Gulmira Özek[1]
Margarita Ishmuratova[2]
Nurhayat Tabanca[3]
Mohammed M. Radwan[4,5]
Fatih Göger[1]
Temel Özek[1]
David E. Wedge[6]
James J. Becnel[7]
Stephen J. Cutler[5,8]
Kemal H. Can Başer[1,9]

[1]Department of Pharmacognosy, Faculty of Pharmacy, Anadolu University, Eskişehir, Turkey
[2]Zhezkazghan Botanical Garden, Zhezkazghan, Karagandinskaya Oblast, Kazakhstan
[3]National Center for Natural Products Research, The University of Mississippi, Oxford, MS, USA
[4]Department of Pharmacognosy, Faculty of Pharmacy, University of Alexandria, Alexandria, Egypt
[5]National Center for Natural Products Research School of Pharmacy, University of Mississippi, Oxford, MS, USA
[6]US Department of Agriculture Agricultural Research Service Natural Products Utilization Research Unit, University of Mississippi, Oxford, MS, USA
[7]USDA-ARS-Center for Medical, Agricultural and Veterinary Entomology, Gainesville, FL, USA
[8]Department of Medicinal Chemistry School of Pharmacy, University of Mississippi, Oxford, MS, USA
[9]Department of Botany and Microbiology College of Science, King Saud University, Riyadh, Saudi Arabia

Research Article

One-step multiple component isolation from the oil of *Crinitaria tatarica* (Less.) Sojak by preparative capillary gas chromatography with characterization by spectroscopic and spectrometric techniques and evaluation of biological activity

Gas chromatographic analysis revealed that the oil of *Crinitaria tatarica* was rich in sabinene (32.1%), β-pinene (8.8%), and two unknown (M+200) compounds (**I**) and (**II**) (21.4% and 3.4%). One-step multiple fractionation of the oil and separation of two unknown constituents were performed using preparative capillary gas chromatography connected to preparative fraction collector system. This combination allowed separation and recover of sufficient quantities of two unknown compounds with high purity from complex oil matrix. Separation conditions (column temperature, cooling temperature, flow rate, injection volume, cut time) were optimized to achieve the best isolation and successful collection. The target compounds were separated from the oil using a HP Innowax (Walt & Jennings Scientific, Wilmington, DE, USA) preparative capillary column in rapid one-step manner with 95.0% purity. Trapping of the isolated compounds in collector system was facilitated by cooling with liquid nitrogen. Structure determination was accomplished by spectral analysis including ultraviolet, nuclear magnetic rezonance, and high-resolution electrospray ionization mass spectrometry. *Z*- (**I**) and *E*-artemidin (**II**) were isolated for the first time from this species. *Crinitaria tatarica* oil and *Z*- (**I**) and *E*-artemidin (**II**) were evaluated for biological activity.

1 Introduction

The genus *Crinitaria* (Asteraceae), encompassing *Crinitaria tatarica* (Less.) Sojak (*russ.* Grudnitsa tatarskaya) is wide-

Correspondence: Dr. Gulmira Özek, Anadolu University, Faculty of Pharmacy, 26470 Eskişehir, Turkey
E-mails: gozek@anadolu.edu.tr, gulmiraozek@gmail.com
Fax: +90 222 3306809.

Abbreviations: CIS, cooled injection system; **HR-ESI-MS**, high-resolution electron spray ionization-mass spectrometry; **PCGC**, preparative capillary gas chromatography; **PFC**, preparative fraction collector

spread in Europe and Central Asia [1]. There are several scientific opinions about *Crinitaria* and close systematic relatives *Galatella* and *Lynosyris* genera. Some scientist considered them as three independent genera [2–4]. However, Merxmuller combined them in one genus *Aster* L. [5]. *Linosyris tatarica* (Less.) C.A. Meyer and *Galatella tatarica* (Less.) Novopokr. are mentioned as synonyms of *C. tatarica* [6]. Other scientists considered *Crinitaria* as subdivision of the genus *Lynosyris* [7]. Novopocrovskyi included *Crinitaria* and *Lynosyris* into the *Galatella* genus [8]. In the last botanical revision, Czerepanov considered *Crinitaria* to be in the *Galatella* genus (*russ.* Soloneshnik) [9]. A search in NAPRALERT database did not reveal any report on the volatiles of *C. tatarica*

1. REPORT DATE **2012**	2. REPORT TYPE	3. DATES COVERED **00-00-2012 to 00-00-2012**

4. TITLE AND SUBTITLE **One-step multiple component isolation from the oil of Crinitaria tatarica (Less.) Sojak by preparative capillary gas chromatography with characterization by spectroscopic and spectrometric techniques and evaluation of biological activity**	5a. CONTRACT NUMBER
	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)	5d. PROJECT NUMBER
	5e. TASK NUMBER
	5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) **US Department of Agriculture,Agricultural Research Service,1600 SW 23rd Drive,Gainesville,FL,32608**	8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
 Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT **Same as Report (SAR)**	18. NUMBER OF PAGES **11**	19a. NAME OF RESPONSIBLE PERSON
a. REPORT **unclassified**	b. ABSTRACT **unclassified**	c. THIS PAGE **unclassified**			

and only one report on the volatiles of its synonym plant *G. tatarica* from Altaisky Krai, Russia, was found [10].

Our work aimed to elucidate further knowledge on the secondary metabolites of *C. tatarica* (from Kazakhstan) volatiles and search for the novel biological properties for the species. In the present study, gas chromatography–flame ionization detector (GC-FID) and gas chromatography–mass spectrometry (GC/MS) analysis of the *C. tatarica* essential oil revealed the presence of two unknown compounds with M+200. Absolute chemical identification requires compounds of high purity for characterization by a combination of spectroscopic techniques, principally MS and nuclear magnetic resonance (NMR) spectroscopy. However, isolation of pure compound can be time consuming and tedious using classical preparative chromatographic procedures for oil fractionation, complicated by the risk of the compounds being lost, altered, or contaminated. Consequently, the isolation of the target constituent(s) from the oil through the use of automated preparative capillary gas chromatography (PCGC) connected to preparative fraction collector (PFC) was considered to be a valuable approach. This combination allowed separation and recover of sufficient quantities of individual compound(s) of high purity quickly from complex oil matrix with minimal prior fractionation. PCGC was reported to have following advantages: efficient harvesting of individual compounds as well as mixtures of several compounds, accelerated separation experiment, simultaneous and multiple separations of several constituents from the complex mixture [11–13]. Previous studies demonstrated that this technique allowed the harvest of pure individual constituents in microgram level from the complex mixtures such as alkanes and fatty acids from archaeological samples and polycyclic aromatic hydrocarbons from environmental materials [14] as well as individual enantiomers [15–17].

In an effort to identify novel classes of natural products with biological activity, the oil of *C. tatarica* and two isolated constituents were evaluated for antifungal activity against plant pathogens *Colletotrichum acutatum*, *Colletotrichum fragariae*, and *Colletotrichum gloeosporioides* and toxicants against *Aedes aegypti* larvae and adults as well. The oil was also subjected to investigation for antioxidant activity (AOA) by three methods: β-carotene bleaching test, Trolox equivalent, and 1,1-diphenyl-2-picrylhydrazyl (DPPH) tests. To the best of our knowledge, the present work is the first report about the composition and biological activity of *C. tatarica* essential oil.

