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Antifungal and antibacterial activities of *Cannabis sativa* L. resins.

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A R T I C L E I N F O

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A B S T R A C T

Ethnopharmacological relevance: *Cannabis sativa* L. (Cannabaceae) is a plant native to Eastern Asia spread throughout the world because of its medicinal properties. Despite being used for thousands of years as a palliative therapeutic agent for many pathologies, in many countries research on its effects and properties could only be carried out in recent years, after its legalization.

Aims of the study: Increasing resistance to traditional antimicrobial agents demands finding new strategies to fight against microbial infections in medical therapy and agricultural activities. Upon legalization in many countries, Cannabis sativa is gaining attention as a new source of active components, and the evidence for new applications of these compounds is constantly increasing.

Methods: Extracts from five different varieties ofCannabis sativa were performed and their cannabinoids and terpenes profiles were determined by liquid and gas chromatography. Antimicrobial and antifungal activities against Gram (+) and Gram (−) bacteria, yeast and phytopathogen fungus were measured. To analyze a possible action mechanism, cell viability of bacteria and yeast was assessed by propidium iodide stain.

Results: Cannabis varieties were grouped into chemotype I and II as a consequence of their cannabidiol (CBD) or tetrahydrocannabinol (THC) content. The terpenes profile was different in quantity and quality among varieties, with (−)b-pinene, b-myrcene, p-cymene and b-caryophyllene being present in all plants. All cannabis varieties were effective to different degree against Gram (+) and Gram (−) bacteria as well as on spore germination and vegetative development of phytopathogenic fungi. These effects were not correlated to the content of major cannabinoids such as CBD or THC, but with the presence of a complex terpenes profile. The effectiveness of the extracts allowed to reduce the necessary doses of a widely used commercial antifungal to prevent the development of fungal spores.

Conclusion: All the extracts of the analysed cannabis varieties showed antibacterial and antifungal activities. In addition, plants belonging to the same chemotype showed different antimicrobial activity, demonstrating that the classification of cannabis strains based solely on THC and CBD content is not sufficient to justify their biological activities and that other compounds present in the extracts are involved in their action against pathogens. Cannabis extracts act in synergy with chemical fungicides, allowing to reduce its doses.

1. Introduction

Cannabis sativa L. plant is native to East Asia and has been used by humans as medical and psychotropic drug for many thousands of years. The first archeological references shown that it was used in China at least since the Neolithic period, 6000 years ago (Ben Amar, 2006; Bonini et al., 2018). The first historical reports on the use of cannabis in traditional medicine also come from the Chinese Pharmacopoea, written in the first century BC, in which all remedies used and transmitted orally from thousands of years were recorded (Bonini et al., 2018; Pisanti and

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Bifulco, 2019). According to ancient references, cannabis was mostly used for rheumatic pain, constipation, as analgesic, antiparasitic, to treat convulsions and to purify blood, not only in China, but also in other Asian cultures as Indian and Japanese (Bonini et al., 2018; Pisanti and Bifulco, 2019). From Asia the plant spread to Africa and Europe and then to America. As a result of dispersion and hybridization made by growers and breeders, thousands of different varieties are now available worldwide. Although is a plant native to Asia, it is currently found, cultivated and used throughout the world, and is part of the cultural heritage of many communities. However, and despite the popular knowledge about cannabis benefits and wide medical applications, during the last century its cultivation, use and even research was prohibited in most of countries. Since 1961 Cannabis sativa L. was included in the Single Convention on Narcotic Drugs, Schedule I ("FINAL ACT OF THE UNITED NATIONS CONFERENCE," n.d.) for being considered an addictive and hazardous substance. Several decades and a great social demand throughout the world were necessary for its use to be legalized and its research to be allowed.

Cannabis sativa L. is the only species in the cannabis genus. The cannabis varieties often differ in the proportion of the active principles as cannabinoids, including tetrahydrocannabinol (THC), cannabidiol (CBD), and terpenes, which make them useful for different medical or industrial purposes. Based on their THC/CBD ratio, cannabis plants are classified into three chemotypes: chemotype I (high THC), chemotype II (1:1 ratio), and chemotype III (high CBD) (Pacifico et al., 2008). Terpenes are unsaturated hydrocarbons present in numerous plant families where they exert functions as mediators in ecological interactions, defense mechanisms, and signal transduction between other cellular processes. Indeed, many of these molecules serve as pharmaceutical agents because of their anticancer, antimicrobial, and anti-inflammatory activities (Bergman et al., 2019; Hanuš and Hod, 2020). In the last years, the role of terpenes has become more evident; however, their content is not yet considered when classifying plants by their chemical composition (Ibrahim et al., 2019). Because almost all cannabis strains are rich in terpenes, their content is relevant for a complete chemical profile and associated biological effects.

There is increasing evidence of a biological synergy between cannabinoids and secondary molecules co-extracted from the cannabis plant, for example, terpenes, originally proposed as an entourage effect by Mechoulam and Ben-Shabat in 1999 (Janatová et al., 2022; Mechoulam and Ben-Shabat, 1999; Russo, 2011, 2019). This can also be extrapolated to the antimicrobial activity and reinforces the idea of using complete extracts instead of purified cannabinoids to achieve the best results. In this direction, recent evidence indicates that total cannabis extracts present selective antibacterial activity, depending on their chemical composition (Sionov and Steinberg, 2022).

Some studies show that cannabinoids alone or in combination with common antibiotics exert antimicrobial action on Gram-positive and Gram-negative bacteria (Ali et al., 2012; Appendino et al., 2008; Blaskovich et al., 2021; Farha et al., 2020; Schofs et al., 2021; van Klingeren and ten Ham, 1976; Wassmann et al., 2020). However, this knowledge has no application in medicine, industry, or agriculture. As antimicrobial-resistant microbes become increasingly prevalent due to the overuse of classical antimicrobial drugs, new strategies are demanded for antimicrobial control to prevent human illness, food contamination, or plant and animal diseases. The demand includes urgent identification of new molecules exerting novel modes of action (Karas et al., 2020) that can work alone or in combination with synergistic compounds. So far, studies about the antifungal activity of the extracts are even more limited (Feldman et al., 2021; Lone and Lone, 2012; Radwan et al., 2009). Although the medical properties from cannabis components are known from long time ago, it is important to note that due to cultural and political reasons that prohibited the study of this plant, the great diversity and functions of the potentially active compounds of cannabis varieties remain virtually unknown.

This work reports the chemical profile of cannabis extracts from inflorescences of five different Argentinean landraces. Selection of varieties was made among those most used in our region with medical purposes and extracts were performed following the instructions of growers and civil organizations, which were formalized in the lab. It is important to note that in this research, the complete cannabis extracts are studied since it is hypothesized that the observed activity comes from the synergistic effect between cannabinoids, terpenes, and other compounds present in each resin of the different varieties. The antifungal and antibacterial activity of resin was evaluated *in vitro* and *in vivo*. The correlation between the observed effect and their cannabinoid and terpene content is discussed.

