Allelochemicals and esters from leaves and inflorescences of *Sambucus nigra* L.

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Highlights

- Allelochemicals are described in leaves and inflorescences from *Sambucus nigra*.
- First-ever report of the pheromone L-isoleucine methyl ester present in Adoxaceae plants.
- The cyanogenic compound mandelonitrile is the most abundant in leaf extracts of *Sambucus nigra*.
- *n*-Alkyl and benzylacyl esters, mandelonitrile and isoleucine methyl ester are characterized by GC-EIMS of their trimethylsilyl derivatives.

Abstract

Allelochemical compounds were detected in leaves and inflorescences of *Sambucus nigra* L. by means of GC-EIMS. The identified compounds were characterized by their mass spectra and relative retention times as their trimethylsilyl derivatives. The pheromone L-isoleucine methyl ester extracted from inflorescences can attract pollinator insects during the flowering period and together with uncommon *n*-alkyl and benzyl esters were firstly identified in *S. nigra*. On the other hand, mandelonitrile, which is used by the plant to avoid herbivore attack, was observed as the most abundant compound from the leaf extracts. In the present work are described 154

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compounds from leaves and 196 from inflorescences including alkanes, alcohols, acids and terpenoids from elder aerial parts.

Keywords: *Sambucus nigra*; Adoxaceae; elderberry; esters; allelochemicals; GC-EIMS.

1. Introduction

The elder (*Sambucus nigra* L.) is an important berry worldwide crop plant widely transformed in jam, jellies and beverages broadly used in gastronomy (Baudar, 2016) and has been used for a long time for his potential benefits to health (Schmitzer *et al*., 2016). Lipids from this plant additionally contain allelochemicals which have been reviewed by De Albuquerque *et al*. (2011).

The elder exhibits allelopathic strategies in order to interact with other species which are harmful, as herbivorous and fungi, or allow it to attract entomophile pollinators. The elderberry contains harmful cyanogenic compounds (Senica *et al*., 2016) which should be taken into account for the consumption. Specifically, the cianolipid mandelonitrile is synthesized by the catalytic activity of the enzyme mandelonitrile lyase over benzaldehyde and cyanide substrates. Its functional role has been screened from several plants including *S. nigra* in order to determine its cyanogenic activity (Asano *et al*., 2005). Hydrolysis of mandelonitrile releases HCN, which is a potent respiratory poison (Swain *et al*., 1994). On the other hand, methyl esters of several amino acids have been described as powerful allelochemicals with sexual pheromone activity in animal kingdom. For example, Robbins *et al*. (2009) used valine methyl ester released by the female from the scarab *Phyllophaga georgiana* for capturing the male of the same species and L-isoleucine methyl ester in order to trap three other species of *Phyllophaga*.

The elder (*Sambucus nigra* Linn.) also known as elderberry is a deciduous and perennial tall shrub or small tree belonging to the Adoxaceae family and original from Asia, North Africa and Europe. This plant has narrow, dark green leaves arranged oppositely, 10-30 cm long and pinnate. The inflorescence which is produced from April to August groups hundreds of individual odorous white flowers having five petals and five stamens and possesses a pleasant strong smell.

The phytochemical description of elderberry including bark, pollen, leaves, flowers and berries, have been previously described by Lawrie *et al*. (1964), Stránský *et al*. (2001), Jäger *et al*. (2009), López-García *et al*. (2013) and Salvador *et al*. (2015) respectively.

The aim of this paper was the identification and quantitation of lipids from the total extracts of leaves and inflorescences by GC-EIMS. We have also focused our characterization on the ester distribution due to their interest because of their scarce description in natural products reports in food plant chemistry. The occurrence of *n*-alkyl and benzylacyl esters has been previously described in few species e.g. *Simmondsia chinensis* (Gülz and Marner, 1986) and *Solanum tuberosum* (Szafranek and Synak, 2006). We report here 5 families of these uncommon esters together with the allelochemicals mandelonitrile and L-isoleucine methyl ester.

2. Material and methods

2.1. Samples

Branches of *S. nigra* were collected during the flowering period (27^{th} 5, 2014) at Manresa (Catalonia; location: latitude 41º43'12.21'' N, longitude 1º49'16.45'' E, 210 m altitude). A voucher specimen (BCN 130076) was deposited at the herbarium of the Faculty of Pharmacy, Universitat de Barcelona (Herbari BCN, Centre de Documentació de Biodiversitat Vegetal).

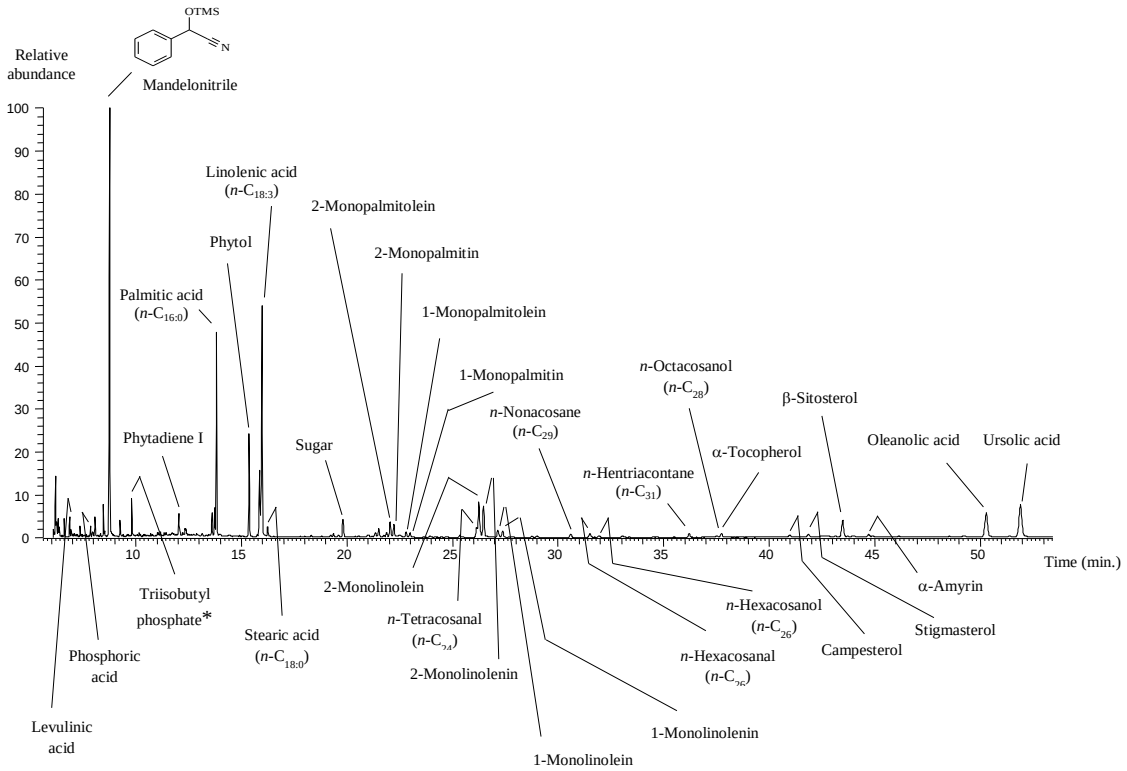


Fig. 1. Total Ion Chromatogram (TIC) obtained by GC-EIMS for extracts of leaves from *Sambucus nigra*. Compounds have been analyzed as trimethylsilyl derivatives. The name and structure of compounds are also shown (* are contaminants).

