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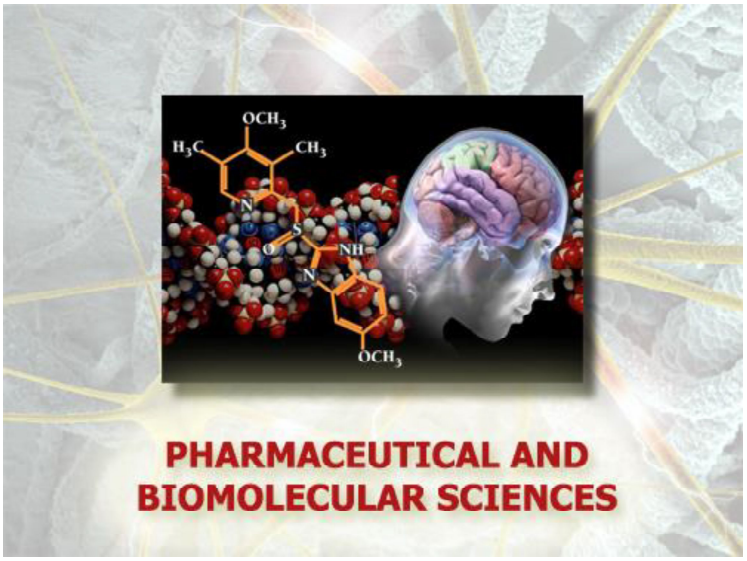

Extraction and analytical techniques for the quality control of food and dietary supplement

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Contents

Foreword

In the last years, because of the increased consumer interest in more natural health-care products a widespread use of dietary supplement has been recorded. Often, the nature of dietary supplements is associated with that of "health supplements", or rather substances that support specific deficiency diseases. This new generation of supplements are products that can be considered as something between drugs and foods, including vitamins, minerals, herbals and botanicals, amino-acids, enzymes and many other compounds.

In contrast to drugs, dietary supplements are not subjected to the stringent and regulatory process of quality/safety control of drugs, and may also be sold outside the pharmacy, herbalists or hospitals.

All products categorized as "dietary supplement" always carry a table/panel, a dosage information. The first one lists the contents, amount of each active ingredient per serving and other excipients. However, in most cases, labels are prudently formulated to avoid any clash with the few rules issued. Indeed, due to the lack of a strict legislation, the chemical characterization of dietary supplements is object of controversial debates. Very often the dietary supplements market is characterized by claims on the content of bioactives present in the raw material that sometimes are far from being reliable. In most cases, wrong titrations occur when the identification of the main bioactive molecules is based on simple chemical characterization or on wrong and amateur protocols.

When based on a rigorous scientific method, dietary supplements may provide beneficial effect to human and animal health as a support of health. Moreover, the scientific approach must be accompanied by a solid scientific quality control policy. In particular, quality control should verify not only the absence of toxic and dangerous substances (such as toxins, pesticides, heavy metals, etc…) but also provide tools for the identification and geographic origin of raw materials.

In this contest, the topic of my doctoral thesis focused on the quality control of food and dietary supplements using advanced analytical methods coupled with biomolecular techniques.

My main project involved the combination of genetic and chemical approaches in order to provide an unequivocal identification of raw materials to support the identification when a simple evaluation of the morphological characteristic is not possible. Objects of this thesis were a typical Mediterranean species: pistachio (*Pistacia vera*). Here, spectrophotometric assays were

combined with mass spectrometric analyses in order to investigate the phytochemical diversity within six pistachio verities of different geographic origin (Bronte, Larnaka, Mateur, Mawardi, Kern and Kerman). Moreover, DNA fingerprinting coupled with RFLP analysis was used to determine the differences in the genome non-transcribing regions (ITS and NTS).

Due to the presence of a large amount of antioxidants in the skin of pistachios, these particular waste products of the manufacturing industry were also used in further studies in order to evaluate their biological activity, with the aim to use this waste material as an innovative component. In particular, antioxidant (AOA) and antiproliferative (APA) activities, and their potential mechanism of action was studied by qRT-PCR.

Another interesting aspect of the dietary supplement market is the standardization of bioactive compounds. During my doctorate period, I found that the most of the supplements made with *Boswellia* extracts were object of chemical overestimation. Indeed, in both *Boswellia sacra* and *Boswellia serrata* gum resin extracts, the label of these products frequently reports a total concentration of boswellic acids equal to or higher than 70% or AKBA contents of 30%. Due to the importance of these extracts in the treatment of several diseases, such as inflammation and osteoarthritis, asthma and age related disorders, the qualitative and quantitative determination of the bioactive boswellic acids is crucial. By using HPLC-DAD-MS/MS we provided a solid guideline for the chemical standardization and validation of all dietary supplements containing *Boswellia* gum resin extracts.

During my last PhD year, I had the opportunity to collaborate with the group of myrmecologists of the department of Life Sciences and Systems Biology. We evaluated how the administration of two simple Volatile Organic Compounds (VOCs) of *Origanum vulgar* plants (thymol and carvacrol) could affect the behavioural changes in ants. HPLC-MS/MS technology was instrumental for the identification and quantification of the biogenic amines (such as dopamine, tyramine, serotonin, etc…), that were the main responsible compounds involved in ant behaviour changes.

In conclusion, during the doctorate period I confirmed that only analytical methods based on gas and/or liquid chromatography coupled to mass spectrometry allow the precise identification and quantification of bioactive compounds in different plant and animal extracts. Moreover, coupling this methodology with biomolecular methods was instrumental to obtain precise information useful for the unequivocal identification of plant species.

Part I.

Chemical and biomolecular quality control for the correct geographic identification of pistachios varieties and valorisation of its waste products.

1 – State of the Art

1.1 Description, origin and traditional use of *Pistacia vera*

Pistachio (*Pistacia vera* L.) is a subtropical plant and member of the cashew family, also known as the *Anacardiaceae*. Among *Anacardiaceae*, the genus *Pistacia* consists of at least 12 tree and shrub species, of which only *P. vera* produces edible nuts (pistachio). *Pistacia vera* is an important product in several fields, such as cosmetic, medical, pharmaceutical and food industry (Fig. 1). The fruits are enriched with many health-benefiting nutrients, essential for health. It is one of the most important and popular world agricultural crops, thanks to its high resistance to extreme environmental conditions, such as warm and moist environments. Originating in arid zones of Western Asia, especially Iran, Iraq, Syria and Turkey, in the last centuries its cultivation has spread outside the traditional geographical regions throughout the Mediterranean area, finally entering in Europe. Here several local varieties were selected, including Bronte in Italy, Larnaka in Greece, Mateur in Spain, which were commercialized all over the world. During the 1880s, due to immigration from the Middle East, pistachio became very popular in the USA. For the favourable climate, dry conditions and moderately cold winters, California turned out to be ideal place for pistachio cultivation and became, with Iran and Turkey, the main pistachio producer in the world. Currently, over 80% of pistachio production is located in these countries (FAOSTAT 2017). Despite the cost of importation, pistachio from California and Iran can be purchased in European markets at a relatively low price. On the contrary, varieties with higher quality, such as the Sicilian variety from Bronte, are appreciated for their aromatic taste and flavour, immediately followed by the Turkish varieties, Mawardi and Red Aleppo.

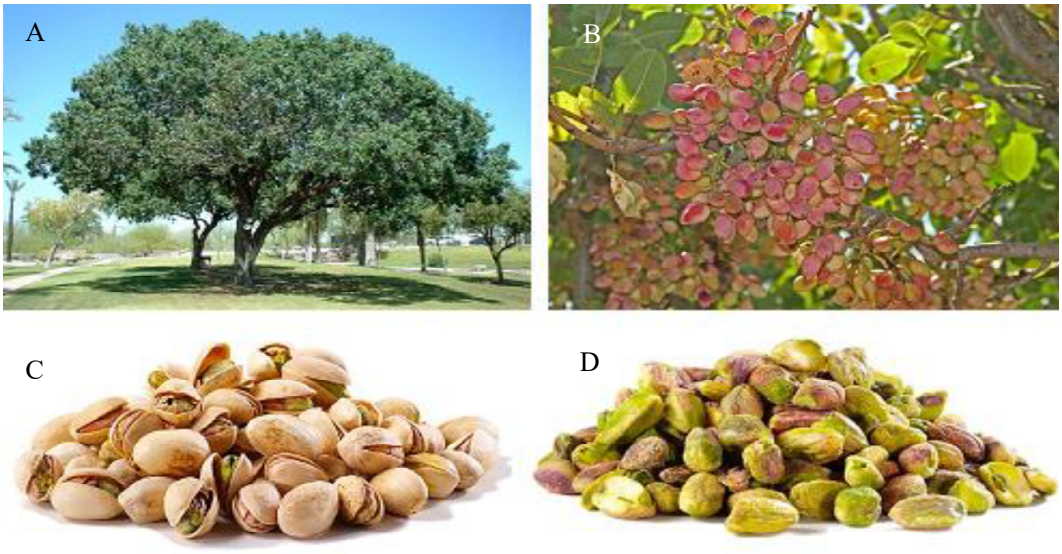


Figure 1. Pistachio (*Pistacia vera*) tree (**A**) and its fruits before the harvest (**B**). During the manufacturing process the mesocarp and the shell (**C**) is industrially removed,to obtain the *Kern*els (**D**).

1.2 Strategy for the correct identification of pistachio varieties

Despite the different flavour and taste, a correct identification of pistachio variety origin, simply based on the phenotypic parameters, is not always possible. Therefore, the use of chemical and molecular profiling methods has been studied in pistachio, in order to help discrimination of varieties from different geographical origin. Chemical partitioning allowed pistachio geographical discrimination thought the identification of specific markers or entire metabolite profiling (Sobolev *et al.*, 2017) using elemental analysis (Anderson and Smith, 2005), carbon and nitrogen isotope analyses (Anderson and Smith, 2006), heavy metals (Taghizadeh *et al.*, 2017), phenolic profile (Saitta *et al.*, 2014; Taghizadeh *et al.*, 2018), essential oils (Dragull *et al.*, 2010) and triacylglycerols (Ballistreri *et al.*, 2010). Biomolecular characterization of pistachio also revealed to be a potent tool for variety discrimination through analysis of chloroplast DNA (Parfitt and Badenes, 1997; Sarra *et al.*, 2015), RFLP analysis (Parfitt and Badenes, 1998), RAPD analysis (Hormaza *et al.*, 1994), SSR-based genetic linkage map (Khodaeiaminjan *et al.*, 2018) and retrotransposon markers (Kirdok and Ciftci, 2016). ITS sequence is widely used in plant molecular systematics at the generic and species levels because of its potentially high resolution of inter- and intraspecific relationships (Cheng *et al.* 2016).

1.3 Phytochemistry and beneficial properties of pistachio

In the last decades, due to the increasing attention of consumers to the beneficial properties of nut consumption, the commercial impact of pistachio strongly increased. Results from *in vivo* studies showed a positive correlation between pistachio intake and reduced risk of cardiovascular disease (Edwards K. *et al.*, 1999; Kocyigit A. *et al.*, 2006; Sheridan MJ *et al.,* 2007; Gebauer SK. *et al.*, 2008). This protection has been specially ascribed to ideal nut fatty acid profile that can have a favourable influence on serum concentration and profile of triglycerides, and total and low-density lipoprotein cholesterol content (Cuschieri and Maier, 2007; Appeldoorn *et al.*, 2009; Lu *et al.*, 2008; Lee *et al.*, 2008; Wang *et al.,* 2010).

Fatty acids (FAs) are very important compounds for both plant and animal health. These compounds are involved in several biochemical pathways. In comparison to other molecules, such as sugars and proteins, the breaking down of FAs carbon-carbon bounds yields a two-fold higher energy (Nelson and Cox, 2006). FAs are also important precursors of other structural components (such as phospholipids, pigments and transporters) and physiological mediators (such as hormones and prostaglandins) (Lehninger *et al.*, 1992). Moreover, experimental data showed that pistachio nut consumption significantly improves the oxidative status of healthy individuals and lowers the levels of circulating inflammatory biomarkers (Sara *et al.*, 2010). Due to this reason, pistachio nut has been ranked among the first 50 food products with the highest antioxidant potential (Sara *et al.*, 2010).

Polyphenols (PCs) contribute significantly to the total antioxidant potential of most foods (Wu, X. *et al.,* 2004). PCs are a diverse class of secondary metabolites that include flavonols, flavones, flavanols, isoflavonoids, the phenolic acids, stilbenes, proanthocyanidins, catechols, anthocyanins, tannins and catechins. These compounds may act both in biochemical process and in defence mechanism from UV light, insects and animals menaces (Siqueria, 1991). From human nutrition point of view, PCs are often linked to a wide range of health benefits, thank to act as potent antioxidants in vitro. These antioxidant properties potentially offer protection against inflammation, heart disease, and cancer (Ferguson, 2001), but an excessive consumption of these compounds may have negative health effects as well (Ferguson, 2001). Notwithstanding the dietary intake of moderate amounts of botanical phenols is generally recommended (Robards *et al.*, 1999). Concerning pistachio, polyphenols compounds are mostly found in the nut skin, which is usually removed and treated as a waste during its manufacture (Aslan *et al.*, 2002; Catalan *et al.*, 2017). Hydrophilic extracts from the Bronte variety was found to contain substantial amounts of PCs with radical-scavenging and anti-oxidative properties (Gentile,

2007), and possessed anti-inflammatory activities in *in vitro* models (Gentile *et al.*, 2012; Gentile, 2015).

1.4 Pistachio skin as a new potential source of natural antioxidants

During the manufacture and processing of pistachio its skin is industrially removed becoming a waste product. However, from a circular economy point of view, wastes from several fruits may represent a potential natural source of antioxidant compounds (Peschel *et al.*, 2006; Vasco *et al.*, 2008; Wijngaard *et al.*, 2009). Indeed, human homeostasis is largely linked to the correct balance between pro-oxidant and antioxidant compounds. This "redox balance" is essential for the survival of organisms and their health, and oxidative stress conditions produce reactive oxygen species (ROS) that cause oxidative damage to membrane lipids, DNA molecules, and proteins (Halliwell, 1990) as well as extensive damage to cells and tissues, degenerative disorders, mutations and cancer (Giovannini and Masella, 2012). In recent years, to help biodefense, there has been a worldwide trend towards the use of the natural phytochemical antioxidants present in fruits and vegetables (Halliwell, 1990; Maritim *et al.*, 2003; Ou *et al.*, 2002; Rauf *et al.*, 2017). These compounds, which are mostly present in the external coatings, are able to scavenge ROS or prevent their overproduction (Ribeiro *et al.*, 2008). Several phytochemicals have shown various effects not only on the direct reduction of free radicals originating in mitochondria, but also on the modulation of both ROS-metabolizing gene expression (Toyokuni *et al.*, 2003) and enzymatic activities (Havsteen, 2002).

2 – Materials and Methods

2.1. Plant material

Seeds of different varieties of *Pistacia vera* L. (Bronte from Sicily, Mawardi from Turkey, Larnaka from Greece, Kern from Iran, Kerman from U.S.A., California and Mateur from Spain) were kindly provided by Pistacchio dell'Etna Srl (*Bronte*, Italy) and by Di Sano Srl (Rozzano, Italy). Seeds were stored in the dark at 4°C before extraction. At least three technical replicates were performed for each lot of seeds.

2.2. Extraction of phenolic compounds

The seed skin and flash of each variety was manually separated and extracted in 75:25 v/v ethanol:water solution, for 3 days in the dark at room temperature, using a 1:20 w/v extraction ratio. After centrifugation (10 min at 10,000 g, 4°C) and filtration through a Millex HV 0.45 µm filter (Millipore, Billerica, MA), the supernatants were recovered and stored at -80°C until analysis. For each variety, the extraction was performed in triplicate. Lipophilic extracts of seed flash were obtained by Soxhlet extraction by using cyclohexane (1:10, w/v). After extraction, the solvent was removed with a nitrogen flow.

2.3. Total phenolic compounds content

The total phenolic compounds content (TPC) was determined by the Folin-Ciocalteu method (Singleton *et al.*, 1999). Briefly, for each extract, 1ml of opportune dilution of the crude extract were mixed with 7.5 mL of Folin-Ciocalteu reagent. The absorbance was read after 30 min with a UV/Vis spectrophotometer (Agilent Cary 60 Spectrophotometer). Gallic acid (GA) was used for the preparation of the calibration curve and the results were expressed as mg GA g^{-1} d.wt. All measurements were repeated three times.

2.4. Total anthocyanin content

The total anthocyanin content (TAC) was measured using the differential pH method (Cheng and Breen 1991). Briefly, 1 mL of extracts were diluted in 9 mL of two different buffers (0.025 M potassium chloride pH = 1.0 and 0.4 M sodium acetate pH = 4.5, respectively). After 30 minutes of incubation at room temperature, absorption (A) was measured at 510 and 700 nm (Agilent Cary 60 Spectrophotometer). For calculation of total anthocyanins as cyanidine equivalent, the

molar extinction coefficient (ε) values 26900 $M^{-1}cm^{-1}$ (Meyers *et al.* 2003) and 29600 $M^{-1}cm^{-1}$ (Cao *et al.* 2011) and a 449 molecular weight was used.

The Abs employed for the calculation of the TAC were obtained as follows:

$$Abs_{sp} = (Abs_{510nm} - Abs_{700nm})_{pH1.0} - (Abs_{510nm} - Abs_{700nm})_{pH4.5}$$

The total anthocyanin content was expressed as mg anthocyanin 100 g^{-1} fresh weight. All measurements were performed in triplicate.

2.5. Total proanthocyanidin (PAC) content

The 4-(dimethylamino)-cinnamaldehyde (DMAC) assay was used to evaluate the total amount of PAC according to Prior *et al.* (2010) with minor modifications (Occhipinti *et al.*, 2016). Briefly, after dilution of extracts in Acetone/Water/Acetic acid mix (75:24.5:0.5, v/v/v), 280 µL sample was mixed with 840 µL 0.1% DMAC solution in a test tube and shortly vortexed. The absorption (640 nm) of the mixture was then analysed after 20 min using a UV/Vis spectrophotometer (Agilent Cary 60 Spectrophotometer). The total PAC content was quantified via an external calibration curve made with a pure PAC-A2 standard and was expressed as mg PAC-A2 g^{-1} d.wt. The measurements were performed in triplicate.

2.6. HPLC-DAD-ESI-MS/MS analysis of phenolic compounds

The HPLC system consisted of an Agilent Technologies 1200 coupled to a DAD and a 6330 Series Ion Trap LC-MS System (Agilent Technologies, USA) equipped with an electrospray ionization (ESI) source. The chromatographic separation was carried out at constant flow rate (0.2 ml min^{-1}). The column was a reverse phase C18 Luna column (3.00 µm, 150 × 3.0 mm i.d., Phenomenex, USA), maintained at 25°C by an Agilent 1100 HPLC G1316A Column Compartment. The UV/Vis spectra were recorded between 220 and 650 nm and the chromatographic profiles were registered at 220, 280, 360 and 520 nm. Tandem mass spectrometry analyses were performed operating either in negative mode (for flavonoids) or in positive mode (for anthocyanins). The nitrogen flow rate was set at 5.0 ml min^{-1} and maintained at 325°C, whereas the capillary voltage was set at 1.5 kV. Helium was used as a collision gas. Compound identification was carried out by comparison of the retention time and UV-VIS/MS spectra with those of authentic reference compounds or using literature data. 4.6.1 Flavonoid analysis. The binary solvent system for flavonoid analysis was MilliQ H_2O acidified with 0.1% v/v (Solvent A) (Millipore, Billerica, MA, USA) and ACN acidified with 0.1% v/v formic acid (Solvent B). The samples were separated by the following gradient: 97% A and 3% B as initial

conditions, 70% A and 30% B for 35 min, and then 2% A and 98% B for 5 min. The concentration of A was maintained at 2% for 5 min and eventually was raised to the initial condition before the next injection. Sample injection volume was 5 µl. 4.6.2 Anthocyanin analysis. The binary solvent system for anthocyanin analysis was MilliQ H_2O acidified with 0.1% (v/v) formic acid (Solvent A) and MetOH 50% v/v acidified with 10% v/v formic acid (Sigma-Aldrich, USA) (Solvent B). The elution method involved a multistep linear solvent gradient changing from an initial concentration of 85% A and 15% B to 55% A and 45% B in 15 min. Finally, the gradient was 30% A and 70% B in 20 min. The concentration of solvent A was decreased to 2% and was maintained for 5 min before the next injection. Sample injection volume was 15 µl.

2.7. Fatty acid analysis

Fatty acids portion was obtained in a Soxhlet apparatus by flushing the samples for 5 h with *n*-Hexane at 69°C. A ratio of crushed nuts to solvent of 1:10 (w/V) was used. Subsequently, the solvent was evaporated under reduced pressure and the amount of extracted material evaluated by weighting. The yields of each fatty acid was expressed on a dry basis.

The Soxhlet extract was then esterified with boron tri-fluoride (10% w/v in methanol) by adding 50 µg heptadecanoic acid (C17:0) as internal standard (Maffei and Peracino, 1993). The fatty acid methyl esters (FAME) were obtained according to Christie and Han (2010), dehydrated with anhydrous $MgSO_4$ and FAME identification and quantification was performed by GC-MS (5975T, Agilent Technologies, USA) and by GC-FID (GC-2010 Plus, SHIMADZU, Japan), respectively. The GC carrier gas was helium with a constant flux of 1 ml min^{-1}, and separation was obtained with a non-polar capillary column ZB5- MS (30 m length, 250 µm diameter and stationary phase thickness of 0,25 µm, 5% phenyl-arylene and 95% poly-dimethyl siloxane) (Phenomenex, USA). Same column and chromatographic condition were used for both GC-MS and GC-FID analyses. The following temperature condition was used: injector 250°C, oven initially at 60 °C, held for 1 minute and raised to 180°C (10.0°C min^{-1} and held for 1 minute). Then the temperature was brought to 230 °C (1.0 °C min^{-1} and held for 2 minutes) and to 320 °C (15 °C min^{-1}) held for 5 minutes. MS parameters were: ionization energy of the ion source was set to 70 eV and the acquisition mode was set to 50–350 m/z. Compounds were identified through comparison of mass fragmentation spectra with reference NIST 98 spectra or by comparison of Kovats indexes and internal standard co-injection of pure standards (Sigma-Aldrich, USA). FAME quantification was obtained by internal standard. At least three biological replicates were run.

2.8. DNA fingerprinting

2.8.1. DNA extraction, PCR amplification, subcloning and sequencing.

Whole pistachio seeds were pulverized in liquid nitrogen using a mortar and pestle. Genomic DNA was extracted and quantified according to Capuzzo and Maffei (2014). Briefly, twenty ng of genomic DNA were used as a template for PCR amplification with specific primers for ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (3'-CCGCAGGTTCACCTACGGA-5'). PCR products were separated by 1.0% (w/v) agarose gel electrophoresis and visualized by GelRed (Biotium) staining under UV, and purified from the gel using the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel). The purified product was used for subcloning using the TOPO-TA Cloning Kit (Thermo Fisher Scientific) and then transformed in *Escherichia coli* Subcloning DH5α Efficiency Competent Cells (Invitrogen, Paisley, UK). Colonies containing DNA inserts of the correct size were picked and grown overnight in 5 mL Luria- Bertani liquid medium. The mini-preparation of plasmid DNAs was carried out using NucleoSpin Plasmid Miniprep Kit (Macherey-Nage). Plasmid DNAs were used as a template for sequencing (Macrogen, Wageningen, Holland). Both DNA strands were sequenced.

2.8.2 PCR-RFLP analysis.

PCR products of the ITS gene were digested at 37°C for 15 min with either 10 U 335 *RsaI*, *PstI* (NEB, New England Biolabs, Ipswich, AM, USA) or *TaqI* (NEB, New England Biolabs, 336 Ipswich, AM, USA) at 65°C for 60 min. One microliter of each digestion reaction was analyzed by 337 capillary gel electrophoresis (CGE) using the Agilent 2100 Bioanalyzer (Agilent Technologies) and 338 the DNA 1000 LabChip Kit (Agilent Technologies) following the manufacturer's instructions.

2.9. Evaluation of Cellular Antioxidant Activity

2.9.1. Cell culture.

Cancer cell line HepG2 (hepatocarcinoma cells, American Type Culture Collection ATCC, Rockville, MD, USA) were cultured in RPMI supplemented with 5% FBS, 2 mM L-glutamine, 50 IU/ml penicillin, and 50 µg/ml streptomycin and maintained in a humidified atmosphere with 5% CO_2 at 37 °C. Cells were routinely cultured in 75 ml culture flasks and were trypsinized using trypsin-EDTA.

2.9.2. CAA assay.

Ethanolic extracts were assayed by the cellular antioxidant activity (CAA) according to Wolfe and Liu (2007). In each experiment, the concentration of ethanol (EtOH) never exceeded 0.25%, culture medium with 0.25% EtOH was used as a control. CAA was calculated as follows:

$$CAA = 100 - [\frac{\int SA}{\int CA}] * 100$$

Where: ∫SA is the integrated area of the sample curve and ∫CA is the integrated area of the control curve. The concentration necessary for 50% of DCF formation inhibition (CAA50) for each extract or pure GA was calculated from concentration−response (CAA) curves using linear regression analysis. GA was used as a standard.

2.10. Evaluation of Antioxidant Capacity

2.10.1. Evaluation of scavenging activity.

The radical scavenging activity of the extracts were evaluated using the ABTS radical cation bleaching assay (Re *et al.*, 1999) and the DPPH radical scavenging assay (Kedare and Singh, 2011). $ABTS^{\bullet+}$ was prepared by reacting of ABTS with potassium persulfate. Samples were analyzed at five different dilutions, within the linearity range of each assay, as previously described (Floegel *et al.*, 2011). Radical scavenging activity was expressed as mmol TE 100 g^{-1} of d.wt material. All measurements were repeated three times.

2.10.2. Evaluation of ferric reducing antioxidant power.

The reducing activity of the ethanolic extracts was assessed using FRAP method as previously reported (Benzie and Strain, 1996). FRAP reactive was fresh prepared by mixing (8:1:1, v/v) 0.3 M acetate buffer pH 3.6, 10 mM TPTZ and 20 mM $FeCl_3$. Ferric reducing power was expressed as mmol TE 100 g^{-1} of d.wt material. All measurements were repeated three times.

