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Antibacterial activity of cannabis (*Cannabis sativa L.*) female inflorescence and root extract against *Paenibacillus larvae*, causal agent of American foulbrood

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ABSTRACT - *Paenibacillus larvae* is a gram-positive bacterium, and the etiologic agent of American Foulbrood (AFB), a disease that affects honey bee larvae. In the search for natural products capable of controlling the disease, we tested the antibacterial activity of inflorescences and roots extracts of Cannabis (*Cannabis sativa l*) against *P. larvae*, *Escherichia coli*, and *Staphylococcus aureus* using the microdilution broth method. We chemically characterized the extracts by the content of polyphenols, flavonoids, saponins, and volatile compounds, and analyzed the antioxidant capacity. Finally, their possible toxic effect on bee larvae was evaluated. Our research showed differences in the chemical composition between flower and root extracts as well as in their antioxidant potential; high potency and efficacy against (AFB) *in vitro*, but low toxicity to honey bee larvae.

Keywords: *Cannabis sativa L., Apis mellifera, Paenibacillus larvae,* antibacterial activity, plant extracts.

1-INTRODUCTION

Paenibacillus larvae is the etiological agent of American Foulbrood (AFB), a bacterial disease that affects the breeding of honeybees (*Apis mellifera*) during the larval or pupal stages (De Graaf, et al. 2013; Genersch, et al. 2006). This gram-positive bacterium, which the main characteristic is the formation of highly infective endospores and withstand adverse environmental conditions for long periods (Morse, et al. 1990). In order to control this disease, beekeepers use antibiotics as an alternative to burning the infected bee hives (Alippi, et al. 2006) being oxytetracycline the most frequently used agent. However, in many countries, including Argentina, this antibiotic suppresses clinical signs by controlling only vegetative cells, but bacterial spores accumulate in the hive and honey, remaining infective for many years (Alippi, et al. 2004). Widespread and wrong use of these substances placed strong selective pressure on different bacterial populations emerging resistant populations in several countries worldwide, including Argentina (Alippi, et al. 2006; Gende, et al. 2010). At the present, natural pesticides became a preferred option to synthetic drugs (Bilikova et al. 2013; Eguaras, et al. 2005) because of their lower toxicity in mammalians, little polluting effect in the environment, and wide public acceptance (Isman, 2000). In the last years several studies were performed to

identify natural compounds with antibacterial activity against *P. larvae*. Those studies included essential oils (Alippi, et al. 1996; Ansari, et al. 2016; Gende, *et al*. 2014; Tutun et al. 2018); pure compounds extracted from plants, bacteria, or fungi (Flesar, et al. 2010; Fuselli, et al. 2006; Lokvam, et al. 2000; Sabate, et al. 2012); honey bee by-products, such as royal jell (Biliková, et al. 2013) and propolis (Antúnez, et al. 2008; Isidorov, et al. 2017; Fangio, et al. 2019); and plants extracts (Boligon, et al. 2013; Chaimanee, et al. 2017; Damiani, et al. 2014; Fernandez, et al. 2019; Kang et al. 2018).

Cannabis sativa L. is an annual herbaceous plant, belonging to the Cannabaceae family, native to Central Asia where it played a historically important role in medical treatments (Frassinetti, *et al*. 2020). Among its compounds, the cannabis plant contains classic cannabinoids, a unique group of secondary metabolites, which are responsible for the peculiar pharmacological effects of the plant (Fischer, *et al*. 2015). The main psychoactive compound delta-9-tetrahydrocannabinol (Δ^9-THC) and the non-psychoactive component cannabidiol (CBD) have been the focus of studies for the pharmaceutical industry, however, the increase in the discovery of new cannabinoids is currently around 115 compounds (Martinenghi, et al. 2020). The discovery of these metabolites present in the Cannabis plant has focused research on cannabinoids and their properties as bactericidal agents in recent years (Karas, et al. 2020; Martinenghi, *et al.* 2020; Wassman, *et al*. 2020). The plant *C. sativa* has secretory structures, namely glandular trichomes, which produce, in addition to cannabinoids, an essential oil composed of monoterpenes and sesquiterpenes with a bitter taste (Happyana and Kayser, 2016). In general, monoterpenes provide various medicinal properties such as antimicrobial, antioxidant, anticancer, and antiarrhythmic, among others (Koziol, et al. 2014); these properties can also be attributed to sesquiterpenes found in both, hops and cannabis (Booth, et al. 2019). While the antibacterial properties of monoterpenes such as linalool (Koziol, et al. 2014; Nuutinen, 2018) and sesquiterpenes such as alpha caryophyllene are known (Booth, et al. 2019; Nuutinen, 2018), there are other natural cannabis metabolites with this property, such as phenolic compounds, such as carvacrol, eugenol, thymol, etc. (Dorman and Deans, 2000; Knobloch, et al. 1986; Lambert, et al. 2001), polyphenols (Frassinetti, et al. 2020; Mkpenie, et al. 2012) y saponins (Sparg, et al. 2004). In the plant, they fulfill the role, among others, to provide resistance and protection against pathogens and predators (Bravo, et al. 1998; Osbourn, 2003; Treutter, 2005).