2. Materials and methods

2.1. Biological material

2.1.1. Plant material

Samples of *Cannabis sativa* L. (marihuana) flowers came from plants of five varieties belonged to the collection of Biología de Cannabis Laboratory, from Universidad Nacional de Mar del Plata. Voucher specimens for each variety were kept and codified as C1 to C5. Plants were grown under controlled conditions in a culture room with 60% humidity, 25 °C, and 18/6 (light/dark) photoperiod for vegetative growth and 12/12 for flowering (Fig. S1). The plant name, first described by Carolus Linnæus in 1753, was checked with World Flora Online, wfo id 0000584,001 (Cannabis sativa L. (worldfloraonline.org)).

2.1.2. Bacterial and fungus growth conditions

The bacterial and fungus strains used in this work were: Bacillus thurigensis, kindly provided by Dr. Corina Berón from INBIOTEC, Argentina; Micrococcus luteus, kindly provided by Dr. Erika Wolsky from Universidad Nacional de Mar del Plata, Argentina; Escherichia coli RP437, kindly provided by Dr. Claudia Studdert from Instituto de Agrobiotecnología del Litoral, Argentina and Pseudomonas protegens, kindly provided by Dr. Claudio Valverde from Universidad Nacional de Quilmes, Argentina. The yeast Saccharomyces cerevisiae was kindly provided by Dr. Cecilia Terrile from Universidad Nacional de Mar del Plata, Argentina. The fungus Fusarium solani f. sp. eumartii (F. eumartii) was kindly provided from Estación Experimental Agropecuaria INTA-Balcarce, Argentina.

The Gram-positive bacteriaBacillus thurigensis and Micrococcus luteus and the Gram-negative bacteria Escherichia coli RP437, and Pseudomonas protegens were grown in Lysogeny Broth (LB) broth (yeast extract 5 g/L, tryptone 10 g/L, and NaCl 10 g/L) with agitation at 37 °C for M. luteus and E. coli or 30 °C for B. thurigensis and P. protegens. Growth was followed by Optical Density at 600 nm (OD_{600}) in a GeneQuant™ 1300 spectrophotometer.

The yeastSaccharomyces cerevisiae was grown in Yeast-Peptone-Dextrose (YPD) culture medium (yeast extract 10 g/L, peptone 20 g/L, and dextrose or glucose 20 g/L) with agitation at 30 °C. Growth was followed by Optical Density at 600 nm (OD_{600}) in a GeneQuant™ 1300 spectrophotometer.

The fungusFusarium solani f. sp. eumartii (F. eumartii) was grown on solid potato dextrose agar (PDA) medium at 25 °C in darkness. Spores from 8-day-old culture plates were collected and suspended in sterile distilled water (Mesas et al., 2021).

2.2. Cannabis resin extraction

Female inflorescences were dried at 18 °C in the dark for 15–20 days and then were processed by mechanical disruption with ethanol in a ratio of 1:10 (w/v). Then, the solvent was eliminated by vacuum rotary evaporation at low temperature, following a procedure developed in our laboratory. Obtained resins were resuspended in dimethyl sulfoxide (DMSO) to have the same final concentration (1 μg resin/μL DMSO).

2.3. Analysis of resins chemical composition

Cannabinoids (THC, CBD, and CBN) of the five cannabis varieties were identified and quantified by High-Performance Liquid Chromatography (HPLC) in a SHIMADZU Prominence chromatograph, with Liquid Chromatography LC Solution software, low-pressure quaternary pump, diode array detector, and manual injector. We used a C18 column of 250 mm length, 4.5 mm diameter, and 5 μm particle size with a 0.7 mL/min flow. Solvents methanol and acetonitrile used in the mobile phase were of HPLC grade. The cannabinoids reference standards were from Cerellian®.

Terpenes were identified and quantified by Gas Chromatography in a Shimadzu QP2020 NX Chromatograph with FID (Flame Ionization Detector). The terpenes reference standards were from Restek®.

2.4. Antimicrobial assays

2.4.1. Antibacterial and anti-yeast assays

Liquid cultures (OD_{600} = 0.1) of the four bacterial strains and the yeast S. cerevisiae were exposed to different concentrations of the resin from each cannabis variety (bacteria treatment: 1, 2, and 4 μg resin/mL, yeast treatment: 0.05; 0.25; 0.5 and 1 μg/mL). Growth curves development was followed by OD_{600} every 40 min for bacteria and every 60 min for yeast (Muñoz et al., 2010). Dimethyl sulfoxide (DMSO) was used as a control. Each assay was performed in triplicate. To evaluate the inhibitory activity of the resins against bacteria and yeasts, the minimum inhibitory concentration (MIC) was adapted according to the Clinical and Laboratory Standards Institute (CLSI) as described in (Ghavam et al., 2021) and (Rojas et al., 2014), respectively.

2.4.2. Cell viability assay

Cell viability was assessed using the dye propidium iodide (PI) (Sigma–Aldrich, St. Louis, MO, USA). Bacterial and yeast cells in the exponential growth phase (1 ml of each culture) were obtained by centrifugation and resuspended in 100 μl of culture medium. Aliquots of 1 μg/μL PI were added, and the cells were incubated for 15 min at room temperature. For positive control, cultures were treated with 70% ethanol for 15 min, and then PI was added (Mesas et al., 2021). Fluorescence and brightfield images were visualized with the Eclipse E200 fluorescence microscope (Nikon, Tokyo, Japan). Assays with each culture were performed twice, and ten images from each independent assay were analysed.

2.4.3. Fungal inhibitor activity

Spores (3×10^5 spores/ml) were resuspended in distilled water, and 5 μl of this suspension were exposed to the corresponding dose of cannabis resin (0.05, 0.1, 0.11, 0.12, 0.15, 0.2, and 0.4 μg/μL) in 2% sucrose solution. The assay was performed in a 12 wells glass plate and incubated at 25 °C in darkness for 24 h (Mendieta et al., 2006). Germinated spores were quantified in a hemocytometer under a light microscope Eclipse E200 (Nikon, Tokyo, Japan). Spores were considered germinated when the germ tube length was longer than one-half of the reproductive structure (Plascencia-Jatomea et al., 2003). Minimum inhibitory concentration (MIC) was based on CLSI as described in (Carrizo et al., 2020).

2.4.4. Combined effect of resins and mancozeb on spore germination

The assay was performed in the same conditions described above, but spores were exposed to the fungicide Mancozeb (Brometan®) in concentrations of 0.1 and 0.2 μg/mL alone or in combination of different doses of cannabis resins (D'Ippólito et al., 2017).

