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Chemical Composition and Antimicrobial Activity of *Gymnolaena oaxacana* (Greenm.) Rydb. (Asteraceae) Essential Oil

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Abstract: *Gymnolaena oaxacana* is an endemic species from Puebla and Oaxaca states (Mexico). This plant is used to treat gastrointestinal diseases; however its medicinal properties have not been investigated. In this study, chemical composition and the antimicrobial effect of *G. oaxacana* essential oil was determined. The essential oil was obtained by hydrodistillation and analyzed by GC-MS. 84.6% of its constituents were monoterpenes: γ-terpinene, β-Ocimene and β-pinene were the most abundant. The oil showed broad-spectrum antimicrobial effect by inhibiting population growth of nineteen bacterial strains, seven strains of yeasts and four strains of filamentous fungi. The most sensitive strains were: *Vibrio cholerae* INDRE 206, *V. cholerae* No. 01 (MIC=0.13 mg/mL) and *Trichophyton mentagrophytes* (FC_{50}=1.03 mg/mL). This is the first report of the chemical composition and antimicrobial activity of the essential oil and validated the ethno botanical use of *G. oaxacana* by the people for the treatment of gastrointestinal infections.

Keywords: Antibacterial Activity, Antifungal Activity, Essential Oil, Gymnolaena oaxacana, Monoterpenes

1. Introduction

Some medicinal plants produce a wide variety of essential oils which are used in traditional medicine for its antimicrobial, antioxidant, immunomodulatory, anti-inflammatory and antirheumatic effects [1, 2]. Biological activity of essential oils depends on their chemical composition, which is determined by the genotype and influenced by environmental conditions [3]. Several studies mentioned application of essential oils as antimicrobial agents that inhibit the growth of a wide range of microorganisms including bacteria, filamentous fungi and yeasts [4, 5]. Asteraceae family is relevant in this regard, since it includes a wide variety of plants that produce essential oils [2] that have compounds with antibacterial and antifungal properties, which are important because the resistance of various microorganisms have been developed [6].

Gymnolaena is a genus belonging to the Asteraceae family, it includes three species: *G. oaxacana* (Greenm.) Rydb, *G. chiapasana* Strother, *G. serratifolia* (DC.) Rydb [7]. In the literature there are no reports about the chemical composition of essential oil of any species of the genus.

Gymnolaena oaxacana is endemic to States of Oaxaca and Puebla (Mexico) [8]. This plant is known as "Zempoalxóchitl chiquito". It is used by the inhabitants of the village of San Rafael Coxcatlán, Puebla (México) for the treatment of gastrointestinal diseases caused by microorganisms. An ethnobotanical study reported that infusions of aerial parts of *G. oaxacana* are used as antidiarrheal. This effect was confirmed because the hexane extract inhibited the growth of ten bacterial strains [9]. *G. oaxacana* produced essential oil but there are not studies about its chemical constituents and

its biological properties. The aim of this study was to determine the chemical composition of the essential oil of *G. oaxacana* and to know its antimicrobial effect for validate the medicinal use of the plant.

2. Materials and Methods

2.1. Plant Material

Aerial parts of *G. oaxacana* were collected in September (2009) in San Rafael Coxcatlán, Puebla. A voucher specimen was deposited in the IZTA herbarium (Voucher no. HMC14/2009).

2.2. Extraction and Analysis of Essential Oil

Essential oil was obtained by hydrodistillation (1.265 kg of fresh aerial parts) during 4 hours in the Cleavenger-type apparatus and stored at 4°C until tested and analyzed, the yield and density were determined. The yield of the essential oil was 0.22% (w/w), d^{25}= 0.91 g/mL.

The essential oil was analyzed in an Agilent Technologies 6850 gas chromatograph equipped with a HP-5MS capillary column (30m × 0.25 mm; film thickness 0.25 μm). The temperature of the column was 325°C. Injector and detector temperatures were set at 230°C and 280°C, respectively. Oven temperature was kept at 70°C for 2 minutes, then programmed to 280°C at a rate of 8°C/minute and finally raised to 280°C. Helium was the carrier gas at a flow rate of 1 mL/minute. The sample was manually injected in the split mode and a volume of 1 μL. Peak areas were measured by electronic integration. The relative amount of the individual components was based on the peak areas. Mass analysis was performed on an Agilent Technologies 5975C. The temperature of the column and the injector were the same as those from GC. Mass spectra were recorded at 70 eV. The oil components were identified by comparison of their retention indices and mass spectra with the NIST08.L Mass Spectral of the internal device library (Match ≥ 90%). Retention indices were calculated by linear interpolation relative to retention times, of a series of *n*-alkanes (alkane's standards Sigma-Aldrich) and through the determination of the respective Kovats retention indices (KI). The KI were compared with those reported in literature [10].

