Biological Activity of *Bunium persicum* Essential Oil from Western Himalaya

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ABSTRACT

The essential oil of *Bunium persicum* collected from cultivated sources in the cold desert area of Lahaul-Spiti is described for its antimicrobial, larvicidal, and biting deterrent activities. Additionally, odor characterization is given. The chemical composition of the essential oil was analyzed by simultaneous GC-MS and GC-FID. γ-Terpinene and p-cymene were found to be the major compounds. Antibacterial testing by an agar dilution assay revealed low activity of the oil against all tested bacteria. Antifungal activity was evaluated against *Candida albicans* as well as three species of the strawberry anthracnose causing plant pathogen *Colletotrichum*. *Bunium persicum* essential oil demonstrated antifungal activity against all four pathogens. Biting deterrent activity against *Aedes aegypti* was greater than the solvent control, but significantly lower than DEET. The essential oil of *B. persicum* exhibited larvicidal activity with an LC_{50} value of 58.6 ppm against *Ae. aegypti* larvae.

Introduction

Many important pathogens causing severe diseases are transmitted to humans by insects. Malaria, dengue, yellow fever, and the West Nile virus are considered the most important mosquito-borne diseases in Asia, Africa, and South America [1]. However, due to global warming, these infectious pathogens spread to new areas threatening even more people and challenging academic and industrial communities [2]. Although many methods have been developed for mosquito control, the most effective method is the use of pesticides. Besides several synthetic pesticides that are available on the market, plant extracts and essential oils (EOs) have become important to research and industry due to their safety to the environment and non-target organisms [3]. The synergistic effect that has been found between some EOs and synthetic larvicides [1] would additionally offer an opportunity to develop more effective chemical control agents that are more valuable and less toxic to the environment.

Not only has the increasing presence of mosquitoes caused concern in the population, but also resistances against well-established substances have become challenging. Because of widespread indiscriminate use of antibiotics and agricultural pesticides since the 1950s, the development of chemical resistance in many bacteria, fungi, and insects, including mosquitoes, is frequent. Additionally, the use of synthetic food preservatives to prevent the growth of food-borne and spoilage bacteria and fungi is being criticized by customers because of their growing concerns over food safety. Therefore, the search for new natural antibiotics, fungicides, pest management agents, and food preservatives that have activity against multiple target sites and a low chance for the development of chemical resistance has led to an increased interest in EOs. Various studies, summed up and described in detail in a recent review by Reyes-Jurado et al. [4], have confirmed the activity of some EOs against many microbes.

In the Himalayan region, many plants and their EOs are economically important and have a history in traditional medicinal use, particularly by the populations living in rural areas where little medical care is available. *Bunium persicum* (Boiss.) B. Fedtsch. (Apiaceae), commonly known as black caraway or black zira, is an aromatic perennial native to the Indian subcontinent (India, Pakistan) and western to middle Asia. *Bunium* seeds are generally black in color, develop a characteristic pleasant aroma, and are widely used as condiments and flavoring agents in local foods [5, 6]. Seeds and the EO are well known for their digestive, anticonvulsive, diuretic, and anthelmintic effects [7, 8]. Due to the wide scope of pharmacological effects, the plants were extensively collected and sold by the Himachal Pradesh people. This led to a highly threatened population status for *B. persicum*. Subsequent attempts at cultivation of the plant demonstrated difficulties in germination because the seeds possess a deep dormancy mechanism. Various techniques were established to meet the complex dormancy requirements suitable for *B. persicum* germination [9]. Today the plants are being successfully cultivated by local farmers in the cold desert of Lahaul-Spiti, an area also with suitable farming conditions for black caraway.

In continuation of our studies on screening EOs from medicinal plants growing wild [10, 11] or cultivated in the western Himalayan region [12], the main purpose of the present investigation was to examine the EO of *B. persicum*. This species was collected from cultivated sources in Lahaul-Spiti at an altitude of 3 500 m, and tested for antimicrobial activities, mosquito biting deterrence, and larvicidal effect against *Aedes aegypti*.