2.5. Statistical analysis

The values shown in each figure are mean values ± SD. Data were subjected to analysis of variance (one-way ANOVA) and post hoc comparisons were done with Tukey's multiple range test at $p < 0.05$, $p < 0.02$ or $p < 0.01$ level. GraphPad Prism 8 (GraphPad Software Inc., San Diego, CA, USA) was used as statistical software program.

Table 1
Chemical composition of the cannabis varieties.

Resin (g/g DF)	C1	C2	C3	C4	C5
	0.3	0.26	0.54	0.25	0.24
Cannabinoids (mg/g DF)					
CBD	ND	ND	ND	ND	75
THC	190.9	130.4	396.9	132.8	74.7
CBN	1.8	1.4	3.5	1.6	1.4
Terpenes (mg/g DF)					
Alpha-Pinene	2.8013	<0.50	1.9911	ND	<0.50
Camphene	ND	ND	ND	ND	ND
(−)-Beta-Pinene	0.9396	<0.50	1.5449	<0.50	<0.50
Beta-Myrcene	8.951	<0.50	7.2635	0.6885	2.0601
Delta-3-Carene	ND	ND	<50	ND	ND
Alpha-Terpinene	ND	ND	ND	ND	ND
p-Cymene	1.2094	<0.50	0.9662	0.8456	1.8597
d-Limonene	ND	ND	0.60598	ND	ND
Ocimene	0.6899	ND	0.6083	ND	ND
Gamma-Terpinene	ND	ND	ND	ND	ND
Terpinolene	ND	ND	2.3916	ND	ND
Linalool	0.8505	ND	<0.50	0.6268	0.8748
(−)-Isopulegol	ND	ND	ND	ND	ND
Geraniol	ND	ND	ND	ND	ND
Beta-Caryopyllene	2.1115	<0.50	2.5652	3.5851	3.2029
Alpha-Humulene	0.5	ND	0.6581	1.2364	1.1366
Nerolidol	ND	ND	<0.50	0.5412	ND
(−)-Guaiol	ND	ND	ND	ND	0.716
(−)-Alpha-Bisabolol	ND	ND	<0.50	ND	ND

3. Results and discussion

3.1. Active compounds of the cannabis varieties

The chemistry of cannabis is very complex due to the large number of its components and their possible interactions. These compounds represent almost all chemical classes, e.g., mono- and sesquiterpenes, sugars, hydrocarbons, steroids, flavonoids, nitrogenous compounds, and amino acids (ElSohly and Slade, 2005). In this work, to obtain information on the chemical profile of five unstudied cannabis strains (C1 to C5), we analysed the content of the three main cannabinoids, CBD, THC, and CBN, and 19 terpenes (Table 1). THC content in each resin varied from 74.7 mg/g of dried flower (DF) to 396.9 mg/g DF (varieties C5 and C3, respectively) (Table 1). The other three varieties exhibited intermediate values. CBD content was below the detection limit in four out of five varieties (C1 to C4), while in the C5 variety, it was 75 mg/g DF. The content of CBN was about 1:100 of that of THC, except for variety C5, which presented a 1:50 ratio (Table 1). According to the THC:CBD ratio, varieties C1 to C4 can be assigned to chemotype I, while variety C5 belongs to chemotype II (Pacifico et al., 2008).

Terpenes are known to contribute to the fragrance and flavour of cannabis flowers. The particular mixture of these compounds will determine the viscosity of cannabis resin which represents an advantage as the notable stickiness of cannabis exudations traps insects (McPartland et al., 2000). Some of them exert antimicrobial effects (Guimarães et al., 2019; Russo, 2011). As can be seen in Table 1, the distribution and content of the 19 analysed terpenes were markedly different in every cannabis variety. Variety C2 contained only five terpenes at a relatively low level, while variety C3 contained 13 distinct molecules, some of which showed a relatively high level (Table 1). Four terpenes (monoterpenes (−)b-pinene, b-myrcene, p-cymene, and the sesquiterpene b-caryophyllene) were present in all varieties. Three of them, pinene, b-myrcene and b-caryophyllene are among the most commonly abundant terpenes found in a large set of samples (Sarma et al., 2020). The dominant terpene in the C1 and C3 varieties was b-myrcene, while

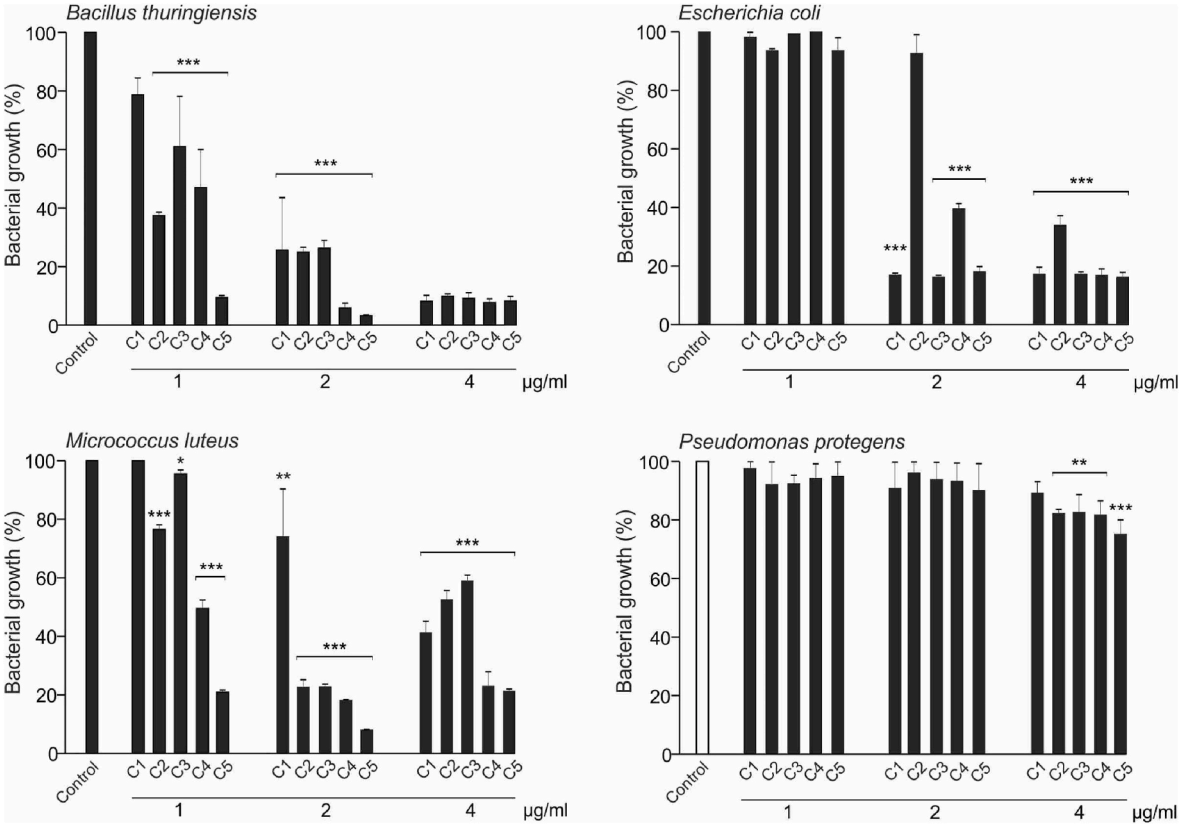

Fig. 1. Effect of cannabis resins on growth inhibition of Gram (+) and Gram (−) bacterial strains. Growth percentage was measured at the end of the growth curve and relativized to control assay (without resin), which was considered as 100% growth. Asterisks point out statistically significant differences. (*: $p < 0.05$, **: $p < 0.02$, ***: $p < 0.01$).

