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Antifungal Activity of the Volatiles of High Potency *Cannabis sativa* L. Against *Cryptococcus neoformans*

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Abstract: The *n*-hexane extracted volatile fraction of high potency *Cannabis sativa* L (Cannabaceae). was assessed *in vitro* for antifungal, antibacterial and antileishmanial activities. The oil exhibited selective albeit modest, antifungal activity against *Cryptococcus neoformans* with an IC_{50} value of 33.1 µg/mL. Biologically-guided fractionation of the volatile fraction resulted in the isolation of three major compounds (**1-3**) using various chromatographic techniques. The chemical structures of the isolated compounds were identified as α-humulene (**1**), β-caryophyllene (**2**) and caryophyllene oxide (**3**) using GC/FID, GC/MS, 1D- and 2D-NMR analyses, respectively. Compound **1** showed potent and selective antifungal activity against *Cryptococcus neoformans* with IC_{50} and MIC values of 1.18 µg/mL and 5.0 µg/mL respectively. Whereas compound **2** showed weak activity (IC_{50} 19.4 µg/mL), while compound **3** was inactive against *C. neoformans*.

Keywords: Volatile fraction; *Cannabis sativa*; antifungal; *Cryptococcus neoformans;* α-humulene.

1. Introduction

The cannabis plant is one of the oldest known medicinal plants and has been described in almost every ancient handbook on plant medicines [1]. More than 750 constituents have been reported in *Cannabis sativa* L. (Cannabaceae) of which the cannabinoids are the most characteristic class [2]. These as well as other constituents, including terpenoids, flavonoids, nitrogenous compounds, sterols, pigments and phenols, have been reviewed [3]. Although cannabis is one of the most chemically

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studied plants, most of these constituents have not yet been evaluated for biological activity [4]. Cannabis contains a significant number of terpenoids that make up a large percentage of its essential oil, where were the subject of several investigations [5-8].

To date more than 120 terpenoids have been found in cannabis, including 58 monoterpenes, 38 sesquiterpenes, one diterpene, two triterpenes, and four other terpenoids. Two comprehensive reviews have been published summarizing these compounds and their identification [9, 10]. Terpenoids display a wide range of biological activities and hence may play a role in some of the pharmacological effects of various cannabis preparations [8, 11].

The essential oil of cannabis obtained by steam distillation is a clear and slightly yellow-colored liquid with a characteristic smell, a property that may be used by law enforcement agents to identify cannabis [6]. Few studies were focused on the antimicrobial activity of the volatiles of cannabis. The essential oil of industrial hemp was shown to control spoilage of food and phytopathogenic microorganisms [12, 13].

Cryptococcus neoformans is encapsulated yeast that can live in both plants and animals. Infection with *Cryptococcus neoformans*, known as Cryptococcosis, primarily manifested as a lung infection. However, fungal meningitis and encephalitis, especially as a secondary infection of AIDS patients, are often caused by *Cryptococcus neoformans* making it a particularly dangerous fungus. In human infection, *Cryptococcus neoformans* is spread by inhalation of aerosolized spores (basidiospores) and can disseminate to the central nervous system where it can cause meningoencephalitis [14].

The purpose of this report is to follow up on the finding that the essential oil of cannabis has antifungal activity and to identify the oil components responsible for such activity.

2. Materials and Methods

2.1. General experimental procedures

NMR spectra were recorded on a Varian Mercury 400 MHz spectrometer at 400 (^{1}H) and 100 MHz (^{13}C). GC/FID analyses were carried out on Varian CP-3380 gas chromatograph equipped with a Varian CP-8400 automatic liquid sampler, a capillary injector and flame ionization detector. The column was a 30 m x 0.25 mm DB-5, 0.25 μ film (J&W Scientific, Inc.). Data are recorded using a Dell Optiplex GX1 computer operating with Microsoft Windows XP and Varian Star (version 6.41) workstation software. Helium is used as the carrier gas. An indicating moisture trap and an indicating oxygen trap located in the helium line from upstream to downstream, respectively, were used. Helium was used as the "make-up" gas at the detector. Hydrogen and compressed air were used as the combustion gases. The instrument parameters used for monitoring samples were: Air - 30 psi (400 mL/min); Hydrogen - 30 psi (30 mL/min.); column head pressure - 14 psi (1.0 mL/min); split flow rate - 50 mL/min; split ratio - 50:1; septum purge flow rate - 5 mL/min; make up gas pressure - 20 psi (20 mL/min); injector temp - 220 ^{0}C; detector temp - 240°C; initial oven temp- 60 ^{0}C; initial temperature hold time - 1 min; temperature rate - 3 ^{0}C/min.; final oven temperature - 240 ^{0}C and final temperature hold time - 4 min. 1.0 μL was injected.

