

Antibacterial activity of cannabis extract (*Cannabis sativa* L. subsp. *indica* (Lam.)) against canine skin infection bacterium *Staphylococcus pseudintermedius*

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ABSTRACT: Superficial bacterial skin infections caused by *Staphylococcus pseudintermedius* are common in dogs. The cannabis plant has shown potential antibacterial effects, but there is limited information about anti-*S. pseudintermedius* activity. This study investigated the antibacterial activity of the ethanolic extract and the topical formulations of *Cannabis sativa* L. subsp. *indica* (Lam.) against 23 *S. pseudintermedius* isolates obtained from canine pyoderma using broth microdilution and time-kill assays. The cannabis extract's minimum inhibitory concentrations for 50% (MIC₅₀) and 90% (MIC₉₀) of the tested *S. pseudintermedius* isolates were 6.25 and 12.50 µg/ml, respectively. The MIC for *S. pseudintermedius* ATCC 49051 was 6.25 µg/ml. The time-kill test revealed that the bactericidal effect of the cannabis extract was time- and not concentration-dependent. Topical formulations containing 0.5, 1, 2, 5, and 10% w/w cannabis extract showed limited *in vitro* antimicrobial activity in the time-kill test. Only the formulations with cannabis extract of 10% and 5% w/w could reduce the number of viable *S. pseudintermedius* ATCC 49051 by more than 3-log₁₀ (99.9%) at 6 and 24 h, respectively. This study demonstrated that the cannabis extract has a good potential to be developed as an antibacterial agent against *S. pseudintermedius*. However, a clinical trial should be conducted to determine its *in vivo* therapeutic efficacy.

KEYWORDS: antibacterial activity, *Cannabis sativa* subsp. *indica*, *Staphylococcus pseudintermedius*, topical solution formulations

INTRODUCTION

Canine superficial pyoderma is a common dermatological disease caused by the bacterium *Staphylococcus pseudintermedius* [1]. It presents initially as erythematous papules and pustules that develop into dry, scaly, and crusted skin; and it is often associated with hair loss and itches [2]. Current treatments for canine superficial pyoderma include systemic and topical antibiotics such as amoxicillin, cephalexin, clindamycin, and mupirocin given over a three to four-week period [3]. The long treatment times and increasing incidence of antibiotic resistance in *S. pseudintermedius* isolates means that many animals suffer from recurrent infections [4, 5]. Moreover, antibiotics not only cause adverse effects such as vomiting, diarrhea, and anorexia but also affect hepatic and renal function [3]. The use of non-antibiotic topical agents as a first-line treatment for superficial pyoderma has some advantages. Topically applied treatments with lotions, shampoos, ointments, gels, and sprays containing benzoyl peroxide, sulfur, chlorhexidine, and povidone iodine can be used at high concentrations at the site of infection; and crusts, scales, and debris from the infected skin surface can be removed during the application. However, these products may cause skin irritation, erythema, and photosensitivity [6]. Therefore, there is a need to develop topical treatments with good

antimicrobial activity against staphylococci bacteria for canine superficial pyoderma and without associated adverse effects.

The cannabis plant, *Cannabis sativa*, has been shown to have many pharmacological properties as well as antibacterial activity against Gram-positive bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA) [7]. This antibacterial activity is proposed to derive from the cannabinoids present in cannabis extracts [8]. A single previous investigation of the antibacterial activity of *C. sativa* essential oil against eight clinical *S. pseudintermedius* strains found modal minimum inhibitory concentrations of 1/16 and 1/32 dilutions of the oil, but the cannabinoid content of the oil was not described [9]. Here, we describe the antibiotic activity of cannabis extract against *S. pseudintermedius* strains isolated from canine superficial pyoderma and the bactericidal effect and time-kill kinetics of topical formulations of the extract.