b-caryophyllene dominated the C4 and C5 profiles, in agreement with the results reported for other cannabis varieties (Booth and Bohlmann, 2019; Sarma et al., 2020; Sommano et al., 2020). Only the variety C5 showed co-dominance between two terpenes, with a ratio of the pair b-caryophyllene/b-myrcene of 1.55. Ratios lower than 2 between terpenes content means co-dominancy (Sarma et al., 2020).

3.2. Antibacterial activity

To obtain information about the antibacterial activity of cannabis resins, we tested the sensitivity of two strains of Gram (+) bacteria (*Micrococcus luteus* and *Bacillus thuringiensis*) and two Gram (−) bacteria (*Pseudomonas protegens* and *Escherichia coli*) to increasing resin concentrations. Growth curves of every bacterial strain are shown in Fig. S2. Cultures to which DMSO was added instead of cannabis resins and cultures without any addition were included as controls.

As evaluated by the maximal OD_{600} reached by bacterial cultures, resins from the five cannabis varieties presented some degree of antibiotic activity against the four bacterial strains tested (Fig. 1). The effect was maximum on the two Gram (+) bacteria, reaching about 90–95% of inhibition at the higher doses tested. Within Gram (−) bacteria, the growth inhibition was moderate to high on *E. coli* (66–83.8%) depending on the cannabis variety and very low on *P. protegens* that was almost insensitive to all tested resins at any concentration, except by the higher one (4 µg/mL) that showed an 11–25.5% of growth inhibition depending on the variety (Fig. 1). The differential effect of cannabinoids against Gram (+) and Gram (−) bacteria was previously reported, resulting in almost all works more sensitive those strains from the Gram (+) group.

Table 2
Growth rate (%) of each bacterial strain in presence of different concentrations of cannabis resins (final resin concentration in culture medium). A value of 100 equals maximum growth rate of each strain. Asterisks point out statistically significant differences (*: $p < 0.05$, **: $p < 0.02$, ***: $p < 0.01$).

		CONTROL	DMSO	C1	C2	C3	C4	C5
Bacillus thurigensis H14	1 µg/mL	100	100	70.9 ± 11.00	20.06 ± 1.74 ***	50.84 ± 25.82 **	30.10 ± 11.57 ***	0.00 ***
	2 µg/mL	100	56.45 ± 10.69	12.70 ± 9.31 *	13.31 ± 1.81 **	15.12 ± 3.96 *	0.00 *	0.00 **
	4 µg/mL	100	100	0.00 ***	3.09 ± 1.93 ***	0.00 ***	0.00 ***	0.00 ***
Micrococcus luteus	1 µg/mL	100	97.37 ± 17.96	87.37 ± 9.65	85.26 ± 9.86	100	53.68 ± 12.63 **	0.00 ***
	2 µg/mL	100	81.89 ± 4.15	100	46.06 ± 7.37 **	55.51 ± 4.72 *	39.37 ± 4.15 ***	0.00 ***
	4 µg/mL	100	99.00 ± 27.78	31.5 ± 5.19 ***	42.00 ± 2.60 ***	55.50 ± 3.00 **	6.50 ± 7.69 *	0.00 ***
Escherichia coli	1 µg/mL	100	100	97.11 ± 12.46	98.19 ± 8.20	100	100	100
	2 µg/mL	100	100	17.26 ± 0.88 ***	62.09 ± 6.12 **	11.17 ± 2.32 ***	30.29 ± 7.93 ***	16.24 ± 3.17 ***
	4 µg/mL	100	100	15.31 ± 1.02 ***	27.23 ± 1.07 ***	13.95 ± 1.64 ***	12.74 ± 1.02 ***	17.21 ± 2.31 ***
Pseudomonas protegens	1 µg/mL	100	100.00 ± 2.96	100.00 ± 5.92	86.66 ± 7.56	79.22 ± 6.04	88.63 ± 17.22	62.74 ± 16.73 **
	2 µg/mL	100	92.07 ± 1.96	100	100	100	100	100
	4 µg/mL	100	94.57 ± 8.81	100	100	100	100	100

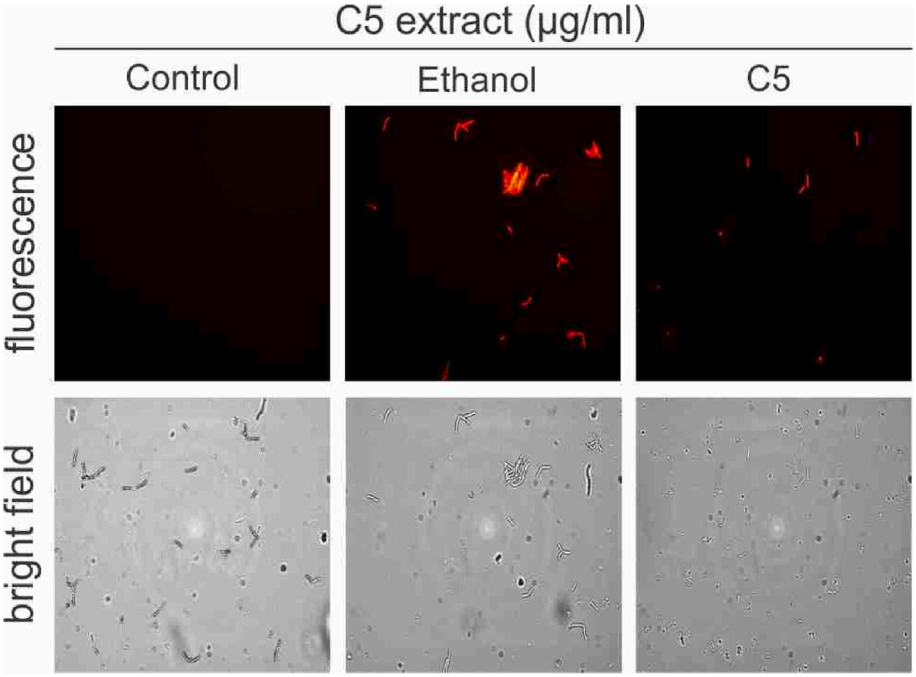

Fig. 2. Representative images of fluorescent Propidium Iodide staining and bright field corresponding to the same image, using 63X magnification. Selected images were those in which the effect is most clearly seen and correspond to *B. thuringiensis* exposed to resin from chemotype II (C5).

