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Chemotyping and Determination of Antimicrobial, Insecticidal and Cytotoxic Properties of Wild-Grown *Cannabis sativa* from Nepal

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ABSTRACT

***Cannabis sativa* was collected from a wild-growing population in Biratnagar, Nepal. The essential oil was obtained by hydrodistillation and analyzed by gas chromatography – mass spectrometry. A total of 107 constituents were identified in the oil accounting for 94.2% of the composition. This Nepalese chemotype is characterized by a predominance of sesquiterpenoids (68.1%) dominated by (*E*)-caryophyllene (20.4%), α-humulene (7.0%), and α-bisabolol (5.8%), but a paucity of monoterpene hydrocarbons (0.9%). In particular, neither myrcene nor terpinolene were detected. The oil in the Nepalese *Cannabis* plants did contain small amounts of cannabidiol (1.6%), cannabichromene (0.2%) and Δ^9-tetrahydrocannabinol (0.4%). The essential oil from Nepalese *Cannabis sativa* was screened for antimicrobial, cytotoxic, larvicidal, and insecticidal activity, and it appears as though *C. sativa* is relatively non-toxic.**

INTRODUCTION

Cannabis sativa L. is one of the three species of genus *Cannabis* that includes *C. sativa*, *C. indica*, and *C. ruderalis* (Guy *et al.*, 2004). *C. sativa*, commonly known as "hemp" or "marijuana" in English and "bhang" or "ganja" in Nepali language, is an annual herbaceous, dioceous plant (Kriese *et al.*, 2004). The origin of wild *Cannabis* is not known with certainty, but the plant, which appears to have been cultivated in northern China since 4000 BC (Mabberley, 2008), is widely distributed and cultivated throughout the world (Nigam *et al.*, 1965).

Some confusion and controversy regarding the taxonomy of *Cannabis* does exist. Small and Cronquist (1976) had proposed a monotypic genus, *C. sativa* with two subspecies, *sativa* and *indica*, while several others have recognized three different species, *C. sativa*, *C. indica*, and *C. ruderalis* (Schultes *et al.*, 1974; Anderson, 1974, 1980; Emboden, 1974). Recent, genetic, morphological, and biochemical investigations have helped to delineate the three species of *Cannabis* (Hillig and Mahlberg, 2004; Hillig, 2004, 2005). *C. sativa* generally has lower levels of Δ^9-tetrahydrocannabinol (THC) than cannabidiol (CBD), while *C. indica* has less CBD than THC (Hillig and Mahlgerg, 2004). Wide-leaf varieties of *C. indica* have shown relatively high ratio of guaiol, γ-eudesmol, and β-eudesmol, while the narrow-leaf varieties of *C. indica* have a relatively high ratio of (*E*)-β-farnesene (Hillig, 2004).

The essential oil of *Cannabis* has demonstrated antimicrobial activity (Novak *et al.*, 2001; Nissen *et al.*, 2010) and mosquito larvicidal activities (Pavela, 2009). In the current study, the composition, chemotype, and bioactivities of the essential oil from a wild-growing *Cannabis* plant, tentatively identified as *C. sativa*, from Biratnagar, Nepal, were investigated.

MATERIALS AND METHODS

Plant material. Flowering *Cannabis* plants collected from city of Biratnagar (26°28′ N, 87°16′ E,

72 m above sea level) in Morang district in Koshi Zone in Nepal on 18 May 2011 were used in this study. The plant material was identified by Tilak Gautam as male, narrow-leafed *Cannabis sativa* L. plants based on the plant morphology. A voucher specimen (4304) has been deposited in the herbarium of the Tribhuvan University, Post-Graduate Campus, Botany Department, Biratnagar.

The essential oil was extracted from fresh leaf samples (100 g) that were crushed and hydrodistilled using a Clevenger type apparatus for 4 h. The clear pale yellow essential oil (0.5 g) produced was stored at 4°C until analyzed.