2.2. Analytical procedures

2.2.1. GC-EIMS pre-analytical conditions: sample treatment, extraction and derivatization

Fresh leaves and inflorescences were crushed and homogenized in a glass mortar, using a glass pestle together with 25 g of previously cleaned sea-sand. All inert materials and tools were previously cleaned and rinsed with acetone before use. The ground samples were introduced into cellulose thimbles, and then extracted in a Söxhlet apparatus over 24 h, using a 7:3 (*v/v*) mixture of pentane/dichloromethane. Milled leaves (5.51 g) and inflorescences (5.74 g) were extracted in darkness. A 300 μL aliquot of internal standard (friedeline; Aldrich) (200 mg/L) was added to the extract, which was subsequently concentrated to a volume of 0.5 mL, and finally, further concentrated under an N_2 stream. Derivatization of hydroxyl and carboxyl groups of extracted compounds were performed with 300 μL of N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA; Merck) by heating the resulting mixture at 70 ºC for 1 h prior to analysis according to Basas-Jaumandreu *et al*. (2014).

2.2.2. GC-EIMS analysis

Aliquots of 1 μL of silylated total extracts of the leaves and inflorescences samples were analyzed within 24 h using a Fisons Instruments gas chromatograph that was operating in splitless mode and coupled to a mass detector (GC 8000/MD 800), which was operating in electronic impact (EI) ionization mode (70 eV). The injector temperature was maintained at 275 ºC. The compounds were separated on a fused silica capillary column (DB-5ms; length: 30 m; i.d.: 0.32 mm) coated with a 0.25-μm low-polarity liquid-phase film (5% methylpolysiloxane; J&W Scientific, Folsom, California). The mass scanning in Total Ion Current (TIC) was acquired from 50 Daltons to 650 Daltons over a period of 1 s. The oven temperature was programmed as follows: start at 40 ºC (1 min); ramp up to 230 ºC (20 ºC/min); ramp up to 300 ºC (2 ºC/min); and finally, hold at 300 ºC (20 min). Helium was used as the carrier gas (flow rate: 1.0 mL/min). The inlet temperature was 300 ºC; the transfer-line temperature, 310 ºC; the ion-source temperature, 200 ºC; and the interface temperature, 300 ºC.

2.2.3. Identification of compounds

Compounds were analyzed by GC/EI-MS. The analytes were identified by comparing their characteristic mass fragmentation patterns and retention times to those reported in the literature. The phenylmethyl and phenylethyl esters were identified according to Gülz and Marner (1986) based on their molecular ion patterns (base peak at *m/z* 91 and 104 respectively). Valeric acid *n*-alkyl esters and benzoyl esters (base peak at *m/z* 103 and 123 respectively) were described through the similarity with some short-chain homologues included on the NIST database.

2.3. Quantitation

Compounds were quantified from the integrated peak area in the TIC using MassLab software. The semi-quantitative results were normalized according to the internal standard (friedeline, Aldrich). Dry weight was calculated after drying at 105ºC to constant weight in an oven (Selecta).

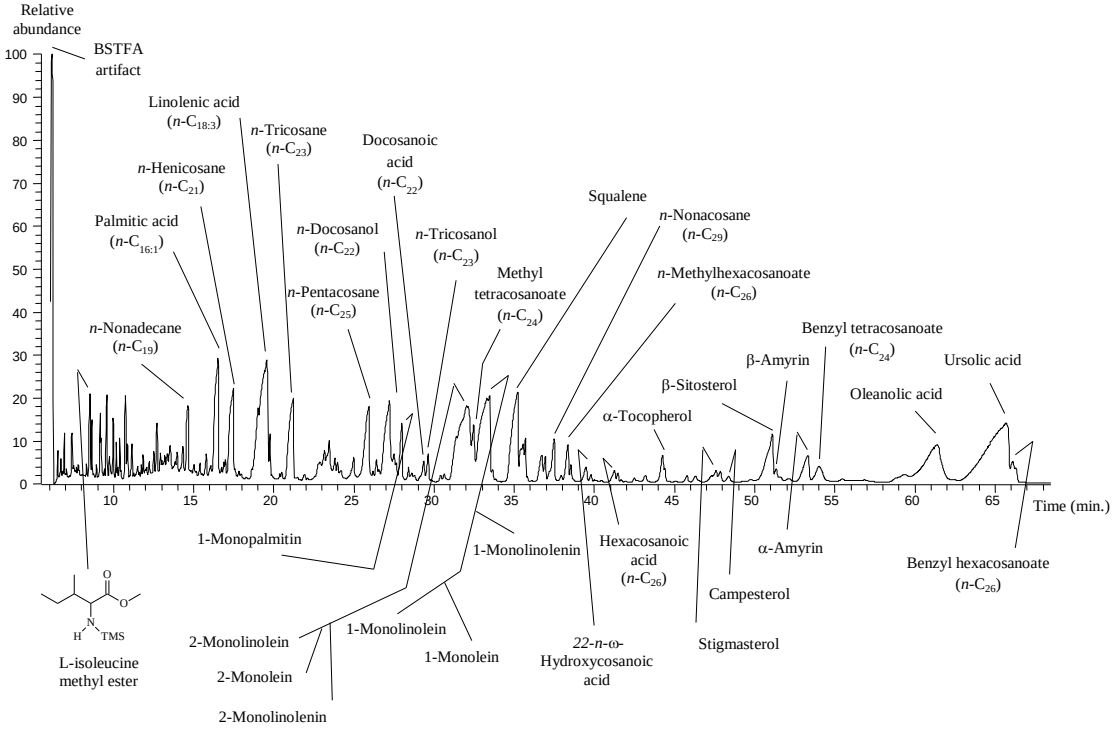


Fig. 2. Total Ion Chromatogram (TIC) obtained by GC-EIMS for extracts of inflorescences from *Sambucus nigra*. Compounds have been analyzed as trimethylsilyl derivatives. The name and structure of compounds are also shown.