However, a recent work by Blaskovich and co-workers (Blaskovich et al., 2021) demonstrated the efficacy of pure CBD against four Gram (−) bacteria belonging to the genera *Neisseria* and *Moraxella*, responsible for serious diseases, and suggests that this sensitivity could be due to structural differences in the composition of the bacterial outer membrane (Blaskovich et al., 2021), demonstrating that not all microbial strains behave in the same way against a harmful compound. Our results agreed with both tendencies of existing references, given that despite being in less proportion, the Gram (−) bacterium *E. coli* was also susceptible to the effect of the cannabinoid. This is a promising result suggesting that the use of whole cannabis extracts could be an effective strategy in healthcare implementations. On the other hand, it is noteworthy that *P. protegens* was almost insensitive to all the resins tested. Considering that this bacterium is a known producer of biocontrol compounds that are active against other bacteria and fungi (Ramette et al., 2011), the lack of inhibitory effects of cannabis resins on this strain is in favour of the potential use of cannabis derivatives against plant pathogens.

Inhibition of bacterial development may be a consequence of a reduced growth rate. To explore this point, the growth rate of each bacterial strain under the effect of each resin at the time of exponential growth of the control cultures was calculated and expressed as a percentage growth rate over this reference (Table 2). In complete agreement with the results in Fig. 1, a dose-dependent reduction in growth rate was observed for *M. luteus*, *B. thuringiensis*, and *E. coli* but not for *P. protegens*.

Among the cannabis strains, the most potent inhibitory effect was produced by the resins of C5, the only variety belonging to chemotype II and containing CBD. Purified CBD is very effective against Gram (+) bacteria exhibiting a Minimal Inhibitory Concentration (MIC) ranging between 1 and 4 µg/mL on more than 20 different strains (Blaskovich et al., 2021; Wassmann et al., 2020). In good agreement with these references, our results showed almost complete inhibition of the two Gram (+) strains tested with a final dose of CBD calculated at 3.15 µg/mL in the bacterial culture. The same concentration was also active on *E. coli*, as a representative model of Gram (−) bacteria (Fig. 1), thus supporting the role of CBD as a broad-spectrum antibacterial agent. As previously mentioned, *P. protegens* was insensitive to virtually all treatments, including those with the C5 variety. It has been described that this strain shows a multidrug-resistant (MDR) phenotype, which is not affected by antibiotics of different chemical characteristics (Luczkiewicz et al., 2015). According to the results presented here, CBD and the other active principles of cannabis are no exception.

It is worth noting that a pronounced bacterial growth inhibition activity was also observed when applying the other four cannabis resins (C1 to C4), in which CBD was not detected. THC is the most abundant molecule in these resins. Depending on the variety, it is accompanied by different terpenes (Table 1). In variety C2, the terpene content is the lowest. It offered the opportunity to test the effect of increasing concentrations of THC on bacterial growth. As shown in Fig. S3, *B. thurigensis* was strongly inhibited (>20% growth) by THC, also at a relatively low dose. The sensitivity of E. coli and M. luteus was lower as it was necessary to double the THC doses to reduce the growth rate to about 50–60%. Notably, they grew at a relative rate of 40–50% of the maximum, even after increasing the dose four-fold. On the other hand, *P. protegens* was virtually insensitive to THC. MIC values are shown in Table S1.

As presented in Table 1, variety C4 has a THC content similar to that of variety C2. However, its inhibitory effect was deeper against sensitive bacterial strains (Fig. 1), thus suggesting the role of accompanying terpenes. Importantly, their diversity and relative contents were similar to those found in C5, which was the more effective cannabis strain.

The THC content in C1 and C3 was higher than in C2 and C4. Nevertheless, their inhibitory effect was lower. It is probably related to the results in Fig. S3, which show that increased THC concentrations do not always imply a deeper inhibition and would depend, at least in part, on the content and diversity of terpenes. In this sense, although the total terpene content in C1 and C3 was highest, the relative abundance of sesquiterpenes was lower than in C4 and C5, suggesting a role of these molecules in growth inhibition.

The mechanisms of action of antimicrobial compounds often involve the destabilization of the cell membrane. Propidium iodide (PI) dye cannot pass through intact membranes but diffuses into cells with damaged envelopes to react with DNA and show red fluorescence. The cell membrane of all strains tested was affected to some extent. While *P. protegens* showed slight alteration, *B. thuringiensis* was visibly

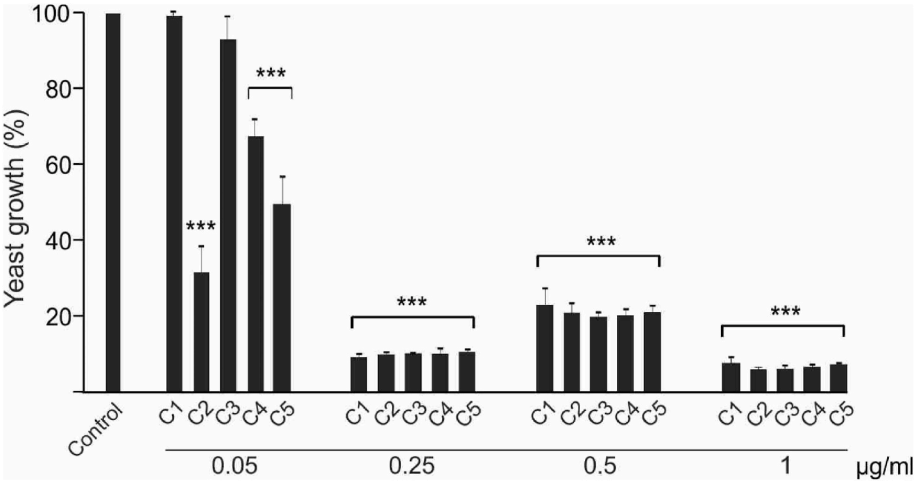

Fig. 3. Effect of cannabis resin from different varieties on growth inhibition of the yeast *Saccharomyces cerevisiae*. Growth percentage was measured at the end of the growth curve and relativized to control assay, which was considered as 100% growth. Asterisks point out statistically significant differences (*: $p < 0.05$, **: $p < 0.02$, ***: $p < 0.01$).

damaged, as denoted by the irregular morphology of the cells and the amount of cell debris found in the culture, which were indeed similar to results obtained after incubating with ethanol as positive control (Fig. 2). These results suggest that the mechanisms of action of the active principles of cannabis resins are related to the disruption of cellular integrity, thus acting as bactericidal agents.

