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Chemical composition and larvicidal activity of the essential oils from *Minthostachys spicata* (Benth) Epling and *Clinopodium bolivianum* (Benth) Kuntze against *Premnotrypes latithorax* Pierce

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Abstract
The larvicidal activity of leaf essential oils (EOs) from *Minthostachys spicata* (Benth) Epling and *Clinopodium bolivianum* (Benth) Kuntze against *Premnotrypes latithorax* Pierce was evaluated. EOs were analyzed by GC-FID and GC-MS. Major compounds in *M. spicate* EO were pulegone, isomenthone, and menthone, while in *C. bolivianum* EO were isomenthone, thymol, and menthone. Different concentrations of the EOs were dissolved in aqueous solution containing 2% of Tween 80, and 1 mL of each concentration was impregnated on 10 cm-diameter disk of filter paper previously introduced into separate polyethylene containers. The larval mortality was observed for 24 h of post exposure. Both EOs exhibited significant larvicidal activity, with LC_{50} and $LC_{90}$of 0.040 and 0.095 µL/cm^2 for *M. spicata* and 0.088 and 0.248 µL/cm^{2}for *C. bolivianum*, respectively. Both leaf EOs, but particularly *M. spicate* EO, may be considered as potent source to produce natural larvicides against *P. latithorax.*

Keywords: *Minthostachys spicata*, *Clinopodium bolivianum*, essential oil, larvicidal, potato protection, *Premnotrypes latithorax*

1. Introduction
The application of chemical pesticides is a cost-effective method of controlling insect pests, but these chemicals are highly toxic to other species in the environment. There is a growing concern worldwide over the indiscriminate use of these chemicals, which cause environmental pollution and posit a toxicity risk to non-target organisms [1]. Therefore, increased efforts are being made to find safe, effective and viable options. The search for new solutions to chemical treatments is nowadays a worldwide necessity to achieve more sustainable control. In this context, plant-based insecticides may represent a useful tool less toxic to humans, readily biodegradable, environmental safe, suitable for use by small-scale farmers, and yet capable of protecting crops from attack by a wide range of insect pests [2, 5].
Essential oils (EOs) derived from aromatic plants are a promising new class of ecological products for the control of insect pests. In Peru, more than 300 plants have been reported with bioplaguicide activities [6]. Aromatic plants have been historically used in Peru to protect stored products against insect pests in traditional agricultural systems [7, 8].
Premnotrypes latithorax Pierce, commonly called Andean potato weevil or potato worm, is associated with potato (*Solanum tuberosum* L.) andit is one of the most important plagues from highland Andes. Ten of the dozen existing species of *Premnotrypes* are native from Peru [9]. *P. latithorax* adults feed on young plants, but larval damage to tubers is also important.
Minthostachys spicata (Benth.) Epling (syn. *Bystropogon spicate* Benth.) is an aromatic scandent shrub distributed in coastal cordillera of Peru and southernmost Ecuador [10]. The aerial parts of the plant have many traditional uses, including as carminative, digestive, antispasmodic, and to produce liqueur [11]. There are only few unreliable reports on the chemical composition of the EO from this species, mainly due to the lack of voucher specimens. According to the most reliable report [12], the major constituents were menthone, 2-hydroxy-*p*-1-menthen-3-one, and 3, 4, 5-trimethoxytoluene; while in a recent study, pulegone, isomenthone, and menthone were found the main compounds [13]. The preservation of potato tubers has been tested with dried leaves of *Minthostachis mollis* [14], and dried leaves and EO

[15]. In both studies the treatments deterred the potato tuber moth (*Phthorimaeao perculella*) from laying eggs on stored potatoes. While the study plants, all from the same locality near Cuzco, were said to belong to "*Minthostachys spicata*, *Minthostachys glabrescens* and *Minthostachys mollis*", they most likely represented other *Minthostachys* spp. [10], but no voucher specimen was available.

