Linking chemical phenotypes of different varieties of *Cannabis sativa* to their antimicrobial activities against a range of microorganisms

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Abstract

Ethanolic, methanolic and water extracts of different chemical phenotypes of *Cannabis sativa* belonging to the Cannabaceae family were tested for their antimicrobial activity against two Gram negative species, seven Gram positive species and two fungal species using the Kirby-Bauer disc diffusion method. Successful extracts were tested for their minimum inhibitory concentration (MIC). All extracts tested proved ineffective against both Gram negative species and both fungal species. Gram positive organisms were inhibited by all extracts, to varying degrees, and produced MICs of under 10 µl of extract per ml of inoculated broth. The average zones of inhibition (ZOI) produced for the successful species, when all extract inhibitions were combined, varied from 12.52 mm for *Enterococcus faecium* to 21.08 mm for *Streptococcus pyogenes*. The CBG (cannabigerol) type *Cannabis* extract proved the largest ZOI producer with results averaging 21.62 mm when all species' inhibition was combined, and CBCV (cannabichromavarin) proved to produce the smallest ZOIs against all species with only an average of 7.29 mm.

Synthetic cannabinoids that act on the same CB_1 and CB_2 receptors in animals were also tested for their antimicrobial activity. Ten in total were tested with one, 5F – PB -22, producing very slight inhibition against all Gram positive species except MRSA, and two more (5f – NPB – 22 and STS – 135) producing very slight inhibition against MRSA but no other species.

Bactericidal testing was performed on all successful extracts and the results indicated that nearly all cannabinoids, against all species, are bacteriostatic. However, these results are thought to not be accurate as the method used could result in potential false positive results that show bacteriostatic activity.

The results obtained in this study were corroborated by some of the studies undertaken by other researchers, but also contradicted by others. In total the amount of studies for comparison is very small and the results they produce are contradictory. The data indicates that *Cannabis sativa* has antimicrobial activities but the differing results from the studies performed so far show that experimental variation between the studies may influence the species upon which the cannabinoids are successful.

Acknowledgments

I would like to thank my supervisor, Dr. Mikhajlo Zubko, for his support, guidance and knowledge in the field of microbiology when I needed assistance, and also for training me to use equipment that I had not used before. Next, I would like to thank Dr. Oliver Sutcliffe for sourcing synthetic cannabinoids for the project and also for information and help regarding their nature and preparation. I would also like to thank the Microbiology Technicians at MMU who were always very helpful with sourcing materials and equipment and were always willing to offer any help that was requested.

Special thanks must go to Etienne De Meijer of G. W. Pharmaceuticals for allowing me to use his selectively bred *Cannabis sativa* strains for the research and for providing the percentage of cannabinoid in each plant sample.

Finally, I would like to thank my family and friends for encouraging me throughout my Masters degree.

1 Introduction

1.1 The History of *Cannabis sativa*

Cannabis sativa is a plant in the Cannabaceae family that has been utilised for textiles and medicine by humans for thousands of years (Andre et al, 2016), starting in Asia prior to 2400 BC (Jiang *et al,* 2006). Some of the earliest traces of humanity's interaction with the *Cannabis sativa* were found in Japan where an archaeological site in the Oki Islands had *Cannabis* achenes from 8000 BC (Long *et al*, 2016) probably signifying use of the plant. There is evidence of *Cannabis* being present in Europe as early as the 12th century where *Cannabis sativa* was introduced by Muslims in paper manufacture techniques (Aldrich, 1997).

Cannabis was used by humans as a source of textiles, food and was also used for its psychoactive properties. Throughout the 1800s the use and cultivation of *Cannabis* was widespread across the globe but toward the latter end of the 19th century, many countries/states had criminalised its use, especially for slave workers in the likes of British Singapore and British Mauritius (Mauritian Government, 1867), Greece and several countries in the Islamic World. *Cannabis* began to re-attract attention medicinally in the 1970s and 1980s in the USA, mainly due to its use by cancer and AIDS patients undergoing chemotherapy and wasting syndrome (Joy *et al*, 1999) and in 1996 California legalised medicinal *Cannabis*. In the last decade the world has begun to embrace *Cannabis,* with many countries and states now allowing *Cannabis* for medicinal and/or recreational use. This has greatly increased the availability of the plant for research into a potentially huge variety of medicinal applications.

Cannabis can be separated into two varieties *Cannabis sativa* cultivated for its cannabinoids, mainly delta-9-tetrahydrocannabinol (THC), and *Cannabis sativa* grown as hemp with less than 0.2% THC but rich in fibre, oils and molecules (Andre *et al*, 2016). Hemp is widely grown around the world in countries where the psychoactive varieties are illegal. In the UK large amounts of hemp are processed for their cannabinoids and shipped across the globe, medicinal global sales also occur from the UK with a UN International Control Board finding 44.9% of the world's *Cannabis* for medicinal and scientific use was produced in the UK.

Now there is a rapidly growing industry emerging around the *Cannabis sativa* plant, with a projected global revenue of $31.4 billion by 2021 (Zhang, 2017) and 32.7% of Canadians

aged between 15 and 64 having tried *Cannabis* in the year 2015/2016 according to the World Drug Report 2016 produced by the United Nations Office on Drugs and Crime. Some economies in some states have earned large sums of tax revenue, with Colorado alone generating $927,000,000 in tax revenue since 2014 from Marijuana taxes, licenses and fee revenues according to the Marijuana Tax Data (Colorado Department of Revenue, 2019).

1.2 Bioactive Compounds in *Cannabis sativa*

Antibiotic resistance is becoming a widespread problem throughout the world and new antibiotics are desperately needed. Plants have been looked at for sources of natural antimicrobials for millennia. Fernandez *et al* (1996) found that the thionins found in barley and wheat were toxic to types of fungi, Gram positive and negative bacteria. Mendoza *et al* (1997) found terpenes isolated from the *Pseudognaphalium* genus showed antimicrobial properties. Terpenes, which are found abundantly in *Cannabis sativa* and other essential oil producing plants, have also been shown to have strong inhibition against *Campylobacter* spp. (Kurekci *et al*, 2013) and *E. coli* and *S. aureus* (Zengin and Baysal, 2014). Flavones and flavonols have been known to share properties with terpenes and cannabinoids and have been shown to present anti-inflammatory, anti-cancer and neuroprotective properties (Andre *et al*, 2010)

The *Cannabis sativa* plant contains many of these compounds, with a wide variety of phenols, terpenes, flavonoids, flavonols and cannabinoids. Phenols have been shown to have a useful antimicrobial ability against *Xylella fastidiosa* (Maddox *et al*, 2010) and *Lactobacillus plantarum, Staphylococcus aureus* and *Candida albicans*, as well as other species (Cueva *et al*, 2010).

Cannabis contains many aromatic compounds that contribute to its distinctive, yet variable smell. Some of these molecules are found to be antibiotic against some microorganisms and have a wide scope of health benefits. Limonene, or D-limonene, is a potent antioxidant and anti-inflammatory agent, it has been shown to reduce stress in mice subject to environmental stress (d'Alessio *et al*, 2014), it may also boost the immune system and studies have shown it has anti-cancer properties (Bodake *et al*, 2002). Myrcene, another aromatic compound found in *Cannabis* also shows anti-inflammatory properties, is thought to help prevent cancer and in a study performed on mice, myrcene increased sleep duration by around 2.6 times (do Vale *et al*, 2002).

These compounds can be increased or decreased in their concentrations through selective breeding of the traits that are desired (De Meijer, 1994). This is beneficial to the medicinal industry as potentially adverse chemicals, for example: delta-9-tetrahydrocannabinol (THC) which is psychoactive, can be bred out of future plants, while the beneficial compounds for a specific purpose can be enhanced. This is particularly important for childhood illnesses that cannabinoids may help with; a strain of *Cannabis sativa* was created specifically for a child with severe seizures, suffering hundreds a day. The *Cannabis* strain (named "Charlotte's web" after the little girl who it was created to help), was selectively bred to have large cannabidiol (CBD) concentrations and very low levels of THC and it reduced the seizures in the child to only 2 to 3 times a month, with no adverse THC related side-effects reported (Young, 2013). This is even more impressive because conventional medicines were totally ineffective and the parents of the child had been told that the child would not live for much longer, the CBD saved the child's life.

1.3 Cannabinoids and their synthesis

Cannabinoids are a class of secondary products in the *Cannabis sativa* plant, they are terpenophenolic substances produced in the glandular trichomes of the plant (Hammond and Mahlberg, 1997).

The biosynthetic pathways of phytocannabinoids have only recently been fully uncovered. Cannabigerolic acid (CBGA), the central precursor for several cannabinoids is synthesised by the alkylation of olivetolic acid (OLA), an alkylresorcinolic acid that forms the polyketide nucleus of the cannabinoids (Gagne *et al*, 2012) with Geranyl Diphosphate (GPP) via the enzyme geranylpyrophosphate:olivetolate geranyltransferase (GOP) (Fellermeier and Zenk, 1998). Three oxidocyclases are then responsible for differentiation into other cannabinoids: THC synthase, CBD (cannabidiol) synthase and CBC (cannabichromene) synthase all convert CBGA to THCA, CBDA & CBCA respectively (Taura *et al*, 2007; Sirikantaramas *et al*, 2004). THCA is the acid form of THC, a carboxylic acid group breaks off from the acid form when it is heated and becomes THC. This means that all *Cannabis* extracts used in the study will have contained a large proportion of the acid forms as they were only heated to 37 ˚C during the study, and 70 ˚C for one set of water extracts.

Through analysis over the decades, many sub types and variations of cannabinoids have been found. Figure 1 shows the 4 main cannabinoid types, the R groups can be one of several variations that determine the exact sub-type of cannabinoid.

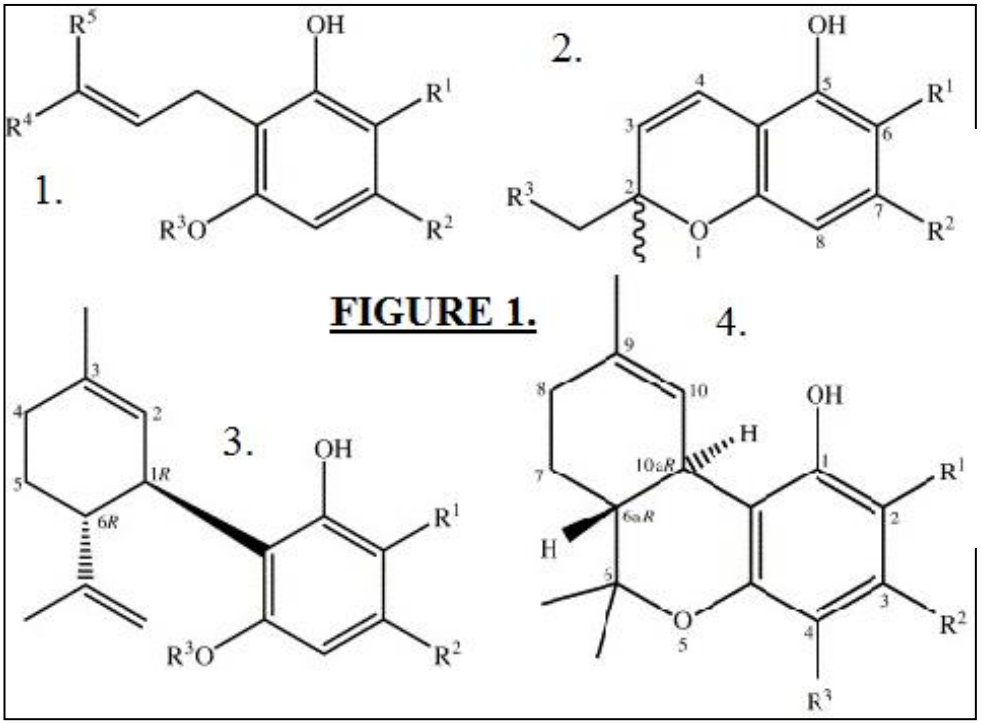


Figure 1. The 4 main types of cannabinoids. 1 – CBG type, 2 – CBC type, 3 – CBD type & 4 – THC type. Derived from ElSohly and Slade (2005).

The molecular layout and formulae of the cannabinoids and other compounds within *Cannabis sativa* have been known for a long time. T. Wood (1898) was the first to isolate a compound from *Cannabis sativa*, isolating Cannabinol. The next cannabinoid to be isolated was Cannabidiol (CBD) by Mechoulam and Shvo (1963) and then delta-9-tetrahydrocannabinol was isolated by Gaoni and Mechoulam (1964).

Our knowledge of the biosynthesis of main type cannabinoids has become so detailed that experiments have been performed that resulted in the production of a wide variety of cannabinoids by heterologous host organisms (Carvalho *et al,* 2017). Another by Luo *et al* (2019) showed that *Saccharomyces cerevisiae* could be engineered to completely synthesise several major cannabinoids, including THC, CBG and CBD. With more understanding of the synthetic pathways of rare cannabinoids, they too could be grown in microorganism species much more easily and then researched to discover any possible health benefits they may possess. These applications could also be applied to the plants themselves with advances in CRISPR (clustered regularly interspaced short palindromic repeats) technology. Research should be performed trying to manipulate normal *Cannabis* tissue (not just the trichomes) into synthesising a large volume of cannabinoids, massively increasing the yields of the plants.

A study by Ross and ElSohly (1996) reported 483 individual natural compounds inside C. *sativa* L. Knowledge of the structures of antimicrobials is important in the field of antimicrobials, because if a compound is inhibitory of microorganisms then it will be due to its particular molecular structure and its subsequent effects. Knowing exactly which structures are effective against microorganisms will make synthesising antimicrobial molecules much easier in the future.

Synthetic cannabinoids are manmade variants of cannabinoids which bind to CB_1 and CB_2 receptors in humans and animals. However, toxicity and hospital admissions are much higher for synthetic cannabinoids than their natural counterparts, this is thought to be due to synthetic cannabinoids being direct agonists of the cannabinoid receptors, whereas THC is only a partial agonist (Mills *et al*, 2015).

1.4 Medicinal uses of *Cannabis sativa* to date

The medicinal activities of *Cannabis sativa* are broad, particularly in regard to the cannabinoids they produce. They have been shown to alleviate nausea symptoms and increase appetite in chemotherapy patients. Machado Rocha *et al* (2008) performed a meta-analysis study that found Dronabinol (an oily resin containing THC) had better acute anti-emetic efficacy than conventional anti-emetic drugs. It has also been used for decades by many HIV/AIDS patients to alleviate nausea symptoms and help improve quality of life, however as of 2013, current studies suffer from effects of bias, small sample size, and lack of long-term data (Lutge *et al*, *2013)*. De Petrocellis *et al* (2011) found THC to be a potent anti-inflammatory, anti-cancer, analgesic, muscle relaxant and neuro-antioxidative.

Cannabidiol has been shown to have beneficial effects comparable to clozapine, an atypical antipsychotic drug in a study performed by Gomez *et al* (2014)

As early as 1888 it was reported that ‘indian hemp’ resulted in benefits on a Parkinsonian syndrome (Gowers, 1888; Russo, 2007). Further research has shown 22/28 Parkinson’s disease patients tolerated smoking *Cannabis* and benefitted from acute improvements in bradykinesia and tremors (Lotan *et al*, 2014). CBD has been shown to be a very promising molecule in neurodegenerative diseases, schizophrenia, multiple sclerosis and epilepsy (Hill *et al,* 2012)

Chong *et al* (2006) found that 68% of multiple sclerosis patients questioned had used *Cannabis* to alleviate symptoms of multiple sclerosis and Chen *et al* (2019) found that

Epidolex (Cannabidiol (CBD)) significantly reduces seizures in combination with standard antiepileptic therapies in infants less than 2 years old with Dravet and Lennox Gastaut syndromes. *Cannabis* is also thought to possess potential anti-cancer properties. Pokrywka *et al* (2016) showed that, in animal models and cell lines *in vitro*, that phytocannabinoids, endocannabinoids, synthetic cannabinoids and their analogues can lead to inhibition of the growth of many tumour types. Another study performed by Leanza (2016) showed that intraperitoneal applications of 5mg/kg of bodyweight CBD in nude mice every 3 days for 28 weeks resulted in almost completely reduced development of metastatic nodules caused by the injection of human lung carcinoma A549 cells. It can also be a powerful antimicrobial (Wasim *et al,* 1995).

Studies have been performed that show that CBC presents antibacterial and antifungal ability (Eisohly *et al*, 1982) and sedative and analgesic properties (Davis and Hatoum, 1983).

Tetrahydrocannabivarin (THCV) has been shown to restore insulin signalling in insulin-resistant hepatocytes and myotubes, as well as improving glucose tolerance and increased insulin sensitivity in genetically obese mice (Wargent *et al*, 2013).

1.5 Detrimental health effects of *Cannabis sativa*

While *Cannabis sativa* has myriad health benefits, there are studies highlighting potential dangers of *Cannabis* use. It possesses antimicrobial activity but a review by Elsohly (2007) showed that smoking of *Cannabis* can impair lung immune functionality, thus increasing susceptibility to bacterial lung infections by inhibiting alveolar macrophages' nitric oxide production, causing immunosuppression (Roth *et al*, 2004).

In the 1970s it was found that a combination of lipopolysaccharide (obtained from *E. coli* and 3 species of the genus *Salmonella, S. Minnesota, S. typhi* and *S. abortus*), THC and a commercial *Pseudomonas* vaccine proved to be highly toxic in mice (Bradley *et al*, 1977) and that this enhanced toxicity could arise in humans as humans have pathogenic, food-borne, and enteric bacteria that can produce lipopolysaccharides.

It has long been argued that *Cannabis* use, like cigarettes, can cause lung cancer and other pulmonary diseases such as emphysema. A survey at a large health maintenance organisation found that marijuana users were more likely to seek help for a variety of respiratory illnesses (Polen *et al*,1993). However, it also found that users who had consumed *Cannabis* for more than 10 years visited no more frequently than those who

had smoked *Cannabis* for less than 10 years. Jett *et al* (2018) reviewed *Cannabis* use and the data behind its relationship with lung cancer, their studies found that smoking cannabis has so far not been definitively proved to be a risk factor in the development of lung cancer. The data, however, are limited by small studies, misclassification due to self-reporting of use, small numbers of heavy cannabis smokers, and confounding of the risk associated with other factors like using *Cannabis* alongside chronic tobacco use.

