

RESEARCH ARTICLE

Metabolomics and chemometrics of seven aromatic plants: Carob, eucalyptus, laurel, mint, myrtle, rosemary and strawberry tree

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Abstract

Introduction: *Arbutus unedo* L. (strawberry tree), *Ceratonia siliqua* L. (carob), *Eucalyptus camaldulensis* Dehnh. (eucalyptus), *Laurus nobilis* L. (laurel), *Mentha aquatica* L. (water mint), *Myrtus communis* L. (common myrtle), and *Rosmarinus officinalis* L. (rosemary) are aromatic plants from the Mediterranean region whose parts and preparations are used for their nutritional properties and health benefits.

Objectives: To evaluate and compare the metabolites profile, total phenol content (TPC), and antioxidant activity of plant leaves for their future use. Gas chromatography–mass spectrometry (GC–MS) was used for metabolomics. Data comparison was performed by chemometrics.

Methodology: Polar and apolar extracts were analysed using untargeted GC–MS metabolomics followed by chemometrics (principal component analysis, heatmap correlation and dendrogram) to identify, quantify and compare the major organic compounds in the plants. Additionally, nuclear magnetic resonance (NMR) spectroscopy was used for the laurel polar extract to identify D-glucose-L-glycero-3-octulose whose presence was unclear from the GC–MS data. TPC and antioxidant assays were performed using classical methods (Folin–Ciocalteu, 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH)) and correlated to the phytochemical profiles.

Results: Forty-three metabolites were identified including amino acids, organic acids, carbohydrates, phenols, polyols, fatty acids, and alkanes. Eight metabolites (D-fructose, D-glucose, D-mannose, gallic acid, quinic acid, myo-inositol, palmitic and stearic acids) were in common between all species. D-Glucose-L-glycero-3-octulose ($37.29 \pm 1.19\%$), D-pinitol ($31.33 \pm 5.12\%$), and arbutin ($1.30 \pm 0.44\%$) were characteristic compounds of laurel, carob, and strawberry tree, respectively. Carob showed the highest values of TPC and antioxidant activity.

Conclusion: GC–MS metabolomics and chemometrics analyses are fast and useful methods to determine and compare the metabolomics profiling of aromatic plants of food and industrial interest.

KEYWORDS

dendrogram, GC–MS, heatmap, NMR, PCA

1 | INTRODUCTION

Aromatic plants, known as herbs and spices, are species characterised by a distinctive smell due to the presence of volatile compounds including essential oils. This characteristic has made them popular since the fifth millennium BC when they were used in the Middle East to enhance the aroma and flavour of food as well as for their preservative and medicinal properties.¹

Over the last two decades, worldwide interest in substituting synthetic compounds with natural products has increased both the demand and the use of aromatic plants in pharmaceutical, food and feed industries.^{2,3}

Aromatic plants contain natural products belonging to different classes of metabolites, such as polyphenols, quinones, flavonoids, alkaloids and polypeptides, all exhibiting biological properties.⁴ Several bioactive compounds in the aromatic plants show therapeutic potential as antioxidants^{5,6} and are used to inhibit oxidative rancidity.⁷ In addition, plant biomasses can actually be recycled and converted in a source of value-added products.⁸

Many aromatic plants originate from the Mediterranean area and their chemical composition has been explored using several targeted analysis, but very little research has been conducted on their whole metabolite content. To this end, non-targeted metabolomics is a very

powerful approach to achieve a comprehensive analysis of metabolite profiles of the plant.^{9,10} Recently, nuclear magnetic resonance (NMR)-based metabolomics approach has been applied to analyse seven aromatic Mediterranean plants traditionally used in cuisine to study the metabolic changes during different seasons.¹¹ However, to the best of our knowledge, there have been no studies based on a gas chromatography–mass spectrometry (GC–MS) metabolomics approach of aromatic plants from the Mediterranean area.

In a search of bioactive metabolites used as food ingredients, we selected different aromatic plant species: *Arbutus unedo* L. (strawberry tree), *Ceratonía siliqua* L. (carob), *Eucalyptus camaldulensis* Dehnh. (eucalyptus), *Laurus nobilis* L. (laurel), *Mentha aquatica* L. (water mint), *Myrtus communis* L. (common myrtle), and *Rosmarinus officinalis* L. (rosemary) (Table 1).

Arbutus unedo L. (Ericaceae), commonly known as the strawberry tree, is an evergreen shrub of increasing interest because of its widespread traditional, industrial, chemical and pharmaceutical use. Plant leaves are used as an infusion for their diuretic, antiseptic, anti-diarrhoeal, astringent, depurative and antihypertensive properties.¹² *Ceratonía siliqua* L. (Caesalpiniaceae) is a flowering evergreen tree with high nutritional value for its unique composition.¹³ Leaves are used in folk medicine for their antidiarrhoeal and diuretic properties, but, to date, no metabolomics research has been carried out on carob

TABLE 1 Studied plants with their related voucher specimens, uses and properties

Species	Voucher specimens	Family	Common name	Uses and properties
<i>Arbutus unedo</i> L.	0120	Ericaceae	Strawberry tree	Leaves used as an infusion for their diuretic, urinary antiseptic, antidiarrhoeal, astringent, depurative and antihypertensive properties. ¹²
<i>Ceratonía siliqua</i> L.	0220	Caesalpiniaceae	Carob tree or carob	Leaves used in folk medicine as antidiarrhoeal and diuretic. ¹⁴
<i>Eucalyptus camaldulensis</i> Dehnh.	0320	Myrtaceae	Eucalyptus	Leaves used as food additives and as a decoction for sore throat and other bacterial infections of the respiratory and urinary tracts. ¹⁵
<i>Laurus nobilis</i> L.	0420	Lauraceae	Laurel	Laurel is traditionally used as food spice to season roast meats, stews, snails, fish, sauces, soups, and boiled chestnuts. ¹⁷
<i>Mentha aquatica</i> L.	0520	Lamiaceae	Water mint	Leaves used as flavouring foods, the essential oil has antimicrobial and antioxidant activities. ²⁰
<i>Myrtus communis</i> L.	0620	Myrtaceae	Common myrtle or myrtle	In folk medicine, a decoction of leaves and fruits is used as stomachic, hypoglycemic, antimicrobial, cough and oral diseases, for constipation, antihemorrhagic and externally for wound healing. ¹⁶
<i>Rosmarinus officinalis</i> L.	0720	Lamiaceae	Rosemary	Leaves used as food spice to season meat, fish, and vegetable and in folk medicine for anti-inflammatory, diuretic and antimicrobial applications. ¹⁸

leaves.¹⁴ *Eucalyptus camaldulensis* Dehnh and *Myrtus communis* L. belong to the Myrtaceae family. Decoction of the eucalyptus leaves is used to treat asthma, sore throat and bacterial infections of the respiratory and urinary tracts.¹⁵ The astringent, tonic and antiseptic characteristics of myrtle leaves justify its use for healing wounds or disorders of the digestive and urinary systems.¹⁶ *Laurus nobilis* L. (Lauraceae) is used in the kitchen as a spicy fragrance to flavour meat, fish, broths, and vegetables. The leaves are also traditionally used to reduce high blood sugars, and protect against fungal and bacterial infections, and gastrointestinal pains.¹⁷ *Rosmarinus officinalis* L. and *Mentha aquatica* L. are widely consumed aromatic plants that belong to the Lamiaceae family. Rosemary is an evergreen shrub; leaves are traditionally used to flavour baked potatoes or meat dishes.¹⁸ Rosemary has been found to have several biological activities, such as antioxidant, anti-inflammatory, antimicrobial, and anti-cancer properties, as well as being useful for anxiety, stress, and memory.¹⁹ *Mentha aquatica* L. is a perennial herbaceous plant and its essential oil has shown very strong antioxidant and antimicrobial activities, in particular, against *Escherichia coli* strains.²⁰ However, to date, there has been no research on the metabolomics profile of *Mentha aquatica* L. leaves.