MATERIALS AND METHODS

Microbial strains and culture conditions

A total of 23 *S. pseudintermedius* isolates were obtained from the Veterinary Pharmacology Laboratory, Faculty of Veterinary Medicine, Khon Kaen University, Thailand. The isolates were identified and confirmed using the polymerase chain reaction-restriction frag-

ment length polymorphism (PCR-RFLP) method by the Veterinary Diagnostic Laboratory, Animal Hospital, Faculty of Veterinary Medicine, Khon Kaen University, Thailand. The control bacterium *S. pseudintermedius* ATCC 49051 was obtained from the American Type Culture Collection (ATCC), Virginia, USA. All strains were cultured in Mueller Hinton broth (MHB) (Becton Dickinson, France) and incubated at 37°C for 24 h. Inocula were prepared by adjusting overnight cultures to 10^6 – 10^7 CFU/ml by measuring the optical density (OD) at 600 nm [10].

Cannabis extract

Cannabis oil (ethanolic extract of *C. sativa* L. subsp. *indica* (Lam.)) was obtained from the Government Pharmaceutical Organization, Ministry of Public Health, Thailand and approved for research uses by the Food and Drug Administration (FDA), Thailand. The major cannabinoid constituents of the oil were tetrahydrocannabinol (THC, 317.4 ± 34.9 mg/g of crude oil), cannabidiol (CBD, 36.6 ± 4.9 mg/g of crude oil), and cannabidiol (CBD, 36.1 ± 4.0 mg/g of crude oil) as determined, using high-performance liquid chromatography (HPLC), by the Center for Research and Development of Herbal Health Products, Faculty of Pharmaceutical Sciences, Khon Kaen University, Thailand.

Topical cannabis formulations

The composition of 5 topical cannabis formulations (F2–F6) and a control (F1) are shown in Table 1. The cannabis extract was formulated as solutions containing 0.5% (F2), 1% (F3), 2% (F4), 5% (F5), and 10% (F6) w/w cannabis oil. The control formulation (F1) contained all ingredients except cannabis oil.

Determination of cannabis extract antibacterial activity by broth microdilution

A stock solution of cannabis extract was prepared by dissolving 160 mg of the cannabis oil in 100 ml of dimethyl sulfoxide (DMSO) (V.S. Chem House, Ayutthaya, Thailand). The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) of the cannabis extract against the 23 *S. pseudintermedius* isolates and *S. pseudintermedius* ATCC 49051 were performed by the broth microdilution method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines [11], with some modifications. Briefly, 100 μ l of the cannabis extract stock solution was serially 2-fold diluted with MHB in 96-well round-bottomed microtiter plates (Costar®, Corning Incorporated, USA) to obtain a final concentration range of 1.56–800 μ g/ml. Then, 100 μ l of the bacterial suspension (1×10^6 CFU/ml) was added into tested wells, and the microtiter plates were incubated at 37°C for 18 h. Wells containing bacteria without cannabis extract served as growth controls. Cephalexin (Sigma-Aldrich, US; 0.5–256 μ g/ml) and

DMSO (0.49–250 μ l/ml) were used as antibacterial and solvent controls, respectively. The MIC was defined as the lowest concentration of the extract that inhibited visible growth after 18 h of incubation. One hundred microliter samples from the MIC wells were inoculated onto Mueller Hinton agar (MHA) plates (Becton Dickinson, France) and incubated at 37°C for 24 h. The MBC was determined from the lowest concentration of the extract that showed no growth on MHA. The MIC_{50/90} and MBC_{50/90} were defined as the lowest concentrations of cannabis extract that inhibited visible growth or killed bacteria in 50% or 90% of tested isolates, respectively. All tests were performed in triplicate.

Time-kill assay

The time-kill kinetics of the cannabis extract and the topical formulations were determined by the time-kill test method previously described by Aiamsaard et al [12]. Briefly, 100 μ l of *S. pseudintermedius* ATCC 49051 suspension (1×10^7 CFU/ml) was mixed with 900 μ l of either the stock 1600 μ g/ml cannabis extract diluted with normal saline to give final concentrations of 1, 5, 10, and 20-times the MIC or individual cannabis extract formulations (F1–F6). After incubation at 37°C for 15 and 30 min; 3, 6, and 24 h at 37°C; 100 μ l of the mixture was serially 10-fold diluted with normal saline solution and 100 μ l samples of 10^0 to 10^{-4} dilutions were inoculated onto MHA plates. After incubation at 37°C for 24 h, bacterial colonies were counted and recorded. Each experiment was performed in triplicate.

