***Ridolfia segetum* (L.) Moris (Apiaceae) from Portugal: a source of safe antioxidant and anti-inflammatory essential oil**

C. Cabral¹, J. Poças², M.J. Gonçalves¹, C. Cavaleiro¹, M.T. Cruz¹, L. Salgueiro^{1*}

¹Faculty of Pharmacy/CEF and Centro de Neurociências e Biologia Celular, Universidade de Coimbra, 3000-295 Coimbra, Portugal

²ESTM, GIRM, Polytechnic Institute of Leiria, Leiria, Portugal

*Corresponding author: Prof. Dr Lúcia Salgueiro, Laboratório de Farmacognosia, Faculdade de Farmácia da Universidade de Coimbra, Pólo das Ciências da Saúde, Azinhaga de Santa Comba, 3000-548 Coimbra, Portugal. Tel.: 00351 239488400; Fax: 00351 239488503; E-mail: ligia@ff.uc.pt

1 **Abstract**

2 *Ridolfia segetum* (L.) Moris essential oil was obtained by hydrodistillation and analysed by GC and
3 GC/MS. The antioxidant properties were evaluated through the TBARS assay and the *in vitro* anti-
4 inflammatory activity was assessed through inhibition of NO production triggered by the *Toll*-like
5 receptor 4 (TLR4) agonist, lipopolysaccharide (LPS), in mouse macrophages. Assessment of cell
6 viability was made through a colorimetric assay using MTT on macrophages, keratinocytes and
7 hepatocytes.

8 The main compounds are α -phellandrene (53.0-63.3%), terpinolene (11.9-8.6%), β -phellandrene
9 (5.5-6.0%), and dillapiol (1.9-8.0%). The oil demonstrated high antioxidant capacity in the
10 concentrations tested (0.05-0.20 μ L/mL) and significantly inhibited NO production without
11 cytotoxicity in concentrations up to 1.25 μ L/mL.

12 The plants showed a high essential oil yield, which confers an industrial potential interest. Also, the
13 high content of α -phellandrene (53.0-63.3%) makes it an important natural source of this
14 compound.

15 These results provide evidence of the therapeutic effects of this oil, and suggest that the anti-
16 inflammatory activity in doses without cytotoxicity on mammalian cells, combined with its
17 antioxidant potential and pleasant smell, could be of great value in the development of new
18 phytopharmaceuticals.

19

20 **Keywords.** *Ridolfia segetum*, essential oil, α -phellandrene, antioxidant activity, anti-inflammatory
21 activity, cytotoxicity

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2 **1. Introduction**

3 Aromatic plants are used for their large therapeutic potential and benefits as natural preservatives
4 (Bakkali *et al.*, 2008) and since antiquity, essential oils have been traditionally used for their
5 biological properties: antioxidant, bactericidal, fungicidal, virucidal, antiparasitical, insecticidal,
6 anti-inflammatory (Timsina *et al.*, 2012), being the antioxidant and anti-inflammatory activities of
7 interest in the food, cosmetic and pharmaceutical industries (Miguel, 2010a; 2010b). The biological
8 properties of the essential oils seem to be the result of a synergism between these compounds.

9 The antioxidant activity of essential oils is of great interest because they may be useful to preserve
10 foods from the toxic effects of oxidants (Maestri *et al.*, 2006). Moreover, essential oils being able of
11 scavenging free radicals may also play an important role in some disease prevention such as brain
12 dysfunction, cancer, heart disease and immune system decline. Increasing evidence has suggested
13 that these diseases may result from cellular damage caused by free radicals (Aruoma, 1998;
14 Kamatou & Viljoen, 2010). Indeed, the formation of reactive oxygen species (ROS) induce lipid
15 peroxidation that, in some circumstances, can overcome the cellular defense system. The membrane
16 structures are destroyed and the function of the cell organelles is loosed with the lipid oxidation.
17 Induction of lipid peroxidation is linked to several diseases: rheumatoid arthritis, atherosclerosis,
18 ischemia, carcinogenesis, and aging (Jadhav *et al.*, 1995). If essential oils are able to scavenge some
19 free radicals, they can also act as anti-inflammatory agents, because one of the triggers of the
20 inflammatory responses is the oxidative burst that occurs in diverse cells (monocytes, neutrophils,
21 eosinophils, and macrophages).

22 Macrophages produce a variety of pro-inflammatory mediators, such as nitric oxide, upon activation
23 with the pro-inflammatory stimulus and TLR4 agonist LPS. Since NO is a well-established
24 inflammatory marker, the inhibition of its production is a good approach to screen anti-
25 inflammatory molecules or phytochemicals.

1 Plants of the Apiaceae family (carrot or parsley family) possess a range of compounds with many
2 biological activities, such as the ability to induce apoptosis, the antimicrobial, hepatoprotective and
3 vaso-relaxant activities, cyclooxygenase inhibitory effect and anti-tumor action (Pae *et al.*, 2002).
4 This family is very well represented in the Portuguese flora and includes well-known aromatic and
5 medicinal plants with characteristic hollow stems.