This work aims to trace and compare the metabolites profiles of the leaves from the selected aromatic plants by using an untargeted metabolomics GC-MS approach followed by principal component analysis (PCA) and chemometrics. This will enable collection of qualitative and quantitative information on polar and non-polar compounds in the plant extracts for future use. In addition, total polyphenol content and antioxidant activity have been determined to evaluate the antioxidant potential of the selected aromatic plants.

2 | EXPERIMENTAL

2.1 | Chemicals

Anhydrous methanol, 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), methoxyamine hydrochloride and *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA), the analytical standards cycloleucine (CYC) and heptadecanoic acid, (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), and potassium persulphate, were obtained from Sigma-Aldrich (Poole, UK). The anhydrous sodium carbonate, anhydrous pyridine, hydrochloric acid (37%), 2,2-diphenyl-1-picrylhydrazyl (DPPH), Folin-Ciocalteu's reagent and gallic acid were obtained from Fischer Scientific (Loughborough, UK).

2.2 | Plant materials

Mature leaves of *A. unedo* L., *C. siliqua* L., *Eucalyptus camaldulensis* Dehnh., *L. nobilis* L., *Mentha aquatica* L., *Myrtus communis* L., and *Rosmarinus officinalis* L. were collected in Cicerale (40°19'41.76"N, 15°07'23.55"E), an Italian municipality belonging to the Cilento, Vallo

di Diano and Alburni National Park. Cicerale is situated at an altitude of 250 m above sea level (a.s.l.). The study site has a typical Mediterranean climate with a mean annual temperature of 16.9°C and 1328 mm of annual rainfall well distributed in winter, spring, and fall, but with a pronounced dry summer. For each species, samples were collected in triplicates from three different plants. Each replicate consisted of 50 g of mature leaves collected from the same plant (50 g × 3). The plants were identified by one of the authors, and voucher specimens were deposited at the Department of Agricultural Sciences, University of Naples Federico II with the following numbers: No. 0120 (*A. unedo* L.), 0220 (*C. siliqua* L.), 0320 (*Eucalyptus camaldulensis* Dehnh.), 0420 (*L. nobilis* L.), 0520 (*Mentha aquatica* L.), 0620 (*Myrtus communis* L.), and 0720 (*Rosmarinus officinalis* L.) (Table 1).

2.3 | Metabolite extraction

Leaves of the selected Mediterranean plants were extracted using the method described by Grauso *et al.*⁹ Briefly, leaves were dried for three days under controlled temperature in a forced air circulation oven, at 30°C, and powdered finely with a pestle and mortar. Then, 4 g of each sample were extracted with 20 mL of *n*-hexane at room temperature under stirring for 1 h. Then, the non-polar extract was filtered, evaporated, and stored at 4°C until analysis. The plant material was further extracted with 20 mL methanol-water (6:4) solution under stirring for 1 h, followed by centrifugation at 3000 rpm for 5 min at 25°C. After separation, the polar extract was collected, dried by a rotary evaporator, and stored in a refrigerator at 4°C until analysis. All samples were analysed in triplicate to ensure reproducibility. Validation of the extraction and identification were performed using a standardised sample preparation protocol previously developed and applied for plant analysis.⁹ This enabled the creation of compound libraries that allowed effective compound identification and efficient dereplication. This protocol was based on two analytical methods complementary for polarity allowing a broad range of organic compounds to be identified. One sample preparation was used for polar compounds and another sample preparation was used for non-polar compounds.

2.4 | Total phenol content

The total phenol content (TPC) of aqueous extracts of seven aromatic plants was determined using the Folin-Ciocalteu method described by Singleton and Rossi²¹ with some modifications: sample (125 µL), water (500 µL) and Folin-Ciocalteu reagent (125 µL) were mixed and left to react for 6 min. Then, 7.5% sodium carbonate solution (1.25 mL) was added to the mixture and brought to a final volume of 3 mL with distilled water. Samples were then placed in darkness for 90 min at room temperature. The absorbance was read at 760 nm (Thermo Scientific Genesys 10S ultraviolet-visible [UV-vis] spectrophotometer; ThermoFisher Scientific, Waltham, MA, USA) and TPC was expressed as gallic acid equivalents (mg/g GAE). A calibration

curve ranging from 0 to 100 µg/mL was used to quantify the TPC in leaf extracts. All determinations were performed in triplicate.

2.5 | Antioxidant activity

The free radical-scavenging activity of plant aromatic aqueous extracts was determined according to previous methods using the reduction of radicals: ABTS and DPPH.^{22,23} For the ABTS assay a mixture of 7 mM ABTS (2.5 mL) and 140 mM potassium persulphate (44 µL) was prepared and left in darkness overnight. The stock solution of ABTS was diluted to 1:80 until the OD (optical density) reached a value between 0.7 and 0.8 nm when read at a wavelength of 734 nm. Samples (100 µL) were added to 1 mL of ABTS solution and after 2.5 min the reduction was measured as the percentage of inhibition. Results were expressed in mmol Trolox equivalent of dry weight (TE/g DW) and a calibration curve ranging from 25 to 250 µM of Trolox was used for measurements. For the DPPH assay, a stock solution was prepared by dissolving 4 mg DPPH in 10 mL of methanol and stored in a freezer at −20°C until needed. The working solution was obtained by diluting the stock solution with methanol (1:20) to obtain an absorbance of 0.9 ± 0.02 units at 517 nm. Samples (20 µL) were dissolved in 1 mL of DPPH working solution and after 10 min the antioxidant capacity was measured using a spectrophotometer with a set wavelength of 517 nm. Results were expressed as mmol Trolox equivalent of dry weight (TE/g DW). All determinations for ABTS and DPPH assays were performed in triplicate and submitted to statistical and multivariate analysis.

2.6 | GC–MS analysis

GC–MS analysis were performed according to the method of de Falco and Lanzotti²⁴ with some modification described later: for polar fractions, 30 µL of internal standard (IS) CYC (2 g/L) was added to an aliquot (90 µL) of a diluted sample and evaporated to dryness in a vacuum centrifuge (Eppendorf Concentrator 5301). Samples were oximated with 50 µL of methoxyamine hydrochloride in pyridine (20 mg/mL) at 60°C for 45 min and then silylated with 150 µL of MSTFA at 60°C for 45 min. For non-polar fractions, total lipids were quantified as fatty acid methyl esters (FAMES) using heptadecanoate (C17:0) as the IS (20 µL; 0.5 mg/mL). Heptadecanoate was added to an aliquot of dissolved sample in *n*-hexane and evaporated in a fume cupboard overnight. Fatty acids were esterified by heating with anhydrous methanol in the presence of an acidic catalyst (1 mL of methanol–hydrochloric acid 93:7) in a sealed vessel at 50°C overnight for methylation. After the reaction, the sample was evaporated under nitrogen flow and then the FAMES were extracted with 1 mL of hexane.

Both polar and non-polar extracts were analysed in a similar way by GC–MS. Then, 1 µL of each derivatised sample was injected in a pulsed splitless mode into an Agilent-7820A GC system (Agilent Technologies, Santa Clara, CA, USA) with 5977E MSD operating in electron ionisation (EI) mode at 70 eV. The injection temperature was set

at 270°C. Helium was used as carrier gas at a constant flow rate of 1 mL/min. All spectra were recorded in full scan with a mass range 50–800 *m/z*.