3. Results and discussion

3.1. Overall description of phytochemicals of Sambucus nigra leaves and inflorescences.

Compared overall chemotaxonomic GC-EIMS analysis of leaves and inflorescences from *S. nigra* is reported for the first time. The respective chromatograms of the total extracts of both aerial parts are presented in Figures 1 and 2 including the most common name of the major compounds. The reported phytochemical analysis of semivolatile compounds from *S. nigra* in the literature is summarized in Supplementary S1 in the Appendix section. Then, the total chemical composition of extracts from both plant parts investigated in this work is summarized in Supplem. S2 and S3 which includes molecular weight (M^+), retention time (min.) and concentration (mg/kg dry weight) of all compounds identified. The GC-EIMS allowed us the

identification of 154 compounds from the leaves and 196 compounds from the inflorescences of *S. nigra*. We have focused on alkyl and phenylacyl ester compounds because they are unusual components of cuticular wax scarcely reported as far as we know from the phytochemical analysis of food plants (Bianchi *et al*., 1995; Szafranek and Synak, 2006). *S. nigra* extracts includes a collection of different series of fatty acid methyl esters (FAMEs), valeric acid *n*-alkyl esters as well as phenylmethyl, phenylethyl and benzoyl esters. Cianolipids and unsaturated fatty acids respectively accounted for 23 and 21% of the identified compounds from the leaves while *n*-alkanes and pentacyclic triterpenoids were the major components from the inflorescences (25 and 24%, respectively). These results on flowers are similar to those reported by Salvador *et al*. (2015) on berries from this plant with triterpenoids and fatty acids as the main chemical families of compounds. According to previous considerations on plant flowering (Torras *et al*. 2007) oxygenic compounds are more abundant in flowers being more appetizing during food digestion.

3.2. Ester compounds

Lipophilic extracts from *S. nigra* are characterized by the presence of several well-known esters together with others which are unusual phytochemicals and investigated here because of the scarcity of their presence in plant kingdom. Figs. 3 and 4 consist on mass chromatograms of most uncommon esters identified from the leaves and inflorescences of *S. nigra* and the mass spectra of the most representative homologue of each series.

3.2.1. *n*-Alkyl esters

3.2.1.1. Fatty acid methyl esters (FAMEs)

Six methyl esters of saturated FAMEs ranging from methyl docosanoate (*n*-C_{22}) to methyl octacosanoate (*n*-C_{28}) were identified and quantified from the leaves of *S. nigra* (55 mg/kg dry weight). The concentration profiles of these compounds both in leaves and inflorescences are presented in Supplem. S4. Methyl tetracosanoate (*n*-C_{24}; 63%) was the most abundant homologue and those with even carbon atom number were more abundant than the odd ones. In the inflorescences, these compounds were more abundant (2959 mg/kg dry weight) and the profile was identic with C-24 (62%) as the major one. As far as we know, this is the first report about the FAMEs of *S. nigra*.

3.2.1.2. Valeric acid *n*-alkyl esters ≡ Esters of valeric acid and *n*-alkanols

Five pentanoic acid alkyl esters are reported for the first time in *S. nigra*. These compounds have not been published excepting by the inclusion of some short chain homologues gathered

by NIST database. Pentacosyl pentanoate (M^+ = 452), tetracosyl pentanoate (M^+ = 438), tricosyl pentanoate (M^+ = 424), docosyl pentanoate (M^+ = 410) and eicosyl pentanoate (M^+ = 382) were detected in the extract from the inflorescences of *S. nigra* but not in the leaves (Figure 3a). Supplem. S4 shows the histogram of concentrations of these ester compounds and S5 their GC-EIMS structural characteristics. Mass spectrum of valeric acid docosyl ester (Figure 4a) shows a base peak at *m/z* 103 that appears from the arrangement of the alkoxy portion of the ester commonly referred as McLafferty rearrangement with double hydrogen transfer (Pavia *et al*., 2015). Another ion corresponds to the loss of *m/z* 102 from the molecular ion ($[M]^+$ = 410) (*m/z* 308).