Results and Discussion

B. persicum EO was characterized by olfactory evaluation as aromatic spicy, green herbal, and cumin-like. The results of the quantitative and qualitative oil analyses by simultaneous GC-MS and GC-FID using 2 different columns are listed in ▶ **Table 1**. Thirty-one compounds were identified from *B. persicum* EO, accounting for 97.7–97.9 % of the total oil. γ-Terpinene was present in the highest amount (40.2–40.4 %), followed by p-cymene (25.8 %), cumin aldehyde (12.8–12.9 %), p-mentha-1,4-dien-7-al (9.1–9.2 %), and p-mentha-1,3-dien-7-al (4.5–4.7 %) (▶ **Table 1**). Several investigations of *B. persicum* oil samples from plants collected or cultivated in Iran are described in the literature. All showed the same main components, but with different concentrations, e.g., the aromatic monoterpene p-cymene occurred at much lower amounts (5–16 %) in the Iranian samples [7, 13–16], whereas cumin aldehyde was higher (24 %) compared to our sample from India [13, 17]. An older investigation on *Bunium* fruits collected in Tajikistan reported p-mentha-1,4-dien-7-al (29.0 %), γ-terpinene (25.7 %), β-pinene (15.6 %), and cumin aldehyde (11.7 %) as major components [18]. These findings once more demonstrate the influence of climatic and local factors on EO composition [7, 19, 20] and possibly the difference between cultivated and wild growing species [21]. Therefore, plants from different origins or genetic backgrounds should be studied for their chemical composition in order to be able to conclude or link to their biological activities.

In the context of screening *B. persicum* EO for antimicrobial activity, the oil was tested against the Gram-positive bacterium *Staphylococcus aureus*, and Gram-negative bacteria *Escherichia coli*, *Salmonella abony*, and *Pseudomonas aeruginosa*. Growth inhibition testing of *Candica albicans* was performed in a liquid agar broth-microdilution assay [10]. Minimum inhibitory concentrations (MICs) in µg/mL are given in ▶ **Table 2**.

B. persicum EO showed medium to low antimicrobial activity against all tested strains with MICs between 1 000 and 8 000 µg/mL (▶ **Table 2**). *P. aeruginosa* was the least sensitive bacterium, while *C. albicans* proved to be the only strain sensitive to the tested oil. Essential oils generally show greater antimicrobial activity against Gram-positive bacteria than Gram-negative bacteria, which is probably related to the bacterial cell membrane structure [4, 22]. Literature reports on antibacterial activity of *B. persicum* EO show diverse results. An investigation of Iranian samples attributed very high antibacterial activity against *E. coli*, *P. aeruginosa* (Gram-negative bacilli), and *S. aureus* (Gram-positive cocci) to the oil, reporting MIC values around 15 µg/mL [23]. In another Iranian study, *B. persicum* EO was found to be inactive against *E. coli* [24]. Results of Oroojalian et al. [25] on Iranian *B. persicum* EO showed an antimicrobial effect with an MIC value of 1 500 µg/mL against *E. coli* and were comparable to our outcome. Activity against *S. aureus* (750 µg/mL) reported in the same publication was higher. The use of different strains and testing methods is the most probable explanation for these varied findings, but there are differences in EO origin and chemical composition that may play a role. In general, *p*-cymene and γ-terpene, the main components of *B. persicum* EO, are regarded as monoterpenes with low antimicrobial activity [26, 27]. These two compounds accounted for 66 % of the oil tested in the present study. Investigations on pure γ-terpinene against *E. coli*, *S. aureus*, and *P. aeruginosa* revealed no activity against any of these bacterial species [26, 28]. In another study, p-cymene showed growth inhibition zones between 17–21 mm against *C. albicans* and between 9.6–11 mm against *S. aureus*, depending on the specific strains [29]. The authors concluded that none of the single compounds tested were as active as the whole EO. Therefore, the effect of an EO seems to be related to trace compounds or potential synergistic effects of various compound combinations. Using methods and conditions described by Mann et al. [25, 26] and Longbottom et al. [30], p-cymene and γ-terpinene have demonstrated activity against *P. aeruginosa*.