For the analyses of the polar phase, the system was equipped with a 30 m × 0.25 mm inner diameter fused-silica capillary column with 0.25 µm DB-5MS stationary phase, following the temperature programme: 2 min of isothermal heating at 70°C, followed by a 5°C/min oven temperature ramp to 260°C, and the 10°C/min to 300 and held for 5 min. The system was then temperature equilibrated for 1 min at 70°C before injection of the next sample. For the analyses of the non-polar phase, the system was equipped with a 30 m × 0.25 mm inner diameter fused-silica capillary column with 0.25 µm DB-23 stationary phase (Agilent Technologies) and the temperature programme was set as follows: 1 min of isothermal heating at 50°C, followed by a 25°C/min oven temperature ramp to 175°C and then 4°C/min to 230 and held for 5 min. Data analysis for metabolite identification and quantification was performed using MassHunter Qualitative Analysis B.07.00 (Agilent Technologies). The identification of metabolites was achieved using mass spectra and retention times of standard compounds, and comparison with data literature and spectra from the National Institute of Standard and Technologies library (NIST MS Search 2.2). The peak areas of multiple peaks identifying the same compound, such as D-glucose, 2,3,4,5,6 pentakis-O-(trimethylsilyl)-, *o*-methyloxy (1E and 1Z isomers), were summed. Deconvolution was applied to all spectra for co-eluting peaks by using AMDIS software (Agilent Technologies), artefact peaks such as those ones from the derivatising agent and solvent were excluded from the analysis. All spectra were normalised using the IS, CYC and C17:0 for polar and non-polar extracts, respectively. Relative quantification for metabolomics comparison of seven aromatic plants was calculated from total ion chromatogram peak area integration of single metabolite and IS.

2.7 | Data analysis

All analyses (ABTS, DPPH, TPC and GC–MS) were performed in triplicate. Results are expressed as mean ± standard deviation. Significant differences between samples were evaluated through the analysis of variance (ANOVA) and shown with different lowercase letters for $P < 0.05$. All statistical procedures were computed using the statistical package SPSS (IBM SPSS Statistics 25; IBM, Armonk, NY, USA). The relationship between ABTS and DPPH with the 22 most abundant chemical compounds was assessed using Pearson's correlation analysis. Data from the Pearson's correlation were mapped using heatplots. Cluster analyses were used to test the similarity of the chemical composition of the seven plant species studied. PCA was also performed to assess the capability of the 22 most abundant compounds to explain chemical differences between the seven plant species and the associated antioxidant activity. In accordance with the approach proposed by Legendre and Legendre,²⁵ ABTS and DPPH were also plotted as a loading vector on the bi-dimensional PCA space even if it was not used to compute the eigenvalues of the same ordination.

3 | RESULTS AND DISCUSSION

3.1 | Total phenol content

The TPC of *A. unedo* L., *C. siliqua* L., *Eucalyptus camaldulensis* Dehnh., *L. nobilis* L., *Mentha aquatica* L., *Myrtus communis* L., *Rosmarinus officinalis* L. is reported in Figure 1. Among the seven different plants, leaves from carob, myrtle and rosemary showed the highest values of TPC (16.43 ± 0.24 ; 16.01 ± 1.32 ; 16.47 ± 0.05 mg/g GAE, respectively) followed by arbutus and eucalyptus (5.97 ± 0.75 and 5.88 ± 0.31 mg/g GAE, respectively), while laurel and mint had the lowest phenol content (3.27 ± 0.44 and 1.23 ± 0.24 mg/g GAE, respectively) (Supporting Information Table S1). These findings agreed with Wong-Paz *et al.*²⁶ who reported similar phenol content for eucalyptus leaves (12.8 ± 0.96 mg/g GAE). Myrtle has been studied by Aidi Wannes *et al.*²⁷ for its chemical composition and antioxidant activity. In their work, the TPC of leaves was found to be higher than that of the stems

and flowers (33.67, 15.70 and 11.11 mg/g GAE, respectively). Although, we did not compare different parts of the plant, our TPC data for myrtle, eucalyptus, rosemary and laurel leaf extracts are in the same order of magnitude of those reported from previous work.^{26–28} However, we found lower TPC values for carob and arbutus compared to those reported by Hsouna *et al.*²⁹ (130 ± 5.62 mg GAE/g) and Mendes *et al.*³⁰ (170.3 ± 1.4 mg GAE/g), respectively. This could be due to the different extraction methods used for the assay and the age of the plants used for the experiments. Shan *et al.*²⁸ evaluated and compared total antioxidant capacity and phenolic content of 26 common spice extracts, including *L. nobilis* and *Rosmarinus officinalis*. They found that laurel leaf extract had a lower phenol content than rosemary leaf extract, as our data indicate in Figure 1.

3.2 | Antioxidant activity

The antioxidant activity of the seven leaf extracts was evaluated using the ABTS and DPPH methods. Results are expressed as mM TE/g DW and reported in Figure 1 (middle and bottom histograms). Data showed significant differences between the antioxidant capacities of the seven leaf extracts. In the DPPH assay, *C. siliqua* and *Myrtus communis* showed the highest values (2.03 ± 0.05 and 1.77 ± 0.03 mM TE/g, respectively) followed by *Rosmarinus officinalis* (1.04 ± 0.01 mM TE/g). Similarly, in the ABTS assay *C. siliqua* along with *Rosmarinus officinalis*, *A. unedo* and *Eucalyptus camaldulensis* showed the highest antioxidant capacity followed by *Myrtus communis*. Our data regarding *A. unedo* are in agreement with a previous study, which reported that the Trolox equivalent antioxidant capacity of methanol and ethanol extracts of *A. unedo* leaves were 1.71 ± 0.01 and 2.25 ± 0.04 mM TE/g, respectively.³¹ Shan *et al.*²⁸ determined the antioxidant capacity and characterised the phenolic constituent of 26 common spice extracts including *Rosmarinus officinalis* L. and *L. nobilis* L. They found very similar antioxidant activity values for rosemary and laurel (0.38 ± 0.021 and 0.34 ± 0.001 mM TE/g, respectively). These values are in the same order of magnitude as our data, but in our study the radical scavenging activity of *Rosmarinus officinalis* is two times higher than that of *L. nobilis* (0.83 ± 0.06 and 0.46 ± 0.04 mM TE/g, respectively).

DPPH values are positively correlated to TPC ($r = 0.88$) meaning that the phenol content is strongly related to antioxidant capacity of the seven leaf extracts. However, there is a poor correlation between TPC and ABTS ($r = 0.53$), for example the *Eucalyptus camaldulensis* extract has the highest antioxidant activity as shown from the ABTS assay (0.96 ± 0.11 mM TE/g) but not the highest phenol content (Figure 1). This could be due to the fact that different radicals have different antioxidant potentials and the DPPH assay is likely more selective than the ABTS assay and does not react with aromatic rings containing only one hydroxyl group.³² This could suggest the presence of other classes of compounds different from phenols (e.g. polyols, oligosaccharides, flavonoids) which can play an important role in radical scavenging activity in the *Eucalyptus camaldulensis* leaves extract compared to other species.