Thermo Finnegan Trace MS interfaced to a Trace 2000 GC equipped with an AS2000 autosampler and a single capillary injector and electron impact (EI+) source was used. High purity helium was used as the carrier gas and a high capacity oxygen trap was located in the helium line. Dell Optiplex 745 workstation operating with Microsoft Windows XP. Data was collected and processed using a ThermoQuest Xcaliber software (Ver. 1.2). The column was a 30 m x 0.25 mm DB-5, 0.25 μ film (J&W Scientific, Inc.) with a 50:1 split injection. The injector temperature was set at 220 ^{0}C and the oven temperature programmed at 60 ^{0}C for 1 minute, then ramped to 240 ^{0}C at a rate of 3 ^{0}C/ minute and held at the final temperature for 4 minutes. 1.0 μL was injected. Column chromatographic separations were performed on silica gel 60 (0.04-0.063 mm). TLC was performed on precoated TLC plates with silica gel 60 F254 (0.2 mm, Merck). The solvent system used for TLC analysis was: *n*-hexane: EtOAc (95:5).

Reference standards of different monoterpenes, sesquiterpenes, and alkanes were obtained from Aldrich Chemical Co. Inc. (Milwaukee, WI), Fluka Chemical Corp. (New York, NY), Roth Co. Chemische Fabrik (Karlsruhe, Germany), Sigma Chemical Co. (St. Louis, MO), and Varian Associates (Houston, TX). Solutions were prepared in methanol at concentrations of 10 mg/mL. For GC/MS analysis each standard solution was diluted by mixing 0.1 mL of the standard solution with 0.9 mL of methanol.

2.2. Plant Materials

Cannabis sativa plants were grown from high potency Mexican seeds (variety code CHPF-01). The seeds and plants were authenticated by Dr. Suman Chandra, The University of Mississippi, and the specimen (S1310V1) was deposited at the Coy Waller Complex, The University of Mississippi. Whole buds of mature female plants were harvested in 2007, air-dried, packed in barrels (#1239) and stored at low temperature (-24 °C).

2.3. Extraction and Isolation

The plant material (1.0 kg.) was extracted with *n*-hexane (2×3 L), at room temperature and evaporated under reduced pressure (40°C) to afford an *n*-hexane (80 g) extract. The extract was heated at 130°C for 30 min. for complete decarboxylation. The extract was then subjected to fractional distillation using Pope-2`` Wiped Film Still apparatus (Internal condenser temp. 200°C, vacuum 0.1 torr, flow rate 2 mL/min. and external condenser temp.70°C) to produce 10 g of the volatile fraction.

The volatile fraction (10.0 g) was subjected to Vacuum Liquid Chromatography (VLC) over silica gel eluting with *n*-hexane/EtOAc (100:0,75:25, 50:50, 40:60, 30:70, 20:80, 10:90 and 0:100). Seven fractions (A – G) were collected, fraction A (3.0 g) showed antifungal activity and antileishmanial activity. Fraction C (1.8 g) showed antibacterial activity while fractions D (1.49 g) and F (156 g) displayed antileishmanial activity.

Fraction A was chromatographed over a silica gel column (120g, 5x100 cm), using a step-gradient elution with a solvent consisting of n-hexane and EtOAc mixtures to yield seven sub-fractions A1-A7. Fraction A4 yielded a pure compound (**1**) identified as α-humulene (60 mg).

Sub-fractions (A_{1-3}, 2.9g) were further purified on a silica gel column (120g, 5x100 cm) using n-hexane/ EtOAc solvent system with gradient elution to yield compound 1 (40.6 mg), compound **2** (45.1 mg) and compound **3** (53.5 mg).

Fraction C (1.8 g) was fractionated on silica gel column (80 g, 2x100 cm) using *n*-hexane/ EtOAc solvent system with gradient elution, producing 15 sub-fractions. Bioassay guided fractionation of these subfractions resulted in five active subfractions against bacteria, leshmania and fungi. (Table 1)

2.3. Antimicrobial Assays

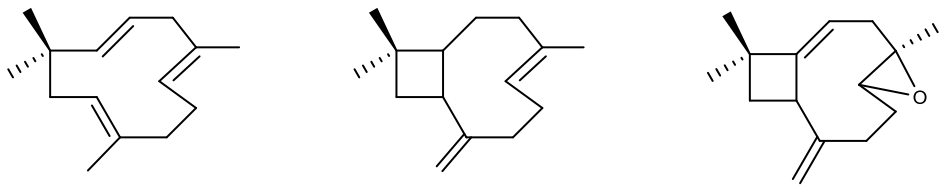
The antimicrobial evaluation of the volatile oil, the isolated fractions, the subfractions as well as the isolated pure compounds (**1-3**) were performed against *Staphylococcus aureus* ATCC 29213, methicillin-resistant *S. aureus* ATCC 33591 (MRSA), *Escherichia coli* ATCC 35218, *Pseudomonas aeruginosa* ATCC 27853, *Mycobacterium intracellulare* ATCC 23068, *Candida albicans* ATCC 90028, *Candida glabrata* ATCC 90030, *Candida krusei* ATCC 6258, *Cryptococcus neoformans* ATCC 90113, and *Aspergillus fumigatus* ATCC 204305 [15]. Ciprofloxacin and amphotericin B were used as positive controls for bacteria and fungi, respectively.