Cannabis has an age restriction in countries where it is recreationally legal because research has shown its detrimental effects on those who smoke at an early age. Studies have shown that those who smoked *Cannabis* during adolescence had fewer neural fibres in specific brain regions, including the precuneus, involved in functions requiring a high degree of integration such as self-conscious awareness and alertness, and the fimbria, located in the hippocampus that is used in learning and memory (Zalesky *et al,* 2012). A reduction in functional connectivity, responsible for executive function (including inhibitory control) and subcortical networks which are thought to process routines and habits, was also reported in a study by Filbey and Yezhuvath (2013).

There is also a link between *Cannabis sativa* use and an increased risk of mental illness, including anxiety and depression, but causality is hard to establish because factors other than *Cannabis* may be directly associated with the risk of mental illness. However, studies have shown that *Cannabis* use correlates with an increased risk of mental illness (Patton, 2002) especially amongst people with pre-existing genetic vulnerability to mental illness (Caspi *et al*, 2005).

1.6 Antimicrobial properties of *Cannabis sativa*

Cannabinoids have shown antimicrobial activity against a variety of different species, notably it has shown high activity against a variety of clinically relevant strains of MRSA (Appendino et al, 2008). Two particularly effective antimicrobial cannabinoids are Cannabichromene (CBC) and Cannabigerol (CBG) with CBG being a more potent antibacterial and CBC being a better antifungal agent.

Ali *et al* (2011) found that the petroleum ether of the seeds, whole plant methanol extract and the oil of *Cannabis sativa* seeds, all produced inhibition against a small range of microorganisms.

As of yet, there has been little study into the potential antimicrobial properties of synthetic cannabinoids. Also, very little is known about the impact of cannabinoids on virulence properties of bacteria.

1.7 Aims for the Experiment

1. Preparation of ethanol, methylated spirit and water extracts from dry tissues of different chemotypes of *Cannabis sativa* provided by Dr. E. De Meijer (GW Pharmaceuticals, Kent). The extractions will be done at 37 °C.

2. Testing antimicrobial activities of the extracts against a range of microorganisms including: *Bacillus cereus, Enterococcus faecium, Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus* (including MRSA), *Staphylococcus epidermidis, Streptococcus pneumoniae, Streptococcus pyogenes, Candida albicans & Candida glabrata.*

This will be achieved by:

a) measuring zones of inhibition (ZOI) of microbial growth in Kirby-Bauer disc diffusion assays, and

b) determining minimum inhibitory concentrations (MIC) using serial dilutions of the extracts at constant concentrations of the microorganisms grown in liquid media in 96-well plates.

3. Looking at possible interactions between different cannabinoids by testing levels of their antimicrobial activities (by measuring ZOI) after mixing different extracts. If features of synergy, antagonism or addictiveness will take place, those effects will be verified by measuring MIC of the mixes.

4. Checking whether any discovered antimicrobial effects are bacterio/fungicidal or bacterio/fungistatic (based on viability assays after growth inhibition).

5. Any effects discovered for objectives 1-3 will be verified by using pure synthetic cannabinoids for CBC, CBD, CBG & THC, CBCV (cannabichromavarin), CBDV (cannabidavarin), CBGV (cannabigerovarin) & THCV. Dr. O. Sutcliffe agreed to supervise this part of the work. The synthetic cannabinoids tested were: 5F-NPB-22, 5F-PB-22, AB-FUBINACA, AB-PINACA, BB-22, EG-018, MDMB-CHMICA, MDMB-CHMCZCA, STS-135 & THJ-018.

6. Investigating effects of the extracts on the expression of *pykA* and *argF* genes (which encode the most important virulent proteins in MRSA).

The research planned in this study will focus on non-antibiotic resistant strains (except MRSA) as the laboratory cannot accommodate many of those organisms, but they can still be pathogenic, especially in immunocompromised individuals. Cannabinoid extracts will be used to produce inhibition, with extracts being individual or combined in pairs to compare inhibition. Synthetic cannabinoids will also be tested to determine if they produce any antimicrobial results. If the cannabinoids prove successful, it would be interesting to apply them to antibiotic-resistant strains in the future. Attempts will also be made to initiate studying the effect of cannabinoids on the expression of genes (in particular *argF & pykA)* encoding important virulence proteins in MRSA.

2 Methodology

All tests were performed in triplicate using the same method, equipment and materials.

2.1 Agar and Broth preparations

Nutrient Agar / Broth (OXOID – CM0003 / CM0001) was used for all species except for: E. *faecium*, S. *pneumoniae* and S. *pyogenes* which were grown on Brain Heart Infusion Agar / Broth (OXOID – CM1136 / CM1135), and C. *albicans* and C. *glabrata* which were grown on Sabouraud Dextrose Agar (OXOID – CM0041). 500 ml of purified water was added to the required amount of agar or broth, as per the container's instructions, and autoclaved at 121 °C for 20 minutes. Agar plates were poured in a sterile flow cabinet using approximately 25 ml of agar per plate and allowed to set before storage and/or use.

2.2 Preparations of agar and broth cultures for testing

For agar plates approximately 2-3 ml of a saline solution, made up of a saline tablet dissolved in 500 ml of purified water, was mixed with a species and scanned in a spectrophotometer (Jenway 6305 spectrophotometer) at 600 nm, that had been calibrated with a saline solution blank, until an Optical Density (OD_{600}) of around 0.3 was achieved, this indicates approximately 10^6 cfu (colony forming units) per ml. A sterile swab was then submerged in the saline–bacteria solution and streaked evenly across the agar three times, rotating after each streak to ensure even coverage.

For the 96 well plate testing broth was used in place of saline for spectrophotometry. When an OD_{600} of around 0.3 was achieved the culture was added to the 96 well plate.

2.3 Extract preparations

150 mg of *Cannabis sativa* raw material was added to 1350 mg of solvent. Two water extracts and a methylated spirit extract were made using the most effective cannabinoid, CBG. Ethanol extracts used for the majority of the experiment were made for all cannabinoids. The extracts were vortexed and then incubated for 24 hours at 37 °C, one water extract was heated for 2 hours at 70 °C before being incubated at 37 °C for 22 hours. After incubation all of the extracts were centrifuged at 1.7x1000g for 12 minutes, twice. The supernatant was then pipetted out and stored in 2 ml Eppendorf tubes; ethanol and methylated spirit extracts were stored in a freezer; water extracts were stored in a refrigerator.

Combined extracts were performed by pipetting an equal volume of each *Cannabis* extract into an Eppendorf tube and vortexed for several seconds before use.

One set of extracts were made using a 1:1 ratio of ethanol and *Cannabis*, by weight, for testing against Gram negative and fungi species. All solvents were left to evaporate from the extracts before being utilised in the study.

2.4 Testing for antimicrobial activity (Kirby-Bauer disc diffusion method)

Approximately 2 ml of saline solution was inoculated with a microorganism until an OD_{600} value of around 0.3 was obtained. Then a sterile swab, having been submerged fully in the saline solution, was streaked across an agar plate from top to bottom. The plate was rotated and re-streaked twice more to ensure full plate coverage.

Blank antibiotic discs were loaded with 15 microlitres of extract and left until the solvent had evaporated, either in a fume cupboard or near a Bunsen burner, then they were placed onto the agar and incubated at 37 °C for 24 hours. Both Streptococcus species were incubated at 37 °C with 5% CO_2 and with slight humidity.

2.5 Testing for antimicrobial activity (MIC 96 well plate assays)

96 well plates were used, with lane 1 containing 90 µl of double concentration broth, 45 µl of extract and 45 µl of water. Lanes 2-10 had 150 µl of broth inoculated with a species (with an OD_{600} of 0.3, diluted 500x). Lanes 11 and 12 were filled only with 150 µl of broth. 150 µl from lane 1 was transferred to lane 2 with a multi-channel pipette, resuspended 3 times and then 150 µl transferred to the next lane, etc. Lane 10 did not have any extract added to it. Instead lane 9 had 150 µl removed, and disposed of, to create the same volume as all other wells. This resulted in their being half as much extract in each consecutive lane. Lane 2 contained 22.5 µl of extract in a total volume of 150 µl, lane 3 contained 11.25 µl, lane 4 contained 5.625 µl, etc.

Each row contained a separate extract corresponding with its numerical equivalent, with Row A containing extract 1, Row B containing extract 2 (CBC), etc.

96 well plates were scanned at 0 hours, T=0, using 500nm and 600nm wavelengths and then incubated at 37 °C, with both Streptococcus species being incubated at 37 °C with 5% CO_2 with slight humidity, the 96 well plates were then scanned at T=24 and T=48.

2.6 Testing for combined extract properties

Each main cannabinoid type's extract was combined with its '-Varin' counterpart in a 1:1 ratio by volume: CBC & CBCV, CBD & CBDV, CBG & CBGV and THC & THCV). Also, a set of extracts were made using the following pairs: CBCV & THCV, CBC & THC, CBDV & CBGV and CBD & CBG, in the same 1:1 ratio. These were then applied to blank antibiotic discs, dried in a fume cupboard or in close proximity to a Bunsen burner and then applied to an agar plate evenly streaked with a microorganism to determine inhibition. Once applied the completed agar was incubated at 37 °C for 24 hours. Both Streptococcus species were incubated at 37 °C with 5% CO_2 and with slight humidity.

2.7 Bacteriostatic/bactericidal testing

After successful inhibition was produced by an extract, a sterile swab, or sterile toothpick (if the ZOI was very small), was applied to the ZOI and then transferred to a fresh agar plate. A Line was drawn across the plate with the swab/toothpick and the plate was then incubated overnight at 37 °C for 24 hours (both Streptococcus species were incubated at 37 °C with 5% CO_2 and with slight humidity) to determine if growth would occur or not.

2.8 Synthetic cannabinoid testing

Synthetic cannabinoids provided by Dr O. Sutcliffe were used, using the Kirby-Bauer disc diffusion method. Approximately 10 mg of synthetic cannabinoid was stored in a glass bijou and dissolved in 10 ml of dimethyl sulfoxide (DMSO), per 10 mg synthetic cannabinoid. 10 µl of which was then added to a blank antibiotic disc and placed on inoculated agar and incubated at 37 °C. Both Streptococcus species were incubated at 37 °C with 5% CO_2 with slight humidity.

2.9 Measuring antimicrobial activity

For the Kirby-Bauer disk diffusion method photographs were taken of all plates, after incubation, on a black tile alongside two perpendicular rulers. The photographs were then used to measure the ZOI, using a pair of dividers the diameter was measured and compared against the ruler. Extracts with very little antimicrobial activity produce values of 6.5+ mm due to the disc being 6 mm in diameter.

The 96 well plates were scanned in a 96 well plate spectrophotometer at 500 and 600 nm absorbencies at T=0, T=24 and T=48. Photographs were taken of the plates at each incubation stage, the MIC was determined by identifying the volume of extract needed to halt bacterial growth after 24 or 48 hours.

2.10 Aseptic technique:

All research was performed within close proximity to one or more Bunsen burners or in a flow cabinet to try to ensure a sterile environment and reduce contamination risks.

All glassware and relevant material such as antibiotic discs, pipette tips and solutions like water and saline were autoclaved before use to ensure sterility and reduce the chance of contamination.

2.11 Statistical analysis

Standard deviation was calculated to determine how consistent any results were, and T-tests were performed between similar results to determine if there is any significant difference between the values.

3 Results

Candida albicans, Candida glabrata, Escherichia coli, and *Psuedomonas aeruginosa* all were uninhibited by all extracts tested. Therefore, they were excluded from tables to save space.

Figure 2 shows each of the species listed above after incubation with all *Cannabis* extracts. No inhibition was produced in all plates.

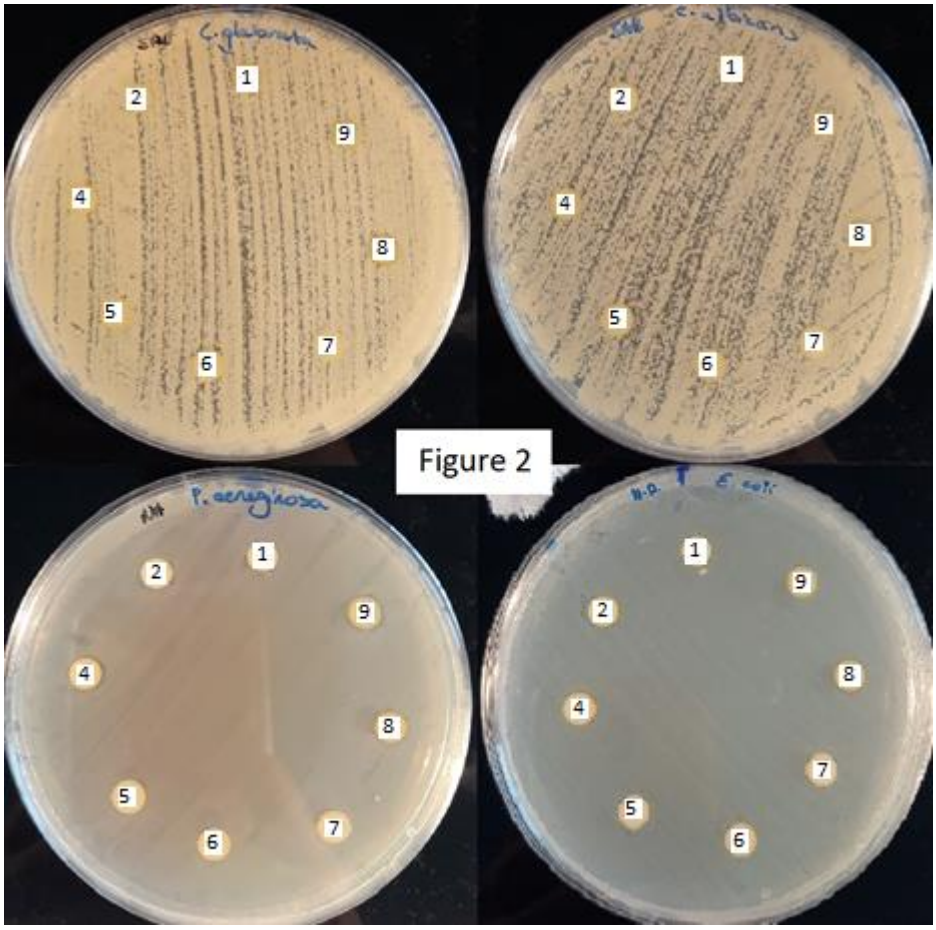


Figure 2. *C. glabrata* (top left), *C. albicans* (top right), *P. aeruginosa* (bottom left) and *E. coli* (bottom right) plates showing that no inhibition occurred from any extract used. Each disc contained 15 µl of an extract. The top disc contains extract 1. With extracts 2, 4, 5, 6, 7, 8 and 9 in an anticlockwise direction.

3.1 Solvent testing against MRSA using CBG

Table 1 shows the results of each solvent type against MRSA using the cannabigerol extract and shows that methylated spirit extracts were slightly better inhibitors of MRSA than ethanol extracts with a 2-3% ZOI diameter increase for volumes 30, 45 and 60 µl. The water extracts produced smaller inhibition, only achieving 2/3rds the inhibition of

methylated spirit at 60 µl. Water extracts that were incubated at 70 °C for 2 hours, and then 22 hours at 37 °C, produced slightly smaller ZOIs than water extracts incubated solely at 37 °C for 24 hours.

Table 1. Zones of Inhibition (mm) against MRSA using methylated spirit, ethanol and water as the solvent. All exctracts utilised CBG (9) cannabis at a 1:9 ratio of cannabis to solvent.

Species	Solvent	Volume (µl)	ZOI 1 (mm)	ZOI 2 (mm)	ZOI 3 (mm)	Average ZOI (mm)	SD
MRSA	Methylated spirit	15	28.00	26.00	27.00	27.00	1.00
MRSA	Methylated spirit	30	29.00	29.00	30.00	29.33	0.58
MRSA	Methylated spirit	45	30.00	29.00	29.00	29.33	0.58
MRSA	Methylated spirit	60	30.00	30.00	30.00	30.00	0.00
MRSA	Water (2 hours @ 70°C)	15	12.00	14.00	14.00	13.33	1.15
MRSA	Water (2 hours @ 70°C)	30	16.00	17.00	16.00	16.33	0.58
MRSA	Water (2 hours @ 70°C)	45	19.50	18.00	19.00	18.83	0.76
MRSA	Water (2 hours @ 70°C)	60	19.75	20.00	19.50	19.75	0.25
MRSA	Water	15	14.00	14.00	14.00	14.00	0.00
MRSA	Water	30	17.00	17.00	18.00	17.33	0.58
MRSA	Water	45	18.00	19.00	19.00	18.67	0.58
MRSA	Water	60	19.00	21.00	20.00	20.00	1.00
MRSA	Ethanol	15	27.00	27.00	27.00	27.00	0.00
MRSA	Ethanol	30	28.00	29.00	29.00	28.67	0.58
MRSA	Ethanol	45	28.00	29.00	29.00	28.67	0.58
MRSA	Ethanol	60	29.00	29.00	29.00	29.00	0.00

All extracts produced inhibition within 1 standard deviation of the mean except for the 15 µl water extracts incubated at 70 °C for 2 hours and then 22 hours at 37 °C which was slightly more varied.

Figure 3 shows the results of each solvent tested, with ethanol, methylated spirit and both of the water extracts. It clearly shows the distinct difference in ZOI produced, especially between the water extracts and the non-water extracts. This is represented in Graph 1, showing the smooth curves for all 4 solvents at each volume.