2.11. RNA isolation, cDNA synthesis and qRT-PCR

HepG2 cells were seeded at a density of $5x10^5$ cells/well in 24-multiwell plates. The media were discarded after 24 h and the cells were treated for 2 h with the extracts in fresh FBS-free DMEM. Then, cells were exposed to 200 μM H_2O_2 for 24 h. After the incubation time, the cells were collected and total cellular RNA was isolated with a commercial kit (RNA-XPress™ Reagent, HiMedia), according to the manufacturer's instructions. One microgram of the total RNA was reverse-transcribed using oligo (dT) and OneScript® Reverse Transcriptase (HiMedia, China), according to the manufacturer's instructions. The resulting cDNA was used as template for a

quantitative real-time PCR, using the BrightGreen 2X qPCR MasterMix-Low ROX (Abm, Canada) and a Stratagene® Mx3000 Real-Time PCR system, according to the manufacturer's instructions. Primers for human CuZnSOD, MnSOD, GPx and CAT genes and of the housekeeping gene β-Actin are listed in Table 1. Real Time PCR was performed according to Sowndhararajan *et al.* (2015), and the gene relative expression levels of a were estimated using the method of Pfaffl (2001). The measurements were performed in triplicate.

Primer(s)		Nucleotide sequence
CuZnSOD	F	5'-ACGGTGGGCCAAAGGATGAA-3'
	R	5'-TCATGGACCACCAGTGTGCG-3'
MnSOD	F	5'-AGAAGCACAGCCTCCCCGAC-3'
	R	5'-GGCCAACGCCTCCTGGTACT-3'
GPx	F	5'-TCGGTGTATGCCTTCTCGGC-3'
	R	5'-CCGCTGCAGCTCGTTCATCT-3'
CAT	F	5'-CCAACAGCTTTGGTGCTCCG-3'
	R	5'-GGCCGGCAATGTTCTCACAC-3'
β-Actin	F	5'-CGGGAAATCGTGCGTGACAT-3'
	R	5'-GGACTCCATGCCCAGGAAGG-3'

Table 1. Nucleotide sequences of cDNAs coding for antioxidant enzymes in HepG2 cell line.

2.12. Evaluation of antiproliferative activity

The antiproliferative activity of all hydrophilic extracts was evaluated by MTT assay. The MTT assay is a measurement of cell metabolic activity, used to estimate cell proliferation, which is based on a protocol first described by Mossmann (1983). The cells were seeded into a series of standard 96-well plates in 100 µL of complete culture medium at 1.0×10^4 cells/cm^2. Cells were incubated for 24 h under 5% CO_2 at 37 °C and the medium was then replaced with 100 µL of fresh medium supplemented by 5% (v/v) FBS containing the treatments. Twenty-four h after seeding, 100 µL aliquots of different solutions at the appropriate concentration were added to the wells and the cells were incubated for 48 h without renewal of the medium. In each experiment, EtOH concentration never exceeded 0.25% and culture medium and 0.25% EtOH was used as control. After the incubation time, cells were washed and 100 µL FBS-free medium containing 0.5 mg/mL of MTT were added. The medium was discarded after a 4 h incubation at 37 °C and formazan blue formed in the cells was dissolved in DMSO. MTT-formazan absorbance was measured at 570 nm with a microplate reader. As the absorbance is directly proportional to the number of living, metabolically active cells, the percentage of growth (PG) with respect to

untreated cell control for each drug concentrations was calculated, according to one of the following two expressions:

$$\text{If } (OD_{test} - OD_{tzero}) \geq 0, \text{ then } PG = 100\ x\ \frac{(OD_{test} - OD_{tzero})}{(OD_{CTRL} - OD_{tzero})}$$

$$\text{If } (OD_{test} - OD_{tzero}) < 0, \text{ then } PG = 100\ x \frac{(OD_{test} - OD_{tzero})}{OD_{tzero}}$$

Where:

- OD_{tzero} is the average optical density measure before exposure of cells to the test compound;
- OD_{test} is the average optical density measure after the desired period of time;
- OD_{CTRL} is the average optical density measure after the desired period of time with no exposure of cells to the test compound.

The concentration necessary for 50% growth inhibition (GI_{50}) for each extract was calculated from a dose–response curve using the linear regression analysis to fit the test concentrations that give PG values above and below the reference value (50%). Each result was the mean value of three separate experiments performed in quadruplicate.

2.13. Statistical analyses

Statistical analyses were performed in order to assess the errors related to the analytical procedures, not to assess the internal variability among the different varieties. Data are expressed as the mean of three biological technical replicates for each lot of seeds. Three technical replicates were run for each biological replicate. ANOVA followed by Tukey–Kramer's HSD post-hoc test ($P < 0.05$) was used to determine significant differences. Principal Component Analysis (PCA) was performed by using covariant matrix of extraction and varimax rotation. All statistical analyses were performed by using the SYSTAT 10 software. The cladogram of gene sequences was performed with ClustalX software by using the Neighbour Joining (NJ) method. Bootstrap values were calculated from 100 resamplings of the alignment data.

3 – Results and Discussion

3.1. Spectrophotometric assays show different content of phytochemicals in the skin of the pistachios varieties under study.

3.1.1. Total phenolic content.

The total phenolic content (TPC) of the six pistachio varieties was quantified in both skins and nuts. The skin TPC ranged between 91.37 (± 01.04) and 363.75 (± 16.50) mg gallic acid equivalents (GAE) g^{-1} d.wt., whereas the concentration in nuts was about 500 fold lower (Table 2). Considering that skin represent about 11% total nut weight, the TPC in pistachio nuts as one or two orders of magnitude higher than in the majority of fresh fruits and vegetables (Deng, 2013; Fu, 2011). Significant differences were found among the six pistachio varieties. Concerning the skins, the highest TPC value was found in Bronte followed by Larnaka and Mawardi, while Kerman had the lowest value. Similar results have been reported by Martorana *et al*. (2013) for the TPC content in Bronte pistachio skin, and were higher than those reported for the Bronte variety by Tomaino *et al.* (2010). Same results have also been obtained for the Kerman variety (Yang, 2009). Moreover, TPC values for some Turkish pistachio varieties (Arcan *et al*., 2009) were at least six times lower than the values obtained in our study.

3.1.2. Evaluation of the total anthocyanin content.

Pistacia vera is the only nut containing anthocyanins (Bellomo and Fallico, 2007). These polyphenolic compounds are mainly stocked in the skin (Tomaino *et al*., 2010). In the present study, we quantified the total anthocyanin content (TAC) in the hydrophilic extracts of the observed six pistachio varieties by the differential pH method. This protocol exploits the pH-dependence of anthocyanin pigmentation due to a pH-dependent change in the C ring structure. TAC of skin extracts are shown in Table 2. Significant differences were found among the six observed genotypes. The highest value was recorded in Bronte, followed by Larnaka. The other observed genotypes showed lower values. Although Mawardi showed a TPC value about twice than Kern and Mateur, its anthocyanin content was comparable to Kern and lower than Mateur. Finally, the moderately high correlation between TAC and TPC ($\rho = 0.86$), suggests an important but not exclusive contribution of anthocyanin derivatives to TPC. On the other hand, no

anthocyanins were detected in the seed flash. Our results are consistent with previously reported data (Bellomo and Fallico, 2007; Liu *et al.*, 2014; Seeram *et al.*, 2006).

3.1.3. Evaluation of the total proanthocyanidin content.

PACs are the major polyphenol compounds in hydrophilic extract from Bronte pistachio nut (Gentile, 2007) and they play a major role as bioactive component of this extract in *in vitro* inflammatory models (Gentile, 2012; 2015). Nowadays, several different procedures, including colorimetric, gravimetric, chromatographic, and mass spectrometric methods, are used to quantify PACs in different raw materials, such as cranberry (Cunningham *et al.*, 2002; Gu *et al.*, 2003; Hammerstone *et al.*, 1999) and cocoa (Hammerstone *et al.*, 1999). However, due to the complexity of the PACs structure and to the different type of linkages, the results can often be erroneous or not reproducible. In order to evaluate the real content of total PACs, both skin and seed extracts were assayed by the colorimetric BL-DMAC assay. DMAC, reacting almost exclusively with compounds having simultaneously a free meta-oriented hydroxyl groups in the flavonoid molecule and a single bond at the 2,3-position of the C ring, yields more accurate measurements (Prior *et al.*, 2009). The highest contents of total PACs were measured in skin extracts, meanwhile seed flash extracts showed no-detectable PACs (Table 2).

	TPC		TPACs		TAC	
Variety	**Seed flesh**	**Seed Skin**	**Seed flesh**	**Seed Skin**	**Seed flesh**	**Seed Skin**
Bronte	1.55 (± 0.08)^{a}	363.75 (± 16.5)^{a}	n.d.	177.57 (± 0.40)^{a}	n.d.	27.31 (± 1.11)^{a}
Kerman	1.93 (± 0.03)^{b}	91.37 (± 1.04)^{b}	n.d.	88.51 (± 2.71)^{b}	n.d.	2.84 (± 0.12)^{b}
Larnaka	1.74 (± 0.04)^{c}	334.64 (± 15.41)^{c}	n.d.	155.09 (± 3.63)^{c}	n.d.	24.24 (± 0.24)^{c}
Kern	0.24 (± 0.01)^{d}	140.91 (± 11.6)^{d}	n.d.	54.48 (± 0.45)^{d}	n.d.	6.34 (± 0.36)^{d}
Mateur	0.18 (± 0.01)^{d}	181.55 (± 5.07)^{e}	n.d.	95.20 (± 3.35)^{b}	n.d.	9.79 (± 0.64)^{e}
Mawardi	0.18 (± 0.02)^{d}	290.28 (± 5.82)^{f}	n.d.	159.69 (± 2.35)^{c}	n.d.	6.74 (± 0.37)^{d}

Table 2. Total polyphenolic content (TPC), total proanthocyanidins content (TPACs) and total anthocyanin content (TAC) of some pistachio varieties of different geographical origin. Mean values are expressed as mg g^{-1} d.wt. (±SD). For each column, different letters indicate significant ($P \leq 0.05$) differences.

Among the skin extracts, Bronte showed the highest value followed by Mawardi and Larnaka, while Kern showed the lowest content. Kerman and Mateur showed about half of the Bronte content. Moreover, a strong correlation between PACs content and TPC (ρ=0.986) suggests that PACs are the major contribution to the TPC.

3.2. Polyphenols and fatty acid profiles support the correct discrimination of the different varieties of *Pistacia vera.*

3.2.1. HPLC-DAD-MS/MS analysis.

Chromatographic analysis performed by liquid chromatography, revealed the presence of 12 different phenolic compounds in pistachio skin extracts. Figure 1 shows the chemical structure of the identified phenolic compounds. Overall, only small differences were detected in the qualitative profile whereas a quantitative significant difference was found among varieties. In general, the most abundant compound was cyanidin-3-glucoside (**1**), followed by idein (**2**), eriodictyol-7-glucoside (**3**), eriodictyol-7-galactoside (**4**) and catechin (**5**). Other common compounds were peonidin-3-glucoside (**6**), hyperoside (**7**), quercetin-3-glucoside (**8**), and quercetin-4'-glucoside (**9**). Luteolin-glucoside (**10**) and marein (**11**) were absent in Mawardi and Larnaka varieties, whereas okanin 4'-O-galactoside (**12**) was absent in the variety Kern (Table 3).

Compound	RT	$[M-H]^-$ / $[M-H]^+$	m/z	λ	Varieties					
					Bronte	Kerman	Larnaka	Kern	Mateur	Mawardi
Eriodictyol-7-galactoside	16.4	449	288	360	71.71 (± 1.14)^a	43.67 (± 0.53)^b	366.67 (± 9.46)^c	135.14 (± 4.68)^d	116.9 (± 5.54)^e	88.28 (± 2.87)^f
Idein	18.7	449	286	520	1885.06 (± 23.58)^a	90.58 (± 1.72)^b	1774.73 (± 39.88)^c	661.66 (± 16.5)^d	739.3 (± 12.47)^e	416.85 (± 9.44)^f
Cyanidin-3-glucoside*	18.7	449	286	520	5297.52 (± 109.31)^a	737.62 (± 12.57)^b	5063.01 (± 97.01)^c	2219.66 (± 46.64)^d	2515.56 (± 45.92)^e	1675.92 (± 24.66)^f
Eriodictyol-7-glucoside	22.7	449	288	360	1194.42 ±(27.91)^a	168.71 (± 1.11)^b	1116.88 (± 22.44)^c	425.76 (± 3.61)^d	562.18 (± 17.40)^e	347.34 (± 8.05)^f
Peonidin-3-O-glycoside*	23.9	463	301	520	120.03 (± 3.56)^a	23.46 (± 0.64)^b	244.31 (± 7.07)^c	103.06 (± 1.72)^d	82.32 (± 5.42)^e	21.23 (± 4.22)^b
Catechin	25.0		289	280	1298.14 (± 35.78)^a	230.05 (± 5.68)^b	1931.68 (± 45.81)^c	172.61 (± 2.38)^b	204.57 (± 8.42)^b	2144.88 (± 22.11)^d
Okanin 4'-O-galactoside	26.8	449	288	280	325.42 (± 6.48)^a	66.55 (± 20)^b	398.75 (± 4.10)^c	n.d.	67.53 (± 2.46)^b	180.57 (± 4.72)^d
Hyperoside	26.8	463	302	360	314.47(± 6.27)^a	82.35 (± 1.72)^b	533.95 (± 5.3)^c	131.47 (± 3.2)^d	151.8 (± 6.36)^e	131.85 (± 4.07)^d
Quercetin-3-O-Glucoside	29.8	463	302	360	248.6 (± 4.96)^a	96.4 (± 3.56)^b	195.04 (± 1.24)^c	139.15 (± 7.76)^d	179.48 (± 5.36)^e	89.52 (± 2.9)^b
Marein	32.8	449	288	360	221.23 (± 4.41)^a	107.91 (± 2.57)^b	n.d.	48.23 (± 0.82)^c	76.26 (± 6.32)^d	n.d.
Luteolin-glucoside	33.0	447	286	360	1029.3 (± 21.46)^a	19.24 (± 0.73)^b	n.d.	237.06 (± 6.32)^c	327.26 (± 8.75)^d	n.d.
Quercetin-4'-O-Glucoside	34.0	463	302	360	57.43 (± 0.1)^a	44.95 (± 1.08)^b	89.17 (± 2.23)^c	68.42 (± 3.21)^d	74.6 (± 4.87)^d	29.52 (± 1.70)^e

RT, retention time; λ, wavelength expressed in nm.

Table 3. Qualitative and quantitative chemical analysis of the phenolic compounds present in the seed skin of some pistachio varieties of different geographical origin. Mean values are expressed as μg g^{-1} d.wt. (± SD). Within the same line, different letters indicate significant ($P \leq 0.05$) differences.

The identified compounds belong to the flavonoid class, apart from the two Okain-glycosides (**11** and **12**) that are chalcones. Among the identified flavonoid we found two flavanones (**3** and **4**), three anthocyanins (**1**, **2** and **6**), three flavonols (**7**, **8** and **9**) and the flavone luteolin-glucoside (**10**). A similar polyphenolic profile was previously detected in hydrophilic extracts from pistachio nut skin by Fabani *et al*. (2013) and Martorana *et al.* (2010).

Concerning the quantitative profile, as detected by HPLC-DAD/MS, we found significant differences among the six pistachio varieties. The total amount of phenolic compounds exceeded 1% (p/p) in Bronte e Larnaka, while it was around 0.4% (p/p) for Kern, Mateur and Mawardi. Kerman contained a significantly lower content, less than 2%, according to data from the colorimetric assay.

The most abundant compounds in the observed skin extracts are the two form of cyanidin (**1** and **2**), Catechin (**5**) and eriodictyol-7-glucoside (**3**). These flavonoids represent about 70% by weight of the total amount of phenolic compounds in Kern and over 80% in all the other genotypes. The luteolin-glucoside (**10**) is present in high quantity in skin extract from Bronte and in discrete amounts in those from Kern and Mateur. Luteolin-glucoside (**10**) has not been identified in the extracts from Mawardi and Larnaka that, on the other hand, are also the only genotypes that do not contain the 4-O-glucoside of okain (**11**). Quercetin glycosides (**7**, **8** and **9**) are present in small amounts and the 3-O-glycosides (**7**) are more abundant than quercetin-4'-O-glucoside (**9**).

The Principal Component Analysis (PCA) calculated on the data matrix of Table 2 and Table 3 with varimax rotation explained 57.59% and 20.57% of the total variance for PC1 and PC2, respectively (Figure 2A). Positive factor scores for PC1 discriminated the Mediterranean varieties Larnaka and Bronte because of high TPC and the highest content of cyanidin-3-glucoside (**1**) and idein (**2**). Negative PC1 factors scores separated all other varieties. The varieties Kerman and Mawardi were separated by both PC1 and PC2 negative factor scores because of the low content of luteolin-glucoside (**10**), whereas the Bronte variety was separated by both PC1 and PC2 positive factor scores because of the highest TAC, TPC and TPACs values. Figure 2B shows the partitioning of the different phenolic compounds based on PC1 and PC2 factor scores.

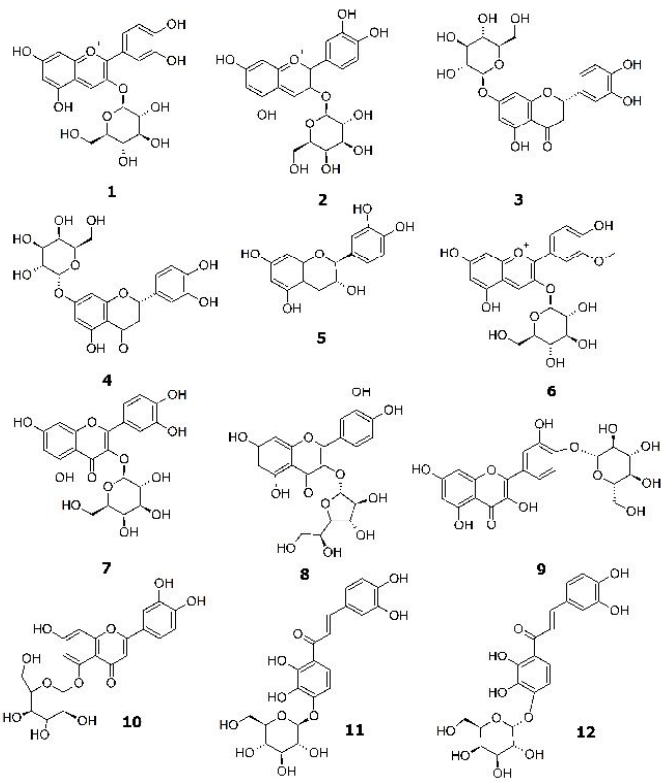


Figure 1. Structure formulae of the phenolic compounds characterizing the pistachio varieties under study.

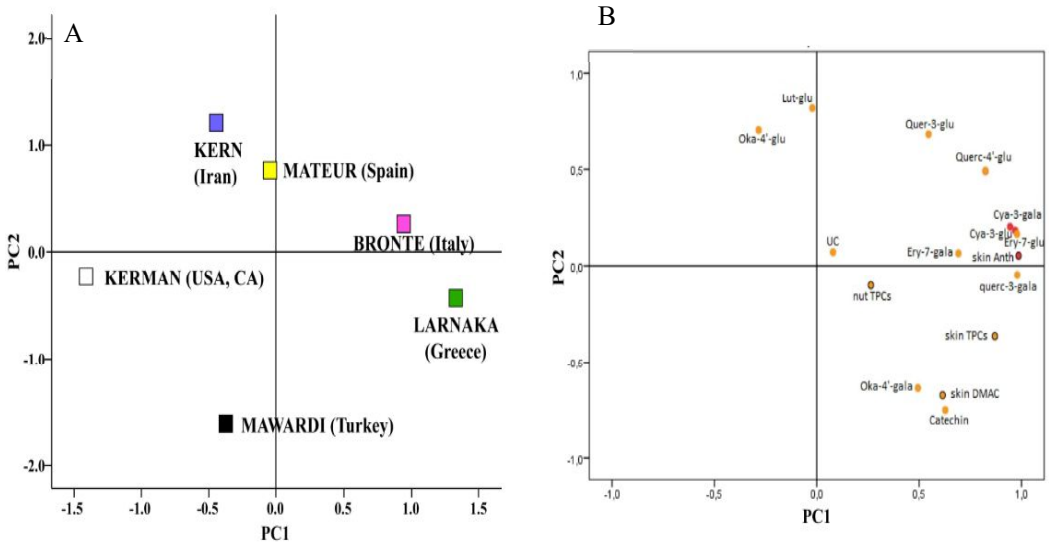


Figure 2. (**A**) Scatter plot of PC1 and PC2 factor scores calculated on phenolic compounds of the pistachio varieties using the data matrix of Tables 2 and 3. A clear separation is obtained from the Mediterranean varieties Bronte and Larnaka, the Californian variety Kerman and the other varieties. (**B**) Chemical partitioning of compounds.

On the other hand, HPLC analysis performed on skin extract after defatting by Soxhlet with *n*-Hexane, revealed the completely absence of flavonols, anthocyanins, proanthocyanidins or other polyphenolic compounds. Only an unknown compound with an absorption wavelength of 240 and 280nm and a specific pattern of fragmentation (MW=451, m/z=432.9, 335.0, 291.9 and 172.7), was found in discrete quantities in all nut extract of pistachio (Figure 3). This compound, never reported in literature form *Pistacia vera*, is currently under analysis for its chemical and structural characterization.

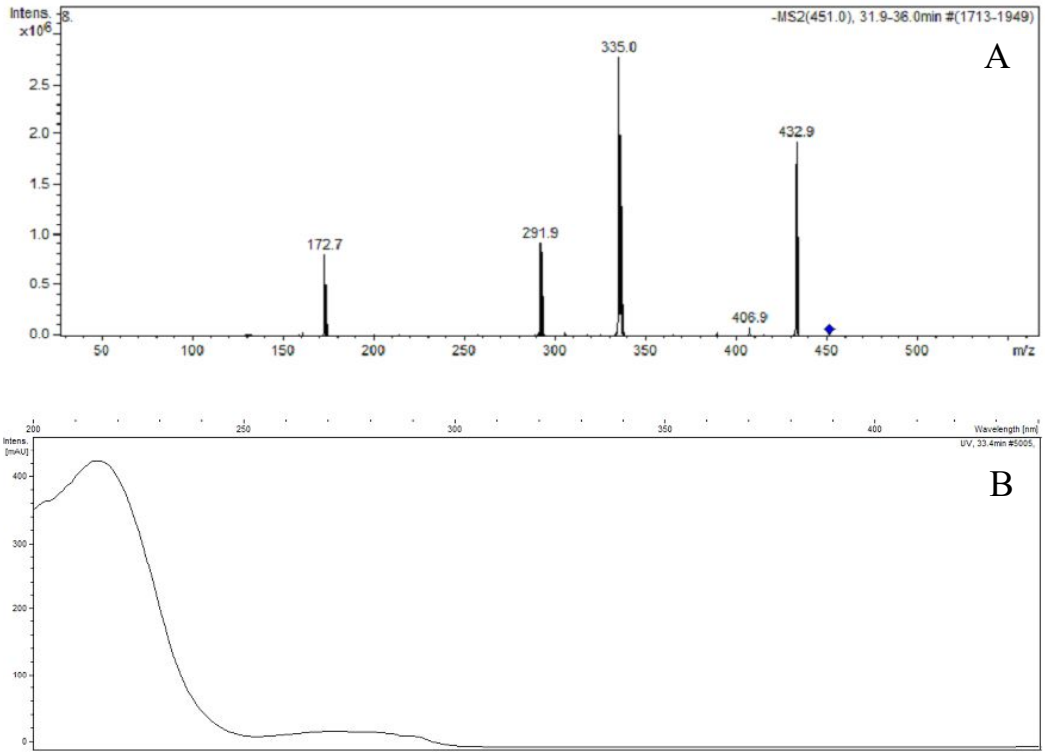


Figure 3. (**A**) Molecular weight (451) and its ion daughter (m/z) of an unknown compound not yet reported in pistachio. (**B**) UV/Vis spectra reordered with a diode array detector (DAD).

3.2.2. GC-MS-FID analysis.

The pistachio fatty acid composition has been used for the differentiation of varieties of different geographical origin (Acar *et al.*, 2008; Arena *et al.*, 2007; Aslan *et al.*, 2002; Chahed *et al.*, 2008; Rabadan *et al.*, 2017; 2018), providing useful criteria for authentication of pistachio seed origin. As expected, the fatty acid content of the six pistachio variety was mainly present in the seed flash. In general, a significantly ($P<0.05$) higher total amount of the identified fatty acids was found in the varieties Mateur, Kern and Bronte, followed by the varieties, Larnaka and Mawardi, whereas the variety Kerman showed the lowest total fatty acid amount (Table 4).