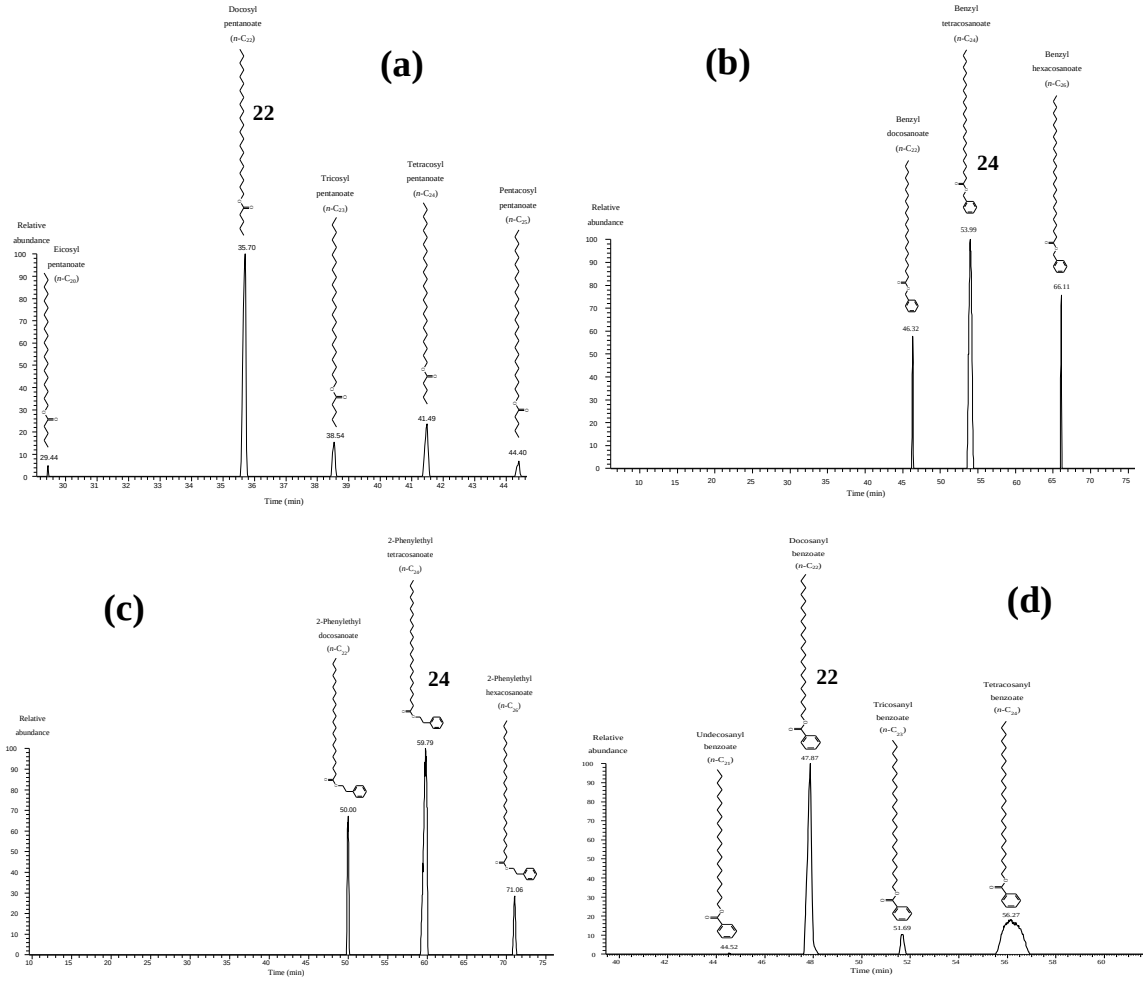


Fig. 3. Mass chromatogram corresponding to: the base peak at *m/z* 103 for all pentanoic acid *n*-alkyl esters (a), *m/z* 108 for benzyl esters (b), *m/z* 104 for phenylethyl esters (c) and *m/z* 123 for *n*-alkyl benzoates (d) identified in the inflorescences from *S. nigra*. In bold is indicated the number of carbon atoms of the *n*-alkanol or *n*-alkanoic acid moiety esterified to valeric acid (a), benzylic alcohol (b), 2-phenylethanol (c) and benzoic acid (d), respectively.

3.2.2. Benzene ring-linked *n*-alkylesters

Three classes of aromatic esters have been identified from the leaves and inflorescences of *S. nigra*.

3.2.2.1. Benzyl acyl esters ≡ Phenylmethyl esters

Three and five aromatic esters of benzyl alcohol and long-chain fatty acids (*n*-C_{22} – *n*-C_{26}) constituting a homologous series have been recognized through the extracted tissues of the leaves and inflorescence from *S. nigra*, respectively. Supplem. S4 shows the histogram of concentrations of these ester compounds and S6 GC-EIMS structural characteristics of these compounds. Benzyl docosanoate (docosanoic acid, phenylmethyl ester) ($[M]^{+\cdot}$ 430), benzyl tetracosanoate (tetracosanoic acid, phenylmethyl ester) ($[M]^{+\cdot}$ 458) and benzyl hexacosanoate (hexacosanoic acid, phenylmethyl ester) ($[M]^{+\cdot}$ 486) were always present and the most abundant was benzyl tetracosanoate (Figure 3b). This mass chromatogram was obtained from a major fragment at *m/z* 108 corresponding to the radical cation of the benzyl alcohol moiety. Fig. 4b shows the mass spectrum of the benzyl tetracosanoate (tetracosanoic acid, phenylmethyl ester) as the maximum member of the distribution of benzyl esters identified in the inflorescences and leaves from *S. nigra*. First fragmentation implies an elimination of a neutral ketene ion (*m/z* 350) together with the radical cation of benzyl alcohol at *m/z* 108 that is transformed in the also abundant tropylium cation (*m/z* 91) which is the base peak. Other fragments include a peak at *m/z* 367 ($[M-91]^{+\cdot}$) (loss of a benzyl radical), *m/z* 351 ($[M-107]^{+\cdot}$) and *m/z* 349 ($[M-109]^{+\cdot}$) (loss of a benzyloxy radical through α-cleavage) (Pavia *et al*., 2015), *m/z* 440 which is the loss of a water molecule and *m/z* 367 (loss of the tropylium ion). Mass spectrum of this compound also shows the molecular peak *m/z* 458. Rapley *et al*. (2004) reported benzyl tetracosanoate from the foliar wax of *Eucalyptus globulus*. Gülz *et al*. (1989) reported from epicuticular waxes of beech (*Fagus sylvatica*) a series of benzylacyl esters with a fatty acid chain length of *n*-C_{24} – *n*-C_{28}. Gülz and Marner (1986) isolated these compounds from the epicuticular waxes from *Simmondsia chinensis* leaves and were the first authors which partially described its MS data.

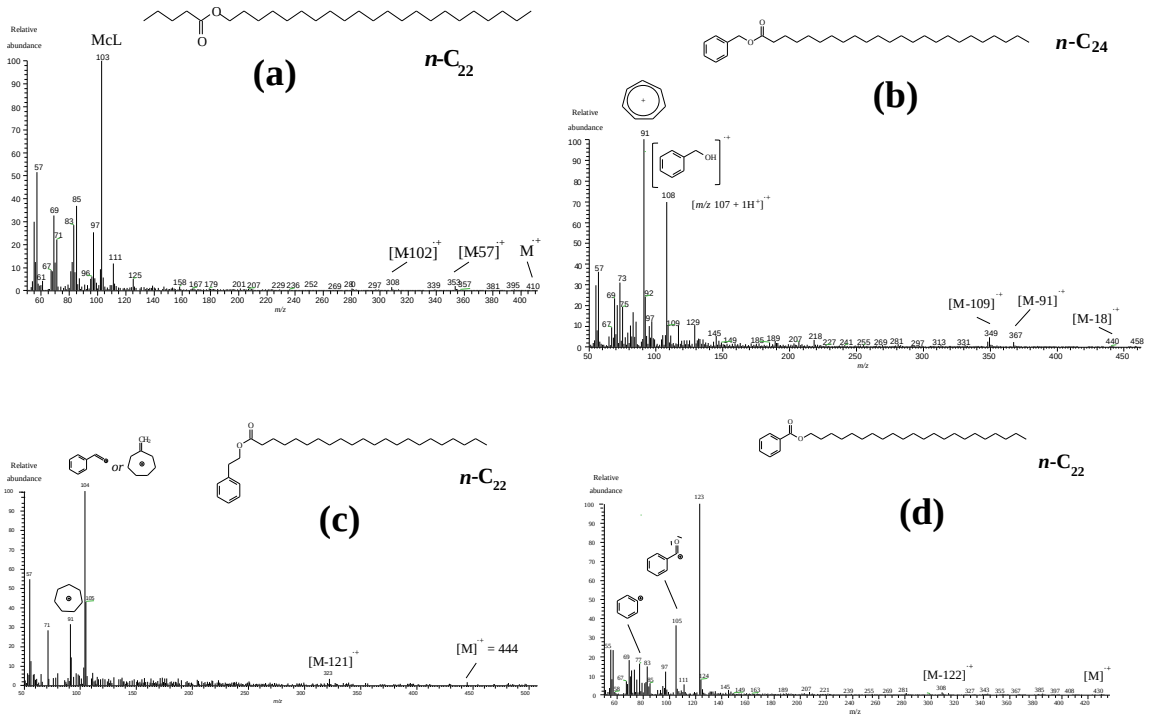


Fig. 4. Electron impact (EI) mass spectrum of the main representaives of 4 uncommon esters: (a) docosyl pentanoate, (b) benzyl tetracosanoate, (c) docosanyl benzoate and (d) 2-phenylethyl docosanoate identified from the inflorescences of *Sambucus nigra* extracts.

