Variation in Essential Oil Content and Composition of *Ridolfia segetum* Moris based on 30-hour Prolonged Fractionated Extraction Procedure

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SUPPLEMENTARY MATERIAL
Section S1: Taxonomic characterization and uses of RS

RS, false fennel or corn parsley (synonyms: Anethum s. L., Foeniculum s. Presl., Meum s. (L.) Guss.) is a glabrous Apiaceae annual weed growing in the Mediterranean region with height up to 1 m (Figure S1-S4). Its stem is erect, striate and banched, and the glabrous leaves are finely divided several times with long, filiform, divaricate lobes. The upper leaves are often reduced to the inflated petiole. Umbels are with ten to sixty slender and almost uniform rays. Bracts and bracteoles are absent, as well as sepals. Petals are yellow, ovate, with inflexed and truncate apex. Laterally compressed ovoid-cylindric fruits are 1.5 x 2.5 mm, with scarcely prominent slender ridges and solitary vittae (Pignatti 1982; Tutin et al. 1968). The species is distributed throughout the Mediterranean region, extending to Portugal, inhabiting cultivated land and waste places. Thriving in heavy soils, it represents one of the most common weeds (known as the Bishop’s weed). RS blossoms in the late spring, from April to June. It has strong odor, sometimes described as unpleasant (Pignatti 1982).

Corn parsley is used in folk medicine to prevent constipation, coughing, gas and respiratory tract infections (Cabral et al. 2015), as well as getting rid of lice and increasing production of breast milk in nursing mothers or to regulate women’s menstrual periods (Thormar 2010). Various plant parts contain EO with a very pungent fragrance. The oil has been assayed for inhibition of two enzyme-associated activities of the HIV-1 reverse transcriptase (Bicchi et al. 2009). RS also demonstrates high antioxidant capacity and significantly inhibits NO production without cytotoxicity, highlighting the great value of this plant in the development of new phytopharmaceuticals (Cabral et al. 2015). Its leaves can be used as a spice, and the herb is also used in pickle industry.
Section S2: Experimental part

Plant material

Aerial parts (herba) of RS were collected in a wild area about 15 km from Tarquinia city (Viterbo Province, Italy), in the archaeological zone near the Etruscan temple *Ara della Regina* (42°15’31.8”N, 11°48’08.7”E). RS is a summer annual completing its life cycle within June/July. Thus, all the material was collected in the end of July 2016, with flowering and fruiting stages mixed together. Taxonomic identification of the species was conducted according to the official European flora (Tutin et al. 1968) and the national Italian one (Pignatti 1982). Voucher specimen (number SDCT-RS/01) has been deposited in the Department of Drug Chemistry and Technology at Sapienza University of Rome. Air-drying of collected RS was performed in a shady place for approximately 20 days.

EO Extraction

The extraction method used is similar to that previously reported and described in detail (Božović et al. 2017a, 2017b, 2018; Garzoli et al. 2018a, 2018b). Accordingly, EOs have been isolated by direct steam distillation using a 62 L steel distillator apparatus (Albrigi Luigi E0131, Verona, Italy). About 1.5 kg of dried RS material was subjected to fractionated steam distillation collecting the EOs at interval times of 1, 2, 3, 6, 12 and 24 hours. However, since there was still a considerable amount of EO after 24 hours, the process was prolonged for additional 6 hours giving a total of seven RSEO fractions. To mimic parallel extraction process with no fractioning, the fractions were mixed as already reported (Božović et al. 2017b, 2018; Garzoli et al. 2018a).
**EO Analysis**

The gas chromatographic-mass spectrometric (GC-MS) analysis was carried out with a GC-MS and flame ionization detector (FID) using a turbomass Clarus 500 GC-MS/GC-FID from Perkin Elmer instruments (Waltham, MA, USA) as reported previously (Božović et al. 2017a, 2017b, 2018; Garzoli et al. 2018a, 2018b). A Stabilwax fused-silica capillary column (Restek, Bellefonte, PA, USA) (60 m × 0.25 mm, 0.25 mm film thickness) was used with helium as carrier gas (1.0 mL/min). GC oven temperature was kept at 60°C for 5 min and programmed to 220°C at a rate of 5°C/min, and kept constant at 220°C for 30 min. Solvent delay 0–2 min and scan time 0.2 s. MS was taken at 70 eV. Mass range was from 30 to 350 m/z. 1 μL of the extract was diluted in 1 mL of methanol and 1 μL of the solution was injected into the GC injector at the temperature of 280°C.

The main components of the EO were identified at first by comparison of their MS spectra with those present in the NIST/NBS and Wiley libraries. A second confirmation was achieved by calculating the GC linear retention indeces (LRI) in accord with the definition of Van den Dool and Kratz from temperature-programming measurements. LRIs of each compound were calculated using a mixture of aliphatic hydrocarbons (C8-C30, Ultrasci). Relative percentages of the components separated were calculated by electronic integration peak areas utilizing the same instrumentation with a FID detector configuration.

**Antifungal Activity**

As previously reported, the MIC was determined by micro-broth dilution method (microsterile plate) according to the Clinical and Laboratory Standards Institute/National Committee for Clinical Laboratory Standards (Božović et al. 2017a,
Miconazole (0.5 mg/mL), a well-known antifungal synthetic drug, was prepared by dissolving the agent in endotoxin free water. EO solutions (100 mg/mL) were prepared in RPMI 1640.

Shortly, to determine the MIC of RSEO fractions extracted at different times, or miconazole, RPMI-1640 supplemented with MOPS at pH 7 was used. EO was diluted in RPMI-1640 supplemented with Tween 80 (final concentration of 0.001% v/v). Dilutions, 11 increasing concentrations, ranging from 0.012 to 12.48 mg/mL of the EO, were prepared in ninety six well plates. The inoculum size was about $2.5 \times 10^3$ cells/mL. The plates were incubated at 30°C for 24–48 hours.

**Section S3: Extended discussion and comparison with literature data**

**Chemical composition**

The extraction method applied gave fractions that differ greatly in their chemical compositions. There is a substantial difference between the first and the second half of the extraction. Monoterpene OCI has been found to prevail in the EOs extracted up to 6 hours, while the phenylpropanoid DAP was a characterizing compound in the later fractions.

OCI is one of the three naturally occurring isomers from the group of cymenes: its benzene ring is $o$-substituted with a methyl and an isopropyl groups. Generally, $p$-isomer is the most common one, and it is a constituent of a number of EOs. Contrary to this, OCI is not so common and is usually not present in high amount. In the oil of RS collected in Tarquinia, the first two fractions (1h and 2h) were particularly rich in OCI content displaying 40.1% and 25.7%, respectively. This component was present in all the fractions with the lowest % in the later ones (12h, 24h and 30h). OCI has been reported for several species such as *Foeniculum vulgare* Mill. (Bowes and Zheljazkov 2017b, 2018; Garzoli et al. 2018a, 2018b).
2005), *Oenanthe pimpinelloides* L. (Evergetis et al. 2009), *Rosmarinus officinalis* L. (Socaci et al. 2010), *Achillea filipendulina* Lam. (Mosayebi et al. 2008) and *Salvia africana-lutea* L. (Kamatou 2006). Its high percentage has been reported for *Lippia origanoides* H.B.K. (up to 31%) (Andrade et al. 2014), *Trachyspermum ammi* Sprague (up to 37.5%) (Mahboubi and Kazempour 2011) and *Satureja rechingeri* Jamzad (59%) (Attaran et al. 2015). However, the literature survey has shown that RS usually contains *p*-isomer (Jannet and Mighri 2007; Pala-Paul et al. 2002) making the record presented herein particularly valuable.

DAP is a phenylpropene mostly found in dill, *Anethum graveolens* L (Saleh et al. 1985; Zhang et al. 2006). Some other Apiaceae species have been also found to contain considerable amounts of this compound: *Pimpinella acuminata* (Edgew.) CB Clarke. up to 20.4% (Babri et al. 2012), *Carum graecum* Boiss. et Heldr. up to 29% (Chowdhury et al. 2009), *Hydrocotyle sibthorpioides* Lam. up to 21.7% (Kapetanos et al. 2008) and *Crithmum maritimum* L. (Pavela et al. 2017). DAP has been reported for some *Piper* species as well (Vernin et al. 1999). The RSEO from Tarquinia is particularly rich in DAP. Its percentage achieved the maximum (56.1%-69.9%) after 6 hours, making the chemical profiles of the later fractions completely different. Comparing to OCI, the opposite evolution (with the extraction development) of these two compounds can be observed.

Although the majority of the literature data reports more or less the same chemical constituents, their relative yield percentage varies widely. Furthermore, comparison is even more difficult since some of the analyses included only one part of the plant (fruit, leaf or root), and it is well known that EOs of Apiaceae species differ greatly in that respect. However, RS could be marked as a plant rich in monoterpenoids, with *α*-phellandrene (up to 65%), *cis*-β-ocimene (up to 38.5%), terpinolene (up to
21.4%), \( \beta \)-pinene (up to 12.1%), \( p \)-cymene-8-ol (up to 15.1%) as the most often reported ones, depending on the part of the plant from which the EO was isolated (Bicchi et al. 2009; Jabrane et al. 2010; Jannet and Mighri 2007; Marongiu et al. 2007; Pala-Paul et al. 2002; Cabral et al. 2015). Additionally, previous reports revealed the presence of phenylpropene DAP, as well as myristicin, the compound closely related to DAP, which is also reported for some other important Apiaceae species (Afshar et al. 2017; Benelli et al. 2017). Some authors (Jannet and Mighri 2007) have marked them as the main constituents of the EO from flowers, whose amounts varied depending on the duration of the distillation time (up to 85.4% and 31.5%, respectively). The study presented herein included the whole plant material collected in July during the reproductive period, thus covering both flowering and fruiting stages. The prevalence of OCI has not been previously reported for RS, while DAP is usually not the main compound. Not only because of that, but also due to the comparatively lower presence of the compounds previously reported for this species (such as \( \alpha \)-phellandrene, \( cis-\beta \)-ocimene and terpinolene), the RSEO from Tarquinia seems to be considered as a quite unique chemotype.

**Anti-
*Candida* Activity**

According to the results of the chemical analysis, RSEO samples are rich in OCI and DAP. Although varying in the content of these main compounds, which makes the fractions quite different between each other, the antifungal assay results presented herein showed the lack of significant activity for this plant species. 7 samples revealed a weak anti-*Candida* activity at 24 hours, whereas none of the samples was active after 48 hours.

From a literature survey, little data was found concerning the antimicrobial
analysis of RS. The phenylpropanoid-rich root EO of this plant was analysed, and the
study showed inhibition of a number of common human pathogenic bacteria, as well as
of some clinical and environmental isolated strains with (Jabrane et al. 2010). Another
study included a selection of Gram(-) and Gram(+) bacteria (Jannet and Mighri 2007).
Different EO samples from flowers rich in DAP and myristicin were screened, and it
was found that the antibacterial efficacy was directly related to the proportions of these
two phenylpropanes. Some other DAP-rich EOs showed a plenty of biological
activities, including the fungicidal one (Johann et al. 2010; Saleh et al. 1985; Zhou et al.
2007). In addition, DAP and its analogues have been extensively investigated showing
moderate anti-phlogistic (Tomić et al. 2009) and good antileishmanial properties
(Camarda et al. 2008). The results herein represent the first report of antifungal
evaluation for this Apiaceae species.

Section S4: Additional references

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Table S1. Yield % of RSEOs.

|       | 1h | 2h | 3h | 6h | 12h | 24h | 30h |
|-------|----|----|----|----|-----|-----|-----|
| cy y  | 0.20 | 0.30 | 0.44 | 0.64 | 0.74 | 0.80 | 0.82 |
| y     | 0.20 | 0.10 | 0.14 | 0.20 | 0.10 | 0.06 | 0.02 |

Note: a extraction hours; cy: cumulative yield % of RSEOs over time; y: yield % calculated on the dried RS plant material.
Table S2. Chemical composition (%) of RSEOs.

| #  | Name                          | Rt¹ | Rtit⁴ | Sample² | 1h  | 2h  | 3h  | 6h  | 12h | 24h | 30h |
|----|-------------------------------|-----|-------|---------|-----|-----|-----|-----|-----|-----|-----|
| 1  | α-pinene                      | 1019| 1022  | 3.9    | 1.2 | 3.6 | 2.6 | 1.2 | -   | -   | -   |
| 2  | β-pinene                      | 1116| 1114  | 4.6    | 2.4 | 3.4 | 1.7 | 1.5 | 0.2 | 0.1 | -   |
| 3  | β-myrcene                     | 1157| 1156  | 0.9    | 0.3 | 1.2 | 1.1 | 0.4 | -   | -   | -   |
| 4  | α-phellandrene                | 1170| 1169  | -     | -   | -   | -   | 16.7| 5.4 | 0.1 | 0.6 |
| 5  | limonene                      | 1206| 1205  | 7.4    | 4.9 | 6.7 | 3.5 | 1.2 | 0.2 | 0.3 | -   |
| 6  | β-terpinene                   | 1219| 1206  | 3.0    | 1.0 | 4.9 | 4.9 | 1.8 | 0.2 | 0.3 | -   |
| 7  | cis-β-ocimene                 | 1250| 1252  | 0.5    | -   | 1.3 | 1.6 | 0.8 | 0.1 | 0.2 | -   |
| 8  | o-cymene                      | 1281| 1287  | 40.1   | 25.7| 3.8 | 13.0| 7.4 | 4.1 | 4.2 | -   |
| 9  | terpinolene                   | 1292| 1290  | -     | -   | 2.1 | 5.6 | 1.6 | -   | -   | -   |
| 10 | borneol                       | 1710| 1704  | 2.0    | 3.3 | 1.8 | 3.0 | 4.2 | 3.0 | -   | -   |
| 11 | pulegone                      | 1677| 1665  | -     | -   | 0.6 | 0.8 | 1.5 | 2.6 | -   | -   |
| 12 | neral                         | 1697| 1697  | 0.8    | 1.0 | 0.4 | -   | -   | -   | -   | -   |
| 13 | cryptone                      | 1711| 1713  | 6.2    | 2.5 | 0.9 | -   | -   | -   | -   | -   |
| 14 | p-menth-1(7)-en-2-one         | 1713|       | *     | -   | 2.2 | 0.9 | 2.1 | 5.4 | 9.7 | -   |
| 15 | cis-sabinol                   | 1822| 1820° | -     | 2.2 | 5.8 | 3.9 | 4.3 | 1.5 | 12.9| -   |
| 16 | p-cymen-8-ol                  | 1858| 1851  | 9.2    | 10.1| 13.4| 4.8 | 3.0 | 3.7 | 6.4 | -   |
| 17 | piperitenone oxide            | 1990| 1984° | 6.5    | 5.2 | 3.6 | 1.4 | 1.0 | 1.5 | 1.9 | -   |
| 18 | 2,3-pinanediol                | 2207| 2197° | 9.6    | 10.7| 1.8 | 6.8 | 2.1 | -   | -   | -   |
| 19 | myristicin                    | 2296| 2289° | -     | 1.5 | -   | -   | 3.2 | 2.6 | 1.7 | -   |
| 20 | dill-apiol                    | 2388| 2380° | 6.5    | 12.8| 21.1| 23.9| 59.2| 69.9| 56.1| -   |

| Unidentified compounds | 7.8 | 13.0 | 18.3 | 3.9 | 0.0 | 4.8 | 0.0 |
|------------------------|-----|------|------|-----|-----|-----|-----|

Monoterpene hydrocarbons                  | 53.0| 30.6 | 20.3 | 47.2| 20.1| 4.7 | 5.4 |
Monoterpene alcohols                       | 18.8| 21.6 | 16.2 | 12.0| 5.1 | 3.7 | 6.4 |
Oxygenated Monoterpenes                    | -   | 2.0  | 3.3  | 2.4 | 3.8 | 5.7 | 5.6 |
Cyclic Monoterpenes                        | 7.4 | 4.9  | 6.7  | 3.5 | 1.2 | 0.2 | 0.3 |
Ketonic Monoterpenes                       | -   | 6.2  | 2.5  | 0.9 | -   | -   | -   |
Other                                   | 13.0| 21.7 | 32.7 | 30.1| 69.8| 80.9| 82.3|

¹ # indicates the compound identification number; ² samples names indicate the extraction time as reported in Table S1; ³ Retention indices measured on polar column; ⁴ Retention indices from literature; *Rlt⁴ not available for polar column. °Normal alkane RI; °Kovats RI
Table S3. Chemical composition (%) of RSEO mixtures.

| #   | Name                  | RI\(^3\) | RI\(^{lit 4}\) | M1 | M2 | M3 | M4 | M5 | M6 |
|-----|-----------------------|----------|----------------|----|----|----|----|----|----|
| 1   | α-pinene              | 1019     | 1022           | -  | 1.3| 3.0| 2.2| 2.4| 2.2|
| 2   | β-pinene              | 1116     | 1114           | 1.4| 2.0| 3.0| 2.2| 2.3| 2.2|
| 3   | β-myrcene             | 1157     | 1156           | -  | 0.4| 1.1| 0.8| 0.9| 0.8|
| 4   | α-phellandrene        | 1170     | 1169           | -  | -  | 14.1| 3.6| 3.7| 2.8|
| 5   | limonene              | 1206     | 1205           | 2.3| 4.2| 5.9| 4.1| 4.2| 4.1|
| 6   | β-terpinene           | 1219     | 1206           | 0.7| 1.3| 4.1| 3.6| 3.9| 3.7|
| 7   | cis-β-ocimene         | 1250     | 1252           | -  | -  | 1.0| 1.0| 1.0| 0.9|
| 8   | α-cymene              | 1281     | 1287           | 29.9| 36.7| 25.6| 20.7| 24.2| 22.0|
| 9   | terpinolene           | 1292     | 1290           | -  | -  | 1.8| 2.4| 2.2| 1.8|
| 10  | borneol               | 1710     | 1704           | 2.0| 1.9| 2.0| 2.2| 2.8| 3.0|
| 11  | pulegone              | 1677     | 1665           | 0.8| 1.0| 0.6| 0.6| 0.7| 0.8|
| 12  | neral                 | 1697     | 1697           | -  | -  | 0.5| 0.5| 0.5| 0.7|
| 13  | cryptone              | 1711     | 1713           | 7.4| 5.0| 1.4| 1.3| 1.0| 1.1|
| 14  | p-menth-1(7)-en-2-one | 1713     | *             | -  | -  | 0.7| 0.8| 1.2| 1.4|
| 15  | cis-sabinol           | 1822     | 1820\(^o\)    | 2.3| 2.4| 3.9| 4.4| 4.8| 4.9|
| 16  | p-cymen-8-ol          | 1858     | 1851           | 18.8| 13.1| 6.0| 5.8| 5.9| 5.8|
| 17  | piperitenone oxide    | 1990     | 1984\(^+\)    | 9.4| 6.3| 2.9| 2.9| 2.2| 2.4|
| 18  | 2,3-pinanediol        | 2207     | 2197\(^+\)    | 12.7| 10.1| 6.0| 6.4| 5.8| 5.6|
| 19  | myristicin            | 2296     | 2289\(^+\)    | 1.6| 1.4| 1.0| 1.9| 1.6| 1.7|
| 20  | dill-apiole           | 2388     | 2380\(^+\)    | 10.7| 11.4| 13.0| 31.1| 26.9| 30.8|

|                | Unidentified compounds | 0.0 | 1.5 | 2.4 | 1.5 | 1.8 | 1.3 |
|----------------|------------------------|-----|-----|-----|-----|-----|-----|
| Monoterpene hydrocarbons |                        | 34.3| 41.7| 53.7| 36.5| 40.6| 36.4|
| Monoterpenes alcohols   |                        | 31.5| 23.2| 12.5| 12.5| 12.2| 12.1|
| Oxygenated Monoterpenes |                        | 2.8 | 2.9 | 2.6 | 2.8 | 3.5 | 3.8 |
| Cyclic Monoterpenes     |                        | 2.3 | 4.2 | 5.9 | 4.1 | 4.2 | 4.1 |
| Ketonic Monoterpenes    |                        | 7.4 | 5.0 | 1.4 | 1.3 | 1.0 | 1.1 |
| Other                   |                        | 21.7| 21.5| 21.5| 41.1| 36.7| 41.2|

\(^{1}\) # indicates the compound identification number; \(^{2}\) samples names were given by merging the letter M (mixture) and serial number of the mix; \(^{3}\) Retention indices measured on polar column. \(^{4}\) Retention indices from literature; \(^{+}\) RI\(^{lit}\) not available for polar column; \(^{o}\) Normal alkane; RI\(^{°}\)Kovats RI
Table S4. Anti-*Candida albicans* activities of 13 RSEO samples.

| Sample | MIC mg/mL | 24h | 48h |
|--------|-----------|-----|-----|
| 1h     | 12.48     | na  | na  |
| 2h     | 12.48     | na  | na  |
| 3h     | 12.48     | na  | na  |
| 6h     | na        | na  | na  |
| 12h    | 12.48     | na  | na  |
| 24h    | 12.48     | na  | na  |
| 30h    | na        | na  | na  |
| M1     | na        | na  | na  |
| M2     | na        | na  | na  |
| M3     | 12.48     | na  | na  |
| M4     | 12.48     | na  | na  |
| M5     | na        | na  | na  |
| M6     | na        | na  | na  |

Note: ¹ sample names indicate the extraction time or the serial number of prepared mixtures; na - non active; ² 0.016 is the MIC value of miconazole (the standard used); The data are result of 3 independent experiments.
Figure S1. Natural habitat in Tarquinia countryside where the plant material of RS was collected (photo by Mijat Božović).

Figure S2. Close-up shot of RS flowers/inflorescence (photo by Mijat Božović).
Figure S3. Close-up shot of RS fruits (photo by Mijat Božović).

Figure S4. RS in full blossom on its natural habitat (photo by Mijat Božović).
Figure S5. Yield curve for RS.