Constituent identification. The essential oil from the *Cannabis* plant was analyzed by GC-MS using an Agilent 6890 GC with Agilent 5973 mass selective detector [MSD, operated in the EI mode (electron energy = 70 eV), with ascan range = 40-400 amu, and scan rate = 3.99 scans/sec], and an Agilent ChemStation data system. The GC column was an HP-5ms fused silica capillary with a (5% phenyl)-polymethylsiloxane stationary phase, a film thickness of 0.25 μm, a length of 30 m, and an internal diameter of 0.25 mm. The carrier gas was helium with a column head pressure of 48.7 kPa and a flow rate of 1.0 mL/min. The injector temperature was 200°C, and detector temperature was 280°C. The GC oven temperature was programed with an initial temperature of 40°C, hold for 10 min, followed by an increase in temperature at 3°C min to 200°C, and then increased at 2°/min to 220°C. A 1% w/v solution of each sample in CH_2Cl_2 was prepared, and 1 μL of the sample was injected using a 10:1 split ratio.

Identification of the oil components was based on the retention indices determined by reference to a homologous series of *n*-alkanes and by comparison of the mass spectral fragmentation patterns with those reported in the literature (Adams, 2007) and stored on the MS library [NIST database (G1036A, revision D.01.00)/ChemStation data system (G1701CA, version C.00.01.080)]. The percentages of each component were determined as raw percentages based on total ion current without standardization.

Antimicrobial activity. The essential oil was screened for anti-microbial activity against Gram-positive bacteria, *Bacillus cereus* (ATCC No. 14579), and *Staphylococcus aureus* (ATCC No. 29213), Gram-negative bacteria, *Pseudomonas aeruginosa* (ATCC No. 27853) and *Escherichia coli* (ATCC No. 10798), and the fungi *Candida albicans* (ATCC No.10231) and *Aspergillus niger* (ATCC No. 16888). Minimum inhibitory concentrations (MICs) were determined using the microbroth dilution technique as previously described (Satyal *et al.*, 2012).

Toxicity. The essential oil was screened for cytotoxicity using human MCF-7 breast adeno-carcinoma cells (ATCC No. HTB-22), along with tests of lethality using brine shrimp (*Artemia salina*), nematicidal activity using the nematode (*Caenorhabditis elegans*), larvicidal activity using glass worm (*Chaoborus plumicornis*), and insecticidal activity using termites (*Reticulitermes virginicus*) and fruit flies (*Drosophila melanogaster*) as previously described (Satyal *et al.*, 2012). LC_{50} values were determined using the Reed-Muench method (Reed and Muench, 1938).

Hierarchical cluster analysis. To develop a hierarchical cluster analysis, a selection of 68 *Cannabis* essential oil compositions, taken from published literature (Ross and ElSohly, 1996; Mediavilla and Steinemann, 1997; Novak *et al.*, 2001; Rothschild *et al.*, 2005; Bertoli *et al.*, 2010; Nissen *et al.*, 2010; Fischedick *et al.*, 2010), were treated as operational taxonomic units (OTUs). The percentage composition of 16 terpenoid essential oil components (α-pinene, β-pinene, δ-3-carene, myrcene, limonene, β-phellandrene, (*Z*)-β-ocimene, (*E*)-β-ocimene, terpinolene, α-bergamotene, (*E*)-caryophyllene, α-humulene, (*E*)-β-farnesene, β-selinene, selina-3,7(11)-diene, and caryophyllene oxide) were used to determine the chemical relationship among the *Cannabis* essential oil samples by the agglomerative hierarchical cluster (AHC) analysis using the XLSTAT software, version 2014.4.09. Pearson's correlation was selected as a measure of similarity, and the unweighted, pair-group method with arithmetic average (UPGMA) was used for cluster definition and to develop a dendrogram for the *Cannabis* selections.