Clinopodium bolivianum (Benth) Kuntze [syn. *Satureja boliviana* (Benth.) Briq.], commonly named Inca muña or Incas's oregano, is an aromatic undershrub distributed mainly in the central and south Andean region, between 3400 and 3850 m above sea level [16].The aerial parts are used to treat indigestion, nausea, diarrhea, anemia and respiratory illnesses [17], while the flowers and leaves are used as a condiment in meats, soups and stews [16].The literature reports some works on the composition of the EO from plants grown in Peru, Bolivia, and Argentina, showing a significant difference in the chemical composition of Peruvian and Argentine EOs [18, 24]; however no report has been found on the larvicidal activity of this EO.

Therefore, the aim of this study was to evaluate the larvicidal activity of two EOs grown in Cusco (Peru), *Minthostachys spicata* (Benth) Epling and *Clinopodium bolivianum* (Benth) Kuntze against larvals of *Premnotrypes latithorax* Pierce.To our knowledge, this study is the first to report on the toxicity of these EOs to *P. latithorax*.

2. Materials and methods

2.1 Plant material

Aerial parts of different specimens of *Minthostachys spicata* (Benth) Epling and *Clinopodium bolivianum* (Benth) Kuntze were collected at San Sebastian district, in Cusco, Peru (3200 m height above sea level), during April 2015. Authentication was performed by botanists of the Herbarium Vargas at the Biological Sciences Faculty of the Universidad Nacional de San Antonio Abad del Cusco, and voucher specimens were deposited at the herbarium, under the register numbers 24.275 CUZ for *M. spicata* and 24276 CUZ for *C. bolivianum*.

2.2 Essential oils

Leaves of each species (200 g each) were submitted to hydro distillation for over 3 h in a Clevenger-type apparatus. Subsequently, the EOs were collected and stored at 4 °C for further analyses. Distillation processes were repeated in triplicate.

2.3 GC-FID and GC-MS analysis

For quantitation the EOs were analyzed in a Konik 4000A (Konik, Barcelona) gas chromatograph equipped with a flame ionization detector and a DB-5ms fused-silica capillary columns (30 m x 0.25 mm i.d. x 0.25 mm) (J & W Scientific, Folsom, CA, USA). The analyses were conducted under the following conditions: oven temperature program, 60 ºC (2 min), 60–220 ºC (4 ºC/min) and 220 ºC (5 min); carrier gas hydrogen flow rate 1 mL/min; injector and detector temperatures 250 ºC, injection volume 0.2 µL and split ratio 20:1.Quantitative analysis of the components was performed using relative percentage abundance and normalization method with correction response factors based on grouping the EO components by their functional groups [25]. Percentage data are the mean values of three injections per sample.

Analysis of the chemical composition of the EOs were performed by gas chromatography coupled to mass spectrometryusing a Hewlett Packard 6890 gas chromatograph, fitted with the same column interfaced with an Hewlett Packard mass-selective detector 5973 (Agilent Technologies, Palo Alto, CA, USA). Helium was used as the carrier gas at a flow rate of 1 mL/min. Other GC parameters were similar to GC-FID and the MS conditions were voltage 70 eV, mass range 35-400 *m/z* and scan rate 1 scan/s. Lineal retention indices (LRIs) were calculated by interpolation to the retention times of a mixture of *n*-alkanes (C_8-C_{24}) analyzed in the same conditions. Identification of the compounds was performed by comparison of their LRIs and mass spectra with those reported in the literature [26]. Mass spectra were compared with corresponding reference standard data reported in the literature [26] and mass spectra from NIST 05, Wiley 6, NBS 75 k, and in-house Flavorlib libraries. In many cases, the EOs were subject to co-chromatography with authentic compounds.

