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Original scientific paper

PHENETHYL ANGELATE – A NEW ESTER FROM IMMORTELLE ESSENTIAL OIL?

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Abstract. *Esters of angelic, senecioic and tiglic acids with various saturated/ unsaturated/aromatic alcohols contribute to the aroma of many essential oils. However, mass spectrometry with electron-impact ionization sometimes fails to distinguish these regio-/geometric isomers and this was the case with the minor constituent of* Helichrysum italicum *(immortelle) essential oil that was tentatively identified as the ester of 2-phenyl-1-ethanol with one of the mentioned acids. Our efforts to identify this phenethyl ester were also hampered by the inconsistency or by the lack of appropriate RI data in the literature. Therefore, we prepared and fully spectrally characterized (1D- and 2D-NMR, IR, MS) synthetic samples of all three isomeric esters. Subsequent GC analyses of immortelle oil samples with spiked synthetic phenethyl esters unambiguously confirmed that the compound in question was phenethyl angelate. This rare plant secondary metabolite has been previously reported only twice as a constituent of samples of natural origin. However, the outcomes of our study strongly imply that this molecule was misidentified in these earlier studies with the corresponding senecioate/tiglate. Thus, the existing libraries of RI/MS data for tiglates and angelates have to be upgraded with appropriate data for senecioates to avoid these kinds of errors in the future.*

Key words: Helichrysum italicum, *essential oil, phenethyl angelate, phenethyl tiglate, phenethyl senecioate, spectral characterization*

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1. INTRODUCTION

Helichrysum italicum (Roth) G. Don (Asteraceae) is a dwarf aromatic shrub with yellow flower bracts that is also called immortelle or everlasting plant because it never rots, retaining its signature curry-like scent in perpetuity even as a dry flower. The essential oil of this iconic plant of the Mediterranean basin has been used since ancient times due to its diverse medicinal properties and, even nowadays, continues to play an important role in alternative medicine, aromatherapy, perfume, and cosmetics industries (Andreani et al., 2019; Guinoiseau et al., 2013; Sarkić and Stappen, 2018). Therefore, the chemical composition of immortelle essential oil has been extensively studied and several chemotypes of *H. italicum* characterized by distinct essential-oil profiles were described. Among them, the most widespread are the Corsican neryl acetate/β-diketones and the Balkan α-pinene/γ-curcumene chemotypes (Andreani et al., 2019).

During our ongoing study of the antimicrobial and anti-inflammatory potential of different immortelle essential-oil chemotypes (Aksić et al., 2019; Genčić et al., 2018; Genčić et al., 2021), a minor constituent (RI = 1538), detected in the sample of the Corsican oil, caught our attention. Although the molecular ion was not observed, its fragmentation pattern in the EI-MS spectrum of this compound gave us some valuable information about its possible structure: (*i*) the base peak at *m/z* 104 (styryl related fragment formed by McLafferty rearrangement), accompanied with ions at *m/z* 91 (tropylium ion) and *m/z* 77 (phenyl ion) pointed to the presence of 2-phenylethyl group in the molecule (Wang et al., 2014), while (*ii*) the presence of intense ions at *m/z* 83 and 55 suggested that this could be a derivative of one of the isomeric $C_5H_8O_2$ unsaturated carboxylic acids (*i.e.* ((*Z*)-2-methyl-2-butenoic (angelic), (*E*)-2-methyl-2-butenoic (tiglic), or 3-methyl-2-butenoic (senecioic); Radulović et al., 2015). Indeed, a comparison of this MS spectrum with those available in several MS libraries (Wiley 6, NIST11, MassFinder 2.3, and a homemade MS library) revealed that the compound in question could be phenetyl-tiglate (83% match) or -senecioate (76% match). MS spectrum of the ester of 2-phenyl-1-ethanol with the remaining isomeric acid, angelic acid, is also available in the literature (Adams and Dev, 2010) and there are almost no differences between this spectrum and the spectra of our immortelle oil constituent and the esters of other two isomeric acids. A previous study disclosed that angelates and tiglates of straight and branched-chain aliphatic alcohols (C_2-C_6) could be easily distinguished by the relative proportions of the ions at *m/z* 100 (free acid) and *m/z* 101 (protonated acid). Unfortunately, this did not apply to the corresponding phenylethyl esters that showed only small differences in their MS spectra with the most notable difference in the intensity of the [R – H] radical ion (Thomas and Willhalm, 1976). However, when these esters are present in small amounts in the analyzed sample (as in our immortelle essential oil), this [R – H] radical ion, as well as the molecular ion, are usually not observed.

Usually, this uncertainty could be resolved if retention index (RI) values for the proposed structures are available in the literature for comparison with the RI value of the unknown compound. Phenethyl tiglate is a common constituent of essential oils and literature data imply that it elutes on DB-5 (or equivalent column) in the RI interval from 1584 to 1591 (NIST) which are considerably higher RI values than that found for our phenethyl ester (RI = 1540). On the other hand, phenethyl angelate has been previously reported only as a minor constituent of *Pinus halepensis* Miller essential oil (Fekih et al., 2014) and as a volatile of Malay rose apple (Pino et al., 2004). Alongside the angelate,

the corresponding tiglate was identified in these samples that according to authors eluted (from Rtx-1 and SPB-5 columns, respectively) *ca*. ten RI units before or after the angelate. Adams and Dev (2010) reported that a synthetic standard of phenethyl angelate eluted faster from a DB-5 column) than the corresponding synthetic sample of tiglate and found a significantly higher RI gap of *ca*. 50 units (Table 1). Moreover, there is no RI data for phenethyl senecioate in the literature and this is not surprising as this ester has been so far only detected in the volatile leaf oil of *Pinus pinaster* Ait (Pauly et al., 1973).

