Original Research Article

Chemical profiling of the fruits of *Styrax officinalis* L. from Monti Lucretili (Latium region, Central Italy): Chemotaxonomy and nutraceutical potential

Alessandro Venditti[1, ✉], Claudio Frezza[2], Ilaria Serafini[1], Sabina Pulone[1], Giorgina Scardelletti[3], Fabio Sciubba[1], Armandodoriano Bianco[1] and Mauro Serafini[2]

[1]Dipartimento di Chimica, Università di Roma "La Sapienza", Piazzale Aldo Moro 5, 00185, Rome, Italy
[2]Dipartimento di Biologia Ambientale, Università di Roma "La Sapienza", Piazzale Aldo Moro 5, 00185, Rome, Italy
[3]Parco Naturale Regionale dei Monti Lucretili, Via A. Petrocchi 11, 00018 Palombara Sabina, Italy

ABSTRACT

In this work, the first phytochemical analysis ever performed on the fruits of *Styrax officinalis* L. (Styracaceae) collected from a population vegetating in the Mounts Lucretili National Park (Italy) was reported. Fifteen compounds were identified: tri-α-linolenoyl-*sn*-glycerol (**1**), 1,2-di-α-linolenoyl-3-linoleoyl-*sn*-glycerol (**2**), 1-α-linolenoyl-2-palmitoyl-*sn*-glycerol (**3**), 1,2-di-α-linolenoyl-*sn*-glycerol (**4**), egonol (**5**), demethylegonol (**6**), homoegonol (**7**), 1,5-anhydro-D-mannitol (**8**), glucose (**9**), sucrose (10), 6'-*O*-benzoyl-sucrose (**11**), raffinose (**12**), lactic acid (**13**), succinic acid (**14**) and glutamic acid (**15**). These compounds belong to seven different classes of natural metabolites and most of them have chemotaxonomic relevance. Moreover, *S. officinalis* might be an useful source of enantiopure 1,5-anhydro-D-mannitol which has several medicinal potentialities and is a versatile building block in organic synthesis, in particular for what concern the "Green" approaches, of valuable and potentially biologically active molecules. The presence of compounds (**1-15**) provides also a phytochemical rationale for the ancient ethnopharmacological uses of the species and affords evidences on its nutraceutical potentialities even for their consumption as food in human nutrition as it actually happens for animals.

1. Introduction

The phytochemical studies of spontaneous species are of primary importance in different fields i.e. pharmaceutical, chemical, botanical, medicinal, traditional and folk medicines.

The medicinal and health promoting properties of plants are generally correlated to the phytochemicals present in their secondary metabolite pattern and the phytochemical studies may substantiate the ancient ethno-medicinal applications by giving a rationale from the chemical standpoint (Venditti et al., 2016a; Mohammadhosseini, 2017; Mohammadhosseini et al., 2017; Ganesan and Xu, 2017; Nunes and Miguel, 2017). Yet, they are also very useful to reveal new and unexpected potential uses by assessing their biological activities in both *in vitro* and *in vivo* studies. (Esposito et al., 2013; Cardile et al., 2015; Parenti et al., 2016; Venditti et al., 2016b; Camilo et al., 2017; Les et al., 2017). The phytochemical analysis may also be employed to detect the presence of toxic constituents, such as in the case of hepatotoxic *neo*-clerodanes from *Teucrium polium* L. (Frezza et al., 2017a; Venditti et al., 2017a, 2017b), and revealed to be a useful tool for the quality control of plant raw materials collected for botanical purposes (Toniolo et al., 2014; Frezza et al., 2017a). The secondary metabolite analysis is an irreplaceable mean of investigation also in Botany and, in particular, for taxonomic purposes mainly because several classes of secondary metabolites have chemosystematic relevance. Actually, in general, this is useful to deepen the knowledge on correlations between the chemical composition and the taxonomical classification of the species particularly in the case of entities of unresolved

✉ Corresponding author: Alessandro Venditti
Tel: +39-0649913229; Fax: +39-0649913841
E-mail address: alessandro.venditti@gmail.com

and problematic classification (Venditti et al., 2016c, 2016d). It also allows to recognize different chemotypes and intraspecific phytochemical variability (Frezza et al., 2017b), and obviously to identify new natural compounds (Venditti et al., 2016e, 2016f, 2016g, 2017b, 2017c). Last but not least, the composition of secondary metabolites can also give useful indications regarding the aspects related to plant physiology and to the interactions between different species at an ecological level (Venditti et al., 2016h, 2017d).

In this paper, our attention was focused on the fruits of *Styrax officinalis* L. This species is commonly known as Storax tree and is a perennial brushy plant belonging to the Styracaceae family. Its name derives from the combination of two words: the first word, related to the genus, is Syrian and refers to a specific place in Syria where this species abundantly grew in ancient times; the second word, related to the species, is Latin and mentions the fact that this species was widely used in medicine. In fact, its aromatic (as incense) and therapeutic properties were well-known by Romans, Egyptians, Phoenicians and Ionions (Vardar and Oflas, 1973; Yayla et al., 2002).

From the morphological standpoint, this species is characterized by a tall stem. The leaves are lucid, glabrous and shortly petiolate. The flowers are odorous and collected in small racemes blooming from April to May. Lastly, the fruits, are light green colored, covered with a velvety film and present a small appendix in the lower part. They are also quite small (about 3 cm) and contain only one seed which has a sub-oval shape. This is woody and quite sharp and presents a central filling. These seeds are produced mainly in Autumn (Fig. 1).

This is a typical Eastern Mediterranean species with a growing area ranging from Albania to Lebanon, Syria and Israel. In Italy, it is characteristic of Mounts Lucretili and Mounts Tiburtini (Latium region, Central Italy) where it grows along streets margins and woods up to 600 m a.s.l..