Compound		Δn	ω−n	*Bronte*	*Kerman*	*Larnaka*	*Kern*	*Mateur*	*Mawardi*
C16:1 t	**Palmitoleic acid**	**Δ9**	**ω−7**	4.83 ± 0.05a	3.88 ± 0.09c	4.88 ± 0.01a	4.74 ± 0.02a	4.53 ± 0.01b	2.43 ± 0.08d
C16:0	**Palmitic Acid**			58.58 ± 0.42c	48.24 ± 0.83d	57.41 ± 0.32c	60.92 ± 1.31b	64.74 ± 0.52a	46.14 ± 0.52e
C18:2	**Linoleic acid**	**Δ9,12**	**ω−6**	97.26 ± 2.74bc	117.48 ± 19.3b	73.06 ± 4.16c	179.08 ± 17.01a	168.72 ± 3.64a	78.92 ± 3.47c
C18:1 c	**Oleic acid**	**Δ9**	**ω−9**	431.86 ± 15.26a	245.69 ± 30.11c	408.15 ± 20.77ab	355.3 ± 26.74c	378.18 ± 9ab	384.69 ± 8.06ab
C18:1 t	**Elaidic acid**	**Δ9**	**ω−9**	16.46 ± 0.14c	13.89 ± 0.02e	14.52 ± 0.15d	18.35 ± 0.22a	17.28 ± 0.03b	12.33 ± 0.04f
c18:0	**Stearic Acid**			9.35 ± 0.06b	5.41 ± 0.05e	8.83 ± 0.07c	6.99 ± 0.05d	8.68 ± 0.06c	10.95 ± 0.03a
c18:3	**γ-Linolenic acid**	**Δ6,9,12**	**ω−6**	1.32 ± 0.03ab	1.05 ± 0.05d	1.26 ± 0.06bc	0.89 ± 0.01e	1.42 ± 0.09a	1.18 ± 0.02cd
c20:0	**Arachidic Acid**			0.72 ± 0.07ab	0.42 ± 0.02c			0.66 ± 0.01b	0.77 ± 0.01a
Total content of Fatty Acid (TFAC)				620.61 ± 15.38b	435.46 ± 0.55e	568.14 ± 4.94c	626.46 ± 8.16ab	643.58 ± 3.93a	537.05 ± 4.39d
UFA %				88.9 ± 0.73	87.72 ± 11.27	88.33 ± 3.65	89.12 ± 5.86	88.58 ± 1.44	89.29 ± 1.44
MUFA %				73.01 ± 0.25b	60.5 ± 0.12d	75.25 ± 0.1a	60.4 ± 0.3d	62.15 ± 0.55c	74.37 ± 1.25ab
PUFA %				15.88 ± 0.02d	27.21 ± 0.63b	13.08 ± 0.08f	28.72 ± 0.07a	26.43 ± 0.02c	14.91 ± 0.01e
SFA %				11.11 ± 0.13	12.28 ± 1.02	11.68 ± 0.23	10.92 ± 0.55	11.41 ± 0.44	10.71 ± 0.89
MUFAs/PUFAs				4.59 ± 0.02c	2.23 ± 0.11de	5.75 ± 0.04a	2.1 ± 0.04e	2.35 ± 0.01d	4.99 ± 0.11b
c18:1/c18:2				47.94 ± 1.33ab	47.94 ± 5.12ab	47.85 ± 1.98ab	53.43 ± 3.47a	45.55 ± 0.72b	36.25 ± 0.64c
c16:0/c16:1				12.12 ± 0.03de	12.43 ± 0.07cd	11.76 ± 0.04e	12.85 ± 0.22c	14.29 ± 0.08b	18.99 ± 0.41a

Table 4. Fatty acid composition of some pistachio varieties of different geographical origin. Mean values are expressed as mg g^{-1} d.wt. (± SD). In the same line, different letters indicate significant ($P<0.02$) differences.

In particular, the total fatty acid (TFAC) content ranged from 435.46 ± 0.55 to 643.58 ± 8.16 mg/g d.wt. Eight fatty acids were identified and quantified. Unsaturated fatty acids (UFAs) were the main fatty acids in our samples. The major fatty acid was oleic acid (C18:1ω9), followed by linoleic acid (C18:2ω6), and palmitic acid (C16:0). Oleic acid (C18:1ω9) represents over 50% of the TFAC, exceeding 70% in Mawardi, Kern and Bronte. On the contrary, the content of the other identified monounsaturated fatty acids (MUFAs), palmitoleic acid (C16:1ω9), did not exceed 1%, ranging from 0.45% (Mawardi) to 0.90% (Larnaka). The high oleic acid content accounts for the high MUFA% in all samples, by exceeding always 60%. MUFAs content in food has important nutritional implications. Indeed, several epidemiological and experimental studies demonstrated cardiovascular protection and anticarcinogenic effects of these unsaturated fatty acids (Peter, 2016; Grundy, 1989; Mukherjee, 1990). In all the analysed samples we also identified elaidic acid (trans C18:1ω9), the trans isomer of oleic acid. Several studies clearly showed that trans fatty acids increase the risk of coronary heart disease and the WHO recommends that the trans fatty acid intake does not exceed 1% of the total caloric amount (Russell E, 2016). On the other hand, we found very modest amounts of elaidic acid, ranging between 2.3% (Mawardi) and 3.2% (Kerman), in our samples. Concerning saturated fatty acids, we found considerable amount of palmitic acid (C16:0) and stearic acid (18:0). On the contrary, arachidonic acid (C20:0) was found in very low quantities and not found in Larnaka and Kern. Polyunsaturated fatty acids (PUFAs) are incorporated into membrane phospholipids where they are important to support cell membrane integrity and function.

We identified linoleic acid (C18:2ω6) and γ-linolenic acid (C18:3ω6). Linoleic acid amount is negatively correlated to oil stability because of its high susceptibility to oxidation. In Mateur, Kerman, and Mawardi linoleic acid accounts for over 25% TFAC. On the contrary, γ-linolenic acid was found in very low quantities, never exceeding 1.5 mg each g of d.wt. Similar amounts of individual fatty acids were found in pistachios grown in Turkey (Satil *et al*., 2003) and in Iran (Abdoshahi *et al*., 2011).

Together to TFAC other parameter, such as SFA%, UFA%, MUFAs%, PUFA%, and MUFAs/PUFAs, C18:1/C18:2 and C16:0/C16:1 ratios, were calculated and reported in Table 4. SFA% and UFA% did not vary significantly, ranging between 10.71 ± 0.89 - 12.28 ± 1.02 and 87.72 ± 11.27 - 89.29± 1.44 respectively. However, Mawardi variety was distinguishable from the others due to its considerably higher C16:0/C16:1 ratio. On the other hand, it was also the variety with the lowest C18:1/C18:2 ratio (19.41 and 35.62 respectively). Among UFAs, the contribution given by PUFAs ranged from 13.08% to 28.74%. Larnaka, showed the highest

MUFA%; Kerman and Kern had the highest PUFA% and were the varieties with the highest linolenic acid content. MUFAs/PUFAs ratio can be a relevant descriptor for the samples. This ratio is higher in Larnaka and Mawardi (5.80 and 5.10 respectively) than in the other varieties. Similar results have been reported for the evaluation of fatty acid compositions in the work of Dogan (2010), which showed a similar fatty acid profile for seven verities of pistachios from Turkey.

The PCA calculated on the data matrix of Table 4 with varimax rotation explained 40.95% and 32.60% of the total variance for PC1 and PC2, respectively (Figure 4A). Positive factor scores discriminated the Mediterranean varieties Larnaka and Bronte because of the higher content of oleic acid, whereas negative factors scores separated the Californian variety Kerman because of the lowest total fatty acid content. The Mawardi variety was separated by positive PC1 and Negative PC2 factor scores because of the lowest content of linoleic acid whereas *Kern* and Mateur varieties were separated by positive PC2 and negative PC1 factor scores because of similar fatty acid contents. Finally, Figure 4B shows the partitioning of the different fatty acids based on PC1 and PC2 factor scores.

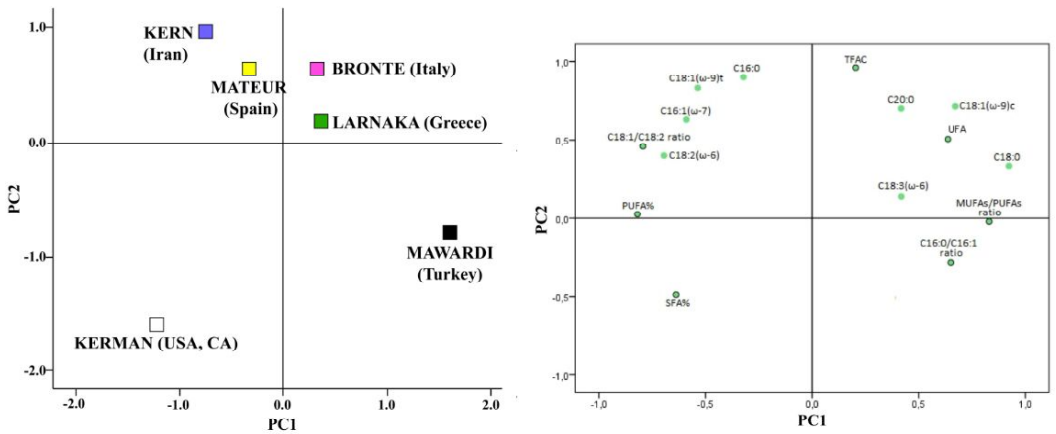


Figure 4. (**A**) Scatter plot of PC1 and PC2 factor scores calculated on fatty acids composition of the pistachio varieties using the data matrix of Table 4. A clear separation is obtained from the Mediterranean varieties Bronte and Larnaka, the Californian variety Kerman and the other varieties. (**B**) Chemical partitioning of fatty acid compounds.

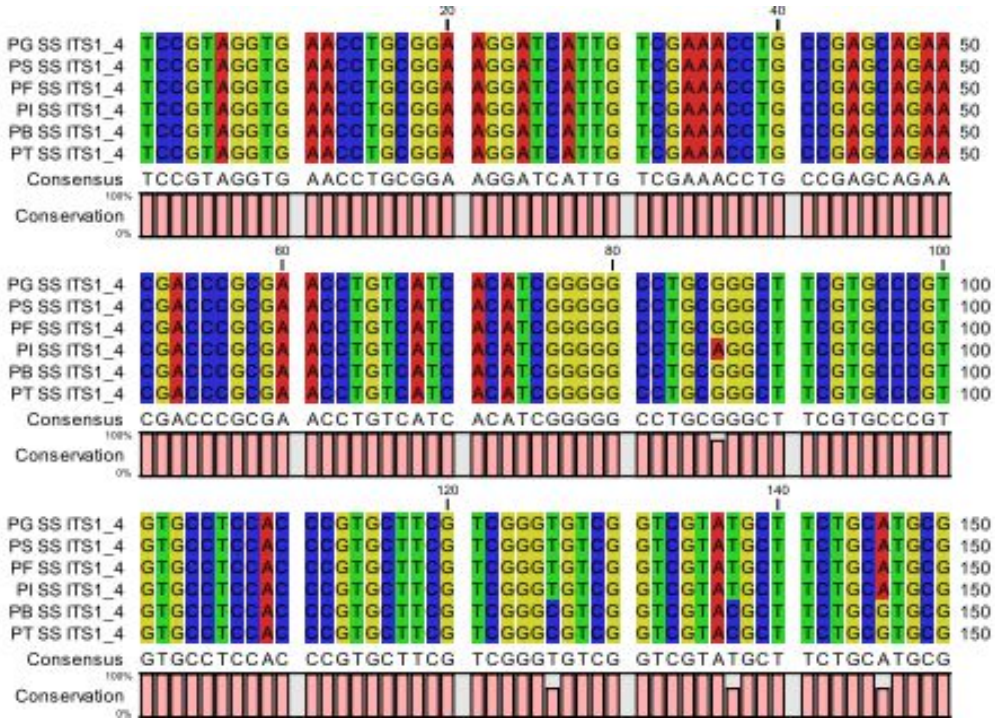
3.3. DNA fingerprinting using PCR-RFLP analysis reveals significant differences in pistachio

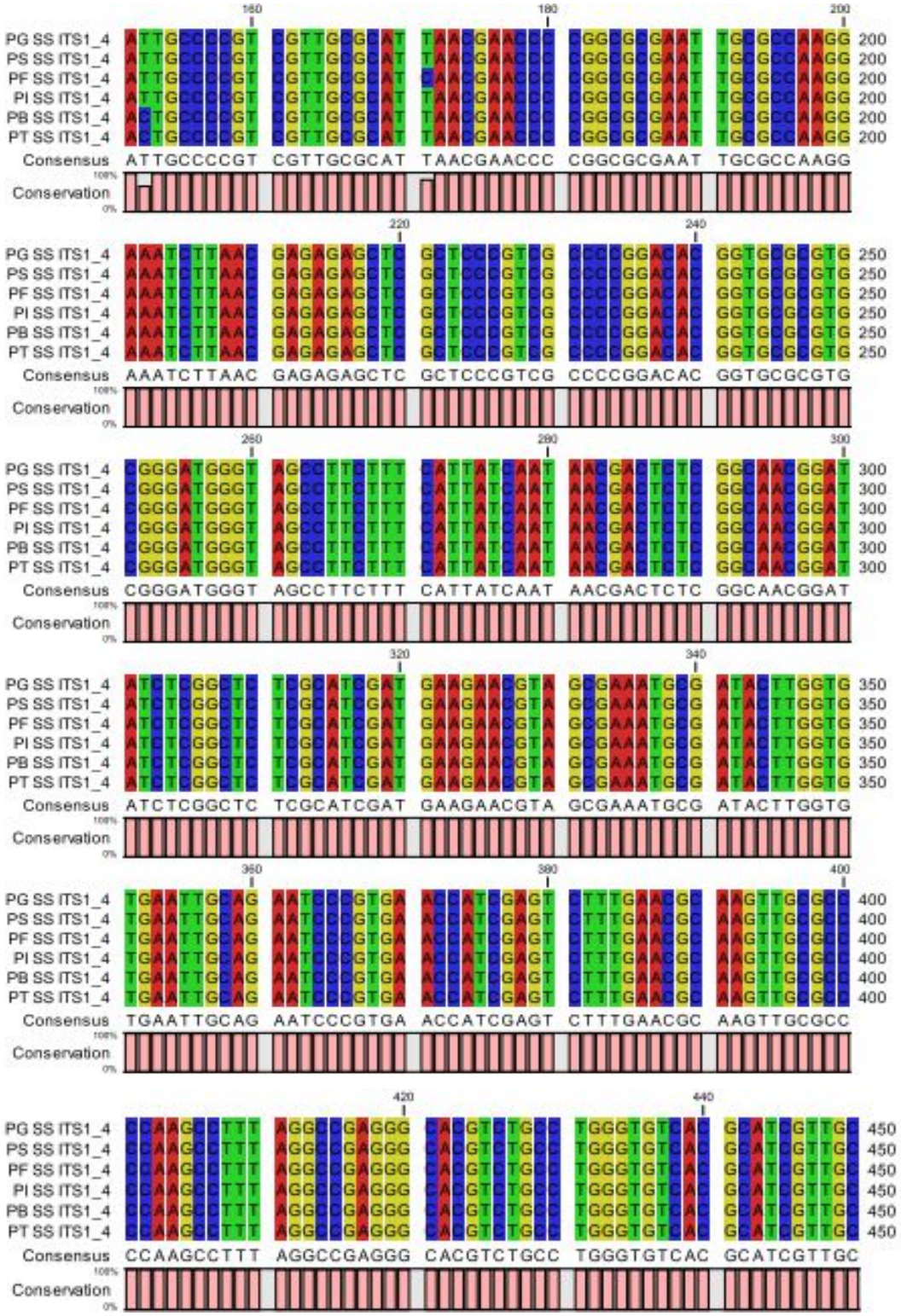
In order to provide a molecular fingerprinting of the six pistachio varieties, ITS-1 coupled with ITS-4 was used for PCR amplification. These sequences are well conserved in higher plants,

showing changes in nucleotides quality and/or quantity of closely related species (Cheng *et al.*, 2016). Figure 5 shows the nucleotide sequence of the ITS regions of the six varieties.

The ITS amplified sequences were 722bp long (Fig. 6 lanes 1-6) (NCBI GenBank Accession Nos: **MH444649**, ITS1-4 Bronte; **MH444689**, ITS1-4 Kerman; **MH444724**, ITS1-4 Kern; **MH444735**, ITS1-4 Larnaka; **MH444780**, ITS1-4 Mateur; **MH444793**, ITS1-4 Mawardi) and the alignment of the six verities sequences shows that 98.75% of the sites are conserved. In particular, out of the 1.25% variable sites, 0.83% provide little information and 0.42% are singleton sites. The ITS fragments were compared by BLAST alignment to other sequences deposited in GeneBank, and the analysis provided a match almost identical to *P. vera* (Sequence ID: AY677201.1) with a 99% identity query score.

In order to better characterize the varieties showing DNA fragments of similar size, a PCR–RFLP method was applied. Three different restriction enzymes (*RsaI*, *TaqαI* and *PstI*) were used to selectively cleave the resulting amplicons. From the identified sequences, a *TaqαI* site could be selectively found in the varieties Kerman (Fig. 6 lane 7), Larnaka (Fig. 6 lane 8) and Mateur (Fig. 6 lane 9), giving five fragments of 76, 86, 90, 185 and 280 bps.

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PF SS ITS1_4
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PB SS ITS1_4
PT SS ITS1_4
Consensus
Conservation
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CGGGATGGGT AGCCTTCTTT CATTATCAAT AACGACTCTC GGCAACGGAT
ATCTCGGCTC TCGCATCGAT GAAGAACGTA GCGAAATGCG ATACTTGGTG
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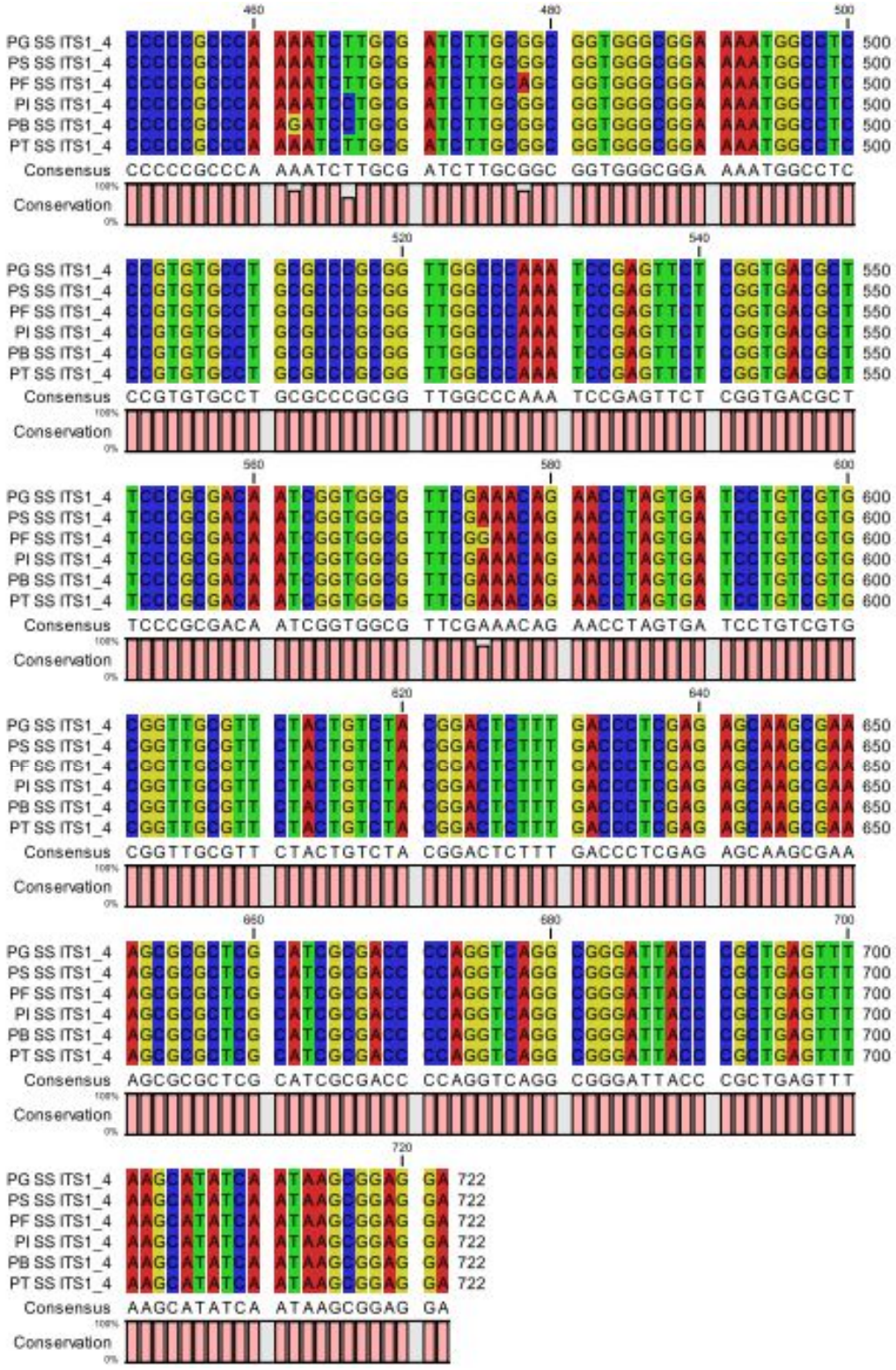

Figure 5. Nucleotide sequence of the ITS regions of the six varieties of *Pistacia vera*.

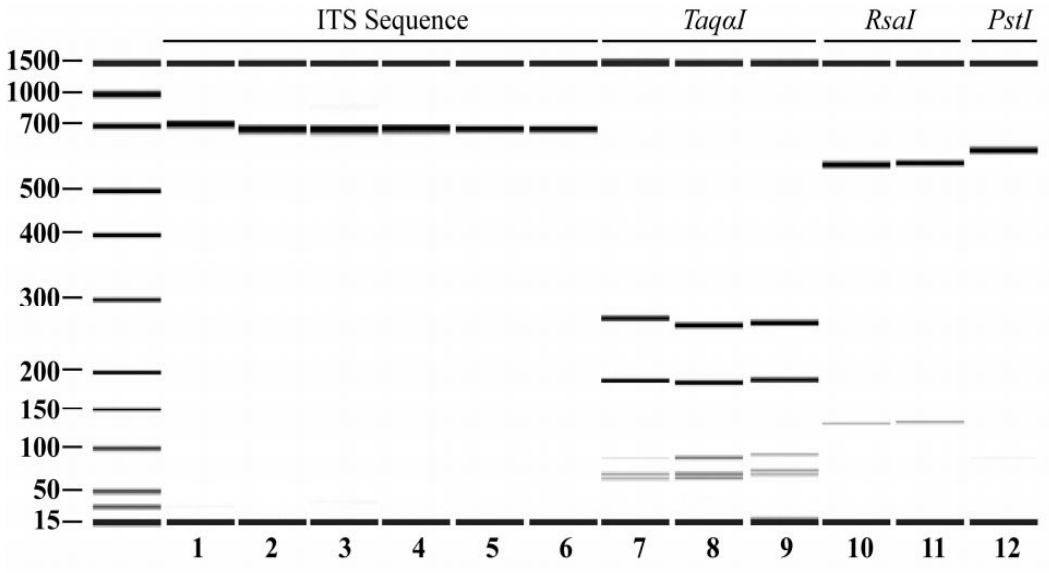

Figure 6. PCR products after capillary gel electrophoresis analysis of the ITS region of some *Pistacia vera* varieties of different geographical origin. Whole ITS sequence of Bronte (lane 1), Kerman (lane 2), Larnaka (lane 3), Kern (lane 4), Mateur (lane 5) and Mawardi (lane 6) varieties. All sequences have a length of about 720 bp. PCR–RFLP analysis using TaqαI pistachio digested PCR products produces five fragments of 75, 85, 90, 185 and 280 bp in Kerman (lane 7), Larnaka (lane 8) and Mateur (lane 9) varieties. Digestion of the PCR products from RsaI restriction enzyme activity on *Bronte* (lane 10) and Mawardi (lane 11) gives two fragments of 180 and 550 bp. Digestion of the PCR products from PstI produces two fragments of 90 and 630 bp on the Kern (lane 12) variety. L = bp markers. The PCR products were separated by using the Agilent 2100 Bioanalyzer and the DNA 1000 LabChip Kit (Agilent Technologies).

Digestion of the PCR products by *RsaI* gave specific patters exclusively on Bronte (Fig. 6 lane 10) and Mawardi (Fig. 6 lane 11) variety sequences, by producing two fragments of 182 and 550 bps. Finally, PCR products from the different varieties were digested by *PstI*, which produced two fragments of 92 and 630 bps exclusively on the Kern variety (Fig. 6 lane 12). These results show that it was possible to differentiate among the six varieties investigated, not exclusively by chemical characterization, but also by fingerprinting analysis.

The sequences were further analyzed by the neighbour joining (NJ) method to infer phylogenetic relationship among the pistachio varieties. Figure 7 shows the phylogenetic tree where the Mawardi and Bronte varieties and Mateur and Larnaka form independent clusters, which robustness is supported by high bootstrap scores. Our data are in agreement with DNA-RAPD markers on *P. vera* phylogenetics (Hormaza *et al.*, 1994).

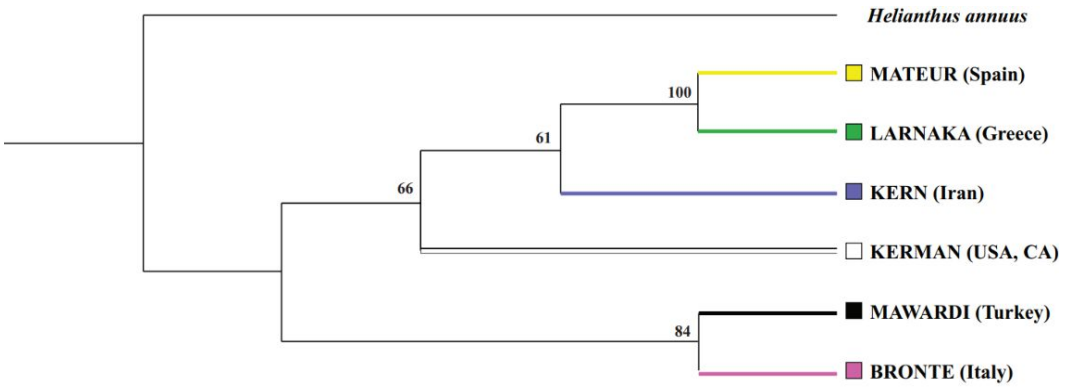

Figure 7. Cladogram of gene sequences performed with ClustalX software by using the Neighbour Joining (NJ) method of some *Pistacia vera* varieties of different geographical origin. A close phylogenetic relationship is present between the Mediterranean Mateur and Larnaka varieties. These two varieties are phylogenetically related to Kern and Kerman varieties. A close relationship is found between Bronte and Mawardi varieties. Bootstrap values were calculated from 100 resamplings of the alignment data.

