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Antifungal and repellent activities of the essential oils from three aromatic herbs from western Himalaya

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Abstract: In this investigation we examined the essential oils of three aromatic plants, *Zanthoxylum armatum*, *Juniperus communis*, and *Dysphania ambrosioides*, which are used by the local population of the western Himalayan region for medicinal purposes. These plants were studied for their antifungal, larvicidal and biting deterrent activities. Additionally, chemical compositions of the oils were determined by GC-MS and their odor evaluated. The main compounds were 2-undecanone (47.7%) and 1,8-cineol (20.5%) from *Z. armatum* and α -pinene (43.2%) and limonene (34.7%) from *J. communis*. p-Cymene (36.3%) and ascaridole (31.2%) were found as the main components of *D. ambrosioides* essential oil. Antifungal activity was evaluated against three *Colletotrichum* species. *Dysphania ambrosioides* oil showed the best activity against these strawberry anthracnose causing fungi. It also demonstrated biting deterrent activity against *Aedes aegypti* similar to DEET as positive control,

whereas *Z. armatum* and *J. communis* were less effective against *Colletotrichum* and mosquitoes.

Keywords: Terpenes; antifungal; larvicidal; biting deterrent; mosquitoes; ascaridole.

1 Introduction

The Himalayan region is known for its rich and great variety of medicinal plants. Western Himalaya refers to the western half of the Himalayan Mountain region, stretching from northeastern Afghanistan, southern Tajikistan, through Kashmir to central Nepal. The Eastern Himalayas extend from the Kaligandaki Valley in central Nepal to northwest Yunnan in China, including Bhutan, North-East (Sikkim) India, southeastern Tibet, and parts of northern Myanmar. This region is widely considered one of the most important biodiversity hotspots in the world [1-5]. These mountain regions are comprised of high altitude ecosystems (3,000 - 4,000 M), steep slopes into deep mountain valleys and thus create numerous river/stream systems (Yarlung-Tsangpo, Brahmaputra, and Mekong) with mild summer temperatures and high rainfall from monsoons [6].

Sikkim, for example, is the least populous and second smallest among the Indian states. As part of the Eastern Himalayas region, Sikkim is notable for its biodiversity, including alpine and subtropical climates. The Kingdom of Sikkim was founded on the Silk Road by the Namgyal dynasty in the 17th century. It is inhabited by native mountain tribes living across the Indian, Tibetan, Bhutan, and Chinese borders. The human inhabitants of these regions live in extremely isolated areas where trees, plants and herbs are an important source for drugs used against various diseases, insects, and ailments. Plants form the most basic components of their daily life from meals, medicines, mouth and teeth treatments, cosmetics

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and physical ornamentation to each tribe's traditional 'alcoholic drink' which is used for pain relief [7].

While other various plant components form the basis for plant defense (mechanical, physical, chemical), the biological activities of a plant may often be attributed to compounds within the essential oil profile. Depending on the plant species, the plant parts (leaves, stems, flowers) of which the essential oil was extracted, and the equipment used for analyses, up to 400 chemicals or even more can be identified within individual oils [8]. Terpenes, mainly mono- and sesquiterpenes, make up the largest group of chemicals from these multi-component mixtures. Natural products, especially essential oils, are important sources to discover potent new lead fungicides. A number of plant essential oils such as rosemary (*Rosmarinus officinalis*) oil (Sporan™) and thyme (*Thymus vulgaris*) oil (Promax™) are marketed as fungicides for organic farmers [9].

Due to global warming insects transporting infectious pathogens like Malaria or yellow fever are appreciably spreading to the northern hemisphere. Besides killing the adult mosquitoes using insecticides, larviciding is another approach to reduce mosquito densities by dispatching the larvae before they emerge into adult individuals. In the last decades customers generally tended to favor natural products over synthetic insecticides and repellents which might possess toxic side effects or trigger allergies when applied onto the skin. That is why the repellent and larvicidal activity of terpenes called attention to essential oils as potent pest management agents [10].

In our previous studies we screened essential oils of medicinal plants typical for the western Himalayas, either from wild growing sources [e.g. 11] or from cultures [e.g. 12,13], for their biological impact. In the present manuscript we focused on the essential oils of three herbs:

Zanthoxylum armatum DC. (Syn.: *Zanthoxylum bungeanum* DC., Rutaceae) is an erect, thorny shrub or small tree that grows in the hot valleys of subtropical Himalaya from Jammu to Butan ascending to 2,200 m of altitude. In the Khasi hills in the north-east it can be found at lower altitudes between 700 and 1000 m. The seeds, leaves and bark are highly aromatic. The natives use the soft twigs as toothbrush for the plant's toothache relieving activity and against insects due to its repellent impact [14]. The essential oil of the fruits further exhibits antiseptic and disinfectant as well as antimicrobial and anthelmintic properties [15].