6 *Ridolfia segetum* (L.) Moris belongs to the Apiaceae and it is the only species of the monotypic
7 genus *Ridolfia* Moris. This species is commonly known as *corn parsley*, *false fennel*, or *false*
8 *caraway*, and it is distributed throughout the Mediterranean region. In Portugal, it is commonly
9 called *andrage* or *endrão*, and it occurs in roadsides and cultivated fields (Aedo, 2003). *Ridolfia*
10 *segetum* is used in traditional medicine to prevent constipation, coughing and respiratory tract
11 infections. In a recent ethnobotanical survey in Sicily, this species was indicated as being eaten raw
12 in salads, cooked and as medicinal-food in cases of gastric acidity (Lentini & Venza, 2007).

13 Although *R. segetum* is used as food and in traditional medicine, there is a lack of information
14 regarding the validation of its uses through evaluation of biological activities and proofs of its
15 safety.

16 The essential oil of this species has been the subject of previous studies of chemical
17 characterization, being possible the distinction of two types of oils: those largely dominated by
18 monoterpene hydrocarbons, usually α -phellandrene, terpinolene and *p*-cymene (Bicchi *et al.*, 2009;
19 Fleisher & Fleisher, 1996; Marongiu *et al.*, 2007; Palá-Paúl *et al.*, 2002; Palá-Paúl *et al.*, 2005); and
20 those which also contain phenylpropanoids as major compounds or in appreciable amounts, usually
21 myristicin and dillapiol (Jannet & Mighri, 2007; Jabrane *et al.*, 2010).

22 Concerning the evaluation of biological activities, Jabrane *et al.*, 2010, evaluated the antioxidant
23 and antibacterial activities. In 2007, Jannet & Mighri evaluated the antibacterial properties of the
24 essential oil of Tunisian *R. segetum*. Latter, in 2009, Bicchi *et al.* reported the HIV-1-inhibiting
25 activity of *R. segetum* from Sardinia, Italy.

1 Bearing in mind the traditional medicinal uses of *R. segetum*, the present study was designed to
2 elucidate the antioxidant activity of its essential oil, as well as, the anti-inflammatory potential and
3 the safety on three types of mammalian cells, macrophages, keratinocytes and hepatocytes.

5 **2. Materials and methods**

6 **2.1 Plant material and reagents**

7 Aerial parts of *R. segetum* were collected during flowering stage in May 2012 (RS_Fl) and fruiting
8 stage in July 2012 (RS_Fr), in Rabaçal (Central Portugal). Voucher specimens were identified by a
9 plant taxonomist and deposited, under the numbers C. Cabral 12012 and C. Cabral 24012, in the
10 Herbarium of Medicinal Plants, Faculty of Pharmacy, University of Coimbra.

11 Thiobarbituric acid (TBA), 2,2-azobis-(2-amidinopropane) dihydrochloride (ABAP), sodium
12 dodecyl sulphate (SDS), butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT)
13 were purchased to Sigma Aldrich[®]. α -phellandrene (>90%) was purchased to the British Drug
14 Houses, Lda.

16 **2.2 Essential oil isolation**

17 Essential oil was isolated by hydrodistillation for 3 h using a Clevenger-type apparatus, according
18 to the procedure described in the European Pharmacopoeia (Council of Europe, 1997).

20 **2.3 Gas chromatography (GC)**

21 Analytical GC was carried out in a Hewlett-Packard 6890 (Agilent Technologies, Palo Alto, CA,
22 USA) chromatograph with a HP GC ChemStation Rev. A.05.04 data handling system, equipped
23 with a single injector and two flame ionization detectors (FID). A graphpak divider (Agilent
24 Technologies, part no. 5021-7148) was used for simultaneous sampling to two Supelco (Supelco,
25 Bellefonte, PA, USA) fused silica capillary columns with different stationary phases: SPB-1

(polydimethylsiloxane 30 m × 0.20 mm, film thickness 0.20 μm), and SupelcoWax-10 (polyethyleneglycol 30 m × 0.20 mm, film thickness 0.20 μm). Oven temperature program: 70–220°C (3°C/min), 220 °C (15 min); injector temperature: 250°C; carrier gas: helium, adjusted to a linear velocity of 30 cm s⁻¹; splitting ratio 1:40; detectors temperature: 250°C.

2.4 Gas chromatography-mass spectrometry (GC-MS)

GC-MS was carried out in a Hewlett-Packard 6890 gas chromatograph fitted with a HP1 fused silica column (polydimethylsiloxane 30 m × 0.25 mm, film thickness 0.25 μm), interfaced with an Hewlett-Packard mass selective detector 5973 (Agilent Technologies) operated by HP Enhanced ChemStation software, version A.03.00. GC parameters as described above; interface temperature: 250°C; MS source temperature: 230°C; MS quadrupole temperature: 150 °C; ionization energy: 70 eV; ionization current: 60 μA; scan range: 35–350 units; scans per second: 4.51.