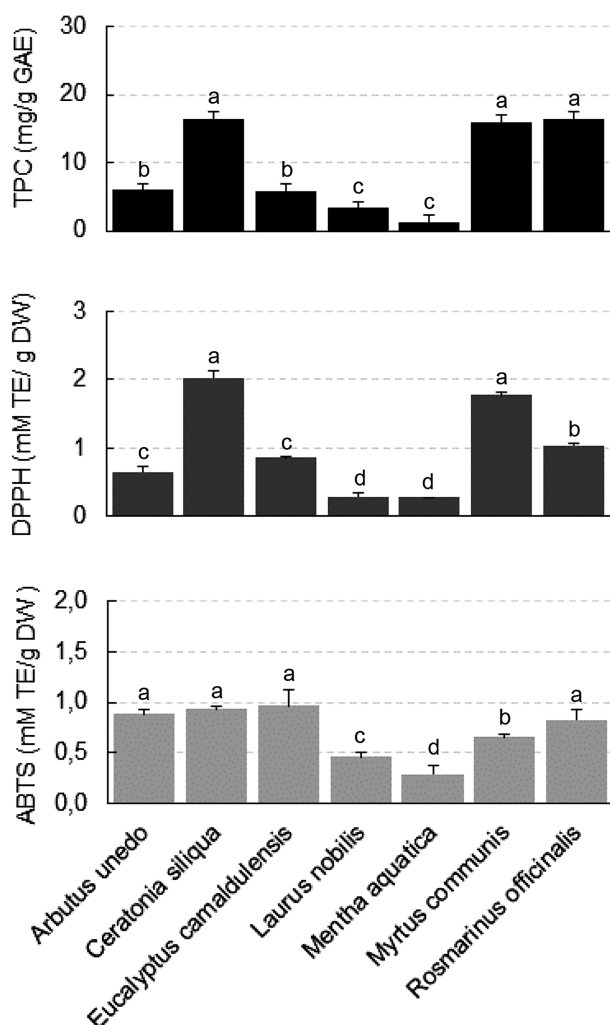


FIGURE 1 TPC, DPPH, and ABTS activities in the extracts of seven selected aromatic plant species. Values are the average of three replicates, different letters indicate statistically significant difference (Duncan test, $P < 0.05$)

3.3 | Metabolomics profile

GC–MS chromatograms of polar and non-polar fractions of the plant extracts are shown in Figures S1 and S2. A total of 43 metabolites were tentatively identified and they can be grouped in the following class of compounds: amino acids (4), carbohydrates (11), organic acids (4), phenols (5), polyols (5), indole (1), alkanes (4), and fatty acids (9) (Table 2). The chemical contribution of each identified metabolite is reported in Table 3 as relative quantification for all species.

In non-polar extracts, 15 metabolites were identified according to level 1 identification of the metabolomics standards initiative.³³ The organic phase extracts are mainly composed of fatty acids, detected as FAMES, and alkanes. The major constituent of the non-polar phase is palmitic acid, ranging from $25.56 \pm 6.54\%$ to $50.96 \pm 3.23\%$, except in *Rosmarinus officinalis* where 3-hydroxy-octadecanoic acid is the major component. Within the plant species analysed in this study, myrtle and mint have the highest content of palmitic acid ($50.96 \pm 3.23\%$ and $45.75 \pm 0.68\%$, respectively) followed by strawberry tree ($41.24 \pm 1.81\%$). Palmitic acid is used to produce soaps, cosmetics, and industrial mould release agents.³⁴ Thus, the use of mint, myrtle, and strawberry tree by-products as a potential source of value-added compounds might have an economic impact for those countries that are large producers of these crops in their agro-food profile, such as the countries of the Mediterranean maquis, United States, Canada and Chile. The compound 3-hydroxy-octadecanoic acid methyl ester was detected only in *Rosmarinus officinalis* extract. Its mass spectrum showed the base peak at $m/z = 103$ which is indicative of the characteristic cleavage at the alpha carbon of the hydroxyl group. The 3-hydroxy fatty acids are an important class of microbial lipids that have been extensively used as biomarkers to aid microbial characterisation.³⁵ This class of compound is an unusual plant component, but the 3OH-C18:0 was previously detected in leaves and flowers of some *Hypericum* species.³⁶ Besides 3OH-C18:0, two other long chain fatty acids, C18:1 and C18:2, were identified only in rosemary leaf extracts. Previous authors have detected these compounds in the essential oil of rosemary leaves.³⁷ Fatty acids and triglycerides are primarily found in seeds, making them one of the main sources for oil production.^{38,39} However, using other organic extraction methods, such as pressurised liquid extraction, might improve the concentration of this class of compound in leaf extracts.⁴⁰ Within the alkanes identified in the organic phase, nonacosane has been detected only in *Rosmarinus officinalis*. Previously, this metabolite has been identified in rosemary leaves by Reverchon and Senatore⁴¹ who compared two extraction techniques to obtain rosemary leaf oil: hydro-distillation and supercritical carbon dioxide (CO₂) extraction. This alkane has been reported as the main wax component in Brassicaceae and apples and it has been associated with plant resistance to water stress.^{42–44} Therefore, the presence of nonacosane only found in rosemary might suggest higher water stress tolerance of this plant compared to the other plant species analysed in this study.

Laurel leaves have the highest content of alkanes, the aliphatic subfraction of the organic extract in *L. nobilis* is 25.04% compared to 23.59% of *Rosmarinus officinalis* represented only by nonacosane.

Table 3 shows that the organic phase extract of *L. nobilis* leaves is composed of a wider range of long-chain hydrocarbons such as tetracosane, hexacosane and heptacosane. Laurel essential oil is used mainly in cosmetic and pharmaceutical products but our results, also supported by a previous finding, showed that they could also be used as a potential source of renewable energy to produce bio-oil and biochar.⁴⁵ Interestingly, tetracosanoic acid (C24:0), better known as lignoceric acid, was detected only in laurel leaves ($3.97 \pm 0.30\%$). This compound was also found in *L. nobilis* leaves also by Dias *et al.*⁴⁶ who reported higher content in cultivated samples compared to wild samples ($11.96 \pm 0.03\%$ and $5.71 \pm 0.31\%$, respectively). There is still little information on the physiological role of lignoceric acid; a few experimental studies reported an altered content of this metabolite in peroximal disorders,⁴⁷ diabetes⁴⁸ and cardioembolic stroke.⁴⁹ Another study demonstrated that lignoceric acid suppresses apoptosis, which is the main pathophysiological cause of atrial fibrillation.⁵⁰ More research is needed to clarify the effect of lignoceric acid on human health.

The metabolite tentatively identified as 2,4-bis(dimethyl benzyl)-6-butylphenol (2,4BDTBP) was detected in non-polar extracts of the following species laurel > eucalyptus > carob > rosemary > mint. Alkylphenols have been found in marine sediments and aquatic samples and have been attributed to pollution.⁵¹ These compounds are used as surfactants, detergents, pesticides and plasticisers. In particular 2,4BDTBP is used industrially in antioxidant blends in the manufacture of rubber and other polymers.⁵² The 2,4BDTBP was also found to be the main volatile compound of castor essential oil (*Ricinus communis* L.) and *Parkinsonia aculeate*, and its antimicrobial activity has been reported.^{53,54} There is still a lack of knowledge in the scientific literature regarding this compound. More research is needed to better understand the relation between plants and 2,4BDTBP and its related activity.

The oxygenated hydrocarbon 6,10,14-trimethyl-2-pentadecanone was found only in mint and laurel organic extracts (Table 3). This compound has a herbal taste, and it has been reported previously as one of the major compounds of the essential oil of the *Mentha spicata* species and *Herniaria incana* Lam.^{55,56} Therefore, it could be used as a potential biomarker for the consumption of these food products.

Concerning the water phase extract, 29 metabolites were tentatively identified (Table 2). Free amino acids (total content ranges from 0 to 0.73%) and organic acids (total content ranges from 0 to 8.28%) were detected in lower amounts compared to other classes of compounds. Sugars are the major constituent of all polar extracts with myrtle leaves having the highest value (77.58%). Polyols are the second class of compounds that affect the semi-quantitative analysis, especially in mint, strawberry tree, and carob leaf extracts where they reach the highest content (36.32%, 36.17% and 32.45% respectively).