Juniperus communis L. (Cupressaceae) is a large shrub or small tree native to Europe, South Asia and North America. In India it grows in temperate or subarctic zones in western Himalaya at altitudes between 2,600 to 4,500 m. *Juniperus communis* is a well-known and well

described medicinal plant that possesses a wide range of pharmacological activities and thus has been used in traditional as well as academic medicine for centuries. The native population in India considers the plant as sacred and uses its needles for incense. The essential oil of the fruits exhibits carminative, stimulant and diuretic properties [16]. Furthermore its antimicrobial and antioxidant impact have been studied. The chemical composition of the essential oils from leaves and berries derived from plants from various countries all over Europe and Asia has been characterized. However, depending on the plants' origin their composition varied distinctively: Although α -pinene was the main component in most European leave oils, sabinene was dominated in several samples from India, Iran and Morocco [17].

Dysphania ambrosioides (L.) Mosyakin & Clemants (Syn.: *Cheopodium ambrosioides* L., Amaranthaceae) is an annual herb native to Mexico but it also grows in tropical and subtropical parts in India. Because of its anthelmintic activity it is known as "wormseed". Cattle generally do not graze this plant due to its strong pungent aroma. The Indian native population uses it for its anthelmintic activity but also the activities against influenza and pneumonia. The essential oil is often rich in ascaridole, a bicyclic monoterpene-peroxide, but several chemotypes containing other monoterpenes as main components have been described in the literature. Further, the essential oil exhibits antifungal, antioxidant, antiviral, antimicrobial and insecticidal activities [18].

In continuation of our investigations on plants typical for the Himalayan region, the main purpose of the present study was to describe the chemical composition of the essential oils of *Z. armatum*, *J. communis*, and *D. ambrosioides* collected from wild and evaluate these samples for their antifungal, larvicidal as well as repellent activity.

2 Experimental Procedures

2.1 Plant material and isolation

Leaves and inflorescence of *Z. armatum* as well as *D. ambrosioides* were collected from wild at the area of Palampur in the Kangra district at an altitude of 1,300 m during July and August 2010. Leaves of *J. communis* were collected from Lahaul valley of Himachal Pradesh at an altitude of 2,800 m during the same time period.

The voucher specimens (*Z. armatum* PLP 1224, *J. communis* PLP 4820, *D. ambrosioides* PLP 1216) were

deposited in the herbarium-PLP of the institute of Himalayan Bioresource Technology, Palampur. All samples were identified by Brij Lal, taxonomist of the Biodiversity division of IHBT, Palampur.

Plant materials of *Z. armatum* (2.021 kg) and *D. ambrosioides* (1.350 kg) as well as the fresh green needle-like leaves of *J. communis* (1.562 kg) were air dried separately in the shade at 25°C. In a glass Clevenger type apparatus of 5 kg capacity plant material was hydrodistilled until total recovery of essential oils. *Juniperus* leaves were chopped before hydrodistillation. *Z. armatum* yielded in 0.4% (fresh wt. basis) of yellow oil. For *D. ambrosioides* 0.1% (fresh wt. basis) of light yellow oil was obtained, and 1.2% (fresh wt. basis) of colorless oil from *J. communis* was collected. All oil samples were dried by anhydrous sodium sulphate, filtered, and stored at 4°C until analyses.

2.2 Essential oil analysis

GC-FID and GC-MS analyses were performed in one run and one GC with the help of a MS-FID-splitter consisting of a quartz Y-splitter and a short (ca. 20 cm) 0.1 mm ID fused silica restrictor column as an inlet to the GC-MS interface and a ca. 1 m X 0.25 mm deactivated fused silica column serving as a transfer line to the FID detector. The restriction column limits the flow into the MS vacuum and prevents entering combustion gases from the FID which is operated at atmospheric pressure. The flow in the analytical column must be greater than the inflow to the MS detector which is limited to about 1 mL/min by means of the restriction line. The GC column flow must be held constant otherwise the FID-MS split ratio changes with temperature. The following instrumentation was used: A Thermo Fisher Scientific Trace GC with a split/splitless injector heated at 250°C and connected to a 50 m X 0.25 mm X 1.0 µm SE-54 (95% Polydimethyl-, 1% Polydivinyl-, 4% Polydiphenylsiloxan) capillary column (made and tested for deactivation and separation efficiency in our lab [19]), a FID detector operated at 250°C. 0.1 µL essential oil samples were injected neat with a 0.5 µL plunger-in-needle syringe at a split ratio of 1:100.