2.5 Qualitative and quantitative analyses

Components of the volatile oil were identified by their retention indices on both SPB-1 and SupelcoWax-10 columns, calculated by linear interpolation relative to retention times of C₈–C₂₄ of *n*-alkanes and compared with those of reference compounds included in CEF laboratory database or literature data (Adams, 2004), and by their mass spectra by matching with reference spectra from the CEF laboratory own spectral database, Wiley/NIST database or literature data (Joulain & Konig, 1998; Adams, 2004; Wiley Registry, 2006). Relative amounts of individual components were calculated based on GC raw data areas without FID response factor correction.

2.6 Antioxidant activity evaluation

2.6.1 TBARS

1 Two sets of experiments were based on a modified thiobarbituric acid (TBA) reactive substances
2 assay (TBARS) to measure the antioxidant ability of the samples, without (1) and with (2) a lipid
3 peroxidation inducer.

4 (1) Egg yolk homogenate was used as the lipid-rich medium, obtained as described by Dorman *et*
5 *al.* (1995), i.e. an aliquot of yolk material was made up to a concentration of 10% w/v in KCl
6 (1.15% w/v). The yolk was then homogenized for 30 s followed by ultrasonication for further 5
7 min. Homogenate (500 μ l) and 100 μ l of sample or positive controls of BHA and BHT (in the
8 concentrations 0.05, 0.1, 0.15 and 0.20 μ g/mL), dissolved in methanol, were added to a test tube
9 and made up to 1 ml with distilled water, followed by the addition of 1.5 ml 20% acetic acid (pH
10 3.5) and 1.5 ml 0.8% w/v TBA in 1.1% w/v sodium dodecyl sulphate (SDS). This mixture was
11 stirred in a vortex and heated at 95°C for 60 min. After cooling to room temperature (20 min in
12 water), 5 ml of *n*-butanol was added to each tube, stirred and centrifuged at 4500 rpm for 15 min.
13 The absorbance of the supernatant was measured at 532 nm using a spectrophotometer (Cintra 101
14 GBC) in the software Cintral General Applications.

15 For set (2), 50 μ l of 2,2-azobis-(2-amidinopropane) dihydrochloride (ABAP) (0.07 M) were added
16 to induce lipid peroxidation, soon after the addition of sample, the remaining procedure being as
17 reported above.

18 The percentage of inhibition was calculated using the following equation: % inhibition = $[(A_C -$
19 $A_S)/A_C] \times 100$.

20 Where A_C is the absorbance of the control (without sample) and A_S is the absorbance in the
21 presence of the sample. Tests were carried out in triplicate.

22

23 **2.7 Anti-inflammatory evaluation**

24 **2.7.1 Cell culture and materials**

1 Raw 264.7 (ATCC number: TIB-71), a mouse macrophage cell line kindly supplied by Dr Otilia
2 Vieira (Centro de Neurociências e Biologia Celular, Universidade de Coimbra, Coimbra, Portugal),
3 was cultured in Iscove's Modified Dulbecco's Eagle Medium supplemented with 10% non-
4 inactivated fetal bovine serum, 100 U/ml penicillin, and 100 $\mu\text{g/mL}$ streptomycin at 37°C in a
5 humidified atmosphere of 95% air and 5% CO_2 .

6

7 **2.7.2. Nitric oxide (NO) measurement**

8 The anti-inflammatory activity of the oil from samples RS_Fl and RS_Fr was evaluated in the
9 mouse macrophage cell line Raw 264.7.

10 The production of NO was measured by the accumulation of nitrite in the culture supernatants,
11 using a colorimetric reaction with the Griess reagent (Green *et al.*, 1982). The cells were plated at
12 0.6×10^6 cells/well in 48-well culture plates, allowed to stabilize for 12h, and then incubated with
13 culture medium (control), or stimulated with 1 $\mu\text{g/mL}$ LPS, or with 1 $\mu\text{g/mL}$ LPS in the presence of
14 different concentrations of the essential oil during 24h. Briefly, 170 μL of culture supernatants were
15 collected and diluted with equal volume of the Griess reagent [0.1% (w/v) N-(1-naphthyl)
16 ethylenediamine dihydrochloride and 1% (w/v) sulphanilamide containing 5% (w/v) H_3PO_4] during
17 30 min, in the dark. The absorbance at 550 nm was measured using an automatic plate reader (SLT,
18 Austria). Nitrite concentration was determined using a sodium nitrite standard curve.

19

20 **2.8 Evaluation of cytotoxicity**

21 **2.8.1 Cell culture and materials**

22 The human keratinocyte cell line HaCaT, obtained from DKFZ (Heidelberg), was kindly supplied
23 by Dr Eugénia Carvalho (Centro de Neurociências e Biologia Celular, Universidade de Coimbra,
24 Coimbra, Portugal). Keratinocytes were cultured in Dulbecco's Modified Eagle Medium (high

1 glucose) supplemented with 4 mM glutamine, 10% heat inactivated fetal bovine serum, 100 U/ml
2 penicillin and 100 µg/ml streptomycin, at 37°C in a humidified atmosphere of 95% air and 5% CO₂.
3 The human hepatocyte cell line HepG2 (ATCC number: 77400), kindly supplied by Professor
4 Conceição Pedroso Lima, was cultured in Dulbecco's Modified Eagle Medium (low glucose)
5 supplemented with 10% heat inactivated fetal bovine serum, 100 U/ml penicillin and 100 µg/ml
6 streptomycin, at 37°C in a humidified atmosphere of 95% air and 5% CO₂. Along the experiments,
7 cells were monitored by microscope observation in order to detect any morphological change.