3.4. Cellular antioxidant activity shows a strong correlation with the total polyphenol and anthocyanin contents.

The oxidative stress results from the imbalance between reactive oxygen species and the cellular ability to neutralize them (Benavente-García *et al.*, 1997). The resulting oxidative damage concurs to the pathogenesis of several human diseases, including cardiovascular and neurodegenerative diseases and cancer. One of the biological methods used to evaluate the antioxidant activity (AOA) of natural extracts is the Cellular Antioxidant Activity assays (CAA). AOA estimated by CAA is the result of different kind of mechanisms, which encompass a reduction of the initial oxidative stress. Indeed, phytochemicals present in extracts can either directly interact with free radicals or regulate both the gene expression and the activity of antioxidant enzyme, such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX). We used the CAA assay to evaluate the antioxidant properties of both skin and seed extracts. Whereas seed flash extracts did not show any AOA, CAA_{50} values calculated for skin extracts ranged from 33.40 ± 1.04 µg FW / mL to 136.72 ± 2.11 µg FW / mL medium (Figure 8). These values are similar to those determined by Wolfe *et al.* (2008) from fresh fruits extracts. The highest activity was recorded for Bronte and Larnaka, the lowest for Kern. Regression analyses revealed a strong correlation between CAA_{50} and TPC ($\rho = -0.82$). We found a very strong correlation between CAA_{50} values and TACs ($\rho = 0.97$). In this regard, it should be noted that the total amount of anthocyanins as well as the content of the three anthocyanin derivatives identified in skin extracts is very similar for Kern and Mateur extracts while it is lower in Kerman.

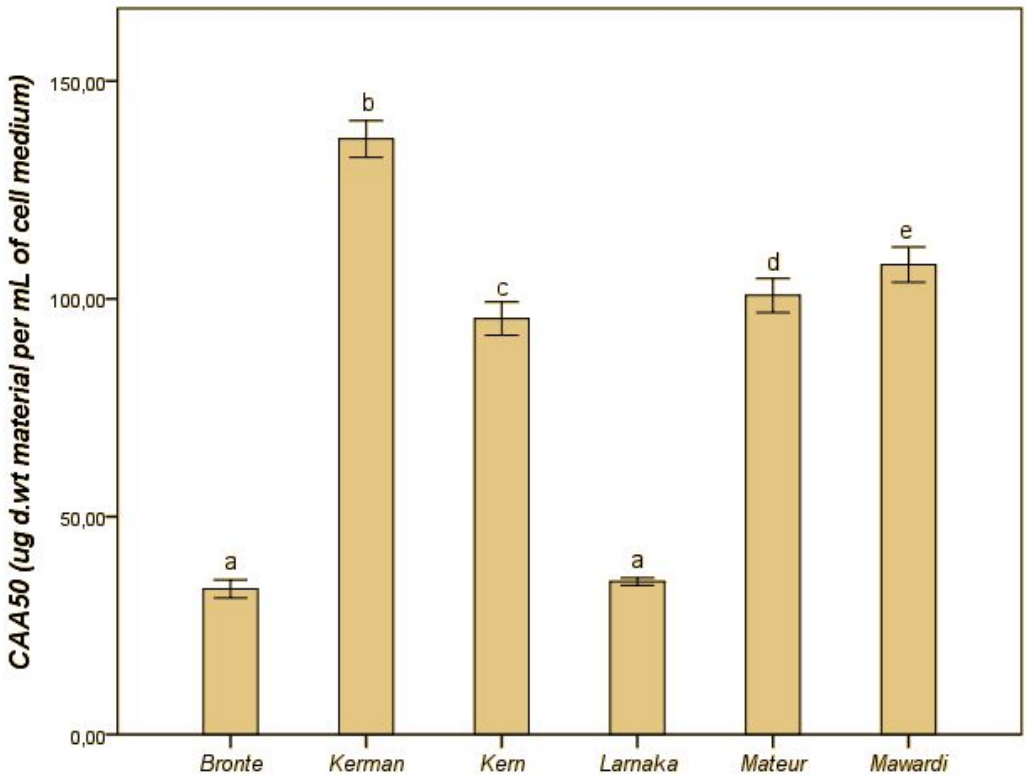

Figure 8. Cellular Antioxidant Activity (CAA) of pistachio seed skin. CAA_{50} is expressed as µg d.st material per mL of cell medium. Different letters indicate significantly different at $p \leq 0.05$ as measured by Tukey's multiple range test.

3.5. Phytochemicals of pistachio skin extracts reduce oxidative stress by both biochemical and biomolecular ways.

The antioxidant activity evaluated by CAA can be the result of free radical reduction by both direct oxidation of phytochemicals and though catalysis operated by antioxidant enzyme (AOE), such as SOD, CAT and GPX.

In order to understand the possible mechanism of action of the skin phytochemicals, we assessed biomolecular and biochemical assays. The antioxidant potential of a plant extract is usually assessed by the use of multiple methods (Ou *et al.*, 2002; Wootton-Beard *et al.*, 2011; Wu *et al.*, 2004), because results from different essays may help to elucidate the mechanisms involved in the observed antioxidant activities. In this study, we used three different assays (FRAP, ABTS and DPPH) to assess the radical scavenging activities and the metal-reducing potential of the six pistachio extracts. However, these assays do not generate radicals and do not take into account the ability of antioxidant compounds to interact with DNA or other compounds, with the

consequent promotion of gene expression of AOE or enhancing of their enzymatic activity. Due to this fact, we also decided to evaluate the expression of genes coding for SOD, CAT and GPX by qRT-PCR, and the EA of the same enzymes by spectrophotometric assays.

3.5.1. Antioxidant activities is mainly associated to the TPACs.

The radical scavenging activity and ferric reducing antioxidant power of the six skin extracts of pistachios were respectively evaluated by DPPH/ABTS and FRAP. Table 5 reports the data obtained from these assays.

	ABTS	DPPH	FRAP
Bronte	288.91±7.13^{a}	456.16±15.02^{a}	181.73±2.21^{a}
Kerman	78.88±2.67^{b}	164.44±2.12^{b}	65.42±0.63^{b}
Larnaka	207.26±10.52^{c}	340.41±2.64^{c}	140.41±2.71^{c}
Kern	138.03±7.74^{d}	158.95±1.46^{b}	39.52±0.75^{d}
Mateur	199.32±10.26^{c}	329.81±14.29^{c}	73.16±2.28^{e}
Mawardi	233.89±10.98^{e}	389.33±10.58^{d}	149.81±3.21^{f}

Table 5. Radical scavenging activity (ABTS and DPPH) and Ferric reducing antioxidant power (FRAP) of the observed six extracts of pistachio skin. Result are expressed as mmol TE per 100g of d.wt material, and the values represented as mean ± SD. Different letters indicate significantly different at $p \leq 0.05$ as measured by Tukey's multiple range test.

In general, the DPPH assay provided higher antioxidant values when compared to ABTS and FRAP assays. Moreover, while data obtained by ABTS and DPPH assays were strongly correlated (p= 0.960), a lower correlation was found between the two assays and FRAP (ρ = 0.848 with ABTS and ρ = 0.907 with DPPH assays). DPPH assay is more suitable for samples with a higher lipophilic antioxidant content (Ozgen *et al.*, 2006). Regardless of the antioxidant method used, Bronte showed always the highest antioxidant capacity. Moreover, a lower correlation between CAA_{50} and the radical-scavenging assays was observed (CAA50/DPPH, ρ = -0.63; CAA50/ABTS, ρ = - 0.72; CAA50/FRAP, ρ = -0.67).

The antioxidant capacity is tightly correlated to the phenolic content of plant extracts (Piluzza and Bullitta, 2011; Sricharoen *et al.*, 2015; Zheng and Wang, 2001). We found that the antioxidant capacities of pistachio extracts were highly correlated with their TP values (TPC vs. ABTS, ρ = 0.920; TPC vs. DPPH, ρ = 0.908; and TPC vs. FRAP, ρ = 0.950). Interestingly, FRAP values showed a very strong correlation (ρ = 0.992) with the total PAC content. On the contrary, TAC showed a lower correlation with all tested antioxidant assays ($0.70 > \rho < 0.75$). Therefore, the highest antioxidant capacity showed by pistachio extracts could be mainly associated to the total PAC content and to a lesser extent to the TAC content.

3.5.2. Skin extracts promote the gene expression of MnSOD and GPX.

Experimental evidence shows that dietary polyphenols can stimulate both the transcription of antioxidant-related genes and the detoxification defence systems through antioxidant responsive elements (ARE) (Masella *et al.*, 2004; 2005). HepG2 cell line is actually used in several experimental protocols to test oxidative stress because show functional characteristics similar to normal hepatic cells, including the normal and induced gene expression of antioxidant enzymes. Superoxide anion is one the most active ROS, which is generated by the incomplete reduction of molecular oxygen in the mitochondria and by the action of the plasma membrane NADH/NADPH oxidases. Therefore, superoxide anion scavenging by SOD is extremely important. Dismutation of the superoxide anion by SOD generates H_2O_2, which in turn is reduced by CAT or GPX to water. Animal cells express two intracellular isoform of SODs: MnSOD, localized in the mitochondria matrix, and the cytosolic CuZnSOD (Valentine *et al.*, 2005).

We evaluated the effect of pistachio extracts on the expression of *CuZnSOD, MnSOD, CAT* and *GPX* genes by qRT-PCR in HepG2 cells subjected to the pro-oxidant action of H_2O_2. The target genes were not expressed in control HepG2 cells. Oxidative stress induced by treatment with 200 μM H_2O_2 exerted a significant upregulation of *CAT* and a stronger downregulation of *MnSOD* and *GPX*, whereas the expression of *CuZnSOD* was not regulated (Figure 9).

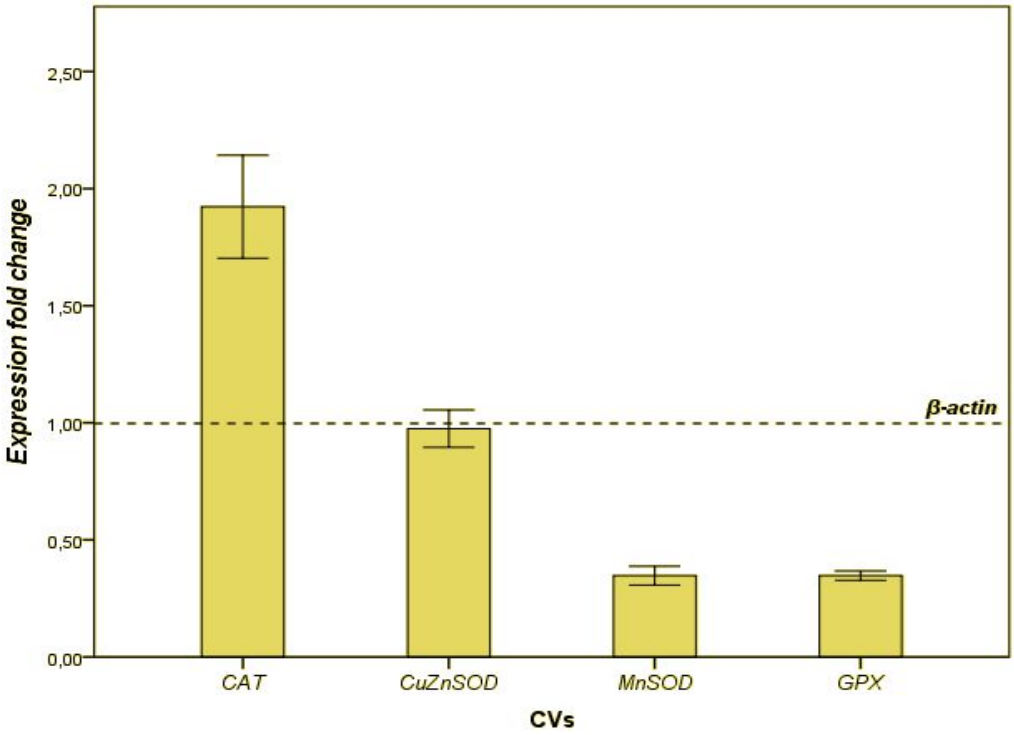

Figure 9. Effect of oxidative stress induced by treatment with 200 µM H_2O_2 on the gene expression of *CAT*, *CuZnSOD*, *MnSOD* and *GPX* in HepG2 cells. Data represent means ± SD of the expression fold change of each gene.

Pistachio extracts added to the cell medium and stressed with H_2O_2 induced a strong up-regulation of *MnSOD* and *GPX*, whereas a lower up-regulation of *CuZnSOD* and *CAT* was observed (Figure 10). A direct comparison of data from Figure 9 and Figure 10 shows that *CAT* expression was not modulated by the different pistachio extracts, whereas extracts from the varieties Bronte, Kerman, Kern and Mawardi showed a small but significant upregulation of *CuZnSOD*.

Our results also show significant differences among the extracts of the different pistachio varieties. Concerning *MnSOD*, the highest upregulation was found in Kerman followed by Kern and Larnaka. A lower, although consistent, upregulation was found for the remaining varieties.

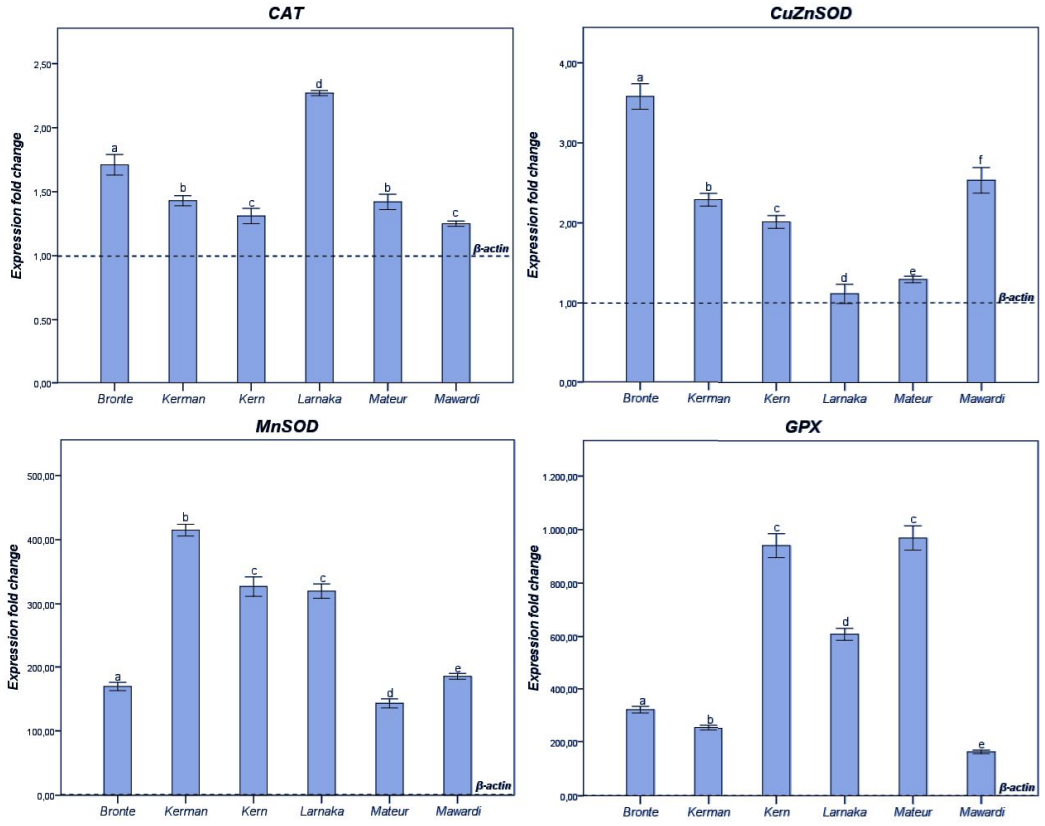

Figure 10. Quantitative real-time PCR determination of the indicated antioxidant enzyme after treatment with the observed six extracts of pistachio skin. Data represent means ± SD of the expression fold change of each gene. Different lowercase letters indicate significantly different at P≤ 0.05 as measured by Tukey's multiple range test.

3.6. Skin and seed extracts of pistachio displayed high antiproliferative activity against three different kind of tumour cell lines.

Consumption of fruits and vegetables has been highly associated with the reduced risk of cancer and a number of experimental data show cancer-preventive properties of several phytochemicals (Manach, 2015). In the present study, we assessed the anticancer potential of both skin and seed flash of pistachio. The antiproliferative effect of the six *P. vera* ethanolic extracts against human cervical cancer cell line (HeLa), human epithelial colorectal adenocarcinoma (Caco2) and human liver cancer cell line (HepG2) was evaluated using the MTT bioassay. This assay detects the reduction of the yellow dye MTT by mitochondrial succinate dehydrogenase to a formazan blue product, which reflects the normal functioning of mitochondria and hence the cell viability (Mosmann, 1983). Moreover, considering a gastrointestinal volume of 600 mL, the concentrations of each extract were consistent with amounts that can be obtained at the gut level after dietary ingestion of 30-50 g pistachios.

When the tumour cells were exposed to both skin and seed extracts the proliferation decreased significantly in a dose-dependent manner. Significant differences were found both among the different varieties and between skin and seed flash extracts. The IC_{50} values of each extracts on the proliferation of examined tumour cells are reported in Figure 12. In general, the antiproliferative activity (APA) of skin extracts was higher when compared to seed flash extracts, with a higher activity against Caco2 cells and a lower activity against HepG2 cells. Moreover, among skin extract series, a resilient correlation between AOA and APA on HepG2 cell line ($\rho_{DPPH/APA}$=-0.901; $\rho_{ABTS/APA}$=-0.829; $\rho_{FRAP/APA}$=-0.709) and especially with TPC (ρ=-0.748) was found. On the other hand, APA on HeLa cell line showed a strong correlation with CAA_{50} (ρ=-0.877). The sensitivity of HeLa cells to different extracts was quite different, with GI_{50} ranging from 50.23 to 250.73 mg dw.t/mL medium. This value is comparable to that measured, in similar experimental conditions, for extracts from other fruits against the same cell lines (Allim, 2009).

Even though the growth inhibitory effects of the extract rich in polyphenols on tumour cells, due to anthocyanins, proanthocyanidins and flavonols, have been largely investigated (Chow, 2002; Malta, 2014), our data suggest that the sole correlation between AOA and APA, is not sufficient to explain the antiproliferative activity of fruits extracts (Gentile, 2016; Tomaino 2014; Sun *et al.*, 2014). Indeed, specific components present also in little concentrations may play a particular biochemical and/or biomolecular role. Therefore, further identification of the possible mechanism of action is currently under study.

Finally, in all tumour cell lines tested, an interesting correlation between APA and an unknown compound identified in seed flash extracts was found (ρ=-0.874). For this reason, after the structure characterization by NMR and IR, our future aim is the chemical synthesis and the evaluation of the bioactivity of the pure and isolated compound.

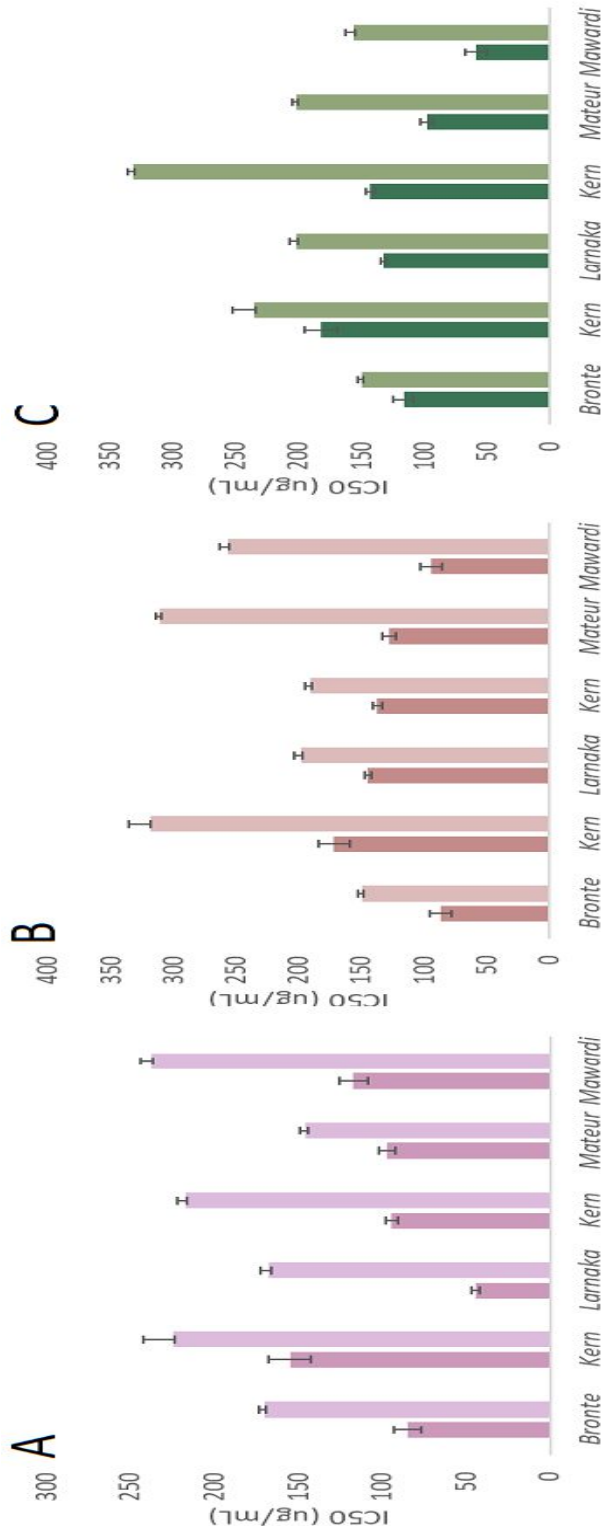

Figure 12. Antiproliferative activities (APA) of skin (darker colour series) and seed (lighter colour series) evaluated on HeLa (**A**), Caco2 (**B**) and HepG2 (**C**) cell lines. Data are express as IC_{50} (µg/mL) ± SD.

4 – Conclusions

The combination of DNA analysis and phytochemical analyses is increasingly used to provide new tools for the unequivocal identification of plants. The stability of DNA fingerprinting is a solid method that supports the chemical partitioning. Despite some controversy exists over the value of DNA barcoding, largely because of the perception that this method would diminish rather than enhance traditional morphology-based taxonomy, an increasing number of gene sequences is now available for DNA barcoding of flowering plants (Cheng *et al.*, 2016).

In this part of the thesis I showed that different varieties of pistachio, a plant with a high food value and phytochemical potential, show a remarkable variability, both at the genomic and gene products (phenolic compounds and fatty acids) levels. By using both molecular and chemical data it is possible to partition the different pistachio varieties according to their geographical origin. In particular, the Mediterranean varieties (Mateur, Bornate and Larnaka) show similar chemical patterns and (in the case of Mateur and Larnaka) a close phylogenetic relationship. Owing to the increased interest and relevance of *P. vera* as a food plant and as a source of interesting phytochemicals with pharmaceutical properties, the identification of bioactive phenolic compounds and specific gene sequences by PCR-RFLP described in this work offers a valuable tool for a rapid and unequivocal identification of pistachio varieties of different geographical origin.

Moreover, our study showed that pistachios skin, actually considered by manufacturing industry as a waste product, are an interesting source for several phytochemicals that may act as antioxidants. Data obtained from the reported experiments suggests that these compounds not only are able to reduce free radicals through a radical scavenging or metallic ion chelating mechanisms, but also promote the gene expression of SOD, CAT and GPX. Finally, the AOA measured showed a good correlation with APA on three different tumour cell lines (HeLa, Caco2 and HepG2).

Part II.

Quantification of bioactive compounds in *Boswellia* species.

1 – State of Art

1.1. Origin, description and traditional use of *Boswellia* species

The Burseraceae family consists of 17 genera and about 500 different species. Most of them contain latex, gum-resins or oils, which are rich in volatile organic compounds (VOCs). The origin of this family of plants is to be found in tropical regions, and with time they extended into the subtropical regions. The gum resins of several species of this family are of considerable commercial value as raw material of incense and balm (Friis *et al.*, 1987) (Fig. 13).

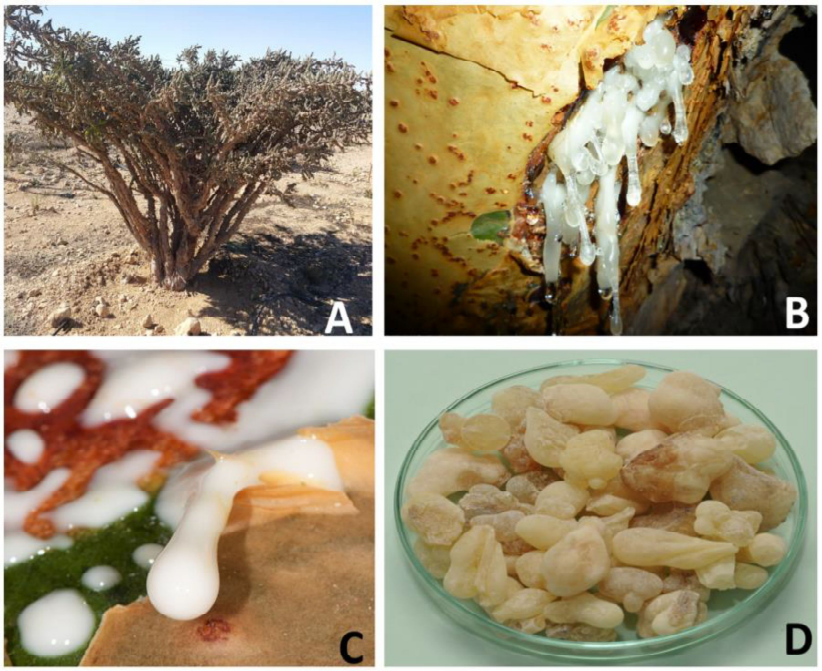

Figure 2. (**A**) Resin extraction from *B. sacra* tree. (**B**) *Boswellia* milky substance is oozing out after the first incision. (**C**) the milky of frankincense after a few days of incision. (**D**) *Boswellia* gum resin as final product of frankincense

Among Burseraceae, *Boswellia* species are trees or shrubs known since antiquity for their interesting pharmacological action (Michie and Cooper, 1991). Between December and May, the pale whitish resin is either scraped off the tree with an iron implement or collected on palm mats on the ground as it drips off (Marshall, 2003). The gum resin of the different species of *Boswellia* contains more than 200 phytochemicals, and its content and composition may vary from species to species, depending on age, geographical and environmental conditions. The gum resins of *Boswellia* species contain terpenoid compounds, such as pentacyclic and

tetracyclic triterpenes, which are commonly considered to be responsible of its pharmacological effects (Al-Harrasi *et al.*, 2013; Gerbeth *et al.*, 2011).