2.4 Larval bioassays

P. latithorax larvae were collected in May 2015 from a natural infested organic potato crop. Active larvae were recovered using a No. 16 ASTM-E-11 sieve (14 mesh). Larvae with mean length of 10-13 mm were selected [27] and maintained at 16 ± 2 °C with relative humidity 60 ± 5% for 48 h. Entomological identification was made in the laboratory of Entomology from the Universidad Nacional de San Antonio Abad del Cusco. The specimens were classified as fourth-instar larvae of *Premnotrypes latithorax* Pierce (Col. Curculionidae).

The bioassay employed a reported methodology [28], with slight modifications and taking into account the technical specifications [29]. It consisted of applying 1 mL of 0.25, 0.5, 1.0, 1.5, and 2.0% v/v of each EO dissolved in aqueous solution containing 2% of Tween 80 on circular N° 642 filter paper (Ahlstrom, Helsinki, Finland) 10-cm in diameter, which had been adhered to the bottom of covered polyethylene containers (air volume equivalent to 0.5 L), with 20 insects. The evaluated doses were 0.032, 0.064, 0.127, 0.191 y 0.255 µL/cm^2. The EO of clove (*Eugenia caryophyllata* Thumb.) (With 79.3% eugenol), an unquestionable larvicide, at the same doses and conditions as used as a positive control ([42], páginas 310, 311 y 321, 322, 323). The same procedure was used for the control with 1 mL of an aqueous solution 2% Tween 80 on the filter paper. There were six replicates for each treatment. The experimental units were kept at 16 ± 2 °C, relative humidity 60 ± 5% and a photoperiod of 9 h with light followed by 15 h darkness (9L:15D). Assessments of mortality were made at 24 h of exposure. An insect was considered dead when there was no movement after prodding it with a dissection needle. Corrections for the larval sensitivity test were performed according to the Abbott formula [30].

2.5 Statistics

The calculation of the extract lethal concentration (LC) in the *in vitro* tests was performed by fitting regression using normal and logistic distribution, with the parameters estimative of these equations obtained by maximum likelihood. The procedure used was the probit-log [31] to estimate the LC_{50} and LC_{90} with the independent variables (dose) transformed by natural logarithm (log dose). Other statistics at 95% confidence limits of upper and lower confidence limits, and chi-square values were calculated using SPSS 18.0 and Statgraphics 15.0 software. Results with $P<0.05$ were considered to be statistically significant.

3. Results and discussion

The larvicidal activity by contact of the EOs from *M. spicata* y *C. bolivianum* against *P. latithorax* is presented in Table 1. Mortality rose with increased concentration of both EOs. Doses 0.064 µL/cm^2 (0.5%) for *M. spicata* EO and 0.127 µL/cm^2 (1%) for *C. bolivianum* EO exceeded 50% mortality at 24 h. Based on this, both EOs can be considered as effective as contact larvicides at dose of 0.255 µL/cm^2 (2%), given that at 24 h both showed a 100% efficacy. Results indicated that EO of *M. spicata* was significantly more toxic against *P. latithorax* than *C. bolivianum*. The LC_{50} and LC_{90} values were 0.040 and 0.095 µL/cm^2 for *M. spicata*; 0.088 and 0.248 µL/cm^2 for *C. bolivianum*, respectively. The EO of clove included as positive control with LC_{50} of 0.016, showed greater larvicidal effectiveness than EOs. No comparable data are present in the literature. To the best of our knowledge, this is the first report on the larvicidal activities of these EOs.

Table 1: Larvicidal activity of *Minthostachys spicata* and *Clinopodium bolivianum* essential oils against *P. latithorax*