The reason of its presence in Italy has been long debated with two main theories. The first one is based on the intentional introduction by the Romans and the subsequent spontaneous naturalization of the species (Rikli, 1942). The second one, instead, is based on the spontaneous penetration of the species from Balkans, during the geological phases that connected the Dalmatian platform with Garganic Apulia, together with other ones present in this area. Actually, many scientists adhere to the latter hypothesis which considers the actual populations of this species as a relict from those of the pre-Quaternary Era which have been progressively decimated in southern Europe during Ice Ages (Montelucci, 1946; Spada, 1988). The presence of particular morphological and climatic characteristics in the Lucretili mountains seems to have provided a suitable shelter for the survival and proliferation of *S. officinalis* L. until present times.

The species itself is well known in Latium region for the production of a very good honey. The resin of the stem is still used in traditional medicine as an expectorant and antiseptic balm (Pignatti, 1982). The fruits are particularly appreciated in cuisine and also as food for animals and the plant itself is locally used to produce brooms, chairs, poles and whips (Pignatti, 1982).

Literature data are full of studies about some particular constituents of *S. officinalis* L. such as benzofuranoids (Anil, 1980; Akgul and Anil, 2003a, 2003b; Pazar and Akgül, 2015), lignans (Ulubelen et al., 1978) and saponins (Akgul et al., 2002; Zehavi et al., 1986) while only one is about the composition of the essential oil (Tayoub et al., 2006). Nevertheless, none of these studied the total polar fraction content as well as the fruits and any sample coming from Monti Lucretili (Latium region, Central Italy).

This represents the main reason why this work was started with the final objective to provide phytochemical data about this population which grows isolated and quite far from the original growing area.

2. Experimental

2.1. Plant material

The fruits of *S. officinalis* L. for the weight of 1.20 Kg were collected on June 26th, 2015 inside the territory of the National Park of Mounts Lucretili and more precisely, in the locality called "Valle Scoperta" (geographical coordinates 42º07'08'' N, 12º75'79'' E), near the town of Palombara Sabina (Latium region, Central Italy), at an altitude of 550 m a.s.l. along the border between cultivated lands and the woods.

The botanical recognition was performed by one of us (G.S.) together with the botanists of the Park by using

Fig. 1. Flowering plant and fruits of *Styrax officinalis* L.* *Photographs are by: Luciano Capasso, from 2005 on - "*Styrax officinalis* L. - Storace". In Acta Plantarum, Forum. (Available on line: http://www.floraitaliae.actaplantarum.org/viewtopic.php?f=95&t=8409)

available literature (Pignatti, 1982).

A representative sample of this collection is stored in our laboratory for further references under the accession number SO26062015.

2.2. Chemicals

During our work, the following reagents and solvents were utilized: ethanol 96% for the extraction procedure; n-butanol, distilled water, dichloromethane and methanol as pure solvents or as mixtures at different concentrations among them all to be used as eluting systems for the chromatographic separations on silica gel 60 (70-230 mesh ASTM) columns; deuterated solvents such as $CDCl_3$ (deuterated chloroform), CD_3OD (deuterated methanol) and D_2O (deuterium oxide) for the identification of the metabolites by NMR Spectroscopy; methanol having RS purity grade for the identification of the compounds by Mass Spectrometry.

All the solvents were at RPE purity grade if not diversely specified and were purchased from Sigma Aldrich (Milan, Italy) as well as the deuterated solvents, while silica gel was bought from Fluka Analytical (St. Louis, MO, USA).

2.3. Instrumentation

NMR spectra were recorded on a Varian Mercury 300 MHz instrument and/or on a Bruker AVANCE III 400 MHz instrument. The chemical shifts were expressed from TMS for spectra in $CDCl_3$, from the internal solvent signal (m5) at 3.31 ppm for spectra in CD_3OD and from the HDO signal (s) at 4.79 ppm for spectra in D_2O.

MS spectra were performed on a Q-TOF MICRO spectrometer (Waters, Manchester, UK) equipped with an ESI source operating in the negative and/or positive ion mode. The flow rate of the sample infusion was 10 µL/min with a minimum of 50 acquisitions per spectrum. Data were analyzed using the MassLynx software developed by Waters.

2.4. 2D NMR experiments

2D NMR spectra were performed on a Bruker Avance III 400 MHz instrument, operating at 9.4 T at 298 °K. HSQC experiments were acquired with a spectral width of 15 and 250 ppm for the proton and carbon respectively, an average $^1J_{C-H}$ of 145 Hz, recycle delay of 2.0 s and a data matrix of 4Kx256 points. HMBC experiments were acquired with a spectral width of 15 and 250 ppm for the proton and carbon respectively, long range coupling constant of $^nJ_{C-H}$ of 8 Hz and 4 Hz, recycle delay of 2.0 s and a data matrix of 4Kx256 points. COSY spectrum was acquired with a spectral width of 10 ppm in both dimensions and performed using gradient pulses with multiple quantum filter and gradient ratio optimized for artifact suppression. NOESY spectra were acquired with a spectral width of 10 ppm

in both dimensions using phase sensitive experiment with presaturation during relaxation delay and mixing time. The spectra were acquired with 100, 350, 500 and 750 ms as mixing times.

2.5. Specific rotation

Specific rotation was measured by using a Jasco DIP-370 polarimeter. The dimensions of the cell was 1 cm high, 1 cm wide and 1 cm deep. The optical path length was 1 cm. The wavelength was set at 589 nm. The temperature was 25 °C.

2.6. Extraction, isolation and identification of metabolites

The fresh fruits for the weight of 1.18 Kg were macerated in the same flask with ethanol 96% (1.5 L each; 48 hours) four times in order to have an exhaustive extraction. The deriving dark green colored solutions were put altogether and concentrated at reduced pressure to remove ethanol. This gave a final water suspension which was then frozen and lyophilized to preserve also the temperature-sensitive compounds eventually present. During the first of these concentrations, pH value of the solution was checked and this resulted to be 6.5 pH units comprised in the optimal range. In fact, one too acidic (pH<5.5) or one too basic (pH>8.5) solution might cause unwanted secondary reaction on the metabolites such as the hydrolysis of ester and glycosidic bonds and these are particularly favored when in presence of water and heating. The obtained dried crude extract colored in dark brown, weighed 49.8 g and was stored in the dark at 4 °C until analysis.