3.2.2.2. Phenylethyl esters ≡ 2-phenyl ethyl-1-acyl esters

Three esters of 2-phenyl-ethanol were identified from the leaves and inflorescences of *S. nigra*. Fig. 3c shows as retention time of these compounds is shifted to the right respect to benzyl esters in full agreement with those previously reported for *Simmondsia chinensis* (jojoba) leaf waxes by Gülz and Marner (1986). Another study conducted by Isidorov and Vinogorova (2003) found 8 homologues (*n*-C_{20} – *n*-C_{26}) from the buds of *Populus nigra*. A histogram of concentrations (S4) and the main spectral data of the fatty acid methyl esters (FAMEs), esters of valeric acid and *n*-alkanols, phenylmethyl esters, phenylethyl esters and *n*-alkyl benzoates (S7) were established for the pentane-dichloromethane extract of *S. nigra* leaves and inflorescences. MS data of these compounds was first described by Gülz and Marner (1986). Mass spectrum of 2-phenethylethyl docosanoate (Fig. 4c) shows a base peak at *m/z* 104 which results from the loss of a phenylethyl fragment from the molecular ion ($[M]^+$ = 444) and the also prominent ion at *m/z* 91 which is a tropylium moiety. The loss of *m/z* 121 is also characteristic of these unusual esters.

3.2.2.3. *n*-Alkyl benzoates

Benzoic acid esters of higher fatty alcohols have been reported in a low number of studies from cuticular waxes. In pentane-dichloromethane extracts of leaves and inflorescences of *S. nigra* series of homologous esters of benzoic acid consisting of four components (Fig. 3d) were identified through distinctive ions at *m/z* 123 (base peak) and *m/z* 105. Table 4 includes the chromatographic and spectrometric properties of these benzoyl esters. Supplem. S4 shows the histogram of concentrations of these ester compounds and S8 the main data observed in the mass spectra of long chain alkyl benzoates identified in *S. nigra* inflorescences extract. Fig. 4d shows the mass spectrum of the benzoyl ester homologue docosanyl benzoate together with its fragmentation pattern. Several peaks can be observed: the molecular ion at *m/z* = 430; a fragment at *m/z* = 308, which corresponds to loss of the moiety which includes de benzene ring; a very prominent peak at *m/z* 105 corresponding to the fragment [Ph-C=O] originated from an α-cleavage adjacent to the carbonyl group; then the fragment at *m/z* 77 consists on the aromatic nucleus; the base peak is at *m/z* 123 corresponding to the protonated benzoic acid [$C_6H_5COOH_2^+$] (Hesse *et al*., 2008).

3.2.3. ters of glycerol

3.2.3.1. Monoglycerides (MG)

A series of saturated and unsaturated 1- (α-) and 2- (β-) monoglycerides were found in leaves and inflorescences of *S. nigra* (Figs. 1 and 2). The chromatographic elution pattern was as follows: 1,3-dihydroxy-MG eluted before 2,3-dihydroxy-MG and unsaturated homologues also before saturated ones. Di- and triunsaturated compounds were the most abundant as is shown in Supplem. S9. Interpretation was according to mass data from Isidorov *et al*. (2007) and del Río *et al*. (2009).

3.2.3.1.1. MG of saturated fatty acids

3.2.3.1.1.1. 2,3-Dihydroxy-MG

Both in leaves and inflorescences, 1-monopalmitin (hexadecanoic acid, 2,3-bis-(OTMS) propyl ester or α-glyceryl palmitate (α-*n*-$C_{16:0}$)) was the most abundant 1-monoglyceride (97 and 96% respectively).

3.2.3.1.1.2. 1,3-Dihydroxy-MG

Hexadecanoic acid, 1,3-bis-(OTMS) propyl ester (β-glyceryl palmitate; (β-*n*-$C_{16:0}$)) was almost exclusively, the only saturated 2-monoglyceride present on the tissues from leaves (34 mg/kg dw) and inflorescences (904 mg/kg dw).

3.2.3.1.2. MG of unsaturated fatty acids

3.2.3.1.2.1. 2,3-Dihydroxy-MG

Four α-monoglycerides identified from the lipid extracts of leaves from *S. nigra* are presented with their concentrations. The most abundant was the unusual α-glyceryl hexadecadienoate (α-*n*-$C_{16:2}$) (34%). Nine homologues were detected from inflorescences with α-glyceryl linoleate (α-*n*-$C_{18:2}$) (42%) as the major component.

3.2.3.1.2.2. 1,3-Dihydroxy-MG

Five β-monoglycerides were identified from the lipophilic extract of the leaves of *S. nigra* and four from the inflorescences. β-glyceryl linoleate (β-*n*-$C_{18:2}$) (32%), β-glyceryl palmitoleate (β-*n*-$C_{16:1}$) (30%) and β-glyceryl linoleaidiciate (β-*trans*-*n*-$C_{18:2}$) (17%) were the most abundant from the leaves and β-glyceryl palmitoleate (β-*n*-$C_{16:1}$) (58%), β-glyceryl oleate (β-*n*-$C_{18:1}$) (18%) and β-glyceryl linoleate (β-*n*-$C_{18:2}$) (15%) from the inflorescences.

3.2.4. Methyl esters of amino acids

Relatively high amounts of leucine methyl ester (1854 mg/kg dry weight) were found in the inflorescences from the elderberry. The *L*- isomer of this compound has been recognized as a female-produced sex pheromone of the scarab beetle *Phyllophaga lanceolate* (Nojima *et al*., 2003). These authors used this compound recovered from the glands of this beetle to caught in traps males and recognized it a as a potent chemical attractant of the females. We propose here that during the floral season the inflorescences of *S. nigra* may release these compounds in order to accomplish its entomopollination. Supplem. S10 shows mass spectrum of L-isoleucine methyl ester as its trimethylsilyl derivative. Fragmentation pattern of this monotrimethylsilylated compound (mass peak at $[M]^+$ = 217) shows two prominent ions at *m/z* 158 (M-59) which corresponds to the α-cleavage of the molecule that releases the carboxymethyl moiety $[M\text{-}C(=O)OMe]^+$ and at *m/z* 160 (M-57) resulting of the β-cleavage from the isopropyl group $[CH_3\text{-}CH(CH_3)\text{-}CH_2]^+$. The γ-cleavage gives a peak at *m/z* 174 (M-43) formed by the loss of $[CH_3\text{-}CH(CH_3)]^+$ also from the hydrocarbon chain site. Base peak corresponding to the TMS group was at *m/z* = 73.

3.3. Phenolic compounds

This is the first report on the compared study of free phenolic compounds from the leaves and inflorescences of *S. nigra*.