Table 1 Literature RI values of phenethyl-angelate and -tiglate

	Phenethyl angelate	Phenethyl tiglate	Reference
RI (Rtx-1)	1568	1546	Fekih et al., 2014
RI (SPB-5)	1589	1600	Pino et al., 2004
RI (DB-5)	1534	1585	Adams and Dev, 2010

This inconsistency in the literature RI values for phenethyl-angelate and -tiglate, as well as the lack of RI data for corresponding senecioate, prompted us to make synthetic standards of all three isomeric esters, to fully characterize them by spectral means (RI, MS, NMR, and IR) and to co-injected them with the sample of immortelle oil to ambiguously confirm the identity of our phenethyl ester.

2. Material and Methods

2.1. Chemicals

For the determination of retention indices, a hydrocarbon mixture (Sigma-Aldrich, USA) ranging from *n*-heptane to *n*-eicosane was used. All solvents (HPLC grade), were purchased from Sigma-Aldrich as well. Chemicals used for syntheses, including 2-phenyl-1-ethanol, 4-(dimethylamino)pyridine (DMAP), *N*,*N*′-dicyclohexylcarbodiimide (DCC), anhydrous $MgSO_4$, as well as senecioic (3-methyl-2-butenoic) and angelic ((Z)-2-methyl-2-butenoic) acids, were of analytical grade, commercially available, and used as received (Sigma-Aldrich, USA; Merck, Germany; Carl Roth, Germany; Fluka, Germany; TCI, Japan).

2.2. Essential oil sample

A commercial sample of *Helichrysum italicum* ssp. *italicum* essential oil was produced by „Casa Vecchia Corsa“ (Sartène, Corsica, France). The details about the plant material and isolation of essential oil are given in Andreani et al. (2019).

2.3. General experimental procedures

Chromatographic separations were carried out using silica gel 60 (particle size distribution 20–45 mm) purchased from Carl Roth GmbH + Co. KG (Karlsruhe, Germany), whereas TLC experiments were performed on precoated Al-backed silica gel 40 F254 plates (Merck, Germany). The spots on TLC were visualized by UV light (254 nm) and by spraying with phosphomolybdic acid (12 g) in EtOH (250 ml) followed by heating.

The GC–MS analyses were repeated three times for each sample using a Hewlett-Packard 6890N gas chromatograph equipped with a fused silica capillary column DB-5MS (5%-diphenylpolysiloxane and 95%-dimethylpolysiloxane, 30 m × 0.25 mm, film thickness 0.25 μm; Agilent Technologies, Santa Clara, CA, USA) and coupled with a 5975B mass selective detector from the same company. The injector and interface were operated at 250 and 300 °C, respectively. The oven temperature was raised from 70 to 290 °C at a heating rate of 5 °C min^{-1} and then isothermally held for 10 min. As a carrier gas, helium at 1.0 mL min^{-1} was used. The samples, 1 μL of the corresponding solutions in diethyl ether (1 : 100), were injected (injection volume 1 μL) in a split mode (split ratio 40:1). The mass selective detector was operated at the ionization energy of 70 eV, in the 35–650 amu range, and scanning speed of 0.32 s.

^{1}H NMR (400 MHz; including ^{1}H NMR selective homonuclear decoupling experiments), ^{13}C NMR (100.6 MHz; with and without heteronuclear decoupling), DEPT-90, DEPT-135, NOESY, and gradient ^{1}H–^{1}H COSY, HSQC, and HMBC spectra were recorded on a Bruker Avance III spectrometer (Bruker, Fällanden, Switzerland), equipped with a 5-mm dual $^{13}C/^{1}H$ probe head. All NMR spectra were measured in $CDCl_3$ at 25 °C. Chemical shifts are reported in ppm (δ) and referenced to TMS (δ_H = 0 ppm) in ^{1}H NMR spectra and/or to solvent signals (δ_H = 7.26 ppm and δ_C = 77.16 ppm) in ^{13}C NMR and heteronuclear 2D spectra.

The IR measurements (ATR-attenuated total reflectance) were carried out using a Thermo Nicolet model 6700 FTIR instrument (Waltham, USA).

2.4. Synthesis of phenethyl esters

The esters of 2-phenyl-1-ethanol and senecioic, tiglic or angelic acid were prepared following the general Steglich procedure (Scheme 1; Radulović et al., 2015). Briefly, 2-phenyl-1-ethanol (1 eq.), the appropriate acid (1.1 eq.), DCC (1.1 eq.), DMAP (0.1 eq.) anhydrous CH_2Cl_2 were mixed and stirred overnight under anhydrous conditions. The precipitated *N*,*N*'-dicyclohexylurea was filtered off and the filtrate was concentrated under vacuum. The resulting residue was purified by column chromatography on SiO_2 using a mixture of 3% Et_2O in *n*-hexane (*v/v*) as the eluent. The purity of the ester fractions was checked by TLC and GC-MS.