Essential oil	Dose (µL/cm^2)	24 h mortality (%) (Mean ± SD)	CL_{50} (µL/cm^2)	95% Confidence limits (µL/cm^2)		CL_{90} (µL/cm^2)	χ^2
				Lower	Upper		
	Control	0.0 ± 0.0					
	0.032	31.7 ± 2.6					
M. spicata	0.064	84.7 ± 3.8	0.040	0.024	0.054	0.095	408.8*
	0.127	94.2 ± 4.9					
	0.191	97.5 ± 4.2					
	0.255	100.0 ± 0.0					
	Control	0.0 ± 0.0					
	0.032	14.2 ± 4.9					
C. bolivianum	0.064	37.7 ± 4.1	0.088	0.043	0.145	0.248	352.0*
	0.127	54.2 ± 4.9					
	0.191	80.0 ± 0.0					
	0.255	100.0 ± 0.0					
E. cariophyllata (positive control)	0.255 Control 0.032 0.064 0.127 0.191 0.255	100.0 ± 0.0 0.0 ± 0.0 74.7 ± 2.6 90.3 ± 4.9 100.0 ± 0.0 100.0 ± 0.0 100.0 ± 0.0	0.016	0.012	0.021	0.063	994.4*

*Significant at $P<0.05$ level

The yields of EO were 0.5% v/m and 0.8% v/m fresh weight for *M. spicata* and *C. bolivianum* leaves, respectively. An analysis of the chemical composition of these EOs (Table 2) showed 55 compounds (98.5% of total composition) and 74 compounds (99.1% of total composition) for *M. spicata* and *C. bolivianum* EOs, respectively. The major constituents of *M. spicata* EO were pulegone (43.2%), isomenthone (15.0%), and menthone (14.2%). The percentage composition of the remaining compounds was lower than 5%. These results agree with the composition of *M. spicata* EO from plants collected in Cusco, Peru [13].

Table 2: Composition of the essential oils from *Minthostachys spicata* and *Clinopodium bolivianum* leaves

Compound	LRI[a]	LRI_O[b]	*M. spicata* (%)	*C. bolivianum* (%)
(*E*)-2-hexenal	855	856	tr[c]	0.1 ± 0.0
(*Z*)-3-hexen-1-ol	859	859	tr	tr
α-thujene	930	931	tr	1.0 ± 0.1
α-pinene	940	939	-	0.3 ± 0.0
α-fenchene*	952	953	0.4 ± 0.0	-
camphene	954	956	tr	tr
sabinene	973	972	0.6 ± 0.0	0.5 ± 0.0
β-pinene	979	979	-	0.3 ± 0.0
octan-3-one*	983	982	tr	-
myrcene	987	989	0.3 ± 0.0	0.2 ± 0.0
3-octanol	991	992	1.1 ± 0.1	0.1 ± 0.0
(*E*)-3-hexenyl acetate	1002	1002	tr	tr
δ-3-carene	1011	1010	-	0.1 ± 0.0
α-terpinene	1016	1014	tr	0.5 ± 0.0
p-cymene	1023	1024	0.2 ± 0.0	9.5 ± 0.3
limonene	1027	1029	1.0 ± 0.1	1.0± 0.1
1,8-cineole	1033	1033	1.2 ± 0.1	2.8± 0.2
(*E*)-β-ocimene	1050	1051	0.5 ± 0.0	5.7 ± 0.4
γ-terpinene	1060	1059	tr	-
cis-sabinene hydrate	1070	1070	0.1 ± 0.0	0.6 ± 0.0
cis-linalool oxide (furanoid)	1074	1075	tr	tr
methyl 2-phenethyl ether*	1083	1083	0.1 ± 0.0	tr
terpinolene	1088	1086	tr	-