3.3.1. Phenolic acids and phenols

Although the polyphenolics such as anthocyanins have been extensively investigated in *Sambucus* genus (Lee and Finn, 2007) simple phenolic acids and phenols remains not well known. We report here a detailed study of 25 of these compounds both from leaves and inflorescences. The most abundant were benzyl alcohol, phenethyl alcohol (2-phenylethanol) and benzoic acid which are the metabolite substrates for the synthesis of several classes of aromatic esters also identified from the extracts of *S. nigra* namely phenylmethyl esters, phenylethyl esters and benzoyl esters, respectively. Fig. 6 shows the histograms of these compounds from the leaves and inflorescences extracts. Benzoic acid was the most abundant phenolic (54%) extracted from the leaves and benzyl alcohol (26%) and phenethyl alcohol (17%) were the major phenols from the inflorescences of *S. nigra*. Turek and Cisowski (2007) found 6 phenolic acids in the bark from *S. nigra* with caffeic, ferulic and 4-hydroxybenzoic acids as the most abundant.

The lignan lariciresinol (M^+ = 576) as its trimethylsilyl derivative has been identified from the leaves extract of *S. nigra* in minor amounts. D'Abrosca *et al*. (2001) also found this lignan in the air dried leaves of *S. nigra*.

3.3.2. Cyanogenic benzene derivatives

Cyanogenesis accounts for the ability of plants and arthropods like centipedes and millipedes to release hydrogen cyanide. Mandelonitrile is a cianolipid synthesized by many plant species including economically important food crops. The tissue disruption releases this compound and after its hydrolysis hydrogen cyanide is liberated causing cyanide poisoning of herbivore animals (Poulton, 1990). To the best of our knowledge this α-hydroxynitrile has not been previously described in *S. nigra* although several species of the genus *Sambucus* have previously been shown to be cyanogenic and among these are *S. glauca*, *S. racemose* and *S. sieboldiana* (Buhrmester *et al*., 2000) due to their cyanogenic derivatives glycosides (D'Abrosca *et al*., 2001). Supplem S11 shows mass spectrum of mandelonitrile as its trimethylsilyl derivative also recently reported by Vujisić *et al*. (2013) in the mediterranean centipede *Himantarium gabrielis*. While these compound represented a percentage less than 0.1 from the tissue extract of the centipede investigated by these authors it was the most abundant compound present in the leaves from *S. nigra* (890 mg/kg dry weight) (Fig. 1). Molecular ion at *m/z* 205 together the fragments corresponding to the loss of a methyl group (*m/z* 190) were prominent. This last ion was the base peak. Other abundant fragments were at *m/z* 105 ($[M\text{-}TMS\text{-}CN]^+$) and *m/z* 116, the even peak which resulted from the cleavage of the silylated hydroxyl group at C(1) on the side chain ($[M\text{-}OTMS]^+$).

3.4. *n*-Alkanes and *n*-alkenes

The profile from the inflorescences was very different from that of the leaves and has not been previously described. This includes homologues with short chain (*n*-C_{15} to *n*-C_{31}) and maximizing at tricosane (*n*-C_{23}) (22%) followed by heneicosane (*n*-C_{21}) (20%). A series of 11 alkanes (range: *n*-C_{23} to *n*-C_{33}) were found in the leaves from the black elder. Homologues are included in Supplem. S2 and S3 and their distributions are shown in Supplem. S12. The major *n*-alkane identified was hentriacontane (*n*-C_{31}) (34%); this distribution pattern with predominantly odd carbon number homologs is similar to that reported by Inoue and Sato (1975) also in the leaves from *S. nigra* (*n*-C_{25} to *n*-C_{31}; C_{max} *n*-C_{29} and *n*-C_{31}). Stránský *et al*. (2001) showed also a typical pattern from cuticular lipids of higher plants (*n*-C_{15} to *n*-C_{31}; C_{max} *n*-C_{25} and *n*-C_{27}) in the pollen extract from *S. nigra*.

Fourteen unsaturated hydrocarbons were identified in the inflorescences of *S. nigra* (Fig. 5). These alkenes ranged from *n*-$C_{17:1}$ to *n*-$C_{31:1}$ and the maximum of the distribution was at *n*-9-nonacosene (*n*-9-$C_{29:1}$). The results are similar to those previously described by Stránský *et al*. (2001) on *S. nigra* pollen (*n*-9-$C_{19:1}$-*n*-9-$C_{33:1}$, $C_{max.}$ *n*-9-$C_{29:1}$; *n*-7-$C_{21:1}$-*n*-7-$C_{31:1}$, $C_{max.}$ *n*-7-$C_{29:1}$) who determined the double bond positions of olefins. The unimodal distribution maximized at 9-nonacosene (*n*-9-$C_{29:1}$) is also in agreement with the cited work. 9-Alkenes are more abundant than 7-alkenes in the major homologues (*n*-$C_{29:1}$ and *n*-$C_{25:1}$) (Supplem. S12).

3.5. *n*-Alkanols, secondary alcohols and alkane-1,3-diols

Lipophilic phytochemicals from *S. nigra* also includes a homologous series of aliphatic alcohols. These molecules are reported here both for leaves and inflorescences. Salvador *et al*. (2015) found octadecanol (*n*-C_{18}) and hexacosanol (*n*-C_{26}) in the berries from *S. nigra*. Stránský *et al*. (2001) described *n*-octacosanol (*n*-C_{28}) as the main aliphatic alcohol from the pollen elder and these results were different from ours. The profile of the distribution of *n*-alkanols from leaves ranging from octanol (*n*-C_8) to dotriacontanol (*n*-C_{32}) was trimodal and maximized at *n*-C_{12} (9%), *n*-C_{22} (19%) and *n*-C_{26} (17%). These compounds represented a minor percentage (1.4%; 43 mg/kg dry weight) of those identified in the total extract. *n*-Alkanols from *S. nigra* inflorescences (*n*-C_6 – *n*-C_{30}) differs from that expected from terrestrial plants because major fatty alcohols occur in most cases either at *n*-C_{26} or *n*-C_{28} while here were centered at tetracosanol (*n*-C_{24}) (22%). Another abundant alkanol was triacontanol (*n*-C_{30}; 9%). They account for a higher percentage (2.7%; 2641 mg/kg dry weight). In both tissues investigated an even over odd chain length dominance was observed.

Only one secondary alcohol was found. Minor amounts of *n*-nonacosan-10-ol were recovered from the leaves of *S. nigra*. While gimnosperms usually present this compound together with several others homologues which belongs not only to 10- secondary alcohols but also to other classes of secondary alcohols with the hydroxyl group located in other positions gimnol is often the only secondary alcohol included in the parenchyma of angiosperm tissues. This compound has not been previously described in *S. nigra* although its concentration was low (0.5 mg/kg dry weight).

Three homologues of a series of 1,3-diols (*n*-C_{26} - *n*-C_{28}) were identified in the leaves and inflorescences of *S. nigra*. This is the first time these diols are described from this plant. Buschhaus *et al*. (2013) reported these uncommon alkanediols from the petal wax of *Cosmos bipinnatus* (Asteraceae) (*n*-C_{20} – *n*-C_{24}). Supplem. S12 shows the histograms of concentrations of these aliphatic alcohols.