8

9 ***2.8.2 MTT assay for cell viability***

10 Cell viability was assessed for the oil of samples RS_Fl and RS_Fr using the mouse macrophage
11 cell line Raw 264.7, the human keratinocyte cell line HaCaT and the cell line Hep G2.

12 Assessment of cell viability was made through a colorimetric assay, using MTT (Mosmann, 1983).

13 HaCaT and HepG2 cells (0.2×10^6 cells/well, cultured in 48-well microplates) were incubated in a
14 final volume of 600 µL, allowed to stabilize for 12h, and then incubated for 24h with different
15 concentrations of the essential oil. After adding 60 µL of MTT solution (5 mg/mL in PBS) to each
16 well, the cells were further incubated at 37°C for 15 and 60 min for HaCaT cells and Hep G2 cells,
17 respectively, in a humidified atmosphere of 95% air/5% CO₂. Supernatants were then discarded and
18 300 µL of acidified isopropanol (0.04N HCl in isopropanol) were added to the cultures and mixed
19 thoroughly to dissolve the dark blue crystals of formazan. Formazan quantification was performed
20 using an ELISA automatic microplate reader (SLT, Austria) at 570 nm, with a reference wavelength
21 of 620 nm.

22 Concerning the cell line Raw 264.7, and after collection of 170 µL of culture supernatants for NO
23 measurement, 43 µL of MTT solution (5 mg/mL in PBS) were added and cells were further
24 incubated at 37°C for 15 min, in a humidified atmosphere of 95% air and 5% CO₂. Supernatants
25 were then discarded and 300 µL of acidified isopropanol (0.04N HCl in isopropanol) were added to

1 the cultures and mixed thoroughly to dissolve the dark blue crystals of formazan. Formazan
2 quantification was performed as described above.

4 **2.9 Data analysis**

5 All the experiments were performed in duplicate, being the results expressed as mean $\pm$ SEM of
6 three independent experiments. The means were statistically compared using two-way ANOVA,
7 with a Dunnett's multiple comparison test. The differences between the means were considered
8 significant for values of $p < 0.001$. The statistical tests were applied using GraphPad Prism, version
9 6.01 (GraphPad Software, San Diego, CA, USA).

11 **3. Results**

12 **3.1 Essential oil composition**

13 The essential oil was obtained with yields of 1.6% in flowering sample (RS_Fl) and 1.9% in
14 fruiting sample (RS_Fr). The qualitative and quantitative composition of the oil is presented in table
15 1, where the compounds are listed by order of their elution on a polydimethylsiloxane column.

16 In total, 26 compounds were identified accounting for 96.0-98.7%. The oil did not show a
17 significant difference in composition between flowering and fruiting stages, with the monoterpene
18 hydrocarbons representing the major percentage (95.2 and 85.0%, respectively), with some
19 variations in the amounts of the main compounds: α -phellandrene (63.3 vs 53.0%), terpinolene
20 (11.9 vs 8.6), β -phellandrene (6.0 vs 5.5), and dillapiol (1.9 vs 8.0%).

22 **3.2 Antioxidant activity**

23 The antioxidant activity was evaluated through the thiobarbituric acid reactive substances (TBARS)
24 assay. Different concentrations of the essential oil and one of its main compounds (α -phellandrene),
25 BHA and BHT (two standard antioxidants) were tested in the absence and in the presence of the

radical inducer 2,2'-azobis-(2-amidinopropane) dihydrochloride (ABAP). The results showed in table 2 demonstrate that both essential oil samples possess a strong antioxidant activity, possibly due in part to the action of α -phellandrene.

3.3 Effect of the essential oil on nitric oxide production induced by LPS

The effect of essential oil on NO production triggered by LPS was evaluated in the mouse macrophage cell line Raw 264.7. After macrophages stimulation with LPS in the presence of the oil, nitric oxide production induced by LPS was significantly reduced with the four concentrations tested (Figure 1). For α -phellandrene (α -phell), only the higher concentration tested (1.25 μ L/mL), reduced significantly the nitric oxide production, thus demonstrating that the anti-inflammatory action of the essential oil is not only due to the action of α -phellandrene (Figure 1).

3.4 Evaluation of cell viability

3.4.1 Effect of the essential oil on macrophages viability

To evaluate the possible cytotoxic activity of the oil in different mammalian cell types, the MTT assay was used in macrophages, keratinocytes and hepatocytes. Twenty four hours of LPS exposure had no significant effect on macrophages viability. As shown in Figure 2, the oil did not show significant cytotoxicity in macrophages compared with control. However, α -phellandrene, the main compound of the EO, demonstrates a slight cytotoxicity.