linalool	1095	1097	4.8 ± 0.2	2.8 ± 0.1
trans-sabinene hydrate	1098	1099	-	0.3 ± 0.0
1-octen-3-yl acetate	1111	1109	-	0.3 ± 0.0
thujol*	1115	1114	-	0.1 ± 0.0
trans-thujone	1118	1120	-	0.1 ± 0.0
cis-2-*p*-menthen-1-ol*	1121	1122	-	tr
3-octyl acetate	1123	1124	0.7 ± 0.0	tr
trans-sabinol*	1141	1141	0.1 ± 0.0	0.1 ± 0.0
isopulegol	1150	1148	0.2 ± 0.0	-
menthone	1153	1152	14.2 ± 0.4	14.8± 0.5
iso-isopulegol*	1160	1160	0.1 ± 0.0	-
iso-menthone	1161	1163	15.0 ± 0.3	20.8 ± 0.5
borneol	1169	1167	-	tr
menthol	1172	1170	1.9 ± 0.1	-
umbellulone	1172	1170	-	tr
terpinen-4-ol	1176	1174	-	1.8 ± 0.1
trans-isopulegone	1182	1181	0.9 ± 0.0	-
p-cymen-8-ol	1183	1183	-	tr
decan-3-one	1185	1184	0.1 ± 0.0	-
neoiso-menthol*	1187	1186	0.2 ± 0.0	tr
decan-3-ol	1189	1190	0.2 ± 0.0	-
α-terpineol	1191	1193	0.4 ± 0.0	0.3 ± 0.0
myrtenal*	1196	1196	tr	-
β-citronellol	1225	1226	tr	0.3 ± 0.0
pulegone	1235	1237	43.2 ± 0.8	6.7 ± 0.3
neral	1238	1238	-	tr
geraniol	1250	1247	-	0.2 ± 0.0
piperitone	1254	1253	1.8 ± 0.1	1.1 ± 0.1
geranial	1267	1269	-	0.1 ± 0.0
iso-piperitenone*	1272	1270	-	0.1 ± 0.0
neo-menthyl acetate	1274	1274	0.3 ± 0.0	-
thymol	1291	1290	0.1 ± 0.0	16.1 ± 0.5
menthyl acetate	1295	1298	0.6 ± 0.0	tr
carvacrol	1298	1300	-	1.1 ± 0.1
methyl (*Z*)-cinnamate	1300	1299	-	0.1 ± 0.0
piperitenone	1342	1340	1.2 ± 0.1	0.5 ± 0.0
thymol acetate*	1352	1352	0.2 ± 0.0	tr
eugenol	1357	1359	tr	tr
piperitenone oxide*	1368	1369	1.2 ± 0.1	-
carvacrol acetate	1371	1374	-	tr
α-copaene	1377	1374	tr	-
methyl (*E*)-cinnamate	1379	1380	-	0.3 ± 0.0
geranyl acetate	1381	1382	-	0.1 ± 0.0
β-bourbonene	1388	1384	0.2 ± 0.0	-
β-elemene	1390	1391	-	0.1 ± 0.0
2-phenylethyl isobutanoate	1395	1391	-	0.1 ± 0.0
α-gurjunene	1410	1408	-	tr
(*E*)-caryophyllene	1419	1418	2.2 ± 0.1	1.8± 0.1
β-copaene	1432	1430	tr	-
nerylacetone*	1437	1436	tr	-
α-humulene	1453	1455	0.3 ± 0.0	0.1± 0.0
allo-aromadendrene	1460	1459	tr	0.2 ± 0.0
α-amorphene	1485	1485	-	0.1 ± 0.0
germacrene D	1485	1486	0.8 ± 0.0	-
2-phenylethyl isopentanoate	1489	1490	-	tr
(*Z*,*E*)-α-farnesene	1491	1492	-	0.1 ± 0.0
trans-muurola-4(14),5-diene*	1493	1491	-	0.2 ± 0.0
epi-cubebol	1497	1497	-	0.1 ± 0.0
bicyclogermacrene	1505	1500	1.9 ± 0.1	2.0 ± 0.1
γ-cadinene	1512	1514	-	0.2 ± 0.0
endo-1-bourbonanol*	1520	1517	-	0.2± 0.0
δ-cadinene	1521	1523	-	0.5± 0.0
α-cadinene*	1539	1539	-	tr
(*E*)-nerolidol	1563	1567	-	0.1 ± 0.0
germacrene D-4-ol*	1575	1575	-	1.3 ± 0.1
spathulenol	1578	1578	0.9 ± 0.0	0.9 ± 0.1
caryophyllene oxide	1583	1584	0.2 ± 0.0	0.1 ± 0.0
α-cadinol	1654	1654	-	0.1 ± 0.0

oplopanone*	1742	1742	-	0.1 ± 0.0
Total identified			98.5	99.1

[a]LRI, experimental linear retention indices on DB-5ms column. [b]LRI_O, linear retention indices from standard or literature on DB-5ms column. [c]trace (<0.1%). *tentatively identified compound by comparison with literature data.