B. persicum EO was evaluated for its antifungal activity against the strawberry anthracnose, causing fungal plant pathogens *Colletotrichum acutatum*, *Colletotrichum fragariae*, and *Colletotrichum gloeosporioides*. Testing was performed with two concentrations of EO at 80 µg and 160 µg applications against three *Colletotrichum* species in the direct overlay bioautography assay (▶ **Table 3**). At the highest tested concentration of 160 µg/spot, *B. persicum* EO demonstrated better activity against all three *Colletotrichum* species with clear zones of 9.0–10.0 mm. A recent study showed growth inhibition activity against the soilborne phytopathogenetic fungus *Fusarium osysporum* [31]. In another investigation, high antifungal activity of *B. persicum* EO against the same fungus was determined. The authors attributed this high inhibitory activity to cumin aldehyde and p-cymene [32]. In our previous antifungal studies, we demonstrated that EOs rich in non-oxygenated mono- or sesquiterpenes did not show good antifungal activity [33, 34]. Therefore, the antifungal effect of *B. persicum* oil might either be

▶ **Table 1** Chemical composition of B. persicum EO (in % peak area) determined by GC-FID and GC-MS.

No.	Compound	RI#	%	RI##	%
1	cumene	928	tr.	1151	tr.
2	α-thujene	931	0.3	1018	0.3
3	α-pinene	941	0.1	1015	0.2
4	sabinene	980	0.7	1109	0.7
5	β-pinene	986	0.1	1100	0.1
6	myrcene	992	0.7	1142	0.7
7	δ-3-carene	1018	0.1	1134	0.1
8	α-terpinene	1023	0.2	1163	0.2
9	p-cymene	1032	25.8	1252	25.8
10	limonene	1036	0.3	1184	0.3
11	β-phellandrene	1038	0.2	1196	0.2
12	1,8-cineole	1039	0.3	1202	0.2
13	(E)-β-ocimene	1049	tr.	1216	tr.
14	γ-terpinene	1067	40.4	1233	40.2
15	cis-sabinene hydrate	1070	tr.	1444	tr.
16	terpinolene	1096	0.3	1261	0.4
17	fenchone	1097	0.3	1376	0.3
18	linalool	1102	tr.	1523	tr.
19	trans-sabinene hydrate	1106	tr.	1530	tr.
20	fenchol	1124	0.1	1555	0.1
21	terpinen-4-ol	1188	0.2	1579	0.2
22	p-cymen-8-ol	1192	0.1	1820	0.1
23	α-terpineol	1201	0.1	1675	0.1
24	p-menth-3-en-7al	1203	0.5	1540	0.4
25	(E,E)-nona-2,4-dienal	1209	tr.	1549	0.1
26	neral	1243	tr.	1661	0.1
27	carvone	1251	tr.	1708	0.1
28	cumin aldehyde	1254	12.9	1751	12.8
29	p-mentha-1,3-dien-7-al	1299	4.7	1767	4.5
30	p-mentha-1,4-dien-7-al	1304	9.1	1772	9.2
31	cuminic acid	1374	0.3	2350	0.4
	Sum		97.9		97.7

#50 m × 0.25 mm × 1.0 µm SE-54; ##60 m × 0.25 mm × 0.25 µm CW20M

▶ **Table 2** Antimicrobial activity of B. persicum EO (agar dilution assay, MIC given in µg/mL).

Tested compounds	Test microorganism				
	S. aureus ATCC 6538	E. coli ATCC 25922	S. abony ATCC 6017	P. aeruginosa ATCC 27853	C. albicans ATCC 10231
B. persicum EO	4000	2000	2000	8000	1000
Ciprofloxacin	0.25	0.15	0.25	1	–
Cefazolin	0.50	2	2	4	–
Amphotericin B	–	–	–	–	0.25
Fluconazole	–	–	–	–	0.25

correlated to p-mentha-1,4-dien-7-al, p-mentha-1,3-dien-7-al, or cumin aldehyde in accordance with [32], or attributable to possible synergistic effects of one or more compounds present in the oil. Further research is needed to conduct bioassay-guided fractionation to separate and isolate the active compounds against *Colletotrichum* species.

