *Quercus ilex*, *Typha latifolia*. polar leaf and root extracts. Quantification was determined by integrating diagnostic peak areas of each metabolite in ^{1}H-NMR spectra.

	A. mollis		***D. viscosa***		***F. sylvatica***		***F. ornus***		***H. helix***		***Q. ilex***		***T. latifolia***		***F. drymejia***	
Metabolite	**Leaves**	**Roots**	**Leaves**	**Roots**	**Leaves**	**Roots**	**Leaves**	**Roots**	**Leaves**	**Roots**	**Leaves**	**Roots**	**Leaves**	**Roots**	**Leaves**	**Roots**
Trigonelline	1.1±0.2	0.4±0.0	0.1±0.0	0.1±0.0	0.1±0.0	nd	0.1±0.0	0.1±0.0	0.2±0.0	1.1±0.0	nd	nd	0.1±0.0	0.4±0.1	0.2±0.0	0.1±0.0
Choline	0.7±0.1	0.2±0.0	0.7±0.1	0.3±0.0	0.2±0.0	0.5±0.0	0.4±0.1	0.3±0.0	0.5±0.1	0.3±0.0	0.3±0.0	0.7±0.1	1.5±0.1	0.3±0.0	0.2±0.0	0.6±0.1
Betaine	9.8±2.3	2.5±0.2	3.5±0.3	1.4±0.2	0.6±0.0	0.5±0.0	0.5±0.1	0.8±0.0	1.2±0.2	0.9±0.1	0.7±0.0	0.5±0.1	0.4±0.0	8.1±1.5	1.9±0.1	1.7±0.2
Aromatics	12.7±2.0	7.0±0.6	19.5±1.5	37.6±4.0	12.8±0.5	17.1±1.7	12.7±2.0	22.0±1.9	3.8±0.3	5.1±0.3	2.6±0.1	20.9±4.3	21.6±1.5	32.4±3.6	3.7±0.1	6.8±1.0
Sucrose	14.2±7.9	12.7±12.9	2.0±1.4	2.9±2.4	0.9±0.3	8.9±4.9	1.2±0.5	28.5±13.1	5.0±4.2	3.9±1.9	23.4±12.7	3.0±1.5	0.9±0.2	3.0±0.6	0.5±0.2	12.5±5.9
β-Rhamnose	2.5±0.4	2.4±0.4	0.1±0.0	0.1±0.0	1.2±0.1	3.5±0.4	0.1±0.0	0.3±0.0	2.7±0.4	8.6±0.1	3.1±0.1	3.0±0.6	10.6±0.9	0.7±0.1	0.1±0.0	2.6±0.3
α-Rhamnose	2.2±0.6	1.3±0.1	0.5±0.1	2.0±0.3	0.5±0.0	1.2±0.1	0.6±0.1	4.5±0.4	0.9±0.1	0.8±0.0	0.8±0.1	1.3±0.3	0.2±0.0	0.4±0.0	1.0±0.0	0.2±0.1
Raffinose	2.2±0.1	36.5±4.0	2.9±0.3	6.9±0.5	nd	3.1±0.1	0.8±0.1	7.8±0.2	7.8±0.2	8.5±1.5	4.2±0.1	3.4±0.5	0.1±0.0	0.1±0.1	0.3±0.0	1.8±0.1
β-Glucose	2.8±0.6	6.7±0.5	0.1±0.0	0.4±0.0	0.6±0.0	4.5±0.4	1.3±0.2	1.9±0.2	0.5±0.1	0.2±0.0	3.9±0.3	3.6±0.6	13.0±1.9	1.8±0.3	0.1±0.0	4.4±1.1
α-Glucose	1.4±0.2	2.8±0.3	0.7±0.1	3.3±0.2	1.7±0.2	2.6±0.2	3.5±0.1	5.4±0.1	7.3±0.4	0.7±0.1	5.2±0.8	1.8±0.3	0.2±0.0	8.9±0.8	1.2±0.0	9.6±0.7
β-Galactose	2.0±0.5	5.1±0.5	0.2±0.0	1.3±0.2	0.1±0.0	4.4±0.3	0.9±0.1	1.4±0.1	0.4±0.0	0.3±0.0	2.3±0.3	4.0±0.7	7.8±0.6	0.6±0.1	0.1±0.0	1.2±0.2
α-Galactose	0.3±0.0	1.7±0.2	1.0±0.1	2.2±0.3	0.2±0.0	0.9±0.1	0.1±0.0	0.7±0.1	4.0±0.2	6.1±0.8	1.0±0.1	0.9±0.2	0.4±0.0	1.1±0.1	0.7±0.0	0.3±0.0
Fructose	2.7±1.4	2.6±1.1	1.2±0.4	3.8±0.9	2.8±0.4	2.6±0.5	1.7±0.4	4.5±0.8	4.2±1.6	18.9±2.4	2.5±1.0	1.6±1.2	2.3±0.4	7.6±4.1	2.3±0.6	9.2±3.5
β-Arabinose	3.6±0.8	7.0±0.5	1.0±0.0	2.6±0.3	0.4±0.0	7.1±1.0	1.4±0.1	2.5±0.3	12.1±0.9	3.8±0.2	3.1±0.2	6.9±1.1	4.5±0.4	2.4±0.4	1.5±0.2	1.6±0.2
α-Arabinose	0.5±0.1	1.6±0.1	0.8±0.1	2.3±0.6	13.8±0.6	4.3±0.4	2.5±1.0	0.9±0.4	1.9±0.2	0.8±0.1	0.9±0.0	1.3±0.3	0.4±0.0	3.6±0.8	15.0±1.3	5.0±1.0
Quercitol	nd	nd	nd	nd	nd	nd	0.4±0.1	4.4±0.4	nd	nd	18.9±1.4	9.9±1.9	nd	nd	9.3±0.5	2.6±0.4
Mannitol	nd	nd	nd	nd	nd	nd	45.1±6.6	nd	nd	nd	nd	nd	nd	nd	nd	nd
Succinic acid	0.3±0.0	nd	0.4±0.0	0.1±0.0	2.4±0.3	0.3±0.0	nd	nd	nd	nd	nd	nd	nd	nd	2.8±0.1	nd