In more detail, valine, proline, 5-oxoproline and gamma-aminobutyric acid (GABA) were detected as free amino acids in the aqueous extracts. However, valine was only detected in mint extract ($0.32 \pm 0.04\%$); proline was found in carob and rosemary leaves but with no significant difference between the two species ($P < 0.05$), and 5-oxoproline was only detected at a low level in rosemary leaves

TABLE 2 Identification of major metabolites in polar and non-polar extracts of seven aromatic plants. The detected metabolites are related to their retention time (RT), molecular formula and *m/z* and reported as trimethylsilyl (TMS) and methyl ester (ME) derivatives for aqueous and organic extract, respectively

Detected metabolite	Abbreviation	RT (min)	Molecular formula	<i>m/z</i>
<i>Polar</i>				
Lactic acid, 2TMS	LA	7.76	C ₉ H ₂₂ O ₃ Si ₂	73, 117, 147, 191, 219
Valine, 2TMS	Val	11.91	C ₁₁ H ₂₇ NO ₂ Si ₂	59, 66, 73, 100, 114
Glycerol, 3TMS	GLY	13.56	C ₁₂ H ₃₂ O ₃ Si ₃	59, 73, 89, 103, 117
Proline, 2TMS	Pro	14.20	C ₁₁ H ₂₅ NO ₂ Si ₂	59, 73, 84, 100, 142
Butanedioic acid, 2TMS	ButA	14.75	C ₁₀ H ₂₂ O ₄ Si ₂	73, 147, 247
Glyceric acid, 3TMS	GlyA	15.04	C ₁₂ H ₃₀ O ₄ Si ₃	73, 89, 103, 133, 147
Malic acid, 3TMS	MA	19.13	C ₁₃ H ₃₀ O ₅ Si ₃	55, 73, 101, 133, 147
5-Oxoproline, 2TMS	PCA	19.88	C ₁₁ H ₂₃ NO ₃ Si ₂	59, 73, 84, 100, 112, 121
Gamma-aminobutyric acid, 3TMS	GABA	20.08	C ₁₃ H ₃₃ NO ₂ Si ₃	59, 68, 73, 86, 100
Threonic acid, 4TMS	ThA	20.85	C ₁₆ H ₄₀ O ₅ Si ₄	59, 73, 83, 89, 103, 292
1H-Indole-2,3-dione, 5-ethyl-1-(TMS)-, 3-[O-(TMS)oxime]	I	21.17	C ₁₆ H ₂₆ N ₂ O ₂ Si ₂	73, 245, 334
Ribonic acid, 5TMS	Rib	25.38	C ₂₀ H ₅₀ O ₆ Si ₅	73, 147, 217, 292, 307
Citric acid, 4TMS	CA	26.63	C ₁₈ H ₄₀ O ₇ Si ₄	59, 73, 147, 183, 191
Pinitol, pentakis (TMS) ether	Pin	26.84	C ₂₂ H ₅₄ O ₆ Si ₅	59, 73, 81, 89, 95, 103
Quinic acid, 5TMS	QA	27.39	C ₂₂ H ₅₂ O ₆ Si ₅	59, 73, 115, 141, 147
Fructose, 5TMS-methyloxime	Fru	27.67	C ₂₂ H ₅₅ NO ₆ Si ₅	73, 89, 103, 204, 217, 307
Glucose, 5 TMS-methyloxyme	Glc	28.19	C ₂₂ H ₅₅ NO ₆ Si ₅	73, 89, 103, 147
Mannose, 5 TMS-methyloxyme	Man	28.52	C ₂₂ H ₅₅ NO ₆ Si ₅	73, 103, 117, 147, 205, 319, 365, 448
Mannitol, 6TMS	Mannit	28.77	C ₂₄ H ₆₂ O ₆ Si ₆	73, 103, 117, 147, 189, 205, 217, 291, 307, 319, 345, 421
Gallic acid, 4TMS	GA	29.43	C ₁₉ H ₃₈ O ₅ Si ₄	73, 179, 207, 237
Gluconic acid, 6TMS	GlucA	30.14	C ₂₄ H ₆₀ O ₇ Si ₆	73, 103, 117, 147, 157
Catechollactate, 4TMS	Cat	31.42	C ₂₁ H ₄₂ O ₅ Si ₄	73, 147, 179, 267, 396
Myo-inositol, 6TMS	Myo	31.86	C ₂₄ H ₆₀ O ₆ Si ₆	59, 73, 81, 87, 103, 117
Caffeic acid, 3TMS	CafA	32.85	C ₁₈ H ₃₂ O ₄ Si ₃	73, 191, 219, 381, 396
Glyceryl-glycoside, TMS ether	Gly-G	35.90	C ₂₇ H ₆₆ O ₈ Si ₆	73, 204, 361
Galacturonic acid, 5TMS	GalA	36.95	C ₂₁ H ₅₀ O ₇ Si ₅	73, 147, 204, 217, 292
D-Glucose-L-glycero-3-octulose, 7TMS-, 2-[methyloxime]	Octul	37.67	C ₃₀ H ₇₅ NO ₈ Si ₇	73, 89, 103, 133, 147, 191, 205, 217, 244, 277, 305, 319, 364, 434
Arbutin, 5TMS	Arb	40.28	C ₂₇ H ₅₆ O ₇ Si ₅	73, 103, 129, 147, 169
Sucrose, 8TMS	Sucr	40.89	C ₃₆ H ₈₆ O ₁₁ Si ₈	73, 103, 147, 361, 437
<i>Non-polar</i>				
Myristic acid, ME	C14:0	8.68	C ₁₅ H ₃₀ O ₂	43, 55, 69, 74, 87
6,10,14-Trimethyl-2-pentadecanone	C15, 2 one	10.23	C ₁₈ H ₃₆ O	43, 58, 71, 85, 95, 109, 124, 210, 250
Palmitic acid, ME	C16:0	10.33	C ₁₇ H ₃₄ O ₂	43, 74, 87, 143, 227
Tetracosane	24sane	11.67	C ₂₄ H ₅₀	43, 57, 71, 85, 99
Stearic acid, ME	C18:0	12.44	C ₁₉ H ₃₈ O ₂	73, 87, 143, 255, 298
11-Octadecenoic acid, ME	C18:1	12.76	C ₁₉ H ₃₆ O ₂	41, 55, 69, 74, 87
Heptacosane	27sane	12.77	C ₂₇ H ₅₆	43, 57, 71, 85, 99
Linoleic acid, ME	C18:2	13.36	C ₁₉ H ₃₄ O ₂	41, 55, 67, 81, 95
Hexacosane	26sane	13.95	C ₂₆ H ₅₄	43, 57, 71, 85, 113
Eicosanoic acid, ME	C20:0	14.94	C ₂₁ H ₄₂ O ₂	43, 55, 69, 74, 87

TABLE 2 (Continued)

Detected metabolite	Abbreviation	RT (min)	Molecular formula	m/z
Nonacosane	29sane	17.85	C ₂₉ H ₆₀	43, 57, 71, 85, 99
3-Hydroxy-octadecanoic acid, ME	3OH-C18:0	18.89	C ₁₉ H ₃₈ O ₃	41, 55, 74, 103
Tetracosanoic acid, ME	C24:0	20.51	C ₂₅ H ₅₀ O ₂	43, 74, 87, 143, 339
2,4-Bis(dimethylbenzyl)-6-butylphenol	2,4BDTBP	21.28	C ₂₈ H ₃₄ O	91, 119, 293, 371, 386

(Table 3). GABA was found in three species showing the highest value in laurel followed by mint and eucalyptus.