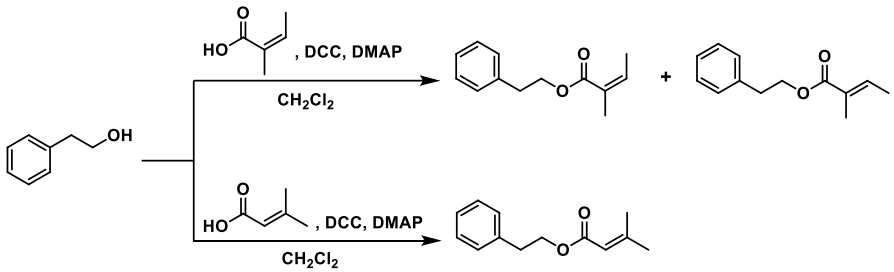
Phenethyl angelate – RI (DB-5MS) = 1540; MS (EI, 70 eV), *m/z* 105 (27.9%), 104 (100), 91 (38.4), 83 (23.8), 77 (27.3), 65 (20.5), 55 (49.6), 39 (24.1); FTIR-ATR (neat) cm^{-1}: 3029 (C–H stretching in olefins and aromatics (*ν*(=CH)), 2954 (asymmetric stretching of CH_3– groups, $\nu_{as}(CH_3)$), 1713 (C=O stretching (*ν*(C=O)), 1649 (C=C stretching in olefins (*ν*(C=C)), 1454, 1350, 1230, 1147, 1042, 846, 747 and 698 (C–H wagging in aromatics (ω(=C–H)).

Phenethyl senecioate – RI (DB-5MS) = 1574; MS (EI, 70 eV), *m/z* 105 (18.6%), 104 (100), 91 (14.3), 83 (58.7), 77 (10.5), 65 (8.3), 55 (20.9), 39 (12.2); FTIR-ATR (neat) cm^{-1}: 3029 (*ν*(=CH)), 2934, 1715 (*ν*(C=O)), 1651 (*ν*(C=C)), 1452, 1347, 1225, 1141, 1076, 85, 744 (ω(=C–H)) and 698 (ω(=C–H)).

Phenethyl tiglate – RI (DB-5MS) = 1587; MS (EI, 70 eV), *m/z* 105 (21.7%), 104 (100), 91 (50.1), 83 (28.4), 77 (30.6), 65 (24), 55 (57.5), 39 (27.9); FTIR-ATR (neat) cm^{-1}: 3028 (*ν*(=CH)), 2953 ($\nu_{as}(CH_3)$), 1706 (*ν*(C=O)), 1651 (*ν*(C=C)), 1454, 1382, 1252, 1133, 1076, 85, 732 (ω(=C–H)) and 698 (ω(=C–H)).

RESULTS AND DISCUSSION

The reaction of 2-phenyl-1-ethanol with angelic acid under Steglich conditions (Scheme 1) gave two products according to TLC analysis, that were easily separated by column chromatography on SiO_2 (3% Et_2O (*v/v*) in *n*-hexane). Subsequent GC-MS analysis revealed that both products represented phenethyl esters of $C_5H_8O_2$ unsaturated carboxylic acids. Having in mind that angelic acid may isomerize to tiglic acid under certain reaction conditions (Buckles et al., 1955), we supposed that these two esters were phenethyl-angelate and -tiglate. NMR analyses (Table 2) undoubtedly confirmed this as we established that phenethyl angelate was the product that eluted first from the GC column (RI = 1540), while the latter one was phenethyl tiglate (RI = 1587). We prepared a synthetic sample of phenethyl senecioate also following the Steglich protocol (Scheme 1) and characterized it by spectral means (NMR, IR, GC-MS). GC-MS analysis revealed that this isomer elutes after phenethyl angelate, but before phenethyl tiglate, from a DB-5MS column (RI = 1574).



Scheme 1 Products of Steglich reaction of 2-phenyl-1-ethanol with angelic and senecioic acids

After the co-injection of these three synthetic standards with the sample of immortelle essential oil, it was unequivocally corroborated that the unknown phenethyl ester is the one with angelic acid. As we mentioned, phenethyl angelate has a rather restricted occurrence in the samples of natural origin (*e.i.* there are only 2 previous reports in the literature). Moreover, this study was motivated by the inconsistency of RI data for phenethyl-angelate and -tiglate (Table 1), as well as by the unavailability of RI data for the corresponding senecioate in the literature. Our RI data for phenethyl angelate (RI = 1540) and -tiglate (RI = 1587) are in good agreement with the corresponding data (1534 and 1585, respectively) reported by Adams and Dev (2010) who synthesized a library of 141 angelates and tiglates and characterized them by Kováts retention indices and mass spectra intending to offer the data that would aid the identification of these esters in essential oils. We established that among the studied isomeric esters phenethyl angelate eluted first from the GC column, followed by the senecioate ($\Delta RI_1 = RI_{senecioate} - RI_{angelate} = 34$), while the tiglate elutes the last ($\Delta RI_2 = RI_{tiglate} - RI_{senecioate} = 13$). Indeed, we have noted in our previous studies that this (angelate < senecioate < tiglate) is most often the order of elution from DB-5 columns of the esters of these three isomeric pentenoic acids (Blagojević et al., 2017; Radulović et al., 2013).