3.4.2 Effect of the essential oil on keratinocytes viability

As shown in Figure 3, the oil did not show significant cytotoxicity in keratinocytes compared with control. α -phellandrene demonstrated a slight cytotoxicity. A cell-free control was performed in order to exclude non-specific effects of the oil on MTT (data not shown).

3.4.3 Effect of the essential oil on hepatocytes viability

1 As shown in Figure 4, the oil and α -phellandrene did not show significant cytotoxicity in
2 hepatocytes compared with control.

3 A cell-free control was performed in order to exclude non-specific effects of the oil on MTT (data
4 not shown).

5

6 **4. Discussion and conclusions**

7 The essential oil of *Ridolfia segetum* from Portugal, in flowering and fruiting stage, was obtained
8 with very high yields (1.6-1.9%) and with high percentage of monoterpene hydrocarbons, especially
9 α -phellandrene (53.0-63.3%). These results are in accordance with the type of essential oil
10 described by various authors (Bicchi *et al.*, 2009, Fleisher & Fleisher, 1996; Marongiu *et al.*, 2007,
11 Palá-Paúl *et al.*, 2002; Palá-Paúl *et al.*, 2005), that is dominated by monoterpene hydrocarbons,
12 usually α -phellandrene, terpinolene and *p*-cymene. Concerning the antioxidant properties of the
13 essential oil, the results presented here demonstrate that both essential oil samples and α -
14 phellandrene have significant antioxidant activity, even higher than BHA in the absence of the
15 peroxidation inducer (ABAP), and higher than BHT in the presence of the peroxidation inducer.

16 The different behavior of the synthetic antioxidants in the absence/presence of ABAP suggests that
17 BHA is able to operate as good antioxidant when the levels of peroxy radicals are relatively high,
18 whereas BHT does not seem to possess such ability (Miguel *et al.*, 2004). Usually, the essential oil
19 with high content of phenylpropanoids or monoterpene phenols (thymol and carvacrol) possess
20 better antioxidant activity (Dandlen *et al.*, 2010). However, *R. segetum* essential oil, in which the
21 main compound is α -phellandrene, a monoterpene hydrocarbon, showed a very high antioxidant
22 activity, thus highlighting that the presence of phenolic compounds in the essential oils is not
23 mandatory for the antioxidant activity, as already suggested by other authors (Miguel *et al.*, 2004;
24 Viuda-Martos *et al.*, 2010).

1 The oil significantly inhibited NO production elicited by LPS in macrophages which demonstrates
2 its strong anti-inflammatory potential. However, α -phellandrene, that revealed an antioxidant
3 activity similar to that of the oil tested, showed a quite low inhibition of NO production, which
4 suggests that the essential oil of *R. segetum* is more active than its main compound. In fact, essential
5 oils are complex mixtures of multiple constituents, and their biological properties seem to be the
6 result of a synergism between these compounds. Bearing in mind this assumption, it makes sense
7 that α -phellandrene presents a lower effect in the reduction of NO production than the essential oil.
8 This volatile oil is a mixture of various compounds specially α -phellandrene (53-63.3%),
9 terpinolene (8.6-11.9), β -phellandrene (5.5 vs 6.0%), and dillapiol (1.9-8.0%), Terpinolene has a
10 broad spectrum of biological activities such as anticancer (Aydin *et al.*, 2013; Harada *et al.*, 2012),
11 antioxidant (Aydin *et al.*, 2013; Dorman *et al.*, 2000), antifungal (Hammer *et al.*, 2004), and
12 larvicidal (Conti *et al.*, 2012), which contributes for the activity of the oil. *Ridolfia segetum* oil did
13 not affect macrophages, keratinocytes and hepatocytes viability at concentrations at least up to 1.25
14 μ L/mL. Often essential oils possess a significant toxicity at 1.25 μ L/mL or even at lower
15 concentrations (Bouzabata *et al.*, 2013; Cabral *et al.*, 2013). The absence of keratinocyte toxicity is
16 crucial to make an essential oil suitable for inclusion in topical formulations. Also, the absence of
17 toxicity on both macrophages and hepatocytes reveal the safety for internal use of this oil.
18 Cytotoxicity evaluation of α -phellandrene demonstrated higher toxicity when compared to *R.*
19 *segetum* oil at the same concentrations. These results suggest that the complex mixture of molecules
20 present in the essential oil attenuates the cytotoxicity shown by its constituents individually tested,
21 thus emphasizing the benefic use of the essential oil.

22 Considering that *R. segetum* showed a high yield in essential oil, which confers an industrial
23 potential interest, and its volatile oil has a pleasant smell and are safe in bioactive concentrations,
24 the results herein presented emphasize its potential use as antioxidant and anti-inflammatory for the

1 food, cosmetic and nutraceutic industries. Also, the high content of α -phellandrene makes this
2 species an important natural source of this compound.