Among the organic acids tentatively identified in the polar leaf extracts, malic acid was found in all plant species analysed in this study except in *A. unedo*, with the highest levels in laurel ($3.79 \pm 0.09\%$) and rosemary ($2.07 \pm 0.14\%$) leaves. Laurel aqueous extract also showed the highest content of citric acid ($4.49 \pm 1.16\%$); this compound also reached a high value in eucalyptus ($3.25 \pm 0.35\%$) compared to the other species (Table 3).

The carbohydrate fructose, detected as fructose 1,3,4,5,6-pentakis-O-(trimethylsilyl)-O-methyloxime showed in the mass spectrum *m/z* peaks at *m/z* = 73, loss of trimethylsilyl (TMS) group; *m/z* = 89, loss of oxygen-TMS group, *m/z* = 103 and 204 cleavages between C5-C6 and between C4-C5; *m/z* = 217 the fragment with chemical formula C₉H₂₁O₂Si₂²⁺; *m/z* = 307 cleavage between C3-C4. Fructose, followed by glucose, represents the major component in *A. unedo*, *Eucalyptus camaldulensis*, *Mentha aquatic*, *Myrtus communis* and *Rosmarinus officinalis* extracts (Table 3). Both monosaccharides reach the highest values in myrtle leaves ($43.51 \pm 1.24\%$ and 28.82 ± 0.76 for fructose and glucose, respectively), with approximately two-fold increases in comparison with other species. Whereas *C. siliqua* and *L. nobilis* show other carbohydrates as major components in their extracts, sucrose and D-glucosyl-glycerol-3-octulose, respectively. In carob leaves, the most abundant sugar is sucrose ($20.32 \pm 4.56\%$) followed by glucose ($12.91 \pm 4.78\%$). It is worth noting that the saccharide D-glucosyl-glycerol-3-octulose was found only in laurel extract as the major sugar component ($37.29 \pm 1.19\%$) of this species. Although, this compound has the highest impact in the semi-quantitative of the laurel aqueous extract, the NIST mass library alone was not able to identify its TMS derivative, showing a very low score (< 10%) when compared to other mass spectra. Thus, the identification of D-glucosyl-glycerol-3-octulose was supported by NMR experiments acquired on the aqueous extract of *L. nobilis*. Results obtained by proton (¹H)-NMR, heteronuclear single quantum correlation (HSQC) and total correlated spectroscopy (TOCSY) experiments (Figures S3–S7) are in agreement with data literature published by Sakata et al.,⁵⁷ who found the D-glucosyl-glycerol-3-octulose as the main constituent of laurel leaves and presented its structure identification by carbon-13 (¹³C)-NMR.

3.4 | Multivariate data analysis

The dendrogram of the seven plant species based on the 22 most abundant compounds shows that *Rosmarinus officinalis* and *Eucalyptus*

camaldulensis as well as *Myrtus communis* and *Mentha aquatic* are relatively similar to each other. On the contrary *A. unedo*, *C. siliqua* and especially *L. nobilis* showed the largest distance from the other plants analysed (Figure 2).

The multivariate approach based on GC-MS data highlighted remarkable differences among the metabolites related to their antioxidant activities (Figure 3). The correlation analysis performed between the compounds and the antioxidant activity and plotted in the PCA has previously been used to correlate major compounds and antioxidant activity of essential oils of basil chemotypes.⁵⁸ The PCA revealed that the first and second components represent 60.0% of the total variance. The first principal component (PC1) is responsible for 38.1% of the total variance and correlated positively with the methods ABTS and DPPH. The second principal component (PC2) accounted for 21.9% of the total variance and correlated positively with the mentioned methods.

3.5 | Correlation between metabolite profile and antioxidant activity

Figure 4 explains the correlation between ABTS and DPPH with the abundance of each specific metabolite. Of note, the major phenolic compound in *A. unedo* leaves aqueous extract was found to be arbutin (Table 3). This result is in agreement with data literature published by Fiorentino et al.⁵⁹ and Jurica et al.⁶⁰ According to previous works, positive effects of *A. unedo* leaf extracts for gastrointestinal complaints are related to antioxidant compounds, such as arbutin and flavonoids.^{12,61} However, the high antioxidant activity of *A. unedo* shown in the ABTS assay (Figure 1, bottom) is probably due not only to the high level of arbutin but also to the high level of the quinic acid ($33.30 \pm 7.55\%$). Figure 4 shows a positive correlation between ABTS and compounds such as quinic acid and arbutin but a negative correlation between DPPH and quinic acid and arbutin. This might also explain why *A. unedo* shows at the same time both a low level of TPC and high value of ABTS assay. The high ABTS value of *A. unedo* is not related only to phenols but might be related to other classes of compounds. Correlation analysis showed that ABTS was negatively associated with abundance of GABA, valine, threonine acid, lactic acid, butanedioic acid, glycerol and myo-inositol with positive but weak correlations with 1H-indole-2,3-dione, 5-ethyl-3-oxime and gallic acid (Figure 4). Also, DPPH was correlated negatively with GABA and glycerol but positively correlated with gallic acid and pinitol (Figure 4).

In accordance with TPC results, where *C. siliqua* showed the highest value of the TPC (Figure 1, top), the metabolites profile

TABLE 3 Relative abundance (%) of main metabolites detected by gas chromatography–mass spectrometry (GC–MS) analysis in polar and non-polar extracts of seven aromatic plants, as calculated from spectral peak intensity. For each metabolite, abbreviation reported in Table 2 were considered. Data refer to mean $\pm$ standard deviation of three replicates for each plant. Results of analysis of variance (ANOVA) are also reported and lowercase letters from indicate statistically significant differences (Tuckey's test, $P < 0.05$). Compounds not detected in the sample are reported as n.d.

	<i>Arbutus unedo</i>	<i>Ceratonia siliqua</i>	<i>Eucalyptus camaldulensis</i>	<i>Laurus nobilis</i>	<i>Mentha aquatica</i>	<i>Myrtus communis</i>	<i>Rosmarinus officinalis</i>							
Polar														
Amino acids														
CYC (IS)	11.69 ± 0.76	e	6.89 ± 1.43	d	36.89 ± 2.28	a	31.31 ± 2.42	b	31.75 ± 6.86	ab	16.00 ± 0.80	c	30.06 ± 3.04	b
GABA	n.d.		n.d.		0.01 ± 0.001	c	0.58 ± 0.05	a	0.41 ± 0.04	b	nd		n.d.	
PCA	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		0.18 ± 0.11	
Pro	n.d.		0.07 ± 0.02	a	n.d.		n.d.		n.d.		n.d.		0.11 ± 0.08	a
Val	n.d.		n.d.		n.d.		n.d.		0.32 ± 0.04		n.d.		n.d.	
Carbohydrates														
Fru	27.31 ± 3.58	b	9.66 ± 2.78	cd	25.82 ± 1.27	b	6.14 ± 1.88	d	15.29 ± 5.5	c	43.51 ± 1.24	a	24.42 ± 1.24	b
GalA	n.d.		0.06 ± 0.01		n.d.		n.d.		n.d.		n.d.		n.d.	
Glc	11.44 ± 6.42	b	12.91 ± 4.78	b	22.23 ± 1.21	a	8.99 ± 0.35	b	7.80 ± 3.01	b	28.82 ± 0.76	a	11.45 ± 0.75	b
GlucA	n.d.		n.d.		n.d.		n.d.		1.10 ± 0.39	a	n.d.		1.34 ± 0.05	a
GlyA	n.d.		n.d.		n.d.		0.08 ± 0.01	a	n.d.		0.08 ± 0.06	a	0.09 ± 0.04	a
Gly-G	n.d.		n.d.		n.d.		n.d.		0.85 ± 0.45		n.d.		n.d.	
Man	3.40 ± 0.52	b	1.05 ± 0.47	d	2.77 ± 0.13	b	1.36 ± 0.36	cd	1.89 ± 0.07	c	4.24 ± 0.17	a	1.53 ± 0.09	cd
Octul	n.d.		n.d.		n.d.		37.29 ± 1.19		n.d.		n.d.		n.d.	
Rib	n.d.		n.d.		n.d.		n.d.		n.d.		0.03 ± 0.01		n.d.	
Sucr	7.92 ± 1.80	b	20.32 ± 4.56	a	4.11 ± 0.85	bc	n.d.		0.88 ± 0.34	c	0.82 ± 0.17	c	8.42 ± 0.52	b
ThA	n.d.		0.11 ± 0.08	b	0.07 ± 0.02	b	n.d.		0.78 ± 0.23	a	0.08 ± 0.01	c	0.49 ± 0.25	a
Indole														
I	n.d.		n.d.		0.02 ± 0.001		n.d.		n.d.		n.d.		n.d.	
Organic acids														
LA	n.d.		n.d.		n.d.		n.d.		0.46 ± 0.29		n.d.		n.d.	
ButA	n.d.		n.d.		n.d.		n.d.		0.61 ± 0.19	a	n.d.		0.41 ± 0.34	a
CA	n.d.		n.d.		3.25 ± 0.35	b	4.49 ± 1.16	a	n.d.		0.08 ± 0.01	c	0.66 ± 0.37	c
MA	n.d.		0.20 ± 0.07	d	0.13 ± 0.002	d	3.79 ± 0.09	a	0.80 ± 0.16	c	0.08 ± 0.02	d	2.07 ± 0.14	b
Phenols														
Arb	1.30 ± 0.44		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.	
CafA	n.d.		0.02 ± 0.00	b	0.01 ± 0.001	b	n.d.		0.21 ± 0.06	a	n.d.		0.27 ± 0.10	a
Cat	n.d.		n.d.		n.d.		n.d.		0.13 ± 0.05	b	n.d.		1.43 ± 0.07	a