The combination of cannabinoids together and non-cannabinoid components could synergistically enhance the beneficial effects of cannabis formulations (Pollastro, et al. 2018). The antibacterial activity found in *Cannabis sativa* may likely arise from this synergism (Dhifi, et al. 2016; Iseppi, et al. 2019). Chemical composition-bioactivity relationships do not always show a clear correlation between the relative amount of the most representative compounds in the extracts and their bioactivity, (Iseppi, et al. 2019) and even if they do, many researchers maintain that several components may be involved in antimicrobial activity (Frassinetti, et al. 2020; Nissen, et al. 2010; Pellegrini, et al. 2020; Zengin, et al. 2018), even if they are present in lesser abundances (Dhifi, et al. 2016).

Another important characteristic of cannabis metabolites is their antioxidant potential; free radicals and, in particular, reactive oxygen species (ROS) as the main cause of aging and tissue

damage. ROS overproduction, most often caused by excessive stimulation of NAD(P)H by cytokines or by the mitochondrial electron transport chain and xanthine oxidase, leads to oxidative stress (Maietti, et al. 2017). Antioxidants of plant origin are able to eliminate from the organism reactive oxygen and nitrogen radicals that irreversibly damage living tissues and induce serious diseases. Polyphenols, flavonoids such as cannflavins A and B, and terpenes such as alpha-pinene play key roles in antioxidant activity (Frassinetti, et al. 2020; Hasemkamp, et al. 2010; Krofta, et al. 2008**;** Nuutinen, 2018).

The honey bee is one of the dominant species visiting cannabis plants for pollen collection during flowering (Dalio, 2012; O Brien, et al. 2019). Besides these previous studies showing the importance of *C. sativa* as a food resource for bees, until now there are no studies intended to assess the use of cannabis against bee pathologies. Taking into account that cannabis plants extracts had been studied as insecticides within the framework of other pest management programs and food production (McPartland and Sheikh, 2018; Tabari, et al. 2020) and that the research on the potential antimicrobial properties of cannabis-derived compounds is in a stage of exponential growth. This work aims to test whether cannabis extracts can also have antibacterial effects against *Paenibacillus larvae*. To understand the potential antimicrobial properties of cannabis extracts, their chemical composition is analyzed and possible lethal effects on honey bee larvae are tested in vitro. It is proposed to evaluate the potential use of cannabis as a natural alternative for the control of AFB.

2- MATERIALS AND METHODS:

2.1 Extracts and chemical methodology

2.1.1 Plant material:

The plant material was provided by the NGO CBG 2000 from Mar del Plata city and characterized by the MDQ Herbarium of Vascular Plants of the Marine and Coastal Research Institute (IIMyC, UNMdP-CONICET/FCEyN). Plant Diversity Laboratory. Juan B. Justo 2550, Mar del Plata (7600), Buenos Aires, Argentina. Voucher number granted, as recorded in the MDQ Herbarium Database*: IMyCHer:MDQ:00630*. The inflorescences were processed by fine grinding, disposed as a thin bed and subjected to a temperature of 100 °C for 30 minutes in a N2-sealed bottle, according to Moreno et al. (Moreno et al. 2020). Under these conditions, monitoring by TLC (Thin Layer Chromatography) showed no detectable amounts of THCA or CBDA. Subsequently, the material was allowed to cool at room temperature before proceeding to the extraction. The total potency for this variety was 89 mg/g (DW) CBD and 52 mg/g THC (DW). CBN << 0.1 mg/g (DW). Roots were dried under nitrogen atmosphere and grindered before extraction.

2.1.2. *Chemicals and reagents*

2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), Folin-Ciocalteu reagent were purchased from Sigma-Aldrich®. The following standard compounds were purchased from Sigma-Aldrich® United States, and all standards purity ≥ 98.0%: Gallic Acid, Quercetin, Trolox, Vanillin, and Oleanoic acid. Ethanol Hexane, Sulfuric Acid, and DMSO were purchased from RESEARCH AG (Anedra) and used as received. Standard solutions of cannabidiol (CBD) and tetrahydrocannabinol (THC) were purchased from Restek.

2.1.3 Extraction process

To prepare the extracts, 0.5036 g of inflorescences were weighed and placed in 5 mL of Hexane in an ultrasound bath for 30 minutes. After that time, filtering was performed. This procedure was repeated three times. The liquids obtained after filtering were combined and evaporated under reduced pressure using a rotary evaporator at low temperature, and the dry extract was resuspended in 10 ml of Dimethyl sulfoxide (DMSO). The resulting solution had a concentration of 0.01383 g mL^{-1} of extract.

For the preparation of the root extract, 10.0012 g of roots were weighed and placed in 10 ml of the extractant composed of Ethanol/Water (1:1), then placed in an ultrasound bath for 2 hours at 50° C. Subsequently, filtering was carried out with filter paper in a Buchner funnel. The final volume obtained after filtering was 31.5 mL, which was dried until obtaining a solid. After drying, the resulting extract was suspended in 10 ml of DMSO, obtaining a final concentration of 0.04911 g mL^{-1} of extract.