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8 **References**

- 9 Adams, R.P., 2004. Identification of Essential Oil Components by Gas Chromatography/
10 Quadrupole Mass Spectroscopy. Allured Publishing Corporation, Carol Stream, IL.
- 11 Aedo, C., 2003. *Ridolfia* Moris, in: Castroviejo, S., Laínz, M., López-González, G., et al. (Eds.),
12 Flora Iberica, Vol. 10, Araliaceae-Umbelliferae, CSIC, Madrid, pp. 282-284.
- 13 Aruoma, O.I., 1998. Free radicals, oxidative stress, and antioxidants in human health and disease. J.
14 Am. Oil Chem. Soc. 75, 199-212.
- 15 Aydin, E., Türkeş, H., Taşdemir, S., 2013. Anticancer and antioxidant properties of terpinolene in
16 rat brain cells. Arh. Hig. Rada Toksikol. 64(3), 415-424.
- 17 Bakkali, F., Averbeck, S., Averbeck D., Idaomar, M., 2008. Biological effects of essential oils-a
18 review. Food Chem. Toxicol. 46(2), 446-475.
- 19 Bicchi, C., Rubiolo, P., Ballero, M., Sanna, C., Matteodo, M., Esposito, F., Zinzula, L.,
20 Tramontano, E., 2009. HIV-1-inhibiting activity of the essential oil of *Ridolfia segetum* and
21 *Oenanthe crocata*. Planta Med. 75(12), 1331-1335.
- 22 Bouzabata, A., Bazzali O., Cabral C., Gonçalves M.J., Cruz M.T., Bighelli A., Cavaleiro C.,
23 Casanova J., Salgueiro L., Tomi F., 2013. New compounds, chemical composition, antifungal
24 activity and cytotoxicity of the essential oil from *Myrtus nivellei* Batt. & Trab., an endemic species
25 of Central Sahara. J. Ethnopharmacol. 149(3), 613-620.

1 Cabral C., Cavaleiro C., Gonçalves M.J., Cruz M.T., Lopes M.C., Salgueiro L., 2013. *Otanthus*
2 *maritimus* (L.) Hoffmanns. & Link as a source of a bioactive and fragrant oil. Ind. Crop Prod. 43,
3 484-489.

4 Conti, B., Benelli, G., Flamini, G., Cioni, P.L., Profeti, R., Ceccarini, L, Macchia, M., Canale, A.,
5 2012. Larvicidal and repellent activity of *Hyptis suaveolens* (Lamiaceae) essential oil against the
6 mosquito *Aedes albopictus* Skuse (Diptera: Culicidae). Parasitol. Res. 110, 2013-2021.

7 Council of Europe, 1997. European Pharmacopoeia, third ed. Council of Europe. Strasbourg.

8 Dandlen, S.A., Lima, A.S., Mendes, M.D., Miguel, M.G., Faleiro, M.L., Sousa, M.J., Pedro, L.G.,
9 Barroso, J.G., Figueiredo, A.C., 2010. Antioxidant activity of six Portuguese thyme species
10 essential oils. Flavour Frag. J. 25, 150-155.

11 Dorman, H.J.D., Deans, S.G., Noble, R.C., Surai, P., 1995. Evaluation *in vitro* of plant essential oils
12 as natural antioxidants. J. Essent. Oil Res. 7, 645–651.

13 Dorman, H.J.D., Figueiredo, A.C., Barroso, J.G., Deans, S.G., 2000. *In vitro* evaluation of
14 antioxidant activity of essential oils and their components. Flavour Frag. J. 15, 12-16.

15 Fleisher, Z., Fleisher, A., 1996. Volatiles of leaves and flowers of *Ridolfia segetum* L. Moris.
16 Aromatic Plants of the Holy Land and the Sinai. Part XII. J. Essent. Oil Res. 8(2), 189-191.

17 Green, L.C., Wagner, D.A., Glogowski, J., Skipper, P.L., Wishnok, J.S., Tannenbaum, S.R., 1982.
18 Analysis of nitrate, nitrite, and [15N] nitrate in biological fluids. Anal. Biochem. 126, 131–138.

19 Hammer, K.A., Carson, C.F., Riley, T.V., 2004. Antifungal effects of *Melaleuca alternifolia* (tea
20 tree) oil and its components on *Candida albicans*, *Candida glabrata* and *Saccharomyces cerevisiae*.
21 Journal of Antimicrob. Chemoth. 53, 1081-1085.

22 Harada, T., Harada, E., Sakamoto, R., Ashitani, T., Fujita, K., Kuroda, K., 2012. Regio- and
23 substrate-specific oxidative metabolism of terpinolene by cytochrome P450 monooxygenases in
24 *Cupressus lusitanica* cultured cells. AJPS 3, 268-275.

1 Jabrane, A., Jannet, H.B., Mastouri, M., Mighri, Z., Casanova J., 2010. Chemical composition and *in*
2 *vitro* evaluation of antioxidant and antibacterial activities of the root oil of *Ridolfia segetum* (L.)
3 Moris from Tunisia. Nat. Prod. Res. 24(6), 491-499.