3. Results and Discussion

There are currently considerable challenges for the treatment of infections caused by strains of clinically relevant bacteria that show multidrug-resistance (MDR), such as methicillin resistant *Staphylococcus aureus* (MRSA). On the other hand, most infections with *Cryptococcus neoformans* consist of a lung infection. However, fungal meningitis and encephalitis, especially as a secondary

infection for AIDS patients, are often caused by *Cryptococcus neoformans* making it a particularly dangerous fungus. Despite the promising antibacterial activity of many plant secondary metabolites and the ability of some of them to modify the resistance associated with MDR strains [16], plants are still substantially untapped source for antimicrobial agents. These considerations make *C. sativa* an important source of compounds that could be studied for their antimicrobial activity.

In continuation of our search for bioactive compounds from a high potency variety of *Cannabis* [17-20], The antimicrobial activity of the volatile oil was investigated. The oil showed modest antifungal activity with an IC_{50} value of 33.15 µg/mL against *Cryptococcus neoformans*. Bioassay guided fractionation resulted in several fractions. Fraction A displayed antifungal activity of against *Cryptococcus neoformans* with an IC_{50} value of 15.6 µg/mL. Fraction A was further fractionated on a silica gel column to give seven subfractions A_1-A_7, of which subfractions A_{1-3}, A_4 and A_{5-6} displayed good antifungal activities (Table 1). The bioactive subfraction A_4 which possessed potent antifungal activity with IC_{50} values of 1.18 µg/mL, 1.45 µg/mL and 5.17 µg/mL against *Cryptococcus neoformans*, *Candida glabrata* and *Candida krusei* respectively, and moderate antileishmanial activity with IC_{50} 21.34 µg/mL was chemically identified as α-humulene by comparison of its NMR data and the GC/MS analysis with those previously reported [21-25].
Fraction A_{1-3} had good antifungal activity with an IC_{50} value of 3.85 µg/mL against *Cryptococcus neoformans* and moderate activity against *Candida glabrata* with IC_{50} 10.49 µg/mL. The purification using many chromatographic techniques of this fraction yielded three pure compounds identified as α-humulene (**1**), β-caryophyllene (**2**) and caryophyllene oxide (**3**) (Figure 1), based on the comparison of GC/MS and NMR data with those previously published [26].



1 **2** **3**

Figure 1. Isolated and Characterized Chemical Structures (**1-3)** from *C. sativa* Volatile Fraction

Compound **1** showed good and selective antifungal activity against *Cryptococcus neoformans*, *Candida glabrata* and *Candida krusei* with IC_{50} value of 1.18 µg/mL, 3.59 µg/mL and 6.94 µg/mL, respectively, and MIC values of 5.0 µg/mL, 1.45 µg/mL and 10.0 µg/mL respectively. Compound **2** showed weak activity against *Cryptococcus neoformans* with IC_{50} 19.4 µg/mL while compound 3 was inactive.

The antibacterial and antileishmanial activities of the volatile oil fractions were also studied (Table 1). The results showed that fraction A had antileishmanial activity with an IC_{50} value of 34.3 µg/mL. Fraction A was subjected to further fractionation over a silica gel column chromatography to give subfractions A1-3, A4 and A5-6 that displayed good antileishmanial activities with IC_{50} values of 7.47, 21.34 and 11.2 µg/mL respectively (Table 1).

Fraction C exhibited moderate antibacterial activity with IC_{50} values of 10.45 µg/mL and 15.93 µg/mL against *S. aureus* and MRSA, respectively. Also it showed antileishmanial activity with an IC_{50} value of 22.34 µg/mL. Biologically guided fractionation of its subfractions C_{1-15} produced only five active subfractions C_{6-10} (Table 1). Sub-fraction C_6 showed significant antibacterial activity with IC_{50} values of 2.98 µg/mL and 1.78 µg/mL against *S. aureus* and MRSA respectively, It also showed moderate antileishmanial activity with IC_{50} value of 12.47µg/mL and IC_{90} value of 28.26 µg/ml. Subfraction C_7 showed significant antibacterial activity with IC_{50} values of 0.93 µg/mL, 0.82 µg/mL

and 16.59 μg/mL against *S. aureus, MRSA and M. intracellulare*, respectively. Moreover, it showed good antileishmanial activity with IC_{50} value of 8.6 μg/mL and IC_{90} value of 24.19 μg/mL.