1.2. Phytochemistry and beneficial properties of *Boswellia* gum resins

Chemically, the main constituent of *Boswellia* species are monoterpenes and sesquiterpenes (Niebler and Buettner, 2014), diterpenes including incensole, incensole acetate and cembrenol (serratol) (Pollastro *et al.*, 2016), lipophilic pentacyclic triterpene acids of the oleanane-(α-boswellic acids), ursane-(β-boswellic acids) and lupane-type (lupeolic acids), as well as an ether-insoluble fraction containing polysaccharides (arabinose, galactose, xylose) *(Herrmann et al.*, 2007). The six major BAs are α-BAs and β-BA, their acetylated forms (Acetyl-α-BAs and Acetyl-β-BA), 11-keto-β-Boswellic acid (KBA) and 3-O-acetyl-11-keto-β-Boswellic acid (AKBA). These organic acids are present in all *Boswellia* species but in varying quantities depending to verities (Shah *et al.*, 2009). Among all the BAs, the two most active, potent and promising anti-inflammatory agents are AKBA and KBA, that due to their lipophilic nature have relatively poor absorption through the gastrointestinal tract, but a very long retention (Abdel-Tawab *et al.*, 2011; Bagul *et al.*, 2014). The action of AKBA and KBA, and in general of BAs, depends on the interaction with a variety of targets, such as 5-lipoxygenase (5-LO), topoisomerases, angiogenesis, cytochrome p450 enzymes and protein kinases (MAPK). The reported pharmacological effects range from anti-inflammatory (Poeckel and Werz, 2006) and anticancer activity (Casapullo *et al.*, 2016), to hypolipidemic (Zutshi *et al.*, 1986), hypoglycemic (Shehata *et al.*, 2015) and immunodulatory activity (Pungle *et al.*, 2003).

1.3. Common problems with the identification and quantification of boswellic acids

Actually, the analysis of boswellic acids (BAs) is performed by different analytical methods including High Performance Thin Layer Chromatography (HPTLC) (Krohn *et al.*, 2001), although the most used methods are based on HPLC coupled to both photodiode array detection (Büchele *et al.*, 2003) and mass spectrometry detection (Frank and Unger, 2006). In accordance with the spectral properties of the BAs, their analysis is performed at three different wavelengths, 210 nm for αBAs, βBAs as well as lupeolic acid, 250 nm for AKBA and KBA, and 280 nm for 9,11-dehydro-α- and -β-boswellic acids (Büchele *et al.*, 2003). However, a precise identification and quantification of BAs is usually obtained with liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) by using selected ion monitoring (SIM) detection (Frank and Unger, 2006).

In general, the total organic acids from *B. serrata* and *B. sacra* constitute approximately 65%–70% by weight of the total alcoholic extract. Of this fraction, approximately 25% is made of triterpenes. In market products, these percentages are often misinterpreted and it is not unusual to find claims of 70% boswellic acids content or 30% AKBA content, which is obviously misleading. Since both *Boswellia* extracts are used in several formulations, especially in dietary supplement preparation, it is important to express unequivocally the "real content" of boswellic acids in both *B. serrata* and *B. sacra*. Therefore, the aim of this part is to provide a robust instruction that can guide from the extraction process to the accurate identification and quantification of BAs in the most used *Boswellia* species, *B. serrata* and *B. sacra.*

2 – Materials and Methods

2.1. Plant Material and Chemicals

Boswellia serrata Roxb. and *Boswellia sacra Flueck* gum resins were purchased from Bauer S.r.l. (Udine, Italy). The origin of *B. sacra* samples was from Ethiopia, whereas *B. serrata* samples originated from India. The gum resins were milled to coarse powder and used for all extractions. All chemicals were of analytical reagent-grade unless stated otherwise. Pure standards were purchased for the quantification by external calibration curves: 11-Keto-β-boswellic acid, α-Boswellic acid and β-Boswellic acid (ExtraSynthese, Lyon, France), 3-O-Acetyl^{-1}1-keto-β-Boswellic Acid (Merck, Darmstadt, Germany), 3-O-Acetyl-α-boswellic acid, 3-O-Acetyl-β-boswellic acid (Sigma-Aldrich, St. Louis, MO. USA).

2.2. Solvent extraction of *Boswellia serrata* and *Boswellia sacra* gum resin

One hundred grams of ground *B. serrata* and *B. sacra* oleo gum-resins were extracted with 1 L methanol (VWR International, Radnor, PA, USA) (extraction ratio 1:10 w/v). Samples were then placed on an orbital shaker for 5 days in the dark. Extracts were then filtered and the resin was rinsed with 400 mL of methanol. To evaluate the recovery of analyzed compounds, the exhaust gum resin was re-extracted with methanol as previously described. Samples were then concentrated by vacuum evaporation (Rotavapor, Büchi, Flawil, Switzerland). Concentrated extracts were then dried in a ventilated oven at 70 °C for 4 h. The powdered extracts were stored at room temperature in the dark until chemical analysis. Extractions were performed in triplicate.

2.3. Isolation and quantification of BAs by HPLC-DAD-ESI-MS/MS

Boswellic acids were identified and quantified by liquid chromatography (1200 HPLC, Agilent Technologies, Santa Clara, CA, USA) equipped with a reverse phase column, Luna C18 (3 µm, 150 mm × 3.0 mm, Phenomenex, Torrance, CA, USA). *B. serrata* and *B. sacra* powdered extracts were dissolved (30 mg·mL^{-1}) in HPLC-grade methanol and properly diluted. The binary solvent system was: (A) MilliQ H_2O (Millipore, Billerica, MA, USA): Methanol 50:50 containing 5 mM ammonium acetate (Sigma-Aldrich, USA); and (B) Methanol:1-Propanol (VWR International, Radnor, PA, USA) 80:20 containing 5 mM ammonium acetate. The chromatographic separation was carried out at constant flow rate (200 µL·min−1) with the following conditions: linear gradient from 30% to 50% of B in 2 min, then 80% of B in 35 min, then at 47 min B concentration was raised to 98%. The concentration of solvent B was

maintained at 98% for 6 min. The initial mobile phase was re-established for 10 min before the next injection. The temperature of well plate autosampler G1377A was set 4 °C while chromatography was carried out at constant temperature (30 °C) controlled by an Agilent 1100 HPLC G1316A Column Compartment.

Tandem mass spectrometry analyses were performed with a 6330 Series Ion Trap LC-MS System (Agilent Technologies, USA) equipped with an electrospray ionization source (ESI) operating in negative mode. The flow rate of nitrogen was set 325 °C and 5.0 L·min−1, while the Capillary Voltage was 1.5 kV. Helium was used as a collision gas.

Identification of *Boswellia* oleo gum resin compounds was performed by scan analyses with a 50–750 m/z scan range and by monitoring the absorption at 210, 250 and 280 nm. Quantitative analyses were performed by Multiple Reaction Monitoring (MRM) by monitoring the fragmentation of quasi-molecular ions for αBA and βBA and KBA (Table 1) and by Diode Array Detector (DAD) at 250 nm for AKBA and 210 nm for A-αBA and A-βBA. Quantification was performed by external calibration curves with pure standards dissolved in HPLC grade Methanol.

2.4. Validation by ME, LOD and LOQ paramters

Limit of Detections (LOD) and Limit of Quantifications (LOQ) for each compounds were determined as described by Ich (2005). Briefly, LOD were determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be reliably detected, meanwhile LOQ were calculated by the analysis of an appropriate number of blank samples and calculating the standard deviation of these responses.

Moreover, in order to evaluate the matrix effect in the quantification of target compounds, *B. serrata* and *B. sacra* powdered extracts were dissolved (30 mg·mL^{-1}) in HPLC-grade methanol and properly diluted. These sample solutions were used to prepare the calibration curves in the presence of other extracted gum resin compounds (Villagrasa *et al.*, 2007). The slope of standard curves obtained with the solvent (methanol) and in the extracts were used to compare the %ME.

$$\%ME = \frac{Calibration\ slope\ of\ sample}{Calibration\ slope\ of\ standard} x100$$

100% of ME percentage indicates no matrix effect; %ME < 100% indicates ionization suppression; %ME > 100% indicates ionization enhancement.

2.5. Statistical analysis

ANOVA followed by Tukey–Kramer's HSD post-hoc test ($P < 0.05$) was used to determine significant differences among data series. All statistical analyses were performed by using the SYSTAT 10 software.

3 – Results and Discussion

3.1. Identification of BAs

The total yield for *B. sacra* methanolic extract was 598.88 $g \cdot kg^{-1}$ (±11.40 $g \cdot kg^{-1}$) gum resin dry weight whereas the yield for *B. serrata* was 549.78 $g \cdot kg^{-1}$ (±29.31 $g \cdot kg^{-1}$) gum resin dry weight. The total recovery of methanolic extracts from *B. sacra* was 99.17% (±1.93%), whereas the total recovery from *B. serrata* was 98.96% (±1.97%). Our findings indicate that the total content of the lipophilic extracts (excluding the polysaccharide moiety of both species) never exceeded 60%, in agreement with previous works (Büchele *et al.*, 2003; 2005; Frank and Unger, 2006). By considering that the methanolic fraction contains several lipophilic compounds, including mono-, di- and triterpenes, it is evident that a claim of 70% BAs is not sustainable, being the BAs only a portion of the total methanolic extract.

In order to define the content and to identify the BAs present in the two *Boswellia* methanolic extracts, we performed HPLC-DAD-ESI-MS/MS analyses. Several BAs were present in both methanolic extracts. Table 6 reports the molecular mass and the fragmentation pattern of compounds identified in the methanolic extracts. Whereas, Figure 14 shows the chemical structure of the identified BAs. The identification of these compounds was achieved by both mass spectrometry and comparison with pure standards.

In both species, the main BAs were represented by AKBA, KBA, αBA, βBA, acetyl-αBA (A-αBA) and acetyl-βBA (A-βBA), in accordance with the literature data (Krohn *et al.*, 2001; Shah *et al.*, 2009; Siddiqui, 2011).

	Compounds	RT (min)	$[M-H]^-$ (*m/z*)	Ions (*m/z*)	Occurrence in	
					B. sacra	*B. serrata*
1	11-Keto-Ursolic acid	24	469	451, 407, 391	x	x
2	11-Keto-β-boswellic acid	25.8	469	451, 407, 391	x	x
3	3-*O*-Acetyl-11-keto-β-Boswellic Acid	28.8	511	451, 361	x	x
4	3-α-Hydroxy-7,24-dien-tirucallic acid	34.5	455	453, 437, 373		x
5	3-α-Hydroxy-8,24-dien-tirucallic acid	36.5	455	453, 437, 373	x	x
6	3-β-Hydroxy-7,24-dien-tirucallic acid	39.4	455	453, 437, 373		x
7	3-*O*-Acetyl-oleanolic acid	41	497	479, 437	x	x
8	Lupeolic acid	41.6	455	437, 409		x
9	3-*O*-Acetyl-ursolic acid	43.3	497	479, 437	x	x
10	α-Boswellic acid	44.5	455	437, 409, 377	x	x
11	β-Boswellic acid	45	455	437, 409, 377	x	x
12	3-*O*-Acetyl-α-boswellic acid	52	497	459, 437	x	x
13	3-*O*-Acetyl-β-boswellic acid	52.9	497	459, 437, 395	x	x

Table 6. Molecular mass and fragmentation pattern of compounds identified in the methanolic extracts of *B. sacra* and *B. serrata*.

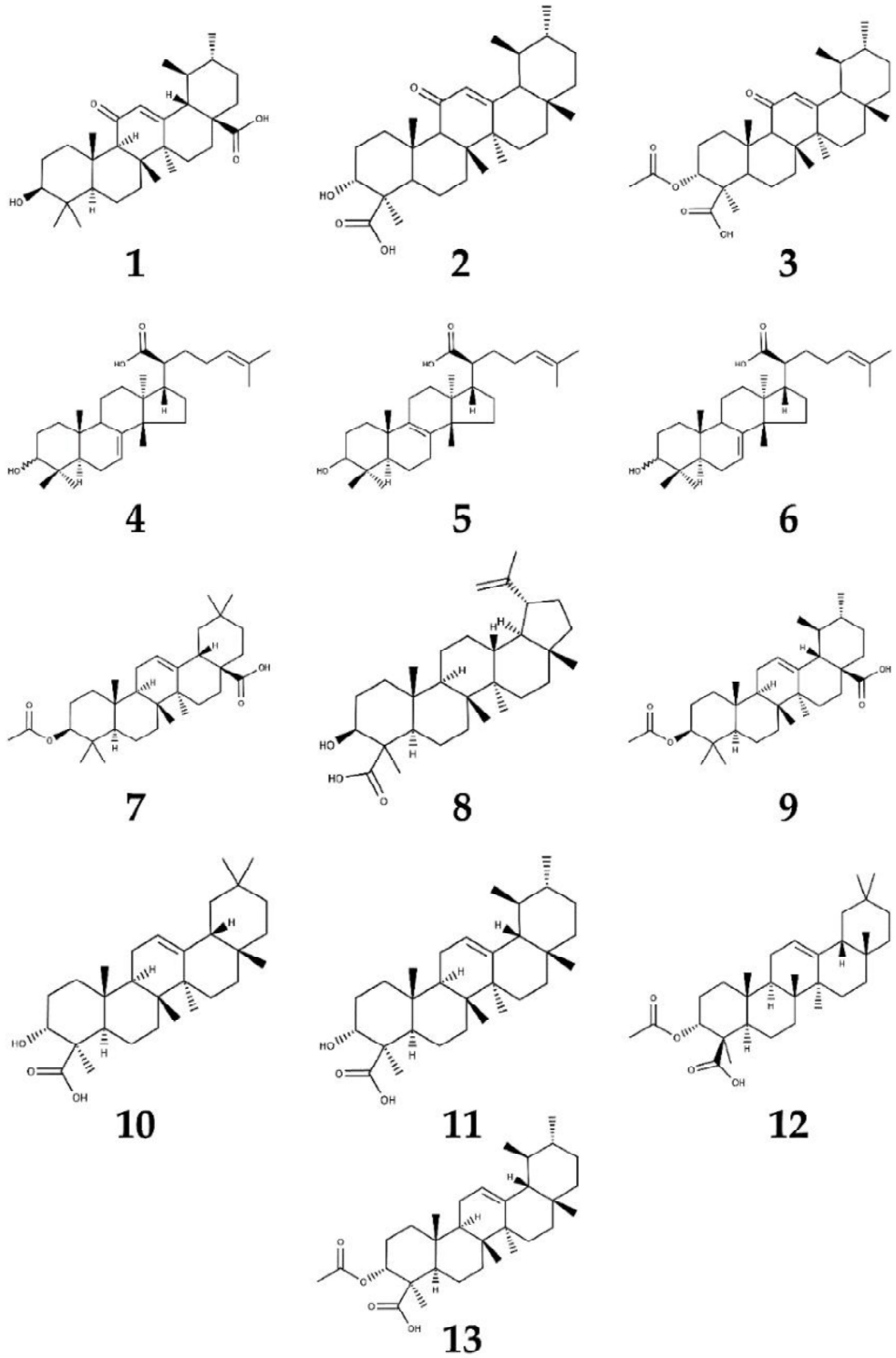

Figure 3. Chemical structure of the boswellic acids identified in *Boswellia serrata* and *Boswellia sacra*. Numbers correspond to compounds listed in Table 6.

3.2. Quantification of BAs

Table 7 shows for both species the quantitative determination of the main BAs. The total amount of the main BAs was statistically ($p < 0.05$) higher in *B. sacra* than in *B. serrata*. In *B. sacra*, the total amount of the main BAs in the methanolic extract was lower than 50% and this value was reduced to about 29% when the amount was considered in terms of the total gum resin dry weight. In *B. serrata* the total content of the main BAs in the methanolic extract was lower than 30% and the value dropped to 16% when BAs were calculated in terms of the gum resin dry weight. In both species, the major BAs were represented by αBA and βBA, in agreement with literature data (Krohn *et al.*, 2001; Shah *et al.*, 2009; Siddiqui, 2011). A direct comparison between the two species shows that the contents of *B. sacra* AKBA (about 10 fold), αBA (1.5 fold) and βBA (1.6 fold) were statistically ($p < 0.05$) higher than in *B. serrata*.

3.3. Validation of the method

In order to validate the quantitative analyses reported in Table 7, we calculated the linearity and precision of the identified BAs standard curves, the detection limit (LOD), the quantification limit (LOQ) and the Matrix Effect (ME). Table 8 shows the validation results for the main identified BAs. All compounds showed a high R^2 value, which indicates a high linearity in the calibration curves. The lowest LOD and LOQ values were found for A-βBA, followed by equal values for KBA and AKBA. The highest LOD and LOQ values were found for A-αBA. In order to complete the validation process, we assessed the ME, to assure that precision, selectivity and sensitivity were not compromised during HPLC-ESI-MS/MS analyses. The absolute ME was calculated by comparing the slope of matrix-matched standard curve with the slope of the standard calibration curve, according to (Villagrasa *et al.,* 2007). Table 8 reports, for each species, the ME accuracy (expressed as percent values) of the main identified BAs. In *B. sacra* extracts, KBA (CV = 14.06) and A-βBA (CV = 11.61) showed the highest percentage of accuracy, followed by AKBA (CV = 7.53) and A-αBA (CV = 1.90). βBA (CV = 8.72) showed the lowest ME accuracy percentages. In *B. serrata*, the highest percentages of accuracy were found for αBA (CV = 0.22), followed by KBA (CV = 14.13), A-αBA (CV = 2.66) and AKBA (CV = 7.74).

Compound	Regression Equation	R^2	LOD μg·μL^{-1}	LOQ μg·μL^{-1}	ME *B. sacra*	ME *B. serrata*
KBA	y = 13891880514x + 180215436.7	0.973	0.006	0.020	100.67 (14.17)	98.83 (13.96)
AKBA	y= 24732.09x − 143.76	0.997	0.006	0.021	99.91 (7.53)	91.53 (7.08)
αBA	y = 1349052089x + 8552264.58	0.989	0.008	0.028	88.52 (9.75)	139.80 (0.31)
βBA	y = 1549451236x + 1749204.16	0.995	0.013	0.042	73.40 (6.40)	77.25 (7.11)
A-αBA	y = 9915.15x − 127.16	0.995	0.017	0.058	93.27 (1.77)	92.91 (2.47)
A-βBA	y = 9179.53x − 101.22	0.995	0.003	0.010	100.23 (11.64)	90.70 (8.74)

Table 7. Validation of boswellic acids (BAs) quantitative analyses of methanolic extracts from *B. sacra* and *B. serrata*. (Standard deviation). R^2, coefficient of determination; LOD, Detection limit; LOQ, Quantification limit; ME, matrix effect, expressed as percentage of accuracy.

Compound	*Boswellia sacra*		*Boswellia serrata*	
	Content in the Methanolic Extract	Content in the Gum Resin	Content in the Methanolic Extract	Content in the Gum Resin
KBA	35.50 (1.26) ^{a}	21.26 (0.76) ^{b}	34.62 (2.57) ^{a}	19.03 (1.41) ^{c}
AKBA	70.81 (4.66) ^{a}	42.41 (2.79) ^{b}	7.35 (0.89) ^{c}	4.04 (0.49) ^{d}
αBA	184.34 (11.27) ^{a}	110.40 (6.75) ^{b}	126.00 (7.77) ^{b}	69.27 (4.27) ^{c}
βBA	186.19 (4.98) ^{a}	111.50 (2.98) ^{b}	113.21 (7.37) ^{c}	62.24 (4.06) ^{c}
A-αBA	5.40 (0.35) ^{a}	3.23 (0.21) ^{b}	2.92 (0.19) ^{b}	1.60 (0.11) ^{c}
A-βBA	9.95 (0.23) ^{a}	5.96 (0.14) ^{b}	11.33 (0.28) ^{a}	6.23 (0.15) ^{b}
Total	491.20 (14.75) ^{a}	294.77 (8.83) ^{b}	295.25 (23.00) ^{b}	162.29 (12.64) ^{c}

Table 8. Quantitative determination of boswellic acids in *Boswellia sacra* and *Boswellia serrata* by High Performance Liquid Chromatography-Diode Array Detector coupled to ElectroSpray Ionization and tandem Mass Spectrometry (HPLC-DAD-ESI-MS/MS), by using calibration curves from pure standards. Data are expressed as g·kg−1 gum resin dry weight. (Standard deviation), in the same row, different letters indicate significant ($p < 0.05$) differences.

Finally, Table 9 shows the recovery of the identified BAs from *B. sacra* and *B. serrata* methanolic extracts. In both species, the total recovery was higher than 98%. In *B. sacra*, the highest recovery was found for AKBA and αBA, whereas in *B. serrata* the highest recovery was found for A-βBA and A-αBA (Table 9).

Compounds	*B. sacra*	*B. serrata*
KBA	96.53	99.06
AKBA	99.68	96.88
αBA	99.39	99.14
βBA	98.75	97.13
A-αBA	88.71	99.38
A-βBA	91.34	99.84
Total	98.68	98.41

Table 9. Percentage of recovery of identified BAs from *B. sacra* and *B. serrata* methanolic extracts. Values are expressed as percentage of recovery.

4 – Conclusions

Boswellia sacra and *Boswellia serrata* extracts are widely used in pharmaceutical and nutraceutical preparations, and their bioactivity is based on the content of BAs. Like for traditional medicines, also for dietary supplements the dose of bioavailable BAs is central to the issue of *Boswellia* efficacy.

Due to absence of analytical protocol, the extraction, isolation and quantification of these organic compounds is object of several misinterpretations. Indeed, is very commonly found on the market, products with claims of 70% BAs or even 30%–40% AKBA. Result obtained from this work confirms that the BAs content never exceeds 50% of the methanolic extract, whereas lower percentages are obtained when BAs are expressed in terms of the gum resin weight. Moreover, the highest percentage of AKBA found in *B. sacra* was below 8%.

Only analytical methods based on HPLC coupled to mass spectrometry allow the precise quantification and identification of BAs in *Boswellia* extracts, whereas other methods based only on HPLC or spectrophotometric methods do not sufficiently allow an accurate quantification of BAs. Therefore, we recommend LC-MS technology for BAs determination and quantification.

Part III.

Pharmacological effects of bioactive compounds in *in vivo* model system: modulation of biogenic amines and behaviour in *Myrmica scabrinodis* ants.

1 – State of Art

1.1. Ant sociality and their tasks in the colony system

Sociality is the main trait characterizing insects like honeybees, ants and termites. Thanks to the complex organization of their community, social insects are considered the most widespread and ecologically dominant animals worldwide, being present in almost all ecological niches. This success can be identified both in the strict organization of the colonies and in the reproductive and ergonomic division of labour and group action.

The order *Hymenoptera* is the most representative group of eusocial animals thanks to over 19.000 species divided in *Formicidae* and *Apoidea* families (~14.000 and ~5.000 species, respectively). Nevertheless, *Formididae* can be considered the most ecologically relevant family. Eusociality in ants is characterized by a strict division in castes of a colony, usually made of many thousands of individuals and a large scale organization of work. In a typical colony life cycle, the winged gyne, or virgin queen, leaves the nest and mates with winged males (whose only task is to breed with the queen). Afterwards, the queen establishes a nest in which to rear the first generation of workers using the energy gained by metabolizing the wings and wings muscles. From this moment, the only task of the queen is to generate the offspring, meanwhile all other duties (nursing, nest maintenance, foraging, and defence) are conducted by workers. In the vast majority of species, workers are monomorphic (there is no morphological difference between individuals), so task specialization may be linked to colony needs, interactions with other nestmates and the environment, age (Seid and Traniello, 2006) or even genetic architecture. In polymorphic species the differentiation in workers tasks may be coupled with morphological differences. For example, large-bodies individuals (called majors) are involved in defence-like activities, territory control etc., and meanwhile male individuals with thin winged bodies have The function of breeding with the queen and generating an offspring, without having any operative duties within the nest.

1.2. Ant behaviour is affected by environment factors

The social organization of ants is based on communication signals, which coordinate large numbers of individuals in collective-decision processes, without a centralised control (Schmid-Hempel, 2002). Although some decisions are individually made, the majority of choices influencing the colony success are performed cooperatively. The decision-making occurs in the selection of the most appropriate food in terms of amount and quality (Beckers *et al.,* 1990), of

the best migration or foraging route (Franks and Fletcher, 1983; Goss *et al.,* 1989), of the most suitable place where to build a new nest (Franks *et al.,* 1992; Theraulaz *et al.,* 1999) as well as in the decision to attack, or not, enemies or competitors (Adams, 1990; Hölldobler, 1981). This peculiar trait of eusocial organisms, together with the ability to regulate the number of individuals performing specific tasks (division of labour), enhance the colony plasticity to react promptly against biotic and abiotic variations (Robinson 1992).

At the same time, however, other organisms that have evolved adaptations to exploit it for their own advantages could also target this coordinated behaviour. The chemical signalling of plants or other organisms can manipulate and influence the decision-making of social insects (Holldobler, 1983; Hölldobler, 1983). For instance, several plants belonging to distinct related families use the multifarious chemical composition of their extrafloral nectar to modify ant behaviour, resulting both in increasing the plant attractiveness for the ant and in playing a significant positive impact on the survivor, growth and reproduction of ants (Apple and Feene,r 2001; Byk and Del-Claro, 2011).