On the other hand, the major constituents of *C. bolivianum* EO were isomenthone (20.8%), thymol (16.1%), and menthone (14.8%). The percentage composition of the remaining compounds was lower than 9.5%. Menthone was the main constituents in the EO of plants from Bolivia [19], while camphene, *p*-cymene, bornyl acetate, neryl acetate, and geranyl acetate [18], or γ-terpinene, β-caryophyllene, germacrene D, bicyclogermacrene, 1, 8-cineol, and linalool [23], or isomenthone and pulegone [24] were the major components of the EO from plants gathered in Argentina. The main constituents in the EO from plants gathered in Cusco were menthone (54.1%), isomenthone (15.1%), carvacrol (6.7%) and isopulegone (5.4%), but no voucher specimen was deposited [20], or isomenthone (29.7%), menthone (24.2%), and pulegone (10.7%) [22]. The major constituents of the Peruvian EO were isomenthone (29.0%), pulegone (12.6%) and menthone (12.6%), while in the Bolivian oil were isomenthone (27.0%), pulegone (20.0%) and thymol (14.7%) [21]. It is evident that the chemical composition of the EOs from Bolivian and Peruvian species differs with those from Argentina. The chemical composition differences of these EOs seem to show chemotype existence and indicate the high influence of environmental factors such as geographic location and light intensity.s

There are numerous reports on the toxicity of the EOs from various species with major constituents similar to the EOs of the present study, such as those from *Hedomea mandonianum* and *Mynthostachys andina* against *Triatoma infestans* [32], *Minthostachys verticillata* and *Hedeoma multiflora* against *Musca domestica* [33, 34], three *Mentha* spp. against *Culex pipiens* [35] in the case of menthone, isomenthone and pulegone.

In the case of thymol-rich EOs, those from *Piper crispum* against *Ochlerotatus caspius* [36], *Thymus satureoides* against *Culexquinque fasciatus* [37], *Thymus vulgaris* against *Anopheles gambiae* [38], *Rhipicephalus* (*Boophilus*) *microplus* larvae [39] and *Xanthogaleruca luteola* larvae [40], and *Lippia sioides* against *Tetranychus urticae* [41]. Furthermore, pulegone and thymol are classified as active toxic compounds for insects [42].

Terpene compounds are known to be active against a range of organisms and the synergy of several terpenes can be effective on several targets because they are a complex mixture of compounds that can interact with multiple molecular targets on various developmental stages of the pest [43, 44]. Therefore, it is quite reasonable to consider that the major constituents of each plant species had some biological activity *in vitro* against *P. latithorax*in the present study. However, synergistic phenomena between the diverse components of the EO may result in a higher bioactivity of the EO compared to its isolated components. Thus, the fact that an EO contains specific major constituents may be an indication of its potential use, but does not warrant its use without confirmation of activity. Also, chemical composition may vary considerably between aromatic plants and varieties, and between the same varieties from diverse geographic zones [45].

4. Conclusions

The larvicidal activity against *Premnotrypes latithorax* of *Minthostachys spicate* and *Clinopodium bolivianum* EOs followed the same pattern in all *in vitro* tests, suggesting both EOs, but particularly *M. spicata* EO, could be interesting candidates for *P. latithorax* control, although *in vivo* studies are necessary to validate the larvicidal properties of these EOs. This communication also recommends for further studies to isolate and to identify the responsible bio-active molecules. These results are useful in search of more selective, biodegradable and ecological larvicide product.

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