Shikimic acid	nd	nd	9.7±1.3	3.4±0.6	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	2.2±0.2
Quinic acid	nd	nd	nd	nd	6.9±0.8	2.3±0.1	0.5±0.1	nd	18.0±0.9	4.9±0.2	13.9±1.0	nd	nd	nd 0	5.8±0.8	3.6±0.7
Malonic acid	0.8±0.2	0.2±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.5±0.1	0.9±0.1	1.1±0.2	0.5±0.0	1.1±0.1	0.2±0.0	1.4±0.4	0.2±0.0	0.7±0.1	0.4±0.0	0.2±0.0
Malic acid	10.4±1.5	3.1±0.2	5.9±0.7	2.8±0.3	2.8±0.1	7.9±0.9	10.5±1.4	1.7±0.1	3.0±0.1	3.6±0.2	4.9±0.5	9.3±2.3	0.5±0.0	3.0±0.2	1.3±0.0	3.8±0.6
Fumaric acid	5.9±1.4	0.7±0.0	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Formic acid	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.4±0.0	nd	0.1±0.0	0.1±0.0	0.5±0.0	0.5±0.0	nd	nd	0.1±0.0	2.6±0.3	0.9±0.0	0.3±0.0
Citric acid	1.4±0.3	0.1±0.0	2.7±0.3	0.8±0.2	3.6±0.2	1.6±0.2	0.5±0.1	0.7±0.0	1.2±0.1	1.0±0.0	2.0±0.1	1.6±0.3	1.9±0.1	0.4±0.0	1.7±0.1	1.4±0.3
Acetic acid	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.6±0.2	0.8±0.3	0.1±0.1
Fatty acids	0.6±0.1	0.5±0.1	1.8±0.2	0.2±0.0	3.3±0.3	0.6±0.1	0.9±0.2	1.2±0.1	5.6±0.6	2.1±0.2	0.5±0.0	1.0±0.2	1.5±0.1	3.0±0.6	2.7±0.2	0.8±0.1
Valine	0.6±0.1	0.1±0.0	1.0±0.1	nd	1.2±0.2	0.1±0.0	0.3±0.0	0.3±0.1	1.5±0.2	4.2±0.1	0.2±0.0	0.5±0.1	0.3±0.0	nd	0.8±0.0	0.2±0.0
Tyrosine	1.8±0.5	0.7±0.3	0.7±0.3	5.0±2.0	2.9±1.7	4.3±2.4	1.6±0.8	3.3±1.9	0.1±0.0	0.2±0.1	0.8±0.4	5.2±2.5	4.8±2.8	5.8±2.6	0.1±0.0	1.2±0.5
Threonine	0.6±0.1	1.0±0.1	2.3±0.3	0.2±0.0	6.9±0.1	0.8±0.0	1.3±0.2	1.1±0.1	3.2±0.2	10.0±0.1	0.6±0.0	1.9±0.3	1.2±0.1	7.0±0.5	6.5±0.2	2.5±0.4
Proline	7.1±1.2	0.8±0.1	nd	nd	nd	1.4±0.2	0.5±0.1	nd	nd	nd	nd	nd	13.0±0.7	1.9±0.4	10.0±0.5	2.9±0.6
Phenylalanine	1.3±0.2	0.5±0.1	2.0±0.1	8.4±0.8	2.6±0.2	2.3±0.2	2.5±0.3	0.3±0.0	0.5±0.0	0.2±0.0	0.1±0.0	1.8±0.3	3.9±0.1	1.6±0.2	1.6±0.1	1.4±0.2
Leucine	1.5±0.1	0.2±0.0	1.3±0.1	0.1±0.0	2.3±0.0	0.3±0.0	0.3±0.0	0.5±0.0	7.3±0.3	2.0±0.1	0.9±0.0	1.0±0.1	1.8±0.1	0.6±0.0	1.8±0.0	0.6±0.0
Isoleucine	1.9±0.2	0.6±0.0	1.3±0.1	nd	1.9±0.1	0.3±0.0	0.2±0.0	0.4±0.0	4.3±0.2	5.9±0.3	0.4±0.0	0.8±0.2	2.5±0.1	0.2±0.0	1.4±0.1	0.5±0.1
Glutamic acid	nd	nd	31.9±3.6	10.7±1.0	24.8±0.8	7.8±0.7	1.5±0.3	nd	nd	nd	nd	5.6±0.9	nd	nd	20.9±0.5	14.2±3.1
GABA	0.9±0.2	0.1±0.0	0.2±0.0	0.4±0.1	nd	1.4±0.2	1.0±0.2	1.0±0.1	0.2±0.0	1.1±0.1	0.8±0.0	1.4±0.2	0.3±0.0	0.3±0.0	0.1±0.0	0.5±0.1
Asparagine	5.2±1.0	0.5±0.0	2.9±0.2	0.6±0.1	0.1±0.0	6.6±0.7	3.8±0.6	2.3±0.3	0.9±0.1	2.9±0.2	1.4±0.2	4.7±0.8	3.6±0.3	0.8±0.1	1.0±0.0	2.4±0.4
Alanine	0.9±0.2	0.1±0.0	1.8±0.2	0.1±0.0	1.8±0.1	0.3±0.0	0.3±0.1	0.2±0.0	0.8±0.1	0.2±0.0	0.6±0.0	0.7±0.1	0.2±0.0	0.2±0.0	1.3±0.1	0.8±0.1

