ISSN 2965-0771

Revista Brasileira de CANNABIS

SOCIEDADE BRASILEIRA DE ESTUDOS DA CANNABIS

DOI: 10.58731/2965-0771.2023.33

EVALUATION OF MICROBIOLOGICAL QUALITY AND ANTIBACTERIAL ACTIVITY OF CANNABIS ARTISANAL OIL USED IN BRAZIL

AVALIAÇÃO DA QUALIDADE MICROBIOLÓGICA E DA ATIVIDADE ANTIBACTERIANA DO ÓLEO ARTESANAL DE CANNABIS UTILIZADO NO BRASIL.

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Submetido em 26 de setembro de 2023

Aceito para publicação em 11 de novembro de 2023

Publicado em 12 de janeiro de 2024

ISSN 2965-0771
Revista Brasileira de CANNABIS
SBEC SOCIEDADE BRASILEIRA DE ESTUDOS DA CANNABIS

RESUMO

O uso medicinal da Cannabis sativa tem se destacado como alternativa no tratamento de doenças neurológicas, devido ao componente canabidiol (CBD), com propriedades anticonvulsivantes. Este estudo avaliou a qualidade microbiológica e a atividade antibacteriana de extratos de Cannabis utilizados por pacientes com epilepsia refratária a fim de trazer mais segurança em seu uso e prescrição. Foram avaliadas cerca de 70 amostras de extratos de Cannabis, 10 delas utilizadas para testes de atividade antibacteriana. A qualidade microbiológica foi medida pela observação do crescimento de bactérias, após a inoculação do extrato de Cannabis nos meios de cultura BHI Agar, BEM Agar, MacConkey Agar e Mueller-Hinton Agar. Para atividade antimicrobiana, um método de Kirby-Bauer modificado foi realizado com 11 isolados bacterianos considerados potencialmente patogênicos. Das amostras analisadas, 70 não apresentaram contaminação bacteriana durante seu processo de preparo, distribuição e armazenamento, tornando-as seguras do ponto de vista microbiológico para consumo humano. Em relação à atividade antimicrobiana, as 10 amostras testadas não apresentaram atividade inibitória contra as bactérias listadas. O produto não pode interferir na microbiota dos usuários em tratamento, pois alguns dos isolados fazem parte da microbiota humana. A análise da qualidade microbiológica e da atividade antibacteriana da cannabis utilizada em pacientes com problemas neurológicos graves é de fundamental importância para garantir maior segurança em seu uso. Portanto, todos os extratos de Cannabis testados estão livres de contaminação microbiana que possa comprometer a qualidade do produto, e nenhum dos extratos utilizados neste estudo inibiu o crescimento in vitro das bactérias testadas.

Palavras-chave: cannabis sativa, qualidade microbiológica, problemas neurológicos, canabidiol.

ISSN 2965-0771

Revista Brasileira de CANNABIS

SBEC SOCIEDADE BRASILEIRA DE ESTUDOS DA CANNABIS

ABSTRACT

The medicinal use of *Cannabis sativa* has been highlighted as an alternative in the treatment of neurological diseases, due to the cannabidiol (CBD), with anticonvulsant properties. This study evaluated the microbiological quality and antibacterial activity of Cannabis extracts used by patients with refractory epilepsy in order to bring more safety in their use and prescription. Around of 70 samples of Cannabis extracts were evaluated, 10 of them used for antibacterial activity tests. Microbiological quality was measured by observing the growth of bacteria, after the inoculation of the Cannabis extract into BHI Agar, BEM Agar, MacConkey Agar and Mueller-Hinton Agar culture media. For antimicrobial activity, a modified Kirby-Bauer method was performed with 11 bacterial isolates considered potentially pathogenic. Of the analyzed samples, 70 showed no bacterial contamination during its preparation, distribution and storage process, making them safe from a microbiological perspective for human consumption. Regarding antimicrobial activity, the 10 samples tested showed no inhibitory activity against the listed bacteria. The product may not interfere with the microbiota of users being treated, as some of the isolates are part of the human microbiota. Analysis of the microbiological quality and antibacterial activity of cannabis used in patients with severe neurological disorder is of fundamental importance to ensure better safety in its use. Therefore, all Cannabis extracts tested are free of microbial contamination that may compromise product quality, and none of the extracts used in this study inhibited the *in vitro* growth of the tested bacteria.