2.1.4 Quantification of total polyphenol content

The total content of phenolic compounds in the different extracts was determined by the Folin-Ciocalteu method, according to the procedure reported by Singleton & Rossi (1965) with some modifications. The Folin-Ciocalteu (F-C) assay is a reaction based on electron transfer that measures the reducing capacity of an antioxidant. The F-C reagent is a mixture of phosphotungstic acid ($H_3PW_{12}O_{40}$) and phosphomolybdic acid ($H_3PMo_{12}O_{40}$) that reacts with phenols and non-phenolic reducing substances to form chromogen. The latter can be detected spectrophotometrically, since under alkaline conditions the oxotungstate and oxomolybdate formed in this redox reaction show a blue coloration proportional to the concentration of polyphenols (Lamuela-Raventós, 2018). For the quantification of phenolic compounds, a calibration curve was made from a standard solution of Gallic Acid (GA) of 200 µg mL-1. The following concentrations were used: 0, 2, 6, 8, 10, 15 and 20 µg ml-1, and the total polyphenol content of each extract was estimated from the linear regression obtained. This determination was performed in a 96-well microplate and the absorbance was measured at 760 nm in quadruplicate, and calculated in mg gallic acid equivalents (GAE) per g of dry extract.

2.1.5 Quantification of total flavonoid content

The total flavonoid content was determined by the Woisky and Salatino method (1998); with some modifications. The calibration curve was constructed from a quercetin (QE) standard solution of 200 µg mL-1. The following concentrations were used; 0, 2, 4, 6, 10, 14, 18, and 22 µg mL-1, and from the linear regression obtained, the total flavonoid content of each extract

was determined. The determinations were carried out in quadruplicate, and the absorbance was measured at 420mn using an Agilent 8453 UV-visible spectrophotometer with a diode array. Total flavonoid content was calculated as mg QE equivalents per g of dry extract.

2.1.6 Quantification of total saponin content

The determination of total saponin content (TSC) was performed using the methodology proposed by Le, et al. 2018, with some modifications. The principle of the method is based on the reaction of triterpene saponins which, after being oxidized by sulfuric acid, react with vanillin. This reaction causes a distinctive change in coloration towards purple-red and can be measured at wavelengths ranging from 473 to 560 nm. The CTS of a plant sample is determined from a calibration curve with a standard saponin (i.e. escin, diosgenin, oleanolic acid, quillaja saponin) and expressed in terms of standard equivalence (Le, et al. 2018). The calibration curve was constructed with Oleanoic Acid (OA) as a reference standard, and concentrations between 0.001- 0.005 µg mL^{-1} of OA were obtained with which the calibration curve was performed. From the linear regression of the calibration curve, the CTS of each extract was obtained. The absorbance at 560 nm was measured in an Agilent 8453 UV-visible spectrophotometer with a diode array and the values were recorded. Treatments were performed in quadruplicate, and the CTS was calculated as mg AO equivalent per g of dry extract.

2.1.7 Determination of antioxidant capacity

Antioxidant capacity for cannabis extracts was determined by comparing the activity of the radical ABTS according to Pires, et al. (2017). The ABTS [2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid], can be used in colorimetric tests to estimate the antioxidant potential of substances or mixtures. In this reaction, the light green ABTS reacts with potassium persulfate to form the green/blue ABTS•+ radical. Finally, the decrease in the absorbance of ABTS•+ before and after reacting with the components of the extracts to be tested is compared, using an Agilent 8453 UV-visible absorption spectrophotometer with a diode array at 734 nm. This difference is visualized by the color turning to light green (Re, et al. 1999). Using 96-well microplates, the calibration curve was prepared with TROLOX as standard compound with the following concentrations: 0, 2, 4, 6, 8, 10, and 12 µg mL^{-1}. From its linear regression, the antioxidant activity of each extract was estimated. The absorbance of the treatments was measured at 734 nm in an ELISA plate reader. The antioxidant activity was calculated as equivalent mg of TROLOX per g of dry extract.

2.1.8 GC-MS analysis of extracts

Samples were analyzed using a Shimadzu GCMS-QP2100ULTRA-AOC20i with a column of 0.25 mm ID, 30 m and 0.1 um phase thickness Zebron ZB-5MS. Samples were injected at pulsed splitless mode and the injection volume was 1ml. The interface and the ionization source were kept at 300 °C and 230 °C respectively. Helium chromatographic grade (99.9999%) was used as the carrier gas with a constant linear velocity of 52.1 cm seg^{-1}. The oven temperature program started at 50 °C, where it was held for 2 min and then increased to 300 °C at 15 °C min^{-1} where it was held for 4 min. Electron impact ionization (EI) was used at 70 eV in full scan. Full-scan EI spectra were acquired under the following conditions: mass range 35–700 m/z, scan time 0.3 s, solvent delay 3.0 min. Characterization of volatile compounds was

performed using NIST and Wiley libraries and retention index. The total Δ^9-THC and CBD content (potency) of the flower and root extracts was quantified using analytical standards and the calibration curve method.