RESULTS

Minimum inhibitory and bactericidal concentrations

MIC and MBC results for the cannabis extract and the cephalexin against the 23 *S. pseudintermedius* canine pyoderma isolates and *S. pseudintermedius* ATCC 49051 are shown in Table 2. The DMSO did not show any antibacterial effect against the 23 tested bacterial isolates at all concentrations of the extract (all MICs > 250 μ l/ml). However, the cannabis extract showed a better antibacterial activity with slightly higher MIC and MBC values than those of cephalexin. For *S. pseudintermedius* ATCC 49051, the cannabis extract and the cephalexin MICs were 6.25 μ g/ml and 4.00 μ g/ml, respectively. The MIC ranges of the 23 tested *S. pseudintermedius* isolates were 3.13–12.50 μ g/ml for the cannabis extract and 1.00–64.00 μ g/ml for the cephalexin, and the cannabis extract MIC₅₀ (6.25 μ g/ml) and MIC₉₀ (12.50 μ g/ml) values were higher than their corresponding cephalexin values (2.00 and 4.00 μ g/ml, respectively). In general, MBC values were higher than MIC values for both the cannabis extract and the cephalexin. The MBC range was 6.25–100.00 μ g/ml for cannabis extract and

Table 1 Ingredients of 100 g of cannabis extract formulation.

Ingredient	Amount (g)					
	F1	F2	F3	F4	F5	F6
Cannabis extract	0	0.5	1	2	5	10
Polyoxyl 35 hydrogenated castor oil	12.5	0.625	1.25	2.5	6.25	12.5
Diethylene glycol monoethyl ether	2.5	0.125	0.25	0.5	1.25	2.5
Poloxamer 407	12	12	12	12	12	12
Water	73	86.750	85.50	83	75.50	63

Table 2 The antibacterial activities of cannabis extract and cephalexin against *S. pseudintermedius*.[†]

Antimicrobial agent	<i>S. pseudintermedius</i> isolates (n = 23)				<i>S. pseudintermedius</i> ATCC 49051	
	MIC ($\mu\text{g/ml}$)		MBC ($\mu\text{g/ml}$)		MIC ($\mu\text{g/ml}$)	MBC ($\mu\text{g/ml}$)
	MIC ₅₀	MIC ₉₀	MBC ₅₀	MBC ₉₀		
Cannabis extract	6.25	12.50	25.00	100.00	6.25	25.00
Cephalexin	2.00	4.00	4.00	16.00	4.00	4.00

[†] Values represent the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) collected from triplicate experiments. MIC₅₀ or MIC₉₀ = MIC for 50% or 90% of tested samples. MBC₅₀ or MBC₉₀ = MBC for 50% or 90% of tested samples.

1.00–256.00 $\mu\text{g/ml}$ for cephalexin, and the cannabis extract MBC_{50/90} values were 6 times higher than the cephalexin MBC_{50/90} values.

Time-kill kinetics of cannabis extract

The time-kill test showed that increasing the concentration of cannabis extract by 5- to 20-times did not increase the killing effect against *S. pseudintermedius* ATCC 49051 (Fig. 1). All concentrations from 1-time

MIC to 20-time MIC reduced the number of viable bacteria from 1.74×10^6 CFU/ml by about 1- \log_{10} or 90% (range 87.41–97.12%) at 15 and 30 min and by 2- \log_{10} or 99% (range 99.57–99.81%) at 3 h. Bactericidal activity corresponding to a 3- \log_{10} or 99.9% reduction in surviving bacteria was seen at 6 h for all concentrations of cannabis extract. Eradication of *S. pseudintermedius* ATCC 49051, which corresponded to a 99.9999% or 6- \log_{10} reduction, occurred at 24 h