Keywords: cannabis sativa, microbiological quality, neurological disorder, cannabidiol

INTRODUCTION

Different people and cultures, presenting indications for treatment of numerous medical conditions such as epilepsy, tuberculosis, malaria, intestinal constipation, pain, expectoration, among others (Zuardi, Adams, Hunt, & Clark, 1940; Pernoncini, 2014), have used *Cannabis sativa* for medicinal purposes for thousands of years. Cannabis belongs to the *Cannabaceae* family, consisting of only one species, known as *Cannabis sativa* L., and several subspecies, among them *C. sativa* spp*. sativa, C. sativa* spp*. indica, C. sativa* spp*. ruderalis, C. sativa* spp*. spontanea, C. sativa* spp*. kafiristanca*. It has over 500 identified chemicals and over 100 of them classified as phytocannabinoids (Bonini et al., 2018; Bruna et al., 2017; Aizpurua-Olaizola et al., 2016; From Backer, Maebe, Verstraete, & Charlier, 2012).

This plant has several active chemical constituents, among them terpenes and phytocannabinoids. These two associated constituents, through the typical committal effect of herbal medicines, can produce a synergy regarding the treatment of pain, inflammatory processes, depression, anxiety, epilepsy, cancer, fungal and bacterial infections (Russo, 2011). The main phytocannabinoids found in *Cannabis sativa* are Δ9-tetrahydrocannabinol (THC), responsible for the hallucinogenic and psychoactive effect of the plant, followed by cannabidiol (CBD), cannabinoid free of this psychotropic activity and which has several pharmacological properties, including anticonvulsant action (Cunha et al, 1980; Rektor et al, 2015; Reddy & Golub, 2016); anti-inflammatory action (Honorio, 2006; Pernoncini, 2014), neuroprotective effect (Hampson, Grimaldi, & Axelroad, 1998; Gontijo, 2016), anxiolytic and antipsychotic (Zuardi, 2006).

The therapeutic potential of cannabis gained prominence with the discovery of the endocannabinoid system and its knowledge in modulating multiple physiological effects, which may be key to several diseases affecting the central nervous system, such as neurodegenerative diseases, cognitive deficits and epilepsy (Pertwee, 2012; Scotter, Abood & Glass, 2010; Katchan, David & Shoenfeld 2016). Studies show that imbalance in the endocannabinoid system generates neuronal excitability, which leads to the development of epileptic seizures (Romigi et al, 2010). The standard treatment of this disorder is classic anticonvulsants that provide symptomatic relief in about 70% of patients. However, 30% of patients with this disease do not obtain a satisfactory clinical response and seizures become difficult to control, known as refractory epilepsy (Gloss & Vickrey, 2014; Kwan et al, 2010; Reddy & Golub, 2016).

According to the World Health Organization (WHO), in February 2017, about 50 million people worldwide suffer from epilepsy, of which 1.9 million are

Brazilians. In this context, the secondary metabolites of *Cannabis sativa* L., known as cannabinoids, are becoming a potential therapeutic alternative for the treatment of refractory epilepsy (Reddy & Golub, 2016).

Currently, the use of *Cannabis sativa* and its cannabinoids in the treatment of various diseases, including refractory epilepsy, has become increasingly present worldwide. In Brazil, there is still no regulation for the use of *Cannabis sativa* or its constituents in the treatment of diseases. On October 11, 2022, the Federal Council of Medicine authorized the use of cannabidiol to treat epilepsy in children and adolescents refractory to conventional treatments in Brazil. Importantly, all products for human consumption must be free of contamination by disease-causing pathogenic microorganisms. The microbiological quality control of non-sterile products aims to prove the absence of pathogenic microorganisms that may compromise product stability, alter physicochemical characteristics, inactivate the active principles of the formulation and aggravate or cause disease in patients who are often already debilitated (Yamamoto et al, 2004).

Thus, the aim of this study was to evaluate the microbiological quality and antibacterial activity of artisanal cannabis oil used by patients with refractory epilepsy, providing greater safety for these patients and for physicians when prescribing.

MATERIAL AND METHODS

Microbiological and antibacterial analyzes of *Cannabis* artisanal oils used by patients with refractory epilepsy were performed in the Microbiology laboratory of the Department of Physiology and Pathology (DFP) and in the Endemic / Tropical Medicine Center laboratory, both from the Federal University of Paraíba.