2 Materials and methods

2.1 Plant material

The aerial parts of *C. tatarica* were collected during flowering on June 2009 on the Bektauata Mountain of Karagandinskaya oblast of the Central Kazakhstan. The identity was confirmed by anatomical examination in comparison with the herbarium specimen retained in the Zhezkazgan Botanical Garden (ZBG), Kazakhstan. A voucher specimen (1997.07.12.03.02)

representing this collection was retained in the herbarium of ZBG (Zhezkazgan, Kazakhstan). The plant material was identified by Dr. Margarita Ishmuratova (ZBG, Kazakhstan).

2.2 Chemicals

All organic solvents and reagents used for PCGC were of analytical or chromatographic grade. Anhydrous sodium sulfate (ACS-ISO, for analysis), *n*-hexane (ACS, for analysis), and dimethyl sulfoxide were purchased from Carlo Erba (Italy). Deuterated chloroform (CDCl₃) was purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). DPPH, β-pinene, and butylated hydroxytoluene (BHT) were supplied from Sigma-Aldrich Chemie (Steinheim, Germany). Technical-grade commercial fungicides benomyl, cyprodinil, azoxystrobin, and captan (Chem Service, Inc., West Chester, PA, USA) were used as fungicide standards at 2 mM in 2 µL of 95% ethanol. Sabinene was purchased from Roth (≥96%, Carl Roth GmbH & Co., Karlsruhe, Germany). For the antifungal assay, potato-dextrose broth (Difco, Detroit, MI, USA), glass silica gel thin layer chromatography (TLC) plates with a fluorescent indicator (250 mm, Silica Gel GF Uniplate, Analtech, Inc., Newark, DE, USA), and a moisture chamber (398-C, Pioneer Plastics, Inc., Dixon, KY, USA) were used.

2.3 Essential oil isolation

The essential oil was isolated according to procedure published in European Pharmacopoeia [18]. The dried aerial parts of *C. tatarica* (50.0 g) were hydrodistilled for 3 h using a Clevenger-type apparatus. The oil yield was calculated on dry weight basis, dried over anhydrous sodium sulfate, and stored in sealed vials in refrigerator (4°C) until GC-FID and GC/MS analyses and biological activity testing. The oil was dissolved in *n*-hexane (10%, v/v) to conduct chromatographic determination of its composition.

2.4 Gas chromatography–mass spectrometry (GC/MS)

The whole oil was analyzed by GC-FID and GC/MS techniques before fractionation and isolation of the compounds of interest. The GC/MS analysis was carried out with an Agilent 5975 GC-MSD system (Agilent, USA; SEM Ltd., Istanbul, Turkey). HP-Innowax FSC column (60 m × 0.25 mm, 0.25-µm film thickness, Agilent, Walt & Jennings Scientific, Wilmington, DE, USA) was used with a helium carrier gas at 0.8 mL/min. GC oven temperature was kept at 60°C for 10 min and programmed to 220°C at a rate of 4°C/min, kept constant for 10 min at 220°C, and then programmed to increase at a rate of 1°C/min to 240°C. The oil was analyzed with a split ratio of 40:1. The injector temperature was 250°C. Mass spectra were taken at 70 eV and the mass range was from *m/z* 35 to 450.

2.5 Gas chromatography (GC)

The GC-FID analysis was carried out with capillary GC using an Agilent 6890N GC system (SEM Ltd., Istanbul, Turkey). FID temperature was set at 300°C in order to obtain the same elution order with GC/MS. Simultaneous injection was performed using the same column and appropriate operational conditions.

2.6 Essential oil fractionation and isolation of target compounds

The oil was subjected to fractionation in order to isolate and concentrate the target compounds using PCGC connected to PFC.

2.6.1 PCGC System

The oil containing the compounds of interest was repeatedly injected by an autosampler into GC equipped with a cooled injection system (CIS) and preparative capillary column. The end of the column was connected to a zero dead-volume effluent splitter that diverted a portion (1.0%) of the effluent to the detector while the majority (99.0%) was transferred to and selected fractions were trapped using a PFC unit. Trapping of the isolated compounds in collector vials (cooled with liquid nitrogen) during the course of multiple injections has produced enough quantities to facilitate their subsequent identification with different spectroscopic techniques.

The PCGC system consists of an Agilent 7890 GC (Agilent; SEM Ltd.), equipped with a FID and 5975 MSD with Triple-Axis Detector, Agilent G 4513 autoinjector, integrated with a Gerstel CIS (Gerstel, Mülheim an der Ruhr, Germany; SEM Ltd., Istanbul, Turkey), a zero dead-volume effluent splitter, and a preparative trapping device. The preparative device consists of an eight-port zero dead-volume valve in a heated interface (≈300°C) and seven 200-μL glass U-tube traps (six sample traps and one waste trap) supported in liquid nitrogen cooled (–30°C) units. The Gerstel multicolumn switching system has been used. The autoinjector, CIS, and trapping device are programmable and controlled by Gerstel modular analytical systems (MAS) using Maestro software (Gerstel, Mülheim an der Ruhr, Germany).

2.6.2 Conditions of PCGC procedure

Good PCGC analytical conditions were required for all analyses in the present work. Thus, different flow rates, column temperatures, PFC transfer and PFC distribution temperatures, cooling temperatures, and injection volumes were tested and optimized. Here, we present the PCGC conditions in which the best separation and successful collection were achieved.

The target compounds were separated from the oil using a HP Innowax (30 m × 0.53 mm × 1.0 μm film thickness, USA) preparative capillary column with helium carrier gas (12 min at 12 mL/min, average flow rate 89.532 cm/s). GC oven temperature was kept 0 min at 140°C and programmed to 240°C at a rate of 50°C/min, and then kept constant for 10 min at 240°C. Total time of analysis was 12 min. The oil was analyzed in splitless mode. The injector temperature was 250°C. Mass spectra were taken at 70 eV and the mass range was from m/z 35 to 450. PFC transfer and PFC distribution temperatures were kept at 220°C and 230°C, respectively. PFC trap cooled with liquid nitrogen was kept at –30°C. Retention time selection intervals (cut time) were selected as 8.50–9.90 min and 10.50–11.35 min for unknown constituents (**I**) and (**II**), respectively. Injector volume was 3 μL.

After PCGC isolation, the U-tubes containing the trapped unknown compounds were detached, and the contents were recovered by addition of *n*-hexane (1 mL) and transferred to 2-mL glass vials. An aliquot (50 or 100 μL) was removed and transferred to a GC-FID and GC/MS autosampler vial for determination of purity and yield.

2.7 Identification and quantification of compounds

Compounds were identified by comparison of the chromatographic peaks retention times with those of authentic compounds analyzed under the same conditions, and by comparison of the retention indices (RI) (as Kovats indices) with literature data. Comparisons of MS fragmentation patterns with those of standards and mass spectrum database search were performed using the Wiley GC/MS Library (Wiley, New York, NY, USA), MassFinder software 3.0 (Dr. Hochmuth Scientific Consulting, Hamburg, Germany) [19], Adams Library [20], and NIST Library. Confirmation was also achieved using the in-house "Başer Library of Essential Oil Constituents" database, obtained from chromatographic runs of pure compounds performed with the same equipment and conditions. A C9–C20 *n*-alkane standard solution (Fluka, Buchs, Switzerland) was used to spike the samples for the determination of chromatographic RI. Percent composition was obtained for each constituent on the basis of GC-FID analyses of the oil. One-dimensional and two-dimensional NMR spectra of compounds **I** and **II** were recorded in CDCl$_3$ on a Varian AS 400 spectrometer (Agilent Technologies, Santa Clara, CA, USA). The ultraviolet (UV) spectrum was obtained on a Hewlett-Packard 8453 UV/Vis spectrometer (Agilent Technologies, Santa Clara, CA 95051, USA). High-resolution electrospray ionization-mass spectroscopy (HR-ESI-MS) was obtained on an Agilent Series1100 SL mass spectrometer.