RESULTS

The essential oil of *C. sativa* from Nepal yielded a total of 112 components of which 107 were identified, accounting for 94.2% of the total composition (Table 1). The oil was rich in sesquiterpene hydrocarbons (46.8%) and oxygenated sesquiterpenoids (21.3%), dominated by (*E*)-caryophyllene (20.4%), α-humulene (7.0%), α-bisabolol (5.8%), and caryophyllene oxide (3.8%).

In the screening of the Nepalese *C. sativa* essential oil for antimicrobial activity and toxicity, the oil showed notable activity in the brine shrimp lethality test with a LC_{50} = 13.6 μg/mL (Table 2). Tests for antimicrobial activity indicated the essential oil had essentially no effect on the tested microorganisms.

The hierarchical cluster analysis of the *C. sativa* essential oil compositions indicated the essential oil from the Nepalese plants was chemically distinct from the essential oil of other *Cannabis* plants (Figure 1). Nepalese *Cannabis* oil was high in caryo-phyllene and deficient in myrcene and terpinolene as compared with other *Cannabis sativa* essential oils.

DISCUSSION

The lack of appreciable antimicrobial activity exhibited by Nepalese *C,* sativa is consistent with previous studies that show only marginal activity of *Cannabis* oils against Gram-positive bacteria (Novak *et al.*, 2001; Nissen *et al.*, 2010). In addition, the marginal toxicity of Nepalese *C. sativa* against nematodes, glass worms, fruit flies, and termites is consistent with the reported marginal activity of Canadian *C. sativa* against mosquito (*Culex quinquefasciatus*) larvae (LC_{50} = 127 μg/mL) (Pavela, 2009). Apparently, *C. sativa* essential oil is relatively non-toxic and thus the lack of insecticidal activity of *C. sativa* oil is not surprising. The major component in the oil, (*E*)-caryophyllene, has shown only limited insecticidal activity against *Aedes aegypti* (Silva *et al.*, 2008), *Rhyzopertha dominica*, *Sitophilus oryzae*, *Tribolium castaneum* (Zeng *et al.*, 2010) or *Lycoriella ingenua* (Park *et al.*, 2008). Likewis, (*E*)-caryophyllene is neither notably anti-bacterial (Palazzo *et al.*, 2009) nor cytotoxic (Schmidt *et al.*, 2006; Wright *et al.*, 2007).

The activity of the *C. sativa* essential oil in the brine shrimp lethality test is comparable to many other essential oils (Werka *et al.*, 2007; Setzer *et al.*, 2008) and may be attributed to the major sesquiterpene components (*E*)-caryophyllene (LC_{50} = 0.23 μg/mL) and α-humulene (LC_{50} = 0.75 μg/mL).

In contrast to previously reported *Cannabis* essential oils (Ross and ElSohly, 1996; Mediavilla and Steinemann, 1997; Novak *et al.*, 2001; Rothschild *et al.*, 2005; Bertoli *et al.*, 2010; Nissen *et al.*, 2010), the oil from Nepal was poor in monoterpene hydrocarbons. In particular, the Nepalese *C. sativa* oil had no detectable quantities of mycrene or terpinolene, normally abundant in *C. sativa* (Ross and ElSohly, 1996; Mediavilla and Steinemann, 1997; Novak *et al.*, 2001; Rothschild *et al.*, 2005; Bertoli *et al.*, 2010; Nissen *et al.*, 2010). The sample collected from Biratnagar, Nepal, was identified as *C. sativa* based on plant and leaf morphology, and the cannabidiol (CBD) to Δ^9-tetrahydrocannabinol (THC) ratio is consistent with *C. sativa* (CBD/THC = 3.9) (Hillig and Mahlberg, 2004). The absence of myrcene and terpinolene, the low α-pinene concentration, and the relatively high concentration of (*E*)-β-farnesene in the Nepalese sourced essential oil, however, is consistent with *C. indica*. *C. sativa* essential oils generally contain high concentrations of myrcene and α-pinene (Hillig, 2004).