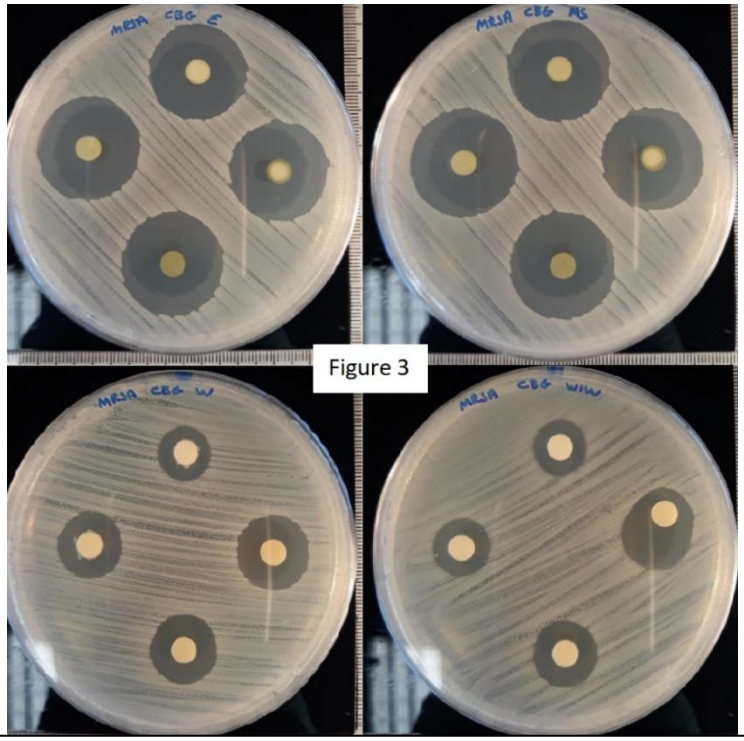
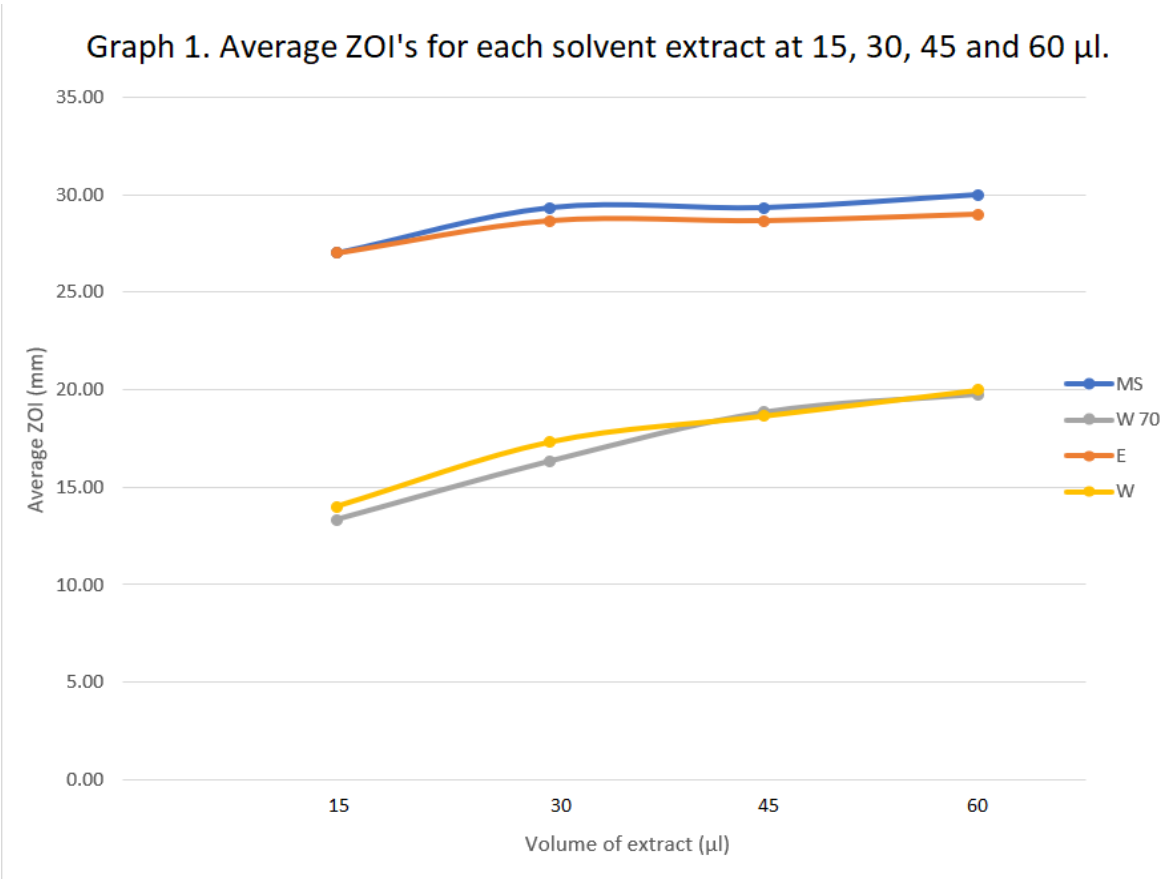


Figure 3. A comparison of MRSA plates with different solvent CBG extracts tested; ethanol (top left), methylated spirit (top right), water extract at 37 degrees (bottom left) and finally water extracts incubated for 2 hours at 70 degrees. The discs, from top anticlockwise, contain 15, 30, 45 and then 60 µl of extract.



Graph 1. A comparison of the average ZOIs for each solvent tested using CBG against MRSA. MS – Methylated spirit. W 70 – Water incubated for 2 hours at 70 °C, then 37 °C for 22 hours. E – Ethanol. W – Water incubated at 37 °C.

3.2 Ethanol extracts against all species

Table 2 contains the inhibition sizes of all extracts, at 15 µl, against all successfully inhibited species and Graph 2 shows the average ZOI for each extract against each species. Extracts 1 (CBCV) and 2 (CBC) proved to be the least effective cannabinoids with average ZOIs of only 6-8 mm for all species except *Streptococcus pyogenes* which had slightly better inhibition, 10.67 mm and 11.67 mm for CBCV and CBC respectively.

Figure 4 shows two plates containing *S. pyogenes* and *S. aureus* and shows the difference in inhibitions between the two. CBCV was loaded on the disc at the top, the other extracts were loaded in numerical order counter clockwise from the top.

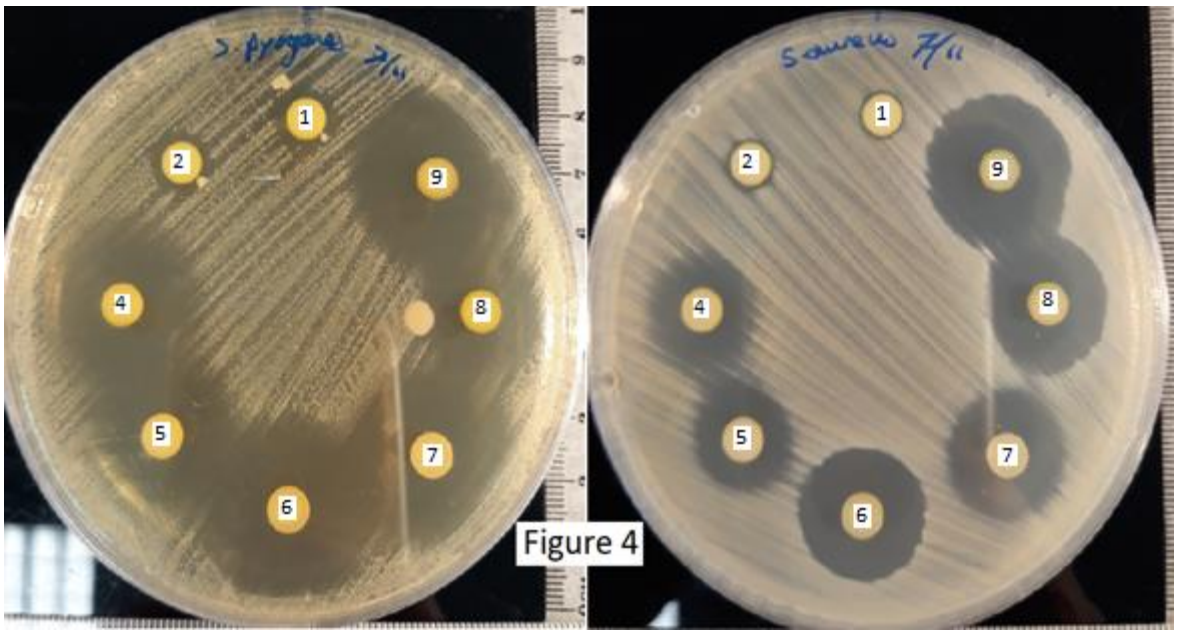


Figure 4. A comparison of an *S. pyogenes* plate (left) and an *S. aureus* plate (right). Discs contain 15 µl of extract, extract 1 is on the disc at the top. With extracts 2, 4, 5, 6, 7, 8 and 9 in an anticlockwise direction. Extract 9 produced the largest ZOI for both species.

The THC type *Cannabis* extracts provided moderate antimicrobial ability against all species with extract 4 (THCV) being worse against all species than its analogue extract 6 (THC). THCV proved least effective against *E. faecium* with only a mean inhibition of 11.67 mm, *S. pneumoniae* was the next most resistant with a mean inhibition of only 12 mm. Counterintuitively, *S. pyogenes* was the least resistant to the THCV extract as it produced an average ZOI of 23.33 mm. Figure 5 shows a comparison of an *S. pneumoniae* and *S. pyogenes* plate, highlighting the differences in ZOIs. Extracts 4 and 6 (left most and

bottom most discs), like most of the different extracts, have a noticeable difference in size.

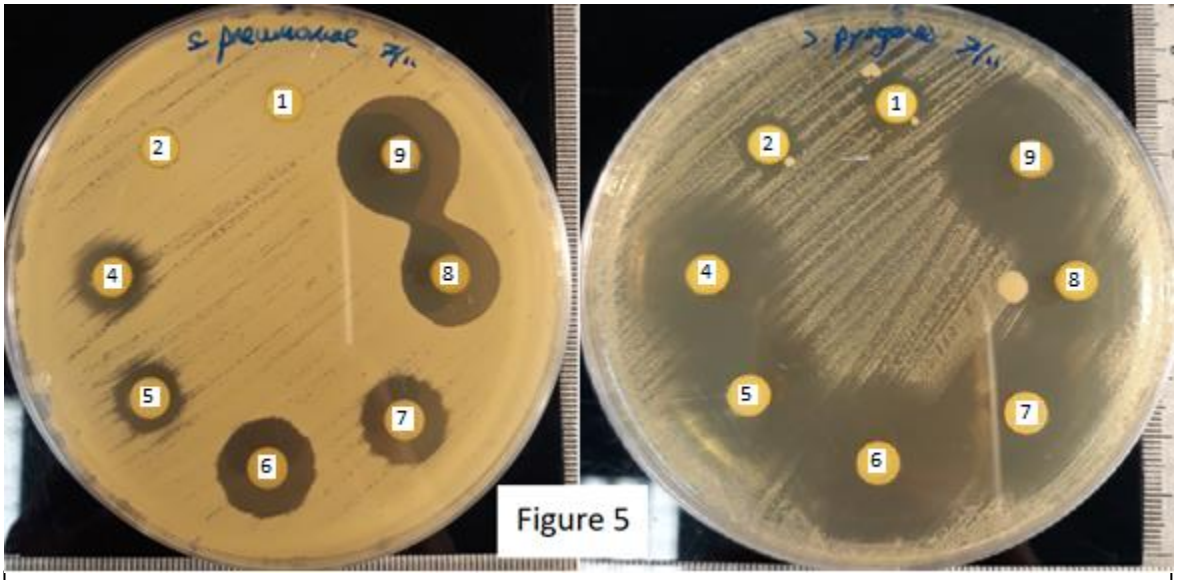


Figure 5. Two plates comparing the inhibition zone sizes of each 15 µl extract against *S. pneumoniae* (left) and *S. pyogenes* (right). The top most disc contains extract 1, with extracts 2, 4, 5, 6, 7, 8 and 9 in an anticlockwise direction.

THC extracts proved a far better inhibitor of *E. faecium*, producing a mean ZOI 146% larger than THCV. Figure 6 shows a plate of *E. faecium* displaying the difference between THCV, 3rd disc anticlockwise from the top, and THC, the 5th disc. Extract 6 was the second strongest inhibitor against *B. cereus, E. faecium, S. aureus, S. epidermidis, S. pneumoniae* and *S. pyogenes*.

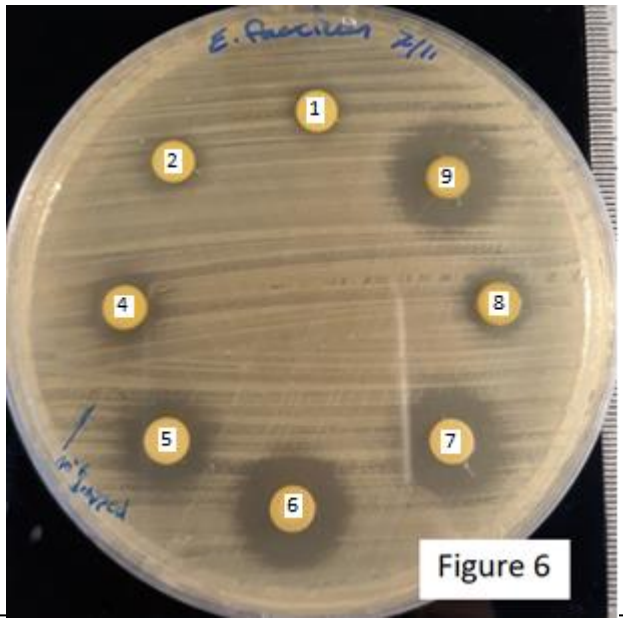


Figure 6. An *E. faecium* plate showing each 15 µl extract's inhibition. The discs follow the same layout as described in Figures 4 and 5.

The most potent extract was extract 9 (CBG) with the strongest inhibition against all species except *B. cereus* with only a 16 mm average ZOI. The inhibition from CBG ranged from 17.33 mm, against *E. faecium*, to an impressive 28.33 mm, against *S. pyogenes*. Extract 8 on the other hand produced, generally, far less inhibitory results than its analogue, CBGV. When compared to extract 9 the results varied from 60% of the mean ZOI, against *E. faecium*, to 96%, against MRSA. The average zone size of CBGV produced 83% the average ZOI of its analogue. The largest inhibitions produced by extract 8 were against MRSA, being only 1 mm less than the strongest extract and 0.33 mm less than extract 7 (CBD), however, extract 7 produced a more varied set of results with a standard deviation being 1.25 whereas CBGV produced very consistent zones of 24 mm. *E. faecium* and *B. cereus* were the least inhibited by extract 8, producing smaller inhibitions of 10.33 mm and 12.33 mm. Figure 7 shows the very small inhibition of CBGV against *B. cereus* and *E. faecium*, the right hand most disc. However, it still produced more inhibition than extracts 1 and 2, central top and top left, respectively.

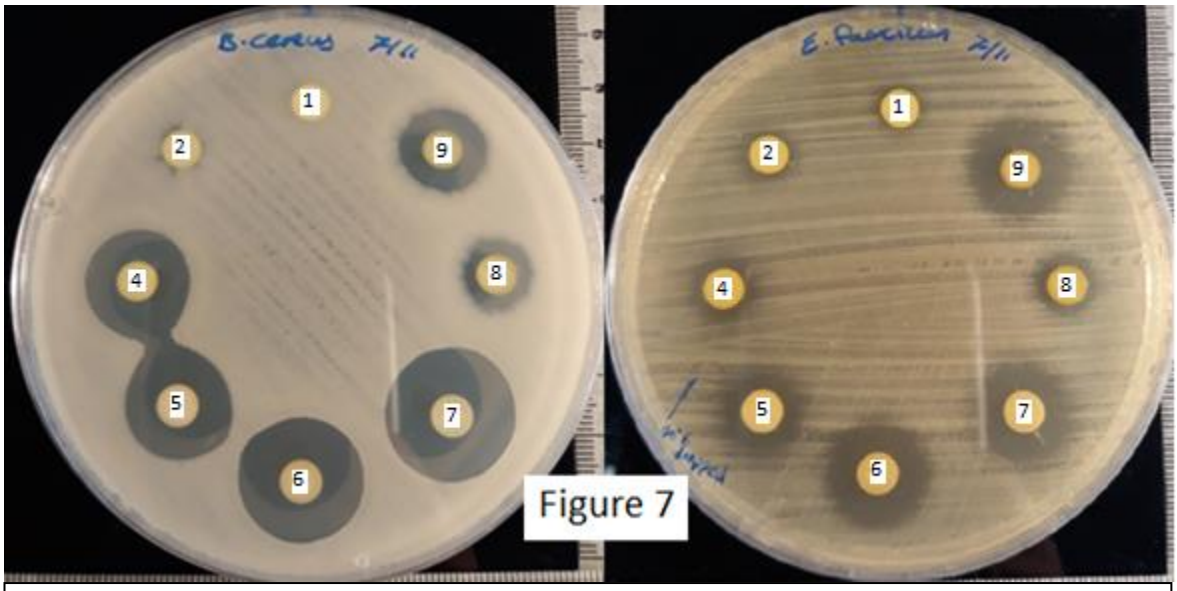


Figure 7. A comparison of *B. cereus* and *E. faecium* plates tested with 15 µl of each extract. The discs follow the same layout as described in Figures 4 and 5.

The CBD type *Cannabis* extracts CBDV and CBD, extracts 5 and 7 respectively, produced successful inhibition. Extract 5 resulted in smaller inhibition zones than extract 7 against all species, with inhibition zones on average 82% the size of extract 7. The largest inhibition produced by extract 5 was against *S. pyogenes* with a mean ZOI of 22 mm however a standard deviation of 2.16 is far higher than all other results and one value of 25 mm raises the average. The smallest inhibition zones were produced against *S. pneumoniae* with only 10.67 mm average ZOI. Extract 7 produced quite strong activity

against all species, with large inhibitions of 24.33 mm and 25 mm against MRSA and *S. pyogenes*. It produced the least inhibition against *S. pneumoniae* and *E. faecium* with only 14.67 mm and 16.33 mm on average.

Table 2. The ZOIs (mm) for all successfully inhibited species when tested with 15 µl of each extract.