Working with whole EOs is difficult because they are a complex oily matrix of chemistry, and meaningful biological conclusions will require specific bioassay methods and approaches. Direct bioautograph with fungal pathogents should identify the number of active compounds in the EO. Purification and subsequent testing of single entity chemicals at µmolar concentrations in a dose-re-

▶ **Table 3** Antifungal activity of B. persicum EO against 3 Colletotrichum species using direct bioautography assays.

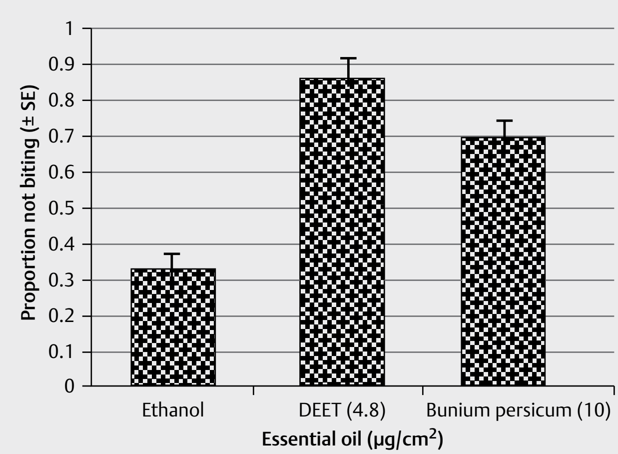
Tested compounds	**Mean fungal growth inhibition§ (mm) ± SD**					
	C. acutatum		**C. fragariae**		**C. gloeosporioides**	
	80 µg/spot	**160 µg/spot**	**80 µg/spot**	**160 µg/spot**	**80 µg/spot**	**160 µg/spot**
B. persicum EO	5.0 ± 0.0	9.5 ± 0.7	5.5 ± 0.7	10.0 ± 0.0	5.0 ± 0.0	9.0 ± 0.0
Standard fungicides *						
Benomyl	Diffuse		21.5 ± 0.7		Diffuse	
Captan	12.0 ± 0.0		18.5 ± 0.7		19.5 ± 0.7	
Cyprodinil	Diffuse		Diffuse		Diffuse	
Azoxystrobin	Diffuse		24.5 ± 1.4		Diffuse	

§Mean inhibitory zones ± SD; * Technical grade of internal standards. Technical grade agrochemical fungicides (without formulation) were applied 2 µL from the 2 mm concentration

sponse format using liquid microdilution broth assays may help determine effects on spore germination and mycelial growth, and may suggest potential structure activity relationships.

Mosquito biting deterrent activity was determined against the yellow fever mosquito, *Ae. aegypti*. *B. persicum* EO showed activity greater than the solvent control (ethanol), but it was significantly less active than DEET, a standard biting deterrent, which was used as a positive control (▶ **Fig. 1**). Toxicity of *B. persicum* EO was determined in mosquito larvicidal bioassays [35]. *B. persicum* EO showed medium to low activity against 1-d old *Ae. aegypti* larvae with an LD_{50} of 58.6 ppm. The LD_{50} value of permethrin, which was used as apositive control, was 0.0034 ppm. Cheng et al. [36] reported that essential oils showing LC_{50} values < 50 ppm can be considered as very active, whereas results > 100 ppm indicated an inactive EO. Making a valid scientific conclusion of results from the literature is difficult because of the variability in testing methods and conditions used by different authors. Since there was no universally accepted guideline to assess larvicidal activity, the use of positive and negative controls is necessary to compare results and make conclusions. Recently, the EO of *B. persicum*, collected in the Kerman region in Iran, was investigated for its larvicidal effects against *Anopheles stephensi* and *Culex pipiens* larvae. Results indicated high toxicity against adult insects with LC_{50} values of 27.72 and 20.61 ppm after 24 h, respectively [17]. However, we report for the first time in this paper on the mosquito biting deterrent and larvicidal activity of *B. persicum* EO against *Ae. aegypti*. Studies using larvicidal activity to drive bioassay-guided fractionation should determine the active compounds. This method will also help determine the number of active compounds in the EO.