2.2 Antibacterial activity

2.2.1 *Bacterial strains*

The effects of the extracts were studied against three strains of *Paenibacillus larvae* identified as ERIC I (UB-CIDEFI strain PL62, UB-CIDEFI strain PL92 and strain Miramar). The PL62 and PL92 strains were acquired from the OIE Reference Laboratories for AFB (UB-CIDEFI) and the isolates of *P. larvae* (Miramar) were collected from brood combs of hives with clinical symptoms of AFB located in the province of Buenos Aires. Aires, Argentina, appropriately isolated and identified according to the methodology used in the bibliography (Bailey, et al. 1991; Fuselli, et al. 2006). The Miramar strain was obtained from *P. larvae* spores and genetically classified as ERIC I. (Moliné et al 2020). In order to better understand the biological properties of the extracts, their bactericidal capacity against other gram-positive bacteria (*Staphylococcus aureus* ATCC 25923) and gram-negative bacteria (*Escherichia coli* ATCC 25922) was analyzed. Cultures were kept isolated and pure on plates with MYPGP 2% (w/v) agar (Mueller-Hinton broth 1% (w/v), yeast extract 1.5% (w/v), glucose 0.2% (w/v).), sodium pyruvate 0.1% (p/v), PO4HK2 0.3% (p/v), bacteriological agar 2% (p/v), distilled water) incubating at 36ºC with 10% (v/v) CO_2 for 48 -72 hours in microaerophilic conditions (Dingman and Stahly, 1983).

2.2.2 Microdilution methodology

To evaluate the antimicrobial activity of flowers and root extracts of *C. sativa*, the microdilution broth method was used, using the Minimum Inhibitory Concentration (MIC) assay (Pellegrini, et al. 2017). The technique is based on the determination of the growth of the microorganism in the presence of decreasing concentrations of the antimicrobial diluted in the culture medium (García-Rodríguez, et al. 2000).

The concentration obtained after the extraction process was used for the root extract, while a dilution of 1:10 in DMSO was used for the flower extract. A volume of brain heart broth (100 µL) was placed in each of the wells (3.7%, w/v) and 100 µL of the extracts were placed in the wells of the first columns. Subsequently, serial dilutions were made to the medium, obtaining, according to this methodology, decreasing concentrations of the tested extract. These ranges varied between 462 to 3.61 µg mL^{-1} (inflorescences), and between 16370 to 128 µg mL^{-1} (root), in the tests against *P. larvae*, and between 462 to 0.9 µg mL^{-1} (inflorescences), and between 8552.38 to 8.35 µg mL^{-1} (root) against *Escherichia coli* and *Staphylococcus aureus*. Finally, the bacterial inoculum (50 µL) was placed in all the wells. *P. larvae* isolates were kept fresh by periodically pecking them onto MYPGP agar. The bacterial inoculum was prepared in sterile peptone water (0.1% w/v) up to an optical density at 600nm (OD600nm) between 0.08-0.1 (Nordström and Fries, 1995). In addition, a viability control of the strain was carried out in wells that only contained culture medium (100 µL) and bacterial inoculum (50 µL), a solvent evaporation control, which contained brain and heart broth (100 µL) and bacterial inoculum

(50 µL), and negative control, containing the culture medium (100 µL) and DMSO (100 µL) with their respective serial dilutions. Microtiter plates were incubated at 36°C in an incubator for 48 hours under microaerophilic conditions (O_2 10% (v/v)). Then, 0.01% (w/v) resazurin salt (10 µL) was added and the same incubation conditions were carried out for 1 hour. Resazurin salt is a redox indicator pigment that allows bacterial growth to be distinguished. It is blue in the absence of bacterial growth and pink in the presence of bacteria. This procedure was performed with three isolates of *P. larvae* in triplicate and for three consecutive days.

2.3 Test on Honeybee larvae

2.3.1 *Honeybee larvae lethality test*

Honeybee larvae used were collected from the Santa Paula experimental apiary (Mar del Plata, National Route 226, Km 10, Argentina (37°55′48″S 57°40′59″W), belonging to the Social Bee Research Center (CIAS) – (IIPROSAM) of the UNMdP. A bioassay based on Medrozky, et al. (2013) and modifications (Iglesias et al. 2020), was used to test the acute toxicity of extracts on bee larvae. Combs with one-day-old larvae (1 st instar, L1) of *A. mellifera* were collected from healthy (non-infested) colonies for *in vitro* experiments. The larvae were transferred from the brood comb to 96-well culture plates, with a single larva added to each well and fifty replicants per treatment. The volume and composition of the diet daily provided to larvae followed the protocol by Aupinel, et al. (2005). The plates were then placed into a desiccator maintained at a relative humidity of 96 % (K2SO4 saturated) in a 34 °C incubator. On day four of incubation (stage L4); the highest MIC obtained from cannabis flower and root was administered on diet. In each experiment, a control group without solvent or treatment was performed and a control group to evaluate the possible effect of the DMSO solvent, incorporating the same volume of DMSO and at the same concentration as the tested extracts. In all cases, the concentration of DMSO in the volume of food administered for each larva did not exceed 1.2%, which is considered non-toxic for honey bee larvae (Pimentel-Betancur, et al. 2021). After administering the extracts at their minimum inhibitory concentration, larval mortality was recorded at 24, 48, and 72 hours. On the last day of the experiment (day 7), the larvae that arrived alive were weighed on an analytical balance to determine if there was any change in weight after the application of the treatments. The larvae used for these tests came from three different hives, and were realized by duplicating.