Species	Cannabinoid Extract #	ZOI 1 (mm)	ZOI 2 (mm)	ZOI 3 (mm)	Average ZOI (mm)	SD
B. *cereus*	1	6.00	6.00	6.00	6.00	0.00
B. *cereus*	2	7.00	7.00	7.50	7.17	0.24
B. *cereus*	4	18.00	19.00	18.00	18.33	0.47
B. *cereus*	5	20.00	19.00	19.00	19.33	0.47
B. *cereus*	6	24.00	22.00	21.00	22.33	1.25
B. *cereus*	7	24.00	24.00	22.00	23.33	0.94
B. *cereus*	8	14.00	11.00	12.00	12.33	1.25
B. *cereus*	9	16.00	16.00	16.00	16.00	0.00
E. *faecium*	1	7.00	7.00	7.00	7.00	0.00
E. *faecium*	2	7.50	7.00	8.00	7.50	0.41
E. *faecium*	4	12.00	10.00	13.00	11.67	1.25
E. *faecium*	5	13.00	13.00	13.00	13.00	0.00
E. *faecium*	6	16.00	18.00	17.00	17.00	0.82
E. *faecium*	7	15.00	17.00	17.00	16.33	0.94
E. *faecium*	8	10.00	11.00	10.00	10.33	0.47
E. *faecium*	9	17.00	19.00	16.00	17.33	1.25
MRSA	1	7.00	7.00	7.00	7.00	0.00
MRSA	2	8.00	7.00	8.00	7.67	0.47
MRSA	4	18.00	19.00	20.00	19.00	0.82
MRSA	5	20.00	22.00	23.00	21.67	1.25
MRSA	6	21.00	23.00	23.00	22.33	0.94
MRSA	7	23.00	24.00	26.00	24.33	1.25
MRSA	8	24.00	24.00	24.00	24.00	0.00
MRSA	9	25.00	25.00	25.00	25.00	0.00
S. *aureus*	1	6.00	6.00	7.00	6.33	0.47
S. *aureus*	2	8.00	7.00	8.00	7.67	0.47
S. *aureus*	4	16.00	17.00	16.00	16.33	0.47
S. *aureus*	5	15.00	15.00	15.00	15.00	0.00
S. *aureus*	6	21.00	20.00	21.00	20.67	0.47
S. *aureus*	7	18.00	19.00	19.00	18.67	0.47
S. *aureus*	8	20.00	19.00	20.00	19.67	0.47
S. *aureus*	9	23.00	22.00	24.00	23.00	0.82
S. *epidemidis*	1	8.00	8.00	8.00	8.00	0.00
S. *epidemidis*	2	7.00	7.00	7.00	7.00	0.00
S. *epidemidis*	4	14.00	15.00	14.00	14.33	0.47
S. *epidemidis*	5	17.00	16.00	16.00	16.33	0.47
S. *epidemidis*	6	21.00	21.00	21.00	21.00	0.00
S. *epidemidis*	7	20.00	19.00	20.00	19.67	0.47
S. *epidemidis*	8	17.00	18.00	17.00	17.33	0.47
S. *epidemidis*	9	21.00	23.00	21.00	21.67	0.94
S. *pneumoniae*	1	6.00	6.00	6.00	6.00	0.00
S. *pneumoniae*	2	6.00	6.00	6.00	6.00	0.00
S. *pneumoniae*	4	12.00	12.00	12.00	12.00	0.00
S. *pneumoniae*	5	11.00	11.00	10.00	10.67	0.47
S. *pneumoniae*	6	17.00	16.00	16.00	16.33	0.47
S. *pneumoniae*	7	15.00	15.00	14.00	14.67	0.47
S. *pneumoniae*	8	18.00	17.00	17.00	17.33	0.47
S. *pneumoniae*	9	20.00	20.00	20.00	20.00	0.00
S. *pyogenes*	1	10.00	10.00	12.00	10.67	0.94
S. *pyogenes*	2	12.00	11.00	12.00	11.67	0.47
S. *pyogenes*	4	23.00	21.00	26.00	23.33	2.05
S. *pyogenes*	5	20.00	21.00	25.00	22.00	2.16
S. *pyogenes*	6	27.00	26.00	29.00	27.33	1.25
S. *pyogenes*	7	25.00	24.00	26.00	25.00	0.82
S. *pyogenes*	8	20.00	19.00	22.00	20.33	1.25
S. *pyogenes*	9	27.00	28.00	30.00	28.33	1.25

Graph 2. All average ZOI's for all extracts, at 15 µl, against all species. Standard deviation is represented as error bars.

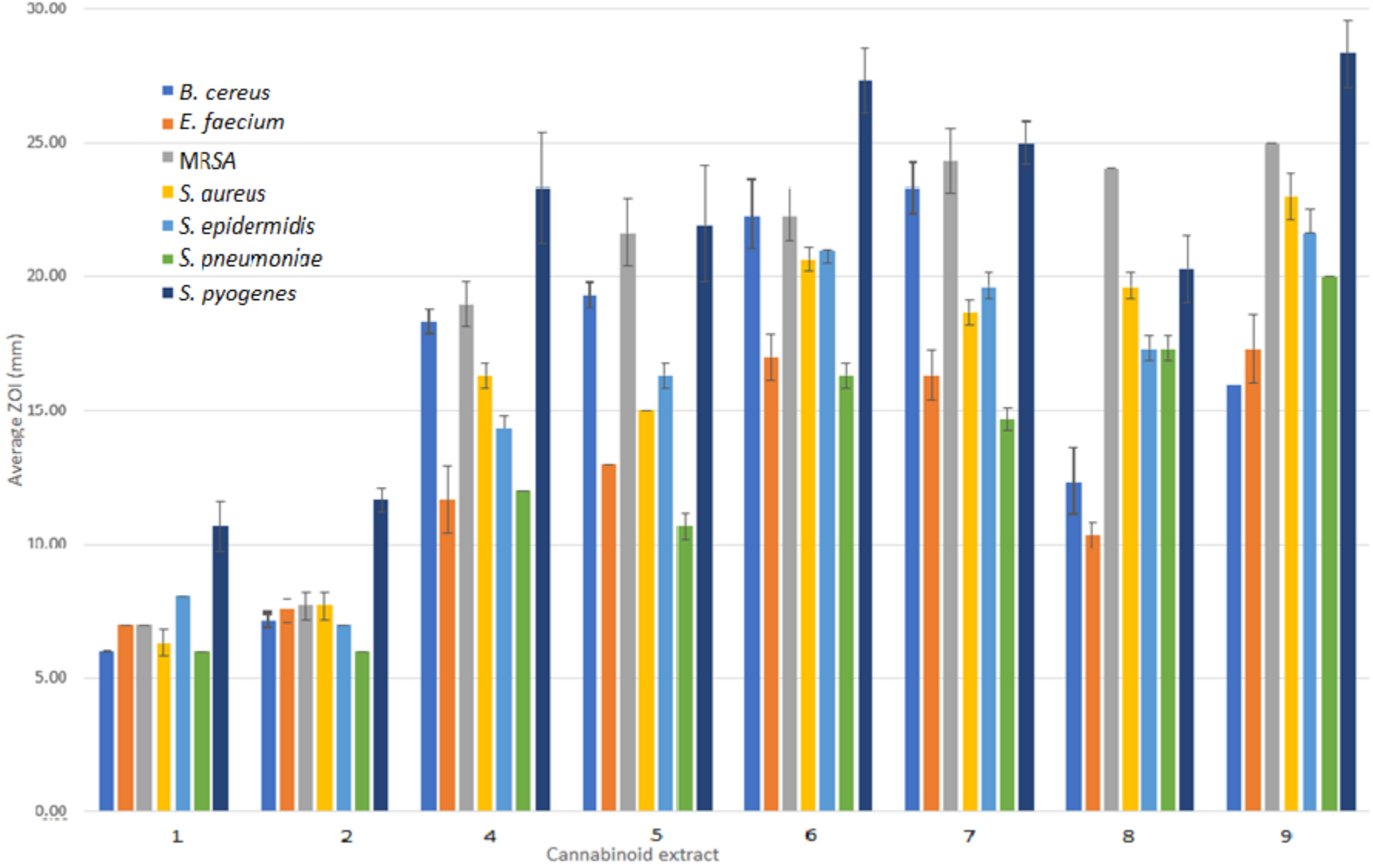


Table 3. Mean inhibition for each species with all extract results combined.

Species	Mean inhibition when all extract values are combined (mm)
B. *cereus*	15.60
E. *faecium*	12.52
MRSA	18.88
S. *aureus*	15.92
S. *epidemidis*	15.67
S. *pneumoniae*	12.88
S. *pyogenes*	21.08

Table 4. Mean inhibition for each extract with all species values combined.

Cannabinoid	Mean inhibition when all species values are combined (mm)
1	7.29
2	7.81
4	16.43
5	16.86
6	21.00
7	20.29
8	17.33
9	21.62

Tables 3 and 4 show the average inhibition against each species when all extract ZOIs (using 15 µl) were combined, and the average inhibition of each cannabinoid extract (using 15 µl) against all species, respectively. This shows extract 9 (CBG) had the highest average inhibition against all species, at 21.62 mm, and extract 1 (CBCV) produced the lowest with 7.29 mm. Table 3 shows that *S. pyogenes* was the most inhibited species when taking into account all cannabinoid extracts ZOIs and that *E. faecium* was the most resistant to the *Cannabis* extract's inhibition.

3.3 MIC 96 well plate testing

The species in Table 2 were tested with serial dilutions of the extracts to determine MICs. 96 well plates were scanned with a spectrophotometer at 600 nm and photographed. MICs were determined visually and via the readings from the spectrophotometer, Figure 8 shows the readings from an MRSA 96 well plate, after being incubated for 48 hours, with emphasis being drawn to squares A6, A7, B6 and B7 showing the sudden increases in absorbance between the adjacent wells. This shows that for CBCV and CBC extracts the MIC was the 6th lane with approximately 1.41 µl of extract per 150 µl total volume or 0.97% concentration.

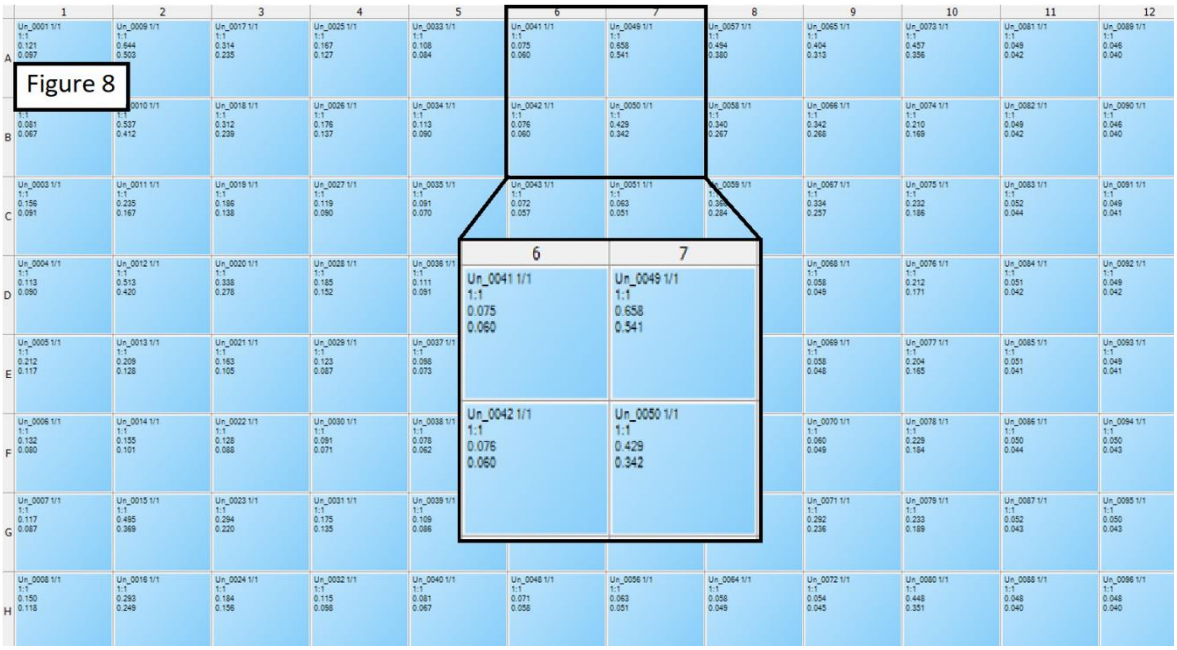


Figure 8. The 96 well plate absorbencies for MRSA after 48 hours of incubation. Zoomed in portion shows the sudden increase of absorbencies between columns 6 and 7, indicating that lane 6 was the MIC for MRSA for extracts 1 and 2.

For all 96 well plates each row contains one extract, from top to bottom (as displayed in figures and spreadsheets) they are: CBCV, CBC, THCV, CBDV, THC, CBD, CBGV and CBG. In the 96 well plate figures the concentration of extract decreases from left to right, from 22.5 µl of extract per 150 µl in lane 2, to approximately 0.18 µl per 150 µl in lane 9, and in spreadsheet figures concentration of extracts start high on the left and decreases in every next right hand column. This equates to MIC results from lanes 2-9 being: 150, 75, 37.5, 18.75, 9.38, 4.72, 2.35, 1.17 µl/ml, respectively.

Figure 9 shows *Streptococcus pneumoniae* at T=0, 24 and 48 hours incubation, from top to bottom. Clear growth is seen in lane 9 for all extracts after incubation, however there is a slight decrease in cloudiness in lane 9 for rows 5 and 6 (THC and CBD) and even less cloudiness in the bottom most column (CBG), indicating that extract 9 was slightly more inhibitory at 0.18 µl per 150 µl than the other extracts.

The top two rows, CBCV and CBC, show the least inhibitory effect as growth is observed in lanes 3 and 4 after 48 hours of incubation. This gives it an MIC against *S. pneumonia* of around 11 µl per 150 µl or 7.3% concentration. CBG on the other hand has an MIC of <0.18 µl per 150 µl or 0.12% concentration.

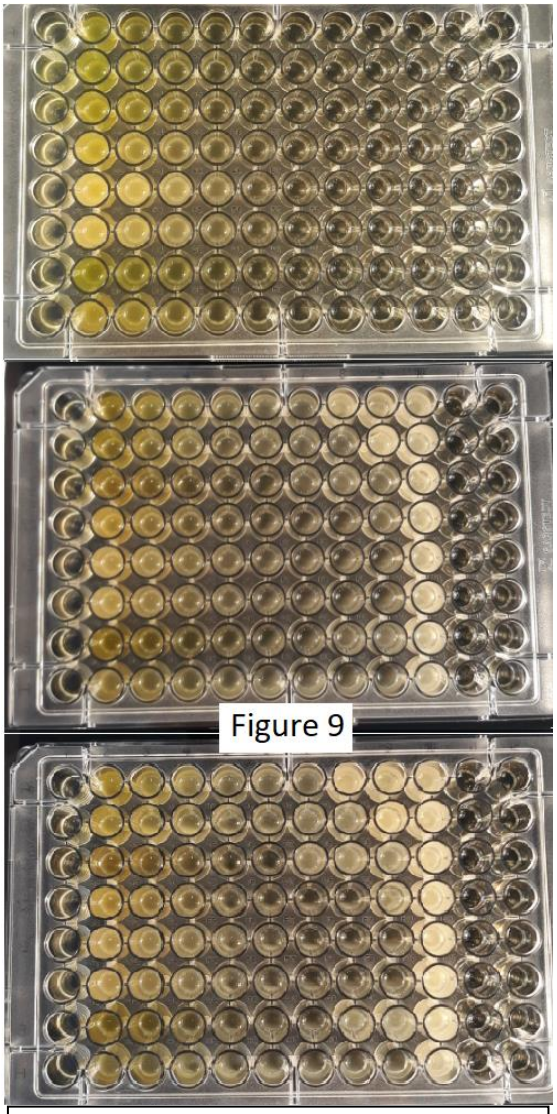


Figure 9. 96 well plates for *S. pneumoniae* at T=0 (top), T=24 (middle) and T=48 (bottom).

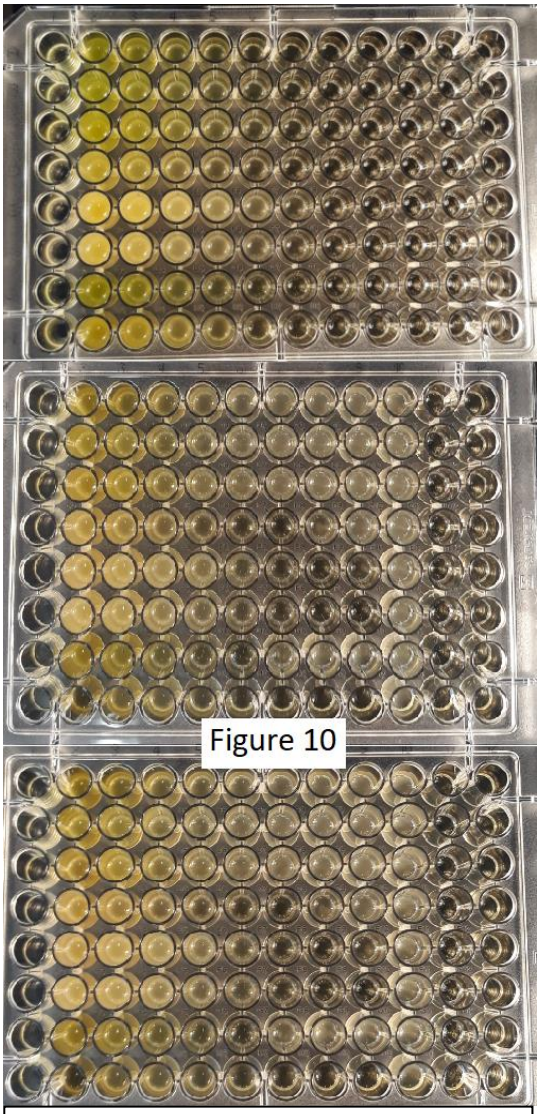


Figure 10. 96 well plates for *E. faecium* at T=0 (top), T=24 (middle) and T=48 (bottom).

Figure 10 shows a 96 well plate containing *Enterococcus faecium* after T=0, 24 and 48, from top to bottom. Results for THC, CBD and CBG (rows 5, 6 and 8) were well defined. They all showed inhibition in lane 9 to some degree, resulting in an MIC of approximately <0.18 µl per 150 µl or 0.12% concentration. CBC and CBCV faired the worst against *E. faecium*, with cloudiness appearing in lanes 3 and even lane 2, this resulted in an MIC of between 11.25 and 22.5 µl per 150 µl or 7.5 – 15% concentration. THCV resulted in an MIC of 1.4 µl per 150 µl or 0.93% concentration.

Table 5. A representation of a 96 well plate that shows at which volume each species was inhibited.