Note: Data refers to mean ± standard deviation of triplicate spectra. n.d.: not dete

Chaper7: Final Conclusion

Considering the potency of plants as sources for antimicrobial drugs, we selected eight species of Mediterranean area and analyzed both leaf and root extracts of each plant for phytochemical property guided by antifungal activity tests. The species pool included two perennial forbs (*Dittrichia viscosa, Acanthus mollis*), two grasses (*Typha latifolia*, *Festuca drymeia*), one vine (*Hedera helix*), one evergreen tree (*Quercus ilex*), and two deciduous trees (*Fraxinus ornus, Fagus sylvatica*). The species selected have been employed as folk remedy for their wound-healing, anti-inflammatory and disinfectant qualities in a long history. The antifungal activity study was established for two extracts (petroleum ether and water/methanol mixture) from leaf and root of each species. The chemical compositions of the tested extracts were analyzed by Gas Chromatography (GC-MS) and Nuclear Magnetic Resonance (NMR) Spectroscopy.

We discussed the results of the bioassay test conducted by measuring the mycelia growth of two fungi, *Trichoderma harzianum* and *Aspergillus niger*, in the apolar and polar extracts of leaf and root samples of eight plants. The inhibition effect against *T. harzianum* were observed on the apolar leaf extracts of *D. viscosa*, polar root extract of *T. latifolia*, polar leaf and apolar root extracts of *H. helix*, apolar leaf, polar leaf and apolar root extracts of *F. ornus*, polar leaf extracts of *F. drymeja*. As for *A.niger*, a resistant phtytopathogen, it was only susceptible to the apolar extract of *D. viscosa* leaf. It was found that the results of bioassay test were consistent with the previous studies.

Through analyzing the apolar phase of leaf and root samples of each species by GC-MS, we found that fatty acids, n-alkanes, terpenoids and steroids were ubiquitous among the samples.The most abundant metabolites among these species were always saturated fatty acids with the relative content from 20.89% (*D. viscosa* leaf) to 81.57% (*A. mollis* root), with four exceptions. In *D.*

viscosa leaf, oxygenated terpenoids were most abundant than fatty acids. In *H. helix* root, the major compounds were unsaturated fatty acids, particularly linoleic acid. These can be counted for the reason why the two apolar extracts have antifungal activity in the bioassy and the concentration of triterpenoids, exceeding the one of fatty acids, was the richest in *A. mollis* leaf and *Q. ilex* root extracts Through analyzing the polar phase of leaf and root samples of each species by NMR and multivariate data analysis, we found that *Q. ilex* and *F. ornus* contained large amounts of specific metabolites, quinic acid, quercitol and mannitol. *D. viscosa* was characterized by a high content of aromatic compounds at the expense of carbohydrate production in the polar fraction. *A. mollis* was separated from the other species due to the presence of betaine and sucrose in leaves and raffinose in roots. All chemical properties of the apolar and polar metabolites presented could, contribute to the phenomenon of antifungal activity at some degree.

All in all, the approach based on GC-MS and NMR spectroscopic techniques and multivariate data analysis proved to be suitable for a rapid investigation of different plant species containing a wild range of metabolites. Guided by bioassay test, the two main objectives of our study were achieved: to describe the chemical composition and diversity of the eight Mediterranean plants and to compare the phytochemical differences among leaf and root organs.

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Publications

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