2.3. Microbial Strains

The following strains of bacteria were used: *Enterobacter agglomerans* ATCC 27155, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922, *E. coli* ATCC 53218, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella typhi* ATCC 19430, *Staphylococcus aureus* ATCC 12398, *S. aureus* ATCC 29213 (donated by the Microbiology Laboratory of FES-Cuautitlán), *Bacillus subtillis*, *Enterobacter aerogenes*, *S. epidermidis*, *Yersinia enterocolitica* (donated by the Clinical Analysis Laboratory of FES Iztacala), *Proteus mirabilis*, *Serratia marcences*, *Streptococcus pneumoniae*, *Y. enterocolitica* (isolated from a clinical case and donated by Hospital Angeles Metropolitano), *Vibrio cholerae* (isolated from a clinical case), *V. cholerae* INDRE 206 (isolated from polluted water), *V. cholerae* (clinical strain pertaining to 01 group, Inaba serotype, El Tor biotype, and enterotoxin producer). These strains were maintained at 4 °C in Mueller Hinton agar (Bioxon), submitted to sensitivity tests (multidiscs Bigaux) and were subcultured twice after bioassays.

The yeasts tested were: *Candida albicans* ATCC 10231, *C. albicans* ATCC 14065, *C. albicans* isolated from a clinical case (donated by the Clinical Analysis Laboratory of FES-Iztacala), *C. albicans*, *C. glabrata*, *C. tropicalis* (isolated from a clinical case and donated by Hospital Angeles Metropolitano), *Criptococcus neoformans* (donated by the Microbiology Laboratory of FES-Cuautitlán). The filamentous fungi tested were: *Fusarium moniliforme* (donated by the Laboratory of Plant Physiology of FES Iztacala), *F. moniliforme* CDBB-H-265, *Rhizoctonia lilacina* CDBB-H-306, and *Trichophyton mentagrophytes* CDBB-H-1112. The stock culture was maintained on potato dextrose agar (PDA).

2.4. Antibacterial Activity

The antibacterial activity was measured by the disk-diffusion method [11]. The microorganisms were grown overnight at 37°C in 10 mL of Mueller Hinton Broth (Bioxon). The cultures were adjusted with sterile saline solution to obtain turbidity comparable to that of McFarland no. 0.5 standard (10^8 CFU/mL) [12]. Petri dishes containing Mueller Hinton agar (Bioxon) were inoculated with these microbial suspensions. Disks of filter paper (Whatman no. 5) of 5 mm diameter were impregnated with 5 μL of essential oil each one (final doses per disk: 4.55 mg of essential oil) and placed on the agar surface. Disks impregnated with chloramphenicol (25 μg) were used as positive controls. The plates were incubated overnight at 37°C and the diameter (mm) of any resulting zones of inhibition of growth, were measured with a vernier. Each experiment was performed in triplicate.

The estimation of the Minimal Inhibitory Concentration (MIC) was carried out by the broth dilution method [11]. Eight dilutions of essential oil (0.062, 0.125, 0.250, 0.5, 0.75, 1.0, 1.5 and 2.0 mg/mL) in broth were evaluated. The tubes were inoculated with 10^8 CFU/mL microorganism suspensions. MIC values were taken as the lowest essential oil concentration that prevents visible bacterial growth after 24 hours of incubation at 37°C. Chloramphenicol was used as reference standard and oil-free plates as controls. Each experiment was repeated at least three times.

The bactericidal kinetic assay was performed using appropriate concentrations of essential oil (corresponding to ½MIC, MIC and MBC) in 10 mL of broth. One control tube was prepared without essential oil. Into each concentration 0.1 mL of microbial suspension (10^8 CFU/mL) was inoculated and incubated at 37°C for four hours. Of each tube 50 μL aliquots were taken in ranges 10 and 30 minutes. Aliquots were plated on Muller-Hinton agar, which were incubated for 24 hours at 37°C. Finally, the number of CFU

was determined. Death kinetics was expressed in $\log_{10}$ reduction time kill plots [13].