2.4 Statistical analysis

Mortality of bee larvae was estimated by comparison of survival curves using the Graph pad software (GraphPad Prism 5 version 5.03 for Windows, www.graphpad.com). The log-rank (Mantel-Cox) Test. Larval weights were compared by an analysis of variance (ANOVA) one way, using the software RStudio. Version 1.3.1056. The values between cones and roots MIC were evaluated with a non-parametric Kruskal-Wallis rank sum test, using the software RStudio. Version 1.3.1056.

3. RESULTS

3.1 Chemical characterization

Both roots and flower extracts presented similar values of flavonoids, however, this was not the case for total polyphenols, with the content of the latter being higher for the inflorescences extract. In addition, a high antioxidant capacity was found in the flower extract. On the other hand, the content of saponins in the root extract was higher. The values are presented as the mean of analyses performed by quadrupled ± standard deviation (Table 1).

Table 1: Content of total phenolic compounds, total flavonoids total saponins and antioxidant capacity of Cannabis extracts. Reference: AG: galic acid; Q: quercetine; AO: oleanoic acid.

	Total phenolic compounds	**Total flavonoids**	**Total saponins**	**Antioxidant capacity**
	mg AG g^{-1} Extract	mg Q g^{-1}Extract	mg AO g^{-1} Extract	mg TROLOX g^{-1} Extract
Roots	29.19 ± (0.04)	0.34 ± (0.02)	22.99 ± (0.09)	81.40 ± (0.20)
Inflorescences	101.98 ± (0.03)	0.35 ± (0.01)	6.34 ± (0.01)	528.92 ± (0.36)

3.2 Volatile organic compounds (VOCs)

The main volatile compounds of the root extraction analyzed by CG-MS were, according to previous reports (Ferrini et al 2021; Jin et al 2020), triterpenoid compounds such as delta-5-ergostenol, beta-sitosterol, stigmasterol, epi-friedelinol and Friedlein having these interesting biological properties. On the other hand, inflorescences showed different chemical profiles, such as Δ^9-THC (delta-9-tetrahydrocannabinol) and CBD (cannabidiol): 2.61 mg mL^{-1} and 4.48 mg mL^{-1} respectively with an entourage of typical cannabis mono and sesquiterpenoid content as shown in Table 2 (a and b).

Table 2-a: Relative percentage of compounds of extracts used in microdilution broth method and toxicity test against *A. mellifera* from flowers of *C. sativa.*

Compounds	Inflorescences		
	Kovats Index Exp	Kovats Index Teo	% Area
α limonene	1028	1027	0,864
nonanal	1105	1104	6,157
benzoic acid	1195	1196	13,51
octanoic acid	1177	1179	7,514
decanoic acid	1360	1364	5,423
β-caryophyllene	1431	1430	1,097
α-bergamotene	1446	1452	3,251
α-caryophyllene	1469	1463	7,288
β-selinene	1486	1485	5,266
α-bulnesene	1507	1505	2,979
β-bisabolene	1510	1509	2,227

α-bisabolene	1545	1549	2,904
nerolidol	1567	1565	5,928
β-caryophyllene epoxide	1596	1606	7,355
guaiol	1608	1602	2,54
10 epi-gamma eudesmol	1611	1609	2,601
β-eudesmol	1650	1654	12,835
α bisabolol	1684	1683	3,045
NI	1902	----	3,825
methyl palmitate	1926	1927	1,71
linoleic acid	2150	2152	1,68

Table 2-b: Relative percentage of compounds of extracts used in microdilution broth method and toxicity test against *A. mellifera* from roots of *C. sativa.*

Compounds	Roots		
	Kovats Index Exp	Kovats Index Teo	% Area
tetradecane	1400	1399	17,095
NI	1450	----	6,487
myristic acid	1735	1737,27	1,625
octadecane	1800	1800	1,953
heptadecanoic acid	2038	2038	12,84
9 octadecenoic acid methyl ester.	2099	2101	1,887
9,12 octadecadienoic acid (Z,Z)	2131	2134	7,096
squalene	2835	2833	14,412
glicerol tricaprilate	2961	2958	5,5
δ-5-ergostenol	3216	3220	2,519
β-sitosterol	3260	3263	5,214
stigmasterol	3295	3306	13,322
epi-friedelinol	3429	3433	4,765
friedlein	3455	3448	5,285

3.3 Antibacterial activity

The extract concentrations necessary to inhibit the growth of *Paenibacillus larvae* were significantly lower for inflorescences than for roots (*P<0.05). This trend is also evident for *Staphylococcus aureus* (Gram positive), while no activity was demonstrated at the concentrations tested for *Escherichia coli* (Gram negative) (Table 3).

Table 3: Antimicrobial activity (MIC) of the cannabis extracts against *Paenibacillus larvae*, *Staphylococcus aureus* and *Escherichia coli*. ND: No MIC was detected at the concentrations tested. The MIC values each, corresponding to the average of triplicates taken during three consecutive days (nine values in total).