Although the antimicrobial mechanisms of cannabinoids remain largely unknown, the combination of CBD and bacitracin induced several septum formations during cell division, along with membrane irregularities, suggesting that CBD may exert antimicrobial activity through mechanisms affecting the cell envelope (Wassmann et al., 2020). Blaskovich and coworkers demonstrated the effect of CBD against Gram-positive bacteria (*Staphylococcus aureus*, *Streptococcus pneumoniae*, and *Clostridium difficile*) (Blaskovich et al., 2021). The activity of CBD against Gram-negative bacteria is limited due to the presence of the outer membrane and lipopolysaccharide (LPS). Therefore, when CBD was used in combination with membrane-altering drugs, susceptibility to CBD increased. Appendino and coworkers examined the effects of structural modification on the bactericidal activity of five major cannabinoids (cannabidiol, cannabichromene, cannabigerol tetrahydrocannabinol and cannabinol). All five cannabinoids demonstrated potent activity against *Staphylococcus aureus*, with MIC values between 0.5 and 2 ug/mL. Methylation and acetylation of the phenolic hydroxyls, esterification of the carboxyl group of the cannabinoid, and introduction of a second prenyl moiety were detrimental to the antibacterial activity of cannabinoids. These changes could reduce aqueous solubility and lead to a loss of antibacterial activity (Appendino et al., 2008), suggesting these structures could be involved in the effect. Concerning the mechanism of action of terpenes as antimicrobial compounds, it remains yet unknown. Guimarães and colleagues investigated the antibacterial activity of 33 free terpenes commonly found in essential oils and demonstrated that the mechanism causing cell death is based on the loss of cellular membrane integrity (Guimarães et al., 2019). Taking into account the results obtained in cell viability assays with Propidium Iodide (Fig. 2), terpenes could be responsible, at least in part, of the antibacterial effect observed.

3.3. Anti-yeast assay

To advance the antimicrobial activity of cannabis resins, we evaluated the inhibitory action against the model yeast *Saccharomyces cerevisiae*. Although most yeasts of the genus *Saccharomyces* are saprobes and live in association with plants and animals, a few species are phytopathogenic. In addition, *S. cerevisiae* is a model organism for testing different molecules in health assays. The results indicate that the antimicrobial effect of the resins was more pronounced in yeast than in bacterial strains. The concentration of 1 µg/mL was the lowest evaluated for bacterial cells, causing a total inhibition of yeast growth. This result prompted the exploration of lower extract concentrations. *S. cerevisiae* showed some development at a dose as low as 0.05 µg/mL. Fig. 3 shows the percentage of growth achieved at the end of the growth curve of *S. cerevisiae* exposed to different concentrations of cannabis resins. All cannabis strains were very active against yeast (Table 3). Fig. S4 shows the complete growth curves of yeast exposed to each resin concentration. There was no detectable growth at 1 µg/mL of extract, but evident yeast growth was observed at 0.05 µg/mL, i.e., 20 times less than the lowest concentration tested for bacterial growth. It is also interesting that yeast growth recovered at the lowest concentration tested. However, it never reached the final OD obtained for the control cells (Fig. S4 panel A). It suggests that even at much lower concentrations, the cannabis components of the resin still affect yeast integrity.

Table 3
Growth rate of *Saccharomyces cerevisiae* in presence of different concentrations of cannabis resins. Different letters point out statistically significant differences (*: $p < 0.05$, **: $p < 0.02$, ***: $p < 0.01$).

Treatment (µg/mL)	Saccharomyces cerevisiae			
	0.05	0.25	0.5	1
CONTROL	100	100	100	100
DMSO	100	100	64.58 ± 12.63	100
C1	100	0.00 ***	0,00 **	0.00 ***
C2	33.87 ± 9.67 **	0.00 ***	8.33 ± 4.77 **	0.00 ***
C3	100	0.00 ***	28.12 ± 26.70 *	0.00 ***
C4	70.97 ± 7.39	0.00 ***	31.25 ± 21.87 *	0.00 ***
C5	45.97 ± 10.55	0.00 ***	11.45 ± 7.22 **	0.00 ***

To analyze the cell membrane integrity, we incubated yeast cultures with PI. Cells exposed to the resins from the five varieties showed very low cell density concerning control assays, being almost all cells red stained, thus confirming membrane disruption that caused the penetration of the dye. Fig. 4 shows representative images of the effect of C5 resin against *S. cerevisiae* cells.

The few references available on the anti-yeast effect of cannabinoids suggest that this effect may depend on the solvent used to prepare the extract or on the plant tissues from which they are extracted (Schofs et al., 2021). Nally and co-workers tested seven compounds isolated from cannabis leaves, with cannabidivarin being the only one effective against the yeast *Candida albicans* (Nalli et al., 2018). On the other hand,

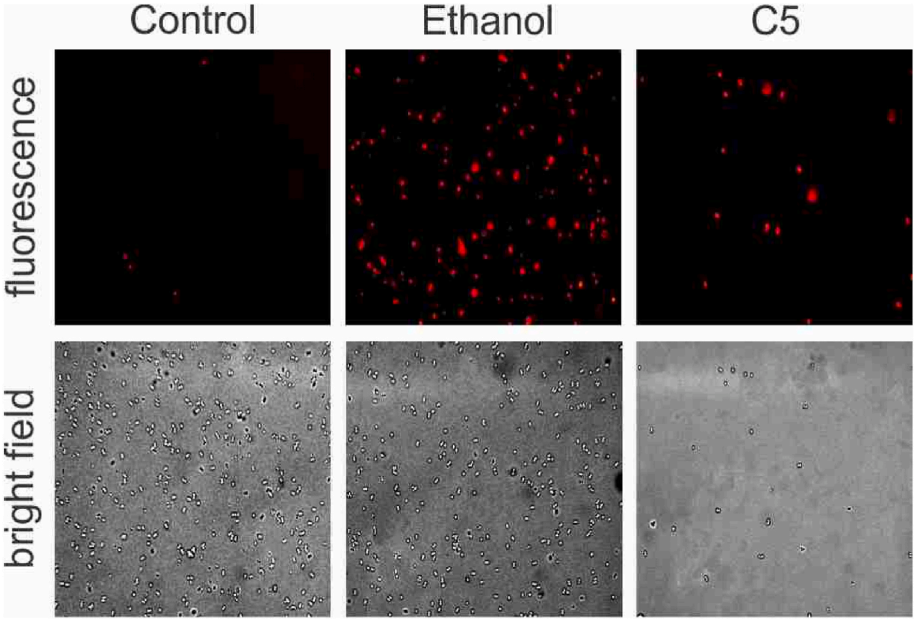

Fig. 4. Representative images of fluorescent Propidium Iodide staining and bright field, using 63X magnification. Selected images were those in which the effect is most clearly seen and correspond to *S. cerevisiae* exposed to resin from chemotype II (C5).