A portion of this dried crude extract (3.1 g) underwent a first chromatographic separation by means of classical Column Chromatography, using a correspective amount of silica gel of 100.1 g (ratio 1:35). The eluting system was a mixture of n-BuOH/H_2O at the concentration 82:18 (v/v). During the chromatographic run, the polarity of the eluting system was increased passing to a solution of n-BuOH/MeOH/H_2O in ratio 70:10:30 (v/v/v). From this chromatographic step, seven compounds were isolated and identified by NMR and MS data comparison with those reported in literature. Mixtures of compounds were analyzed by an NMR-based metabolomic approach already used in our previous works (Sciubba et al., 2014a, 2014b; Venditti et al., 2017e, 2017f). In particular, the identified compounds were: 1,5-anhydro-D-mannitol (styracitol) (**8**) (Bock et al., 1981; Que and Gray, 1974) as almost pure compound from the assembly of fractions 72-116 for the weight of 298.6 mg; glucose (**9**), sucrose (**10**), raffinose (**12**), lactic acid (**13**), succinic acid (**14**) and glutamic acid (**15**) (Venditti et al., 2017f; Sciubba et al., 2014a, 2014b) as an only mixture (ratio not detectable) from the methanol column wash for the total weight of

Table 1

Complete ¹H-NMR and ¹³C-NMR assignments for compound (**8**).

	8				
	¹H-NMR 400 MHz; ¹³C-NMR 100 MHz, D₂O		¹H-NMR 400 MHz; CD₃OD, δH	¹³C-NMR 100 MHz, CD₃OD, δC	HMBC
position	δH, J in Hz	δC, HSQC			
1	3.94 (dd, J=12.8, 2.0); 3.65 (dd, J=12.8, 3.5)	70.8	3.91 (dd, J=12.4, 2.0); 3.56 (d, J=12.4)	71.3	C-3, C-5
2	3.99 (br s)	70.1	3.83 (s)	70.9	C-1, C-3
3	3.60 (t, J=9.5)	74.5	3.47 (dd, J=9.5, 3.2)	75.8	C-1, C-2, C-4
4	3.66 (dd, J=9.5, 3.4)	68.3	3.57 (t, J=9.5)	69.0	C-2, C-3, C-5, C-6
5	3.31 (ddd, J=9.0, 6.6, 2.1)	81.5	3.18 – 3.10 (m)	82.5	C-3, C-4, C-6
6	3.90 (dd, J=12.3, 2.1); 3.70 (dd, J=12.3, 6.6)	62.2	3.85 (dd, J=11.6, 2.2); 3.67 (dd, J=11.6, 5.6)	63.0	C-5
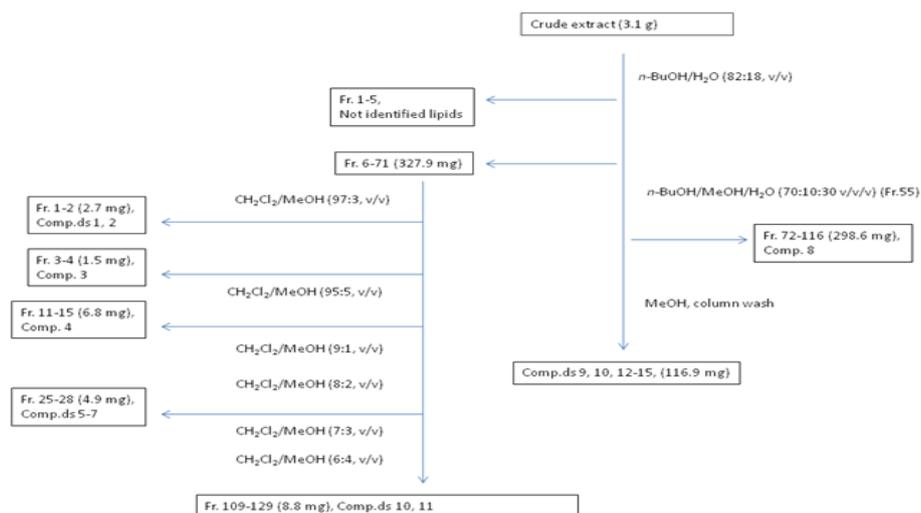
Scheme 1. Chromatographic separation scheme.

116.9 mg.

Since not all metabolites could be clearly identified from this first chromatographic step, a second chromatographic separation was performed on the assembly of fractions 6-71 (for the total weight of 327.9 mg) deriving from the first one. This time, the correspective amount of silica gel was 10.0 g (ratio 1:35) and the eluting system was a mixture of CH₂Cl₂/MeOH at different concentrations. The initial one was 97:3 (v/v) but during the chromatographic run, this was gradually modified in order to raise its polarity and let the elution of the most polar compounds, to 95:5 (v/v), 9:1 (v/v), 8:2 (v/v), 7:3 (v/v) and lastly, 6:4 (v/v). From this chromatographic step, six other components were isolated and identified: tri-α-linolenoyl-sn-glycerol (**1**) and 1,2-di-α-linolenoyl-3-linoleoyl-sn-glycerol (**2**) (Sciubba et al., 2014a; Venditti et al., 2017f) in mixture in ratio 1:1 from the assembly of fractions 1-2 for the weight of 2.7 mg; 1-α-linolenoyl-2-palmitoyl-sn-glycerol (**3**) (Sciubba et al., 2014a; Venditti et al., 2017f) as almost pure compound from the assembly of fractions 3-4 for the weight of 1.5 mg; 1,2-di-α-linolenoyl-sn-glycerol (**4**) as almost pure compound from the assembly of fractions 11-15 for the weight of 6.8 mg; egonol (**5**) (Akgul and Anil, 2003b; Li et al., 2005), demethylegonol (**6**) (Li et al., 2005a) and homoegonol (**7**) (Giesbrecht et

al., 1985; Pauletti et al., 2000; Timmers et al., 2015) in mixture in ratio 5:2:1 from the assembly of fractions 25-28 for the weight of 4.9 mg; 6'-O-benzoyl-sucrose (**11**) (Miyase and Ueno, 1993; Miyase et al., 1999) in mixture with sucrose (**10**) (1:2) in the assembly of fractions 109-129 for the weight of 8.8 mg.

A scheme of the chromatographic procedure is reported in the Scheme 1.

2.7. Acetylation of 1,5-anhydro-D-mannitol (**8**)

A portion of 30.0 mg of the pure compound present in the assembly of fractions 72-116 derived from the first chromatographic step, was put in a 10 mL flask covered with a moisture trap. It underwent an acetylating reaction with acetic anhydride (500 μL) in dry pyridine (250 μL). The reaction mixture was maintained under continuous magnetic stirring at room temperature for 24 hours. The reaction progress was monitored by TLCs and when the starting material completely disappeared, the acetic anhydride still present in the reaction mixture was quenched with methanol (2 mL). Then, the solution was concentrated at reduced pressure at a temperature of about 30 ºC. The eventual left traces of pyridine were removed by treatment with methanol and evaporation under vacuum (3x5.0 mL each). The obtained final dried

tetra-acetylated derivative (**8a**) weighed 20.1 mg (yield 33.15 %).

2.8. NMR and MS data of some identified compounds

5(3''-hydroxypropyl)-7-methoxy-2-(3',4'-methylenedioxyphenyl)-benzofuran, egonol (**5**): ^{1}H NMR (400 MHz, CDCl$_3$) δ: 7.40 (1H, dd, J=8.2, 1.4 Hz, H-6'), 7.32 (1H, d, J=1.4 Hz, H-2'), 6.98 (1H, br s, H-4), 6.87 (1H, d, J=8.2 Hz, H-5'), 6.79 (1H, s, H-3), 6.63 (1H, br s, H-6), 6.00 (2H, s, OCH$_2$O), 4.04 (3H, s, CH$_3$O), 3.71 (2H, t, J=6.3 Hz, H-3''), 2.82 - 2.71 (3H, m, H-1''), 1.99 - 1.89 (2H, m, H-2'').

ESI-MS: m/z 349.23 [M+Na]$^+$, (calc. 349.1046 for C$_{19}$H$_{18}$NaO$_5^+$).

5(3''-hydroxypropyl)-7-hydroxy-2-(3',4'-methylenedioxyphenyl)-benzofuran, demethylegonol (**6**): ^{1}H NMR (400 MHz, CDCl$_3$) δ: 7.45 (1H, dd, J=8.7, 1.7 Hz, H-6'), 7.37 (1H, d, J=1.7 Hz, H-2'), 7.00 (1H, br s, H-4), 6.93 (1H, d, J=8.7 Hz, H-5'), 6.84 (1H, s, H-3), 6.69 (1H, br s, H-6), 6.03 (2H, s, OCH$_2$O), 3.64 - 3.54 (2H, m, H-3''), 2.78 (2H, t, J=6.1 Hz, H-1''), 1.78 - 1.71 (2H, overlapped, H-2'').

ESI-MS: m/z 311. 21 [M-H]$^-$, (calc. 311.0924 for C$_{18}$H$_{15}$O$_5^-$).

5(3''-hydroxypropyl)-7-methoxy-2-(3',4'-dimethyloxyphenyl)-benzofuran, homoegonol (**7**): ^{1}H NMR (400 MHz, CDCl$_3$) δ 7.42 (1H, dd, J=8.1, 1.4 Hz, H-6'), 7.34 (1H, d, J=1.3 Hz, H-2'), 6.87 (1H, d, J=8.1 Hz, H-5'), 6.84 (1H, s, H-3), 6.66 (1H, br s, H-6), 6.02 (2H, s, OCH$_2$O), 4.04 (3H, s, CH$_3$O), 3.99 (3H, s, CH$_3$O), 3.93 (3H, s, CH$_3$O), 3.71 (2H br t, J=6.3 Hz, H-3''), 2.82 - 2.71 (2H, m, H-1''), 1.99 - 1.89 (2H, m, H-2'').

ESI-MS: m/z 365.27 [M+Na]$^+$, (calc. 365.1359 for C$_{20}$H$_{22}$NaO$_5^+$); m/z 381.21 [M+K]$^+$, (calc. 381.1098 for C$_{20}$H$_{22}$KO$_5^+$).

1,5-anhydro-D-mannitol (**8**): white amorphous solid;
^{1}H NMR and ^{13}C NMR see Table 1;

$[α]^{25}_D$ (H$_2$O) = -20.1º (C, 5 mg/mL);
ESI-MS: m/z 187.06 [M+Na]$^+$, (calc. 187.0576 for C$_6$H$_{12}$NaO$_5^+$); m/z 351.12 [2M+Na]$^+$, (calc. 351.1261 for C$_{12}$H$_{24}$NaO$_{10}^+$).

2,3,4,6-tetraacetyl-1,5-anhydro-D-mannitol (**8a**): white amorphous solid;
^{1}H NMR and ^{13}C NMR see Table 2;
$[α]^{25}_D$ (CHCl$_3$) = -12.9º (C, 10 mg/mL);
ESI-MS: m/z 371.15 [M+K]$^+$, (calc. 371.0738 for C$_{14}$H$_{20}$KO$_9^+$).

6'-O-benzoyl-sucrose (**11**): ^{1}H NMR (400 MHz, CD$_3$OD) δ: 8.04 (2H, d, J=7.2 Hz, H-2'' and H-6''), 7.62 (1H, t, J=7.4 Hz, H-4''), 7.49 (2H, t, J=7.7 Hz, H-3'' and H-5''), 5.43 (1H, d, J=3.7 Hz, H-1'), 4.62 (1H, dd, J=12.0, 1.8 Hz, H$_a$-6'), 4.49 (1H, dd, J=12.0, 6.1 Hz, H$_b$-6'), 4.16 (1H, br d, J=10.0 Hz, H-3'), 4.06 (1H, m, H-4), 3.96 (1H, overlapped, H-3), 3.95 (1H, overlapped, H-5), 3.84 (1H, dd, J=11.6, 2.2 Hz, H$_a$-6), 3.77-3.67 (1H, overlapped, H$_a$-1), 3.66 (1H, overlapped, H$_b$-6), 3.68-3.47 (1H, overlapped, H$_b$-1), 3.67 (1H, overlapped, H-5'), 3.48 (1H, overlapped, H-2'), 3.47 (1H, overlapped, H-4'), (overlapped signals were assigned by HSQC correlations).

^{13}C NMR (100 MHz, CD$_3$OD) δ: 168.10 (ArCOO), 134.36 (C-4''), 130.71 (C-1''), 129.72 (C-2'' and C-6''), 129.65 (C-3'' and C-5''), 105.32 (C-2), 93.60 (C-1'), 83.82 (C-5), 78.12 (C-3), 76.32 (C-4), 74.85 (C-5'), 73.00 (C-2'), 72.09 (C-3'), 71.61 (C-4'), 65.8 (C-1), 65.10 (C-6'), 62.80 (C-6).

ESI-MS: m/z 469.27 [M+Na]$^+$, (calc. 469.1316 for C$_{19}$H$_{26}$NaO$_{12}^+$).

3. Results and Discussion

The phytochemical analysis of the fruits of *S. officinalis* L. led to the isolation and identification of fifteen known compounds: tri-α-linolenoyl-*sn*-glycerol (**1**), 1,2-di-α-linolenoyl-3-linoleoyl-*sn*-glycerol (**2**), 1-α-linolenoyl-2-palmitoyl-*sn*-glycerol (**3**), 1,2-di-α-
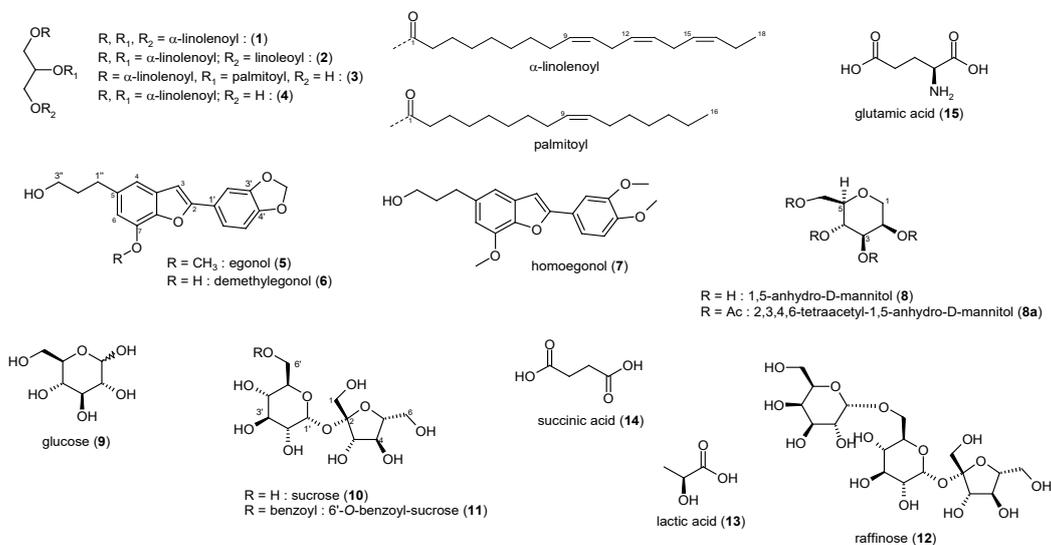
Fig. 2. Structures of the compounds present in the fruits of *Styrax officinalis* L.

Table 2

Complete ^{1}H-NMR and ^{13}C-NMR assignments for compound (**8a**).

position	H, δ, J in Hz	C, δ, HSQC	HMBC	COSY	NOESY
	8a				
	^{1}H-NMR 400 MHz; ^{13}C-NMR 100 MHz, CD$_3$OD				
1	3.98 (dd, J=13.2, 2.0); 3.80 (dd, J=13.2, 1.0)	68.9	C-2, C-3, C-5	H-2	H-2
2	5.36 – 5.30 (m)	70.4	C-3, C-4, Ac	H-1, H-3	H-1, H-3
3	5.14 (dd, J=10.0, 3.5)	73.2	C-2, C-4, C-5, Ac	H-2, H-4	H-2
4	5.21 (t, J=9.9)	67.7	C-3, C-5, C-6, Ac	H-3, H-5	
5	3.72 (ddd, J=9.6, 5.1, 2.3)	77.5	C-1, C-3, C-4, C-6	H-4, H-6	
6	4.23 (dd, J=12.3, 5.1); 4.10 (dd, J=12.3, 2.3)	63.9	C-4, C-5, Ac	H-5	
Ac	2.15 (s)	20.8 172.7	C-6		
Ac	2.07 (s)	20.71; 172.2	C-2		
Ac	2.05 (s)	20.70; 171.8	C-3		
Ac	1.98 (s)	20.66; 171.7	C-4		

linolenoyl-*sn*-glycerol (**4**), egonol (**5**), demethylegonol (**6**), homoegonol (**7**), 1,5-anhydro-D-mannitol (**8**), glucose (**9**), sucrose (**10**), 6'-*O*-benzoyl-sucrose (**11**), raffinose (**12**), lactic acid (**13**), succinic acid (**14**) and glutamic acid (**15**) (Fig. 2).