Table 1. Biological activity of volatiles fraction, fractions and pure compounds **1-3** (IC_{50} /MIC μg /mL)

Name	*C. neoformans* IC_{50} / MIC	*C. glabrata* IC_{50} / MIC	*C. krusei* IC_{50} / MIC	*S. aureus* IC_{50}	*MRSA* IC_{50}	*M. intracellulare*	*L. donovani* IC_{50}	*L. donovani* IC_{90}
V.F.	33.15	[a]NA	[a]NA	44.71	98.79	[a]NA	>40	>40
A	15.6	NA	NA	NA	NA	NA	34.3	NA
A_{1-3}	3.85	10.49	NA	NA	NA	NA	7.47	12.16
A_4	1.18	1.45	5.17	NA	NA	NA	21.34	NA
A_{5-6}	4.46	8.37	5.07	NA	NA	NA	11.2	23.43
A_7	NA	NA	NA	NA	NA	NA	NA	NA
B	NA	NA	NA	NA	NA	NA	NA	NA
C	NA	NA	NA	10.45	15.93	NA	22.43	31.84
C_{1-5}	NA	NA	NA	NA	NA	NA	NA	NA
C_6	NA	NA	NA	2.98	1.78	NA	12.47	28.26
C_7	NA	NA	NA	0.93	0.82	16.59	8.6	24.19
C_8	NA	NA	NA	3.40	2.09	NA	11.75	17.16
C_9	NA	NA	15.79	11.64	4.22	NA	18.06	28.69
C_{10}	NA	NA	NA	19.9	17.34	NA	23.05	32.11
D	NA	NA	NA	NA	NA	NA	20.11	NA
E	NA	NA	NA	NA	NA	NA	NA	NA
F	NA	NA	NA	NA	NA	NA	38.77	NA
G	NA	NA	NA	NA	NA	NA	NA	NA
1	**1.18 / 5.0**	**3.59 / 1.45**	**6.94 / 10.0**	**NA**	**NA**	**NA**	**9.76**	**29.68**
2	**19.4**	**NA**	**NA**	**NA**	**NA**	**NA**	**NA**	**NA**
3	**NA**	**NA**	**NA**	**NA**	**NA**	**NA**	**NA**	**NA**

[a]NA: not active at the highest test concentration.
V.F : Volatile fraction
IC_{50} : The Concentration of the sample that affords 50% inhibition of the target organism relative to growth and blank control.
IC_{90}: The Concentration of the sample that affords 90% inhibition of the target organism relative to growth and blank control.
MIC: (Minimum Inhibitory Concentration is the lowest concentration that allows no detectable growth)
C. neoformans C. glabrata, C. krusei
S. aureus = Staphylococcus aureus
MRSA = Methicillin Resistant Staphylococcus aureus
M. intracellulare = Mycobacterium intracellulare
L. donovani = Leishmania donovani

Subfraction C_8 showed antibacterial activity against *S. aureus* and MRSA with IC_{50} of 3.40 μg/mL and 2.09 μg/mL, respectively. Subfractions C_{9-10} showed weak antibacterial activity against *S. aureus* and MRSA, but C_9 had good activity against MRSA with an IC_{50} value of 4.22 μg/mL and a weak antileishmanial activity (IC_{50} value of 18.06 μg/mL). The characterization of the antibacterial and antileishmanial compounds in those fractions will be subjected to further investigation.

The terpenoids present in *Cannabis* possess a wide range of biological activities, possibly including modulation of the effects of Δ^9-THC via their own anxiolytic, sedative, analgesic, and anti-

depressant effects [27] Other actions of terpenoids include anti-inflammatory, acetyl-cholinesterase inhibition, anti-oxidant, antibiotic, and anti-mutagenic [28].

In conclusion, the volatile oil of a high potency *C. sativa* variety and fractions thereof showed promising antifungal and antibacterial as well as antileishmanial activity. The compound α-humulene (**1**) isolated from the active fraction A showed potent antifungal activity against the human pathogen *Cryptococcus neoformans*. This study suggested that the cannabis volatile oil and its constituents could be a promising source for anti-infective agents.

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Supporting Information

The supporting data include: GC chromatograms of the crude volatile fraction and the active fractions (A, C, D and F), as well as ^{1}H-NMR, ^{13}C and DEPT 135-NMR spectroscopic data of compounds (**1-3**).

Supporting Information accompanies this paper on http://www.acgpubs.org/RNP

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