TABLE 3 (Continued)

	<i>Arbutus unedo</i>	<i>Ceratonia siliqua</i>	<i>Eucalyptus camaldulensis</i>	<i>Laurus nobilis</i>	<i>Mentha aquatica</i>	<i>Myrtus communis</i>	<i>Rosmarinus officinalis</i>							
GA	0.76 ± 0.49	b	16.26 ± 4.61	a	1.44 ± 0.08	b	0.03 ± 0.02	b	0.41 ± 0.16	b	1.43 ± 0.11	b	0.27 ± 0.13	b
Polyols														
GLY	n.d.		0.25 ± 0.08	d	0.05 ± 0.01	d	2.93 ± 0.64	b	4.23 ± 0.28	a	n.d.		1.61 ± 0.10	c
Mannit	n.d.		n.d.		n.d.		n.d.		0.29 ± 0.15	a	n.d.		0.24 ± 0.08	a
Myo	2.88 ± 0.19	c	0.66 ± 0.29	c	1.05 ± 0.11	c	3.11 ± 0.24	c	29.92 ± 4.47	a	1.53 ± 0.10	c	11.05 ± 0.86	b
Pin	n.d.		31.33 ± 5.12		n.d.		n.d.		n.d.		n.d.		n.d.	
QA	33.30 ± 7.55	a	0.20 ± 0.03	cd	2.15 ± 0.14	bc	0.07 ± 0.03	d	1.89 ± 0.88	c	3.28 ± 1.29	b	3.88 ± 0.16	b
Non-polar														
Alkanes														
24sane	n.d.		n.d.		6.65 ± 0.65	a	7.08 ± 0.30	a	4.22 ± 0.08	b	n.d.		n.d.	
26sane	n.d.		11.50 ± 0.24	a	n.d.		8.38 ± 0.34	b	4.36 ± 0.43	c	n.d.		n.d.	
27sane	n.d.		n.d.		n.d.		9.58 ± 0.27		n.d.		n.d.		n.d.	
29sane	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		23.59 ± 0.13	
Fatty acids														
C14:0	2.2 ± 0.23	b	1.52 ± 0.25	cd	2.79 ± 0.57	a	1.19 ± 0.03	de	0.96 ± 0.25	e	n.d.		n.d.	
C15, 2 one	n.d.		n.d.		n.d.		5.37 ± 0.18	b	7.49 ± 0.63	a	n.d.		n.d.	
C16:0	41.24 ± 1.81	b	26.88 ± 1.72	cd	25.56 ± 6.54	c	26.73 ± 0.17	c	45.75 ± 0.68	ab	50.96 ± 3.23	a	18.41 ± 1.82	d
C17:0 (IS)	47.17 ± 1.89	a	39.54 ± 3.26	b	46.94 ± 3.53	a	20.00 ± 0.55	e	24.95 ± 2.10	d	34.41 ± 2.59	c	14.43 ± 1.06	f
C18:0	6.34 ± 0.26	c	8.75 ± 0.33	b	6.22 ± 0.41	c	5.09 ± 0.26	c	8.52 ± 1.26	b	14.64 ± 0.84	a	8.66 ± 1.60	b
C18:1	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		2.72 ± 0.40	
C18:2	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		3.00 ± 0.35	
3OH-C18:0	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		26.52 ± 0.20	
C20:0	3.00 ± 0.53	bc	5.80 ± 0.98	a	2.43 ± 0.21	cd	1.68 ± 0.18	d	2.12 ± 0.17	d	n.d.		n.d.	
C24:0	n.d.		n.d.		n.d.		3.97 ± 0.30		n.d.		n.d.		n.d.	
Phenol														
2,4BDTBP	n.d.		6.01 ± 0.46	abc	9.41 ± 10.68	ab	10.93 ± 0.24	a	1.64 ± 0.19	c	n.d.		2.67 ± 0.11	bc

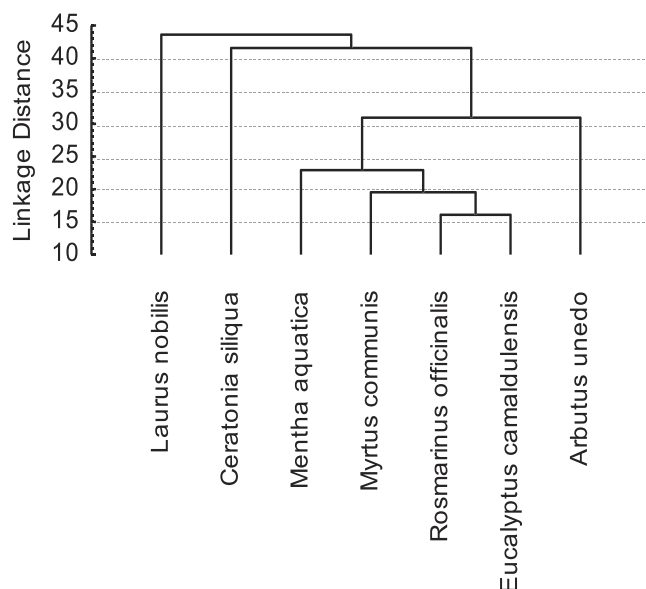


FIGURE 2 Dendrogram of studied aromatic plant based on the 22 main compounds identified in the extracts