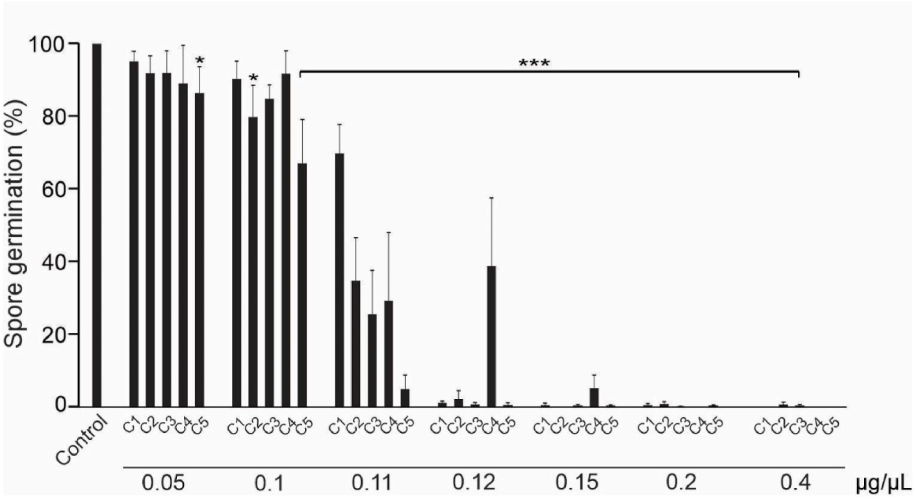

Fig. 5. Germination of *F. eumartii* spores in presence of different concentration of cannabis resins. Asterisks point out statistically significant differences. (*: $p < 0.05$, **: $p < 0.02$, ***: $p < 0.01$).

the synthetic compounds cannabichromene and cannabigerol showed modest antifungal effects when tried against C. albicans, S. cerevisiae, and A. niger (Elsohly et al., 1982). Recently, Feldman and co-workers used commercial CBD to inhibit the biofilm development of Candida albicans, but the concentration of CBD needed to avoid biofilm formation was more than 30 times higher than the doses used in our work. They showed a time and dose dependence of biofilm inhibition by CBD. They used a minimal inhibitory concentration of 12.5 µg/mL of CBD to inhibit 50%, while 100 µg/mL of CBD avoided 90% biofilm formation (Feldman et al., 2021). In our trials, 3.15 µg/mL CBD caused inhibition of yeast growth. However, resins without CBD also showed efficacy against yeast growth. MIC values are shown in Table S1. Our results with whole resins from five cannabis strains suggest that cannabis inflorescences possess bioactive molecules effective against yeast and other microorganisms, even at much lower concentrations.

3.4. Inhibition of fungal spore germination

The agriculture industry loses billions of dollars each year due to plant pathogens. The Food and Agriculture Organization of the United Nations (FAO) estimates that pests cause an annual loss of between 20% and 40% of global crop production. Each year, plant diseases cost the global economy an estimated $220 billion ("FAO - News Article: New standards to curb the global spread of plant pests and diseases," n.d.). Among the most widespread fungal infections is that caused by Fusarium spp. In particular, Fusarium solani is a plant pathogen fungus responsible for severe disease in many agriculturally important crops such as tomato, wheat, banana, and barley (Coleman, 2016; Dusunceli, n.d.), being urgent the development of new weapons to deal with it. Synthetic chemical fungicides are the most widely used strategy to prevent plant infections. Their indiscriminate use implies risks to health and the environment, causing pathogen resistance. Therefore, the search for ecological and effective alternatives is necessary and intensive. The

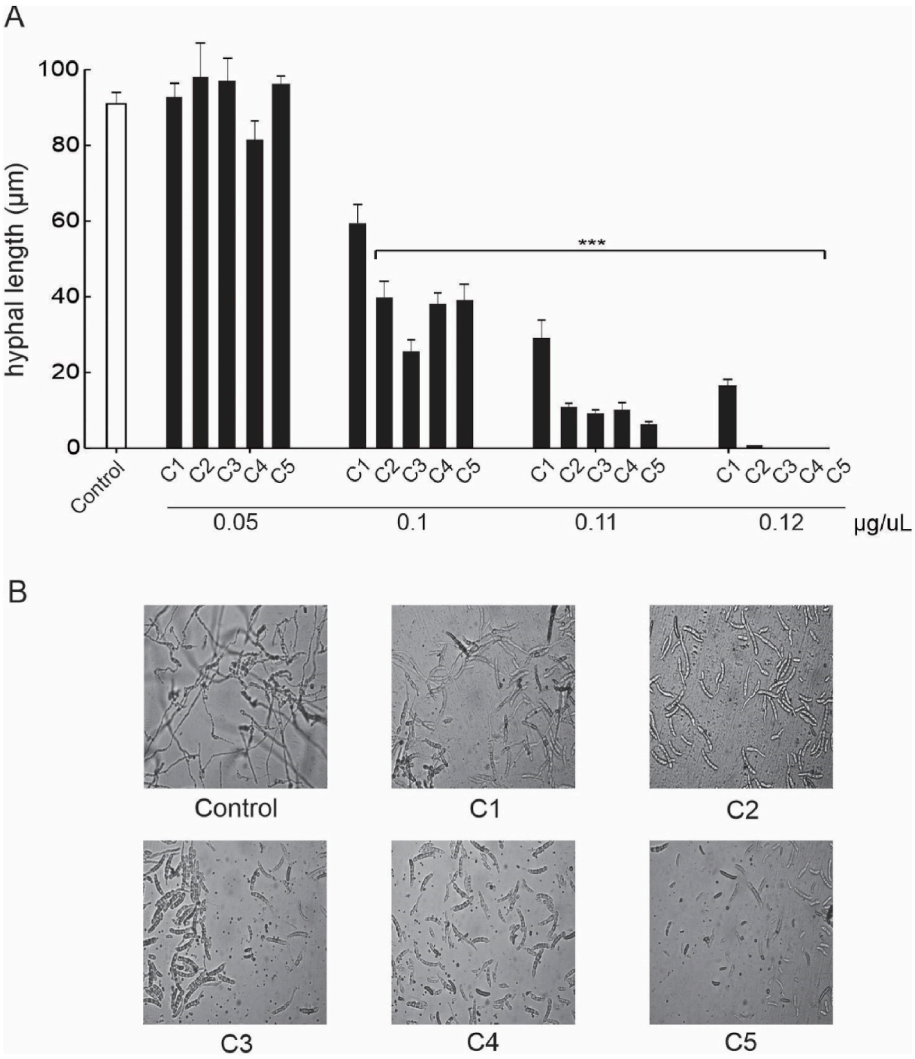

Fig. 6. A: Hyphae length (µm) as a function of resin concentration. Asterisks point out statistically significant differences (*: $p < 0.05$, **: $p < 0.02$, ***: $p < 0.01$). B: *F. eumartii* spores exposed to distilled water (control) or cannabis resin at the concentration 0.11 µg/µL.