1.3. The mechanism of the social manipulation in insects

How the manipulation of social activities is achieved is not fully disentangled and the mechanism is likely to vary in distinct systems. However, a role of biogenic amines (BAs) in driving behavioural changes of both the vertebrates (Wunderlich *et al.,* 2012; Yu *et al.,* 2014), and invertebrates (Huber *et al.,* 1997; Libersat and Pflueger, 2004; McQuillan *et al.,* 2012; Riemensperger *et al.* 2010), including ants (Kamhi and Traniello, 2013; Smith *et al.,* 2013; Szczuka *et al.,* 2013) has been demonstrated. Surveys on ants are still in their infancy, yet some supporting evidence was provided at least for two species. In colonies of the Red Imported Fire Ant, *Solenopsis invicta*, the loss of the queen causes a reduction in the nest-mate recognition of workers, which is a fundamental eusocial trait related to the colony identity (Barbero 2016; Vander Meer *et al.,* 2008). This lower ability in discriminating between members and strangers is determined by a decrease in the brain level of the octopamine. In *Pheidole* ants, another crucial social activity as the cooperative foraging is affected by a depletion in the serotonin titre that modulates the trail-following behaviours of workers (Muscedere *et al.,* 2012).

In interspecific interactions, functional variations in the neurogenic system directly causing a manipulation of the behaviour have been pinpointed in the astonishing case of insects parasitized by hairworms. The infected individuals are driven to suicide by jumping into an aquatic environment, which is essential to accomplish the parasite life cycle. The suicidal instinct is given by a slight day/night variation of dopamine level caused by the production of

undetermined molecules that alters the normal function of the grasshopper's central nervous system (Biron *et al.,* 2005; Thomas *et al.,* 2003).

The behavioural manipulation, however, does not necessarily lead to the death of the individual. The exocrine secretions produced by the caterpillars of *Narathura japonica*, a Lycaenid butterfly, contribute to foster the interaction with the host ant, *Pristomyrmex punctatus* (Hojo *et al.,* 2015). In addition to nutritional substances, by sipping the secretion attendance ants receive some bioactive compounds that are able to reduce their locomotor activities and to increase their aggressiveness. Therefore, the droplets emitted by the dorsal nectary organ of caterpillar are not simply food rewards but can manipulate worker behaviours by altering the dopaminergic regulation thus increasing host cooperation and fidelity (Hojo *et al.,* 2015). Other Lycaenid larvae, belonging to *Maculinea* genus, have evolved chemical and acoustical (Barbero *et al.,* 2009) strategies to exploit their *Myrmica* host ants. In this multitrophic system, however, the ant-butterfly association is obligatory parasitic and involves also the species-specific larval host plant (LHP).

1.4. Central nervous system (CNS) anatomy of social insects

In insects, the CNS is also known as the supraoesophageal ganglion and it consists of three different regions (Protocerebrum, Deuterocerebrum and Tritocerebrum) subsequently subdivided in various neuropils which are amyelinic zones with a large number of synapsis. Hymenoptera brain is highly conserved among the order showing many similarities between individuals of different suborders. Anatomically, the ants protocerebrum comprises the optic lobe, that connect via the lobula (**lo**) and medulla (**me**) and lamina (**la**) to the compound eyes and the ocelli (photosensor cells responsible for the detection of light/dark) and the mushroom bodies (**MB**), which are the best developed neuropil in ants. These are thought to be the centres responsible of the most sophisticated and complex behaviour and computations occurring in ant brains; MB consist of two main regions: the calyx and the peduncle. The calyx (**ca**) includes highly specialised neurons, called Kenyon cells (**Kcs**), presenting a highly branched cellular body with a great number of tree-like dendrites and are thought to be involved in learning, processing memories and chemical, visual and mechanical inputs. The other two neuropils that make up the protocerebrum are the central body (**cb**) (that generates patterns of reaction according to the stimulus received) and the pars intercerebralis (**pc**) whose function is mainly neurosecretory. Deuterocerebrum main neuropil is the Antennal Lobe (**al**) which links to the antennae via antennal nerves and is therefore the region where olfactory inputs are processed. It contains a variable number of computational subregions called glomeruli, where the

interpretation of the gustative and olfactory inputs is taken forward. This neuropil is highly developed and presents a great number of glomeruli in worker ants (Figure 15).

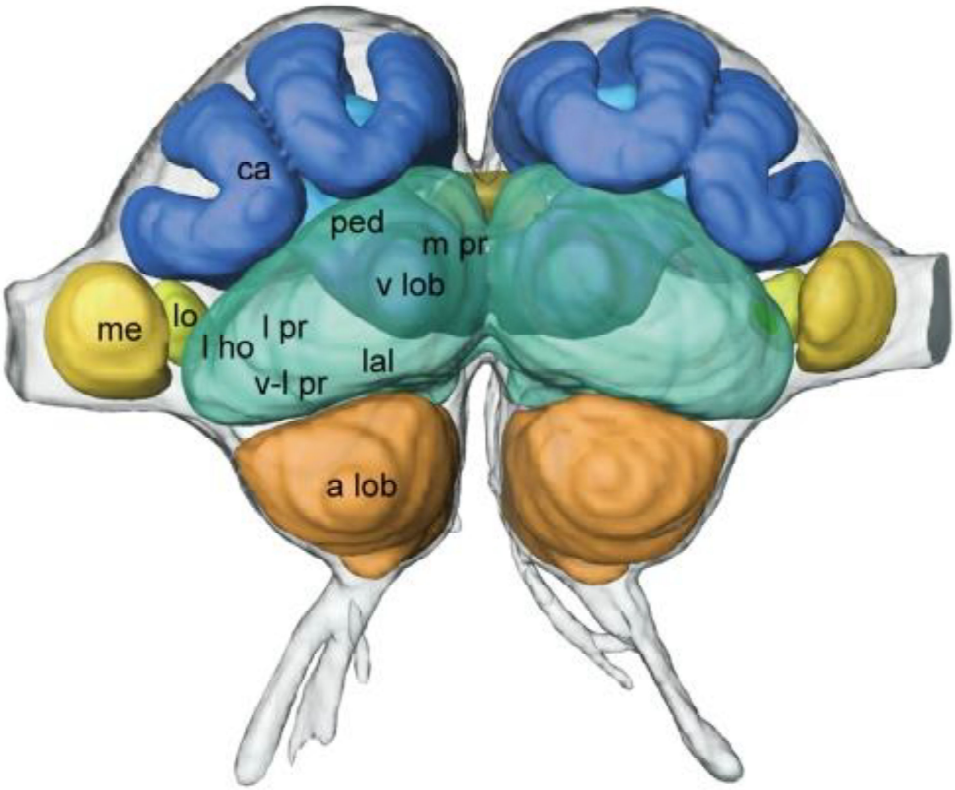

Figure 15. Three-dimensional reconstructions of major neuropils of the ant brain viewed ventrally. The protocerebrum (green) consists of the mushroom body, the lateral PR (l pr) that includes the lateral horn (l ho), the ventro-lateral PR (v-l pr), the medial and dorsal PR (m pr and d pr), the lateral accessory lobe (lal) and the central complex (cc: khaki). The mushroom body consists of calyces (ca: blue), the pedunculus (ped: light blue) and the vertical and medial lobes (v lob, m lob: light blue, broken line). The medulla (med: yellow) and the lobula (lo: light yellow) are the second and third optic neuropils. The deutocerebrum consists of the antennal lobe (a lob: orange) and the dorsal lobe (d lob: magenta).

1.5. Biogenic amines and their physiological role

Biogenic amines such as Dopamine (DA), Tyramine (TA), Octapamine (OA) and Serotonin (5-HT) are the most important ant CNS neuromodulators and have been closely related with sociality and behaviour in numerous studies (Figure 16).

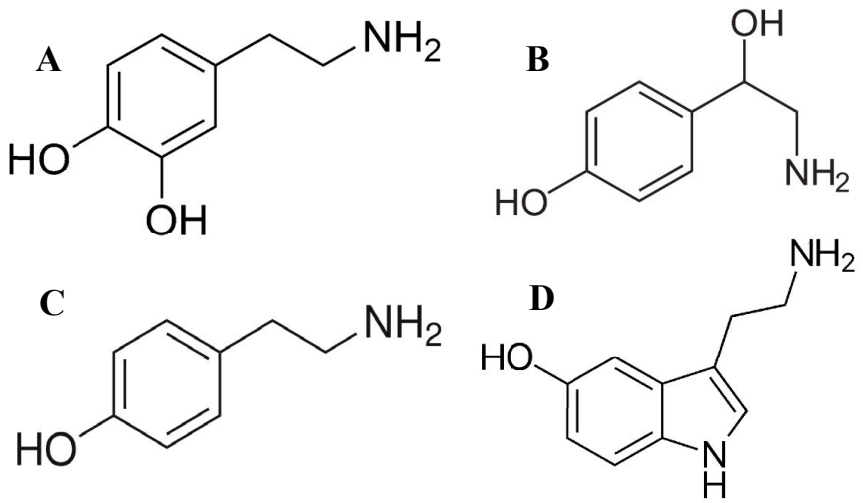

Figure 16. Chemical structure of the main biogenic amines (BAs) that are mainly involved in behaviour changes in ants. **A** Dopamine (DA), **B** Tyramine (TA), **C** Octapamine (OA), **D** Serotonin (5-HT).

Is known that the behavioural modulation by biogenic amines (5-HT, DA and OA) depends on the nature of the receptor to which the monoamines bind, but little is known about these type of receptors in ants. However, the immunolabelling has revealed recurring patterns in various species. For example, in the ponerine ant *Herpegnatos saltator*, the DA fibres have shown an intense immunoreactivity in mushroom body calices, but not the antennal lobe or the optic lobe in which instead are largely present serotonergic fibres. This suggests that 5-HT is closely related to sub-caste specialization by developing the olfactory sensitivity, while DA is used in the modulation of more complex tasks (Hoyer *et al., 2005;* Seid and Smith*, 2013*).

1.6. Interaction ant-plant-ant and its effect on biogenic amine level

The interaction between *Myrmica* ants and *Origanum vulgare* plants (LHP of *Maculinea arion*) leads to a variation in the plant volatile emission. Plants growing in the surroundings of a *Myrmica* nest produce a higher amount of carvacrol, whilst the emission of all the other volatile compounds, including the isomer thymol, does not vary (Fig. 17) (Patricelli *et al.,* 2015). The release of carvacrol is the indirect signal interpreted by the *M. arion* gravid female to locate the food plant growing in the vicinity of its *Myrmica* host colonies thus providing its brood with both sequential hosts, i.e. the source of food, shelter and care in the nest (Patricelli *et al.,* 2011, 2015; Witek *et al.,* 2011).

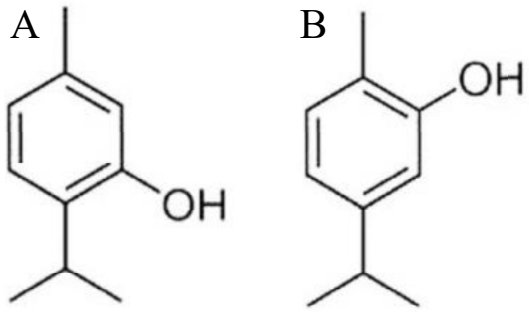

Figure 17. Chemical structure of the two volatile organic compounds (VOCs) used for the experiments: Carvacrol (**A**) and Thymol (**B**).

Carvacrol is known for its insecticide properties and tests of acute exposure revealed that *Myrmica* ants survive longer than other ant species. When exposed to this biocide, *Myrmica* ants upregulate specific genes in order to recognize and deplete the toxicity of the monoterpene compound. Indeed, the occurrence of *Myrmica* nests beneath *Origanum* plants significantly exceeds that of other ants. It has been hypothesized that *Myrmica* ants benefit from being resistant to carvacrol by occupying a competitor-free spaces surrounding the *Origanum*, although they increase their chance of encountering parasite larvae (Patricelli *et al.,* 2011, 2015; Witek *et al.,* 2011).

The mechanism that pushes *Myrmica* ants to forage and found their nests in the proximity of the oreganos has not been investigated yet. We hypothesized that carvacrol acts on ant behaviour by modifying the biogenic amine level in their brain. We explore how the emission of two specific volatile organic compounds, thymol and carvacrol (Figure 17), released by *Origanum vulgare* affect the locomotor activity and the aggressive behaviour of three different ant species (*Myrmica scabrinodis*, *Tetramorium caespitum* and *Formica cinerea*). We tested if the behavioural changes observed can be correlated to variation in biogenic amines levels.

2 – Materials and Methods

2.1. Study Species

We collected colonies of *Myrmica scabrinodis*, *Formica cinerea* and *Tetramorium caespitum* (Fig. 18) at the Parco Fluviale Gesso e Stura, North Italy, where a previous study on acute exposure of ants to specific *Origanum vulgare* volatiles had been made (Patricelli *et al.,* 2015). Four colonies per species were collected and reared in plastic boxes (24×24×9 cm). Ants were fed twice a week on a honey and protein diet.

Figure 18. Ants selected for the current study. *Myrmica scabrinodis* (**A**), *Formica cinerea* (**B**) and *Tetramorium caespitum* (**C**).

2.2. Locomotor Activity

Variations in the locomotor activity were evaluated after treatment of the three ant species with a solution of Carvacrol (C), Thymol (T), a mixture of 3:1 (v/v) Carvacrol/Thymol (CT), or a mixture of 3:1 (v/v) Thymol/Carvacrol. Two controls (blank and DMSO) were use in the bioassays. 20 ⌈L of each solution [9.54 mg mL^{-1}] were poured on a small paper disk (diameter

of 5 mm) placed in the centre of a Petri dish (9 cm internal diameter 1.5 cm height) in order to have a final concentration of 0.1 ppm.

Afterwards, 18 *F. cinerea*, 30 *M. scabrinodis* and 30 *T. caespitum* ants were randomly chosen among foraging workers of one colony. Three *F. cinerea* ants and five workers in the case of *M. scabrinodis* and *T. caespitum* were placed per each plastic Petri dish and allowed to settle for 5 minutes. Ants of each species were tested simultaneously in six Petri dishes (4 treatments and 2 controls). Three colonies per ant species were assayed (N=9) and a total of 234 workers were observed. On the transparent lid of each Petri a line that bisected the dish was drawn. Ant behavior was recorded for one hour and the number of times each ant crossed the solid line was recorded.

2.3. Aggression test

Aggression bioassays were performed on *Myrmica* ants. Foraging workers of 3 colonies per species were tested. All ants were marked on the thorax and allow to recover before the aggression tests were carried out. Bioassays were performed as described by Csata *et al.* (2017). Briefly, two transparent plastic tubes (3 cm long) were joined and separated by a small piece of red plastic foil. One worker was placed per tube and after one minute the plastic foil was removed.

Individuals of the same colony were treated with carvacrol, thymol or a mixture of carvacrol and thymol. Ants were separated in a Petri dish each contained a single worker which has been treated for 30 minutes in 2 ppm atmosphere saturated with one of the aforementioned compounds. Then bioassays were carried out testing non-nestmate (heterocolonial) ants with the following combinations, Carvacrol vs Carvacrol (CC), Thymol vs Thymol (TT), Carvacrol/Thymol vs Carvacrol/Thymol (CT_CT) and Thymol/Carvacrol vs Thymol/Carvacrol (TC_TC). Control tests were carried out using ants left in a Petri dish for 30 minutes with no addition of any compounds (CTRL). Per each treatment, three tests using all the possible combinations of the three colonies were replicated three times. Thus 45 (9 tests per 5 combinations of treatments) aggression tests using 90 ants were performed.

The observations started with the first contact of the workers and lasted for three minutes. All behaviours (biting, pulling, stinging, allogrooming and antennation) were recorded. The latter two were considered as positive or neutral interactions, respectively, whilst the others were categorized as aggressive. An aggression index (AI) for each encounter was calculated as AI = the total number of aggressive behaviours divided by the total number of interactions.

2.4. Ant brain dissection and sample preparation

Sample preparation and the dissection of ant brains were performed following the protocols by Hojo *et al.* (2015). Briefly, after two weeks of freezing, ants were rapidly beheaded under a dissection microscope using micro-scissors. A small medial-lateral incision was made directly behind the mandibles and, in order to prevent contamination by retinal pigments, optic lobes were removed from the rest of the brain. The remainder of the brain was homogenized in 20 µL of a water solution contains 0.05% (v/v) formic acid and 1 ng of 3,4-di-hydroxybenzylamine (DHBA, Sigma-Aldrich) used as an internal standard. After vortexed mixing, samples were centrifuged at 10,000 g for 20 min at 4°C. In order to remove undesirable compounds, a solution of Isopropanol/Chloroform (1:4, v/v) was directly added to supernatant in 1:1 (v/v) ratio. Samples were centrifuged at the same condition described before. The aqueous layer obtained after centrifugation was immediately frozen on dry ice and stored at 80°C until injection into an HPLC-ESI-MS/MS (1200 HPLC, Agilent Technologies, Santa Clara, CA, USA) for monoamine quantification. Each sample contained two brains with each dissection time a*vera*ging less than 1 min.

2.5. Isolation and quantification of biogenic monoamines by HPLC-ESI-MS/MS

Monoamines (serotonin, dopamine, DHBA and tyramine) were identified and quantified by liquid chromatography (1200 HPLC, Agilent Technologies, Santa Clara, CA, USA) equipped with a reverse phase Kinetex® F5 Core-Shell LC Column (2,6 µm, 150 mm × 3.0 mm, 100 Å), Phenomenex, Torrance, CA, USA) thermostated at 40°C during chromatography. The binary solvent system was: (A) MilliQ H_2O (Millipore, Billerica, MA, USA) containing formic acid 0.1% (v/v) and (B) MeCN (VWR International, Radnor, PA, USA) containing formic acid 0.1% (v/v). The chromatographic separation was carried out at a constant flow rate (200 $\mu L \cdot min^{-1}$) with the following conditions: isocratic gradient at 3% of B for the first 15 minutes of analysis, then 40% of B in 5 min, then at 24 min B concentration was raised to 98%. The concentration of solvent B was maintained at 98% for 5 min. The initial mobile phase was re-established for 10 min before the next injection. The ionization of each amine was performed by electrospray ionization (ESI) operating in positive mode and tandem mass spectrometry analyses were performed with a 6330 Series Ion Trap LC-MS System (Agilent Technologies, USA). The flow rate of nitrogen was set at 325°C and 5.0 $L \cdot min^{-1}$, while the Capillary Voltage was set at 1.5 kV. Helium was used as a collision gas. Quantitative analyses were performed by Multiple

Reaction Monitoring (MRM) by monitoring the fragmentation of quasi-molecular ions for serotonin (m/z: 177.1; daughter m/z: 159.9), dopamine (m/z: 154.1; daughter m/z: 137.0), tyramine (m/z: 138.0; daughter m/z: 121.0) and DHBA (m/z: 140.0; daughter m/z: 123.0). Quantification was performed by external calibration curves with pure standards. Limit of Detections (LOD) and Limit of Quantification (LOQ) for each compound were determined as described in complementary guideline of validation of analytical procedures (Ich 2005).

2.6. Statistical analysis

Kolmogorov–Smirnov tests were used to assess the data distribution. Aggression indices were compared using a linear mixed model (LMM) with the colony ID as a random factor and C_C, T_T, CT_CT, TC_TC, CTRL as fixed factors.

For locomotor activity (LA) assays, the data were analyzed using a linear mixed model using cumulated number of crossings as a response variable, ant colony as a random intercept, and treatment (CTRL, DMSO, C, T, C/T, T/C) as fixed factor.

Differences in the amount of each biogenic amine was tested by LMM using ant colony as a random factor and treatments (CTRL, DMSO, C, T, C/T, T/C) as fixed effect.

Tukey's honest significant difference (HSD) post hoc was used to test pairwise comparisons.

Pearson's coefficients were used to test the correlation between chemical and behavioural data.

3 – Results and Discussion

3.1. Oregano terpenoids modulate ant locomotor activity

The two volatile compounds (VOCs) released by *Origanum vulgare* modify the locomotor activity (LA) of ant species belonging to the three genera. Treatments with pure carvacrol and thymol or blended solutions differently affected the ant movements. *Formica cinerea* and *Tetramorium caespitum* workers generally increase their propensity to move in response to the blend with thymol in a higher concentration than carvacrol (Fig. 19 A and C). The increase in *Myrmica* movements only occurs when carvacrol or thymol are administered as a pure solution, whilst the mixture of compounds triggers a slight, but significant depletion of the worker locomotor activity (Fig. 19 B). The increase in the locomotor activity can be used as a proxy for ant escaping behaviour, whereas its reduction could indicate a greater site or partner fidelity (Hojo *et al.*, 2015). We argue that the dissimilar response of ant species is linked to their different success in detoxifying these plant VOCs.

In particular, single or mixed compounds increased LA in *F. cinerea* with respect to controls (Fig. 19 A; $F_{18,5}$ =28.641; $P < 0.001$). Movements were enhanced especially when ants were treated with TC and, to a lesser extent, with C followed and T. Although increased, LA of workers exposed to a blend with a larger amount of CT did not vary significantly from controls. Differences in the LA were also found in treated and untreated *T. caespitum* workers (Fig. 19 C; $F_{18,5} = 13.658$; $P < 0.001$). Overall, treatments elicited an increase in ant movements, but this variation was significant only in response to TC. A different response was found when LA was assayed in *M. scabrinodis*. Treatments affected the movements of *M. scabrinodis* (Fig. 19 C; $F_{18,5} = 19.268$; $P < 0.001$). The exposure to C and T induced an LA increase in workers. In contrast, both CT and TC caused a significant decrease in ant movements.

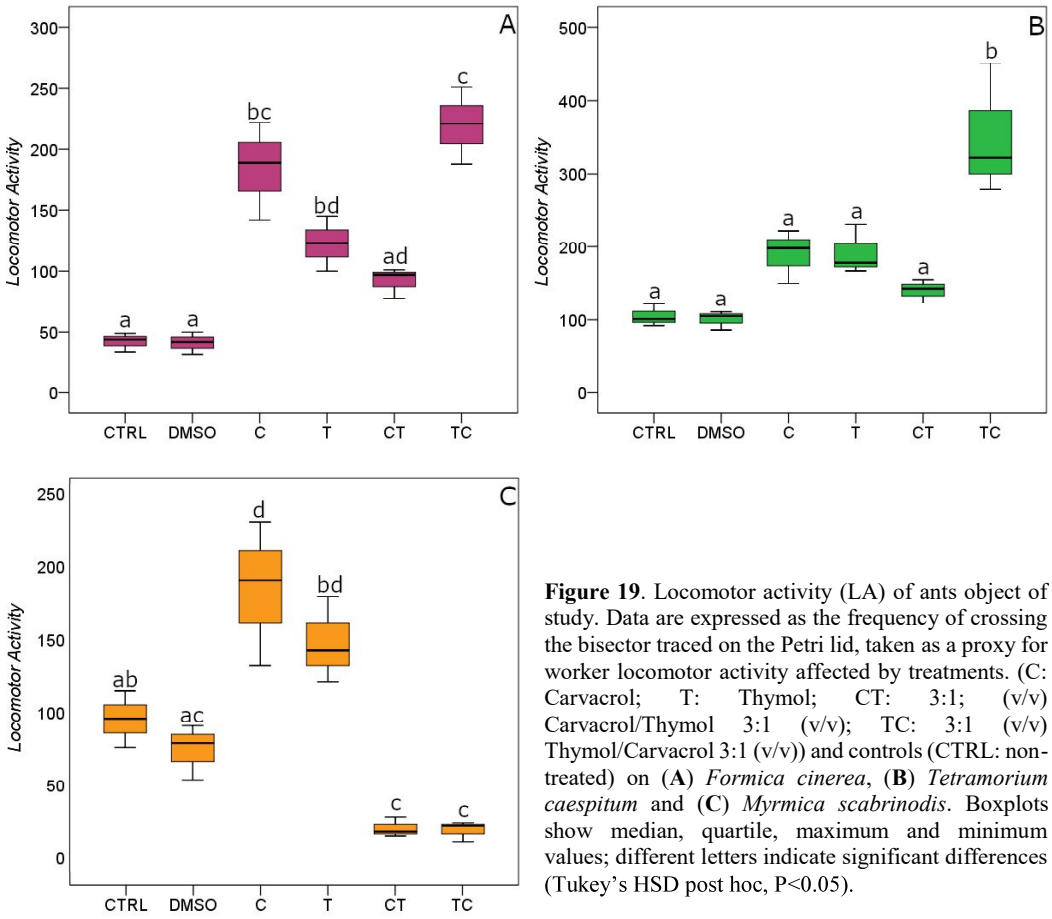

Figure 19. Locomotor activity (LA) of ants object of study. Data are expressed as the frequency of crossing the bisector traced on the Petri lid, taken as a proxy for worker locomotor activity affected by treatments. (C: Carvacrol; T: Thymol; CT: 3:1; (v/v) Carvacrol/Thymol 3:1 (v/v); TC: 3:1 (v/v) Thymol/Carvacrol 3:1 (v/v)) and controls (CTRL: non-treated) on (**A**) *Formica cinerea*, (**B**) *Tetramorium caespitum* and (**C**) *Myrmica scabrinodis*. Boxplots show median, quartile, maximum and minimum values; different letters indicate significant differences (Tukey's HSD post hoc, $P<0.05$).

Both thymol and carvacrol can be detrimental for insects (Kordali *et al.,* 2008). Although it has not been identified yet, several mechanisms of action have been proposed for carvacrol (Monzote *et al.,* 2009; Rao *et al.,* 2010; Xu *et al.,* 2008), and its toxicity against many organism has been allowed its use as insecticide (Ahn *et al.,* 1998), fungicidal (Anderson and Coats, 2012) and acaricide (Regnault-Roger and Hamraoui, 1995). However, some bacteria (Ultee *et al.,* 2000) but also *Myrmica* ants (Patricelli *et al.,* 2015) are able to adapt and survive to environmental doses of carvacrol. In response to carvacrol treatments, *Myrmica* workers upregulated three genes (acetylcholinesterase – *AchE*; glutathione S transferase – *GST*; and a cytochrome P450 – *CYP4509E2*) whose products bind and detoxify the biocide and make the colonies more resilient than other common ant species (Patricelli *et al.,* 2015). *F. cinerea* and *T. caespitum* exposed to carvacrol did not show any significant upregulation of detoxifying genes (only a slight upregulation of *AChE* was observed in *T. caespitum*) and died soon after the treatment. Hence, the increase in the propensity to move (escaping behavior) reported for

these latter species when treated with carvacrol and thymol was expected, as a countermeasure to enhance their chances of survival.