In conclusion, it appears that the EO of *B. persicum* from cultivated sources from western Himalaya had antifungal activity against *Colletotrichum* species, and future studies need to be conducted with active components using 1D-TLC bioautography to identify active individual compounds. The active compounds could be subsequently evaluated in 96-well microdilution broth assays against a broader range of important plant pathogenic fungi. The larvicidal activity of *B. persicum* EO against *Ae. aegypti* could be considered as medium to high. Additionally, the oil exhibited good biting deterrent activity. Based on these results, Himalayan black caraway oil (*B. persicum*) may be a promising potential source for new naturally derived agrochemicals or biopesticides. This research needs to be further investigated to study single entity active molecules and possible synergistic effects of chemical combinations.



▶ **Fig. 1** Proportion of not biting values of B. persicum EO at 10 µg/cm² against female *Ae. aegypti*. DEET at 4.8 µg/cm² was used as a positive control. Ethanol was used as a solvent control.

Materials and Methods

Plant material and isolation procedure

B. persicum, cultivated by local farmers, was harvested from Lauhul-spiti'a cold desert area of western Himalaya, at an altitude of 3 500 m, in October/November 2009. The samples were identified by the taxonomist (Brij Lal) of the Institute of Himalayan Bioresource Technology (IHBT Palampur, India). A voucher specimen (PLP 2845) was deposited at the Herbarium-PLP of the IHBT Palampur. Black seeds were air-dried at room temperature (about 25 °C) in the shade. One kilogram of seed material was hydrodistilled in a clevenger-type apparatus as described in [12], yielding light yellow colored oil (0.52 % on dry wt. basis).

Essential oil analysis

Using a Finnigan ThermoQuest TraceGC with 2 split/splitless injectors, a FID detector, and a Finnigan Automass quadrupole mass spectrometer, GC-MS-FID analyses were carried out in one instrument on a 50 m × 0.25 mm × 1.0 µm SE-52 fused silica column (CS Chromatographie Service) and a 60 m × 0.25 mm × 0.25 µm CW20M (J&W Scientific) column, respectively. Splitting of the column effluent was done with a quartz Y connector, one outlet connected to the MS interface via a short (ca. 20 cm) 0.1 mm ID fused silica capillary flow restrictor. The other outlet was attached to a 1 m × 0.25 mm deactivated fused silica capillary as a transfer line to the FID.

The use of this configuration resulted in an FID and an MS chromatogram with identical chromatographic separation and nearly the same retention times, thus facilitating peak assignments in the FID chromatogram with respect to the MS. The carrier gas was helium 5.0 with a constant flow rate of 1.5 mL/min, the injector temperature was 230 °C, FID detector temperature 250 °C, GC-MS interface heating 250 °C, ion source 150 °C, EI mode at 70 eV, scan range 40–500 amu, and scan rate of 500 µs. The following temperature program was used: 60 °C for 1 min isotherm, then increased at a rate of 3 °C/min to 230 °C. Identification of the compounds was performed as described in [10].

Olfactory evaluation

For olfactory evaluation, one droplet of *B. persicum* EO was applied onto commercially available paper blotters. Each sample was examined by a panel consisting of a professional perfumer and 2 aroma chemists over 90 min to control odor progression. Odor descriptions were compared to our own database of referenced aroma compounds.

Antimicrobial testing

The antimicrobial effects of *B. persicum* EO were tested against the Gram-negative bacteria *E. coli* (ATCC 25922), *S. abony* (ATCC 6017), and *P. aeruginosa* (ATCC 27853) and the Gram-positive bacterium *S. aureus* (ATCC 6538), as well as *C. albicans* (ATCC 10231). The strains were purchased from the National Reference Laboratory of Mycology at the National Center of Infectious and Parasitic Diseases, Sofia; Department of Microbiology and Immunology at the Medical University of Plovdiv and Clinical Laboratory Chronolab Ltd., Plovdiv. They were deposited in the Microbial Culture Collection of the Department of Biochemistry and Microbiology (University of Plovdiv, Bulgaria) and bacterial strains were stored on Nutritional Agar (NA, HiMedia Laboratories Ltd.). The fungal strain was stored on Sabouraud Dextrose Agar (SDA, HiMedia Laboratories Ltd.). Antimicrobial activity of *Bunium* EO was evaluated by broth microdilution tests described earlier [10]. Minimal inhibition concentration (MIC) was defined as the lowest concentration of EO that resulted in an absorbance reduction of > 90 % compared to the observed absorbance of control samples without EO. For positive controls, standard antibacterial antibiotic HiComb™ MIC test strips of Ciprofloxacin and Cefazolin and antifungal HiComb™ MIC test strips of Amphotericin B and Fluconazole (HiMedia Laboratories Ltd., 100 % purity) were evaluated. All tests were performed in duplicate.