The hierarchical cluster analysis of essential oil compositions revealed the essential oil from Nepal is chemically distinct from other *Cannabis* essential oils and represents a new chemotype, rich in caryo-phyllene, but deficient in myrcene and terpinolene. Of the essential oils examined, two other samples of *C. indica*, also showed no terpinolene and small concentrations of myrcene (samples Bedropuur and AN) (Fischedick *et al.*, 2010).

Table 1. Composition of the essential oil of wild *Cannabis sativa* from Biratnagar, Nepal.

RI	Compound	%		RI	Compound	%
777	(2*Z*)-Pentenol	*0.1*		1346	4-Hydroxybenzyl alcohol	0.2
799	Hexanal	0.2		1349	α-Cubebene	tr
830	2-Furaldehyde	0.2		1357	Eugenol	0.2
851	(2*E*)-Hexenal	0.9		1366	Cyclosativene	0.1
853	(3*Z*)-Hexenol	4.7		1375	α-Copaene	0.1
866	(2*E*)-Hexenol	0.6		1391	7-*epi*-Sesquithujene	0.2
869	1-Hexanol	0.6		1408	(*Z*)-Caryophyllene	0.3
893	2-Heptanone	0.1		1410	Sesquithujene	0.2
897	Cyclohexanone	0.4		1422	(*E*)-Caryophyllene	20.4
905	Heptanal	0.1		1430	β-Copaene	0.1
940	α-Pinene	0.1		1438	α-*trans*-Bergamotene	3.0
954	Camphene	tr		1445	6,9-Guaiadiene	0.2
964	Benzaldehyde	0.1		1450	Unidentified	0.8
974	Sabinene	tr		1456	α-Humulene	7.0
979	β-Pinene	0.1		1460	(*E*)-β-Farnesene	3.3
989	6-Methyl-5-hepten-2-one	0.1		1470	4,5-di-*epi*-Aristolochene	0.1
1009	(3*Z*)-Hexenyl acetate	0.2		1478	γ-Muurolene	0.6
1029	Limonene	0.3		1481	γ-Curcumene	0.2
1032	1,8-Cineole	3.8		1485	γ-Selinene	0.4
1033	Benzyl alcohol	0.7		1488	β-Selinene	2.3
1039	(*Z*)-β-Ocimene	tr		1496	α-Selinene	2.3
1043	Phenylacetaldehyde	0.1		1501	α-Muurolene	0.2
1049	(*E*)-β-Ocimene	0.3		1504	(*E*,*E*)-α-Farnesene	0.1
1058	γ-Terpinene	0.1		1511	β-Bisabolene	2.6
1068	*cis*-Sabinene hydrate	1.5		1526	β-Sesquiphellandrene	2.8
1072	1-Octanol	0.1		1529	(*E*)-*iso*-γ-Bisabolene	0.3