In nature however, the carvacrol could only be found in blends with thymol, because this latter compound is typically produced by *O. vulgare* plants (Patricelli *et al.,* 2011), while carvacrol primarily occurs as an outcome of the ant-plant interaction (Witek *et al.,* 2011). The fact that long exposure to carvacrol also leads *Myrmica* ants to death (Witek *et al.* ,2011) could explain why these ants are significantly dispelled only when treated with pure carvacrol (Figure 19B, panel B). Interestingly a reduction in the locomotor activity is observed only in *Myrmica* and as a response to blends. Overall, our results suggest the existence of two non-alterative dynamics, which might contribute to foster this ant-plant association. Workers can use the blends to spot and actively select the foraging area next to *Origanum* plants, or they can be manipulated by the plants to stay and patrol their surroundings (Huber *et al.,* 1997; McQuillan *et al.,* 2012; Riemensperger *et al.,* 2010; Wunderlich *et al.,* 2012; Yu *et al.,* 2014). The ability to survive in the presence of carvacrol benefits *Myrmica* ants with the opportunity to colonise an enemy-free space and perhaps could provide some other advantages in terms of protection against fungal infection (Anderson and Coats, 2012). Simultaneously the exploitation of the ground underneath the plants rises the risk to be parasitized by *Maculinea* larvae (Patricelli *et al.,* 2015). Field data demonstrated that *Myrmica* nests are found closed to *O. vulgare* plants more frequently than other ant species (Patricelli *et al.,* 2015), but the balance between the costs and benefits of this co-occurrence are still under debate.

3.2. Oregano terpenoids induce *Myrmica scabrinodis* aggression

Aggression behaviour was assessed in *M. scabrinodis* workers only, because this species is directly involved in the associations with *O. vulgare* plants and in the interaction with the *Maculinea* butterfly parasite. The level of aggression varied with exposure to different treatments (Fig. 20: $F_{45,4} = 8.031$, $P = 0.007$), and our results show that blends not only decrease worker locomotor ability, but also enhanced *Myrmica* aggression. In particular, aggression was significantly lower when heterocolonial ants were treated with CC, whilst TT caused a reduction in the antagonist behaviour, which was not significantly different from the control tests. Exposure to CT-CT and TC-TC significantly increased the aggression of *M. scabrinodis*, when compared to C and T treatments.

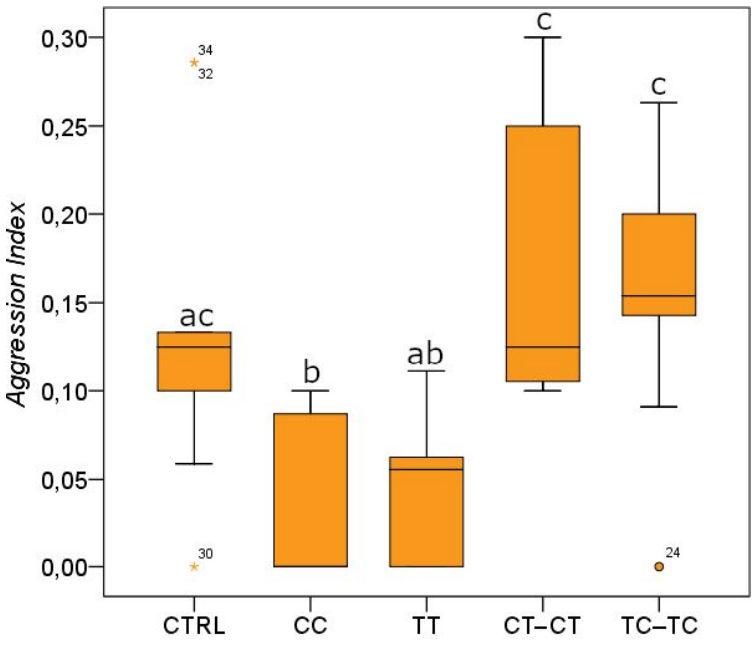

Figure 20. Aggression index between heterocolonial *M. scabrinodis* workers treated with different compounds or pure air (CTRL: non-treated; CC Carvacrol vs. Carvacrol; TT: Thymol vs. Thymol; CT-CT: 3:1 (v/v) Carvacrol/Thymol vs. 3:1 (v/v) Carvacrol/Thymol; TC-TC 3:1 (v/v) Thymol/Carvacrol vs. 3:1 (v/v) Thymol/Carvacrol). Data are expressed as number of aggressive interactions per total interactions. Boxplots show median, quartile, maximum and minimum values; different letters indicate significant differences (Tukey's HSD post hoc, $P < 0.05$).

On the plant side, the emission of carvacrol could play a general function of deterring enemies (Erler and Tunc, 2005), which could not be adequate to control the plethora of insects attacking oreganos as food and nectar source. Therefore, the behavioural manipulation of *Myrmica* ants, which are already resistant to oregano defence, aids in getting further protection. Our findings suggest that the blends of thymol and carvacrol act as a manipulative and reinforcing plant signal to enhance partner fidelity and defence.

3.3. Oregano terpenoids modulate brain biogenic amine content

A similar scenario is described by Hojo and colleagues (2015) who found that a *lycaenid* larvae secreted drugs lowering attendance ant locomotor activity and enforcing their aggressive behaviour, by only altering the worker dopaminergic regulation.

In partial agreement to Hojo *et al.* (2015), we found that the manipulation of locomotor behaviour results to be strictly correlated to a variation in brain levels of both the dopamine and the tyramine, whilst serotonin seems to play no role, irrespectively of the species. Moreover, irrespectively of the species, brain dopamine and tyramine contents were similarly affected by treatments, as they both increase or decrease in response to the same compound (all ant species Pearson correlation; $\rho = 0.97$, $P < 0.001$).

In particular, treatments with C and T significantly affected the brain content of dopamine in *F. cinerea* (Fig. 21 A: $F_{18,5} = 63.744$, $P < 0.001$), *T. caespitum* (Fig. 21 B: $F_{18,5} = 58.104$, $P<0.001$) and *M. scabrinodis* (Fig. 21 C: $F_{18,5} = 6.500$, $P = 0.006$).

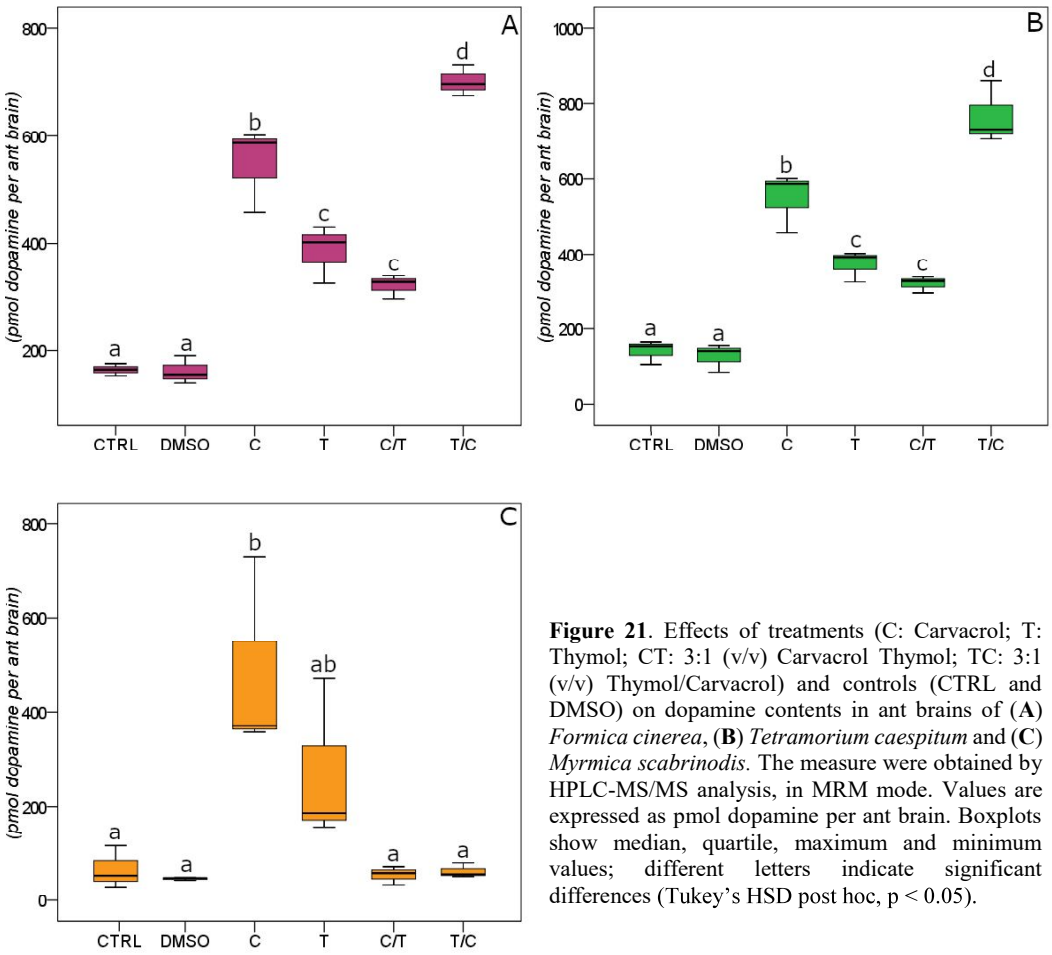

Figure 21. Effects of treatments (C: Carvacrol; T: Thymol; CT: 3:1 (v/v) Carvacrol Thymol; TC: 3:1 (v/v) Thymol/Carvacrol) and controls (CTRL and DMSO) on dopamine contents in ant brains of (**A**) *Formica cinerea*, (**B**) *Tetramorium caespitum* and (**C**) *Myrmica scabrinodis*. The measure were obtained by HPLC-MS/MS analysis, in MRM mode. Values are expressed as pmol dopamine per ant brain. Boxplots show median, quartile, maximum and minimum values; different letters indicate significant differences (Tukey's HSD post hoc, $p < 0.05$).

On the other hand, Tyramine brain content was also significantly affected in *F. cinerea* (Fig. 22 A: $F_{18,5} = 15.799$, $P < 0.001$), *T. caespitum* (Fig. 22 B: $F_{18,5} = 27.315$, $P < 0.001$) and *M. scabrinodis* (Fig. 22 C: $F_{18,5} = 5.963$ $P = 0.008$).

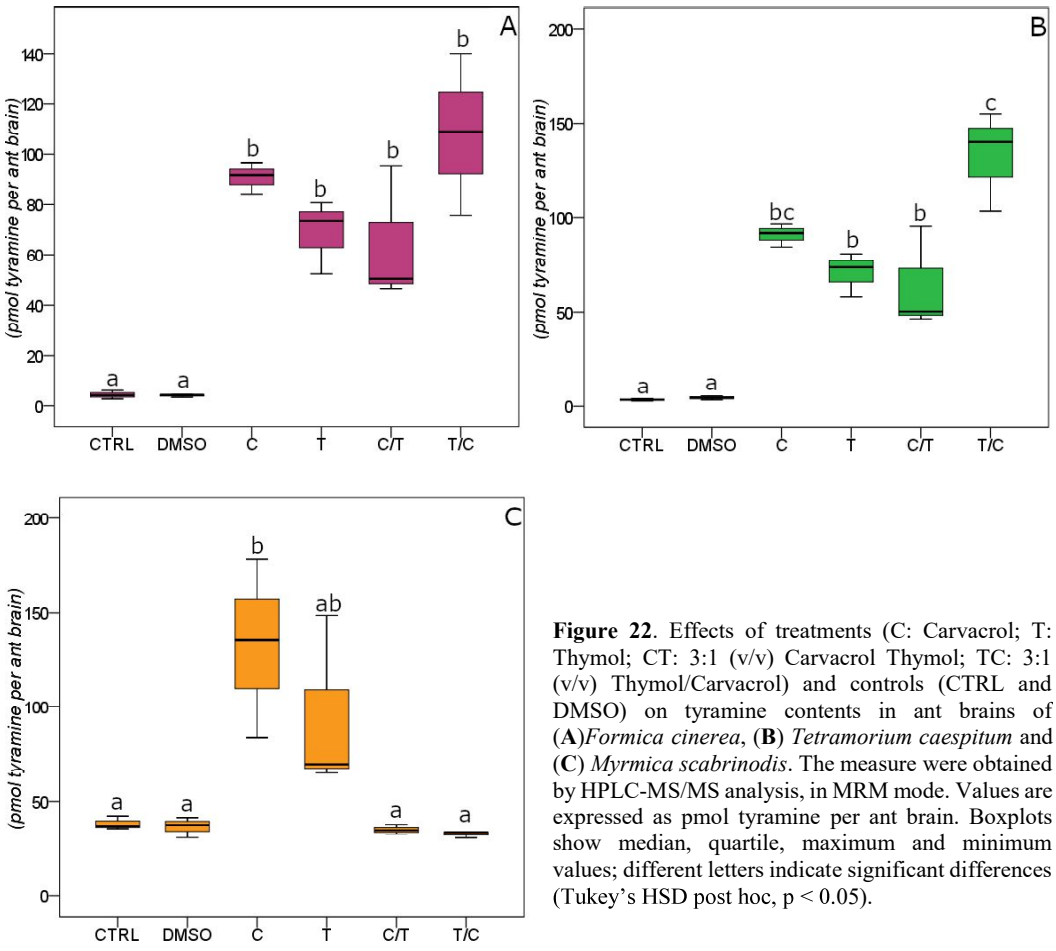

Figure 22. Effects of treatments (C: Carvacrol; T: Thymol; CT: 3:1 (v/v) Carvacrol Thymol; TC: 3:1 (v/v) Thymol/Carvacrol) and controls (CTRL and DMSO) on tyramine contents in ant brains of (**A**)*Formica cinerea*, (**B**) *Tetramorium caespitum* and (**C**) *Myrmica scabrinodis*. The measure were obtained by HPLC-MS/MS analysis, in MRM mode. Values are expressed as pmol tyramine per ant brain. Boxplots show median, quartile, maximum and minimum values; different letters indicate significant differences (Tukey's HSD post hoc, $p < 0.05$).

In contrast to dopamine and tyramine, no significant variation in the serotonin brain content was reported in all ant species (Fig. 23 A, B and C for *F. cinerea*, *T. caespitum* and *M. scabrinodis,* respectively).

The role of dopamine in modulating insect movements and aggression was demonstrated in *Drosophila melanogaster* model species (Alekseyenko *et al.,* 2013; Riemensperger *et al.,* 2010). BAs are also known to be involved in the response to biotic and abiotic stress, such as the variation in environmental temperatures (Apple and Feener, 2001; Byk and Del-Claro, 2011), in population density (Wunderlich *et al.,* 2012; Yu *et al.,* 2014) or in food and water availability. The latter case was studied in ants specifically. It has been proven how some typical social activities (trophallaxis or grooming), but also some colony stress (starvation or isolation) could drastically affect the dopamine levels thus resulting in alterations of eating and drinking

behaviours (Robinson, 1992). In addition, levels of BA can be linked to age or task in social insects (Seid and Traniello, 2005; Smith *et al.,* 2013).

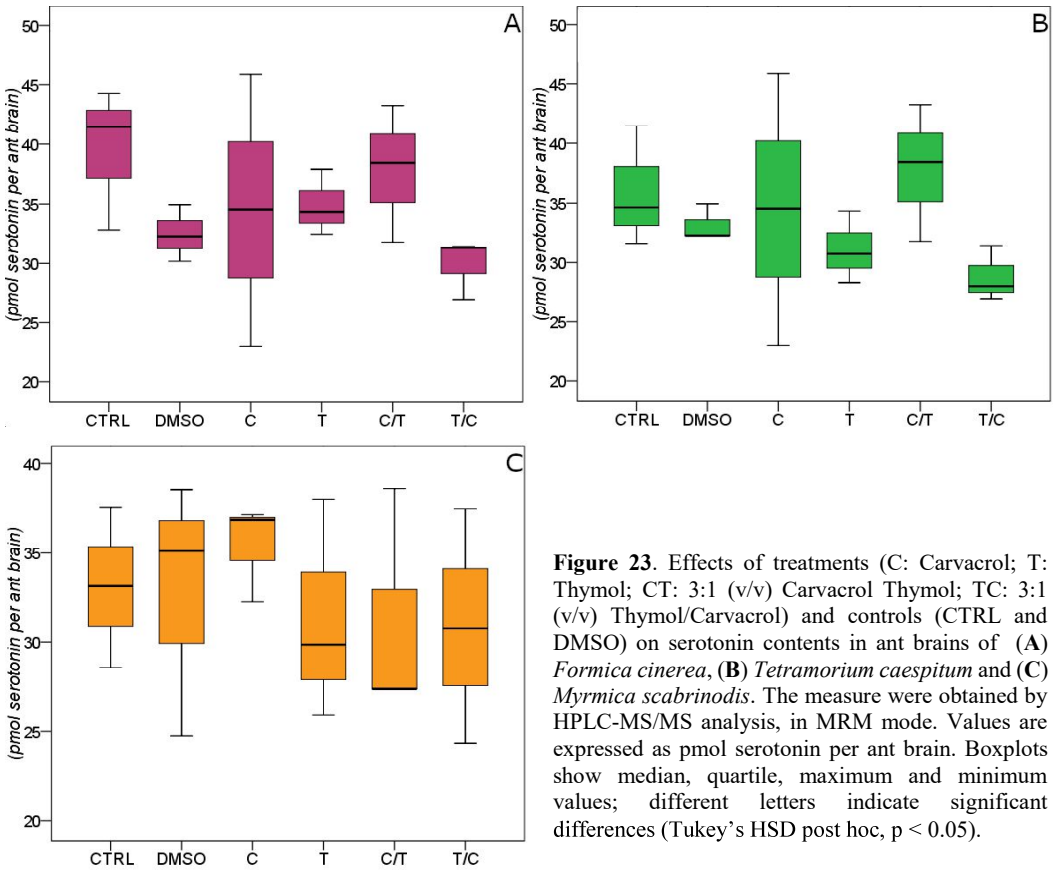

Figure 23. Effects of treatments (C: Carvacrol; T: Thymol; CT: 3:1 (v/v) Carvacrol Thymol; TC: 3:1 (v/v) Thymol/Carvacrol) and controls (CTRL and DMSO) on serotonin contents in ant brains of (**A**) *Formica cinerea*, (**B**) *Tetramorium caespitum* and (**C**) *Myrmica scabrinodis*. The measure were obtained by HPLC-MS/MS analysis, in MRM mode. Values are expressed as pmol serotonin per ant brain. Boxplots show median, quartile, maximum and minimum values; different letters indicate significant differences (Tukey's HSD post hoc, $p < 0.05$).

Biogenic amines could function as neurotransmitters, neuromodulators, or neurohormones but studies showing a direct causal role of these molecules in mediating the behavioural plasticity are rare (Libersat and Pflueger, 2004), mainly performed on invertebrate (Claassen and Kammer, 1986), and especially in social insects (Beninger, 1983; Claassen and Kammer, 1986). Although, the mechanism through which plant volatiles can modulate the titres of biogenic amines in ant forager brain is not unravelled, these data provide evidence of an interspecific behavioural manipulation through neurogenic dopamine and tyramine regulation. The strict correlation we found between biogenic amine levels and changes in locomotor activity or aggressiveness adds knowledge on the physiological basis of social behaviours, but also remarks the need for further and targeted research on how those biogenic amines actually modulate the neuronal response.

4 – Conclusions

Ants represent a good model system for neuroethological studies because of their relatively simple neural architecture. Our work provides new insights on the role of plant terpenoids in dopamine and tyramine modulation for the regulation of the locomotor activity and aggressiveness of ant workers, supporting a direct function of biogenic amines in the control of behaviour and colony organization. In the *Maculinea-Myrmica-Origanum* system the two plant monoterpenes, C and T, play crucial roles by modulating ant behaviour, through aminergic regulation, and fostering bi-level interspecific associations. Understanding how the flow of information among distinct biological levels works will be a fundamental challenge to better understand the interplay between different trophic levels.

It is increasingly clear that several interactions that have been considered to be mutualistic in the past, are nowadays revised as potentially parasitic because the ant partner is not receiving an actual reward but it is rather manipulated. In the future, the identification of specific neural networks on which biogenic amines act and the identification of genes involved in the development of these neurons, along with ethological studies, will be pivotal to understand how behavioural manipulation is widespread and actually achieved in plant-ant interactions.

Concluding remarks

In conclusion, in the present work I confirmed that advanced analytical instrumentation, such as liquid and gas chromatographic system, are essential for quality control policy

In the first part, by coupling of chemical and biomolecular techniques, I showed a rapid protocol for the correct identification of pistachio samples from different geographic origins. In particular, the chemical profile of each pistachio sample, obtained by HPLC-DAD-MS/MS and GC-FID-MS analyses of both hydrophilic and lipophilic portions, was combined with molecular fingerprinting data. The quality control shown in this study, would contribute to valorize pistachio nut skins, a waste product originating from the industrial manufacturing of pistachio nuts. Indeed, the investigation on the chemical and biochemical properties of pistachio skin suggests a strong nutraceutical potential of this waste product because of its consistent amount of phytochemicals, in particular anthocyanins and proanthocyanidins which exhibit interesting functional properties. The reported data showed that, through a radical-scavenging activity and modulation of antioxidant systems, hydrophilic extracts from pistachio skin possess antioxidant properties.

A correct identification and quantification of phytochemicals in dietary supplements or nutraceuticals is essential to obtain *in vivo* a real functional activity and to reduce the potential toxicological effects. In the second part, a quality control protocol was assessed by means of advanced analytical techniques which were used to design a precise guideline for the correct identification and quantification of boswellic acids, both in the raw materials (*Boswellia sacra* and *Boswellia serrata*) and in nutraceutical preparations containing concentrated extracts from the gum resins of these plants. In particular, we demonstrated that HPLC-DAD-MS/MS technology is required for the quality control of dietary supplements containing boswellic acids.

Finally, the last work included in the third part is a clear proof of how plant bioactive components may have important pharmacological effects on organisms. In this study, we used ants as *in vivo* model system, in order to evaluate how two VOCs emitted by *Origanum vulgare* plants (carvacrol and thymol) may change their behaviour, in term of locomotor activity and aggression activity by the modulation of biogenic amines. Also in this case, HPLC-MS/MS technology was essential for the identification and quantification of biogenic amine involved in

the process, and our study proved that tyramine and dopamine were the main responsible of changes in the ant behaviour.

In conclusion, my PhD work confirms that analytical methods based on GC and LC technologies coupled to MS detectors are useful and versatile instrumentations that can be applied to different levels of quality control. These methods are instrumental for the correct identification and quantification of bioactive compounds in the final products.

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molecules

MDPI

Communication

Quantitative Determination of 3-*O*-Acetyl-11-Keto-β-Boswellic Acid (AKBA) and Other Boswellic Acids in *Boswellia sacra* Flueck (syn. *B. carteri* Birdw) and *Boswellia serrata* Roxb

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Abstract: *Boswellia serrata* and *Boswellia sacra* (syn. *B. carteri*) are important medicinal plants widely used for their content of bioactive lipophilic triterpenes. The qualitative and quantitative determination of boswellic acids (BAs) is important for their use in dietary supplements aimed to provide a support for osteoarthritic and inflammatory diseases. We used High Performance Liquid Chromatography (HPLC)-Diode Array Detector (DAD) coupled to ElectroSpray Ionization and tandem Mass Spectrometry (ESI-MS/MS) for the qualitative and quantitative determination of BAs extracted from the gum resins of *B. sacra* and *B. serrata*. Limit of detection (LOD), limit of quantification (LOQ), and Matrix Effect were assessed in order to validate quantitative data. Here we show that the BAs quantitative determination was 491.20 g·kg^{-1} d. wt (49%) in *B. sacra* and 295.25 g·kg^{-1} d. wt (30%) in *B. serrata*. Lower percentages of BAs content were obtained when BAs were expressed on the gum resin weight (29% and 16% for *B. sacra* and *B. serrata*, respectively). The content of Acetyl-11-Keto-β-Boswellic Acid (AKBA) was higher in *B. sacra* (70.81 g·kg^{-1} d. wt; 7%) than in *B. serrata* (7.35 g·kg^{-1} d. wt; 0.7%). Our results show that any claim of BAs content in either *B. sacra* or *B. serrata* gum resins equal to or higher than 70% or AKBA contents of 30% are simply unrealistic or based on a wrong quantitative determination.

Keywords: Acetyl-11-Keto-β-Boswellic Acid; 11-Keto-beta-Boswellic Acid; boswellic acids; standardization; *Boswellia serrata*; *Boswellia sacra*; HPLC-DAD-ESI-MS/MS

1. Introduction

The genus *Boswellia* (Burseraceae), comprises 25 species of trees and shrubs which are widely spread in Arabia, the north-eastern coast of Africa and India [1]. Since ancient times, the natural resin of Boswellia trees has been collected and used to produce the oleo gum resin, frankincense (olibanum). The gum resin is harvested from incisions made on the trunk of the tree and the darkening of resin droplets is an index of oxidation [2]. Among *Boswellia* species, only a few are of economic importance as a natural source of phytopharmaceutical compounds, including *B. serrata* Roxb. and *B. sacra* Flueck (syn. *B. carteri* Birdw, syn. *B. undulatocrenata* Engl.) [3–5].

B. serrata is used for the treatment of oxidative and inflammatory damage [2], rhinitis [6] asthma [7], age-related disorders [8], neurorecovery [9], arthritis [10], skin disorder [11], cancer [12], and against several human pathogenic and plant pathogenic fungi [13]. Recently, the pharmacological properties and clinical effectiveness of *B. serrata* have been studied systematically [3].

B. sacra oleo gum resin is used in the treatment of gastric and hepatic disorders [14], skin disorders [15], for its hepatoprotective activity [16], analgesic effect [4], antiglycation and antioxidant activities [17], tumor suppression [18], anticoagulation effects [19], antinflammatory activity [20], and cardioprotective effects [21].