		Lane number (extract amount present in 150 µl well)							
		2 (22.5µl)	3 (11.25µl)	4 (5.625µl)	5 (2.8125µl)	6 (1.40627µl)	7 (0.70813µl)	8 (0.35256µl)	9 (0.17578µl)
Cannabinoid Extract	1		S.pyogenes S.pneumoniae E.faecium		S.epidermidis B.cereus	MRSA S.aureus			
	2		S.epidermidis S.pneumoniae E.faecium	S.pyogenes	B.cereus	MRSA S.aureus			
	4		E.faecium			S.epidermidis S.pyogenes S.pneumoniae	MRSA	S.aureus B.cereus	
	5						S.pyogenes S.pneumoniae E.faecium	S.epidermidis	MRSA S.aureus B.cereus
	6							S.pyogenes E.faecium	MRSA S.aureus S.epidermidis B.cereus S.pneumoniae
	7								MRSA S.aureus S.epidermidis B.cereus S.pyogenes S.pneumoniae E.faecium
							B.cereus	S.aureus	
	8					E.faecium	B.cereus MRSA S.pyogenes S.pneumoniae	S.aureus S.epidermidis	
	9							B.cereus	MRSA S.aureus S.epidermidis S.pyogenes S.pneumoniae E.faecium

Table 5 shows the MICs of each whole extract for all species, with a species' name being placed into the well representing the lowest concentration that produced no growth, the units are in µl as it represents the MIC of the whole extract, including all components and possible antimicrobials. Table 6 shows the actual amount of cannabinoid present in each extract for each lane, this has units of µg as it accounts for the percentage by mass of the exaggerated cannabinoid in each extract in each lane. These two must be used in conjunction to accurately represent the MIC of each cannabinoid.

Table 6. A representation of a 96 well plate that shows the amount of exaggerated cannabinoid in each extract in each lane.

Cannabis extract & Cannabinoid%	Amount of cannabinoid (µg/ml)							
Extract 1 & 1.8	2.7	1.35	0.6750	0.3375	0.1688	0.0844	0.0422	0.0211
Extract 2 & 2.0	3	1.5	0.7500	0.3750	0.1875	0.0938	0.0469	0.0234
Extract 4 & 5.5	8.25	4.125	2.0625	1.0313	0.5156	0.2578	0.1289	0.0645
Extract 5 & 7.3	10.95	5.475	2.7375	1.3688	0.6844	0.3422	0.1711	0.0855
Extract 6 & 15.8	23.7	11.85	5.9250	2.9625	1.4813	0.7406	0.3703	0.1852
Extract 7 & 13	19.5	9.75	4.8750	2.4375	1.2188	0.6094	0.3047	0.1523
Extract 8 & 3.6	5.4	2.7	1.3500	0.6750	0.3375	0.1688	0.0844	0.0422
Extract 9 & 11.1	16.7	8.35	4.1750	2.0875	1.0438	0.5219	0.2609	0.1305
	2	**3**	**4**	**5**	**6**	**7**	**8**	**9**
	96 Well plate lane number							

Cannabinoid 7 (CBD) produced the lowest MIC for all species, with an MIC less than 0.18 µl per 150 µl or 0.12% by concentration, making it the most effective low concentration extract against the successful Gram positive organisms. Extract 9 (CBG) was the next lowest across all species with only *B. cereus* having an MIC higher than 0.12%, at approximately 0.24% by concentration. CBCV and CBC (extracts 1 and 2) were the extracts with the highest MIC for all species. They proved stronger against MRSA and *S. aureus*, with an MIC of 1.41 µl per 150 µl, but proved weakest against *S. pneumoniae* and *E. faecium* only achieving an MIC of 7.5% by concentration.

CBGV and CBDV extracts (8 and 5) had strong inhibition across the spectrum of species tested with all MICs being above 1.41 µl per 150 µl for CBGV and all MICs being above 0.71 µl per 150 µl for CBDV.

THC proved a better inhibitor than its analogue THCV with extract 6 producing MICs of 0.24% or 0.12% by concentration against all species, whereas extract 4 only had 2 species with an MIC of 0.24%. *E. faecium* proved far more resistant to THCV than THC with an MIC of 7.5% for THCV compared to 0.24% for THCV showing *E. faecium* was 31.25 times more susceptible to inhibition from the THC extract over its analogue's extract.

Figure 11 shows the 96 well plate reading for *S. epidermidis* after 24 hours incubation, emphasis has been made to show the difference in MIC between CBGV, Row G, and CBG, Row H. CBG has a lower MIC with lane 9 producing no detectable bacterial growth, CBGV however has got a much larger absorbance than the blank broth in columns 11 and 12.

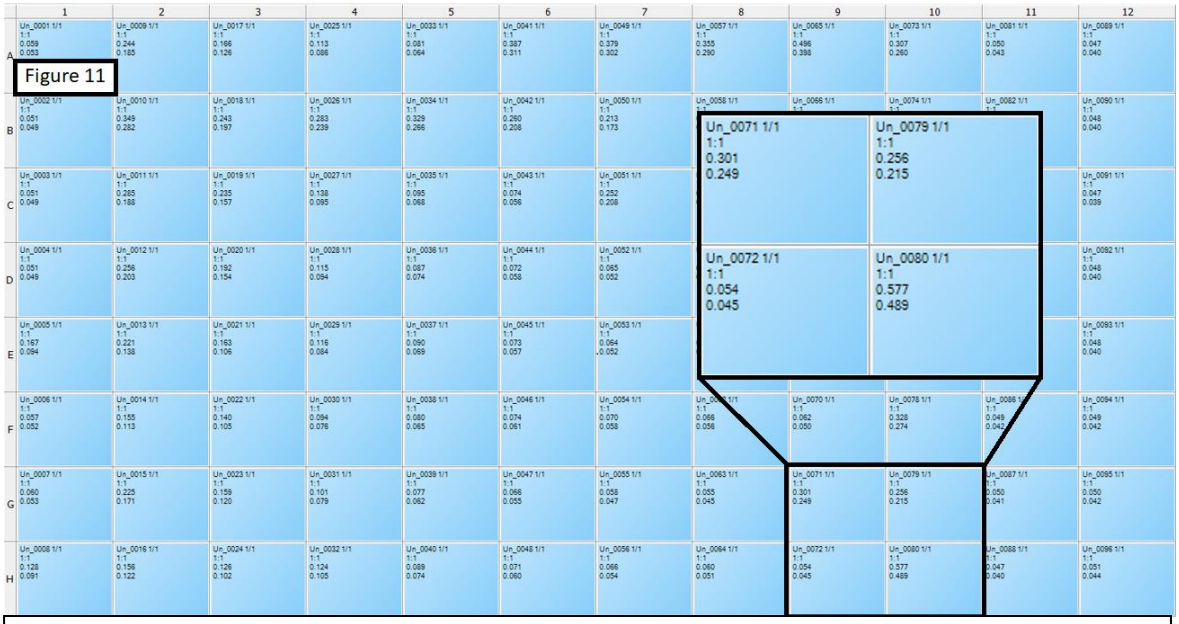


Figure 11. The 96 well plate absorbencies for *S. epidermidis* after at T=24. Zoomed in section highlights the presence of inhibition in lane 9 for extract 9 (bottom row) but not for extract 8.

3.4 Combined extract testing

Each extract was combined with its '-varin' analogue in equal volumes. These were then tested out on all species that had proved successfully inhibited by cannabinoids so far and were included in Table 7 in triplicate alongside 1 antibiotic disk containing each of the individual cannabinoids separately. Figure 12 shows 2 *S. aureus* plates using CBG and CBGV on the left and THC and THCV on the right. There is a blue dot on the top right outer edge of each plate, this indicates the closest disc contains the lowest numbered individual extract. The higher numbered individual extract is the disc to the left and the three discs on the bottom half of the plate are the triplicate combined extract results.

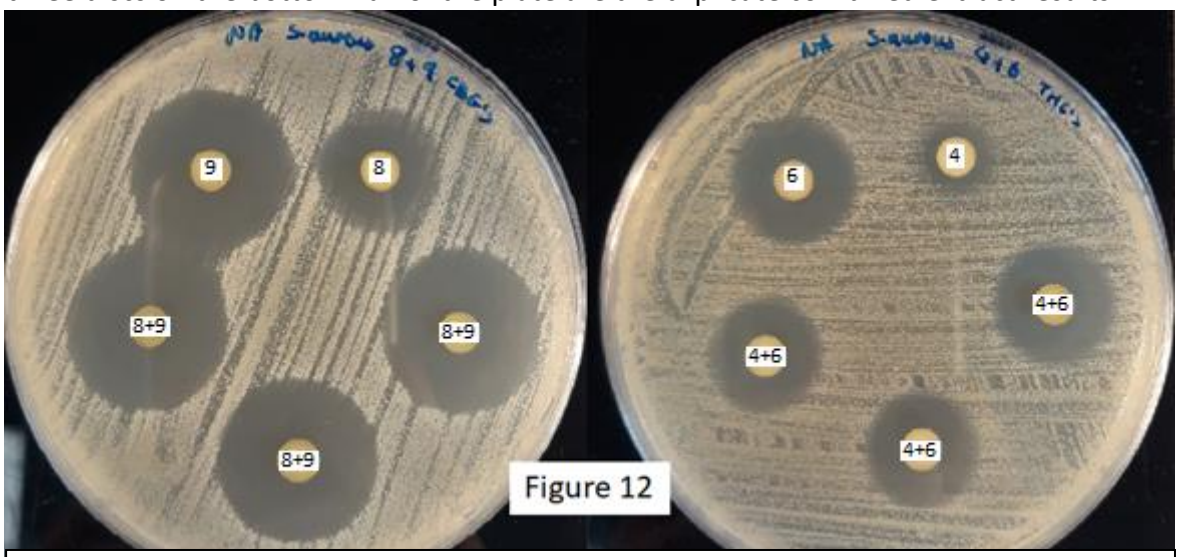


Figure 12. Comparison of *S. aureus* plates when tested with two different combination extracts. The left hand plate has extract 8 (top right), extract 9 (top left) and combination extracts containing both 8 & 9 (bottom three). The right plate has extract 4 (top right), extract 6 (top left) and combination extracts of 4 & 6 (bottom three). All using 15 µl on each disc.

Species	Cannabinoid Extract	ZOI 1 (mm)	ZOI 2 (mm)	ZOI 3 (mm)	Average ZOI (mm)	SD
B. cereus	1	9.00			9.00	
B. cereus	2	10.00			10.00	
B. cereus	1 & 2	8.00	7.00	7.00	7.33	0.47
B. cereus	4	10.00			10.00	
B. cereus	6	17.00			17.00	
B. cereus	4 & 6	16.00	15.00	17.00	16.00	0.82
B. cereus	5	15.00			15.00	
B. cereus	7	19.00			19.00	
B. cereus	5 & 7	18.00	17.00	18.00	17.67	0.47
B. cereus	8	12.00			12.00	
B. cereus	9	17.00			17.00	
B. cereus	8 & 9	16.00	16.00	17.00	16.33	0.47
E. faecium	1	7.00			7.00	
E. faecium	2	7.00			7.00	
E. faecium	1 & 2	6.50	6.00	7.00	6.50	0.41
E. faecium	4	9.00			9.00	
E. faecium	6	19.00			19.00	
E. faecium	4 & 6	17.00	17.00	17.00	17.00	0.00
E. faecium	5	12.00			12.00	
E. faecium	7	19.50			19.50	
E. faecium	5 & 7	16.00	17.00	16.00	16.33	0.47
E. faecium	8	10.00			10.00	
E. faecium	9	20.00			20.00	
E. faecium	8 & 9	19.00	20.00	17.00	18.67	1.25
MRSA	1	10.00			10.00	
MRSA	2	11.00			11.00	
MRSA	1 & 2	9.00	10.00	8.00	9.00	0.82
MRSA	4	14.00			14.00	
MRSA	6	23.00			23.00	
MRSA	4 & 6	22.00	23.00	22.00	22.33	0.47
MRSA	5	21.00			21.00	
MRSA	7	28.00			28.00	
MRSA	5 & 7	27.00	28.00	27.00	27.33	0.47
MRSA	8	26.00			26.00	
MRSA	9	30.00			30.00	
MRSA	8 & 9	31.00	30.00	30.00	30.33	0.47
S. aureus	1	8.00			8.00	
S. aureus	2	9.00			9.00	
S. aureus	1 & 2	7.00	6.50	7.00	6.83	0.24
S. aureus	4	11.00			11.00	
S. aureus	6	21.00			21.00	
S. aureus	4 & 6	18.00	18.00	20.00	18.67	0.94
S. aureus	5	13.00			13.00	
S. aureus	7	18.00			18.00	
S. aureus	5 & 7	17.00	18.00	18.00	17.67	0.47
S. aureus	8	19.00			19.00	
S. aureus	9	27.00			27.00	
S. aureus	8 & 9	27.00	27.00	27.00	27.00	0.00
S. epidermidis	1	7.00			7.00	
S. epidermidis	2	8.00			8.00	
S. epidermidis	1 & 2	8.00	7.00	8.00	7.67	0.47
S. epidermidis	4	10.00			10.00	
S. epidermidis	6	20.00			20.00	
S. epidermidis	4 & 6	18.00	16.00	17.00	17.00	0.82
S. epidermidis	5	17.00			17.00	
S. epidermidis	7	21.00			21.00	
S. epidermidis	5 & 7	22.00	20.00	21.00	21.00	0.82
S. epidermidis	8	19.00			19.00	
S. epidermidis	9	26.00			26.00	
S. epidermidis	8 & 9	25.00	25.00	24.00	24.67	0.47

Table 7. The ZOIs produced by extracts where each cannabinoid was combined with its varin analogue.

Table 7. (cont)

S. pneumoniae	1	NA				
S. pneumoniae	2	NA				
S. pneumoniae	1 & 2	NA	NA	NA		
S. pneumoniae	4	9.00			9.00	
S. pneumoniae	6	15.00			15.00	
S. pneumoniae	4 & 6	16.00	14.00	14.00	14.67	0.94
S. pneumoniae	5	NA				
S. pneumoniae	7	NA				
S. pneumoniae	5 & 7	NA	NA	NA		
S. pneumoniae	8	15.00			15.00	
S. pneumoniae	9	19.00			19.00	
S. pneumoniae	8 & 9	19.00	20.00	19.00	19.33	0.47
S. pyogenes	1	11.00			11.00	
S. pyogenes	2	10.00			10.00	
S. pyogenes	1 & 2	11.00	12.00	12.00	11.67	0.47
S. pyogenes	4	21.00			21.00	
S. pyogenes	6	23.00			23.00	
S. pyogenes	4 & 6	22.00	23.00	22.00	22.33	0.47
S. pyogenes	5	22.00			22.00	
S. pyogenes	7	22.00			22.00	
S. pyogenes	5 & 7	21.00	22.00	20.00	21.00	0.82
S. pyogenes	8	19.00			19.00	
S. pyogenes	9	24.00			24.00	
S. pyogenes	8 & 9	24.00	24.00	24.00	24.00	0.00

Figure 13 shows two different combined extracts against *S. pneumoniae* and *S. epidermidis*, the top two plates show THC and THCV, the bottom two plates show CBGV and CBG. The extract combining THC and its analogue produced an average inhibition of 14.67 mm and 17 mm against *S. pneumoniae* and *S. epidermidis* respectively, larger than the average value of an individual THC and THCV extract. The same applies for the CBGV and CBG extract combination with a mean ZOI of 19.33 mm against *S. pneumoniae* and 24.67 mm average ZOI against *S. epidermidis*, both are closer to the stronger extract's individual inhibition than the weaker individual extract.

S. pneuoniae produced two sets of triplicate plates that had no growth at all (on the entire plate), extracts 1 & 2 and 5 & 7.

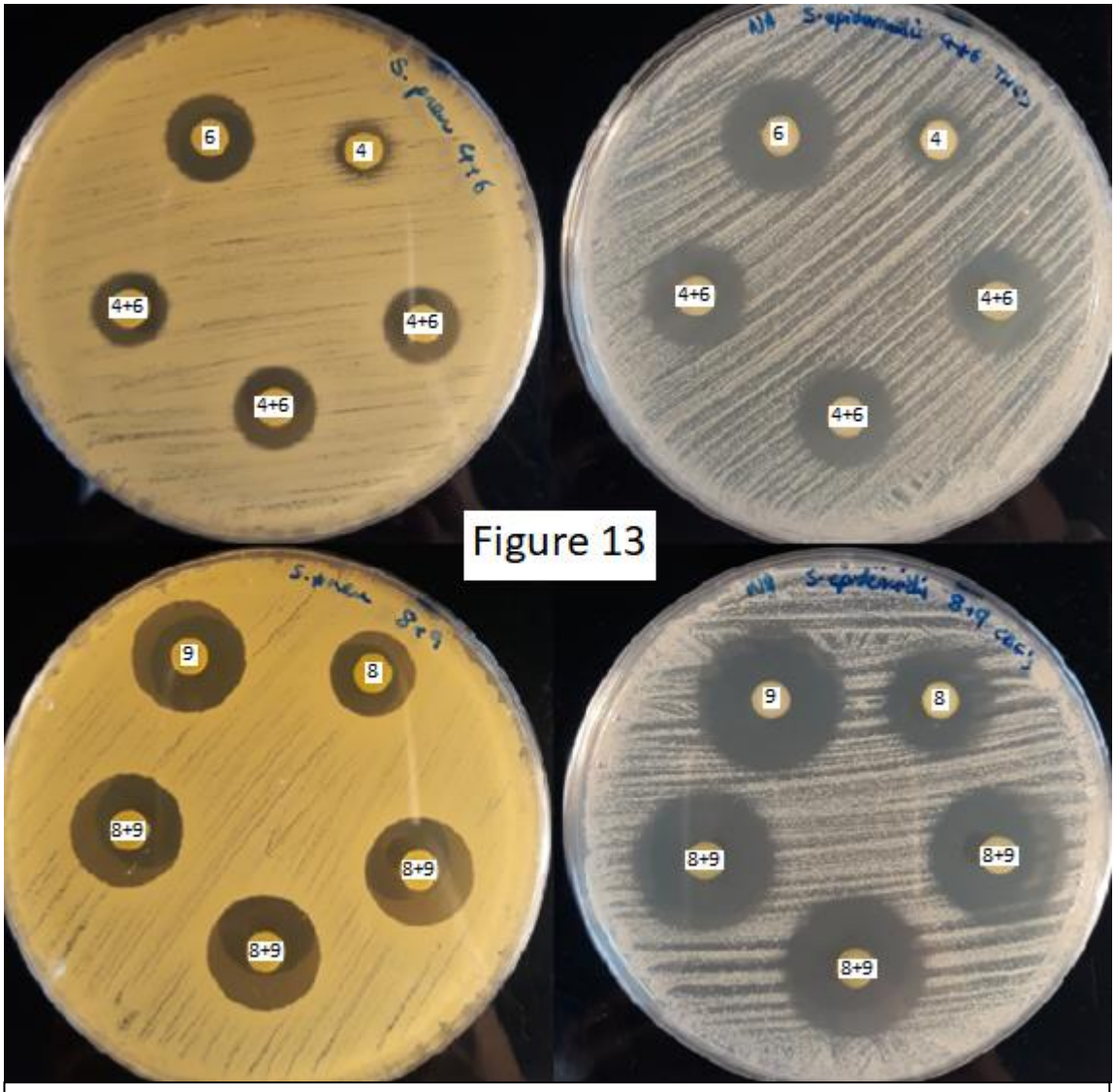


Figure 13. Comparison of *S. pneumoniae* (left hand plates) and *S. epidermidis* (right hand plates) using extract 4, 6 and 4 & 6 (top two plates) and extract 8, 9 and 8 & 9 (bottom two plates). Following the same disc layout as Figure 12. All discs contained 15 µl of extract.