4 Jadhav, S.J., Nimbalkar, S.S., Kulkarni, A.D., Madhavi, D.L., 1995. Lipid oxidation in biological
5 and food systems, in: Madhavi, D.L., Deshpande, S.S., Salunkhe, D. K. (Eds.), Food Antioxidants -
6 Technological, Toxicological, and Health Perspectives. Marcel Dekker, New York, pp. 5-62.

7 Jannet, H.B., Mighri, Z., 2007. Hydrodistillation kinetic and antibacterial effect studies of the
8 flower essential oil from the Tunisian *Ridolfia segetum* (L.). J. Essent. Oil Res. 19(3), 258-261.

9 Joulain, D., Konig, W.A., 1998. The Atlas of Spectral Data of Sesquiterpene Hydrocarbons. E.B.-
10 Verlag, Hamburg.

11 Kamatou, G.P.P., Viljoen, A.M., 2010. A review of the application and pharmacological properties
12 of α -bisabolol and α -bisabolol-rich oils. J. Am. Oil Chem. Soc. 87, 1-7.

13 Lentini, F., Venza, F., 2007. Wild food plants of popular use in Sicily. JEE 3, 15.

14 Maestri, D.M., Nepote, V. Lamarque, A.L., Zygadlo, J.A., 2006. Natural products as antioxidants,
15 in: Imperato, F. (Ed.), Phytochemistry: Advances in Research. Research Signopost, Kerala, India,
16 pp. 105-135.

17 Marongiu, B., Piras, A., Porcedda, S., Tuveri, E., Maxia, A., 2007. Comparative analysis of the oil
18 and supercritical CO₂ extract of *Ridolfia segetum* (L.) Moris. Nat. Prod. Res. 21(5), 412-417.

19 Miguel, M.G., 2010a. Antioxidant activity of medicinal and aromatic plants. A review. Flavour
20 Frag. J. 25, 291-312.

21 Miguel, M.G., 2010b. Antioxidant and Anti-Inflammatory Activities of Essential Oils: A Short
22 Review. Molecules 15, 9252-9287.

23 Miguel, M.G., Simões, M., Figueiredo, A.C., Barroso, J.G., Pedro. L.G., Carvalho, L., 2004.
24 Composition and antioxidant activities of the essential oils of *Thymus caespititius*, *Thymus*
25 *camphoratus* and *Thymus mastichina*. Food Chem. 86, 183-188.

1 Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to
2 proliferation and cytotoxicity assays. J. Immunol. Methods 65, 55–63.

3 Pae, H.O., Oh, H., Yun, Y.G., Oh, G.S., Jang, S.I., Hwang, K.M., Kwon, T.O., Lee, H.S., Chung,
4 H.T., 2002. Imperatorin, a furanocoumarin from *Angelica dahurica* (umbelliferae), induces
5 cytochrome c-dependent apoptosis in human promyelocytic leukaemia, HL-60 cells. Pharmacol.
6 Toxicol. 91, 40-48.

7 Palá-Paúl, J., Velasco-Negueruela, A., Pérez-Alonso, M.J., Ramos-Vázquez, P., 2002. Volatile
8 constituents of *Ridolfia segetum* (L.) Moris gathered in Southern Spain, Andalucia Province. J.
9 Essent. Oil Res. 14(3), 206-209.

10 Palá-Paúl, J., Velasco-Negueruela, A., Pérez-Alonso, M.J., Vallejo, M.C.G., 2005. Volatile
11 constituents of *Ridolfia segetum* (L.) Moris gathered in Central Spain: Castilla la Mancha Province.
12 J. Essent. Oil Res. 17, 119-121.

13 Timsina, B., Shukla, M., Nadumane, V.K., 2012. A review of few essential oils and their anticancer
14 property. Journal of Natural Pharmaceuticals 3(1), 1-8.

15 Viuda-Martos, M., Navajas, Y. R., Zapata, E. S., Fernández-López, J., Pérez-Alvarez, J.A., 2010.
16 Antioxidant activity of essential oils of five spice plants widely used in a Mediterranean diet.
17 Flavour Frag. J. 25, 13-19.

18 Wiley Registry, 2006. NIST 05 Mass Spectral Library, eight ed. John Wiley & Sons, Hoboken, NJ.

Table 1. Chemical composition of *Ridolfia segetum* essential oils from Portugal (RS_Fl - flowering stage; RS_Fr – fruiting stage).