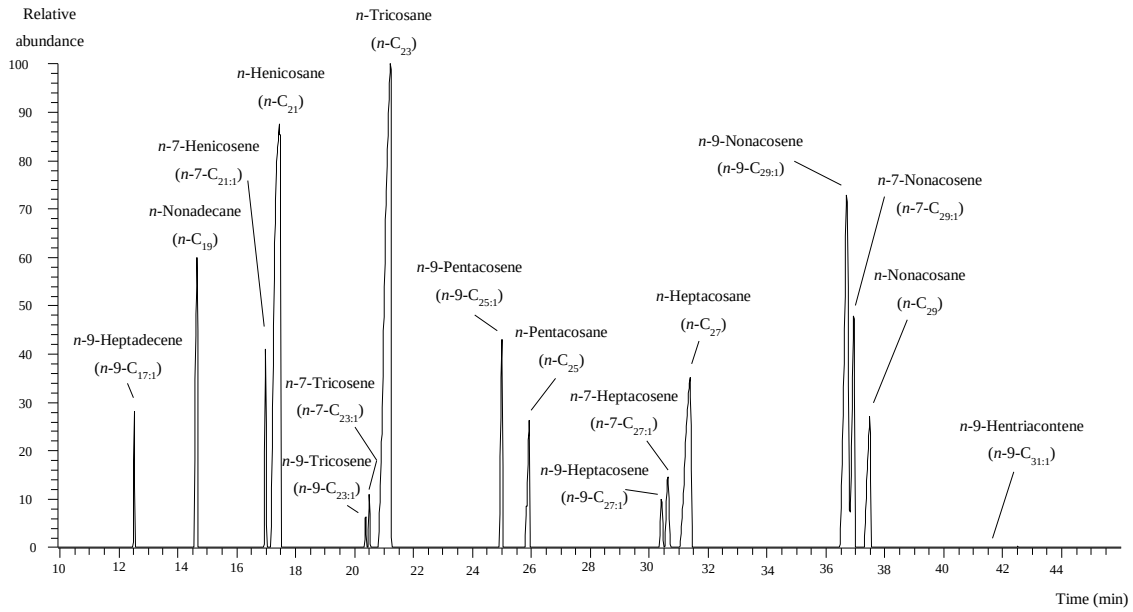


Fig. 5. Gas chromatogram of *n*-alkanes and *n*-alkenes from the inflorescences of *S. nigra*.

3.5.1. *n*-Alkanoic, *n*-alkenoic, *n*-ω-hydroxy- and *n*-dicarboxylic acids

Saturated fatty acids ranging from heptanoic acid (*n*-$C_{7:0}$) to triacontanoic acid (*n*-$C_{30:0}$) were found in the leaves of *S. nigra* (412 mg/kg dry weight). Even chain homologues were much more abundant than the odd ones. The profile could be considered bimodal although hexadecanoic acid (palmitic acid) (*n*-$C_{16:0}$) which is the first maximum was more than fifty

times more abundant (359 mg/kg dry weight; 87%) than the second tetracosanoic acid (*n*-$C_{24:0}$) (6 mg/kg dry weight; 1%). Similar distribution was quantified from the inflorescences (*n*-C_6 – *n*-C_{32}) with a bimodal profile maximizing at palmitic acid (*n*-C_{16}) (65%) and tetracosanoic acid (*n*-C_{24}) (11%). Total amount of these compounds was nearly 18 times higher (7273 mg/kg dry weight). In agreement with our results Salvador *et al*. (2015) also found palmitic acid as the most abundant alkanoic acid in the berries. Stránský *et al*. (2001) described palmitic acid (*n*-C_{16}) as the most abundant *n*-alkanoic acid (*n*-C_{14} – *n*-C_{28}) from the pollen of *S. nigra*. Toulemonde and Richard (1983) also reported from *S. nigra* flowers extracts palmitic acid as the most abundant saturated fatty acid. Supplem. S12 shows the profile abundances of these compounds.

The fatty acid profile of *S. nigra* flowers was recently shown by Barros *et al*. (2011). In such report α-linolenic acid (25%) was the most abundant homologue followed by linoleic acid (24%) and oleic acid (12%). Stránský *et al*. (2001) showed oleic (14%), linoleic (13%) and linolenic (12%) acids as the most abundant unsaturated fatty acids from pollen elder. Previously, Toulemonde and Richard (1983) showed a similar distribution to ours with linolenic (9.1%), linoleic (9%) and oleic (5.7%) acids as the most abundant. In our case ten unsaturated fatty acids (800.6 mg/kg dry weight) were recovered from the leaves of *S. nigra* with homologues including members of 16 and 18 carbon atoms (Supplem. S13). α-Linolenic acid (*all-cis*-$\Delta^{9,12,15}$-*n*-$C_{18:3}$) was the most abundant (39%) followed by linoleic acid (*all-cis*-$\Delta^{9,12}$-*n*-$C_{18:2}$) (20%) and oleic acid (*cis*-Δ^{9}-*n*-$C_{18:1}$) (20%). Very similar distribution was found in the inflorescences with linoleic acid (30%) and vaccenic acid (*cis*-Δ^{11}-*n*-$C_{18:1}$) (30%) as major alkenoic acids followed by α-linolenic (18%) and oleic acids (15%). These compounds have not previously been reported from *S. nigra* leaves.

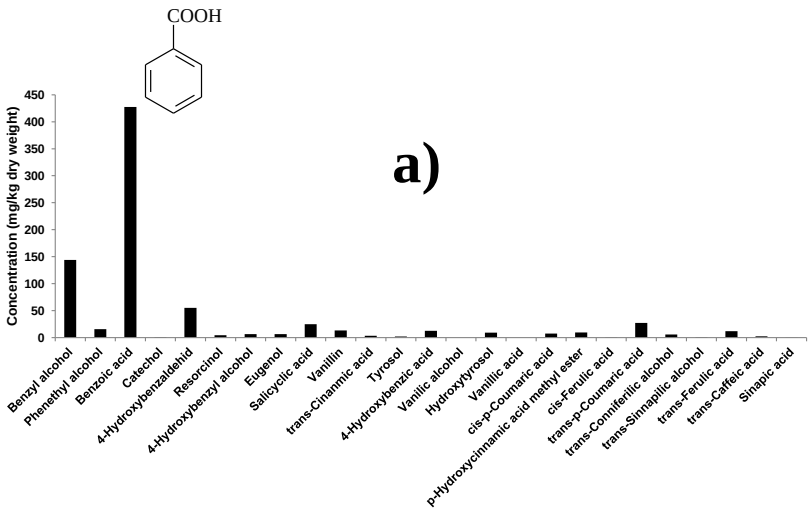
An unprecedented distribution with 3 ω-hydroxycarboxylic acids was detected in the leaves and inflorescences of *S. nigra*. 22-Hydroxydocosanoic acid was by far, the most abundant in both tissues (86 and 88%, respectively). Only one *n*-dicarboxylic acid was detected in the extracts of *S. nigra*. 1,22-Docosanedioic acid was present in the inflorescences. This compound has not been previously reported from this plant. Supplem. S13 shows the profile of these series of lipids.