Table 8 shows the triplicate ZOI values for the next series of combined extracts, which were chosen to combine two main type cannabinoids and two varin analogue cannabinoids. The chosen combinations were: CBCV and THCV, CBC and THC, CBDV and CBGV and finally CBD and CBG.

Extracts 1 and 4 proves the least effective extract against all species, with only minor inhibition against all species except *S. pyogenes* which produced an average inhibition of 15 mm. CBC and THC proved a stronger antibacterial combination than their analogue extracts produced when combined with a mean ZOI against *E. faecium* greater than 2 times the CBCV and THCV extract achieved. Extracts 2 & 6 produced moderate inhibition against all species with average ZOIs in the range of 14.33 mm to 18.67 mm against *E. faecium* and *S. pyogenes* respectively.

The extract combination of CBDV and CBGV was the second weakest combination tested, except against MRSA which it produced a mean ZOI of 18.67 mm which was slightly larger than the pairing of CBC and THC, but far shy of extract 7 & 9's mean inhibition of 24.33 mm against MRSA. CBD and CBG's extract pairing was the strongest in this particular set of extracts against all species except *E. faecium*. The extract combination of 7 & 9 was less effective than the pairing of CBGV and CBG, extracts 8 and 9 from the previous test, against all species except for *B. cereus* against which it proved more effective.

Table 8. The ZOI results of the second series of synergistic extracts that combined main type cannabinoids extracts and varin analogue extracts together.

Species	Cannabinoid Extract	ZOI 1 (mm)	ZOI 2 (mm)	ZOI 3 (mm)	Average ZOI (mm)	SD
B. cereus	1 & 4	9.00	10.00	10.00	9.67	0.47
B. cereus	2 & 6	15.00	17.00	17.00	16.33	0.94
B. cereus	5 & 8	13.00	14.00	14.00	13.67	0.47
B. cereus	7 & 9	16.00	18.00	19.00	17.67	1.25
E. faecium	1 & 4	7.00	7.00	7.00	7.00	0.00
E. faecium	2 & 6	13.00	14.00	16.00	14.33	1.25
E. faecium	5 & 8	15.00	11.00	11.00	12.33	1.89
E. faecium	7 & 9	10.00	16.00	16.00	14.00	2.83
MRSA	1 & 4	11.00	11.00	11.00	11.00	0.00
MRSA	2 & 6	17.00	18.00	18.00	17.67	0.47
MRSA	5 & 8	18.00	19.00	19.00	18.67	0.47
MRSA	7 & 9	24.00	24.00	25.00	24.33	0.47
S. aureus	1 & 4	8.00	10.00	9.00	9.00	0.82
S. aureus	2 & 6	16.00	19.00	17.00	17.33	1.25
S. aureus	5 & 8	15.00	14.00	16.00	15.00	0.82
S. aureus	7 & 9	21.00	24.00	22.00	22.33	1.25
S. epidermidis	1 & 4	9.00	9.00	9.00	9.00	0.00
S. epidermidis	2 & 6	14.00	16.00	17.00	15.67	1.25
S. epidermidis	5 & 8	16.00	13.00	15.00	14.67	1.25
S. epidermidis	7 & 9	19.00	18.00	19.00	18.67	0.47
S. pneumoniae	1 & 4	9.00	8.00	8.00	8.33	0.47
S. pneumoniae	2 & 6	17.00	15.00	14.00	15.33	1.25
S. pneumoniae	5 & 8	16.00	12.00	12.00	13.33	1.89
S. pneumoniae	7 & 9	20.00	19.00	18.00	19.00	0.82
S. pyogenes	1 & 4	14.00	15.00	16.00	15.00	0.82
S. pyogenes	2 & 6	18.00	18.00	20.00	18.67	0.94
S. pyogenes	5 & 8	15.00	18.00	22.00	18.33	2.87
S. pyogenes	7 & 9	22.00	23.00	22.00	22.33	0.47

Figure 14 shows a plate of MRSA, left, and *B. cereus*, right, with all 4 combinations applied with extracts 1 & 4 being at the top, and extracts 2 & 6, 5 & 8 and 7 & 9 in anticlockwise order. Extract 7 & 9 produced an inhibition of 24 mm against MRSA compared with only a 16 mm ZOI against *B. cereus*.

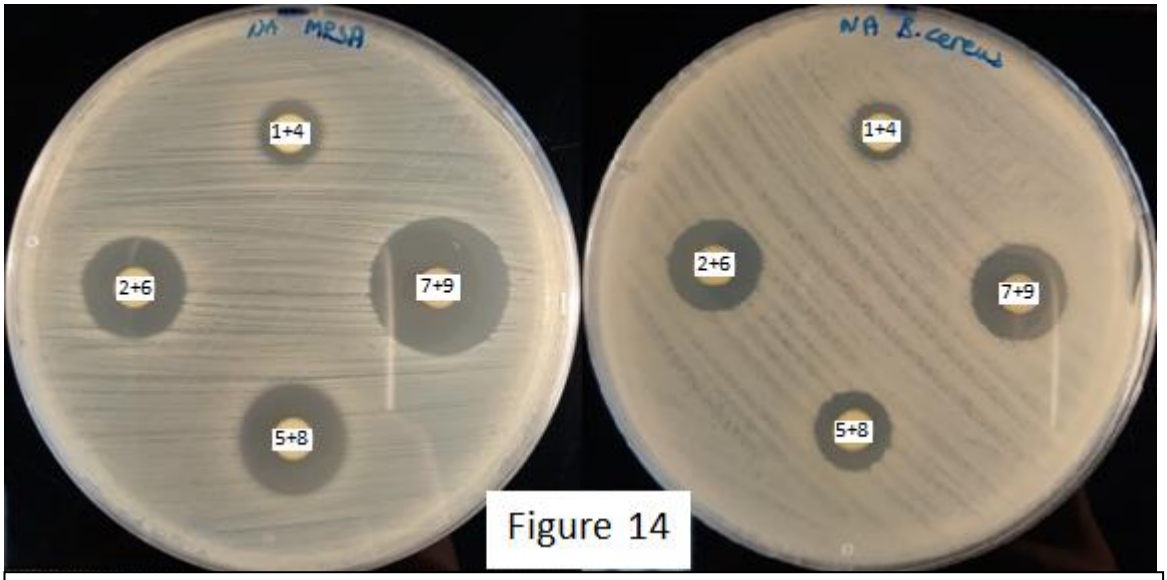


Figure 14. A comparison between MRSA (left plate) and *B. cereus* (right plate) after testing with combination extracts 1 & 4 (top discs), 2 & 6 (left discs), 5 & 8 (bottom discs) and 7 & 9 (right discs). Each disc contained 15 µl of extract.

3.5 Bacteriostatic/bactericidal testing

Table 9 shows whether each successful extract was bacteriostatic or bactericidal, 52 out of 56 samples produced growth when the successful ZOIs were swabbed and streaked on fresh agar. Only 4 results came back bactericidal: extract 7 against *B. cereus*, extract 8 against *E. faecium*, extract 9 against MRSA and extract 4 against *S. epidermidis*.

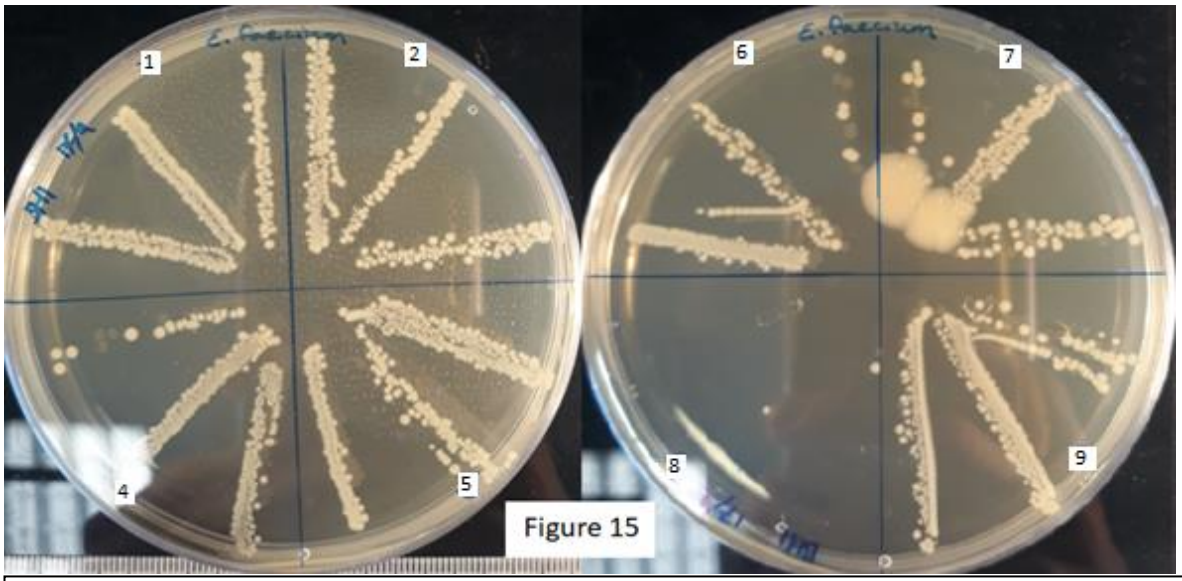


Figure 15. The bacteriostatic/cidal results for *E. faecium*. The left hand plate shows swab streak results from the ZOIs of extract 1 (top left quadrant), extract 2 (top right), extract 4 (bottom left) and extract 5 (bottom right). The right hand plate contains the swab streak results for extracts 6 (top left), extract 7 (top right), extract 8 (bottom left) and extract 9 (bottom right).

Figure 15 shows growth of cells for all swab streaks except extract 8 which showed bactericidal results, however, one small colony is growing, the result is vastly different

than the clear bacteriostatic growth for other extracts, so it is classed as bactericidal. Slight contamination occurred in the quadrant containing extract 7's swab results.

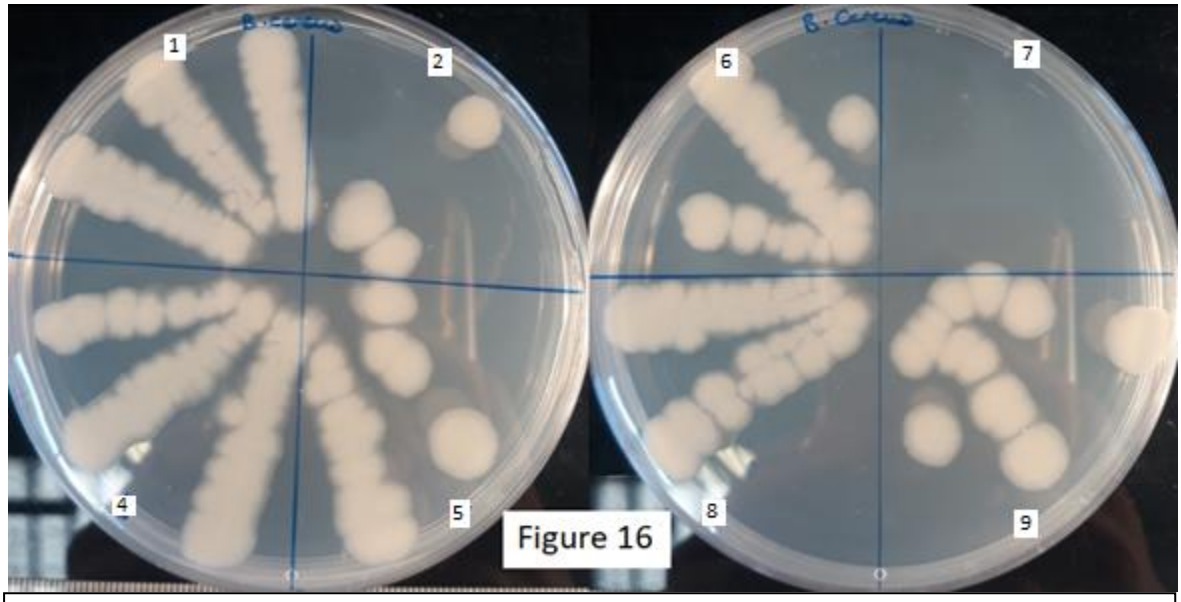


Figure 16. The bacteriostatic/cidal results for *B. cereus*. Following the same layout as Figure 15.

Figure 16 shows the results of the test for *B. cereus* with the upper right hand quadrant of the right hand plate showing no cell regrowth from the ZOIs of extract 7. Notably the one of the swabs from a ZOI of extract 2 produced no cell regrowth, as did one of the streaks for extract 8.

Species	Cannabinoid Extract	Bacteriostatic (static) or Bactericidal (cidal)
B. cereus	1	static
B. cereus	2	static
B. cereus	4	static
B. cereus	5	static
B. cereus	6	static
B. cereus	7	cidal
B. cereus	8	static
B. cereus	9	static
E. faecium	1	static
E. faecium	2	static
E. faecium	4	static
E. faecium	5	static
E. faecium	6	static
E. faecium	7	static
E. faecium	8	cidal
E. faecium	9	static
MRSA	1	static
MRSA	2	static
MRSA	4	static
MRSA	5	static
MRSA	6	static
MRSA	7	static
MRSA	8	static
MRSA	9	cidal
S. aureus	1	static
S. aureus	2	static
S. aureus	4	static
S. aureus	5	static
S. aureus	6	static
S. aureus	7	static
S. aureus	8	static
S. aureus	9	static
S. epidermidis	1	static
S. epidermidis	2	static
S. epidermidis	4	cidal
S. epidermidis	5	static
S. epidermidis	6	static
S. epidermidis	7	static
S. epidermidis	8	static
S. epidermidis	9	static
S. pneumoniae	1	static
S. pneumoniae	2	static
S. pneumoniae	4	static
S. pneumoniae	5	static
S. pneumoniae	6	static
S. pneumoniae	7	static
S. pneumoniae	8	static
S. pneumoniae	9	static
S. pyogenes	1	static
S. pyogenes	2	static
S. pyogenes	4	static
S. pyogenes	5	static
S. pyogenes	6	static
S. pyogenes	7	static
S. pyogenes	8	static
S. pyogenes	9	static

Table 9. Bacteriostatic or bactericidal results for each extract and species.

3.6 Synthetic cannabinoid testing

All species were tested with all 10 synthetic cannabinoid extracts. Table 10 shows the successful synthetic cannabinoids and their ZOIs. 5F – PB – 22 was the only synthetic cannabinoid that worked against more than one species, producing almost negligible ZOIs in some cases but enough to be detectable. It proved most successful against *S. pyogenes* and *S. aureus* with mean ZOIs of 10.33 mm and 9.17 mm respectively, 2 plates of which are found in Figure 17. The inhibition produced against *B. cereus, E. faecium* and *S. pneumoniae* were extremely faint, only just creating a zone of no growth, when compared to the natural cannabinoid extracts they fared poorly.

Table 10. Successful synthetic cannabinoid ZOIs.

Species	Synthetic Cannabinoid	ZOI 1 (mm)	ZOI 2 (mm)	ZOI 3 (mm)	Average ZOI (mm)
B. cereus	5F - PB - 22	6.50	6.50	6.00	6.33
E. faecium	5F - PB - 22	7.00	7.00	6.50	6.83
MRSA	5F - NPB - 22	6.50	7.00	6.50	6.67
MRSA	STS - 135	6.50	6.50	6.00	6.33
S. aureus	5F - PB - 22	9.00	9.00	9.50	9.17
S. epidermidis	5F - PB - 22	9.00	8.00	8.00	8.33
S. pnuemoniae	5F - PB - 22	7.00	7.00	8.00	7.33
S. pyogenes	5F - PB - 22	11.00	10.00	10.00	10.33

5F – NPB – 22 and STS – 135 were effective against MRSA in the testing, but produced no inhibition against any other species, including its non-methicillin resistant species member *Staphylococcus aureus.* The miniscule inhibition of 5F – NPB – 22 is shown in Figure 17, the plate on the far right and highlighted further in Figure 18.

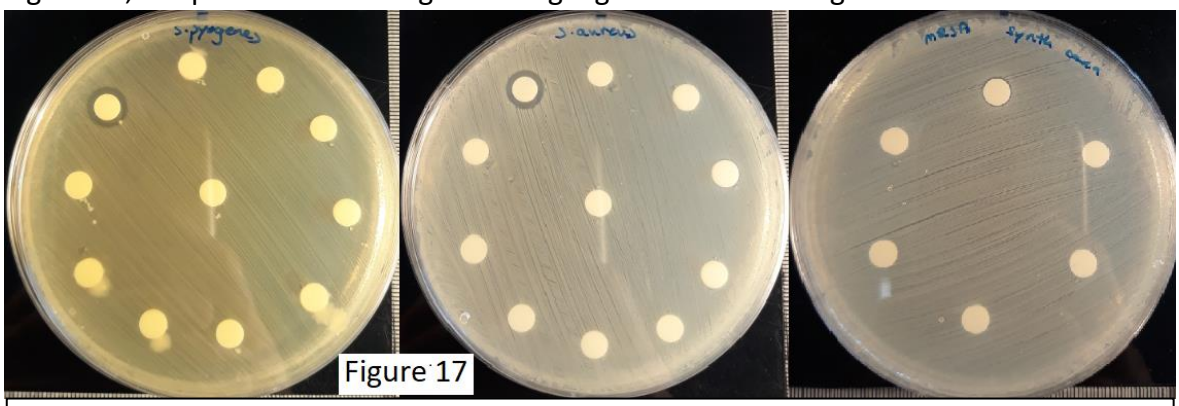


Figure 17. Plates for *S. pyogenes* (left plate), *S. aureus* (middle plate) and MRSA (right plate). *S. pyogenes* and *S. aureus* plates have inhibition on disc 2 (from top, anticlockwise), 5F – PB – 22. MRSA plate shows 5F – NPB – 22 (top disc) with miniscule inhibition. Discs contained 10 µl of extract.