2.5. Antifungal Activity

Antifungal activity assays were carried by inhibition of radial growth method [14]. Inoculate of 5 mm in diameter were placed in the center of petri dishes containing potato dextrose agar (20 mL). Disks of filter paper of 5 mm diameter were impregnated with 5 µL of essential oil each one (4.55 mg/disc) and placed on the agar surface. Disks impregnated with Ketoconazole (56 µg) were used as positive controls. The plates were incubated at 28°C for a period of 72 to 96 hours, until the mycelium covered the agar surface. Disks containing samples that had formed crescents of inhibition were considered with antifungal activity. Each experiment was performed in triplicate. The assay for anti-yeast activity was carried out in accordance with the disk-diffusion method [15] in this case the inoculums were adjusted to 10^5 CFU/mL. The discs were impregnated with 5 µL of essential oil per disc. As a positive control, Nystatin (30 µg/disc) was used.

Dilutions of essential oil from 3.0 to 0.0 mg/mL were added to PDA (2 mL) into 24 well plates for quantitative assays. An inoculum of 1 mm diameter was placed into the center of each well when the agar reached the room temperature. The plates were incubated overnight at 28 °C and the mycelium diameter of each well was measured. Each experiment was performed in triplicate. The inhibition of the fungal growth was determined in percentage; concentration-response curves were constructed with the data to calculate the Medial Fungicidal Concentration (FC_{50}) [16].

2.6. Statistical Analysis

The statistical significance of the results was determined by analysis of variance (one-way ANOVA), *p*-values of 0.01 or less were considered statistically significant. Linear regression analysis was performed to determine the FC_{50} and MFC.

3. Results and Discussion

3.1. Chemical Composition of the Essential Oil

Constituents of the essential oil of *G. oaxacana* are shown in table 1 according to their elution order (Figure 1). A total of nineteen compounds were identified by GC–MS analysis. The essential oil was characterized by high amounts of monoterpenes (84.6%) of which 73.5% are hydrocarbon monoterpenes and 11.1% are oxygen-containing monoterpenes. The most abundant components were: γ-terpinene (36.0%), β-Ocimene (11.9%), β-pinene (7.1%) and (+)-4-carene (5.7%), α-pinene (4.0%) and *Trans*-tagetone (4.0%). Results on the chemical composition of the essential oil of *G. oaxacana* are a significant contribution to the knowledge of the species, since no previous studies in this regard.

Table 1. *Chemical composition of essential oil of G. oaxacana.*

No	Compounds	RI	RIr	Percentage (%)
1	α-Thujene	903	911	1.1
2	α-Pinene	913	917	4.0
3	(+)-4-Carene	996	1002	5.7
4	β-Pinene	963	980	7.1
5	α-Phellandrene	984	996	0.9
6	*o*-Cymene	1007	1017	3.7
7	β-Ocimene	1022	1027	11.9
8	Artemisia alcohol	1090	1080	3.4
9	γ-Terpinene	1058	1064	36.0
10	3-Carene	1024	1011	1.6
11	*Trans*-Tagetone	1127	1146	4.0
12	*p*-Cymenene	1114	1095	1.6
13	3-Isopropenyl-5-methyl-1-cyclohexene	1192	-	0.6
14	2-ethenyl-1,3,3-trimethyl- Cyclohexene	1242	-	0.9
15	Cuminol	1262	1267	2.1
16	3-ethenyl-1,2-dimethyl-1,4-Cyclohexadiene	1276	-	1.6
17	4-Hydroxy-4-(4,6-dimethylcyclohex-3-enyl)butan-2-one	1341	-	1.0
18	Caryophyllene	1448	1428	2.5
19	Germacrene D	1491	1485	1.1
	Total			90.8

Compounds listed in order of elution from a non-polar HP-5 MS capillary column. RI: Retention indices relative to *n*-alkanes on non-polar HP-5MS column. RIr: Kováts Index references.

