Valorization of essential oils from two populations (wild and commercial) of *Geranium macrorrhizum* L.

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20

21 **Abstract**

22 The insecticidal and acaricidal effects of the essential oils (EO) from two populations of
23 *Geranium macrorrhizum* L (ornamental variety Bevan, VB and wild population from Hungary,
24 GH) have been studied against several insect pests of economic importance in
25 agriculture (*Spodoptera littoralis*, *Myzus persicae* and *Rhopalosiphum padi*), and the
26 hard tick *Hyalomma lusitanicum*. The oil from the wild population (GH) was characterized
27 by its content in β -elemenone (30.53%), thymol (18.52%) and germacrone (15.54%), while the
28 oil from the commercial variety (VB) had linalool (26.45%) and linalyl acetate (25.11%) as
29 major components. *S.littoralis* and *R. padi* were significantly affected by the wild
30 population oil (GH-EO). The oil from the commercial variety VB was less effective
31 against insects. The ixodicidal effects of these oils were strong, being the wild population
32 (GH-EO) the most effective. To correlate the composition of these oils with their activity, the
33 essential oil major components have been isolated (germacrone and β -elemenone) and
34 tested (linalool, thymol, germacrone and β -elemenone). The insect antifeedant and
35 ixodicidal effects of thymol and germacrone explained the bioactivity of the GH essential oil.
36 Therefore, the wild variety of *G. macrorrhizum* has been selected for domestication given its
37 potential as a source of bioactive extracts and metabolites.

38

39 **Keywords:** *Geranium macrorrhizum*, essential oil, chemotype, germacrone, antifeedant,
40 ixodicidal.

41

1. Introduction

The genus *Geranium* of the family Geraniaceae is represented by 400 species occurring in temperate and mountainous regions, mainly distributed throughout the northern hemisphere (Aedo *et al.*, 1998). Most of the *Geranium* species are used in folk medicine due to their analgesic, antimicrobial, antioxidant and hepatoprotective properties, while some species are ornamental plants (Calzada *et al.*, 1999). The phytochemical analysis of this genus showed the presence of hydrolyzable tannins, phenolic compounds and flavonoids (Calzada *et al.*, 2005; Okuda *et al.*, 1989).

Geranium macrorrhizum L., commonly known as ‘bigroot geranium’, ‘Bulgarian geranium’ and ‘rock crane's-bill’, is widely distributed in the Southeast Alps and the Balkans. It is a hardy flowering herbaceous and perennial species, with a big rhizome, five-lobed aromatic leaves and pale pink flowers. It is cultivated as an ornamental plant in temperate regions with cultivars selected for flower colors. This plant grows on calcitic soils facing north or northeast because these are moist and shaded environments.

The phytochemical analysis of its organic extract showed flavonoids, sesquiterpenes and phenolic acids and the main components of the essential oil reported are germacrone and geraniol (Chalchat *et al.*, 2002; Ivancheva *et al.*, 1992; Leslie, 1993; Lis-Balchin, 2002). The plant is used in traditional herbal medicine due to its antimicrobial, antioxidant, hypotensive, astringent, cardiogenic and sedative properties (Bate-Smith, 1981; Chalchat *et al.*, 2002; Ivancheva *et al.*, 1992; Leslie, 1993; Lis-Balchin, 2002; Ovcharov *et al.*, 1992). Regarding its biocidal effects, a water extract of leaves and stems of *G. macrorrhizum* has been reported as being effective to control the

aphid *Myzus persicae* in small farms (Velcheva et al., 2001). However, there are no reports on the insecticidal or acaricidal effects of the essential oil of *G. macrorrhizum*.

As part of an ongoing search for plants with biocidal potential for domestication and cultivation, the insecticidal and acaricidal effects of the essential oils from two populations of *G. macrorrhizum* (an ornamental variety Bevan, VB, and a wild population from Hungary, GH) have been studied. Some of the essential oil major components have been isolated and also tested. The target organisms included several insect pests of economic importance in agriculture (*Spodoptera littoralis* Boisd., *Myzus persicae* Sulz. and *Rhopalosiphum padi* L.), and the hard tick *Hyalomma lusitanicum* Koch (Ixodida: Ixodidae) abundant in the Mediterranean region (Apanaskevich et al., 2008; Encinas-Grandes, 1986, Basco-Basco et al., 2008; Barandika et al., 2011). *H. lusitanicum* is a vector of *Theileria annulata*, bacterial agents such as *Anaplasma* spp., *Ehrlichia* spp., *Bartonella* spp., *Borrelia* spp., *Coxiella burnetii*, *Francisella* spp. and *Rickettsia* spp. (Toledo et al., 2008; 2009) and the Crimean-Congo hemorrhagic fever virus (CCHFV) (Estrada-Peña et al., 2012).

2. Material and methods

2.1. General experimental procedures.

NMR spectra (^1H and ^{13}C) were recorded with Varian Direct-Drive 500 (1H 500 MHz/ ^{13}C 125 MHz) spectrometers. For high-resolution MS we used an Autospec-Q VG-Analytical (Fisons) mass spectrometer. Semi-preparative normal-phase chromatography was carried out on a 10 by 250 mm column (5- μm silica particle column, Agilent) with a 15 mm refillable guard pre-column using a Series 1100 liquid chromatograph (Agilent). The column was eluted at room temperature at a flow rate of

2 mL/min. Pure samples (>90% purity) of lilanool and thymol were purchased from Sigma Aldrich.

2.2. *Plant Material*

The wild population (GH) was obtained by vegetative multiplication of plants donated by Dr. Tillyné Mándy Andrea (Budapesti Corvinus Egyetem University), maintained in pots (Humin Substrat® N3) with irrigation for a year and then transferred to an experimental field in a shaded area located in Ejea de los Caballeros (Spain) as described in Navarro-Rocha (2017). A voucher specimen of this population has been deposited in the Herbarium of Instituto Pirenaico de Ecologia (CSIC), Spain (voucher number R297730). The commercial “Bevan” population (VB) was obtained from germinated seeds (Semillas Montaraz S.A.) cultivated as described above. Plants from both populations were collected during the flowering season in 2013.

2.3. *Extraction of essential oils*

Laboratory scale hydrodistillation (essential oil, EO) was performed in a Clevenger-type apparatus (VB= 0.1 and GH 2.5% yield) according to the method recommended by the *European Pharmacopoeia* (<http://www.edqm.eu/en/Homepage-628.html>).

2.4. *Isolation of germacrone and β -elemenone*

Dried ground plant material (410 g, aerial parts) were extracted with methyl tert-butyl ether (TBDME) in a Soxhlet apparatus for 24h to give 61.2 g of extract. The extract (10.2 g) was chromatographed on a Si-gel (250g, 40-63 μ m) column and the eluted with hexane-TBDME 9:1 to give germacrone (419 mg) mixed with fatty acid

esters. This mixture was further chromatographed on a Si-gel column (25g) eluted with hexane-TBDME 95:5 to give 278 mg of pure germacrone.

A germacrone-elemenone mixture (103 mg) obtained by isomerization of 110 mg of germacrone with 37 mg of $\text{PdCl}_2(\text{PhCN})_2$ in 6 ml of toluene during 3 hours was chromatographed on a Si-gel column (5 g) eluted with hexane-TBDME 98:2 to give 23 mg of germacrone, 22 mg of a germacrone-elemenone mixture and 25 mg of elemenone.

These compounds were identified by means of their spectroscopic data by comparison with those of authentic samples.

2.5. Gas Chromatography-Mass Spectrometry (GC-MS)

The essential oils were analyzed by gas chromatography mass spectrometry (GC-MS) using a Shimadzu GC-2010 gas chromatograph coupled to a Shimadzu GCMS-QP2010 Ultra mass detector (electron ionization, 70 eV) and equipped with a 30 m $\times$ 0.25 mm i.d. capillary column (0.25 μm film thickness) Teknokroma TRB-5 (95%) Dimetil-(5%) diphenylpolisiloxane. Working conditions were as follows: split ratio (20:1), injector temperature 300°C, temperature of the transfer line connected to the mass spectrometer 250°C, initial column temperature 70°C, then heated to 290°C at 6°C min⁻¹. Electron ionization mass spectra and retention data were used to assess the identity of compounds by comparing them with those of standards or found in the Wiley 229 Mass Spectral Database. Further, the mass spectra and retention times of authentic compounds (germacrone and β -elemenone isolated from the essential oil, linalool and thymol from Sigma) were also used to confirm the identities of the constituents. The relative amounts of individual components were calculated based on the GC peak area (FID response) without using a correction factor.

2.6. Insect Bioassays

Spodoptera littoralis, *Myzus persicae* and *Rhopalosiphum padi* colonies were reared on artificial diet, bell pepper (*Capsicum annuum*) and barley (*Hordeum vulgare*) plants respectively, and maintained at 22 ± 1 °C, >70% relative humidity with a photoperiod of 16:8 h (L:D) in a growth chamber.

The bioassays were conducted with ten-twenty newly emerged *S. littoralis* L6 larvae and two hundred *M. persicae* or *R. padi* adults. Feeding or settling inhibition (%FI or %SI) were calculated as $FI = [1 - (T/C)] \times 100$, where T and C are the consumption of treated and control leaf disks, respectively, or as $\%SI = [1 - 10 (\%T/\%C)] \times 100$ where %C and %T are percent aphids settled on control and treated leaf disks, respectively as described (Burgueño-Tapia *et al.*, 2008). The organic extracts and pure compounds were tested at initial concentrations of 100 and 50 µg/cm² respectively, and the antifeedant effects (FI/SI) were analyzed for significance by the non-parametric Wilcoxon signed-rank test. Effective antifeedant doses (EC₅₀) (effective dose to obtain 50% feeding inhibition) were determined for extracts and pure compounds with FI/SI values > 70 %, and calculated by logarithmic linear regression (five 1:2 serial dilutions, STATGRAPHICS Centurion XVI, version 16.1.02).

2.7. Ixodicidal Bioassay

Engorged females of the tick *Hyalomma lusitanicum* Koch 1844 (Ixodoidea: Ixodida) were collected from their host (deer) in central Spain (Finca La Garganta, Ciudad Real), and maintained at 22-24°C, 70% RH until oviposition. The eggs were kept under the same environmental conditions until they hatched. Larvae (4-6 weeks old) were used for the bioassay as described in Ruiz-Vásquez *et al.* (2017). The larvicidal activity data are presented as percentage of mortality corrected according to

Schneider-Orelli's formula (Püntener, 1981). Lethal doses (LC₅₀ and LC₉₀) were calculated by Probit Analysis (five 1:2 serial dilutions, STATGRAPHICS Centurion XVI, version 16.1.02).

3. Results and discussion

The Essential oil yield of the wild population (GH-EO) was 25 times higher than that of the variety Bevan (VB-EO). The GC-MS analysis of the essential oils (EOs) showed qualitative and quantitative differences in the composition of the two populations (Table 1). The EO from the wild population (GH-EO) had β -elemenone (30.53%), thymol (18.52%) and germacrone (15.54%) as the major components. A similar composition has been reported for wild individuals of this species collected in Hungary (Radulovic *et al.*, 2010), except for the presence of thymol. The EO from the variety Bevan (VB-EO) had linalool (26.45%) and linalyl acetate (25.11%) as major components. This composition varied from previous reports, and indicates that the selection of the Bevan variety (VB) for ornamental purposes resulted in a different yield and chemical composition of its essential oil with respect to the wild GH population.

TABLE 1

The essential oil composition of aromatic plants is determined by their genotype (Bruneton, 2001). However, variations in essential oil composition are found in morphologically and genetically homogeneous species, and a correlation between chemotypes and plant habitats has been suggested (García-Vallejo, 1990). In this case morphological and physiological differences between the two populations have been observed (Navarro-Rocha *et al.*, 2017) in addition to the chemical variation shown here.

S.littoralis and *R. padi* were significantly affected by GH-EO in a dose-response manner (EC₅₀ values of 2.2 and 4.7 mg/ml respectively) (Table 2). Overall, the oil from the commercial variety VB was less effective against insects, with a moderate effect on *R. padi* (Table 2). Among the main components of the active essential oils tested (Figure 1), linalool, had no effect on the target insect species, thymol was active on both aphid species but not on *S. littoralis*, germacrone exhibited strong antifeedant activity against *S. littoralis* and *M. persicae*, and moderate to *R. padi* and β -elemenone showed no effects (Table 2). Therefore, the insect antifeedant effects of GH-EO could be explained by its content in thymol and germacrone.

TABLE 2

FIGURE 1

Both essential oils were toxic against *H. lusitanicum* larvae, with GH-EO being the most effective (Table 3). Linalool was not toxic to *H. lusitanicum*, suggesting synergistic effects among the components of the VB-EO to explain its ixodicidal activity (Table 3). Thymol and germacrone were very effective against the tick *H. lusitanicum*, with thymol being more effective than germacrone. However, the overall effect of GH-EO was similar to that of germacrone (LD₅₀ of 1.37-1.08 and LD₉₀ of 2.87-3.13 mg/ml for GH-EO and germacrone) (Table3).

TABLE 3

Many essential oils have been described as toxic and/or repellent against insects (Deletre et al., 2016; Yazdgerdian et al., 2016) and ticks (Flores-Fernández et al., 2016; Galvao-Peixoto et al., 2015; Kiss et al., 2012; Kröber et al., 2013), including *Hyalomma* spp. Specifically, *Lavandula angustifolia*, wich essential oil is rich in linalool and linalyl acetate, was repellent to *H. marginatum* (Mkolo and Magano, 2007). Essential

oils from other *Geranium* species have been reported as tick repellents against *Amblyomma americanum* (Tabanca *et al.*, 2013). However, this is the first report on the insect antifeedant and ixodocidal effects of the essential oils from *G. macrorrhizum*.

Previous studies have shown insecticidal, acaricidal and ixodocidal effects for thymol (Calderón *et al.*, 2014; Daemon *et al.*, 2009; Montañó and Novoa, 2007; Monteiro *et al.*, 2009; Novelino *et al.*, 2007; Pino *et al.*, 2011). Germacrone has been reported as being acaricidal against *Tetranychus urticae* (Benelli *et al.*, 2017), however this is the first report on the insect antifeedant and ixodocidal effects of this molecule. Among these two active compounds, germacrone (with lower volatility than thymol) could be used as a building block for the development of new insect antifeedants and ixodocidal agents due to the functionality of the molecule (Catalán, 2007; Pérez-Morales *et al.*, 2013).

4. Conclusion

The essential oils of two *G. macrorrhizum* populations (an ornamental, VB and a wild, GH) had different chemical compositions. The VB population had linalool and linalyl acetate while the GH one had β -elemenone, germacrone and thymol as major components. These oils were antifeedant against insects (*S. litoralis* and *R. padi*) and ixodocidal against *H. lusitanicum*, being the essential oil of the wild population (GH) the most effective. Germacrone and thymol also had antifeedant and ixodocidal effects and explained the activity of the GH essential oil. Therefore, the domestication of the wild *G. macrorrhizum* population (GH) for cultivation and biomass production could represent a potential source of bioactive essential oil and valuable lead compounds such as germacrone.

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366

367 **Figure captions**

368 **Figure 1.** Chemical structures of linalool, thymol, germacrone and β -elemenone.

369

370 **Table 1.** Composition (%) of the essential oil of two *G. macrorrhizum* populations (VB
 371 and GH).
 372

Compound	rt	%VB	%GH
<i>p</i> -Cymene	5.297		1.04
<i>cis</i> -Linalool oxide	6.157	6.17	
<i>trans</i> -Linalool oxide	6.460	4.49	
Linalool	6.657	26.45	
1-Octenyl acetate	6.840	6.55	
<i>endo</i> -Borneol	8.120	2.52	
Linalyl acetate	10.02	25.11	
Geranyl acetate	10.783	7.56	
Thymol	10.827		18.52
Neryl acetate	12.447	1.33	
Neryl acetate	12.867	1.92	
β -Elemene	13.190		0.59
β -Himachalene	13.497		0.56
β -Caryophyllene	13.847		1.14
γ -Elemene	14.087		3.70
γ -Curcumene	15.067		1.61
Cuparene	15.123		2.51
Germacrone iso	17.603		4.79
β -Elemenone	17.71		30.53
Caryophyllene oxide	17.330	3.76	1.90
β -Elemene	18.303		0.97
β -Eudesmol	18.687		1.78
Nerolidol acetate	18.760		5.42
Germacrone Iso	19.013		6.4
Germacrone Iso	19.397		0.69
Germacrone	19.573		15.54

373

374 **Table 2.** Antifeedant effects of the essential oil (EO) of two populations of *G. macrorrhizum*
 375 (VB and GH) and their major compounds.

Essential oil / compound	mg/ml	<i>S. litoralis</i> (%FI) ^a	<i>M. persicae</i> (%SI) ^a	<i>R. padi</i> (%SI)
VB-EO	10	55.5 ± 16.1	31.1 ± 7.6	69.9 ± 7.3*
GH-EO	10	87.8 ± 9.7*	55.1 ± 7.7	77.8 ± 5.4*
	EC ₅₀ ^b	2.2 (1.4-3.4)		4.70 (3.67-6.01)
Linalool	5	45.3 ± 7.2	51.9 ± 8.3	18.9 ± 5.6
Thymol	5	52.4 ± 13.0	98.1 ± 1.4*	80.0 ± 3.5*
	EC ₅₀ ^b		4.6 (3.1-6.7)	3.5 (2.3-5.2)
Germacrone	5	90.5 ± 9.8*	73.9 ± 7.0*	69.6 ± 6.5*
	EC ₅₀ ^b	1.9 (0.1-3.6)	2.8 (2.2-3.4)	
β-Elementone	5	25.2 ± 8.1	55.9 ± 8.3	50.2 ± 7.0

376 ^aPercent feeding (FI) / setting (SI) inhibition. ^bConcentration needed to produce 50% feeding / setting
 377 inhibition (EC₅₀) and 95% confidence limits (lower, upper), * p<0.05, Wilcoxon paired rank test.

378

379 **Table 3.** Ixodicidal effects of the essential oil (EO) of two populations of
 380 *G.macrorrhizum* (VB and GH) and their major compounds.

381

Essential oil / compound	mg/ml	<i>H. lusitanicum</i> (%Mortality) ^a
VB-EO	20	100 ± 0
	LD ₅₀ ^b	6.97 (4.92-8.55)
	LD ₉₀ ^b	16.32 (14.00-20.49)
GH-EO	20	100 ± 0
	LD ₅₀	1.37 (1.15-1.60)
	LD ₉₀	2.87 (2.47-3.54)
Linalool	5	9.73±5.02
Thymol	5	100.0 ± 0.0
	LD ₅₀ ^b	0.18 (0.16-0.20)
	LD ₉₀ ^b	0.27 (0.24-0.31)
Germacrone	5	93.1 ± 4.5
	LD ₅₀ ^b	1.08 (0.70-1.38)
	LD ₉₀ ^b	3.13 (2.66-3.93)
β-Elementone	10	0.0

382 ^aCorrected according to Scheider-Orelli's formula. Values are means of three replicates. ^bDoses needed to
 383 give 50 and 90% mortality (LC₅₀ and LC₉₀) and 95% confidence limits (lower, upper).