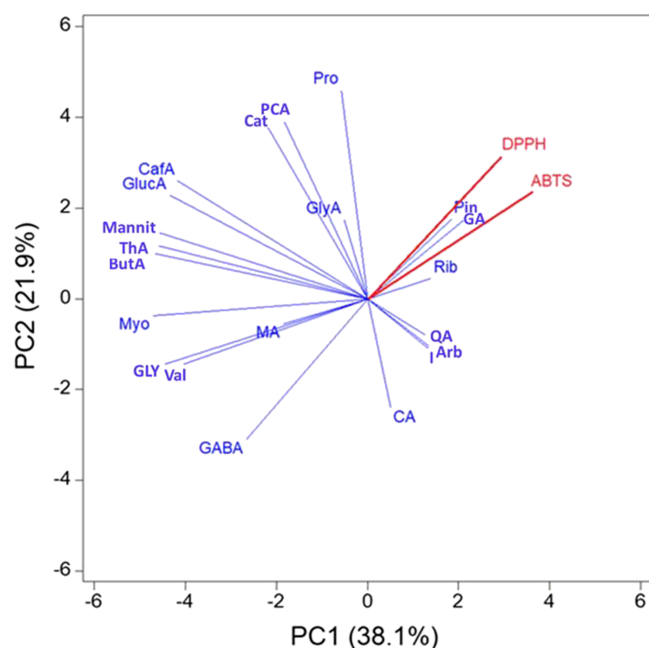


FIGURE 3 Principal component analysis (PCA) between plant chemistry and associate activities (ABTS, DPPH). Panel shows loading vectors of different chemical compounds, with ABTS, DPPH are also plotted as a supplementary variable following Legendre and Legendre.²⁵ Compound abbreviation as follows: Arb, arbutin; ButA, butanedioic acid; CA, citric acid; CafA, caffeic acid; Cat, catechollactate; GA, gallic acid; GABA, gamma-aminobutyric acid; GlucA, gluconic acid; GLY, glycerol; GlyA, glyceric acid; I, 1H-indole-2,3-dione, 5-ethyl-3-oxime; LA, lactic acid; MA, malic acid; Mannit, mannitol; Myo, myo-inositol; PCA, 5-oxoproline; Pin, pinitol; Pro, proline; QA, quinic acid; Rib, ribonic acid; ThA, threonic acid; Val, valine

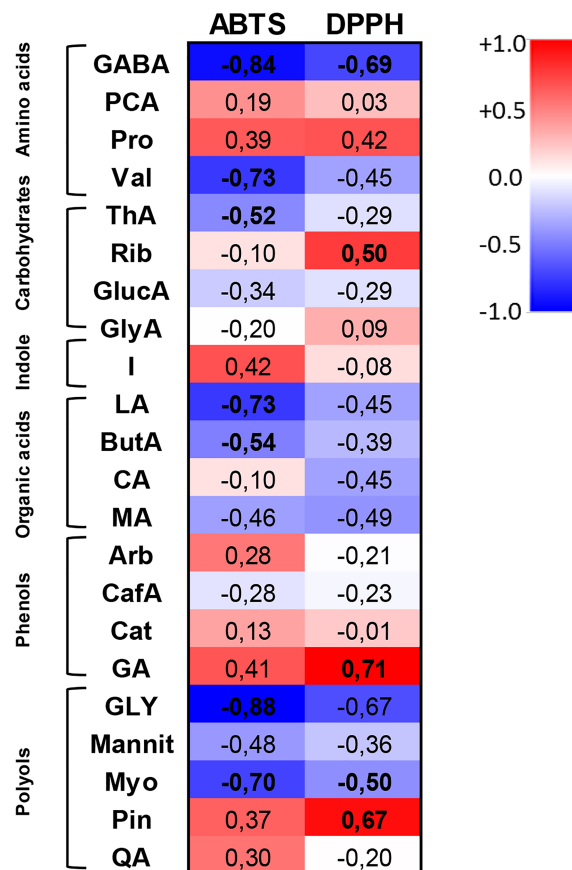


FIGURE 4 Heat correlation map between ABTS and DPPH activities with abundance of the 22 compounds. Values are Pearson coefficient, in bold significant values ($P < 0.05$). Compound abbreviation as reported in Figure 3

indicated that phenols reach the highest values in carob leaves, having gallic acid as the major component ($16.26 \pm 4.6\%$). However, the relative low content of phenols in myrtle and rosemary extract, as indicated in Table 3 (1.43% and 1.97% mostly represented by gallic acid and catechollactate, respectively), does not explain the high values of their TPC results (Figure 1, top). This is probably due to the presence of other phenol compounds in the leaf extract of this species that were not detected at GC-MS. In fact, previous work on myrtle leaves reported, besides gallic acid as main phenol, other phenolic acids (caffeic, syringic, vanillic and ferulic acids) at very low amount ranging from 0.35% to 0.71% .²⁷

Polyols are the second major components after sugars in the aqueous leaf extracts. Among them, quinic acid is a sugar acid while myo-inositol, mannitol and glycerol belong to the sub class of sugar alcohols. Sugar alcohols are frequently found in plants, fruits, and vegetables. They are used as sugary additive in food, drinks and medicines for their sweet taste and low-calories content.⁶² High values of myo-inositol and glycerol were found in mint and rosemary (Table 3). The high content of myo-inositol in mint leaf extract makes this plant a good candidate for the extraction of this metabolite from natural sources. Quinic acid values showed high levels in strawberry tree followed by rosemary with an

eight-fold decrease (Table 3). Interestingly, in each water plant extract the main metabolites are represented by carbohydrates, except for strawberry tree and carob in which quinic acid and pinitol showed the highest values, followed by fructose and sucrose, respectively (Table 3). This might explain the linkage distance of these two species compared to the others (Figure 2). D-Pinitol was only found in carob water extract. Numerous *in vitro* and *in vivo* studies have shown that D-pinitol has several pharmacological activities, such as antidiabetic, anti-inflammatory, anti-cancer, chemopreventive, and antioxidant.⁶³ High content of D-pinitol was found in carob pods and soybean leaves, but so far there are no studies reporting D-pinitol in carob leaves.^{64,65} Thus, carob leaves can be a new potential source for the extraction of D-pinitol as food supplement. Moreover, the high content of this compound in carob leaves water extract can explain the high antioxidant activity of *C. siliqua* on ABTS and DPPH assays (Figure 1). This compound is in the same PCA quadrant of ABTS, and DPPH methods (Figure 3) and it is positively correlated with the antioxidant activities (Figure 4). As a general trend of the PCA (Figure 3) it was observed that PC1 correlated positively with the methods ABTS and DPPH and with metabolites arbutin, ribonic acid, quinic acid, pinitol, gallic acid while PC2 correlated positively with the mentioned methods and metabolites butanedioic acid, threonic acid, mannitol, gluconic acid, caffeic acid, glyceric acid, catechollactate, 5-oxoproline, proline, pinitol and gallic acid.

This study provides comprehensive information on the metabolomics profiles of aromatic plants and their antioxidant activity. Among the plant analysed, *L. nobilis*, *C. siliqua* and *A. unedo* showed the highest linkage distance compared to the other species. Most probably this is due to the high level of D-glucose-L-glycero-3-oxotulose, D-pinitol and quinic acid found in these three species, respectively. *Ceratonia siliqua*, *Myrtus communis* and *Rosmarinus officinalis* have the highest TPC. However, the results of DPPH and ABTS assays revealed that there might be another class of compounds, different from phenols, responsible for the antioxidant activity of the analysed plants. Our method and correlation approach do not consider synergies or interferences in the multicomponent mixture analysed. The non-polar extracts are mostly composed of saturated fatty acids and among them palmitic acid is the most abundant in all species. In conclusion in this work a GC-MS based metabolomics profiling method has been used for seven aromatic plants of great importance in food for their essential oil production. In this study, the water and *n*-hexane extracts of the aromatic plants were explored suggesting the opportunities for further exploitation of these crops due to their metabolite profiles.

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DATA AVAILABILITY STATEMENT

Data available on request from the authors

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