	Paenibacillus larvae			*Staphylococcus aureus*	*Escherichia coli*
	Miramar	Típica	PL62	ATCC 25923	ATCC 25922
	MIC(µg mL^{-1})	MIC(µg mL^{-1})	MIC(µg mL^{-1})	MIC(µg mL^{-1})	MIC(µg mL^{-1})
Inflorescences	20.80	32.81	76.83	115.25	ND
Roots	1136.80	937.90	2955.73	8552.38	ND

3.4 Honeybee larvae lethality test and larvae weight

No statistically significant differences were observed in the survival curves of the larvae up to day seven. Log-rank (Mantel-Cox) test (*P>0.05). The survival graph is shown from day 4 (incorporation of the extracts into the diets) (Figure 1).

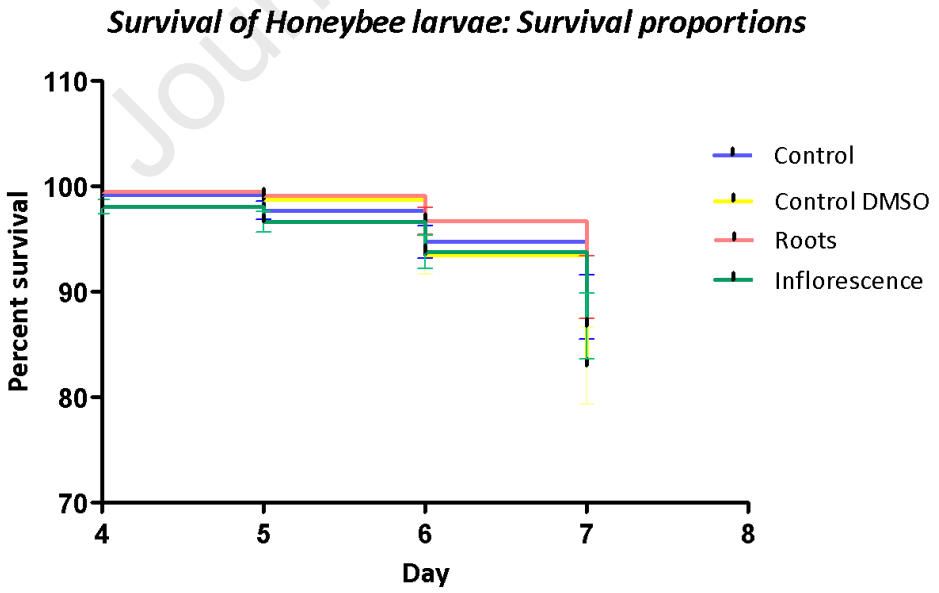

Figure 1: Kaplan-Meier plot of honey bees larvae survival function on different treatment: Control with no solvent (Control), control with solvent (Control DMSO), Roots, Inflorescences.

On the honeybee larvae weight we found no significant differences in the average weights of the larvae on day 7. (*P>0.05) (Figure 2). i.e. Weights: (Flowers: 0,1197g ; Control: 0,1193g; Control DMSO: 0,1281g; Roots: 0,1214g). Min: (Flowers: 0,0709g ; Control: 0,0923g; Control DMSO: 0,0904g; Roots: 0,0709g). Max: (Flowers:0,1468g; Control: 0,1470g; Control DMSO: 0,2522g; Roots: 0,1515g).

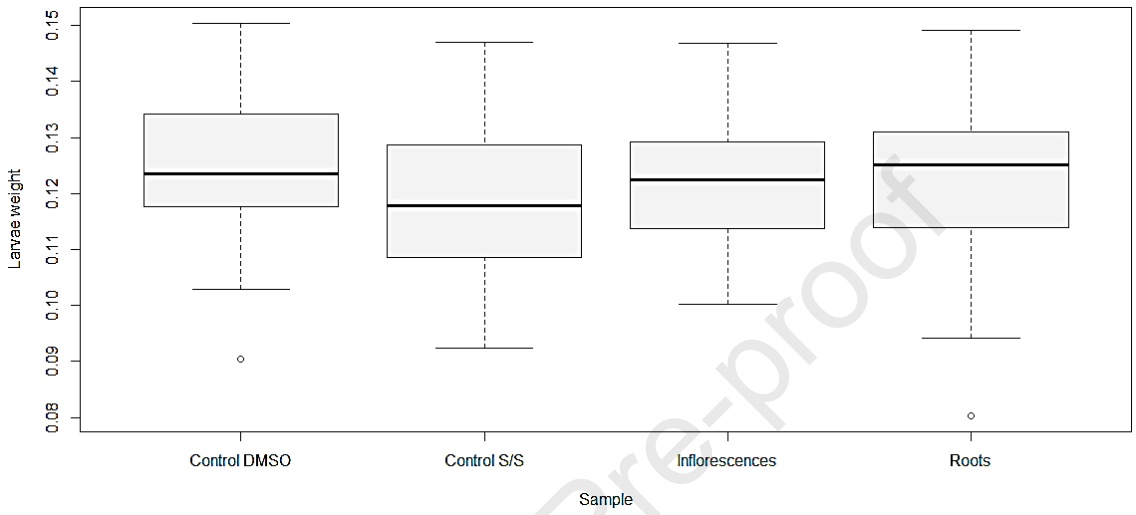

Figure 2: Boxplot of larval weights on day 7 with different treatments: Control with no solvent (Control S/S), control with solvent (Control DMSO), Roots, and Inflorescences.