2.8 Biological assays

The oil and compounds **I** and **II** were evaluated for antifungal activity against plant pathogens [21] and toxicants against *Ae. Aegypti* larvae and adults [22] using procedures described previously. AOA of the oil was evaluated on three tests: TEAC [23], 1,1-diphenyl-2-picrylhydrazyl (DPPH•) radical scavenging test [24], and inhibition of β-carotene/linoleic acid cooxidation [25, 26], which were carried out using procedures described previously.

Table 1. Chemical composition of the essential oil of *Crinitaria tatarica*

No.	RRI[a]	RRI[b]	Compound	(%)[c]	ID method
1	1 032	1 032 [56]	α-Pinene	3.4	d, e
2	1 035	1 035 [56]	α-Thujene	1.6	d, e
3	1 076	1 085 [56]	Camphene	0.3	d, e
4	1 118	1 118 [56]	β-Pinene	8.8	d, e
5	1 132	1 132 [56]	Sabinene	32.1	d, e
6	1 174	1 156 [56]	Myrcene	4.3	d, e
7	1 188	1 179 [56]	α-Terpinene	1.3	d, e
8	1 195	1 200 [57]	Dehydro-1,8-cineole	tr	d, e
9	1 203	1 205 [56]	Limonene	1.5	d, e
10	1 218	1 209 [58]	β-Phellandrene	0.2	d, e
11	1 244	1 237 [59]	2-Pentyl furan	tr	d, e
12	1 246	1 230 [56]	(Z)-β-Ocimene	1.3	d, e
13	1 255	1 256 [56]	γ-Terpinene	2.2	d, e
14	1 260	1 252 [60]	(E)-β-Ocimene	4.4	d, e
15	1 279	1 279 [56]	*p*-Cymene	0.3	d, e
16	1 289	1 283 [56]	Terpinolene	0.5	d, e
17	1 378	1 368 [61]	*cis*-Alloocimene	tr	d, e
18	1 413		Rose furan	0.1	e
19	1 429	1 331 [58]	Perillen	tr	d, e
20	1 449	1 438 [62]	β-Thujone	tr	d, e
21	1 457		Hexyl-3-methyl butyrate (= *Hexyl isovalerate*)	tr	d, e
22	1 460		2,6-Dimethyl-1,3(E),5(E),7-octatetraene	tr	e
23	1 474	1 474 [63]	*trans*-Sabinene hydrate	0.5	d, e
24	1 485	1 480 [62]	Citronellal	tr	d, e
25	1 494		(Z)-3-Hexenyl 3-methylbutyrate	0.1	d, e
26	1 497	1 497 [60]	α-Copaene	tr	d, e
27	1 498		(E)-β-Ocimene epoxide	tr	d, e
28	1 499		α-Campholene aldehyde	tr	e
29	1 532	1 532 [56]	Camphor	tr	d, e
30	1 542		Modhephene	tr	e
31	1 554	1 556 [56]	Linalool	0.1	d, e
32	1 556	1 556 [64]	*cis*-Sabinene hydrate	0.3	d, e
33	1 560	1 560 [65]	*cis*-*p*-Menth-2-en-1-ol	0.2	d, e
34	1 579	1 622 [66]	*trans*-*p*-Menth-2-en-1-ol	tr	d, e
35	1 586	1 585	Pinocarvone	0.1	d, e
36	1 586	1 571	Bornyl acetate	0.7	d, e
37	1 611	1 616	Terpinen-4-ol	2.5	d, e
38	1 612	1 604 [60]	β-Caryophyllene	tr	d, e
39	1 648	1 645	Myrtenal	0.1	d, e
40	1 660	1 645	Pulegone	tr	d, e
41	1 663	1 632	(Z)-β-Farnesene	0.1	d, e
42	1 669	1 646 [66]	*trans*-Pinocarveol	0.1	d, e
43	1 670	1 658	Citronellyl acetate	0.8	d, e
44	1 683	1 680	*trans*-Verbenol	tr	d, e
45	1 687	1 687	α-Humulene	0.1	d, e
46	1 689	1 741	*trans*-Piperitol	tr	d, e
47	1 695		(E)-β-Farnesene	tr	d, e
48	1 706	1 706 [64]	α-Terpineol	0.1	d, e
49	1 726	1 716 [60]	Germacrene D	0.1	d, e
50	1 737	1 728 [60]	(Z,E)-α-Farnesene	tr	d, e
51	1 741		β-Bisabolene	tr	d, e
52	1 755	1 742 [60]	Bicyclogermacrene	0.3	d, e
53	1 758		*cis*-Piperitol	tr	d, e
54	1 758	1 749 [60]	(E,E)-α-Farnesene	tr	d, e
55	1 765	1 742 [65]	Geranyl acetate	0.8	d, e
56	1 772	1 752 [65]	Citronellol	tr	d, e
57	1 773	1 764 [60]	δ-Cadinene	tr	d, e

Table 1. Continued.

No.	RRI[a]	RRI[b]	Compound	(%)[c]	ID method
58	1 776	1 766 [60]	γ-Cadinene	tr	d, e
59	1 780	1 768 [66]	β-Sesquiphellandrene	0.8	d, e
60	1 802		Cumin aldehyde	tr	e
61	1 803	1 790 [66]	Myrtenol	0.1	d, e
62	1 810	1 790 [57]	*p*-Mentha-1,5-dien-7-ol	tr	d, e
63	1 855	1 852 [66]	Geraniol	tr	d, e
64	1 945		1,5-Epoxy-salvial(4)14-ene	tr	e
65	1 948		*trans*-Jasmone	0.1	d, e
66	2 008	2 008 [63]	Caryophyllene oxide	0.1	d, e
67	2 030	2 042 [67]	Methyl eugenol	0.1	d, e
68	2 031	2 016 [68]	Salvial-4(14)-en-1-one	tr	d, e
69	2 073		*p*-Mentha-1,4-dien-7-ol	tr	d, e
70	2 100	2 068 [69]	Cumin alcohol	0.1	d, e
71	2 131	2 132 [70]	Hexahydrofarnesyl acetone	tr	d, e
72	2 146	2 150 [64]	Spathulenol	1.4	d, e
73	2 183		γ-Decalactone	0.5	e
74	2 190		Zingiberenol	tr	e
75	2 232	2 233 [71]	α-Bisabolol	0.1	d, e
76	2 247		*trans*-α-Bergamotol	0.2	d, e
77	2 255	2 231 [66]	α-Cadinol	tr	d, e
78	2 257	2 231 [66]	β-Eudesmol	tr	d, e
79	2 260	2 218 [72]	Intermedeol	0.2	d, e
80	2 266		Guaia-3,9-dien-11-ol	0.2	e
81	2 268		Torilenol	tr	e
82	2 375	2 396 [73]	Eudesma-4(15),7-dien-1β-ol	tr	d, e
83	2 415		6-Dodecen-4-olide	0.2	e
84	2 620	2 606 [74]	Phytol	0.2	d, e
85	2 768		*cis*-Artemidin (Unknown I)	21.4	f
86	2 875		*trans*-Artemidin (Unknown II)	3.4	f
87	2 931		Hexadecanoic acid	0.1	d, e
Total				98.5	
Monoterpene hydrocarbons				62.2	
Oxygenated monoterpenes				6.6	
Sesquiterpene hydrocarbons				1.4	
Oxygenated sesquiterpenes				2.2	
Isocoumarins				24.8	
Others				1.3	

a)Relative retention indices calculated against *n*-alkanes (C9-C20) on HP-Innowax column.

b)Relative retention indices reported in literature.

c)Percentage calculated from FID data; tr, trace ($<$0.1%).

d)Identification based on retention index of genuine compounds on the HP-Innowax column.

e)Identification on the basis of computer matching of the mass spectra from Başer, Adams, MassFinder, Wiley, and NIST libraries.

f)Identification on the basis of NMR and mass spectra.

3 Results and discussion

3.1 Composition of the oil

The present work is the first report on the composition and biological activity of the essential oil of *C. tatarica*. The oil of *C. tatarica* was investigated by means of GC-FID and GC/MS techniques in order to determine its qualitative and quantitative profiles. Hydrodistillation of the aerial parts of *C. tatarica* gave yellowish oil with a specific odor in 0.84% yield. The list of detected compounds with their relative percent-

ages, RI, and percentages of compound classes is given in Table 1 in order of their elution on the HP-Innowax FSC column. GC/FID and GC/MS analysis of the oil resulted in 87 constituents representing 98.5% of the oil with sabinene (32.1%), β-pinene (8.8%), and two unknown (M+200) compounds **I** (21.4%) and **II** (3.4%) (Fig. 1). Monoterpene hydrocarbons, oxygenated monoterpenes, sesquiterpene hydrocarbons, oxygenated sesquiterpenes, and isocoumarins were the main groups present in the oil. Monoterpene hydrocarbons were the most abundant among these groups representing 62.2%, followed by oxygenated monoterpenes 6.6%, and the
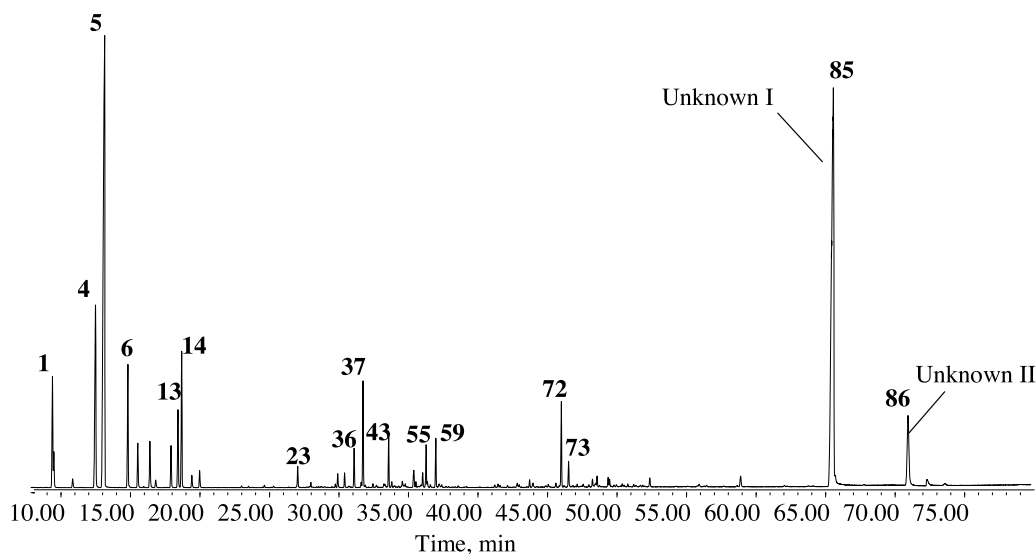
Figure 1. Chromatographic profile (GC/MS) of the essential oil of *Crinitaria tatarica* on analytical HP Innowax column. The peak numbering is given in agreement with Table 1.

sesquiterpenes were presented in scarce amounts (1.4 and 2.2%). The obtained data were evaluated taking into account a report on the oil of *G. tatarica* (collected in Altay Krai/ Russia), which was mentioned as the synonym of *C. tatarica* and α-pinene (42.1%), β-myrcene (12.6%), β-pinene (9.2%), and *trans*-β-ocimene (7.5%) were reported to be the main constituents [10]. The report by Koroljuk seems to show a different composition than the oil composition of *C. tatarica* studied in this present work.

Fractionation of the oil and isolation of two unknown constituents have been performed using PCGC connected with PFC. Selected target regions for compounds **I** (between 8.15 min and 9.90 min) and **II** (between 10.50 min and 11.35 min) are shown shaded on the chromatogram obtained on preparative column (Fig. 2). The target peak **I** was trapped into the first glass collector, while the following peak **II** was trapped into the second one. Both of the glass collectors were cooled by liquid nitrogen (–30°C) to prevent of the trapped constituents from evaporation during isolation procedure. Fraction recoveries were 0.4 mg/injection and 0.1 mg/injection for unknown constituents **I** and **II**, respectively. We demonstrated that it is possible to collect selected components from numerous repeat injections of the oil sample to permit increased mass recovery from an external cryotrap collection device. Peak retention times remained reproducible (<0.3 s) over the repeated injections and switching events. This methodology was utilized to confirm peak identity. The isolated compounds were eluted from the trapping capillaries with *n*-hexane into vials after each set of collections and subsequently controlled for purity by GC-FID and GC/MS. PCGC have generated far superior purity: the target constituents **I** and **II** have been isolated from the oil with 95.0% purity (Fig. 3). Precise heart-cutting allowed effective separation of rather big peak of constituent **I** (21.4%) as well as minor compound **II** (3.4%) from the whole oil in single step. Trapping of

the isolated compounds during the course of multiple injections produced sufficient quantities to facilitate subsequent NMR spectroscopic analysis as well as mass spectrometry.

Compounds **I** and **II** were isolated as colorless oils. Their UV spectra showed typical isocoumarin derivative profile [27]. They were identified as *Z*-artemidin (**I**) and *E*-artemidin (**II**) by spectroscopic analysis including HR-ESI-MS and H-NMR (^{1}H-NMR, ^{13}C-NMR, DEPT-135, COSY, HMQC, and HMBC) and confirmed by comparing their physical data with those reported in literatures [28, 29]. Multiple component isolation from *C. tatarica* oil by the PCGC resulted with *Z*-artemidin (12.0 mg, yield 0.024%) and *E*-artemidin (1.8 mg, yield 0.0036%).

A literature search revealed that *E*-artemidin was earlier isolated from *Anthemis fuscata* Brot. by Bohlmann [30]. Greger et al. isolated *cis*-artemidin from *Artemisia dracunculus* L. and formulated it as 3-(1*E*-butenyl)-isocoumarin on the basis of spectral data [31–33]. Artemidin (stereochemistry not defined) was also isolated by Mallabaev and Sidjakin [34] and eventually synthesized by Batu [35]. It was reported, that *Z*- and *E*-isomers of artemidin occurred in distinct ratios in aerial (10:7) as well as underground organs (3:1) of *A. dracunculus* [36]. The different accumulation rates of different isomers appeared to be the result of specific enzymatic activities rather than a mere consequence of isolation procedures. For instance, *Chamaemelum fuscatum* (Brot.) Vasc. was reported to accumulate only the *E*-isomer of artemidin in its roots [30, 37]. In the present work, *cis*-artemidin and *trans*-artemidin have been reported in *Crinitaria* genus for the first time. The ratio of the *cis* and *trans* isomers in the oil of *C. tatarica* was found as 7:1.

Z-Artemidin [3-(3-butenyl)-isocoumarin] (**I**): colorless oil; UV (MeOH) λ_{max} 224, 334 nm; ^{1}H-NMR (CDCl$_3$, 400 MHz) δ_H 1.13 t (*J* = 7.6 Hz, H$_3$-4'), 2.68 m (H$_2$–3'), 5.82 td (*J* = 7.6, 12 Hz, H-2'), 5.93 d (*J* = 12 Hz, H-1'), 6.35 s (H-4), 7.38 d
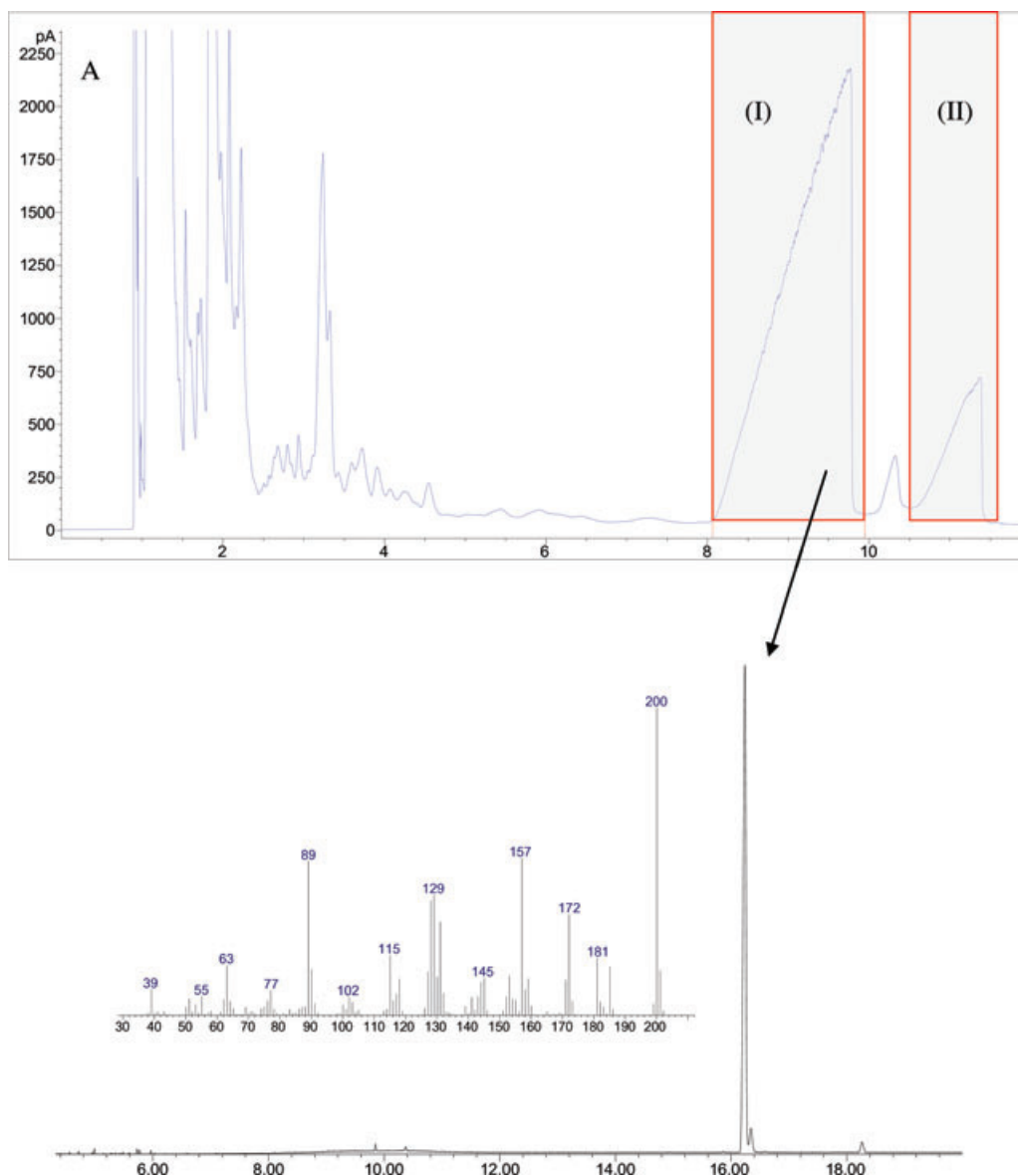
Figure 2. (A) Preparative gas-chromatographic separation of the target constituents **I** and **II** from the essential oil of *Crinitaria tatarica* on preparative column using PFC system. The shaded areas represent regions selected for trapping by PCGC technique: **I**: between 8.15 min and 9.90 min; **II**: between 10.50 min and 11.35 min. (B) Control of the isolated unknown constituent **I** for purity using GC/MS and GC-FID.

(J = 8 Hz, H-5), 7.46 t (J = 7.6 Hz, H-7), 7.66 dt (J = 1.2, 7.6 Hz, H-6), 8.25 d (J = 8 Hz, H-8); ^{13}C-NMR (CDCl$_3$, 100 MHz) δc 162.4 (C-1), 153.9 (C-3), 140.8 (C-2), 137.8 (C-10), 134.9 (C-6), 129.8 (C-8), 128.1 (C-7), 125.8 (C-5), 120.4 (C-9), 120.3 (C-1′), 106.1 (C-4), 23.2 (C-3′), 14.4 (C-4′); GC/MS *m/z* (rel. int.): 201 (14), 200 (100), 185 (16), 181 (18), 172 (37), 157 (53), 131 (32), 129 (42), 128 (39), 118 (14), 115 (21), 89 (54), 63 (18); EI-MS *m/z* (rel. int.): 200 [M, 100%]$^+$; HR-ESI-MS *m/z* 223.0767 (calculated for C$_{13}$H$_{12}$O$_2$Na: 223.0735).

E-Artemidin [3-(3-butenyl)-isocoumarin] (**II**): colorless oil; UV (MeOH) λ_{max} 224, 342 nm; ^{1}H-NMR (CDCl$_3$, 400 MHz) δ_H 1.10 t (J = 7.6 Hz, H$_3$-4′), 2.26 m (H$_2$-3′), 6.04 td (J = 5.6, 15.6 Hz, H-2′), 6.27 s (H-4), 6.68 d (J = 15.6, H-1′), 7.37 d (J = 8 Hz, H-5), 7.46 dt (J = 1.2, 7.6 Hz, H-7), 7.66 dt

(J = 1.2, 8 Hz, H-6), 8.25 dd (J = 0.8, 7.6 Hz, H-8); GC/MS *m/z* (rel. int.): 201 (14), 200 (100), 185 (13), 181 (14), 172 (27), 157 (50), 131 (27), 129 (39), 128 (35), 118 (11), 115 (18), 89 (50), 63 (16); EI-MS *m/z* (rel. int.): 200 (M, 100%)$^+$; HR-ESI-MS *m/z* 223.0764 (calculated for C$_{13}$H$_{12}$O$_2$Na: 223.0735).

3.2 Biological activity

Isocoumarins are a class of naturally occurring lactones with a broad spectrum of biological activity [38]. This group exhibits antifungal [39], antitumor [40, 41], antiallergic, antibacterial [42], antiinflammatory, antiangiogenic [43], antidiabetic, phytotoxic, and immunomodulatory activities
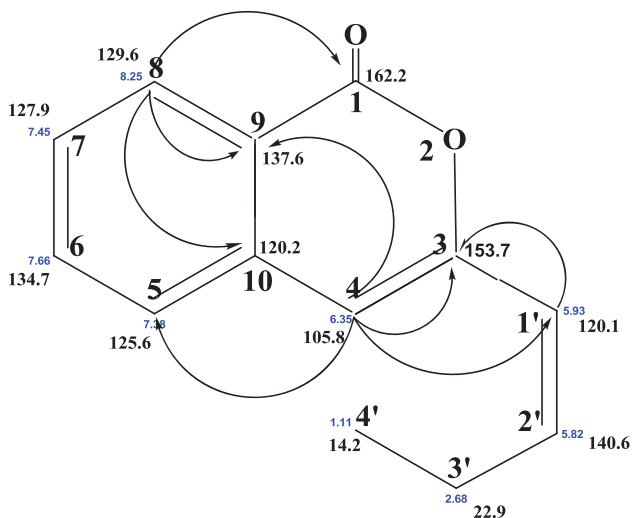
Figure 3. Structure of *cis*-artemidin.

Figure 4. Growth inhibition of *Botrytis cinerea*, *Fusarium oxyspo-rum*, and *Phomopsis viticola* using a 96-well microtiter format in a dose-response to *cis*-artemidin (**I**) and the commercial fungicide standard captan.

[40, 41, 44–46]. Among isocoumarins, capillarin, and cercophorin A have been shown to possess antifungal activity against *Colletotrichum fragariae*, *Colletotrichum gloeosporioides*, *Colletotrichum acutatum*, *Sordaria fimicola,* and *Ascobolus furfuraceus* [39, 44] while capillarin also acts as an insect antifeedant against larva of the cabbage butterfly *Pieris rapae crucivora* [47]. Oosponol and oospolactone exhibit antifungal against *Alternaria maritime*, *Cochliobolus miyabeanus*, *Fusarium splendens*, *Giberella zeae*, *Helminthosporium maydis*, *Penicillium expansum* as well as antibiotic activities [48]. Some isocoumarins exhibited phytotoxic effect for corn, crabgrass, and soybean, and on Barnyard grass and spiny amaranth [49]. Bioassays with several 3-butylisocoumarins of *Artemisia dracunculus* and *Chamaemelum mixtum* (L.) All. demonstrated remarkable antifungal activities [32, 33, 37, 50]. Artemidin and several naturally occurring derivatives demonstrated antifungal activities in a germ-tube inhibition test against a susceptible strain of rice blast fungus *Pyricularia grisea*. It was proved that 3-butyl side-chain is a prerequisite for high antifungal activity [51].

A literature data about antifungal potential of isocoumarins [39, 44] prompted us to evaluate the activity of the isolated *cis-* and *trans-*artemidin as well as the whole oil. Antifungal activity of *C. tatarica* oil was observed against the strawberry anthracnose causing fungal plant pathogens *Colletotrichum acutatum*, *Colletotrichum fragariae,* and *Colletotrichum gloeosporioides* using the direct overlay bioautography assay. Direct bioautography of *C. tatarica* oil on silica gel TLC revealed 5.5 ± 0.71 mm antifungal zone at 80 μg/spots against the plant pathogens *Colletotrichum acutatum*, *Colletotrichum fragariae*, and *Colletotrichum gloeosporioides*. Antifungal activity was evident by the presence of clear zones with a dark background where fungal mycelia or reproductive stroma was not present on the TLC plate. TLC profiles of *C. tatarica* oil in *n*-hexane/diethyl ether (8:2, v/v) were subsequently tested against *Colletotrichum* spp. and R_f
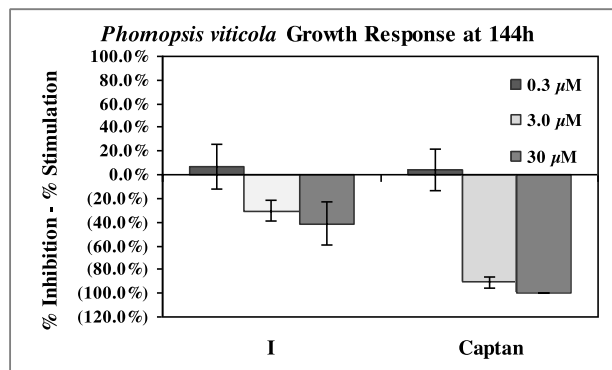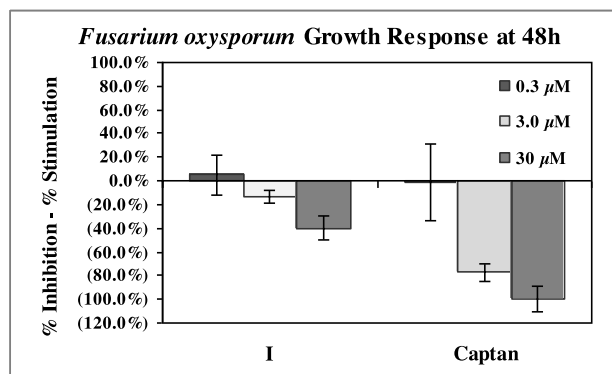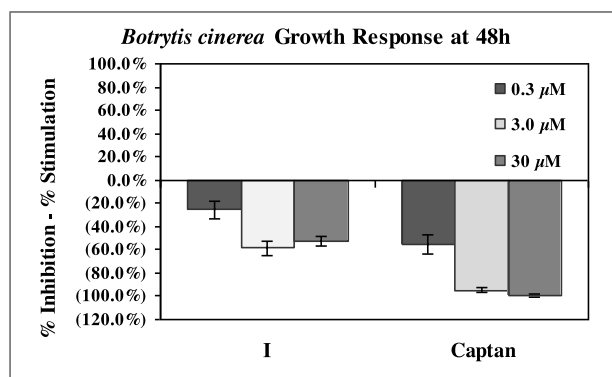
value of 0.41 demonstrated nonselective antifungal activity. Subsequent direct bioautography of the compounds **I** and **II** appeared to correspond with the R_f values of 0.41. Following the procedure, compounds **I** and **II** evaluated using a 96-well microbioassay system in a dose response format for activity against *Colletotrichum acutatum*, *Colletotrichum fragariae*, *Colletotrichum gloeosporioides*, *B. cinerea*, *Phomopsis obscurans*, *P. viticola,* and *F. oxysporum*. Secondary screening of active compounds using this microbioassay system showed that compound **I** possessed its highest activity at 3.0 and 30 μM and inhibited the growth of *B. cinerea* by 58.7 and 52.8%, respectively, after 48-h exposure (Fig. 4) and no activity was observed against other tested fungi. We attempted

Table 2. TEAC Trolox equivalent antioxidant capacity test results

TEAC Trolox equivalent antioxidant capacity after 30 min (mM)				
β-Pinene 10 mg/mL	Sabinene 10 mg/mL	*Z*-Artemidin 10 mg/mL	Essential oil, 10 mg/mL	BHT 10 mg/mL
0.17	1.00	0.28	0.30	2.42

to compare results on antifungal activity of the isolated isocoumarins with others previously reported in literature. So, *Z*-artemidin demonstrated growth inhibition (40–60%) of *B. cinerea* in a dose-response manner similar to capillarin isolated from *A. dracunculus* [39]. At the highest concentration of 30 μM, *Z*-artemidin demonstrated 41.2 and 24.8% fungal growth inhibition against *P. viticola* and *P. obscurans*, respectively, after 144-h exposure (Fig. 4). At 3.0 μM, compound **I** showed 40.0% fungal growth inhibition against *F. oxysporum* after 48-h exposure (Fig. 4). Captan used as a standard fungicide is well known as a multisite inhibitor fungicide with no systemic activity and is used as a protectant fungicide to prevent anthracnose diseases in fruits and ornamentals. Captan at 30 μM showed 99.3, 100, 98.4, and 100% activity against *B. cinerea*, *P. viticola*, *P. obscurans* and *F. oxysporum*, respectively. Compound II demonstrated no antifungal activity against any of the fungi. That is to say, the stereochemistry had an influence on bioactivity of compounds. In literature, there are reports on different biological activities of *cis-* and *trans-*isomers: anethole [52], zearalenone [53], α,β-dimethylstilbene [54], and 4-aminocrotonic acid [55].

In searching for new bioactive, environmentally friendly and biodegradable natural insecticides from botanical sources, *C. tatarica* oil and *Z*-artemidin (**I**) were evaluated to high-throughput larval bioassay and adult toxicity against *Ae. aegypti*. *Crinitaria tatarica* oil was able to kill 100% *Ae. egypti* larvae at 500 and 250 ppm and 30% mortality was observed at the concentration of 125 ppm. There was no mortality at the lower concentration (62.5, 31.25, 15.625 ppm). The unique compound, *Z*-artemidin (**I**), with 21.4% of the total oil in *C. tatarica* oil has been investigated in *Ae. aegypti* larvae. *Z*-artemidin (**I**) showed 100% mortality at 500, 250, and 125 ppm and 30% mortality at 62.5 ppm. There was low adult mortality observed for the oil and compound (**I**) at the screening rate of 3.125 ppm per insect. The results described here for *C. tatarica* oil and *Z*-artemidin (**I**) determined that these samples appear to have limited potential for mosquito control. *E*-artemidin (**II**) could not be evaluated in mosquito bioassays due to an insufficient quantity for the assay.

Evaluations for AOA have revealed that the oil, β-pinene, and *Z*-artemidin demonstrated weak inhibitory activity in 1,1-diphenyl-2-picrylhydrazyl (DPPH•) radical scavenging and inhibition of β-carotene/linoleic acid co-oxidation test-systems. In TEAC test, only sabinene demonstrated activity close to BHT activity (Table 2). There was no possibility to test *E*-artemidin for AOA because of its low yield from the oil.

4 Conclusion

A fast and accurate one-step separation by PCGC using a PFC system of two components, namely, *Z*- and *E*-artemidin with high purity (95%) from the essential oil of *C. tatarica* with subsequent structural elucidation and qualitative analysis was carried out. The present work is the first contribution into chemical composition and antifungal, mosquito larvicidal, adulticidal, and antioxidant activities of the oil of *C. tatarica*.

The authors have declared no conflict of interest.

5 References

[1] Pavlov, N. V., *Flora of Kazakhstan*, Izd. AN KazSSR, Alma-Ata 1956, p. 754.

[2] Boisser, P. E., *Flora Orientais. Suppl.*, Lyon, Geneva, Basel 1898.

[3] Candolle, D., *Asteroideae. Prodromus Systematics Naturalis Regni Vegetabilis*, Treuttel & Wurtz, Paris, France 1836, pp. 241–342.

[4] Ledebour, C. F., *Flora Rossica*, Sumptibus Librariae E. Schweizerbart, Stuttgartiae 1844, p. 936.

[5] Merxmuller, H., Schreiber, A., Yeo, P. F., *Aster L. In: Flora Europaea*, vol. 4, Plantaginaceae to Compositae (and Rubiaceae), T. G. Tutin (Ed), Cambridge University Press, London 1976, pp. 112–116.

[6] Govaerts, R., *World Checklist of Selected Plant Families Database in ACCESS: 1-216203. The Board of Trustees of the Royal Botanic Gardens, Kew* 2003.

[7] Kemularia-Natadze, L. M., *Vestn. Tifl. Bot. Garden* 1927, *3*, 123–148.

[8] Novopokrovskyi, I., *Bot. Mater.* 1949, XI, 211–233.

[9] Czerepanov, S. K., *Sosudistye Rastenia SSSR*, Nauka, Leningradskoe Otd-nie, Leningrad 1981.

[10] Koroljuk, E. A., Pokrovskyi, L. M., Tkachev, A. V., *Khim. Rast. Syr. (rus.)* 2002, *1*, 5–18.

[11] Macnamara, K., Hoffmann, A., *Gas Chromatographic Technology in Analysis of Distilled Spirits*, Elsevier, Amsterdam 1998.

[12] Rühle, C., Eyres, G. T., Urban, S., Dufourc, J.-P., Morrison, P. D., Marriott, P. J., *J. Chromatogr. A* 2009, *1216*, 5740–5747.

[13] Eglington, T. I., Aluwihare, L. I., Bauer, J. E., Druffel, E. R. M., Mcnichol, A. P., *Anal. Chem.* 1996, *68*, 904–912.

[14] Mandalakis, M., Gustafsson, Ö., *J. Chromatogr. A* 2003, *996*, 163–172.

[15] Schurig, V., *J. Chromatogr. A* 2001, *906*, 275–299.

[16] Bicchi, C., D'amato, A., Rubiolo, P., *J. Chromatogr. A* 1999, *843*, 99–121.

[17] Maier, N. M., Franco, P., Lindner, W., *J. Chromatogr. A* 2001, *906*, 3–33.

[18] Pharmacopoeia, E. Council of Europe, Strasbourg, France 2005.

[19] Hochmuth, D. H., MassFinder-4, Hochmuth Scientific Consulting, Hamburg, Germany 2008.

[20] Adams, R. P., *Identification of Essential Oil Components by Gas Chromatography/Mass Spectrometry*, Allured Publishing Corporation, Carol Stream, IL 2007.

[21] Wedge, D. G., Nagle, N. G., *J. Nat. Prod.* 2000, *63*, 1050–1054.

[22] Tabanca, N., Bernier, U. R., Tsikolia, M., Becnel, J. J., Sampson, B., Werle, C., Demirci, B., Baser, K. H. C., Blythe, E. K., Pounders, C., Wedge, D. E., *Nat. Prod. Commun.* 2010, *5*, 1409–1415.

[23] Papandreou, M. A., Kanakis, C. D., Polissiou, M. G., Efthimiopoulos, S., Cordopatis, P., Margarity, M., Lamari, F. N., *J. Agric. Food Chem.* 2006, *54*, 8762–8768.

[24] Gyamfi, M. A., Yonamine, M., Aniya, Y., *Gen. Pharmacol.* 1999, *32*, 661–667.

[25] Oomah, B., Mazza, G., *J. Agric. Food Chem.* 1996, *44*, 1746–1750.

[26] Velioglu, Y., Mazza, G., Gao, L., Oomah, B. D., *J. Agric. Food Chem.* 1998, *46*, 4113–4117.

[27] Engelmeier, D., Hadacek, F., Hofer, O., Kutschera, G. L., Nagl, M., Wurz, G., Greger, H., *J. Nat. Prod.* 2004, *67*, 19–25.

[28] Greger, H., Bohlmann, F, Zdero, C., *Phytochemistry* 1977, *16*, 795–796.

[29] Chatterjea, J. N., Bhakta, C., Mukherjee, S. K., *Indian J. Chem.* 1981, *20B*, 992–993.

[30] Bohlmann, F., Zdero, C., *Chem. Ber.* 1970, *103*, 2856–2859.

[31] Greger, H., *Phytochemistry* 1979, *18*, 1319–1322.

[32] Greger, H., *Phytochemistry* 1979, *18*, 1319–1322.

[33] Greger, H., in: Margaris, N., Koedam, A., Vokou, D. (Eds.), *Aromatic Plants: Basic and Applied Aspects*, Martinus Nijhoff Publishers, The Hague, The Netherlands 1982, pp. 153–163.

[34] Mallabaev, A., Yagudaev, M. R., Saitbaeva, I. M., Sidyakin, G. P., *Chem. Nat. Comput.* 1970, *6*, 549–551.

[35] Batu, G., Stevenson, R., *J. Org. Chem.* 1980, *45*, 1532–1534.

[36] Aglarova, A. M., Zilfikarov, I. N., Severtseva, O. V., *Pharm. Chem. J.* 2008, *42*, 81–86.

[37] Bohlmann, F., Kleine, K.-M., *Chem. Ber.* 1962, *95*, 39–46.

[38] Barry, R. D., *Chem. Rev.* 1964, *64*, 229–260.

[39] Meepagala, K. M., Sturtz, G., Wedge, D. E., *J. Agric. Food Chem.* 2002, *50*, 6989–6992.

[40] Pochet, L., Frederick, R., Masereel, B., *Curr. Pharm. Design* 2004, *10*, 3781–3796.

[41] Canedo-Hernandez, L., Acebal-Sarabia, C., Garcia-Gravalos, D., *Int. Appl.* WO 9627594, 1996.

[42] Rama, N. H., Iqbal, R., Zamani, K., Saeed, A., Iqbal, M. Z., Chaudary, M. I., *Indian J. Chem.* 1998, *37B*, 365–369.

[43] Nakashima, T., Hirano, S., Agata, N., Kumagai, H., Isshiki, K., Yoshioka, T., Ishizuka, M., Maeda, K., Takeuchi, T., *J. Antibiotics* 1999, *52*, 426–428.

[44] Whyte, A. C., Gloer, J. B., Scott, J. A., Malloch, D., Cercophorins, A.-C., *J. Nat. Prod.* 1996, *59*, 765–769.

[45] Bauta, W. E., Lovett, D. P., Cantrell, W. R., Burke, J. B. D., *J. Org. Chem.* 2003, *68*, 5967–5973.

[46] Hirano, S., Mase, T., Agata, N., Iguchi, H., Matsumoto, N., Yoshioka, T., Tone, H., Kumagai, H., Ishizuka, M., Takeuchi, T., Patent WO 9748/693, 1997.

[47] Yano, K., *J. Agric. Food Chem.* 1983, *31*, 667–668.

[48] Nozawa, K., Yamada, M., Tsuda, Y., Kawai, K., Nakajima, S., *Chem. Pharm. Bull.* 1981, *29*, 2689–2691.

[49] Hallock, Y. F., Clardy, J., Kenfield, D. S., Strobel, G., *Phytochemistry* 1988, *27*, 3123–3125.

[50] Harada, R., Noguchi, S., Sugiyama, N., *J. Chem. Soc. Jpn., Pure Chem. Sect.* 1960, *81*, 654–658.

[51] Engelmeier, F. H., Hofer, O., Lutz-Kutschera, G., Nagl, M., Wurz, G., Greger, H., *J. Nat. Prod.* 2004, *67*, 19–25.

[52] Boissier, J. R., Simon, P., Le Bourhis, B., *Therapie* 1967, *22*, 309–323.

[53] Mirocha, C. J., Pathre, S. V., Behrens, J., Schauerhamer, B., *Appl. Environ. Microbiol.* 1978, *35*, 986–987.

[54] Solmssen, U. V., *Chem. Rev.* 1945, *37*, 481–598.

[55] Johnston, G. a. R., Curtis, D. R., Beart, P. M., Game, C. J. A., Mcculloch, R. M., Twitchin, B., *J. Neurochem.* 1975, *24*, 157–160.

[56] Abdelwahed, A., Hayder, N., Kilani, S., Mahmoud, A., Chibani, J., Hammami, M., Chekir-Ghedira, L., Ghedira, K., *Flavour Fragrance J.* 2006, *21*, 129–133.

[57] Vernin, G., Merad, O., Vernin, G. M. F., Zamkotsian, R. M., Parkanyi, C., in: Charalambous, G. (Ed.), *Food Flavors: Generation, Analysis and Process Influence*, Elsevier Science, Amsterdam 1995, pp. 147–205.

[58] Chung, T. Y., Eiserich, J. P., Shibamoto, T., *J. Agric. Food Chem.* 1993, *41*, 1693–1697.

[59] Chen, G., Song, H., Ma, C., *Flavour Fragrance J.* 2009, *24*, 186–191.

[60] Shimizu, Y., Imayoshi, Y., Kato, M., Maeda, K., Iwabuchi, H., Shimomura, K., *Flavour Fragrance J.* 2009, *24*, 251–258.

[61] Lopes-Lutz, D., Alviano, D. S., Alviano, C. S., Kolodziejczyk, P. P., *Phytochemistry* 2008, *69*, 1732–1738.

[62] Raal, A., Orav, A., Arak, E., *Nat. Prod. Res.* 2007, *21*, 406–411.

[63] Taarit, M. B., Msaada, K., Hosni, K., Marzouk, B., *Food Chem.* 2010, *119*, 951–962.

[64] Cardile, V., Russo, A., Formisano, C., Rigano, D., Senatore, F., Arnold, N. A., Piozzi, F., *J. Ethnopharmacol.* 2009, *126*, 265–272.

[65] Choo, L. C., Wong, S. M., Liew, K. Y., *J. Sci. Food Agric.* 1999, *79*, 1954–1957.

[66] Gonny, M., Cavaleiro, C., Salgueiro, L., Casanova, J., *Flavour Fragrance J.* 2006, *21*, 99–106.

[67] Brophy, J. J., Davies, N. W., Southwell, I. A., Stiff, I. A., Williams, L. R., *J. Agric. Food Chem.* 1989, *37*.

[68] Weyerstahl, P., Marschall, H., Thefeld, K., Subba, G. C., *Flavour Fragrance J.* 1998, *13*, 377–388.

[69] Pino, J. A., Marbot, R., Quert, R., Garcia, H., *Flavour Fragrance J.* 2002, *17*, 1–4.

[70] Miyazava, M., Nagai, S., Oshima, T., *J. Oleo Sci.* 2008, *57*, 139–143.

[71] Chisholm, M. G., Wilson, M. A., Gaskey, G. M., *Flavour Fragrance J.* 2003, *18*, 106–115.

[72] Merle, H., Verdeguer, M., Blázquez, M. A., Boira, H., *Flavour Fragrance J.* 2007, *22*, 461–464.

[73] Frum, Y., *Phd. Thesis*, University of the Witwatsrand, Johannesburg 2006.

[74] Pala–Paul, J., Brophy, J. J., Goldsack, R. J., Fontaniella, B., *Biochem. Syst. Ecol.* 2004, *32*, 55–62.