1088	Fenchone	0.3		1531	10-*epi*-Cubebol	0.2
1095	Methyl benzoate	0.1		1538	α-Cadinene	0.1
1098	*trans*-Sabinene hydrate	0.9		1545	Unidentified	3.2
1101	Linalool	0.2		1554	Unidentified	0.6
1105	Nonanal	0.1		1564	(*E*)-Nerolidol	0.5
1113	2-Phenylethyl alcohol	0.4		1569	Caryophyllenyl alcohol	0.2
1120	*trans*-Pinene hydrate	0.4		1580	*trans*-Sesquisabinene hydrate	0.5
1138	*trans*-Pinocarveol	0.1		1584	Caryophyllene oxide	3.8
1141	*cis*-Pinene hydrate	0.2		1600	Guaiol	1.4
1143	4-Ketoisophorone	0.1		1607	Humulene epoxide II	1.3
1147	Ipsdienol	0.4		1621	10-*epi*-γ-Eudesmol	0.5
1149	Myrcenone	0.2		1632	(*E*)-Sesquilavandulol	2.0
1155	(3*Z*)-Nonenol	0.1		1637	Caryophylla-4(12),8(13)-dien-5-ol	0.7
1158	β-Pinene oxide	0.2		1646	α-Muurolol (= Torreyol)	0.1
1162	Pinocarvone	0.2		1652	β-Eudesmol	0.4
1165	Borneol	0.2		1655	α-Eudesmol	0.8
1166	δ-Terpineol	0.1		1657	Selin-11-en-4α-ol	0.4
1177	Terpinen-4-ol	0.6		1660	Intermedeol	0.7
1185	*p*-Cymen-8-ol	tr		1669	Bulnesol	1.2
1187	(3*Z*)-Hexenyl butanoate	0.2		1672	14-Hydroxy-9-*epi*-(*E*)-caryophyllene	0.8
1190	α-Terpineol	0.5		1686	α-Bisabolol	5.8
1193	Hexyl butanoate	0.2		1689	Unidentified	0.9
1208	Verbenone	0.1		2100	(*E*)-Phytol	1.0
1232	(3*Z*)-Hexenyl 2-methylbutanoate	tr		2419	Cannabidiol	1.6
1238	Neral	0.1		2427	Cannabichromene	0.2
1241	Carvone	tr		2518	Δ^9-Tetrahydrocannabinol	0.4
1249	*p*-Anisaldehyde	0.1		-	Total Identified	94.2
1252	Geraniol	tr		-	Monoterpene hydrocarbons	0.9
1260	2-Hydroxybenzenemethanol	0.2		-	Oxygenated monoterpenoids	10.1
1269	Unidentified	0.3		-	Sesquiterpene hydrocarbons	46.8
1296	Perilla alcohol	tr		-	Oxygenated sesquiterpenoids	21.3
1312	*p*-Vinylguaiacol	1.1		-	Benzenoid aromatics	3.1
1325	(3*Z*)-Hexenyl tiglate	tr		-	Cannabinoids	2.2
1339	Piperitenone	0.1		-	Others	9.7