These compounds belong to seven different classes of natural metabolites such as acyl-glycerols (compounds **1-4**), benzofurans (norneolignans) (compounds **5-7**), anhydo-hexitol (compound **8**), carbohydrates (compounds **9-10** and **12**), oligosaccharide ester (**11**), organic acids (compounds **13-14**), amino acids (compound **15**). The presence of these compounds is important from both the chemosystematic and the nutraceutical standpoints as discussed in the next paragraphs.

3.1. Identification of 1,5-anhydro-D-mannitol

The identification of 1,5-anhydro-D-mannitol (**8**) isolated in this study, was achieved by mean of extensive NMR analysis which was conducted on both the pure compound present in the assembly of fractions 72-116 derived from the first chromatographic step and its acetylated derivative (**8a**) obtained after a semi-synthetic approach.

The proton spectrum of compound (**8**) showed resonances comprised between 3.31 and 3.99 ppm compatible with oxymethine and oxymethylene functions (Supplementary Fig. 1) whereas the carbon spectrum showed the presence of six resonances comprised between 81.5 and 62.2 ppm (Supplementary Fig. 2) which are compatible with an anhydro-hexitol moiety.

In particular, from the proton spectrum, the following signals were easily recognizable: a broad singlet at 3.99 ppm, assigned to H-2; a complex signal at 3.31 ppm (ddd, J=9.0, 6.6, 2.1) assigned to H-5 also on the basis of the observed $^3J_{H-5,Hb-6}$ and $^3J_{H-5,Ha-6}$ values (6.6 and 2.1 Hz, respectively). The protons H$_a$-1 and H$_a$-6 resulted to be partially overlapped between 3.94 and 3.90 ppm but their assignments and multiplicity (dd, J=12.8, 2.0 and

dd, J=12.3, 2.1, respectively) were resolved by HSQC experiment (Supplementary Fig. 3).
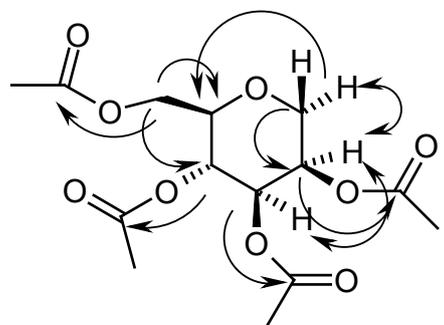
Fig. 3. Selected HMBC (⟶) and NOESY (⟵⟶) correlations for compound **8a**.

The presence of other overlapped signals and complex multiplets in the proton spectrum of compound **8** made the interpretation of the experimental data difficult. In particular, this concerned the correct measurement of coupling constants of the very near and/or partially overlapped proton signals (H$_b$-1, H-3, H-4 and H$_b$-6) even with the aim of bidimensional spectra (i.e. see HSQC, Supplementary Fig. 3). In a first analysis, the values 3.65 (dd, J=12.8, 2.0 Hz), 3.60 (t, J=9.5 Hz), 3.66 (dd, J=9.5, 3.4 Hz) and 3.70 (dd, J=12.3, 6.6 Hz), were assigned to these protons, respectively.

To provide an unequivocal assignment of each resonance a synthetic approach was undertaken and the obtained acetylated derivative **8a** showed the expected results in the NMR experiments. These were a series of signals not overlapped and sufficiently far one from the other (Supplementary Fig. 4) to permit an unequivocally assignment of each resonance on the basis of the mono- and bidimensional experiments (Supplementary Fig.s 5-8). In particular, it was evidenced that only the proton signal of one oxymethylene group (the exocyclic one, H-6) resulted to be shifted at lower fields (4.23-4.10 ppm) due to the direct acetylation and this showed long range correlation with a carboxylic carbon

resonating at 172.7 ppm (HMBC). On the other hand, the chemical shift recorded for the other oxymethylene in C-1 was compatible with a deshielding effect due to a β-acetylation. By the study of the coupling constants, it was also evident that the oxymethine proton in position 5 resulted to be adjacent to the exocyclic oxymethylene ($^3J_{H-5,Ha-6}$=5.1 Hz; $^3J_{H-5,Hb-6}$=2.4 Hz) and to the oxymethyne in position 4 ($^3J_{H-4,H-5}$=9.7 Hz). The value of $^3J_{H-4,H-5}$ is also compatible with a relative *trans* orientation of these protons and this configuration was confirmed by a NOESY experiment. Conversely, the oxymethine in position 2 resulted to be adjacent to the oxymethylene involved in the closing of the tetrahydropyrane ring (C-1). The presence of $^3J_{H-1,H-2}$ of low values ($^3JH_{a-1,H-2}$=2.0 Hz; $^3J_{Hb-1,H-2}$=0.9 Hz) also gave a reason for the broadening of the signal relative to the proton in position 2. The signals relative to H-2, H-3 and H-4 revealed chemical shifts compatible with those of esterified (acetylated) oxymethine being all deshielded over 5.0 ppm. The acetylation in these positions was furtherly confirmed in HMBC experiments by long range correlations with the acetyl carboxylic carbons (Supplementary Fig. 7). The relative positions of protons of the tetrahydropyrane ring were also confirmed by a COSY spectrum, available as supplementary materials (Supplementary Fig. 8). In contrast with what observed for the protons, the effect of acetylation on the chemical shifts of carbons resulted into a shielding effect (0.5-5.0 ppm) for all the carbons with the exception of C-6 which showed a little deshielding effect of a value of 0.9 ppm.

A complete assignment of the resonances recorded in two different solvents (D_2O and CD_3OD) for compound **8** and its derivative **8a** is reported in Table 1 and Table 2 and selected HMBC and NOESY correlations are reported in Fig. 3.