The main constituents of *B. serrata* and *B. sacra* are volatile oils, composed of monoterpenes and sesquiterpenes [22,23], diterpenes including incensole, incensole acetate and cembrenol (serratol) [24], lipophilic pentacyclic triterpene acids of the oleanane-(α-boswellic acids), ursane-(β-boswellic acids) and lupane-type (lupeolic acids), as well as an ether-insoluble fraction containing polysaccharides (arabinose, galactose, xylose) [25]. Among triterpenoids, bioactive boswellic acids are of particular interest, particularly 3-*O*-Acetyl-11-Keto-β-Boswellic Acid (AKBA), 11-Keto-beta-Boswellic Acid (KBA), and the various β-boswellic acids (βBAs), and α-boswellic acids (αBAs) and their esters. The analysis of these triterpenes is performed by different analytical methods including High Performance Thin Layer Chromatography (HPTLC) [26], although the most used methods are based on HPLC coupled to both photodiode array detection [27] and mass spectrometry detection [28]. In accordance with the spectral properties of the boswellic acids, their analysis is performed at three different wavelengths, 210 nm for αBAs, βBAs as well as lupeolic acid, 250 nm for AKBA and KBA, and 280 nm for 9,11-dehydro-α- and -β-boswellic acids [27]. However, a precise identification and quantification of boswellic acids is usually obtained with liquid chromatography electrospray ionisation tandem mass spectrometry (LC-ESI-MS/MS) by using selected ion monitoring (SIM) detection [28].

In general, the total organic acids from *B. serrata* and *B. sacra* constitute approximately 65%–70% by weight of the total alcoholic extract. Of this fraction, approximately 25% is made of triterpenes. In market products, these percentages are often misinterpreted and it is not unusual to find claims of 70% boswellic acids content or 30% AKBA content, which is obviously misleading because the highest amounts so far reported of boswellic acids in *B. serrata* is about 140 mg/g (i.e., 14%) and in *B. sacra* is about 190 mg/g (i.e., 19%) [27]. Since both *Boswellia* extracts are used in several formulations, it is important to express unequivocally the "real content" of boswellic acids in both *B. serrata* and *B. sacra*. Therefore, the aim of this work is to provide a guideline for the accurate identification and quantification of boswellic acids in these two important *Boswellia* species by using HPLC-DAD-ESI-MS/MS.

2. Results and Discussion

2.1. Identification of Boswellic Acids

Gum resin of *B. sacra* and *B. serrata* were extracted using methanol in order to evaluate the total BAs content. The total yield for *B. sacra* methanolic extract was 598.88 g·kg^{-1} (±11.40 g·kg^{-1}) gum resin dry weight whereas the yield for *B. serrata* was 549.78 g·kg^{-1} (±29.31 g·kg^{-1}) gum resin dry weight. The total recovery of methanolic extracts from *B. sacra* was 99.17% (±1.93%), whereas the total recovery from *B. serrata* was 98.96% (±1.97%). Our findings indicate that the total content of the lipophilic extracts (excluding the polysaccharide moiety of both species) never exceeded 60%, in agreement with previous works [27,29,30]. By considering that the methanolic fraction contains several lipophilic compounds, including mono-, di- and triterpenes, it is evident that a claim of 70% BAs is not sustainable, being the BAs only a portion of the total methanolic extract.

In order to define the content and to identify the BAs present in the two Boswellia methanolic extracts, we performed HPLC-DAD-ESI-MS/MS analyses. Several BAs were present in both methanolic extracts. Table 1 reports the molecular mass and the fragmentation pattern of compounds identified in the methanolic extracts, whereas Figure 1 shows the chemical structure of the identified BAs. The identification of these compounds was achieved by both mass spectrometry and comparison with pure standards (see Supplementary Figure S1 for mass spectra of identified compounds and Supplementary Figure S2 for UV chromatograms). In both species, the main BAs were represented by AKBA, KBA, αBA, βBA, acetyl-αBA (A-αBA) and acetyl-βBA (A-βBA), in accordance with the literature data [2,31,32].

Table 1. Molecular mass and fragmentation pattern of compounds identified in the methanolic extracts of *B. sacra* and *B. serrata*.

	Compounds	RT (min)	$[M - H]^-$ (*m*/*z*)	Ions (*m*/*z*)	Occurrence in	
					B. sacra	*B. serrata*
1	11-Keto-Ursolic acid	24	469	451, 407, 391	x	x
2	11-Keto-β-boswellic acid	25.8	469	451, 407, 391	x	x
3	3-*O*-Acetyl-11-keto-β-Boswellic Acid	28.8	511	451, 361	x	x
4	3-α-Hydroxy-7,24-dien-tirucallic acid	34.5	455	453, 437, 373		x
5	3-α-Hydroxy-8,24-dien-tirucallic acid	36.5	455	453, 437, 373	x	x
6	3-β-Hydroxy-7,24-dien-tirucallic acid	39.4	455	453, 437, 373		x
7	3-*O*-Acetyl-oleanolic acid	41	497	479, 437	x	x
8	Lupeolic acid	41.6	455	437, 409		x
9	3-*O*-Acetyl-ursolic acid	43.3	497	479, 437	x	x
10	α-Boswellic acid	44.5	455	437, 409, 377	x	x
11	β-Boswellic acid	45	455	437, 409, 377	x	x
12	3-*O*-Acetyl-α-boswellic acid	52	497	459, 437	x	x
13	3-*O*-Acetyl-β-boswellic acid	52.9	497	459, 437, 395	x	x

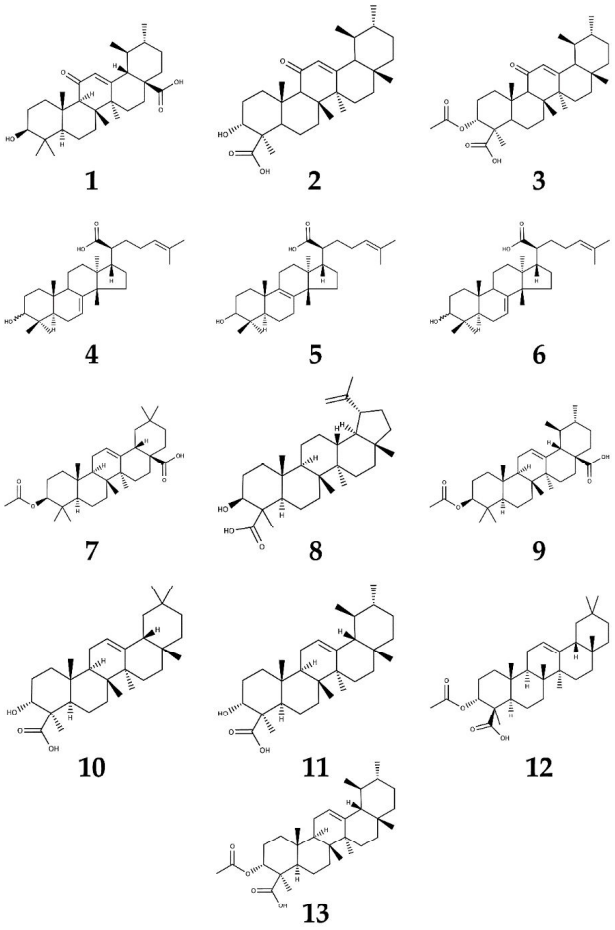

Figure 1. Chemical structure of the boswellic acids identified in *Boswellia serrata* and *Boswellia sacra*. Numbers correspond to compounds listed in Table 1.

2.2. Quantification of Boswellic Acids

Table 2 shows for both species the quantitative determination of the main BAs. The total amount of the main BAs was statistically ($p < 0.05$) higher in *B. sacra* than in *B. serrata*. In *B. sacra*, the total amount of the main BAs in the methanolic extract was lower than 50% and this value was reduced to about 29% when the amount was considered in terms of the total gum resin dry weight (Table 2). In *B. serrata* the total content of the main BAs in the methanolic extract was lower than 30% and the value dropped to 16% when BAs were calculated in terms of the gum resin dry weight (Table 2). In both species, the major BAs were represented by αBA and βBA, in agreement with literature data [32]. A direct comparison between the two species shows that the contents of *B. sacra* AKBA (about 10 fold), αBA (1.5 fold) and βBA (1.6 fold) were statistically ($p < 0.05$) higher than in *B. serrata*.

Table 2. Quantitative determination of boswellic acids in *Boswellia sacra* and *Boswellia serrata* by High Performance Liquid Chromatography-Diode Array Detector coupled to ElectroSpray Ionization and tandem Mass Spectrometry (HPLC-DAD-ESI-MS/MS), by using calibration curves from pure standards. Data are expressed as $g \cdot kg^{-1}$ gum resin dry weight. (Standard deviation), in the same row, different letters indicate significant ($p < 0.05$) differences.

Compound	*Boswellia sacra*		*Boswellia serrata*	
	Content in the Methanolic Extract	Content in the Gum Resin	Content in the Methanolic Extract	Content in the Gum Resin
KBA	35.50 (1.26) [a]	21.26 (0.76) [b]	34.62 (2.57) [a]	19.03 (1.41) [c]
AKBA	70.81 (4.66) [a]	42.41 (2.79) [b]	7.35 (0.89) [c]	4.04 (0.49) [d]
αBA	184.34 (11.27) [a]	110.40 (6.75) [b]	126.00 (7.77) [b]	69.27 (4.27) [c]
βBA	186.19 (4.98) [a]	111.50 (2.98) [b]	113.21 (7.37) [c]	62.24 (4.06) [c]
A-αBA	5.40 (0.35) [a]	3.23 (0.21) [b]	2.92 (0.19) [b]	1.60 (0.11) [c]
A-βBA	9.95 (0.23) [a]	5.96 (0.14) [b]	11.33 (0.28) [a]	6.23 (0.15) [b]
Total	491.20 (14.75) [a]	294.77 (8.83) [b]	295.25 (23.00) [b]	162.29 (12.64) [c]

In order to validate the quantitative analyses reported in Table 2, we calculated the linearity and precision of the identified BAs standard curves, the detection limit (LOD), the quantification limit (LOQ) and the Matrix Effect (ME). Table 3 shows the validation results for the main identified BAs. All compounds showed a high R^2 value, which indicates a high linearity in the calibration curves. The lowest LOD and LOQ values were found for A-βBA, followed by equal values for KBA and AKBA. The highest LOD and LOQ values were found for A-αBA. In order to complete the validation process, we assessed the ME, to assure that precision, selectivity and sensitivity were not compromised during HPLC-ESI-MS/MS analyses. The absolute ME was calculated by comparing the slope of matrix-matched standard curve with the slope of the standard calibration curve, according to [33]. Table 3 reports, for each species, the ME accuracy (expressed as percent values) of the main identified BAs. In *B. sacra* extracts, KBA (CV = 14.06) and A-βBA (CV = 11.61) showed the highest percentage of accuracy, followed by AKBA (CV = 7.53) and A-αBA (CV = 1.90). βBA (CV = 8.72) showed the lowest ME accuracy percentages. In *B. serrata*, the highest percentages of accuracy were found for αBA (CV = 0.22), followed by KBA (CV = 14.13), A-αBA (CV = 2.66) and AKBA (CV = 7.74).

Table 3. Validation of boswellic acids (BAs) quantitative analyses of methanolic extracts from *B. sacra* and *B. serrata*. (Standard deviation).

Compound	Regression Equation	R^2	LOD $\mu g \cdot \mu L^{-1}$	LOQ $\mu g \cdot \mu L^{-1}$	ME *B. sacra*	ME *B. serrata*
KBA	y = 13891880514x + 180215436.7	0.973	0.006	0.020	100.67 (14.17)	98.83 (13.96)
AKBA	y= 24732.09x − 143.76	0.997	0.006	0.021	99.91 (7.53)	91.53 (7.08)
αBA	y = 1349052089x + 8552264.58	0.989	0.008	0.028	88.52 (9.75)	139.80 (0.31)
βBA	y = 1549451236x + 1749204.16	0.995	0.013	0.042	73.40 (6.40)	77.25 (7.11)
A-αBA	y = 9915.15x − 127.16	0.995	0.017	0.058	93.27 (1.77)	92.91 (2.47)
A-βBA	y = 9179.53x − 101.22	0.995	0.003	0.010	100.23 (11.64)	90.70 (8.74)

R^2, coefficient of determination; LOD, Detection limit; LOQ, Quantification limit; ME, matrix effect, expressed as percentage of accuracy.

Finally, Table 4 shows the recovery of the identified BAs from *B. sacra* and *B. serrata* methanolic extracts. In both species, the total recovery was higher than 98%. In *B. sacra*, the highest recovery was found for AKBA and αBA, whereas in *B. serrata* the highest recovery was found for A-βBA and A-αBA (Table 4).

Table 4. Percentage of recovery of identified BAs from *B. sacra* and *B. serrata* methanolic extracts. Values are expressed as percentage of recovery.

Compounds	*B. sacra*	*B. serrata*
KBA	96.53	99.06
AKBA	99.68	96.88
αBA	99.39	99.14
βBA	98.75	97.13
A-αBA	88.71	99.38
A-βBA	91.34	99.84
Total	98.68	98.41

The quantitative determination of *B. sacra* and *B. serrata* BAs content and the validation of the quantitative chemical analysis show that any claim of BAs content in either *B. sacra* or *B. serrata* gum resins equal to or higher than 70% or 30% AKBA are simply unrealistic or based on a wrong quantitative determination. The same is true when the percentage of BAs is calculated in the methanolic extract.

3. Materials and Methods

3.1. Plant Material and Chemicals

Boswellia serrata Roxb. and *Boswellia sacra* Flueck gum resins were purchased from Bauer S.r.l. (Udine, Italy). The origin of *B. sacra* samples was from Ethiopia, whereas *B. serrata* samples originated from India. The gum resins were milled to coarse powder and used for all extractions. All chemicals were of analytical reagent-grade unless stated otherwise. Pure standards were purchased for the quantification by external calibration curves: 11-Keto-β-boswellic acid, α-Boswellic acid and β-Boswellic acid (ExtraSynthese, Lyon, France), 3-*O*-Acetyl-11-keto-β-Boswellic Acid (Merck, Darmstadt, Germany), 3-*O*-Acetyl-α-boswellic acid, 3-*O*-Acetyl-β-boswellic acid (Sigma-Aldrich, St. Louis, MO. USA).

3.2. Solvent Extraction of Boswellia serrata and Boswellia sacra oleo Gum Resins

One hundred grams of ground *B. serrata* and *B. sacra* oleo gum-resins were extracted with 1 L methanol (VWR International, Radnor, PA, USA) (extraction ratio 1:10 w/v). Samples were then placed on an orbital shaker for 5 days in the dark. Extracts were then filtered and the resin was rinsed with 400 mL of methanol. To evaluate the recovery of analyzed compounds, the exhaust gum resin was re-extracted with methanol as previously described. Samples were then concentrated by vacuum evaporation (Rotavapor, Büchi, Flawil, Switzerland). Concentrated extracts were then dried in a ventilated oven at 70 °C for 4 h. The powdered extracts were stored at room temperature in the dark until chemical analysis. Extractions were performed in triplicate.

3.3. Isolation and Quantification of Boswellic Acids by HPLC-DAD-ESI-MS/MS

Boswellic acids were identified and quantified by liquid chromatography (1200 HPLC, Agilent Technologies, Santa Clara, CA, USA) equipped with a reverse phase column, Luna C18 (3 µm, 150 mm × 3.0 mm, Phenomenex, Torrance, CA, USA). *B. serrata* and *B. sacra* powdered extracts were dissolved (30 mg·mL^{-1}) in HPLC-grade methanol and properly diluted. The binary solvent system was: (A) MilliQ H_2O (Millipore, Billerica, MA, USA):Methanol 50:50 containing 5 mM ammonium acetate (Sigma-Aldrich, USA); and (B) Methanol:1-Propanol (VWR International, Radnor, PA, USA)

80:20 containing 5 mM ammonium acetate. The chromatographic separation was carried out at constant flow rate (200 $\mu L \cdot min^{-1}$) with the following conditions: linear gradient from 30% to 50% of B in 2 min, then 80% of B in 35 min, then at 47 min B concentration was raised to 98%. The concentration of solvent B was maintained at 98% for 6 min. The initial mobile phase was re-established for 10 min before the next injection. The temperature of wellplate autosempler G1377A was set 4 °C while chromatography was carried out at constant temperature (30 °C) controlled by an Agilent 1100 HPLC G1316A Column Compartment.

Tandem mass spectrometry analyses were performed with a 6330 Series Ion Trap LC-MS System (Agilent Technologies, USA) equipped with an electrospray ionization source (ESI) operating in negative mode. The flow rate of nitrogen was set 325 °C and 5.0 $L \cdot min^{-1}$, while the Capillary Voltage was 1.5 kV. Helium was used as a collision gas.

Identification of *Boswellia* oleo gum resin compounds was performed by scan analyses with a 50–750 *m/z* scan range and by monitoring the absorption at 210, 250 and 280 nm. Quantitative analyses were performed by Multiple Reaction Monitoring (MRM) by monitoring the fragmentation of quasi-molecular ions for αBA and βBA and KBA (Table 1) and by Diode Array Detector (DAD) at 250 nm for AKBA and 210 nm for A-αBA and A-βBA. Quantification was performed by external calibration curves with pure standards dissolved in HPLC grade Methanol. Limit of Detections (LOD) and Limit of Quantifications (LOQ) for each compounds were determined as described in [34].

To evaluate the ME in the quantification of target compounds, *B. serrata* and *B. sacra* powdered extracts were dissolved (30 $mg \cdot mL^{-1}$) in HPLC-grade methanol and properly diluted. These sample solutions were used to prepare the calibration curves in the presence of other extracted gum resin compounds [33]. The slope of standard curves obtained with the solvent (methanol) and in the extracts were used to compare the ME percentage ($\%ME = Calibration\ Slope_{(sample)}/CalibrationSlope_{(standard)} \times 100$). 100% ME percentage indicates no ME, a ME% < 100% indicates ionization suppression and a ME% > 100% indicates ionization enhancement.

4. Conclusions

Boswellia sacra and *Boswellia serrata* extracts are widely used in pharmaceutical and nutraceutical preparations. The bioactivity of these *Boswellia* extracts is based on the content of BAs. Clearly, the dose of bioavailable BAs is central to the issue of *Boswellia* efficacy. Claims of 70% BAs or even 30%–40% AKBA are currently found, but this work confirms that the BAs content never exceeds 50% of the methanolic extract, whereas lower percentages are obtained when BAs are expressed in terms of the gum resin weight. Moreover, the highest percentage of AKBA found in *B. sacra* was below 8%. Only analytical methods based on HPLC coupled to mass spectrometry allow the precise quantification and identification of BAs in *Boswellia* extracts, whereas other methods based only on HPLC or spectrophotometric methods do not sufficiently allow an accurate quantification of BAs. Therefore, we recommend LC-MS technology for BAs determination and quantification.

Supplementary Materials: The following are available online at http://www.mdpi.com/1420-3049/21/10/1329/s1, Figure S1: mass spectra of boswellic acids isolated in this study; Figure S2: UV chromatograms of boswellic acids isolated in this study.

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Conflicts of Interest: The authors declare no conflict of interest.

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Sample Availability: Samples of the compounds are not available.

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Poster presentation to FISV 2016 Congress, Rome, Italy
P6.20

Chemical characterization and standardization of bioactive boswellic acids from *Boswellia* (Frankincense) species by HPLC-ESI-MS/MS

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Plant extracts are a rich source of secondary metabolites able to exert biological activities on humans. They also have a high economic impact on the dietary supplements market. Environmental factors and extraction methods can strongly affect the chemical composition of plant extracts; therefore, the use of accurate and sensitive analytical techniques for the characterization and quantification of bioactive molecules is a compulsory quality standard to assure to costumers both safety and bioactivity. Traditional medicine uses *Boswellia* spp. resin-gum extracts for anti-inflammatory, anti-proliferative, antiseptic and neuro-protective effects because of the presence of pentacyclic triterpenoids known as boswellic acids (BAs). *B. sacra* and *B. serrata* are characterized by significant amounts of 3-*O*-Acetyl-11-keto-β-boswellic acid (AKBA), α- and β-BAs and their acetylated derivatives. In market products, BAs percentages are often misinterpreted and it is not unusual to find claims of 70% BAs content. This study aims to quantify BAs content by using HPLC coupled to Tandem Mass Spectrometry in two commercial *Boswellia* species to achieve the accurate standardization of bioactive BAs.

Poster presentation to SIBV/SIGA 2017 Congress, Pisa, Italy
P. 6.25

Phytochemical analysis, antioxidant properties and antiproliferative activity of nut extracts from six cultivars of *Pistacia vera*

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Pistacia vera L. is the only species of the *Pistacia* genus producing edible nuts. Pistachios have been ranked among the first 50 food products with a high antioxidant potential. Moreover, pistachios consumption has positive effects in human serum lipid profile, by significantly improving the redox status and reducing the circulating inflammatory biomarkers. Our previous research provided evidence that a hydrophilic *P. vera* nut extract of a Sicilian cultivar (Bronte) contains substantial amounts of polyphenols (including proanthocyanidins) exerting radical scavenging, antioxidant properties and anti-inflammatory activities in *in vitro* cellular models [1,2]. In this work, the phytochemical diversity within six pistachio cultivars was investigated in order to discriminate their nut chemical and antioxidant properties. Hydrophilic extracts from nut skins (SHE) and nut kernels (KHE) were prepared. Total phenolic content varied from 56 to 256 mg rutin equivalent/100g, with the highest values being observed in SHE, in particular in Bronte, Larnaka and Mawardi cultivars. On other hand, KHE of Kern, Mawardi and Mateur displayed the lowest values. The radical scavenging and antioxidant activity of SHE, measured by DPPH, ABTS, FRAP and CAA methods, was always higher (~100-fold) than in KHE, with significant differences among the different cultivars. Qualitative and quantitative chemical analysis by HPLC-DAD-ESI-MS/MS showed significant differences among the six cultivars and between pistachio SHE and KHE. In particular, SHE anthocyanins and proanthocyanidins contributed almost exclusively to total polyphenol content. Moreover, to evaluate the potential bioactivity of nut extracts, MTT assay was used to assessed antiproliferative activity against three human cancer epithelial cell lines, using dietary relevant concentrations of pistachio nut extracts. In general, MTT assays showed a concentration-dependent antiproliferative activity in both SHE and KHE, with a consistent variability among the three different cell lines and the six different cultivars. The highest growth inhibitory effects was found against HeLa cells, whereas CaCo2 cells were the less sensitive to treatments. The sensitivity of tested cancer cells to different extracts was quite different, with GI50 ranging from 18 to 86 μg FW/mL medium, from 43 to 94 μg FW/mL medium and from 61 to 159 μg FW/mL medium for HeLa, HepG2 and CaCo2 cells, respectively. The sole antioxidant properties are not sufficient to explain the antiproliferative activity of pistachios nuts. Indeed, there was no significant linear relationship between antioxidant activity or total phenolic compounds and antiproliferative activity, expressed as GI50 values. This was consistent with previous reports showing that inhibition of cancer cell proliferation by fruit extracts cannot be explained by the total phenolic contents. It is possible that other components may play a specific role. Studies are under way to better characterize these components.

PLANT BIOLOGY EUROPE 2018

Poster presentation to PBE 2018 Congress, Copenhagen, Denmark
P.9.16

Phytochemical analysis, antioxidant properties and DNA fingerprinting of pistachio skin from six Pistacia vera L. kinds growing in the subtropical line

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Among Anacardiaceae family the genus *Pistacia* contains at least 12 tree and shrub species, of which only *Pistacia vera* produces edible nuts. Results from *in vivo* studies showed a positive correlation between pistachio intake and reduced risk of cardiovascular disease. This protection has been ascribed to fatty acid profile that can have a favorable influence on serum concentration and profile of triglycerides, and total and LDL cholesterol. Otherwise, experimental data showed that pistachio consumption significantly improves oxidative status of healthy individuals and lowers the levels of circulating inflammatory biomarkers, suggesting a positive correlation with the content of hydrophilic phytochemicals having antioxidant and anti-inflammatory activities. Due to the kernel contains over 50% of lipids, polyphenol compounds in pistachio are mostly found in the skin, generally removed when pistachio is used as a confectionery ingredient. Instead, this study investigated the phytochemical profile and the antioxidant properties of pistachio nut skin of six kinds of *Pistacia vera* (Bronte, Kerman, Kern, Larnaka and Mawardi). TPC varied from 66.02 to 262.81mg GAE per g of skin, with the highest values being observed in Italian cultivar Bronte (262.81±11.93mg/g). The AOA of SHEs were measured by DPPH, ABTS, FRAP and CAA methods, showing significant differences among the cultivars. Moreover, qualitative and quantitative chemical analysis by HPLC-DAD-ESI-MS/MS showed differences in the amount of phenols, but not in the quality. Moreover, the quantification of anthocyanins and proanthocyanidins by pH-jump and DMAC suggested that these classes of compound contributed almost exclusively to TPC. Finally, in order to investigate the possible action mechanism of CAA, qPCR analyses were carried out on the expression of genes involved in AOA (CuSOD, MnSOD, GPx and CAT). At least, in order to discriminate closely-related kinds of *Pistacia vera*, the chemical data were compared with PCR-RFLP analysis of the ITS and 5SP sequences of nrDNA.

Poster presentation to FISV 2018 Congress, Rome, Italy
P19.9

Chemical profile, antioxidant properties and molecular fingerprinting of six varieties of *Pistacia vera L.*

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Palermo, Italy Pistacia vera is the only species of the *Pistacia* genus producing edible nuts, and it have been ranked among the first 50 foods with high AOA. In this work, spectrophotometric assays (Folin-Ciocalteu and DMAC) were combined to HPLC-DAD-MS/MS analysis to investigate the phytochemical diversity within six pistachio cultivars (Bronte, Larnaka, Mateur, Mawardi, Kern and Kerman) both in skin (SHE) and in fruits (FHE) extracts. Chemical assays (ABTS, DPPH and FRAP) along with *in vitro* assays (CAA50 and MTT) were employed in order to evaluate their radical scavenging, antioxidant and anti-proliferative activity. In particular, SHEs showed always the highest antioxidant activity and polyphenols content respect to fees FHEs, but a completely comparable anti-proliferative activity. In agreement with previous reports, the inhibition of cancer cell proliferation cannot be explained just by the total phenolic contents, but is possible that other components may play a specific role. Studies are under way to better characterize these components. Finally, DNA fingerprinting was assessed to understand if differences in chemical composition and bioactivity could be linked to a different genotype.