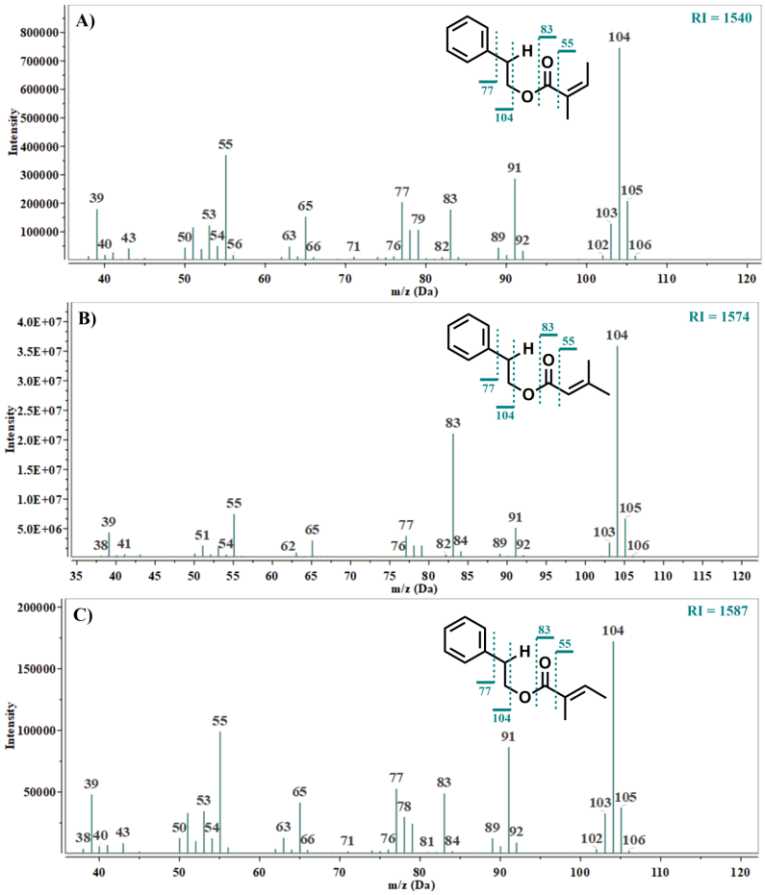


Fig. 1 Comparison of MS spectra of phenethyl-angelate (**A**), -senecioate (**B**) and -tiglate (**C**) with the corresponding structures on which characteristic fragment ions are designated

Therefore, it seems that phenethyl angelate was misidentified in previous studies (Fekih et al., 2014; Pino et al., 2004). As the difference of RI values between the two detected phenethyl esters in these two studies was 12 and 11, respectively, and having in mind our RI data, we supposed that these esters were phenethyl-senecioate (with lower RI value) and -tiglate (higher RI value). The resemblance of mass spectra (Fig. 1) and the lack of RI data in the literature were most probably the reasons for these misidentifications. After closer inspection of the mass spectra of the studied esters, we have noted that the ester of senecioic acid could be discriminated from the other two based on the relative proportions of the ions at *m/z* 55 and 83 that arise through α-cleavage at the carbonyl group. Specifically, in the mass spectrum of phenethyl senecioate, the acyl ion *m/z* 83 is considerably more intense than the ion at *m/z* 55 (58.7% *vs.* 20.9%), while an opposite trend is visible in the mass spectra of phenethyl-angelate and -tiglate (23.8% *vs.* 49.6% and 28.4% *vs.* 57.5%). Not only that the intensity of the tropylium ion (at *m/z* 91) is considerably higher in the mass

spectra of phenethyl-angelate and -tiglate (38.4 and 50.1%) than in the mass spectrum of the corresponding senecioate (14.3), but also it represents the most meaningful difference between the angelate and tiglate.

Besides the MS spectrum, other spectral data for phenethyl angelate are not available in the literature. Tokuyasu and coworkers (2005) reported both ^{1}H- and ^{13}C-NMR data ($CDCl_3$, at 270 and 67.5 MHz, respectively) for phenethyl senecioate, while the corresponding spectra of phenethyl tiglate (recorded also in $CDCl_3$, at 300 and 70 MHz, respectively) are available in the Advanced Chemistry Development database. However, these NMR data are either unassigned or partially assigned. Therefore, we have made an effort to fully assign ^{1}H- and ^{13}C-NMR spectral data for all three isomeric phenethyl esters based on a combination of 1D-, 2D-NMR (^{1}H–^{1}H COSY, NOESY, HSQC, and HMBC) and simulation experiments.

^{1}H- and ^{13}C-NMR data for the studied phenethyl esters are summarized in Tables 2 and 3, respectively. Chemical shifts, multiplicities, and the observed coupling constants were in accordance with the structure of the investigated esters. The angelate and tiglate moieties were readily distinguished by their diagnostic ^{1}H NMR signals, especially by the chemical shifts of H-3, which in the angelate was a quartet of quartets at δ 6.04 (J = 7.2, 1.6 Hz), while in the tiglate the corresponding signal was downfielded to δ 6.83 (J = 7.1, 1.4 Hz) due to the deshielding effect of the ester carbonyl group (C-1; Jackman and Wiley, 1960). The different substitution pattern on the double bond of the senecioate was recognizable by the H-2 quartet of quartets at δ 6.04 (J = 1.3, 1.3 Hz), coupled with two CH_3- groups doublets at δ 1.88 and 2.14 (characteristic 4-bond allylic coupling). In addition, two well-separated methylene triplets for the -CH_2CH_2- group (typical A_2X_2 system) and one complex (second-order) AA'BB'C pattern for the phenyl group were present in ^{1}H NMR spectra of all three esters.