Antifungal testing

Pathogen production and bioautography procedures of Stappen et al. [10] were used to evaluate antifungal activity against fungal plant pathogens. The sensitivity of each fungal species to each test compound was determined by comparing the sizes of the inhibitory zones. Bioautography experiments were performed multiple times using both dose- and non-dose-response formats. Fungicide technical grade standards benomyl (> 98 %), cyprodinil (> 98 %), azoxystrobin (> 98 %), and captan (> 98 %; Chem Service, Inc.) were used as controls at 2 mM in 2 µL of 95 % ethanol. *B. persicum* EO was spotted with 80 and 160 µg/spot in hexane. To detect biological activity directly on the TLC plate, silica gel plates were sprayed with one of the 3 spore suspensions adjusted to a final concentration of 3.0×10^5 conidia/mL with liquid potato dextrose broth (PDB, Difco) and 0.1 % Tween-80. Using a 50-mL chromatographic sprayer, each TLC plate with a fluorescent indicator (250 µm, silica gel GF Uniplate; Analtech, Inc.) was sprayed lightly (to a damp appearance) 3 times with the conidial suspension. Inoculated plates were then placed in a 30 × 13 × 7.5 cm moisture chamber (39 °C, 100 % relative humidity; Pioneer Plastics, Inc.) and incubated in a growth chamber at 24 ± 1 °C and a 12-h photoperiod under 60 ± 5 µmols · m^{-2} s^{-1} light. The inhibition of fungal growth was measured 4 days after treatment. The sensitivity of each fungal species to each test compound was determined by comparing the size of the inhibitory zones on the TLC plate.

Mosquito biting testing

Mosquitoes: *Ae. aegypti* larvae used in these studies were from a laboratory colony maintained at the Mosquito and Fly Research Unit at the Center for Medical, Agricultural and Veterinary Entomology, USDA-ARS, Gainesville, Florida, using standard rearing practices [37]. For biting deterrence bioassays, pupae were maintained in the laboratory at 27 ± 2 °C and 60 ± 10 % RH in a photoperiod regimen of 12:12 h (L:D). For larvicidal bioassays, the eggs were hatched and the larvae were maintained at the above temperature.

Mosquito biting bioassays: Experiments were conducted by using a 6-celled *in vitro* Klun and Debboun (K&D) module bioassay system developed by Klun et al. [38] for quantitative evaluation of biting deterrent properties of candidate compounds. Briefly, the assay system consists of a 6-well reservoir with each of the 3 × 4 cm wells containing 6 mL of blood. As described by Ali et al. [39], a feeding solution consisting of CPDA-1 and ATP was used instead of blood. A green fluorescent tracer dye (www.blacklightworld.com) was used to determine the feeding by the females. Treatments of the EO of *B. persicum* were applied at 10 µg/cm^2, and DEET (97 %, N, N-diethyl-*meta*-toluamide; Sigma Aldrich) at 25 nmol/cm^2 was used as a positive control, while ethanol served as a solvent control. All treatments were conducted as described in [10].

Larvicidal bioassays

Bioassays were conducted to test the EO of *B. persicum* for their larvicidal activity against *Ae. aegypti* by using the bioassay system described by Pridgeon et al. [40]. Further methods and statistical analyses are described in [35]. DMSO was used as a solvent to prepare the treatments and was also used as a negative control. Permethrin (95.7 %; Chem Service, Inc.) was used as a positive control.

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Conflicts of Interest

The authors declare no conflict of interest.

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