World Health Organization (WHO) states that 11% of the 252 essential medicines are exclusive of plant origin (Veeresham, 2012).

Since spores are the structures that filamentous fungi use to reproduce and infect, we studied the antifungal effect of cannabis resins against *Fusarium eumartii* spores. Fig. 5 shows that all varieties exerted inhibitory activity. This effect depended on each variety used. At low resin concentrations (0.05 µg/µL and 0.1 µg/µL), there was little effect, with C5 resin being the most effective. Resins C1 and C4 showed the lowest activity. The percentage of spore germination decreased when increasing the resin concentration. In all varieties, a concentration of 0.12 µg/µL inhibited spore germination completely, except in C4. In this case, at 0.15 µg/uL, the spore inhibition was almost complete (Fig. 5). At resin concentrations of 0.1 µg/µL, germination occurred between 67 and 92%. Notably, treatments with 0.12 µg/µL showed a marked effect for all varieties, reaching almost 100% spore inhibition in four out of five resins. It led us to test an intermediate concentration. At 0.11 µg/µL, spore inhibition was variable depending on the cannabis variety assayed (Fig. 5). Controls with water or DMSO did not affect spore germination. Therefore, the effect was due solely to the cannabis components.

Notably, in all cases, the doses needed to inhibitF. eumartiispore germination were two orders higher than those used in bacteria and yeast assays. Taking into account that spores are reproductive resistance structures, higher doses of antifungal compounds are required to reach a complete inhibition of germination.

Spores germinated under cannabis treatment showed hyphae that did not reach the same length as the control. Then, we evaluated the effect of cannabis extracts on *F. eumartii* hyphae length (Fig. 6). Hyphal length decreased with increasing doses. Considering all these results, it is clear that cannabis resins inhibit spore germination and affect hyphal elongation.

Scarce evidence exists on the antifungal (not yeast) effect of cannabis derivatives. McPartland describes the fungistatic activity of isolated CBD and THC against the pathogen fungusPhomopsis ganja (McPartland, 1984). Recently, the anti-aflatoxigenic properties and fungal growth partial inhibition of cannabis extracts were reported by Al Khouri and co-workers (Al Khoury et al., 2021). Also, Khan and Javaid described the antifungal effect of *Cannabis sativa* L. leaf extracts made with different solvents against the fungus *Aspergillus flavipes* (Khan and Javaid, 2020). However, the cannabis inflorescence resins effect against spore germination and hyphal elongation of the fungus *F. eumartii* has not been reported. Considering the economic relevance of this phytopathogenic fungus, these results are promising for the development of environmentally friendly antifungal strategies focused on agricultural health, making cannabis resin a good candidate as an agent for the control of pathogenic microorganisms.

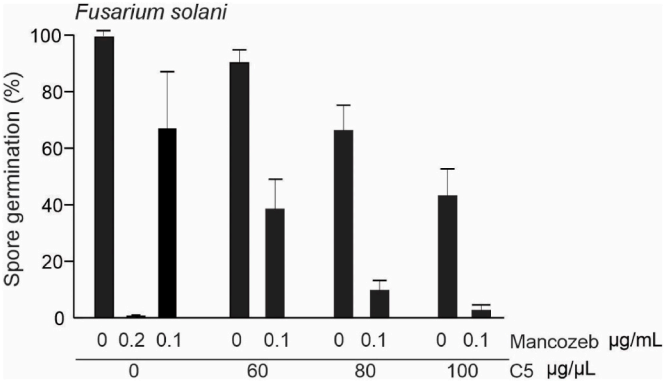

Fig. 7. Germination of *F. eumartii* spores in presence of different concentrations of cannabis resin from C5 variety, in presence of different concentrations of chemical fungicide and in presence of a combination of both resin and fungicide. Asterisks point out statistically significant differences (*: $p < 0.05$).

3.5. Synergistic action between mancozeb and cannabis resins

Considering the antifungal activity and the limited evidence on the effects of cannabis on pathogenic fungi, we decided to deepen the study. We compared the action of cannabis extract with Mancozeb, a chemical fungicide widely used to control fungal diseases in crops, fruits, and vegetables.F. eumartiispores were treated with Mancozeb alone or combined with different doses of C5 resin. After that, the inhibition of spore germination was analysed. Mancozeb completely inhibited spore germination at 2 μg/μL. When the concentration of Mancozeb was reduced by half (1 μg/μL), germination reached 67% of the control (without added fungicide). When 1 μg/μL Mancozeb and 60 μg/mL cannabis resin were combined, spore germination was reduced to 37.4%. As expected, higher doses of cannabis resin (80 μg/mL and 100 μg/mL) combined with the same dose of commercial fungicide (1 μg/μL) caused complete inhibition of spore germination (9.6 and 2.6%, respectively) (Fig. 7). The results show a synergistic effect between the commercial fungicide and the cannabis resins.

4. Conclusions

The results presented in this work add evidence to the broad spectrum of biological systems in which cannabis derivatives have a potential effect, not only because of cannabinoids but also because of the possible action of terpenes. On the other hand, the antimicrobial effect of alternative cannabinoids or other compounds such as sugars, hydrocarbons, steroids, flavonoids, nitrogenous compounds and amino acids, among others, should not be ruled out. The simplicity of cannabis resins preparation and its high performance in fungal and bacterial growth inhibition support using these plant derivatives as natural antimicrobial agents, alone or in combination with chemical biocides, reducing their doses and minimizing the environmental impact. In addition, and in concordance with the entourage effect, using complete extracts seems more effective than pure cannabinoids since lower doses achieve the same inhibitory results, supporting its use in environment-friendly strategies to deal with pathogenic microorganisms.

As can be seen from the results shown, the classification of cannabis strains into chemotypes based solely on THC and CBD content is not sufficient to justify the biological activities of cannabis extracts since varieties belonging to the same chemotype exhibit very different antimicrobial action.

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CRediT authorship contribution statement

María Eugenia Vozza Berardo: Investigation, Formal analysis. **Julieta Renée Mendieta:** Conceptualization, Writing – review & editing, Funding acquisition. **María Daniela Villamonte:** Investigation, Methodology. **Silvana Lorena Colman:** Software, Data curation. **Débora Nercessian:** Conceptualization, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jep.2023.116839.

LIST OF ABBREVIATIONS

THC	tetrahydrocannabinol
CBD	cannabidiol
CBN	cannabinol
LB	broth Lysogeny Broth
OD	Optical Density
YPD	Yeast-Peptone-Dextrose culture medium
PDA	Potato Dextrose Agar medium
w/v	weight/volume
DMSO	dimethyl sulfoxide
HPLC	High-Performance Liquid Chromatography
PI	propidium iodide
SD	standard deviation
DF	dried flower
MIC	Minimal Inhibitory Concentration

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