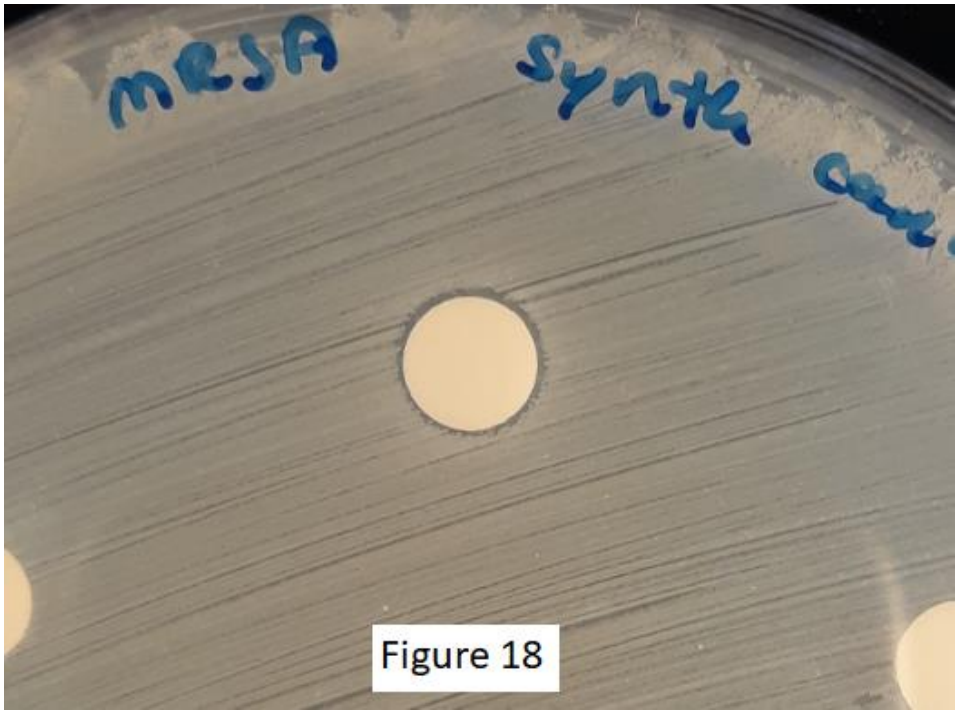


Figure 18. A zoomed in picture of the miniscule, yet apparent inhibition of 5F – NPB – 22 against MRSA. Disc contained 10 µl of extract.

3.7 MRSA genetic testing

Due to time constraints, and personal circumstances, there was not enough time to complete the genetic testing to an acceptable level and so it was excluded from the study. It would be interesting to study this in the future, given more time.

3.8 *E. coli* and *P. aeruginosa* additional extract tests

E. coli and *P. aeruginosa* were both uninhibited by all extracts tested previously. A set of extracts with much higher concentration were made using 150 mg of *Cannabis* with 150 mg of solvent. Ethanol and extracts were prepared.

Figure 19 shows the complete lack of inhibition for all cannabinoids against both species, resulting in the exact same results as the normal extracts which were only $1/9^{th}$ of the concentration.

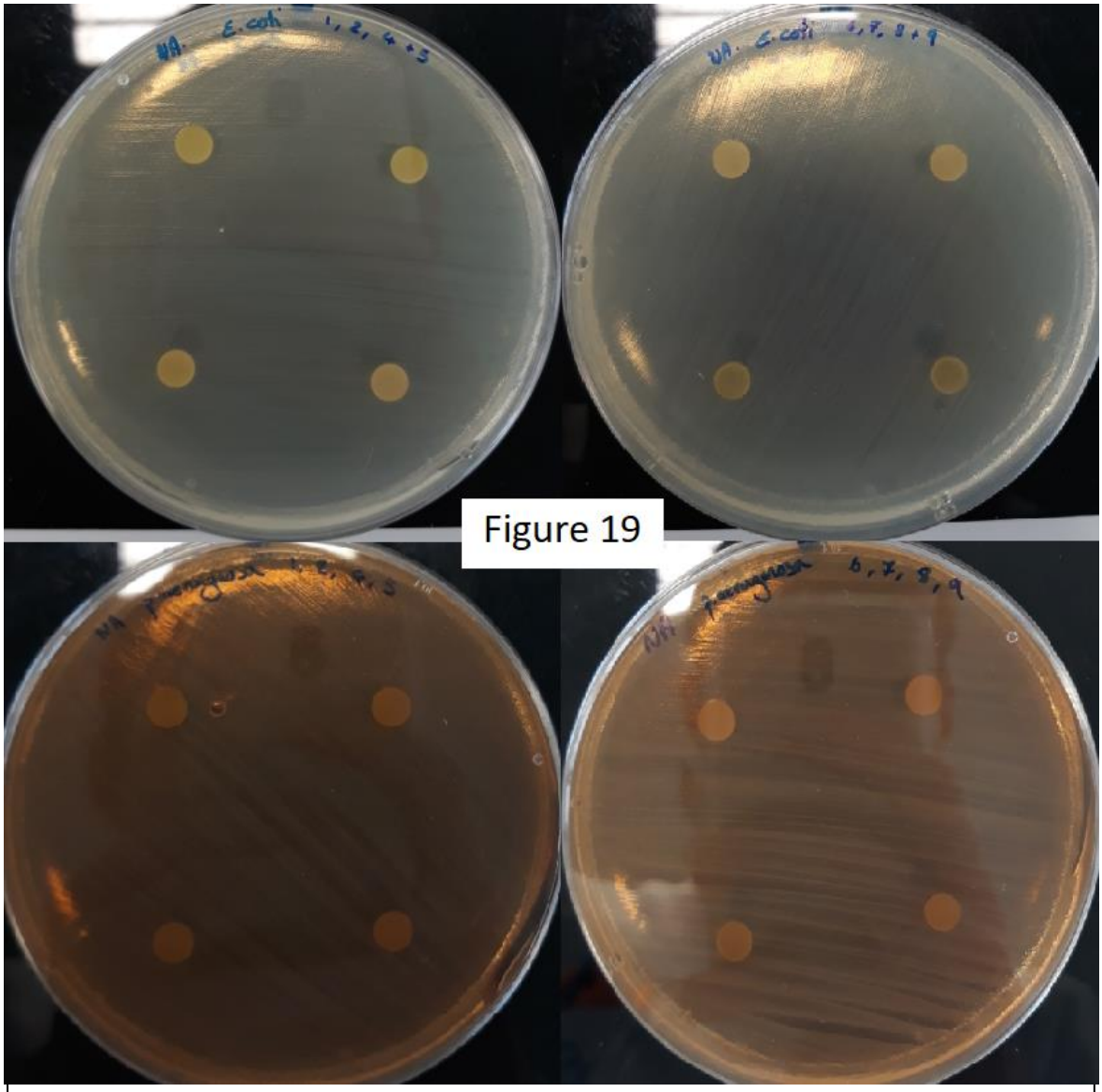


Figure 19. *E. coli* (top plates) and *P. aeruginosa* (bottom plates) plates showing no inhibition when 10 times strength extracts were used.

4 Discussion

4.1 Unsuccessful species

E. coli, P. aeruginosa, C. albicans and *C. glabrata* all proved resistant to all extracts tested, including all solvents tested and all cannabinoid variants, including synthetic cannabinoids. This is in direct contrast with several studies.

Naveed *et al* (2014) produced results that showed inhibition of 24.1 mm, 10.3 mm and 22.2 mm against *Staphylococcus aureus, Pseudomonas aeruginosa* and *Escherichia coli* using a concentration of 5 grams of *Cannabis sativa* leaf material and 50 ml of water or ethanol – a weaker concentration than used in this study, yet inhibition occurred from their weaker extracts. Another study by Sarmadyan *et al* (2013) provided results that

showed inhibition was produced using a disk diffusion method against *E. coli* but did not produce inhibition against *P. aeruginosa*. Radwan *et al* (2009) found that 4 isolated compounds from the *Cannabis sativa* plant had antifungal activity against *Candida albicans,* this was supported by Ali *et al* (2012) who found that methanol extracts of whole plant *Cannabis sativa* produced low activity against *Candida albicans* and high activity against *E. coli* and *P. aeruginosa.* Another study by Mehmedic *et al* (2014) showed that fractions of volatile oil of *Cannabis sativa* containing different terpenoids showed good and selective antifungal activity, despite containing low concentrations of cannabinoids.

However, Vu *et al* (2016) performed a study on a wide variety of plant extracts and found that *Cannabis sativa* had no antimicrobial ability against *E. coli* and *P. aeruginosa*.

Most referenced studies produced results with differing outcomes to the results of this experiment even when utilising the same extracts, sometimes at higher concentrations of *Cannabis* to solvent. In this study extracts were prepared specifically for the species unaffected by the regular extracts. These extracts were in a 1:1 ratio of *Cannabis sativa* material to ethanol, 10 times more concentrated than the standard extracts that proved so successful against Gram positive species. This was unexpected but could potentially be attributed to the widely varying amount of antimicrobial compounds that can be achieved in *Cannabis sativa* plants. The plants used in this study were bred to exaggerate an individual cannabinoid above its normal levels, in the process of doing this there may have been a reduction in the levels of other compounds the plants produced. It may be these non-cannabinoid molecules that allow the inhibition of Gram negative bacteria and fungi species. Alternatively, there could be slight differences between the strains of each species used in the different experiments, it is unlikely, but this study may have utilised a strain of each species that happened to be resistant to cannabinoids. Future testing could be done on multiple strains of each species to ensure this is not the case.

Also, in future research, column chromatography could be used to separate and identify the different components of the extracts to verify if the cannabinoids are the main antimicrobial agent or if it is due to other compounds such as phenols that could be inhibiting Gram negative species and fungi species. Mahmoudi *et al* (2016) showed that methanolic leaf extracts of different varieties of Algerian fig (*Ficus carica* L.), containing many phenolic and antioxidant compounds, produced antibacterial effects against Gram negative bacteria, so these may be the route of inhibition in *Cannabis* extracts against

Gram negative bacteria. This could also explain why studies such as Ali *et al* (2012) and Novak *et al* (2001) have produced inhibition against Gram negative bacteria using *Cannabis sativa* seed oils. In Novak's study the amount of cannabinoids were assessed and found to be very poor, this implies the inhibition arose from other compounds.

4.2 Antimicrobial activity against Gram positive species

The results for Gram positive species were generally very successful. With all *Cannabis* extracts and solvents producing some degree of inhibition, in triplicate, against all Gram positive species.

4.2.1 Different solvent extractions

In the different solvent testing against MRSA using CBG, the methylated spirit extract proved to be the strongest inhibitor when compared with ethanol extracts and the two water extracts incubated at 37 °C for 24 hours or 70 °C for 2 hours and then 22 hours at 37 °C.

The methylated spirit and ethanol extracts had very similar ZOIs for all volumes, with only a 3 mm and 2 mm average ZOI difference between 15 and 60 µl and for methylated spirit and ethanol respectively. This could be due to CBG being a powerful antibacterial, displaying an MIC of 0.13 µg/ml or 1.17 µl/ml of extract, and being close to the limit of its diffusion area which is limiting its ZOI sizes as no zones in the whole study produced more than a 30 mm ZOI. The water extracts proved to be less effective, inhibition zones in all cases were smaller than those for ethanol or methylated spirit. The water that was incubated for 2 hours at 70 °C proved less effective than the water extract incubated solely at 37 °C for volumes of 15, 30 and 60 µl. But for volume 45 µl it proved to be very slightly more effective. A T-test performed for the 45 µl produced a p-value of 0.78, larger than the threshold p-value of 0.05, determining the results are not statistically different. T-tests performed between methylated spirit and ethanol extracts at 15, 30 and 45 µl showed p-values of: 1, 0.23 and 0.23, respectively, showing no statistical differences between the values.

Wasim *et al* (1995) produced results using a well diffusion method showing that ethanolic and petroleum ether extracts inhibited growth of Gram positive and Gram negative bacteria and against the fungi tested. Their water extracts did not produce any

antimicrobial activity at all. This is in contrast to this experiment as the results from this test showed that water extracts both produced inhibition against all Gram positive species tested. However, the results for *Candida albicans* were the same in both studies for aqueous extracts. No inhibition produced. The studies differed in most of the species studied, with only *S. aureus* and *C. albicans* being used in both studies.

4.2.2 Ethanol extracts at 15 µl and MICs

The cannabichromene type *Cannabis* extracts were continuously the smallest zone-producing extracts, this contrasts with some studies. A study published by Turner and Elsohly (1981) produced results showing antibacterial activity was strong against Gram negative and positive species, Appendino *et al* (2008) also found that CBC was a strong inhibitor. This appears contradictory to results obtained in this study for ZOIs, as consistently the CBC and CBCV extracts produced weak inhibition, with a ZOI range against all species of 6 – 10.67 mm for CBCV and 6 – 11.67 for CBC. Appendino *et al* reported MIC values of 2 µg/ml of CBC against *S. aureus* and MRSA, which appears more effective than the extract tested in this study with an MIC of 9.4 µg/ml. However, Appendino *et al*'s study used pure (>98%) cannabinoids from *Cannabis sativa* and so their extracts contained far more cannabinoids than the CBC chemotype *Cannabis* used in this study which had cannabinoid percentages by weight of 1.8 and 2, for CBCV and CBC. This means the extract tested contained at most 1.8-2% CBCV and CBC which equates to an MIC of 0.17-0.19 µg of CBC/ml, as shown in Table 6 which shows the MIC in µg/ml for each extract's actual cannabinoid concentration in each lane. This study found CBC was at a concentration of 0.19 µg/ml when it produced inhibition, a considerably lower MIC than Appendino's study, however this experiment could not discern which compounds within the extract were responsible for any inhibition, only whether inhibition occurred or not. 98% of the botanical raw material used in extract 2 was not cannabidiol, so it was a very real chance that other compounds were contributing to the inhibition, this should be studied further. Ali *et al*'s study (2012) produced an MIC result of 50 µg/ml against *S. aureus* using a methanol whole plant extract, significantly higher than even the highest MIC produced in this experiment where CBCV and CBC both had an MIC of 9.3 µl/ml, Strangely, the study performed by Ali showed that the same whole plant extract had a MIC against *E. coli* and *P. aeruginosa* of 25 µg/ml and 12.5 µg/ml, whereas this study produced no inhibition for any species.

It is important to take into account the concentration of each cannabinoid in the extracts as the *Cannabis sativa* material received from G. W. Pharmaceuticals has a range of 1.8% cannabinoid by weight (for CBCV, extract 1) and 15.8% cannabinoid by weight (for THC, extract 6). Unfortunately, some studies relating to the antimicrobial ability of *Cannabis sativa* do not contain information on the exact amounts of cannabinoids in the extracts, only that they are present.

The THC type extracts produced generally moderate to strong inhibition against most Gram positive species. This has been known for several decades, with Van Klingeren and Ten Ham (1976) producing results showing THC and CBD had an MIC against *Staphylococci* and *Streptococci* of 1-5 mug/ml, this is much higher than the MICs of the THC extract tested in this experiment which had <0.52 µg/ml for the *Staphylococcus* and *Streptococcus* species. This could easily be explained by the large increases in THC yields that are now achievable in comparison to 4 decades ago. Their study also provided results showing that Gram negative bacteria were resistant to THC and CBD, which aligns with the results of this study. THCV has, as far as could be found, no published research specifically stating its efficacy as an antimicrobial, this is unusual as the cannabinoid was isolated and identified at the beginning of the 1970s (Gill *et al*, 1970).

Appendino *et al* (2008) produced results showing MIC ranges of 0.5 to 2 µg/ml against multi-drug resistant *S. aureus* and the major methicillin-resistant *S. aureus* strains, these were occurring in hospitals in the UK at the time (Richardson and Reith, 1993). In this experiment, the THC extract produced an MIC of 4.72 µl/ml against MRSA, equating to an MIC 0.26 µg/ml of pure THC, compared to the MIC of 0.5 µg/ml achieved against one strain of MRSA used in Appendino's study. A similar MIC for the THC extract was achieved in Appendino's study as in this one, both having an MIC of 2 µg/ml.

CBD type *Cannabis* has been known to be antibacterial since at least 1976 when Van Klingeren and Ten Ham showed it produced inhibition against *Staphylococci* and *Streptococci* species, it has been understood to exist since the 1940s and had its structure discovered in 1963 (Burstein, 2015). Yet there is still a lack of research on isolated cannabinoids (or at least *Cannabis* bred to have exaggerated chemotypes as in this study). Appendino *et al* (2008) provided one of the only published research papers studying isolated cannabinoids and not just whole plant materials. Their study provided an MIC for cannabidiol of 1 µg/ml which is slightly lower than the value for the CBD extract in this experiment (1.17 µg/ml).

The CBG extracts proved to have generally strong inhibition against the Gram positive species with inhibition size data ranging from 17 to 28 mm. CBGV was a worse inhibitor than its non-varin analogue for all Gram positive organisms but it must be taken into account that the CBG type *Cannabis* used in this experiment had a CBG concentration of 11.1% by weight of raw material, CBGV on the other hand had a CBGV concentration of 3.6% by weight. This means when comparing the MICs of the two extracts it is important to reference Table 5 and Table 6 which show each species' MIC for the amount of extract per 150 µl well and the amount of cannabinoid present in 1 ml, respectively. In the 96 well plates the CBG extract had lower MICs for all species than those of the CBGV extracts. However, when comparing the actual amount of cannabinoid present in each of the wells for inhibition to take place, CBGV had a lower MIC than CBG against *B. cereus*, with 0.16 µg/ml and 0.26 µg/ml respectively, and also against *S. aureus* and *S. epidermidis* where CBGV had an MIC of 0.08 µg/ml and CBG had an MIC of 0.13 µg/ml. These results were stronger than penicillin against S*. aureus,* where Rubin *et al* (2011) showed an MIC for penicillin was 1 µg/ml. This indicates CBGV is potentially 12.5 times stronger than penicillin for this species. The MIC obtained for CBG was smaller than in Appendino *et al*'s 2008 study. They recorded an MIC of 1 µg/ml for 5 of their 6 strains, with one MRSA strain having an MIC of 2 µg/ml, whereas this experiment showed MICs for CBG of 0.13 µg/ml. A study by Borchardt *et al* (2008) showed that a *Cannabis sativa* ethanol extract produced inhibition of 25 mm against *S. aureus* and found no inhibition against Gram negative species they used in the study. The closest result from this experiment was the CBG extract, which produced a mean inhibition of 23 mm, however only 15 µl of extract was tested in this study while Borchardt *et al* used 50 µl.