For substance identification a Thermo Scientific Automass Solo Mass Spectrometer was used with the GC-MS interface heating at 250°C, ion source 230°C, EI mode at 70 eV, filament 500 µA, scan range 40 - 500 amu. The following oven temperature program was used: 60°C for 1 min. then heated to 230°C at a rate of 3°C/Min, 230°C isotherm for 12.3 min. The carrier gas was helium 5.0, with a constant flow rate of 1.5 mL/min.

Thermo Xcalibur 2.2 software was used for identifying the compounds by correlating mass spectra to databases of NIST 08 [20], Wiley 8th ed. [21], Adams library [22], MassFinder terpenoids library [23] and our own library. Retention indices are determined with the use of the measured retention times of a series of n-alkanes that elute over the whole span of the chromatogram and calculated according to the method of van den Dool and Kratz [24].

Quantification was performed using normalized peak area calculations of the FID chromatogram without (by first approximation) relative FID-response factors.

2.3 Olfactory evaluation

For olfactory evaluation, one droplet of each essential oil was applied onto commercially available paper blotters. The samples were examined by a panel consisting of two aroma-chemists and a professional perfumer over 90 min to control for odor progression. Odor descriptions were compared to our own database of referenced aroma compounds.

2.4 Fungal pathogen production and inoculum

Isolates of *Colletotrichum acutatum* Simmonds, *C. fragariae* Brooks, and *C. gloeosporioides* (Penz.) Penz. and Sacc. were obtained from B. J. Smith, USDA, ARS, Poplarville, MS and isolated from strawberry (*Fragaria x ananassa* Duchesne). Conidia of each fungal species are harvested from 7-10 day-old cultures by flooding plates with 5 mL of sterile distilled water and dislodging conidia by softly brushing the colonies. Conidial suspensions are then filtered to remove mycelia then conidia concentrations are determined photometrically from a standard curve based on the percent of transmittance (%T) at 625 nm and manual hemocytometer counts. Conidial stock suspensions are adjusted with sterile distilled water to a concentration of 1.0×10^6 conidia/mL [13].

2.5 Direct Bioautography

Matrix bioautography was used to screen for *Z. armatum*, *J. communis* and *D. ambrosioides* essential oils. All essential oils were diluted in acetone and spots at 80 and 160 µg/spot were applied onto thin-layer chromatography (TLC) plates. Essential oils were diluted in acetone. Each TLC plate was subsequently sprayed with a spore suspension

(10^5 spores/mL) of these three *Colletotrichum* and incubated in a moisture chamber for four days at 26°C with a 12 h photoperiod. Bioautography experiments were performed twice and technical grade commercial fungicides benomyl, cyprodinil, azoxystrobin, and captan (Chem Service, Inc., West Chester, PA) were used as fungicide standards at 2 mM in 2 μ L of 95% ethanol.

2.6 Mosquito Bioassays

Insects: *Aedes aegypti* larvae and adults used in these studies were from the laboratory colonies maintained at the Mosquito and Fly Research Unit at the Center for Medical, Agricultural and Veterinary Entomology, USDA-ARS, Gainesville, Florida. For biting deterrence bioassays, eggs were hatched and the insects were reared to the adult stage in the laboratory and maintained at $27 \pm 2^\circ\text{C}$ and $60 \pm 10\%$ RH with a photoperiod regimen of 12:12 h (L:D). 8-13 day-old adult females were used. For larval bioassays, the eggs were hatched and the larvae were maintained at the above temperature.

Mosquito Biting Bioassays: Experiments were conducted by using a six-celled *in vitro* Klun and Deboun (K&D) module bioassay system developed by Klun et al. [25] for quantitative evaluation of biting deterrence of candidate compounds. The assay system consisted of a six well reservoir with each of the 3 X 4 cm wells containing 6 mL of blood. Further procedures were performed as described by Ali et al. [26].

The temperature of the solution in the reservoirs was maintained at 37°C by continuously passing the warm water through the reservoir using a circulatory bath. The reservoirs were covered with a layer of collagen membrane (Devro, Sandy Run, SC). The test compounds were randomly applied to six 4 X 5 cm areas of organdy cloth and positioned over the membrane-covered CPDA-1+ATP solution with a Teflon separator placed between the treated cloth and the six-celled module to prevent the contamination of the module. A six celled K&D module containing five female mosquitoes per cell was positioned over cloth treatments covering the six CPDA-1 + ATP solution membrane wells, and trap doors were opened to expose the treatments to these females. The number of mosquitoes biting through cloth treatments in each cell was recorded after a 3 min exposure and mosquitoes were prodded back into the cells to check the actual feeding. Mosquitoes were squashed and the presence of green fluorescent tracer dye (or not) in the gut was used as an indicator of feeding. A replicate consisted of six treatments: four test compounds, DEET (a standard biting

deterrent) and ethanol treated organdy as solvent control applied randomly. Five replications with five females per treatment were conducted using newly treated organdy and a new batch of females in each replication.

Larval Bioassays. Bioassays were conducted to test the essential oils of *D. ambrosioides*, *Z. armatum* and *J. communis* for their larvicidal activity against *Ae. aegypti* by using the bioassay system described by Ali and coworkers [27]. Eggs were hatched and larvae were held overnight in the hatching cup in a temperature-controlled room. Five one day-old *Ae. aegypti* larvae were added in a droplet of water to each well of 24-well plates (BD Labware, Franklin Lakes, NJ) by use of a disposable 22.5 cm Pasteur pipette. Fifty microliters of larval diet (2% slurry of 3:2 Beef Liver powder (Now Foods, Bloomingdale, Illinois) and Brewer's yeast (Lewis Laboratories Ltd., Westport, CT) was added to each well by using a Finn timer (Thermo Fisher, Vantaa, Finland). All chemicals tested were diluted in dimethyl sulfoxide (DMSO). Eleven microliters of the test chemical was added to the labeled wells, while 11 μ L of DMSO was added to control treatments. After the treatment application, the plates were swirled in clock-wise and counter clockwise motions, front and back, and side to side five times to ensure even mixing of the chemicals. Larval mortality was recorded 24 h post treatment. Larvae that showed no movement in the well after manual disturbance of water were recorded as dead. A series of concentrations ranging between 125 and 31.25 ppm were used in each treatment to obtain a range of mortality.

Statistical Analyses: For statistical analyses, proportion not biting (PNB) was calculated using the following formula:

$$\text{PNB} = 1 - \left(\frac{\text{Total no. of biting females}}{\text{Total no. of females}} \right)$$

Data on the PNB values were then analyzed using the ANOVA procedure of SAS (version 9.2; SAS Institute Cary, NC).

Ethical approval: The conducted research is not related to either human or animals use.

3 Results and Discussion

The essential oils were analyzed using simultaneous GC-FID and GC-MS. A total of 59 compounds, accounting for 96.8%, were identified for *Z. armatum* oil. It possessed a high amount of 2-undecanone (47.7%) and 1,8-cineol (20.5%) followed by α -pinene (6.0%) and 2-tridecanone (4.7%) (Table 1). This is in accordance with the literature on *Z. armatum* leaf essential oil from India [28]. In general

Table 1: Constituents identified in the essential oil of *Zanthoxylum armatum* analysed by GC-MS-FID.

compounds	RI [#]	%Area (FID)	compounds	RI [#]	%Area (FID)
(E)-2-hexenal	850	tr.	2-decanone	1191	0.1
(Z)-3-hexenol	853	0.2	cryptone	1195	tr.
hexanol	864	0.1	α-terpineol	1199	0.4
α-thujene	933	0.1	pinocampheol	1205	tr.
α-pinene	942	6.0	trans-piperitol	1215	tr.
camphene	958	0.1	cis-3-hexenyl 2-methylbutanoate	1231	0.1
sabinene	980	2.3	linalyl acetate	1255	0.1
β-pinene	986	0.3	piperitone	1263	0.6
myrcene	992	1.4	cis-chrysanthenyl acetate	1268	0.1
dehydro-1,8-cineol	996	tr.	2-undecanone	1296	47.7
α-phellandrene	1011	0.1	2-undecanol	1300	0.6
α-terpinene	1023	0.1	α-terpinyl acetate	1356	0.1
p-cymene	1030	0.1	geranyl acetate	1380	tr.
limonene	1037	4.7	2-dodecanone	1393	0.1
1,8-cineol	1040	20.5	β-bourbonene	1405	0.1
(E)-β-ocimene	1049	0.6	(E)-β-caryophyllene	1442	1.5
γ-terpinene	1064	0.2	α-humulene	1476	0.3
cis-sabinene hydrate	1073	0.1	2-tridecanone	1495	4.7
2-nonanone	1090	0.2	germacrene D	1502	0.4
terpinolene	1095	0.2	(E,E)-α-farnesene	1509	0.2
linalool	1101	0.9	bicyclogermacrene	1517	0.1
nonanal	1103	0.1	γ-cadinene	1532	tr.
trans-sabinene hydrate	1105	0.1	δ-cadinene	1538	tr.
4,8-dimethyl-1,3,7-nonatriene (isomere 1)	1117	tr.	(E)-nerolidol	1567	0.1
p-mentha-1,3,8-triene	1124	tr.	caryophyllene oxide	1608	0.2
cis-p-menth-2-en-1-ol	1129	0.1	humulene epoxide II	1634	0.1
trans-p-menth-2-en-1-ol	1147	0.1	α-muurool	1660	0.2
citronellal	1154	tr.	τ-muurool	1674	0.1
camphor	1156	0.1	sum		96.8
δ-terpineol	1175	0.1			
terpinen-4-ol	1187	0.5			

[#] 50m x 0.25mm x 1.0 μm SE-54, tr. = trace
(<0.05%)

very little on leaf oil has been published in the literature, most of the research has been done on seed oil with linalool and limonene as major components from India [10,29] and borneol and isobornyl acetate from samples from Pakistan [30]. The odor of *Z. armatum* oil can be described as spicy refreshing odor with a touch of citrus and some terpeny notes. Its heart note is soft spicy with cypress and woody connotations, the fond note herbal-balsamic. In an older investigation *Z. armatum* leaf oil collected at 1,200 m altitude from an area near Palampur in 1992, close to the region our plants were collected, was described by Weyerstahl and coworkers. The authors

found linalool (18.8%), 2-undecanone (17.0%), 1,8-cineole (15.7%) and β-phellandrene (12.7%) as main constituents. 2-Tridecanone, another characteristic component of *Z. armatum* leaf oil, appeared in a ratio of 2.0%. The odor was described as green and fruity with a dominant tropical note as well as sweaty-animalic undertones [31]. This odor-description as well as the high amount of linalool is in contrast to our findings. Since this discrepancy can rather not be due to altitudinal or regional growth differences of the plants, one could assume that a climatic influence might be the cause [32,33]. Even more that almost 20 years lie between these two investigations.

Table 2: Constituents identified in the essential oil of *Juniperus communis* analysed by GC-MS-FID.

compounds	RI [#]	%Area (FID)	compounds	RI [#]	%Area (FID)
octane	800	tr.	linalyl acetate	1258	tr.
(<i>E</i>)-2-hexenal	848	tr.	methyl citronellate	1262	0.5
tricyclene	931	0.1	piperitone	1267	tr.
α -pinene	943	43.2	geranial	1275	0.1
α -fenchene	955	0.1	2-undecanone	1296	0.1
camphene	958	0.3	bornyl acetate	1298	0.4
verbenene	968	0.1	isobornyl acetate	1301	tr.
sabinene	980	0.4	dihydrocarvyl acetate	1309	0.2
β -pinene	986	2.8	methyl geranate	1328	0.2
myrcene	992	3.0	myrtenyl acetate	1337	0.1
ethyl hexanoate	995	tr.	citronellyl acetate	1355	0.1
δ -2-carene	1008	0.5	α -terpinyl acetate	1361	1.3
α -phellandrene	1011	0.1	γ -terpinyl acetate	1365	tr.
δ -3-carene	1018	0.4	α -cubebene	1369	tr.
α -terpinene	1023	tr.	geranyl acetate	1386	0.1
p-cymene	1030	0.2	α -copaene	1399	tr.
limonene	1038	34.7	β -elemene	1412	0.6
γ -terpinene	1065	0.1	sibirene	1428	0.1
cis-sabinene hydrate	1074	tr.	(<i>E</i>)- β -caryophyllene	1448	0.1
terpinolene	1096	0.5	g-elemene	1454	tr.
linalool	1101	0.1	(<i>E</i>)- β -farnesene	1466	tr.
trans-sabinene hydrate	1106	tr.	α -humulene	1483	0.1
α -pinene oxide	1110	tr.	germacrene D	1509	0.2
trans- <i>p</i> -mentha-2,8-dien-1-ol	1128	0.1	β -selinene	1516	0.2
cis- <i>p</i> -menth-2-en-1-ol	1131	0.1	α -muurolene	1523	0.4
cis- <i>p</i> -mentha-2,8-dien-1-ol	1142	0.1	b-bisabolene	1526	0.5
trans-limonene oxide	1146	tr.	γ -cadinene	1540	0.4
trans-pinocarveol	1152	0.1	d-cadinene	1546	1.3
trans-verbenol	1155	0.1	(<i>E</i>)- γ -bisabolene	1550	0.1
camphene hydrate	1163	tr.	(<i>E</i>)- α -bisabolene	1557	0.1
borneol	1179	tr.	α -cadinene	1562	0.1
terpinen-4-ol	1189	0.3	elemol	1571	tr.
<i>p</i> -cymen-8-ol	1192	0.1	(<i>E</i>)-nerolidol	1574	0.3
cryptone	1197	tr.	4 α -hydroxygermacra-1(10),5-diene	1604	0.4
α -terpineol	1201	0.4	1- <i>epi</i> -cubenol	1655	0.1
myrtenol	1208	tr.	τ -muurol + τ -cadinol	1667	0.4
trans-carveol	1211	tr.	α -cadinol	1680	0.4
citronellol	1230	0.2	α -bisabolol	1703	0.4
thymol methyl ether	1240	0.1	sum		97.9
neral	1247	0.1	# 50m x 0.25mm x 1.0 μ m SE-54, tr. = trace (<0.05%)		
carvone	1255	tr.			

The complex mixture of *J. communis* essential oil consisted mainly of monoterpene hydrocarbons with α -pinene (43.2%) and limonene (34.7%) as dominant constituents out of a total of 79 components (97.9%) (Table 2). Although Dahmane and coworkers [34] as well as Kumar and colleagues [35] also detected mainly monoterpenoids in their oils from Algeria and India, respectively, these oils differed in the ratio of the main compounds. Here again, most of available publication described the oil of Juniper berries, just little research was done on the needles/leaves. Olfactory evaluation of *J. communis* essential oil resulted in a fresh and terpeny odor with a touch of gin as a top note, a soft spicy and herbaceous heart note and a balsamic fond odor with a tender woody touch.

For *D. ambrosioides* essential oil, 16 compounds, comprising 82.9% of the oil, were identified. p-Cymene (36.3%) and ascaridole (31.2%) were most prominent (Table 3). The oil exhibited a terpeny, slightly metallic and harsh top note, reminding of myrcene, and a herbal camphorous, rather disgusting heart note. Its fond note was unpleasant, somewhat dusty-minty. A study on *D. ambrosioides* (*Chenopodium ambrosioides*) essential leaf oil derived from the same area in Palampur in 1993 showed high concentration of α -terpinene (65%) and p-cymene (29.4%). Interestingly the ratio of ascaridole was very low (0.7%) which is rather unusual since this monoterpenoid is the lead-component of this oil and responsible for its anthelmintic activity [36]. The reason for this could again be climatic issues. Despite local variation our findings are in accordance with literature on oil samples from Brasil derived from cultivated plants in Lavaras [37]. However, they differ significantly from samples collected from wild in Sao Paolo which, besides p-cymene (14.5%), included cis-piperitone oxide (35.2%) and trans-isoascaridole (14.1%) as main components [18].

In bioautography assays, *D. ambrosioides* essential oil showed the best antifungal activity with fungal growth inhibition zones from 6.5 to 8.0 mm and 11.0 to 14.5 mm using two concentrations of 80 and 160 $\mu\text{g}/\text{spot}$ against *Colletotrichum* species. Antifungal activity was evident by the presence of clear zones with a dark background where fungal mycelia or reproductive stroma were not present on the TLC plate. At the highest tested concentration (160 $\mu\text{g}/\text{spot}$), the zone inhibitions were almost close to commercial fungicide captan (1.2 $\mu\text{g}/\text{spot}$) against all three *Colletotrichum* species (Table 4). Data published by a French research group confirmed this high antifungal activity using different screening methods on several essential oils against cellulolytic mold strains: *D. ambrosioides* showed the best results [38]. The other two essential oils,

Table 3: Constituents identified in the essential oil of *Dysphania ambrosioides* analysed by GC-MS-FID.

compounds	RI [#]	%Area (FID)
hexanol	863	tr.
santene	907	0.1
p-cymene	1033	36.3
limonene	1038	0.3
γ -terpinene	1067	0.1
p-cymenene	1097	0.1
4-hydroxy-4-methylcyclohex-2-enone	1129	4.0
p-cymen-8-ol	1194	1.0
α -terpineol	1202	0.1
iso-ascaridole	1235	2.4
ascaridole	1256	31.2
piperitone epoxide isomere 1	1266	0.7
piperitone epoxide isomere 2	1270	1.0
ascaridol glycol isomere 1	1282	2.4
ascaridol glycol isomere 2	1300	3.2
carvacrol	1308	0.2
sum		82.9
[#] 50m x 0.25mm x 1.0 μm SE-54, tr. = trace (<0.05%)		

Z. armatum and *J. communis*, showed moderate antifungal activity. In our previous antifungal studies, essential oils rich in non-oxygenated monoterpenes such as α -/ β -pinenes, p-cymene, limonene, myrcene and the oxygenated monoterpene 1,8-cineole were not active in bioautography bioassays against *Colletotrichum* species [39-43]. The low antifungal results of *Z. armatum* and *J. communis* oils in the present investigation are another prove of the rather inefficient antifungal impact of oils comprising these terpenoids as main components. However, the essential oil of *D. ambrosioides* was the most antifungal active oil tested. This high antifungal impact might be due to the oil's high amount of ascaridole: It is a bicyclic monoterpene with a rather unusual bridging peroxide functional group, and has long been the only known natural peroxide, first described in 1908. Salimena and coworkers noted high antifungal activity of *D. ambrosioides* oil against spore germination of *Botrytis cinerea* (Syn. *Botrytinia fuckeliana*), a plant pathogen mainly affecting roses. The authors also pointed out that, besides thymol and carvacrol, also ascaridole has a high antifungal impact which has repeatedly been reported in the literature [37]. Other authors also demonstrated its antifungal activity against fungus *Sclerotium rolfsii* [44] as well as its potent allelopathic effect on *Amaranthus hypochondriacus* [45].

Table 4: Antifungal activity of three Himalayan essential oils against *Colletotrichum* test species using direct bioautography.

	Mean fungal growth inhibition [§] (mm) ± SD			
	Essential oils			
Fungal plant pathogens	<i>Dysphania ambrosioides</i>	<i>Juniperus communis</i>	<i>Zanthoxylum armatum</i>	
		80 µg/spot		
<i>C. acutatum</i>	6.5±0	2.5±0.71		4.0±0.71
<i>C. fragariae</i>	8.0±0	3.0±0.71		3.0±0.71
<i>C. gloeosporoides</i>	7.5±0.71	2.5±0.71		3.5±0.71
		160 µg/spot		
<i>C. acutatum</i>	11±1.41	3.5±0.71		5.5±0.71
<i>C. fragariae</i>	14.5±0.71	4.0±0.71		4.0±0.71
<i>C. gloeosporoides</i>	13.5±0.71	3.5±0.71		5.0±0.71
	Technical grade fungicides standards			
	Benomyl	Captan	Cyprodinil	Azoxystrobin
<i>Fungicide standard</i>	1.16 µg/spot	1.2 µg/spot	0.9 µg/spot	1.61 µg/spot
<i>C. acutatum</i>	Diffuse inhibitory zone	12.4±1.67	Diffuse inhibitory zone	Diffuse inhibitory zone
<i>C. fragariae</i>	22.0±1.58	15.4±2.07	Dz	26.0±2.23
<i>C. gloeosporoides</i>	Diffuse inhibitory zone	12.2±1.78	Diffuse inhibitory zone	Diffuse inhibitory zone

[§]Mean inhibitory zones and standard deviations (SD) were used to determine the level of antifungal activity against each fungal species. Acetone was used as a solvent control and had 0 fungal growth inhibition.

Dz: Diffuse zone

Biting deterrent activity of the essential oils of *D. ambrosioides*, *Z. armatum*, and *J. communis* is given in Fig. 1. *Dysphania ambrosioides* essential oil showed highest biting deterrence with PNB value of 0.8 which was similar to DEET with PNB value of 0.88 followed by *Z. armatum* and *J. communis* demonstrating PNB values of 0.48 and 0.52, respectively. Bigoga et al. [46] reported 100% repellency of *C. ambrosioides* essential oil against *Anopheles gambiae* females at a dose of 200 ppm for four hours for both seed and leaf essential oils. These findings indicated that *C. ambrosioides* possesses mosquito larvicidal and repellent potential that might be further exploited in combating mosquito vectored diseases through anti-vector interventions.

Data on the toxicity of *Z. armatum*, *J. communis* and *D. ambrosioides* essential oils against one day-old larvae of *Ae. aegypti* at 24 h post treatment is given in Figure 2. Mortality in DMSO, which was used as solvent control, was 0%. All the three essential oils gave 100% mortality at the dose of 125 ppm. Mortality at 62.5 ppm was 100, 90 and 80%, in the essential oils of *Z. armatum*, *J. communis* and *D. ambrosioides*, respectively, whereas only *Z. armatum* and *J. communis* essential oils gave 100 and 40% mortality, respectively, at the dose of 31.25 ppm. Massebo and colleagues reported LD₅₀ values of 17.5 and 9.1 ppm of *C. ambrosioides* essential oil against 4th instar

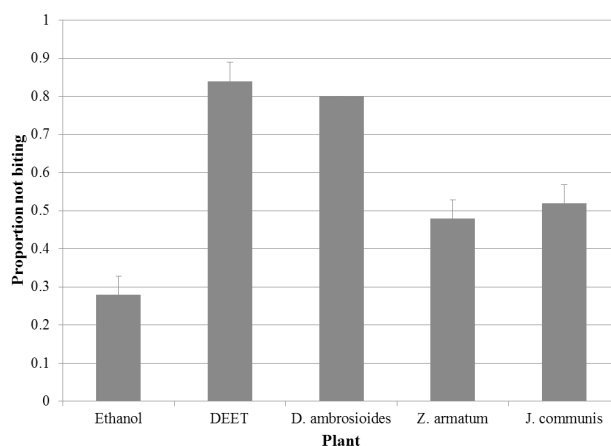


Figure 1: Proportion not biting values (+ SEM) of *D. ambrosioides*, *Z. armatum* and *J. communis* essential oils at 10 µg/cm² against female of *Ae. aegypti*. DEET at 25 nmol/cm² was used as positive control. Ethanol was used as solvent control.

larvae of *An. arabiensis* and *Ae. aegypti*, respectively [47]. Bigoga et al. reported 100% larval mortality at 200 ppm and 300 ppm for the essential oils from seeds and leaves, respectively [46]. Rajkumar and Jebanesan reported 50% mortality of 3rd instar *Culex quinquefasciatus* larvae at a dose of 7.12 ppm whereas mortality was 100% at 25 ppm. The extract also affected the egg laying of the mosquitoes [48]. Tiwary and coworkers reported larvicidal activity

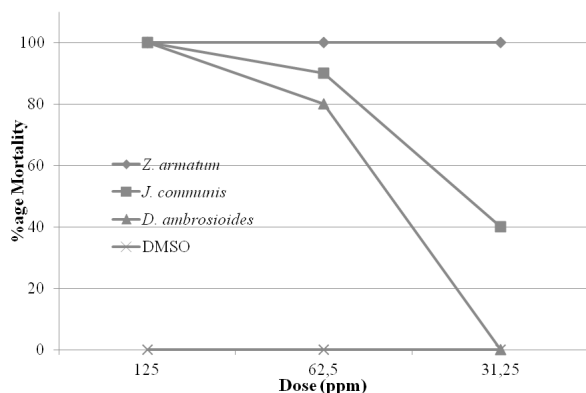


Figure 2: Percent mortality at various dosages of the essential oils of *D. ambrosioides*, *Z. armatum* and *J. communis* against 1-d old *Aedes aegypti*. In this bioassay, permethrin was used at a dose of 0.025 ppm as positive control which gave 100% larval mortality.

of *Z. armatum* essential oil against 3rd instar larvae of *Cx. quinquefasciatus* ($LC_{50} = 49$ ppm), *Ae. aegypti* ($LC_{50} = 54$ ppm) and *An. stephensi* ($LC_{50} = 58$ ppm) [10]. These data corroborate the above findings against *Ae. Aegypti*.

4 Conclusion

From all three essential oils being discussed in this study, only *Z. armatum* gave 100% mortality against *Ae. Aegypti* larvae at all concentrations tested, and therefore can be considered as potent natural insecticide. Ascaridole rich *C. ambrosioides* demonstrated the highest antifungal activity against three *Colletotrichum* species. Additionally, *C. ambrosioides* essential oil showed potential repellent activity against *Ae. aegypti* and ascaridole, again, might be the responsible repellent compound against these mosquitoes [49]. There is no study that reports antifungal activity of this monoterpenoid against *Colletotrichum* species. Therefore, *D. ambrosioides* essential oil should further be studied using 1D-TLC bioautography and the active antifungal compounds should be easily detectable directly on the TLC surface and further structurally identified. Although it has been stated that the whole essential oil usually is more active than its single components [50], isolated ascaridole should be tested on its biological activity in a follow up investigation.

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