The specific rotation values, recorded at 25 °C for compound **8** and its derivative **8a**, were -20,1° and -12.9°, respectively. The observed differences in the magnitude of the specific rotation values in respect to those reported in literature (Coffey, 1967; Fletcher Jr and Diehl, 1952; Gray and Barker, 1967) are likely due to increased intermolecular association between the polyol molecules at higher concentrations which may cause a decrease in specific rotation. The occurrence of this phenomenon was already observed in diols i.e. as malic acid (Bancroft and Davis, 1930) and also in (S)-2-phenylpropanal (Consiglio et al., 1983), and was recently reviewed by Girard and Kagan (2000), while its quantitative description was reported by the workgroup of Baciocchi (Baciocchi et al., 2002).

3.2. Chemosystematic considerations

The norneolignans (benzofurans) (**5-7**) may be useful chemotaxonomic markers. In particular, egonol (**5**) is a widely retained metabolite within the genus *Styrax* having been recognized in the majority of the species i.e. *S. officinalis* L. (Akgul and Anil,

2003a, 2003b), *S. perkinsiae* Rehder (Li et al., 2005a), *S. japonica* (*S. japonicus* Siebold & Zucc.) (Kim et al., 2007), *S. ferrugineus* Nees & Mart, *S. camporum* Pohl and *S. pohlii* A. DC. (Moraes et al., 2012) and *S. obassia* (*S. obassis* Siebold & Zucc.) (Lee et al., 2008). Indeed, demethylegonol (**6**) and homoegonol (**7**) seem to be less widespread metabolites. These two egonol derivatives (**6**, **7**) might have a relevance at a specific level, having been recognized so far only in a few species such as *S. perkinsiae* Rehder (Li et al., 2005a) and *S. macranthus* Perkins (Luo et al., 2007) for what concerns the former compound and *S. ramirezii* Greenm. (syn. of *S. argenteus* var. *ramirezii* (Greenm.) Gonsoulin) (Timmers et al., 2015), *S. ferrugineus* Nees & Mart. (Pauletti et al., 2000; Moraes et al., 2012) *S. camporum* Pohl (Giesbrecht et al., 1985) and *S. pohlii* A. DC. (Moraes et al., 2012) for what concerns the latter one.

Anhydro-hexitols are a rare class of natural occurring compounds (Soltzberg, 1970) since their presence has been recognized in a very limited cases while their synthesis have been more widely investigated in the last decades mainly to obtain acceptable yields, in particular on large scale, (Bock et al., 1981; Kocienski and Pant, 1982) and/or for the synthesis of isosorbide (Soltzberg et al., 1946; Liang et al., 2011; Kobayashi et al., 2015) which has several useful applications in medicinal chemistry and pharmacy. 1,5-anhydromannitol (**8**) (also known with the trivial name of "styracitol") has been initially recognized from the husks of *S. obassia* (Asahina, 1912), a native *taxon* of Japan and more recently from the fruits peel of *S. officinalis* L. (Anil, 1977).

The unequivocal determination of its structure, in particular for what concerns its stereochemistry, has been widely debated in the past, and several synthetic approaches have been applied in order to provide a clear response in this context (Freudenberg and Rogers, 1937; Freudenberg and Sheehan, 1940; Hockett et al., 1943; Richtmyer and Hudson, 1943; Fried and Walz, 1949; Sowden and Oftedahl, 1961). To fill the gap regarding the assignment of the ^{1}H-NMR signals for compound (**8**) and its derivative (**8a**), which are not available in the literature, we took also the occasion to provide a complete assignment of NMR signals obtained in two different deuterated solvents (Table 1 and Table 2).

For what concerns the presence of (**11**), it is notable that benzoyl esters of sucrose are quite rare compounds and have been recognized before in a very few cases, mainly from *Polygala* spp. (Polygalaceae) (Zhang et al., 1998; Wei et al., 2002; Li et al., 2005b) in which several multiesters of di- and oligosaccharides with organic acids bearing the base structure of benzoic acid and/or cinnamic acid, were identified. From the chemosystematic standpoint the co-occurrence of (**11**) in *S. officinalis* L. and in *Polygala* spp. is of interest and deserves further studies since these species belong to families (Styracaceae and Polygalaceae, respectively) which are comprised in Clades belonging to two

different Orders and, taxonomically, are relatively far one from the other: Ericales of Asterids for the former and Fabales of Rosids for the latter. As hypothesis, the presence of **11** in *S. officinalis* L., being the only compound belonging to the oligosaccharide esters of benzoic acid recognized in the present study, might be due to a relictual metabolic pathway, but this should be confirmed also on molecular basis.

3.3. Nutraceutical considerations

The four acyl-glycerols, perfectly divided into two triacyl-glycerols and two diacyl-glycerols are mainly constituted by poly-unsaturated fatty acid chains in their structures. This fact alone makes these compounds potential beneficial elements in humans. It's well documented that poly-unsaturated fatty acids are extremely able to reduce the total level of cholesterol in blood and are associated with a healthy life. For this reason, they should be preferred rather than saturated fatty acids in every diet. In addition to this, it's important to underline that these fatty acids have also other important healthy effects. In particular, α-linolenic acid has a cardioprotective activity (Pan et al., 2012) and linoleic acid is a good antioxidant compound. Compound (**3**) is the only glyceride, evidenced during this study, which contains a saturated fatty acid in its structure. Anyway, it's presence does not affect the total potential beneficial effect of the compound since palmitic acid displays strong antioxidant and selective cytotoxic properties (Harada et al., 2002).

Egonol (**5**) and its derivatives (**6**, **7**), are well known to have interesting pharmacological properties on a wide range of actions, from the antileukemic to the anticomplement, antiasthmatic, cytotoxic, antimutagenic, antifungal, NO- and cyclooxigenase inhibitors, antischistosomal and antibacterial ones (Hirano et al., 1994; Pauletti et al., 2000; Min et al., 2004; Teles et al., 2005; Öztürk et al., 2008; Bertanha et al., 2012; Bertanha et al., 2014; Shin et al., 2014; Cao et al., 2015; De Oliveira et al., 2016a, 2016b). They are also known to exert a synergistic insecticide activity with pyrethrum (Takanashi and Takizawa, 1988; Pauletti et al., 2000).