4- DISCUSSION

The antioxidant capacity found in plant extracts has been attributed by several studies to polyphenols, flavonoids, and phenolic compounds in general, among other secondary metabolites (Karabin, et al. 2015; Masek, et al. 2014; Mongelli, et al. 2016; Schroeter, et al. 2002). Our extracts do not differ in total flavonoids content but in the amount of total polyphenols, which could suggest that the antioxidant activity observed in the flower extract is due to the non-flavonoid polyphenols present in the said extract, or to that the flavonoids present in the flower extract have a higher antioxidant capacity than those present in similar quantities in the root extract. Dan, et al. 2020 searched for flavonoids methylated and prenylated aglycones or conjugated *O*-glycosides or *C*-glycosides of orientin, vitexin, isovitexin, quercetin, luteolin, kaempferol, and apigenin in cannabis root and flower extracts, not finding these compounds family in the root, and a low percentage in flower (0.07–0.14%). This, together with our results, suggests that the antioxidant capacity in cannabis extracts could be related to non-flavonoid polyphenols. It should be noticed that in the quantification of polyphenols there is a major quantity of CBD which also has a polyphenol structure. Our inflorescence extract presented a concentration of 2.61 mg mL^{-1} of CBD, while this

cannabinoid is not found in the root extract. As suggested by Gruschow, (2020) in their work, the antioxidant capacity of flower extracts may be due to CBD concentrations.

The MIC values of cannabis inflorescences (20.80-76.83 µg mL^{-1}) are among the extracts with the highest antimicrobial capacity evaluated against *Paenibacillus larvae*. These values are similar to those found for other extracts, examples: *Eucalyptus gunnii* (16-32 µg mL^{-1}); *Rosmarinus officinalis L*. (16-32 µg mL^{-1}); *Catha edulis Forssk* and *Eucalyptus citriodora Hook*. (32-64 µg mL^{-1}); *Laurus nobilis L* (16-32 µg mL^{-1}) (Flesar, et al. 2010). Lower than other extracts such as *Citrus paradisi* (385 mg mL^{-1}) (Fuselli, et al. 2008); *Scutia buxifolia* (1.56 mg mL^{-1}; 6.25 mg mL^{-1}; 25 mg mL^{-1}) (Boligon, et al. 2013) and essential oils example *Pimenta dioica (L.)* (78 µg mL^{-1}); *Litsea cubeba Pers*. (85 µg mL^{-1}); *Trachyspermum ammi L*. (137µg mL^{-1}); *Mentha arvensis L* (144.7 µg mL^{-1}) (Ansari, et al. 2016). The MIC values found for synthetic antibiotics are variable, for example, for Oxytetracycline (4.2 µg mL^{-1}) (Ansari, et al. 2016); (0.016–0.031µg mL^{-1}) (Flesar, et al. 2010); and for Miyagi, et al. (2000), some strains of *P. larvae* are resistant to oxytetracycline and the MIC values in some cases exceed 32 µg mL^{-1}. This last value is even higher than the one found for cannabis in this work.

A differential antibacterial activity was observed between flower and root extracts, being evident in the ability of flower extract to inhibit bacterial growth. Undoubtedly, the chemical composition of the flower extract justifies this high activity; On the one hand due to the presence of the cannabinoids CBD and THC whose antibacterial properties are well studied (Appendino, et al. 2008; Farha, et al. 2020; Lelario, et al. 2018; Martinenghi, et al. 2020; Van Klingeren, 1976), and even knows its possible mechanism of action; THC together with THCA induce a bacterial response in strains with genotoxic sensitivity, which suggests that its mode of action damages DNA, while other cannabinoids such as CBD produce effects of oxidative and/or cytotoxic damage (Harpaz, et al. 2021). On the other hand, due to the abundance of diterpenes and sesquiterpenes with antibacterial properties known as β-eudesmol, with strong antimicrobial properties against gram-positive and gram-negative bacteria and fungi, (Acharya, et al. 2021), β-caryophyllene epoxide and α-caryophyllene are the most representative compounds in cannabis extracts reported as antimicrobials along with β- caryophyllene and β-myrcene, being more variable the representativeness of compounds such as α-bergamotene, α-selinene, β-bisabolene, nerolidol, β-eudesmol, etc, in these extracts (Benelli, et al. 2018; Issepi, et al. 2019; Nissenen, et al. 2010; Novak, et al. 2001; Zengin, et al. 2018). However, the antibacterial activity of the root extract was not null, although considerably low compared to that of the flower, it is not too far from the inhibitory concentrations of other types of extracts analyzed against *Paenibacillus larvae* (Chu Y-Y, et al. 2013; Fuselli, et al. 2006; Gonzalez, et al. 2010; Khan SU, et al. 2018).