The number of carbon signals corresponded to the expected number of carbon atoms of the studied compounds, while the DEPT90/135 spectra confirmed the existence of four CH groups, two CH_2 and two CH_3 among the protonated carbons in each ester. The ^{13}C–^{1}H coupling constants, previously unavailable in the literature, were determined from the proton-coupled ^{13}C NMR spectra. Many resolved long-range coupling constants (2- and 3-bond couplings) along with one-bond coupling of directly attached carbon and hydrogen atoms were visible in these spectra (Table 3). The observed multiplicities of the signals of the carbon atoms from the phenyl group were crucial for their unequivocal assignment. In general, the signals derived from *ortho* and *meta* carbons of monosubstituted benzenes usually have very similar intensities, and thus it could be difficult to unequivocally assign them. For example, see the carbon signals at δ 128.6 and 129.1 in our ^{13}C NMR spectrum of phenethyl angelate (Fig. 2). The only substantial long-range C–H coupling within a monosubstituted benzene ring is the *meta* 3-bond coupling, and thus, the *ortho*, as well as the *para*, carbon that have two *meta* protons usually appear as a *dt*, whereas the *meta* carbon, with only one *meta* proton to it, should look like a *dd* (Breitmaier and Bauer, 1984; Fig. 2). Indeed, in the proton-coupled ^{13}C NMR spectrum of phenethyl angelate (as well as, in the spectra of the other two esters), one *dd* at δ 128.6 (^{1}J = 159.5 Hz and ^{3}J = 7.5 Hz) and one *dt* at 126.6 (^{1}J = 160.1 Hz and ^{3}J = 7.2 Hz) were observed that were assigned to *meta* and *para* carbons, respectively. These *dd* and *dt* patterns could be sometimes more complex and they are not always strictly first order. This is the case with the *ortho* carbons in our phenethyl esters that appeared as a doublet of pseudo pentuplets (*d pseudo-p*) due to their additional 3-bond coupling with two benzylic protons (Fig. 2) that was of similar strength as the mentioned *meta* 3-bond coupling (^{3}J(C,H) = 6.1 Hz).

Table 2 ^{1}H NMR (400 MHz) spectral data ($CDCl_3$) for phenethyl-angelate, - senecioate, and - tiglate

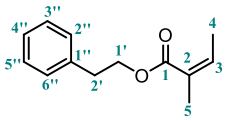
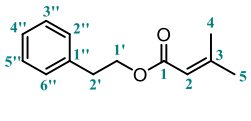
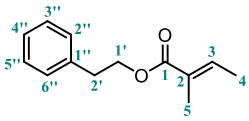
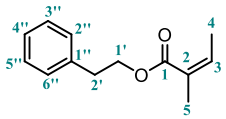
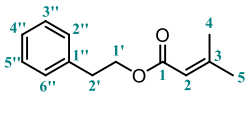
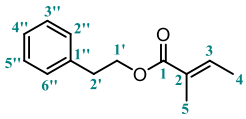
Position			
2	-	6.04 (*qq*, 1H) $J_{2,4}$ = 1.3 Hz $J_{2,5}$ = 1.3 Hz	-
3	6.04 (*qq*, 1H) $J_{3,5}$ = 7.2 Hz $J_{3,4}$ = 1.6 Hz	-	6.83 (*qq*, 1H) $J_{3,5}$ = 7.1 Hz $J_{3,4}$ = 1.4 Hz
4	1.92 (*dq*, 3H) $J_{3,5}$ = 7.2 Hz $J_{4,5}$ = 1.6 Hz	2.14 (*d*, 3H) $J_{2,4}$ = 1.3 Hz	1.77 (*dq*, 3H) $J_{3,5}$ = 7.1 Hz $J_{4,5}$ = 1.3 Hz
5	1.86 (*dq*, 3H) $J_{3,4}$ = 1.6 Hz $J_{4,5}$ = 1.6 Hz	1.88 (*d*, 3H) $J_{2,4}$ = 1.3 Hz	1.81 (*dq*, 3H) $J_{3,4}$ = 1.4 Hz $J_{4,5}$ = 1.3 Hz
1'	4.36 (*t*, 2H) $J_{1',2'}$ = 7.0 Hz	4.30 (*t*, 2H) $J_{1',2'}$ = 7.1 Hz	4.34 (*t*, 2H) $J_{1',2'}$ = 7.0 Hz
2'	2.99 (*t*, 2H) $J_{1',2'}$ = 7.0 Hz	2.95 (*t*, 2H) $J_{1',2'}$ = 7.1 Hz	2.99 (*t*, 2H) $J_{1',2'}$ = 7.0 Hz
2'' and 6''	7.2392 (AA'BB'C, 2H) $J_{2'',3''}$ = 7.8 Hz $J_{2'',6''}$ = 2.2 Hz $J_{2'',4''}$ = 1.2 Hz $J_{2'',5''}$ = 0.5 Hz	7.2327 (AA'BB'C, 2H) $J_{2'',3''}$ = 7.7 Hz $J_{2'',6''}$ = 1.8 Hz $J_{2'',4''}$ = 1.3 Hz $J_{2'',5''}$ = 0.4 Hz	7.2364 (AA'BB'C, 2H) $J_{2'',3''}$ = 7.8 Hz $J_{2'',6''}$ = 1.8 Hz $J_{2'',4''}$ = 1.2 Hz $J_{2'',5''}$ = 0.4 Hz
3'' and 5''	7.2977 (AA'BB'C, 2H) $J_{2'',3''}$ = 7.8 Hz $J_{3'',4''}$ = 7.4 Hz $J_{3'',5''}$ = 1.7 Hz $J_{3'',6''}$ = 0.5 Hz	7.2979 (AA'BB'C, 2H) $J_{2'',3''}$ = 7.7 Hz $J_{3'',4''}$ = 7.45 Hz $J_{3'',5''}$ = 1.8 Hz $J_{3'',6''}$ = 0.4 Hz	7.3014 (AA'BB'C, 2H) $J_{2'',3''}$ = 7.8 Hz $J_{3'',4''}$ = 7.4 Hz $J_{3'',5''}$ = 1.8 Hz $J_{3'',6''}$ = 0.4 Hz
4''	7.2235 (AA'BB'C, 1H) $J_{3'',4''}$ = 7.4 Hz $J_{4'',5''}$ = 7.4 Hz $J_{2'',4''}$ = 1.2 Hz $J_{4'',6''}$ = 1.2 Hz	7.2232 (AA'BB'C, 1H) $J_{3'',4''}$ = 7.45 Hz $J_{4'',5''}$ = 7.45 Hz $J_{2'',4''}$ = 1.3 Hz $J_{4'',6''}$ = 1.3 Hz	7.2273 (AA'BB'C, 1H) $J_{3'',4''}$ = 7.4 Hz $J_{4'',5''}$ = 7.4 Hz $J_{2'',4''}$ = 1.2 Hz $J_{4'',6''}$ = 1.2 Hz