The saccharides glucose (**9**), sucrose (**10**), and raffinose (**12**) and the oligosaccharide ester, 6'-*O*-benzoyl-sucrose (**11**) have also nutraceutical properties. Beside the fact that they all are energy sources for the metabolism, they are endowed with other interesting properties. In particular, glucose (**9**) is important to improve brain activity, sucrose (**10**) exerts preservative and antioxidant effect (Balbani et al., 2006) and raffinose (**12**) is a good sweetener. Benzoyl ester (*p*-hydroxy derivatives) and benzoates (mainly as sodium salt, E211) are widely used as food preservatives and compound (**11**) may have also this kind of property. For what concerns the organic acids, lactic acid (**13**) is a food additive, known as E270, a preservative and a flavoring agent while succinic acid (**14**) (E363) is an acidity regulator and an excipient used in food and beverage industry and pharmaceutics (Zeikus et al., 1999).

In literature there is no reference about the bioactivity of styracitol (**8**), but other anhydro-hexitols, such as 1,5-anhydro-D-fructose and 1,5-anhydro-D-glucitol, have been widely evaluated in both *in vitro* and *in vivo* models for their potential medical application. It resulted that 1,5-anhydro-D-fructose inhibits the growth of the carcinogenic *Streptococcus mutans* (Hisaku et al., 2004) and shows anti-inflammatory effects by reducing the LPS-mediated cytokine release and NO production by down-regulating the inducible nitric oxide synthase (iNOs) (Meng et al., 2009a, 2009b). 1,5-anhydro-D-glucitol is the major polyol after glucose present in human plasma (Yamanouchi et al., 1992) and may be used as a diabetes marker for glycemic control (Yabuuchi et al., 1989). It is also capable to stimulate insulin secretion in MIN6 mouse insulinoma, a cell line which closely resembles normal pancreatic β-cells in regard to glucose metabolism and insulin secretion (Yamanouchi et al., 2003). The structure of styracitol (**8**) is very similar to those of these two anhydro-hexitols and, in particular, its stereochemistry shows differences only in the C-2 position, being so the epimer in C-2 of 1,5-anhydro-D-glucose, while 1,5-anhydro-D-fructose has a pro-kiral keto function in the same position and is metabolized to 1,5-anhydro-D-glucose in several organisms including bacteria, fungi, algae and mammalian (Yu, 2008). Considering the high structure similarity styracitol (**8**) might have similar biological activities. Anhydro-hexitols represent, indeed, interesting building blocks or intermediate for the synthesis of nucleoside analogues (Verheggen et al., 1993, 1995; Pérez-Pérez et al., 1996; Ostrowski et al., 1998; Van Aerschot et al., 2003; Lambertucci et al., 2007; D'Alonzo et al., 2008) which show interesting properties, mainly as antiviral agents (Verheggen et al., 1993, 1995; Pérez-Pérez et al., 1996; Ostrowski et al., 1998; Van Aerschot et al., 2003) but also as a useful probes for the study of natural DNA properties (D'Alonzo et al., 2008) and as a possible universal primer in PCR sequencing analysis (Lambertucci et al., 2007). In this context, *S. officinalis* L. may represent an useful and potential natural source of 1,5-anhydro-D-mannitol (**8**) as an enantiopure compound for the employment in Green Chemistry approaches.

Lastly, glutamic acid (**15**), even if not an essential amino acid, is equally important because it is also used as food additive, in particular as flavor enhancer. In fact, glutamic acid (**15**) is responsible of the "umami" taste, the fifth basic taste (together with sweetness, sourness, bitterness, and saltiness), discovered in 1908 by the Japanese chemist, Kikunae Ikeda (Ikeda, 2002). It is largely used as food additive in the form of monosodium salt (E621) to enhance the perception of dishes since in humans the taste receptors are far more

sensitive to glutamate than to the other amino acids (Zhang et al., 2003). The presence of glutamic acid, together with other taste active compounds recognized as constituent of this species (i.e. acylglycerols, organic acids, saccharides and derivatives), may be the reason why these fruits are highly appreciated as ingredients of traditional culinary recipes.

4. Concluding remarks

The phytochemical analysis of the fruits of *S. officinalis* L. collected in Mounts Lucretili, from an isolated population, led to the isolation and identification of fifteen known compounds. Several of these have a chemotaxonomic relevance and may be considered useful markers at a specific level. Moreover, the present study showed that S. officinalis L. may be an useful source of enantiopure 1,5-anhydro-D-mannitol which has several medicinal potentialities and is a versatile building block in organic synthesis for bulk and fine chemical preparation of valuable and potentially biologically active molecules, in particular for what concern the "Green" approaches. This obviously might result into a limitation of industrial wastes productions with clear advantages at Environmental level. The secondary metabolites content showed also the presence of constituents with health promoting activities which make this species an important source of interesting phytochemicals with nutraceutical and pharmacological properties. The presence of all the compounds with health promoting activities, provides also a phytochemical rationale for both the ancient pharmacological uses of the species and the today nutraceutical ones. These properties are also, and above all, important in the light of the fact that the worldwide demographic growth has contributed to the emergence of new issues regarding the access to food that should be guaranteed to everyone and, new sources of nutrients and molecules with health promoting activity are of primary importance in this context. Moreover, the discovery of potential nutritional and with added value constituents in a spontaneous species, as evidenced in the present case, could lead to the protection and the valorization of the species itself which may result in the cultivation systems with both economic and biodiversity conservation advantages.

Conflict of interest

The authors declare that there is no conflict of interest.

Acknowledgments

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