3.6. Terpenoids

The monoterpene linalool was detected from leaves (9 mg/kg dry weight) and inflorescences (56 mg/kg dry weight). This compound was found in the essential oil from *S. nigra* flowers (3.7%) by Toulemonde and Richard (1983).

Six sterols were found in the leaves from the black elder: cholesterol, campesterol, stigmasterol, β-sitosterol, β-sitostanol and Δ^5-avenasterol and five in the inflorescences, cholesterol, campesterol, stigmasterol, β-sitosterol and Δ^5-avenasterol with β-sitosterol as the most abundant in both tissues (82 and 87%, respectively). Concentrations are showed in Supplem S13. Identification of Δ^5-avenasterol was obtained by comparison with Kamal-Eldin *et al*. (1998) which analyzed the TMS-derivatives and retention times of fucosterol and Δ^5-avenasterol. Stránský *et al*. (2001) showed a comparable profile with β-sitosterol as the most abundant sterol from pollen elder (66%) followed by campesterol (16%) and stigmasterol (2%). Similar results were found by Inoue and Sato (1975) with β-sitosterol as the major sterol from the leaves of *S. nigra* and minor amounts of campesterol and stigmasterol. Results in inflorescences have not been previously described.

Four pentacyclic triterpene acids and alcohols were detected in the leaves and inflorescences of *S. nigra* (Supplem. S13). They included β-amyrin and oleanolic acid which are oleanane triterpenes and α-amyrin and ursolic acid from the ursane family. Supplementary material includes the concentration of these compounds. The most abundant both in the leaves and the flowers of *S. nigra* was ursolic acid (57 and 67%) followed by oleanolic acid (39 and 33%). The occurrence of these four triterpenoids was previously reported by Inoue and Sato (1975) in the leaves from *S. nigra* (Supplem. S1). Jäger *et al*. (2009) found oleanolic and ursolic acids in the leaves and bark of *S. nigra* but not in inflorescences. These secondary metabolites have been associated with several therapeutic effects combined with a low toxicity in humans. Pharmacological properties of triterpenes include inflammatory, tumoral, bacterial and viral protection (Liu, 2005).



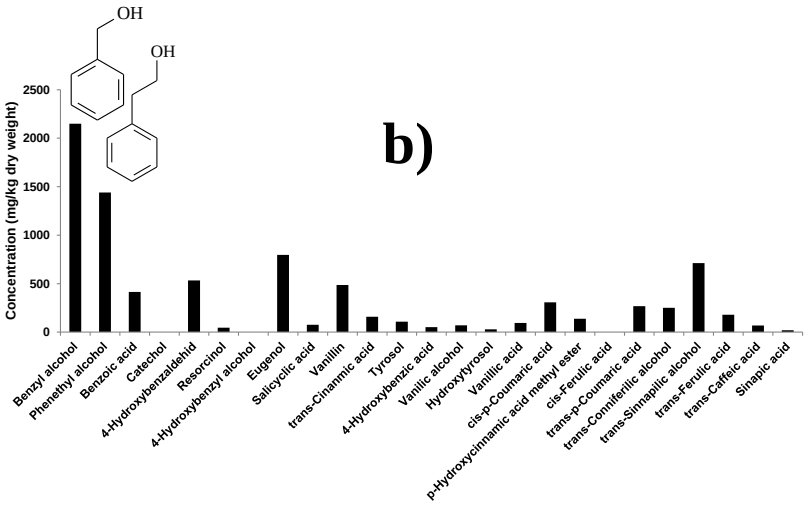


Fig. 6. Histograms of phenolic acids and phenols identified and quantified from the leaves (a) and inflorescences (b) of *S. nigra*.

3.7. Miscellaneous

Two isomers of *gamma*-tocopherol (γ_1- and γ_2-) together with α-tocopherol were detected in the leaves and inflorescences of *S. nigra*. α-Tocopherol was the most abundant representing respectively 97 and 99% of the total amount of this class of lipids (Supplem. S14). These vitamin E compounds are powerful antioxidants found in many different plant species.

Three phytadienes were detected from the leaves of *S. nigra*. Neophytadiene was the most abundant followed by (*E*)-1,3-phytadiene and (*Z*)-1,3-phytadiene (Supplem. S14). These results are similar to those showed by Nguyen *et al*. (2007) in the leaves extracts of *Fagus sylvatica*. Squalene was in higher amounts in the inflorescences than in the leaves. In opposite, phytol was more abundant in the leaves extract (45 mg/kg dry weight) than in the inflorescences one (6 mg/kg dry weight).

Two short-chain *n*-alkylamines were found. Ethylamine and isobutylamine were previously reported by Steiner and Von Kamiensky (1953) in *S. nigra* flowers. In our study, ethylamine was more abundant in the inflorescences (414 mg/kg dry weight) than in leaves (87 mg/kg dry weight). Trace amounts of isobutylamine were detected only in leaves.

Four anhydro sugars isomers characterized by ions at *m/z* 103, 116, 129, 189, 204 and 217 were related to those described by Fabbri *et al*. (2002). Supplem. S17 shows mass spectra of these compounds together with the corresponding TIC chromatogram.

Finally, Supplem. S15 and S16 shows a summary of concentrations of all classes of compounds identified from leaves and inflorescences of *S. nigra* through a table and histogram summary of total concentrations of each class of compounds identified from *S. nigra* extracts.

4. Conclusions

Leaves and inflorescences of *S. nigra* were analyzed for their phytochemical composition. The overall results showed mandelonitrile as the most abundant allelochemical from the leaves. On the other hand, our results also showed several classes of ester compounds including fatty acid methyl esters, valeric acid *n*-alkyl esters, phenyl methyl esters, phenyl ethyl esters and *n*-alkyl benzoates which are all together in the tissues of the same plant not previously described. The high amounts of the insect pheromone L-isoleucine methyl ester could become a great successful allelochemical strategy in order to achieve pollination by insects. There is also a significant presence of saturated and unsaturated monoglycerides. The GC-EIMS analysis of the extracts showed 19 classes of compounds. The most abundant chemical families from leaves extract were cianolipids (23%). *n*-Alkanes (25%) and pentacyclic triterpenes (24%) were the most abundant from *S. nigra* inflorescences.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgements

The authors are grateful to the BCN Herbarium (CRAI, Universitat de Barcelona) through Dr. Josep Vicent Fandos for deposit of plant samples.

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