Table 3 ^{13}C NMR (100 MHz) spectral data ($CDCl_3$) for phenethyl-angelate, -senecioate, and -tiglate

Position			
1	168.1 (*m*, C)	166.7 (*m*, C)	168.1 (*m*, C)
2	128.0 (*br pseudo-sept*, C) ^2J(C,H) = 6.1 Hz	116.1 (*d pseudo-sept*, CH) ^1J(C,H) = 159.3 Hz ^3J(C,H) = 5.0 Hz	128.7 (*pseudo-septd*, C) ^2J(C,H) = 6.1 Hz ^3J(C,H) = 6.1 Hz ^3J(C,H) = 2.1 Hz
3	138.1 (*dqq*, CH) ^1J(C,H) = 150.8 Hz ^2J(C,H) = 7.2 Hz ^3J(C,H) = 2.7 Hz	156.9 (*pseudo-septd*, C) ^2J(C,H) = 6.2 Hz ^2J(C,H) = 1.4 Hz	137.4 (*d pseudo-sept*, CH) ^1J(C,H) = 157 Hz ^2J(C,H) = 6.3 Hz ^3J(C,H) = 6.3 Hz
4	15.8 (*qd*, CH_3) ^1J(C,H) = 127.8 Hz ^3J(C,H) = 2.7 Hz	20.3 (*qdq*, CH_3) ^1J(C,H) = 127.7 Hz ^3J(C,H) = 8.0, 4.2 Hz	14.4 (*qd*, CH_3) ^1J(C,H) = 126.9 Hz ^2J(C,H) = 3.7 Hz
5	20.7 (*qd*, CH_3) ^1J(C,H) = 128.0 Hz ^2J(C,H) = 7.2 Hz	27.5 (*qdq*, CH_3) ^1J(C,H) = 126.9 Hz ^3J(C,H) = 8.8, 4.4 Hz	12.0 (*qd*, CH_3) ^1J(C,H) = 127.7 Hz ^2J(C,H) = 7.8 Hz
1'	64.8 (*tt*, CH_2) ^1J(C,H) = 148.2 Hz ^2J(C,H) = 5.3 Hz	64.2 (*tt*, CH_2) ^1J(C,H) = 148.1 Hz ^2J(C,H) = 5.7 Hz	65.0 (*tt*, CH_2) ^1J(C,H) = 148.4 Hz ^2J(C,H) = 5.5 Hz
2'	35.3 (*br t*, CH_2) ^1J(C,H) = 127.9 Hz	35.4 (*br t*, CH_2) ^1J(C,H) = 127.9 Hz	35.2 (*br t*, CH_2) ^1J(C,H) = 128.1 Hz
1''	138.2 (*m*, C)	138.2 (*m*, C)	138.2 (*m*, C)
2'' and 6''	129.1 (*d pseudo-p*, CH) ^1J(C,H) = 157.0 Hz, ^3J(C,H) = 6.1 Hz)	129.0 (*d pseudo-p*, CH) ^1J(C,H) = 157.7 Hz ^3J(C,H) = 6.6 Hz	129.0 (*d pseudo-p*, CH) ^1J(C,H) = 157.0 Hz ^3J(C,H) = 5.7 Hz
3'' and 5''	128.6 (*dd*, CH) ^1J(C,H) = 159.5 Hz ^3J(C,H) = 7.5 Hz	128.6 (*dd*, CH) ^1J(C,H) = 159.6 Hz ^3J(C,H) = 7.6 Hz	128.6 (*dd*, CH) ^1J(C,H) = 159.5 Hz ^3J(C,H) = 7.6 Hz
4''	126.6 (*dt*, CH) ^1J(C,H) = 160.1 Hz ^3J(C,H) = 7.2 Hz	126.6 (*dt*, CH) ^1J(C,H) = 160.2 Hz ^3J(C,H) = 7.3 Hz	126.5 (*dt*, CH) ^1J(C,H) = 160.4 Hz ^3J(C,H) = 7.3 Hz