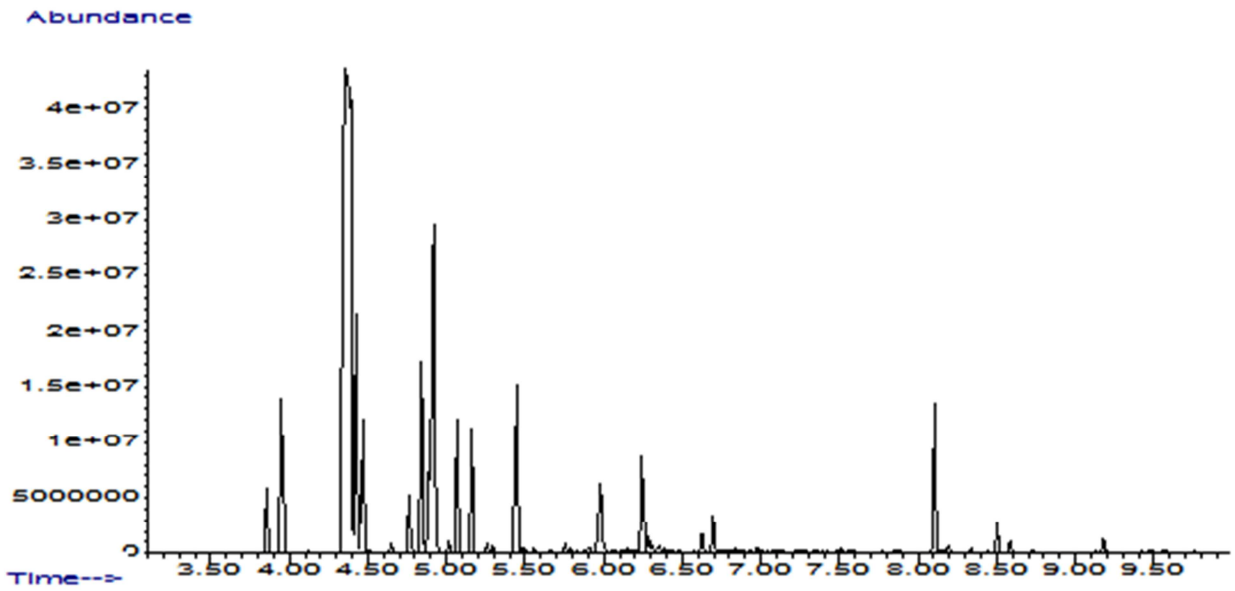

Figure 1. *Chromatogram of the essential oil of G. oaxacana.*

3.2. Antimicrobial Activity of the Essential Oil

Essential oil inhibited the growth of 31 microbial strains; six Gram-positive and thirteen Gram-negative bacterial strains (Table 2), seven yeasts and five filamentous fungi (Table 3). The most sensitive strains to *G. oaxacana* essential oil were: Gram-negative bacterial *V. cholerae* INDRE 206 (MIC=0.13 mg/mL) *V. cholerae* No. 01 (MIC=0.13 mg/mL), *V. cholerae* Tor (MIC=0.75 mg/mL) and *V. cholerae* cc (MIC=1.5 mg/mL); and Gram-positive bacterial *B. subtilis* (MIC=1.0 mg/mL) (Table 2). Antibacterial effect of the essential oil did not show significant differences on inhibition of Gram-positive and Gram-negative bacteria (p<0.01) (Table 2). This suggests that it acts on some common structures for both kinds of bacteria such as cell membranes. It has reported that the constituents of essential oils have lipophilic properties and interact with the cytoplasmic membranes and rendering them more permeable and bacteria lose their viability [17, 18, 19, 20]. Several studies have demonstrated that whole essential oil usually have higher antibacterial activity than the principal components, suggesting that the mixes of compounds are critical to synergistic activity [21, 22].

Table 2. *Antibacterial activity of essential oil of G. oaxacana.*

Organism	Positive control Chloramphenicol		Essential oil	
	Inhibition zone (mm) (25 µg/disc)	MIC (µg/mL)	Inhibition zone (mm) (4.55 mg/disc)	MIC (mg/mL)
B. subtilis FES I*	29.33 ± 2.62	2	8.00 ± 1.00	1.00
E. faecalis ATCC 29212*	25.66 ± 2.08	8	7.67 ± 0.58	3.00
S. aureus ATCC 12398*	24.00 ± 0.82	1	8.33 ± 1.15	2.00
S. aureus ATCC 29123*	30.00 ± 0.00	8	7.33 ± 0.58	2.00
S. epidermidis FES I*	22.67 ± 0.47	2	7.67 ± 0.58	2.00
S. pneumonia HA*	24.50 ± 1.90	16	15.33 ± 4.16	2.00
E. aerogenes FES I	19.33 ± 0.47	4	6.00 ± 0.00	3.00
E. agglomerans ATCC 27155	19.67 ± 0.47	nd	6.00 ± 0.00	3.00
E. coli ATCC 25922	21.67 ± 1.70	4	6.67 ± 0.58	3.00
E. coli ATCC 53218	11.33 ± 1.52	4	6.00 ± 0.00	3.00
P. aeruginosa ATCC 27853	27.60 ± 0.11	8	7.00 ± 0.00	3.00
P. mirabilis HA	13.33 ± 4.16	nd	8.00 ± 1.00	3.00
S. marcences HA	22.30 ± 0.25	nd	6.67 ± 0.58	3.00
S. typhy ATCC 19430	28.00 ± 1.63	2	6.00 ± 0.00	3.00
V. cholerae Tor	32.67 ± 0.47	1	16.00 ± 1.00	0.75
V. cholerae cc	27.67 ± 0.47	1	14.67 ± 3.21	1.50
V. cholerae INDRE 206	30.33 ± 0.47	1	10.67 ± 0.58	0.13
V. cholerae No. 01	35.00 ± 0.50	1	8.67 ± 0.58	0.13
Y. enterocolitica FES I	25.67 ± 0.47	4	6.67 ± 0.58	3.00