To perform the tests, the Brazilian Cannabis Hope Support Association (ABRACE) donated all samples of artisanal Cannabis oil. After receiving these samples, each of them was identified with a specific code to perform the analyzes. Samples used for analysis were taken from aliquots of donated batches immediately after manufacturing, ready for use by patients. For microbiological analysis, 70 samples from the following lots were used: 70.7, 225.5, 225.04, 271.19, 225.8, 261.1, 261.2, 174.2, 276.3, 225.10, 225.7, 70.6, 275.1, 276.2, 225.5, 277, 225.12, 269.3, 275.2 276.1, 276.4, 225.1, 225.2, 225.11, 271.22, 225.03, 271.23, 271.24, 276.1, 261.3, 269.2, 225.9, 271.20, 271.21, 274.3 and 70.8. For the antibacterial activity assays, 10 samples from the following lots used: 19044, 4404, 3429, 3434, 4311, 312.8, 152.6, 263.2, 144.08 and 289.4.

Obtaining Artisanal *Cannabis* Oil

The artisanal oil samples tested were obtained from *Cannabis sativa* crude ethanolic extract (BSE) with a concentration of 2mg / mL. About 10g of *Cannabis sativa* resin flower was dried, crushed and added to 200mL of cereal alcohol. Then a mechanical stirrer was used for 30 minutes, filtered and rotavapped for 1h at a temperature of 71 ° C. After this procedure, BSE was added to 30mL of soy lecithin and vegetable glycerin to obtain the oil.

Evaluation of microbiological quality of artisanal *Cannabis Sativa* oil

The microbiological quality of the 70 samples of crude ethanolic extract (BSE), obtained from 10g of dried and crushed *Cannabis* plant resin, was performed in two stages: (1) growth observation of various microorganisms with the inoculation of the oil in Brain Heart Infusion Agar Broth (BHI); (2) bacterial growth analysis in BEM Agar, MacConkey Agar and Mueller-Hinton agar culture media (Wilkins, 1973).

Each sample was identified with a specific code for the work, consisting of a letter and a number identified in their labeled containers that corresponded to a respective ABRACE nomination.

In the first step, the BHI medium was distributed in test tubes previously autoclaved at 121^{o}C / 20min, one tube for each oil sample, in a titration of 900µL of BHI medium to 100µL of oil. The distribution was performed with sterile disposable pipettes near the Bunsen burner. Finally, the media were kept in a incubator, preserving its cooling temperature between 45-50 ° C for 5 days, with daily observation of all samples to identify the characteristics of the medium, associated with the presence of turbidity in the samples. This procedure was performed in duplicates, in which each BSE-containing vessel received by the team was distributed into two different test tubes, both presenting the BHI medium to obtain greater precision of results.

The second stage was performed with two different culture media (MacConkey and Mueller-Hinton), prepared and distributed in disposable Petri dishes. Then each sample from the vial was evenly distributed on the Petri dish of each of the culture media previously identified with the corresponding sample from a swab close to the Bunsen burner (Henry et al., 1997).

To ensure the viability of the culture medium, a control group was performed using *Klebsiella* sp. and the other was kept free of bacteria. In the final stage, after the distribution of the samples in the plates, they were incubated for 24h until they were positive with or without bacterial growth. The

criterion used was the visualization of colonies. In cases of sample contamination, the experiments were discarded and restarted from the first stage.

Antibacterial activity of artisanal *Cannabis* oil

To determine the antibacterial activity of *Cannabis* oil, only 10 samples out of 70 were used due to the quantity of materials, which were donated by the Association. The bacterial isolates used were provided by the DFP Microbiology Laboratory and the Department of Pharmaceutical Sciences (DCF) of the Federal University of Paraíba. Tests were performed with Gram bacterial isolates: (1) Gram negative bacilli: *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *K. pneumoniae* - carbapenemase (KPC), *K. pneumoniae* - Extended-spectrum beta-lactamase (ESBL), *Salmonella* spp. and (2) Gram positive cocci: *Staphylococcus aureus*, *S. aureus* - Methicillin-resistant (MRSA), *Streptococcus sobrinus*, *S. sanguinis* and *S. mutans*. All of these isolates are considered potentially pathogenic and opportunistic.

To evaluate the sensitivity of bacteria to the oils analyzed, a modified Kirby-Bauer method (disk diffusion method) was used (Peterson et al., 1980). Streptococci were multiplied in blood agar culture medium and incubated in microaerophilia and the other bacteria in Müller-Hinton medium, both in a bacteriological incubator at 36ºC / 24h. Petri dishes containing the respective sterile media were sown with a suspension of approximately $1.5x10^8$ / mL of each bacterium using sterile swab. For antibacterial analysis of the oils, the 6 mm diameter filter paper was autoclaved at 121 ° C for 15 minutes and dried in a drying oven at 36ºC / 24h. After this procedure, the filter paper discs were immersed in different oil concentrations (50, 100, 150 and 200 mg/mL) in dilutions of 0,2 mL. Each disc received a different concentration of oil and was placed equidistant over the seeded medium in triplicate. This procedure was repeated for each oil sample analyzed. For the control test, sterile distilled water was used. The prepared petri dishes were incubated at 36 ° C for a period of 18 to 24 hours. The results obtained were evaluated by measuring the diameter of the inhibition halos, in mm, formed around the discs. The extracts considered active would have to present inhibition halos greater than 10mm.

RESULTS AND DISCUSSION

BHI broth is used for the recovery of fastidious and non-fastidious microorganisms, including aerobic and anaerobic bacteria and fungi. However, even in the face of an ideal culture medium for the detection of numerous potentially contaminating microorganisms, the inoculation of the oil in the culture medium presented a hindrance regarding the visualization of the expected turbidity in case of bacterial growth. This fact resulted from the alteration of the visual characteristics of the culture medium after contact with oil, becoming cloudier with a less citrine characteristic, which made the first stage of the research difficult.

Thus, facing a first attempt that was not conclusive facing the expected objectives, the second step was performed, which resulted in satisfactory data. During culture with BEM Agar, MacConkey and Mueller-Hinton media, capable of identifying, differentiating and isolating pathogens of clinical importance, visible bacterial growth was achieved in only five of the samples at first, but none of them were positive in the two spaces for the duplicate. In the others, the spaces in the petri dish remained without visible bacterial growth. Therefore, a new experiment was carried out, similar to the previous ones, containing the two steps, to certify the veracity of the possible BSE contamination, observed in the first moment in the five samples that presented positivity.

After the tests, the results observed were that no samples showed bacterial growth in the three media used, which characterizes that, in the previous test, there was an external contamination during its realization process and the possible contamination in the process of obtaining the BSE is discarded. In the spaces in which colonies of *Klebsiella* sp. were inoculated, as positive control, there was normal growth and the spaces that remained without any content showed no alteration. Thus, the 70 samples analyzed showed no bacterial contamination during its preparation, distribution and storage process, making it safe from a microbiological perspective for human consumption.

Regarding the antimicrobial activity evaluated, the 10 BSE samples showed no inhibitory activity against the tested microorganisms at all concentrations used (Tables 1 and 2, Fig. 1). The assays were repeated changing the methodology for wells drilled with 5mm diameter tiny punctures in the seeded culture medium and filled with dilutions, however with results similar to the first assay.

ISSN 2965-0771
Revista Brasileira de CANNABIS
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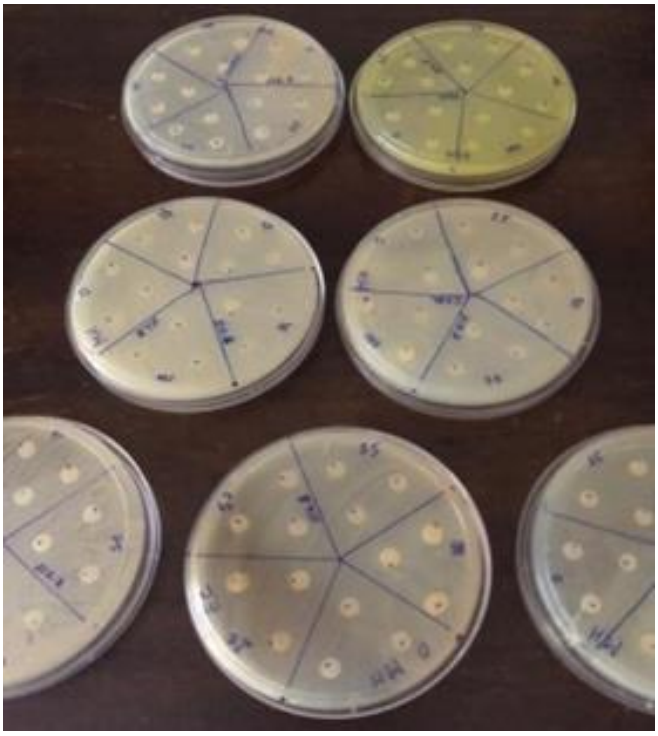
Table 1 – Determination of antimicrobial activity of *Cannabis sativa* cannabinoid extracts against Gram positive bacterial isolates by the modified Kirby-Bauer method. (+) means there was growth and (-) means there was no growth.

Concentration (mg/mL)	*Staphylococcus aureus*	MRSA	*S. sobrinus*	*S. sanguini*	*S. mutans*
0	-	-	-	-	-
50	-	-	-	-	-
100	-	-	-	-	-
150	-	-	-	-	-
200	-	-	-	-	-

Table 2 – Determination of the antimicrobial activity of *Cannabis sativa* cannabinoid extracts against Gram negative bacterial isolates by the modified Kirby-Bauer method. (+) means there was growth and (-) means there was no growth.

Concentration (mg/mL)	*Escherichia coli*	*Pseudomonas aeruginosa*	*Klebsiella pneumoniae*	KPC	K. ESBL	*Salmonella* spp.
0	-	-	-	-	-	-
50	-	-	-	-	-	-
100	-	-	-	-	-	-
150	-	-	-	-	-	-
200	-	-	-	-	-	-



Source: Authors

Figure 1: Petri dishes containing the inhibition test, performed using the modified Kirby-Bauer method.

Although this result does not indicate any antimicrobial activity to the bacteria tested, there is an observed advantage, as the product possibly will not interfere with the microbiota of the users under treatment, considering that some of the isolates are part of the human microbiota.

On the other hand, further research deserves to be carried out as to the effect of extracts, after metabolism, if they have probiotic effects in relation to the intestinal microbiota. In works developed with plant extract, Alves et al (2008) showed antimicrobial activity of *Rosmarinus officinalis* against cariogenic microorganisms, such as *Streptococcus mitis*, *S. mutans*, *S. sanguis*, *S. sobrinus* and *Lactobacillus casei*, evidencing its potentiality against these bacteria. In a study to evaluate the antimicrobial activity of *Syzygium cumini* ethanolic extract in relation to oral cavity microorganisms, Cartaxo-Furtado et al (2015) identified the strong inhibiting activity on *Candida albicans*. Antibacterial action was also studied *in vitro* of ethanolic extract (aerial parts) and phases of *Wissadula periplocifolia* (L.) C. on pathogenic microorganisms, tested by Teles

et al. (2014) against *E. coli*, *S. aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Acinetobacter* spp., to no avail, but there was activity for *Enterococcus faecalis*. As a therapeutic activity, Lessa, Cavalcanti and Figueiredo (2016) conducted a study that showed that synthetic cannabinoids and *C. sativa* extracts have analgesic effects, especially in pain of neuropathic origin, and anxiolytic effects when used to treat pain associated with cancer patients with multiple sclerosis and rheumatoid arthritis.

Therefore, many studies have evaluated the antimicrobial action of plant extracts. However, in the literature, there is still little data on the action of cannabinoid extracts. Therefore, continuing these studies with *C. sativa* can bring many benefits to patients who use extracts, as well as help eradicate the myths that part of the population has about the use of *C. sativa*.

CONCLUSION

Thus, the analysis of the microbiological quality of *Cannabis* extract used in patients with severe neurological problems is of fundamental importance for the guarantee and better safety of the clinical practice of patients who benefit from this compound. Thus, it can be concluded that, according to the data obtained in this study, the use of BSE is in adequate conditions. All cannabinoid extracts tested are free of microbial contamination that may compromise product quality. Concomitantly, none of the extracts used in this study inhibit the *in vitro* growth of the tested bacteria.

The results obtained in this work do not exclude the need to evaluate these same extracts and their actions on other microorganisms, including fungi, also research regarding the intestinal microbiota for the possible effects of metabolites. Further research is needed to study the physicochemical properties and characteristics to complement the information on the extract oil, making the efficacy-safety-quality tripod more certified.

ACKNOWLEDGEMENTS

The authors wish to thank ABRACE (Associação Brasileira de Apoio Cannabis Esperança) for the excellent assistance and important support to the accomplishment of this work.

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