This assignment of phenyl-group carbons enabled us to estimate the chemical shifts of the corresponding protons from cross-peaks in the HSQC spectra (Fig. 3). However, the AA'BB'C phenyl pattern was too complex for manual (first-order) analysis. Therefore, the chemical shifts and coupling constants of this pattern were solved by the use of Global Spectral Deconvolution and Spin Simulation algorithms incorporated in MestReNova software package (ver. 6.0.2–5475, Mestrelab Research, Santiago de Compostela, Spain; Table 3). The initial inputs for these calculations consisted of the estimated chemical shifts of the phenyl-group protons (from the HSQC spectra) and the values of *ortho*, *meta*, and *para* coupling constants available in the literature (Radulović et al., 2017). These starting data were then finely tuned to achieve a as-high-as-possible similarity of the experimental *vs.* simulated NMR spectrum (Fig. 4). The *ortho*, as well as the *meta*, protons represent chemically equivalent but magnetically inequivalent pairs of protons (differing from each

other in the coupling constants values with the same neighboring protons, Table 2). The calculated values of the chemical shifts and coupling constants of the AA'BB'C spin system of the phenyl group were quite similar for all three studied phenethyl esters. It was determined that the vicinal $J_{2'',3''}$ had a slightly higher value than the vicinal $J_{3'',4''}$ (ΔJ = 0.25 – 0.4 Hz) and this is in agreement with the previously observed trend that the replacement of a hydrogen by some substituent on the benzene ring usually causes an increase in $J_{2,3}$ and a decrease in $J_{3,4}$ (Fraser, 1966).

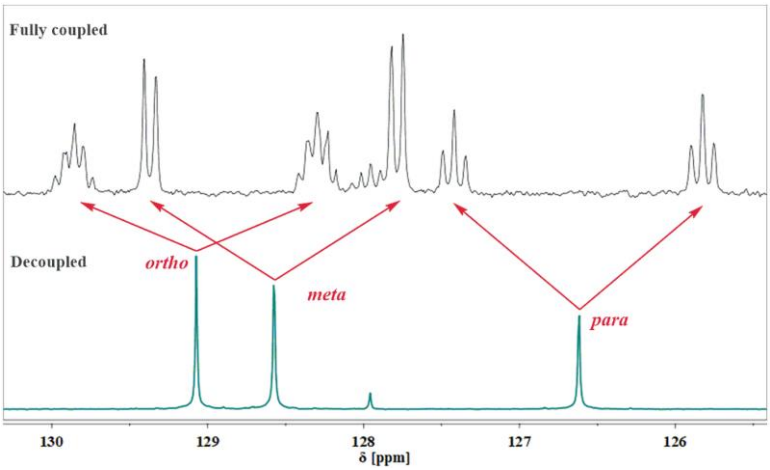


Fig. 2 Fully ^{1}H coupled and decoupled ^{13}C NMR spectrum of phenethyl angelate (expansion of the region between 125.4 and 130.3 ppm) with designated signals of *ortho*, *meta*, and *para* carbons from the monosubstituted benzene ring

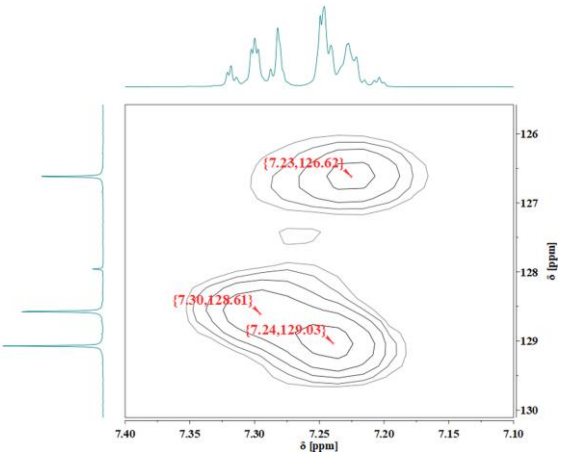


Fig. 3 Expansion (^{1}H: 7.1-7.4; ^{13}C: 125.5-130 ppm) of the HSQC spectrum of phenethyl angelate

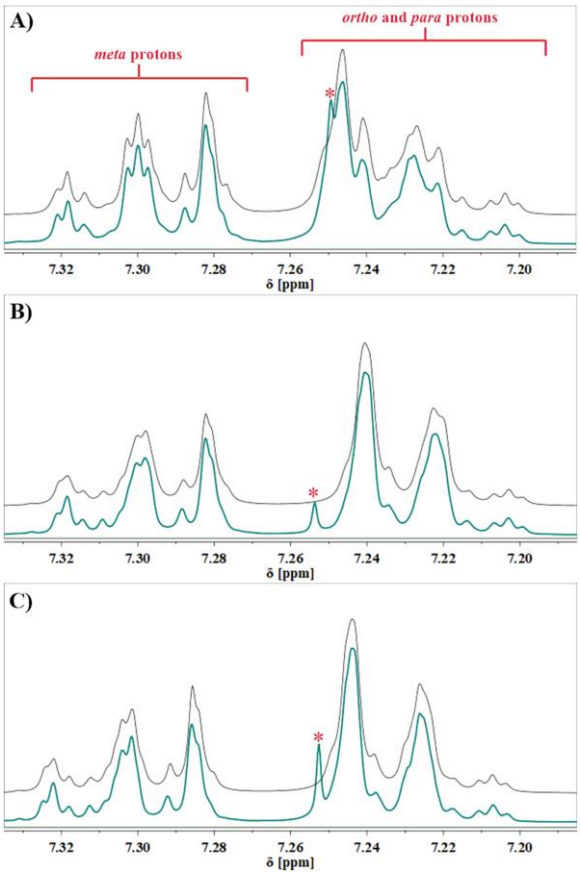


Fig. 4 A comparison of the experimental (green) and simulated (gray) ^{1}H NMR spectra (the higher order phenyl group spin system) of phenethyl-angelate (**A**), -senecioate (**B**), and -tiglate (**C**). The residual solvent signal of $CHCl_3$ is designated with the symbol *