This inhibitory capacity found in the root could be due to the high content of triterpenoid compounds and saponin content. Armah, et al. (1999) explained the mode of action of saponins in the membranes. According to this model, the spontaneous formation of a complex between the saponins and the membranes formed a micellar-type structure in two directions until the formation of the pore, thus allowing the passage of macromolecules into the cell. No THC and CBD contents were observed in the root extract. The absence of cannabinoids, monoterpenes, and sesquiterpenes in root extracts had been previously reported (Dan, et al. 2020). In addition,

the root extract obtained among its main volatile compounds, oleic acid with known antibacterial properties (Ghavam, et al. 2021; Stenz, et al. 2008); also 3-hydroxylauric acid is a hydroxy fatty acid with known antifungal properties (Kaspersen, et al. 2017); stigmasterol, which in *Kalanchoe Pinnata* extracts has wound-healing properties (Puertas Mejía, et al. 2014). To a lesser extent, ergosterol, with antimicrobial properties (Duarte, et al. 2007; Park and Kim. 2020). It is possible, then, that in root extracts a high saponin and other triterpenoid content, acts on the bacterial membrane, breaking it, thus allowing the passage of bactericidal compounds into the cell.

Both in the flower and root extracts, the representativeness of the majority of compounds with antibacterial properties is variable, therefore, we can support the idea of synergism between compounds that determine the bactericidal capacity, without attributing said action exclusively to the compounds that are found in greater proportion in the extracts.

The lower susceptibility of gram-negative bacteria of Cannabis extracts was also previously reported (Sarmadyan, et al. 2014; Vu, et al. 2016), although some investigations present more promising results regarding the spectrum of activity. (Ali, et al. 2012; Lone and Lone, 2012). The variations in the active compounds identified depend on several factors, such as the extraction methods, the type of solvent used, and the part of the plant analyzed (Abubakar, et al. 2020), likewise, the environmental conditions of the field and climatic stress can strongly influence the chemical profile. (Pavela and Benelli, 2016). In addition, it has previously been reported that the presence and amounts of cannabinoids in essential oils depend on various factors such as the state of the plant material to be processed, the harvesting period, and the distillation conditions (Calzolari, et al. 2017; Malingre, et al. 1973; Malingre, et al. 1975; Novak, et al. 2001).

Both the normal survival of the larvae when incorporating the extracts into the diet, as well as the no evidence of changes in weight at the end of the trial, seems to indicate an apparent innocuousness of the cannabis extracts. The lack of an endocannabinoid system in *Apis mellifera* (McPartland, et al. 2001) could be seen as an advantage. The lack of endocannabinoid receptors in bees, and therefore, the non-incorporation of cannabinoids in their system, could allow a greater availability of these for the exclusive participation in synergistic reactions with other metabolites of the plant, perhaps maintaining, more conservatively the bactericidal potential of extracts. Some plant extracts have been previously tested on bee larvae, eg (Chaimanee, et al. 2017; Damiani, et al. 2013; Gashout and Guzmán-Novoa, 2009; Kruszakin and Migdal, 2022; Pimentel-Betancur, et al. 2021), among these natural extracts, hops stands out, which is non-toxic to larvae and adult bees (Iglesias, et al. 2020; Iglesias, et al. 2021; Flesar, et al. 2010) and with which *Cannabis sativa* shares many of its secondary metabolites (Nuutien, et al. 2018).

5- CONCLUSION

Since the discovery of the metabolites present in the Cannabis plant, research has focused on cannabinoids, finding several properties associated with human health and well-being. This

work tries to expand the scope of the properties of plants, such as the antibacterial properties of cannabis, in order to benefit the health of other species, and to repair the damage caused by the indiscriminate use of antibiotics. Thus, we show how, using simple extraction methods, extracts can be obtained that exhibit in vitro bactericidal activity against *Paenibacillus larvae*, as well as against *Escherichia coli* and *Staphylococcus aureus*. Also due to their low toxicity for bees, these plant-derived products could represent a natural alternative to synthetic antibiotics in the control of AFB, decreasing with great controversy about antibiotic residues and resistance. The cannabis extracts tested here represent a good opportunity within the framework of Integrated Pest Management for AFB; for this reason, field studies must be continued to further investigate in field studies.

Declaration of Competing Interest The authors declare there are no conflicts of interest.

Author's contributions: MM and RC conceived this research and designed experiments; FG, IA, OD, FF, RF, MG and FS participated in the interpretation of the data; FG, OD, FF, and RC performed experiments and analysis; FG, OD, MM and RC wrote the paper. All authors read and approved the final manuscript**.**

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HIGHLIGHTS

- Cannabis sativa extracts show in vitro bactericidal activity against *Paenibacillus larvae*, as well as against *Escherichia coli* and *Staphylococcus aureus*.
- Cannabis extracts present low to null lethality on honeybee larvae.
- Cannabis inflorescences containing mono and sesquiterpenoids as well as high concentrations of cannabinoids, present a more efficient antibacterial capacity.
- Cannabis roots present triterpenoid compounds, plausible of having interesting biological properties

Declaration of interests

☒ 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.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: