Antimicrobial Studies of Cannabidiol as Biomaterials against superbug MRSA

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Abstract— **Due to its limited treatment options, multi-drug resistant bacteria such as Gram-positive methicillin-resistant Staphylococcus aureus (MRSA) still remains a serious public health threat. The creation of new compelling antimicrobial materials, antibiotics and optional methodologies, which are successful against resistant microbes, is earnestly required. The legalization of cannabis in Canada has provided a new opportunity to investigate the antimicrobial studies of both extracts and individual cannabinoids.**

This study investigates pure cannabidiol (CBD) isolated from Cannabis sativa by using a methodology of extraction, purification, characterization, and quantification of CBD. The shredded plant material was dissolved in ethanol, with the extract further purified using supercritical fluid chromatography (SFC) to obtain purified CBD. Product purity was confirmed by HPLC and NMR spectroscopy. CBD's antibacterial activity on MRSA strain USA300 bacteria was studied using dilution series in liquid culture and disk diffusion assays to provide the minimum inhibitory concentration (MIC) and minimal bactericidal concentrations (MBC). We have also performed statical analysis between CBD concentration groups with no CBD (control) and found a significant difference in cell counts of these groups. Past papers had not shown any MBC values – we have obtained a novel MBC value for CBD industrially extracted from Canadian grown C. Sativa plants.

The results showed that CBD exhibited a significant bactericidal effect on MRSA with the MIC value of 2.5 µg/mL and MBC of 10 µg/mL. CBD powder form gave a higher antimicrobial activity than its oil form in terms of the inhibition zone. This study shows that CBD exhibits good antimicrobial impact against the MRSA strain showing its utility for enabling a new antibiotic-free method for treating MRSA infections.

Keywords—**CBD, antimicrobial activity, MRSA.**

I. Introduction

Cannabis sativa, grown specifically for medical/recreational use, is of significant clinical interest[1,2]. The initiative of Cannabis research became more feasible in Canada as the Federal *Cannabis Act* on October 2018 made Canada the second country in the world to formally legalize the cultivation, possession, acquisition, and consumption of cannabis and its by-products [22]. Short-term use of oral cannabinoids has been shown to improve multiple sclerosis (MS)-related spasticity symptoms, post-traumatic stress disorder, cancer, epilepsy, cachexia, glaucoma, HIV/AIDS, and degenerative neurological conditions [3]. Several research studies suggest that approximately 2% of Canadian adults used cannabis for therapeutic purposes (CTP) in the past years, primarily to relieve nausea and pain[4]. Patients with MS and chronic pain report similar results; approximately 15% of respondents report high levels of perceived effectiveness of cannabis for treatment against nausea, pain, bad mood, headache and migraine [5,6].

The cannabis plant comes in three main derivatives, Cannabis sativa L., Cannabis indica, and Cannabis ruderdalis [7]. Clinical research has mainly focused on C. sativa L. due to its ease of cultivation and adaptation to many severe climates. *Cannabis* is known to produce over 120 different compounds which unfortunately have unknown or poorly defined pharmacological profiles. The constituents of C.sativa are the psychoactive *tetrahydrocannabinol* (THC), non-psychoactive *cannabidiol* (CBD), mildly psychoactive *cannabinol* (CBN), followed by the parent molecule *cannabigerol* (CBG), *cannabichrom* (CBC) and in low amounts psychoactive *cannabinodiol* (CBND)[8]. Especially, CBD has drawn extensive interest because of its various therapeutic and pharmacological attributes [9,10]. A challenge is it has low solubility in aqueous media and a relatively low bioavailability, enabling it to be utilized as single compound or dissolved with polar solvents [11]. CBD is a non-psychotropic compound with low cytotoxicity [12]. In addition, Cannabis sativa L. contains high-graded oils and its quality in drug extraction can likewise improve cannabinoids absorption.

Many plants and their extracts have significant antimicrobial which can be found in folk medicine, essential oils or isolated compounds [24]. The antimicrobial characteristics of C. Sativa are relatively unknown, and what is their fundamental pharmacological mechanism and biochemical mode of action. Studies are required to find new bactericidal materials that satisfy antiseptic requirements. These novel biocompounds can potentially be used for multi-drug therapy used for complex disease pathogenesis such as AIDS, or

against multi-resistant bacterial infections [13-16]. MIC values in [13] of CBD which was extracted in the lab were 1 µg/mL against *Epidemic Methicillin-Resistant Staphylococcus Aureus* but the purity was unknown. However, our study will focus on industrially extracted 99 % pure CBD and its antimicrobial studies. The exploration of industrial-grade CBD having the power to not only inhibit growth (MIC) but also cause cell death (MBC) has not been studied before as previous papers.

Methicillin-resistant Staphylococcus aureus (MRSA) is a common pathogen found in clinical infections which is a significant challenge. MRSA can enter the human body by skin surface wounds, as well as infections in the central nervous system, or diseases such as osteomyelitis, invasive endocarditis, septic arthritis, septicemia, pneumonia, and infections in the urinary tract. MRSA has become immune to many types of antibiotics and sepsis has become the most common form of staphylococcal infection[20]. Studies in Canada indicate that about 1 in 12 Canadian adults hospitalized are colonized or infected with MRSA[18]. In these hospitals, 67 % of patients were either colonized or infected with MRSA in comparison to other bacterial infections[18].

In 2008, research suggested major cannabinoid compounds of marijuana have shown effective activity against a range of MRSA strains [17]. More recently in 2020, McMaster University uncovered the pathogenesis of cannabigerol (CBG) through targeting the cytoplasmic membrane of gram-positive bacteria and demonstrated *in vivo* efficacy of CBG in a murine systemic infection model caused by MRSA[25]. Based on these studies, the motivation behind this study was to compare the bactericidal activity of CBDs, industrially extracted from Ontario grown C. Sativa in various forms (solid and lipid dissolved) by measuring substantial inhibitory effects with minimal inhibitory concentrations studies and inhibitory zone studies. Time kill analysis and minimal bactericidal concentration were carried out to prove antibiotic effects.

II. Materials & Methods

A. Bacterial Strains

Methicillin-Resistant S. aureus (USA300) was taken from the Heinrichs lab stock. MRSA was cultured in tryptic soy broth at 37ºC for 24 h with shaking. Bacteria were then harvested by centrifugation at 13000 rpm for 1 min at room temperature, then the supernatant was removed and each sample was resuspended with an OD_{600} 1 in 1 mL sterile saline.

B. Bacterial Strains

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C. Isolation and Purification of Cannabidiol

Industrial samples of CBD powder and oil were obtained from Ontario grown Cannabis using an extraction procedure described in [19]. The industrial process of extraction by them involved: shredding the plant material; soaking the shredded material into the ethanol to form a polar solvent/cannabinoid mixture; further filtering the polar solvent/cannabinoid mixture from residual solid plant material; The mixture was subjected to a supercritical fluid to isolate and purify the cannabinoid mixture into individual cannabinoids as stated in [26]. The extracts obtained an orange-colored oil of 95 % CBD. Only CBD powdered form was further purified by evaporative crystallization of CBD to produce a CBD purity > 99 %.

In order to verify the purity of the industrial CBD crystals samples, CBD fractions were guided in the Charpentier's lab by Shimadzu HPLC (Kyota, Japan). The quantification of cannabinoids of interest was achieved by comparison of the peak intensity and integration numbers of our sample CBD to that given in literature [23].

Characterization of purified CBD was measured by ^{1}H NMR spectroscopy, Varian. Inova – 600 (California, USA) as shown in Fig.1, showing no other compounds present. According to [20] the processed cannabis powder extracts, 20 mg, were dissolved in 1 mL DMSO as they retained concentration longer when diluted in DMSO rather than in methanol due to a higher boiling point and, consequently, less volatile properties. The orange oil extracts, 20 mL, were dissolved in 1 ml pure canola oil (Saporito foods). Both mixtures were further sonicated for loosening extracts adhering to surfaces until use as antibiotics. CBD extracts per solvent was 20 mg/ml.

D. In vitro assay for Antimicrobial Activity

a) *MIC Studies:* The MIC for purified CBD powder was determined by dissolving CBD powder in DMSO to make a concentration of 2 mg/mL. 20µL of the dissolved CBD solution was added to 2mL of Tryptic soy broth (TSB) growth media to obtain a starting concentration of

20µg/mL. This prepared sample was then diluted in a two-fold series in TSB as shown in figure 2. The tested concentrations ranged from 20-0.625 µg/mL CBD and a no CBD control. 10µL of USA300 at an OD_{600} 1 was added to each sample, reaching a starting OD_{600} of 0.01, the samples were then incubated at 37 °C with shaking for 24 hours. The OD of the samples were then measured in a 96 well plate using a microplate spectrophotometer from Biotek instruments (USA), and the MIC was determined as the lowest concentration with no discernible growth. The experiment was repeated in three separate trials and the results were plotted using GraphPad Prism software(California, USA) as shown in Fig.4.

b) *Zone of Inhibition Studies:* For the CBD oil-based extracts dissolved in canola oil, it was difficult to dissolve in broth solution for dilution and MIC studies. So the agar disk diffusion method [21] was used to evaluate cannabidiol oil solutions. Agar plates were inoculated with a standardized O.D 1 inoculum of USA300. Then, filter paper discs (6mm dia.), containing 10 µl CBD oil solution at a concentration of 20 mg/ml were placed on the agar surface. The petri dishes were incubated overnight in 37 ºC. The antimicrobial CBD diffused

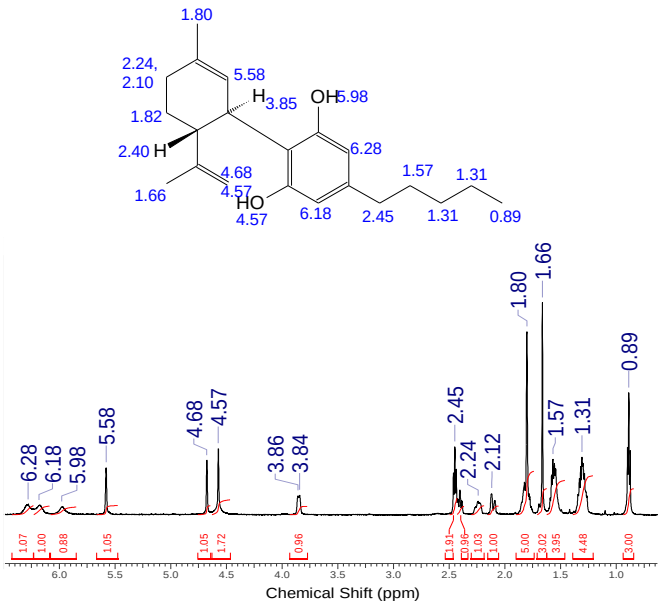

Fig. 1 ^{1}H NMR spectrum of CBD in $CDCl_3$ collected by Inova 600

into the agar media and inhibited growth of the microorganism which was measured by the diameter of inhibition growth zone (Fig. 3). Table 1 shows the measured inhibition zones on the agar plates that were measured in mm. The solvents, DMSO, canola oil and ethanol were used for inhibition zone studies as well.

III. Results & Discussion

A. Analysis of CBD Extracts

The industrial samples of the oil were through further purified CBD from other derivatives. The selection of the purest CBD fractions were guided by HPLC analysis of all fractions. A characteristic curve of CBD was observed at different calibration levels. Peaks were observed against concentrations. Peaks from the analytical standards available

Table 1 Antimicrobial Effect of Antimicrobial Effect of CBD

Compound	Zone of inhibition in 10 ul at 20 mg/mL concentration of CBD against USA300
CBD (powder)	11 mm
CBD (oil)	9 mm
Ethanol (control)	None
DMSO (control)	None
Canola (control)	None

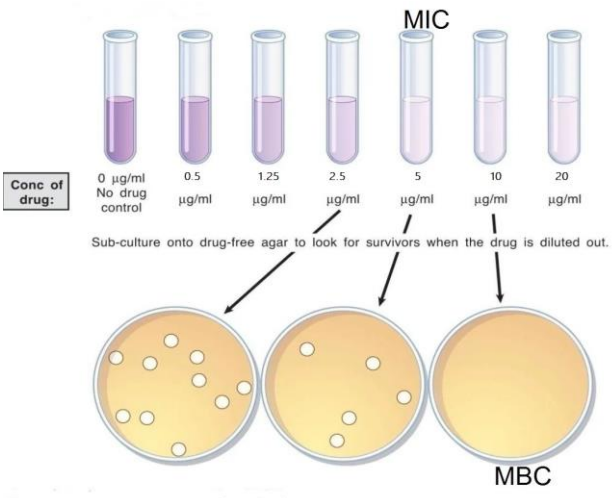

Figure 2. MIC studies of CBD (powder).

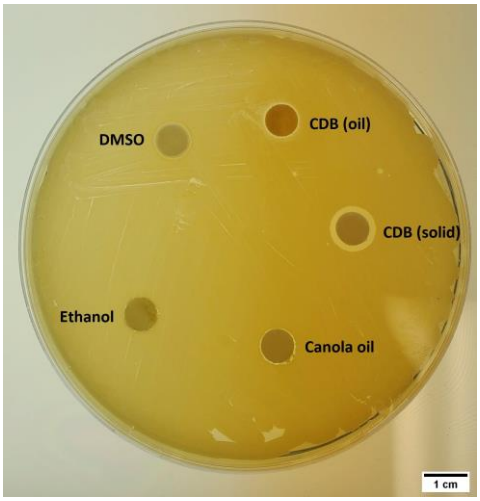

Fig. 3 Zone of Inhibition for CBD(oil & powder), Canola, DMSO & ethanol against MRSA USA300.

in literature [23] align with the purified compounds. Integration of the entire chromatograms of the purities gave clean fractions of >99 % CBD.

The NMR spectrum of CBD exhibited peaks due to $[M]^+$ and $[M\text{-}C_6H_{11}]^+$ at m/z values (base peak) in Fig.1, respectively. These peaks are compatible with the cannabinoid structure (Fig. 1). In the ^{1}H- NMR spectrum of CBD measured in deuterochloroform, broad peaks were observed. These signals were not affected by the addition of deuterium oxide, which reveals that these are aromatic hydrogens rather than hydroxyl groups. The presence of no other compounds was observed in the NMR peaks as all peaks corresponded to the CBD molecule.

B. Antimicrobial Activity

The antimicrobial activities of CBD and its solvents were evaluated against MRSA using the disk diffusion assay method (Table 1). The results show that the EtOH, DMSO, and canola solvents had no significant effect on antimicrobial activity of CBD extracts. The strongest inhibitory activity or the mean diameter of inhibition zone [DIZ] = 11 mm, was observed by the CBD powder (dissolved in DMSO) in comparison to CBD oil which had a DIZ = 9 mm. The DIZ studies demonstrated crystalized CBD powder to have two-fold higher activity against Gram-positive MRSA compared to dry CBD oil due to fact that it was further purified 99 % during the industrial extraction process.

Overall, the CBD powder exhibited good antimicrobial activity against MRSA expressed as the minimum inhibitory concentration. When tested against USA300 the MIC was found to be 2.5 µg/mL. This was a bit higher than the value in [13] for European lab extracted CBD. The observed MBC of CBD was determined by spot plating the bacterial suspensions from the MIC culture tubes on fresh agar plates. The lowest concentration resulting in no viable bacterial colonies was reported as MBC was 20 µg/mL. The number of CFUs was also reduced in the 10µg/mL sample, although not entirely eliminated Fig.4.

C. Statistical Analysis

We used a one-way ANOVA test by PRSM software, to compare the control column with no CBD and the different concentrations of CBD. There was a significant difference between the values with CBD and no CBD ($P < 0.0001$ as shown with ****) as shown in Figure 4. This showed that we can accept the alternative hypothesis (H_A), which means is CBD and no CBD groups means' are statistically significantly different from each other, and CBD concentration has an effect on the log CFU counts of the cell.

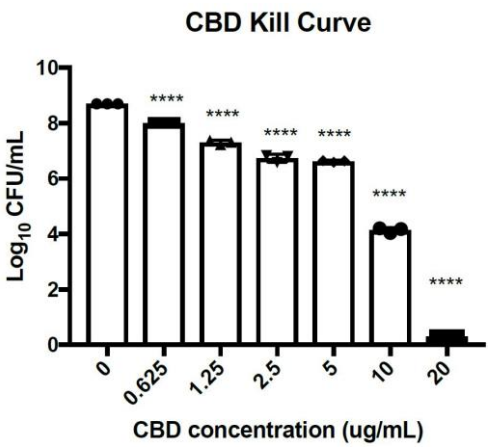

Fig. 4 Antibiotic (CBD powder) kill curve against MRSA. ANOVA comparison using one-way comparison between counts with no CBD(0) and other concentrations were (4 stars ****) referring to significant difference ($P < 0.0001$).

IV. Conclusions

We described a strategy for the quantification of cannabidiol (CBD) from the herb Cannabis sativa L. Industrial CBD oil and powder were obtained as yellowish oil and white powder. Quality characterization was done by chromatography and spectroscopy to verify the purity of particles in

our lab. The goal of this research was to find the antibacterial activity of CBD extracts to the MRSA USA300 strain. Cannabinoids have been found to have intense antimicrobial activity against gram-positive MRSA, as it has an MBC of 20 µg/mL demonstrating pure CBD well capable of resulting microbial death is proved for the first time, in our study. The benefit of using plant-based chemicals. such as CBD, as an alternative to antibiotics is that it is plant-derived and has fewer side effects in comparison to commercial antibiotics. Commercial antibiotic, *vancomycin* had MIC of 2 µg/ml, in USA against MRSA which is also similar to the MIC of CBD found in our studies [27]. This shows that they have the potential to be used as *Biomaterials* in eradicating MRSA biofilms in medical use. Along with combination therapy with bactericidal agents it is evident that other applications could be done using C. Sativa extracts as they have promising antimicrobial activity, demanding further research. As bacteria are rapidly developing resistance against existing drug materials, cannabinoids present a novel and exciting opportunity as a potential new source of bactericidal biomaterials.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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