RI ^a	RI ^b	Compound*	Percentage in samples%	
			RS_Fl	RS_Fr
921	1026	α -Thujene	t	0.4
928	1026	α -Pinene	2.5	2.6
944	1073	Camphene	t	t
962	1125	Sabinene	0.3	0.3
967	1115	β -Pinene	1.5	2.5
980	1159	Myrcene	1.0	1.0
998	1171	α -Phellandrene	63.3	53.0
1009	1188	α -Terpinene	t	0.3
1012	1272	<i>p</i> -Cymene	0.5	1.5
1020	1213	β -Phellandrene	6.0	5.5
1020	1204	Limonene	3.5	2.8
1024	1232	<i>Z</i> - β -Ocimene	4.0	6.0
1035	1250	<i>E</i> - β -Ocimene	0.5	0.4
1046	1246	γ -Terpinene	0.1	0.1
1076	1284	Terpinolene	11.9	8.6
1105	1555	<i>Z-p</i> -Menth-2-ene-1-ol	t	t
1111	1378	<i>allo</i> -Ocimene	t	0.2
1160	1595	Terpinene-4-ol	t	0.1
1183	n.d.	<i>trans</i> -Piperitenol	t	t
1230	1719	Piperitone	t	t
1267	1573	Bornyl acetate	t	t
1328	1948	Piperitenone oxyde	1.1	2.3
1408	1595	<i>E</i> -Caryophyllene	0.1	t
1442	1662	α -Humulene	0.1	0.1
1466	1701	Germacrene D	0.1	t
1586	2348	Dillapiol	1.9	8.0
Monoterpene hydrocarbons			95.2	85.2
Oxygen containing monoterpenes			1.3	2.6
Sesquiterpene hydrocarbons			0.3	0.2
Phenylpropanoids			1.9	8.0
Total identified			98.7	96.0

* Compounds listed in order to their elution on the SPB-1 column

t = traces ($\leq 0.05\%$)

RI^a = Retention indices on the SPB-1 column relative to C₈ to C₂₄ *n*-alkanes.

RI^b = Retention indices on the SupelcoWax-10 column relative to C₈ to C₂₄ *n*-alkanes.

Table 2. Antioxidant index (%) of the essential oils, BHA, BHT and α -phellandrene, in different concentrations (using TBARS assay in the absence or presence of ABAP.

Standard/EO		Concentration, mean $\pm$ SEM (μ g/mL)			
		0.20	0.15	0.10	0.05
Absence of ABAP	BHA	64.10 $\pm$ 1.27	60.57 $\pm$ 1.11	58.03 $\pm$ 1.21	52.81 $\pm$ 0.38
	BHT	95.35 $\pm$ 0.43	93.20 $\pm$ 0.51	89.62 $\pm$ 0.51	84.65 $\pm$ 0.92
	RS_Fl	78.41 $\pm$ 1.81	74.90 $\pm$ 1.90	71.54 $\pm$ 1.28	65.05 $\pm$ 1.16
	RS_Fr	72.11 $\pm$ 1.51	69.13 $\pm$ 0.64	62.57 $\pm$ 3.46	51.84 $\pm$ 3.08
	α -phel	75.78 $\pm$ 1.08	70.94 $\pm$ 1.36	66.97 $\pm$ 1.52	57.70 $\pm$ 0.67
Presence of ABAP	BHA	89.94 $\pm$ 0.96	88.56 $\pm$ 1.27	86.25 $\pm$ 1.57	79.94 $\pm$ 0.80
	BHT	71.77 $\pm$ 0.79	68.34 $\pm$ 0.50	66.14 $\pm$ 0.64	63.99 $\pm$ 0.87
	RS_Fl	77.37 $\pm$ 1.34	75.14 $\pm$ 0.74	71.60 $\pm$ 0.80	64.68 $\pm$ 1.61
	RS_Fr	79.23 $\pm$ 0.74	77.40 $\pm$ 0.76	73.43 $\pm$ 0.58	64.91 $\pm$ 1.14
	α -phel	70.39 $\pm$ 0.89	68.51 $\pm$ 0.66	62.43 $\pm$ 1.16	50.11 $\pm$ 0.88
SEM (standard error of the mean)					

Figure 1

Figure 2

Figure 3

Figure 4

Figure 1. Effect of essential oil of *Ridolfia segetum* (RS_Fl and RS_Fr) and α -phellandrene on NO production in macrophages. Results are expressed as a percentage of nitrite production by cells cultured in the presence of LPS. Each value represents the mean $\pm$ SEM from three experiments, performed in duplicate (**p<0.001, compared to LPS).

Figure 2. Effect of essential oil of *Ridolfia segetum* (RS_Fl and RS_Fr) and α -phellandrene on macrophages viability (MTT assay). Results are expressed as a percentage of MTT reduction by control cells maintained in culture medium. Each value represents the mean $\pm$ SEM from three experiments, performed in duplicate (**p<0.001, compared to control).

Figure 3. Effect of essential oil of *Ridolfia segetum* (RS_Fl and RS_Fr) and α -phellandrene on keratinocytes viability (MTT assay). Results are expressed as a percentage of MTT reduction by control cells maintained in culture medium. Each value represents the mean $\pm$ SEM from three experiments, performed in duplicate (**p<0.001, compared to control).

Figure 4. Effect of essential oil of *Ridolfia segetum* (RS_Fl and RS_Fr) and α -phellandrene on hepatocytes viability (MTT assay). Results are expressed as a percentage of MTT reduction by control cells maintained in culture medium. Each value represents the mean $\pm$ SEM from three experiments, performed in duplicate (**p<0.001, compared to control).