The results from the ethanol compounds against Gram positive organisms produced contradictory results to many studies, particularly focussing on the different results for Gram negative. Eisohly *et al* (1982) found that CBC was a superior antibiotic to THC and CBD, which is the opposite according to the results obtained in this study. CBC was shown to have an MIC against MRSA of 0.19 µg/ml, whereas THC and CBD had MICs against MRSA of 0.18 and 0.15 µg/ml, respectively, showing that CBC was a slightly less effective antibacterial than THC and CBD.

The results obtained in this study showed that there was a noticeable difference between the chemical phenotypes of cannabis that were used to prepare each extract. CBC, and its varin analogue, consistently performed poorly against most species when compared with

the other chemical phenotype extracts, which all proved far more successful. It is not possible from this experiment to deduce whether these differences are specifically due to the different cannabinoids that are exaggerated in each extract, or whether there are other antimicrobials present in varying levels throughout the different plants which could also be responsible. However, there was a noticeable pattern that the main type cannabinoid extracts (CBC, CBD, CBG and THC) all proved more successful inhibitors than their varin analogue extracts did, this adds weight to the argument that the cannabinoids are a reason for their antimicrobial activity. The lower inhibitory ability of the varin analogues may indicate that a propyl side chain attached to the aromatic ring (instead of a pentyl side chain found in the main type cannabinoids) has a detrimental affect on the molecules antibacterial activity.

4.2.3 Combined Extracts

Combined ethanol extract testing produced results that showed slightly unexpected values, at face value. Extracts 8 and 9 (CBGV and CBG) produced inhibition zones of around 10 mm and 20 mm against *E. faecium* and inhibition zones of approximately 19 mm and 27 mm against *S. aureus* when tested individually. But when compared to the combined extract results for 8 and 9, both species had a shift toward the stronger CBG zone sizes than they did to the average of the two individual extracts. This could potentially be explained due to the differing concentration of each exaggerated cannabinoid in the different extracts. When mixed together in equal volumes and pipetted (15 µl) onto a blank antibiotic disc, there was a marked difference in cannabinoid amount between the two, with CBGV being present in a mass of approximately 0.27 µg and CBG being present in 3 times that amount, approximately 0.83 µg. This can be the explanation behind the perceived synergistic antibacterial effect they share as CBG is 3 times more prevalent in the extract. T-tests performed between extracts 8 and 9 showed a p-value of 0.0006, below the threshold of 0.05, meaning the data was significantly different. Between extract 8 and the combination extract, containing 8 & 9, a p-value of 0.0001 was produced, also showing a statistical difference between the extract's ZOIs. Extract 9 produced a p-value of 1 when compared with the combined extract, implying there was no significant difference between the two extract's resulting inhibition sizes and thus helps confirm that any synergistic antibacterial effect that appeared in the test is

highly likely to be a result of CBG in the extract, and not a combination effect of the CBG and its varin analogue CBGV.

Likewise, average ZOI sizes for the combination extract containing extracts 4 & 6 were far closer to the individual extract containing THC (4) than it was to the individual THCV extract. On first inspection this would imply a synergistic antibacterial effect between the two extracts. However, extract 4 had 5% THCV by weight while extract 6 had 15.8% THC. The 3 times more concentrated cannabinoid could easily explain the difference in antibacterial activity between the two, instead of implying that THCV has a less active structure. This could be studied further with equal concentrations of cannabinoids.

T-tests performed showed p-values of 0.0002 and 0.0009 between extracts 4 and 6, and extract 4 against the combined extract, respectively. Showing statistically significant differences in the results. The p-value obtained for extract 9 and the combination extract was 0.057, above the 0.05 threshold, and so is not significantly different.

An interesting set of results for extract combination 7 & 9 were produced. Both cannabinoids are expressed in large quantities compared to most samples, with 13% and 11.1%, a much smaller difference than the other two combined extracts discussed above. The results for some species do not fall near to the average as would be expected. Against *S. pneumoniae* extract 7 produced 14.67 mm average ZOI, while extract 9 produced 20 mm on average. When combined, the results gave an average ZOI of 19 mm. Comparing this with the results against *B. cereus*; extract 7 produced 23.33 mm average ZOI, while extract 9 produced 16 mm on average. When combined, the results gave an average ZOI of 17.67 mm. Despite the CBG extract (9) being a stronger inhibitor of growth in *S. pnuemoniae* and weaker in *B. cereus* than the CBD extract (7), the average ZOI for the combined extract was much closer to extract 9's inhibition size than extract 7's. Even in the case where the CBD-type individually produced a 7 mm larger diameter of inhibition than extract 9 did individually, the combined extract was 5.66 mm smaller than extract 7's. Potentially this could mean that the CBG type inhibited the CBD cannabinoid from being effective, or it could mean that there was not enough CBD present on the disc to diffuse far enough, or cause enough inhibition.

For some reason extracts 1 & 2 and 5 & 7 against *S. pneumoniae* produced no growth at all on their plates and so no results could be obtained. No reason was found for the complete lack of growth for these 2 triplicates.

Unfortunately, there is no discernible research relating to combining particular cannabinoids to try and enhance or decrease antimicrobial activity. Studies that have been used for comparison throughout this study either use whole type *Cannabis* with a natural spectrum of cannabinoids (meaning they contained fewer of the rarer cannabinoids, such as CBG and the varin analogues, than the *Cannabis* samples received from E. De Meijer (G. W. Pharmacetuicals) for this study. CBG was found to be less than 10% cannabinoid fraction and never more than total THC or CBD concentrations in 66 *Cannabis sativa* plants (Welling *et al*, 2016)) or they used isolated or fractions of *Cannabis* extracts.

4.2.4 Bacteriostatic/bactericidal results

Research pertaining to the bactericidal or bacteriostatic properties of cannabinoids has not been performed. Very few studies used isolated, or high concentrations of, cannabinoids. Appendino *et al's* (2008) study was the most in-depth to date, using purer forms of cannabinoids and producing results against a variety of species, but even this has no mention of bactericidal or bacteriostatic properties of *Cannabis sativa*. More research must be done on cannabinoids antimicrobial activity and methods of inhibition.

The results from this study, regarding its bactericidal or bacteriostatic, were unreliable. Contamination occurred in many plates and, when repeated, results proved fairly inconsistent, with only 4 swabs producing bactericidal results in triplicate.

Some results, such as in Figure 16, show that there was a lack of consistency in the results for several species. Extract 8 only produced 2 streaks of growth against *B. cereus* in that plate, yet the swabbing and transferring technique were the same for all the 3 swabs.

4.2.5 Result viability

Some of the inhibition zones produced in these experiments against several species were extremely obvious. *S. pneumoniae, B. cereus, S. aureus* and MRSA all produced very clearly defined ZOIs in almost all cases, *S. epidermidis* also consistently produced quite well defined ZOI edges. *S. pyogenes* and *E. faecium* both produced poorly defined inhibition zones in almost all plates. It could be due to the heterogeneity of the strains with varying levels of susceptibility to the cannabinoids. This made measuring such result

harder, and more prone to personal opinion, than the clearly defined measurements taken for the other species. This means that ZOI sizes for *S. pyogenes* and *E. faecium* should be taken cautiously, instead attention should be paid to the MIC values obtained as these are more accurate considering the lawns of both bacteria seemed resistant to growing on solid agar.

All MIC values should be accurate, occasionally a plate would contain contamination and be removed from the measurements leaving duplicate values to compare and deduce an average. But MIC results were consistent in successful plates, so the data appeared consistent when uncontaminated.

Bactericidal and bacteriostatic testing produced a significant number of bacteriostatic results. This is believed to be erroneous and due to the method used, as a small slip could cause the swab or toothpick to touch the un-inhibited lawn of bacteria and become inoculated with live cells which would then provide a false bacteriostatic result.

4.3 Synthetic cannabinoids

As the synthetic cannabinoids tested in this study were not isomers of the natural compounds being tested from the *Cannabis sativa* plant, and only acted upon the same CB1 and CB2 receptors in animals, positive results were not expected. It was surprising then to see any inhibition at all.

Synthetic cannabinoid 5F – PB – 22 produced very minor, almost negligible, inhibition against *B. cereus* and *E. faecium*, and very slightly larger inhibitions against *S. aureus, S. epidermidis, S. pnuemoniae* and *S. pyogenes*. T-tests performed for results of *B. cereus* and *E. faecium* against the DMSO blank disc produced p-values of 0.116 and 0.007, respectively. Showing a significant difference between the results of 5F – PB – 22 and DMSO with no cannabinoid for *E. faecium*, but not significantly different against *B. cereus*.

5F – PB – 22 had no inhibition against MRSA. Whereas 5F – NPB – 22, an indazole derivative of 5F – PB -22 (Kohyama *et al*, 2017), showed slight inhibition against MRSA. The only difference between the compounds is a nitrogen atom replacing a carbon atom in a pentyl ring.

STS – 135 was the only other synthetic cannabinoid that produced any antibiotic activity, only against MRSA. It produced a slightly smaller ZOI than 5F – NPB – 22 did. A T-test

performed for the two sets of data produced a p-value of 0.23, above the 0.05 threshold, and so are not significantly different.

As the structures of the synthetic cannabinoids tested in this experiment were not closely related to the natural cannabinoids being tested, it does not help determine whether the

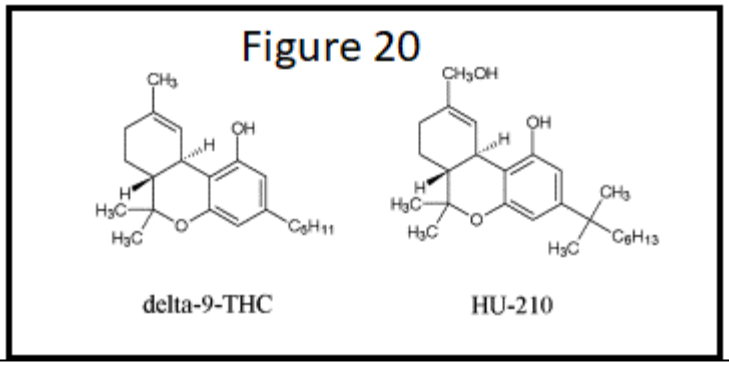


Figure 20. The molecular structure of Delta-9-THC (THC) and HU – 210, a synthetic cannabinoid.

antimicrobial properties of the 8 extracts tested were due to the presence of cannabinoids. It would be interesting in future research to try comparing synthetic cannabinoids that contain very similar structures to natural cannabinoids. Figure 20, derived from Castaneto *et al* (2014), shows the similarities in structure between THC, the main cannabinoid in extract 6 of this study, and HU – 210, a synthetic cannabinoid. In this hypothetical study both compounds could be tested, the *Cannabis sativa* THC chemotype extract against the pure HU – 210 synthetic cannabinoid. Any large differences could imply that there are other antimicrobial compounds working in the whole plant extract than just the main cannabinoid that it has been bred to exaggerate.

There is currently no research available on the applications of synthetic cannabinoids as antimicrobial agents. The efficacy shown in this experiment is low. The concentration of the synthetic cannabinoid extracts were 10mg/ml, much higher than the concentration of the natural cannabinoids in the extracts used in this study, and yet they produced either none, or very little inhibition. This indicates synthetic cannabinoids, or at least the 10 tested in this study, are ineffective antimicrobials.

5 Limitations of the study and future improvements

There were several aspects of the study that could have yielded more meaningful results. Firstly, the selection, and subsequent availability, of only 11 species, 7 of which were Gram positive, allowed for only a small sample size for Gram negative and fungal species. In future studies, purchasing of a wider variety of microorganisms would be a more

meaningful approach over solely using the organisms held by the university technicians. More Gram negative and fungi species were needed in this experiment. The sample size of 2 is considered too low to be able to draw a general conclusion for the extract's efficacy against Gram negative bacteria and fungi. Potentially more than one strain could have been used for each species as this could have identified if there was a difference in antimicrobial ability between strains of the same species and could potentially explain why some *Cannabis sativa* apparently works against Gram negatives and some do not.

Secondly, a few varieties of wild *Cannabis sativa* grown without human intervention to manipulate cannabinoid levels, would have been interesting to incorporate into the study. It could provide a more balanced cannabinoid profile and could help to decipher where the antimicrobial properties of *Cannabis* come from. This could further be supplemented using pure isolates of each of the major cannabinoids, buying them where available, and using column chromatography to isolate any cannabinoids that are too expensive or are not sold. Alternatively, it would be useful if *Cannabis sativa* variants could be found that exaggerated individual cannabinoids, like the *Cannabis* used in this study, but at the same levels. If all *Cannabis* samples contained, for example, 10% of the chosen cannabinoid then it could allow for much more meaningful comparisons between the cannabinoids.

The extracts themselves did not actually prove that the cannabinoids, that were exaggerated differently in each individual plant, were responsible for any antimicrobial inhibition they may have caused. Given more time, it would be interesting to use column chromatography in the future to prepare fractions of each plant chemotype and testing each one's antimicrobial ability. This would allow for genuine deduction of where the inhibitory effects of *Cannabis sativa* come from and whether it is due to a handful of compounds or a large collection of compounds that work better when combined.

The volumes of extracts in the main ethanolic test of this experiment were only 15 µl per disc. In future it would be more useful to use a variety of volumes, at least 3.75, 7.5, 30, and 60 µl, this could allow for weaker extracts to potentially show their inhibition better and stronger extracts to more accurately demonstrate their antimicrobial properties. This could be used to create graphs to help determine MICs.

The synthetic compounds used in this study provided little positive data. In future it would be interesting to test synthetic analogues that are more closely related to the natural cannabinoids as this would help to figure out whether the cannabinoids are the

source of *Cannabis sativa*'s antimicrobial ability, and if so which arrangements are most effective. This could have ramifications for antibiotic synthesis in the future.

In future experiments, bactericidal and bacteriostatic testing should be performed differently than in this study. The method used for this experiment was too easily prone to false negative results for bactericidal results, as the ZOI of some species were small, any swab or toothpick used could accidentally be contaminated by the lawn of surviving microorganism, which could explain why there are so many bacteriostatic results in this experiment. Using a similar technique to the MIC 96 well plates used in this experiment, dilutions of each extract could be used against broth with the same concentration of colonies. The weakest extract that works could then be transplanted to an agar plate and incubated to see if the species regrows, less chance of contamination would mean more meaningful results.

Given the time, it would have been interesting to try many combinations of extracts for the combination testing including in combinations of 3 or more and in higher concentrations. This could produce results proving if a wide variety of cannabinoids is more powerful, or in some cases successful at all, against certain species. There are not a lot of studies that have been performed that combine several cannabinoids, they usually contain either purer isolates or whole *Cannabis sativa* extracts that do not contain minor cannabinoids in relevant concentrations.

Finally, the literature on *Cannabis sativa* and its use as an antimicrobial is sparse. Some of the cannabinoids tested in this study have essentially no studies regarding their ability as antimicrobials, mainly the varin analogues; CBCV, CBDV, CBGV and THCV. In the future it would be ideal for more research to be done in the field, focusing on rarer cannabinoids as well as the major 4 types (CBC, CBD, CBG and THC), this could be used for much stronger comparison and debate.

Conclusion

The results from this study showed that *Cannabis sativa* extracts were effective antimicrobials, *in vitro*, against all species of Gram positive organisms tested and that varying degrees of inhibition were achieved by the different chemical phenotypes of the plant. Gram negative bacterial and fungal species that were tested all proved to be resistant, even to specifically made extracts 10 times the concentration of the successful

Gram positive extracts. The negative results are contrasted by studies that have shown that *Cannabis* and its isolated compounds are antimicrobial against these microorganisms, but the results are corroborated by studies which have also concluded that *Cannabis* extracts do not work against Gram negative species and fungi tested. What makes this more interesting is that the *Cannabis sativa* varieties used in this study contained high levels of the desired cannabinoid. So, when compared with isolated cannabinoids like Appendino *et al* (2008) used in their study, the results would have been expected to have a close relationship. But this was not the case for some species and cannabinoids and so, if the cannabinoids were the source of antimicrobial activity, why did these extracts show no inhibition against Gram negative species when studies like Naveed *et al* (2014) and Ali *et al* (2012) showed whole plant cannabis resulted in inhibition against Gram negative species? The cannabinoids present in the *Cannabis sativa* used here were bred to be exaggerated and so undoubtable in higher levels than the studies that produced inhibition against Gram negative species, yet the results were opposite.

This study provided results that proved different and distinct chemical phenotypes of *Cannabis sativa* produce different antibacterial activity. They showed that extracts containing *Cannabis* bred to exaggerate cannabichromene (CBC) and cannabichromavarin (CBCV) were the least effective against all species on average for ZOIs and MICs. Cannabigerol (CBG) was the *Cannabis* extract that proved the strongest, in most cases, for ZOIs and MICs. Its analogue, CBGV, had equally low MICs but due to it containing only around 1/3 of the desired cannabinoid than the CBG type cannabis, it did not produce equal results regarding ZOIs. Cannabidiol (CBD) and delta-9-tetrahydrocannabinol (THC) both proved to be strong antimicrobials for ZOIs and MICs with their analogues being slightly worse. The results imply that the different cannabinoid emphasised in each extract is responsible for the differing ZOIs and MICs but this cannot be proven as there are too many potentially antimicrobial agents within the plant that may be responsible.

There is a lack of comparable evidence in the field of *Cannabis* antimicrobials, especially regarding varin analogues, the amount of studies is currently small, and the results obtained in said studies are constantly contradictory. This emphasises that the *Cannabis sativa* plant could have an abundance of antimicrobial compounds in its arsenal, not just the popular cannabinoids. These compounds may be dependent on the genetics of each plant, and therefore are widely variable, and thus could be responsible for the contradictory results that have been found. More research needs to be done in this area

to understand the mechanisms and sources of the *Cannabis sativa's* antimicrobial activity, and also whether any of these effects are retained *in vivo*, as nearly all research to date has focused on inhibiting microorganisms *in vitro*.

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