Some previous studies indicated that the distinction between some angeloyl and tigloyl esters could be achieved by their widely different characteristic frequencies in the infrared - specifically, by the use of a hyphenated technique such as GC-FTIR. Precisely, it was found that angelates were characterized by two absorption bands near 1232 cm^{-1} and 1156 cm^{-1} with lower intensity for the former one, while the corresponding tiglate absorptions consisted of the specific band at 1258 cm^{-1} and another at 1140 cm^{-1} with an inverted intensity ratio. According to the authors, this IR-based method enabled differentiation of angelates/tiglates in a less ambiguous way than the one based on the relative ion abundances in EI-MS spectra (Bicchi et al., 1987; Christen et al., 2009). Indeed, these diagnostic bands were present in the IR spectra of both phenethyl-angelate and -tiglate. However, there is a deviation from the previous findings in the IR spectrum of phenethyl angelate as the band at 1230 cm^{-1} was slightly more intense than the band at 1147 cm^{-1}. The corresponding bands in the IR spectrum of phenethyl senecioate were located at 1225 cm^{-1} and 1141 cm^{-1}. All other intense absorption bands (e.g. those associated with the stretching of

C=O or wagging of =C–H bonds) were found at similar wavenumber values in all three studied phenethyl esters.

CONCLUSION

The unknown minor constituent of immortelle essential oil was tentatively identified, according to the MS fragmentation pattern, as the ester of 2-phenyl-1-ethanol with one of the isomeric pentenoic acids - angelic, senecioic, or tiglic acid. The discrepancy in the RI values for phenethyl-angelate and -tiglate in the literature, as well as the lack of the RI value for the senecioate, prompted us to prepare and fully spectrally characterize (RI, MS, IR, and NMR) synthetic samples of all three mentioned esters. After GC-coinjection experiments of the obtained esters and immortelle oil, the compound in question was confirmed as phenethyl angelate.

Although phenethyl angelate has been previously reported twice as the volatile constituent of samples of natural origin, our findings strongly imply that this molecule was misidentified with the corresponding senecioate/tiglate. Therefore, it is necessary to update the existing library of RI data for angelates/tiglates with RI data for senecioates (that usually elute from nonpolar GC columns closely to the corresponding tiglates) to prevent this kind of misidentifications in the future.

The comparison of spectral data of these three related esters revealed that there were no significant dissimilarities in their EI-MS and IR spectra. Expectedly, the different substitution pattern on the double bond in the acid fragment of the ester was readily distinguishable in both proton and carbon NMR spectra. However, NMR-based methods are not always optimal for the identification of minor components in highly complex mixtures such as essential oils. Overall, the most substantial difference between these esters was in their GC retention properties, and therefore a GC-MS method could be considered as the most versatile for their mutual distinction.

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FENETIL-ANGELAT –NOVI ESTAR IZ ETASKOG ULJA BILJNE VRSTE *HELICHRYSUM ITALICUM*?

Estri angelika, senecio i tiglinske kiseline sa različitim zasićenim/nezasićenim/aromatičnim alkoholima doprinose aromi mnogih etarskih ulja. Međutim, nekada je nemoguće razlikovati ove regio-/geometrijske izomere pomoću masene spektrometrije sa elektronskom jonizacijom i to je upravo bio slučaj sa malozastupljenim sastojkom etarskog ulja biljne vrste Helichrysum italicum *za koji je pretpostavljeno da predstavlja estar 2-fenil-1-etanola sa jednom od pomenutih kiselina. Naš pokušaj da identifikujemo ovaj fenetil-estar bio je sputan i nedoslednošću ili nepostojanjem odgovarajući RI vrednosti u literaturi. Stoga smo napravili i potpuno spekralno okarakterisali (1D- i 2D-NMR, IR, MS) sintetske standarde sva tri izomerna estra. GC analiza uzoraka etarskog ulja* H. italicum *obogaćenih dobijenim standardima estara, nesumljivo je potvrdila da je nepoznati estar fenetil-angelat. Ovaj retki isparljivi sekundarni metabolit je prethodno bio pronađen samo dva puta u uzorcima biljnog porekla. Međutim, rezultati našeg istraživanja ukazuju na to da je ovaj molekul najverovatnije bio pogrešno identifikovan u ranijim istraživanjima, odnosno pomešan sa odgovarajućim senecioatom/tiglatom. Imajući ovo u vidu, poželjno je da se postojeće biblioteke RI/MS podataka za tiglate i angelate dopune podacima za senecioate da bi se ovakve greške izbegle u budućnosti.*

Ključne reči: Helichrysum italicum, *etarsko ulje, fenetil-angelat, fenetil-tiglat, fenetil-senecioat, spektralna karakterizacija*