Table 2. Biological activity of *C. sativa* essential oil.

Test organism	Antimicrobial activity MIC (μg/mL)
Bacillus cereus	625
Staphylococcus aureus	2500
Escherichia coli	2500
Pseudomonas aeruginosa	2500
Aspergillus niger	625
Candida albicans	1250
	Toxicity LC_{50} (μg/mL)
MCF-7	103
Artemia salina	13.6
Chaoborus plumicornis	227
Caenorhabditis elegans	232
Reticulitermes virginicus	354
Drosophila melanogaster	500

The dendrogram demonstrated the presence of four well-defined myrcene-rich clusters: (a) very high (*E*)-caryophyllene and high terpinolene, (b) high α-pinene and (*E*)-caryophyllene, (c) very high (*E*)-caryophyllene, and (d) very high myrcene. Other chemotypes were characterized by: (e) high terpinolene, but moderate levels of myrcene and (*E*)-caryophyllene, (f) high levels of β-pinene, limonene, and terpinolene, but moderate myrcene, (g) high limonene, but reduced myrcene, and (h) the essential oil from the present work with high (*E*)-caryophyllene, but no myrcene and no terpinolene.

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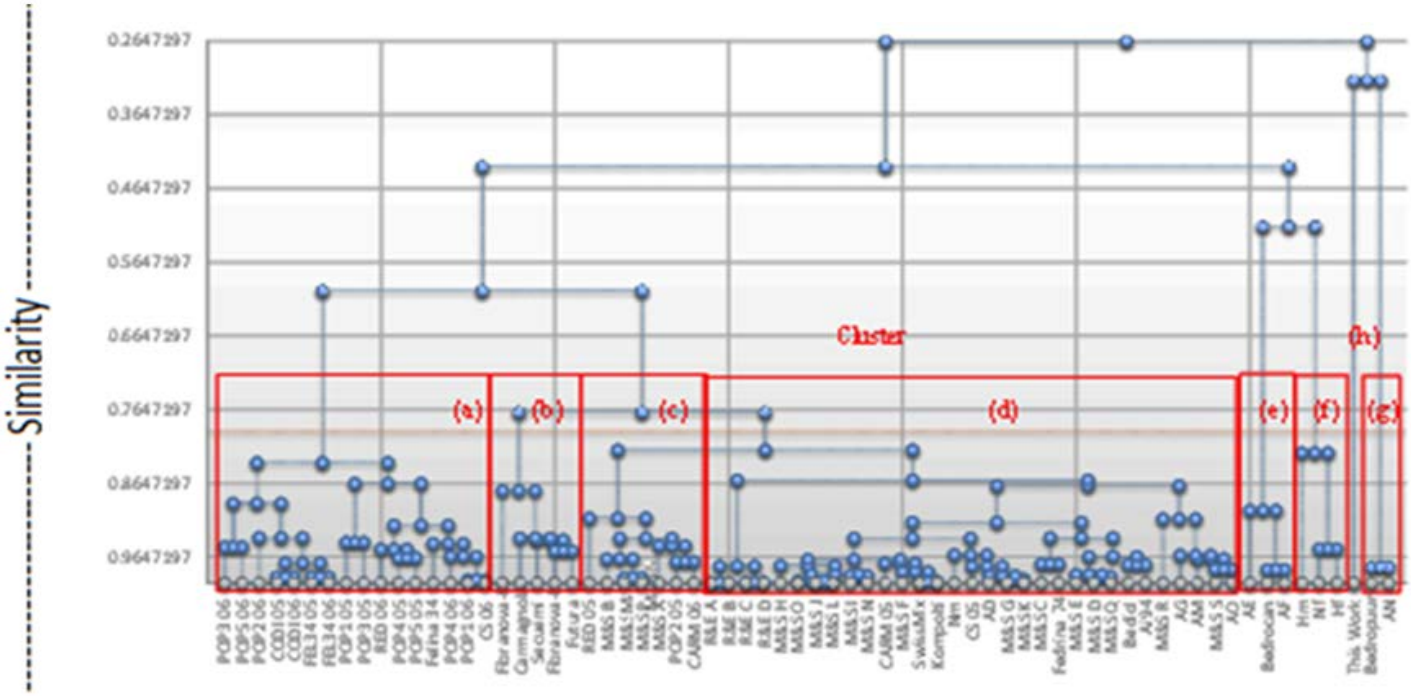

Figure 1. Cannabis dendrogram obtained from the agglomerative hierarchical cluster analysis of 68 *Cannabis* essential oil samples.

Supplemental: Sources

M&S A, M&S B, M&S C, M&S D, M&S E, M&S F, M&S G, M&S H, M&S I, M&S J, M&S K, M&S L, M&S M, M&S N, M&S O, M&S P, M&S Q, M&S R, M&S S (Mediavilla and Steinemann, 1997); R&E A, R&E B, R&E C, R&E D (Ross and ElSohly, 1996); Carmagnola, Fibranova-I, Fibranova-II, Futura (Nissen *et al.*, 2010); Nf, Nm, Hf, Hm (Rothschild *et al.*, 2005); SwissMix, Felina 34, Fedrina 74, Kompolti, Secuemi (Novak *et al.*, 2001); AO, Bedropuur, Bedrocan, Bediol, AG, AE, Ai94, AN, AF, AM, AD (Fischedick *et al.*, 2010); POP1 05, POP1 06, POP2 05, POP2 06, POP3 05, POP3 06, POP4 05, POP4 06, POP5 05, POP5 06, CARM 05, CARM 06, RED 05, RED 06, CS 05, CS 06, CODI 05, CODI 06, FEL34 05, FEL34 06 (Bertoli *et al.*, 2010).

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