FES I= strains donated by the Clinical Analysis Laboratory of FES Iztacala, HA= strains isolated from a clinical case donated by Hospital Angeles (Metropolitano). *Gram positive strains, nd = no determinated.

The bactericidal kinetic assay was performed on two pathogens *V. cholerae* No. 01 and *B. subtilis*. Bacterial survival curve of *B. subtilis* showed that ½MIC, MIC and MBC (0.5, 1.0 and 1.5 mg/mL respectively) of essential oil had a bactericidal effect on bacterial population at 150 minutes (Figure 2). Essential oil of *G. oaxacana* at ½MIC and MIC (0.065, 0.125 mg/mL respectively) had bactericidal effect in *V. cholerae* No. 01 at 60 minutes. *V. cholerae* was highly sensitive to the essential oil because MBC (0.5 mg/mL) reduced the bacterial population by 99.9% in ten minutes (Figure 3).

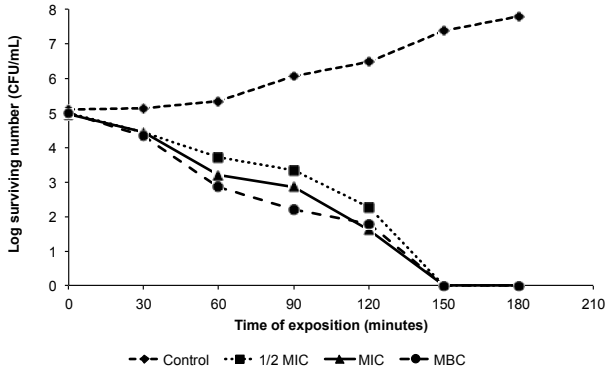

Figure 2. *Survival curve of B. subtilis exposed to G. oaxacana essential oil. The essential oil was added to each experimental culture in zero time. The concentrations used were: 0.5 mg/mL (½ MIC), 1.0 mg/mL (MIC) and 1.5 mg/mL (MBC). The control tube did not contain essential oil. MIC= Minimal Inhibitory Concentration. MBC= Minimal Bactericidal Concentration.*

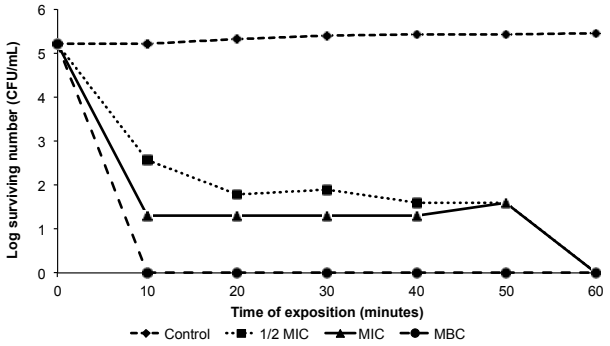

Figure 3. *Survival curve of V. cholerae No. 01 exposed to essential oil of G. oaxacana. The essential oil was added to each experimental culture in zero time. The concentrations used were: 0.065 mg/mL (½ MIC), 0.125 mg/mL (MIC) and 0.5 mg/mL (MBC). The control tube did not contain essential oil. MIC = Minimal Inhibitory Concentration. MBC = Minimal Bactericidal Concentration.*

These results demonstrate the bactericidal effect of essential oil on bacterial strains of medical importance. *B. subtilis* is an opportunistic microbial, usually found in soil and contaminated foods. *V. cholerae* is the etiological agent of cholera [23], it is transmitted through contaminated water and has caused major pandemics in the history of humanity [6]. In Latin America, cholera remains endemic. Untreated, the mortality is approximately 40-60% [24]. Additionally *B. subtilis* and *V. cholerae* are causing diarrheal diseases. According to our results and the traditional use of *G. oaxacana* we suggest that the essential oil content in the infusion of the plant can relieve gastrointestinal diseases of infectious origin.

Table 3. *Antifungal activity of essential oil of G. oaxacana.*

Organism	Positive controls				Essential oil		
	Inhibition zone (mm)		MIC (µg/mL)	FC_{50} (µg/mL)	Inhibition zone (mm)	MIC (mg/mL)	FC_{50} (mg /mL)
	Nystatin (30 µg/disc)	Ketoconazole (56 µg/disc)			(4.55 mg/disc)		
C. albicans ATCC 10231	9.67 ± 0.58	nd	4	nd	10.00 ± 0.00	1.50	nd
C. albicans ATCC 14065	11.83 ± 2.02	nd	11	nd	10.00 ± 0.00	1.50	nd
C. albicans FES I	9.33 ± 0.58	nd	11	nd	9.67 ± 0.58	1.50	nd
C. albicans HA	9.33 ± 0.58	nd	11	nd	8.67 ± 1.53	1.50	nd
C. glabrata HA	7.67 ± 0.58	nd	8	nd	8.00 ± 1.00	>2.00	nd
C. tropicalis HA	9.00 ± 1.00	nd	9	nd	7.00 ± 1.00	>.200	nd
C. neoformans FES C	8.67 ± 0.58	nd	4	nd	15.33 ± 0.58	1.50	nd
F. moniliforme CDBB-H-265	nd	+	nd	0.2	+	nd	2.38
F. moniliforme FES I2	nd	+	nd	0.2	+	nd	2.54
R. lilacina CDBB-H-306	nd	+	nd	0.2	+	nd	2.33
T. mentagrophytes CDBB-H-1112	nd	+	nd	0.1	+	nd	1.03

FES C = strains donated by FES-Cuautitlán, FES I= strains donated by the Clinical Analysis Laboratory of FES Iztacala, FES I2= strains donated by the Laboratory of Plant Physiology of FES Iztacala, HA= strains isolated from a clinical case donated by Hospital Angeles (Metropolitano). + = positive to test inhibition of radial growth. nd= not determined.

In the determination of the antifungal activity, the four strains of *C. albicans* and *C. neoformans* were sensitive to essential oil (FC_{50}=1.5 mg/mL). Among filamentous fungi *T. mentagrophytes* was the most sensitive fungal species (FC_{50}=1.03 mg/mL) (Table 3).

Gymnolaena oaxacana essential oil inhibited the growth of *C. albicans*, *C. glabrata* and *C. neoformans* which are of medical importance [6]. *C. albicans* causes 90% of gynecological infections and *C. glabrata* 5%. Yeast infections are developed by the interaction of these species, however *C. glabrata* is little sensitive to antifungal drugs and remains longer in the body [25]. Moreover *C. neoformans* and *T. metagrophytes* cause skin infections; *C. neoformans* is the species that causes opportunistic infections in AIDS

patients more frequently, affects 25 million people with the disease [26, 27]. *T. metagrophytes* is a dermatophyte fungus that invades and propagates in keratinized skin, often causing contagious infections [28].

Sensibility of fungi to essential oil may be due to some changes in cellular permeability [29], depolarization of mitochondrial membranes and activity of ionic channels affecting the proton pump and the ATP pool, decreasing the synthesis of ergosterol and sterol uptake [29, 30].

The antimicrobial activity of essential oil could be due to its high content of monoterpenes, these molecules have the property of altering the membrane permeability and fluidity, upon insertion between the fatty acyl chains of the phospholipids [29]. This mechanism explains why essential oils are antimicrobial agents with broad-spectrum activity against pathogenic microorganisms that cause diseases to humans [31].

Our results on the chemical composition of the essential oil of *G. oaxacana* and its biological effect represent new knowledge on the genus.

4. Conclusions

The essential oil is composed mainly of monoterpenes (84.6%), and their main components are: γ-terpinene, β-Ocimene and β-pinene. It has broad-spectrum antimicrobial effect against Gram-positive and Gram-negative bacteria, yeasts and filamentous fungi. Subsequent studies are required to evaluate the mode of action by which the main components of essential oil act on the most sensitive microorganisms. The results validate the medicinal use of *G. oaxacana.*

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