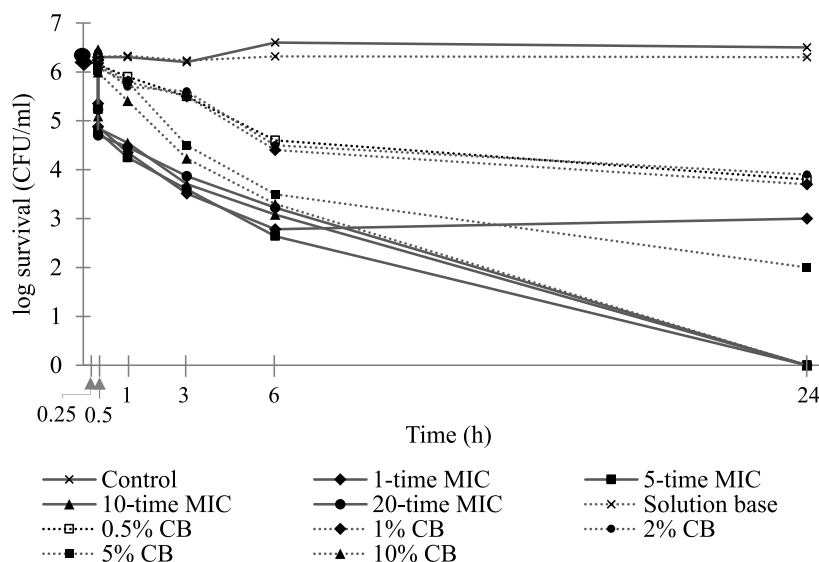


Fig. 1 The time-kill assay of cannabis extract and topical formulations against *S. pseudintermedius* ATCC 49051. MIC = minimum inhibitory concentration for *S. pseudintermedius* ATCC 49051; 1-time MIC = 6.25 $\mu\text{g/ml}$; 5-time MIC = 31.25 $\mu\text{g/ml}$; 10-time MIC = 62.50 $\mu\text{g/ml}$; and 20-time MIC = 125.00 $\mu\text{g/ml}$. Control = normal saline solution. The cannabis extract formulation concentrations (CB) refer to percent weight per weight (% w/w). Solution base = a formulation containing 0% w/w cannabis extract.

for bacteria exposed to concentrations of 5-, 10-, and 20-time MIC. The control showed no antibacterial activity throughout the 24 h of incubation.

Physical characteristics and time-kill kinetics of topical cannabis formulations

The prepared topical cannabis formulations were clear, dark brownish green liquids, and free of fractionation and sedimentation. The color intensity and the viscosity of the formulations increased as the concentration of the extract increased (Fig. S1). The formulation base (control) showed no antibacterial effect in the time-kill test, and each topical formulation of cannabis extract (0.5, 1, 2, 5, and 10% w/w) had different antibacterial activities (Fig. 1). All the cannabis formulations showed reduced antibacterial activity compared with the cannabis extract in DMSO, with less than a 1- \log_{10} reduction in the number of viable bacteria at 15 and 30 min. The 10% w/w cannabis formulation showed the most antibacterial activity, reducing the number of viable bacteria by 90% at 1 h, by 99% at 3 h, by 99.9% at 6 h, and eradicating 99.9999% of the viable *S. pseudintermedius* ATCC 49051 at 24 h. The 5% w/w cannabis formulation reduced the number of viable bacteria by 90% at 3 h, 99% at 6 h, and 99.9% at 24 h but did not eradicate the bacteria. The formulations containing 0.5–2% w/w showed only a maximum 2- \log_{10} or 99% reduction in the number of viable bacteria at 24 h.

DISCUSSION

The current study showed that the medicinal cannabis extract, supplied by the Thai Government Pharmaceutical Organization, showed good antibacterial potential against *S. pseudintermedius* isolates and *S. pseudintermedius* ATCC 49051. The extract was effective at concentrations slightly higher than the antibiotic cephalixin, which is commonly used to treat canine pyoderma associated with *S. pseudintermedius* [3]. Previous studies have indicated that cannabis extract and its cannabinoid constituents have good antimicrobial effects, especially against staphylococcal bacteria. Appendino et al [13] reported that pre-cannabidiol, cannabidiol, cannabichromene, cannabigerol, Δ^9 -tetrahydrocannabinol, and cannabinal isolated from *C. sativa* had MIC values against MRSA in the range of 1–2 $\mu\text{g/ml}$, lower than norfloxacin (4 $\mu\text{g/ml}$), tetracycline, and oxacillin (both 128 $\mu\text{g/ml}$). Similarly, Martinenghi et al [14] showed that cannabidiolic acid and cannabidiol had MICs against MRSA and *Staphylococcus epidermidis* in the range of 1–4 $\mu\text{g/ml}$ (compared with tobramycin 1 $\mu\text{g/ml}$, meropenem 2–16 $\mu\text{g/ml}$, and ofloxacin 1–64 $\mu\text{g/ml}$), while the cannabinoid MICs for *Escherichia coli* and *Pseudomonas aeruginosa* were more than 64 $\mu\text{g/ml}$. In addition, they conducted a time-kill assay showing that cannabidiol had a bactericidal effect at concentrations

2 to 8-times its MIC (2–8 $\mu\text{g/ml}$) against MRSA, and that this effect was both dose- and time-dependent. This finding was in accordance with a study by Farha et al [15] who found that the cannabigerol, cannabidiol, cannabinal, cannabichromenic acid, and Δ^9 -tetrahydrocannabinol MICs against MRSA were 2 $\mu\text{g/ml}$, and that cannabigerol inhibited biofilm formation at 0.5 $\mu\text{g/ml}$ and had a minimal biofilm eradication concentration of 4 $\mu\text{g/ml}$. Once again, this activity was dependent on both dose and time. The time-kill kinetics of the cannabinoids in these studies were different from those described in the current study, which showed only time-dependent anti-*S. pseudintermedius* activity for 5–20 times the MIC of the cannabis extract. This could be due to differences in the type of test organism as our study was the first to examine the effects of cannabis extract against *S. pseudintermedius*. We also used a crude ethanolic extract of cannabis that contained several types of cannabinoids as well as other compounds, which may affect antimicrobial activity [16, 17].

The topical formulations developed in this study showed limited bactericidal activity *in vitro*. The formulations were prepared at concentrations of 0.5–10% w/w, which is many times higher than the concentrations of the extracts that were used to determine the MICs. The highest concentrations of cannabis oil used for the MIC and time-kill experiments were 800 $\mu\text{g/ml}$ and 125 $\mu\text{g/ml}$, respectively, compared with the concentrations of 5000–100 000 $\mu\text{g/ml}$ in the formulations. Despite this increased amount of cannabis extract, the 0.5–2% formulations could only reduce the number of *S. pseudintermedius* ATCC 49051 in the time-kill assay by 2- \log_{10} after 24 h, compared with at 3 h for all the tested crude extracts. Furthermore, the formulations showed both time- and concentration-dependent antimicrobial effects in the time-kill assay, in contrast to the solely time-dependent effects seen for the crude extracts. These *in vitro* differences in the antibacterial effects of the crude cannabis extract and the topical formulations are likely due to the additional ingredients that are required for an effective topical treatment. For topical drugs, the properties of excipients in the formulation may affect the formulation's action because they can affect the release of the active ingredients from the formula [18]. In our time-kill assay experiments, the formulations did not have the same antibacterial effect as the crude extract despite being at much higher concentrations, and this may be because the excipients affected the release of cannabinoids from the formulation. Only a formulation with the highest concentration of cannabis extract was able to eradicate the bacteria after 24 h.

Phytocannabinoids are natural cannabinoids found in the cannabis plant. They are terpeno-phenolic compounds that are sparingly soluble in water, but highly soluble in non-polar and organic

solvents [19,20]. So, to prepare an aqueous formulation using water as a vehicle, we used diethylene glycol monoethyl ether (DEGEE) as the solvent and polyoxyl 35 hydrogenated castor oil and poloxamer 407 as the emulsifier. DEGEE is widely used as a solvent in pharmaceuticals, cosmetics, and food additives because it is non-toxic and biocompatible with the skin. This substance allows the drug to penetrate the skin and provides sustained drug release [21–23]. Poloxamer 407 helps lipophilic agents dissolve better in water and acts as a gelling agent inside the formula forming a hydrogel able to entrap and embed active compounds resulting in controlled release of the drug [24]. Slow-release formulations of topical antimicrobial drugs promote continuous and prolonged antimicrobial activity in the desired area, which is a great advantage for drugs that have time-dependent effects. These types of formulations reduce the number and frequency of applications, which reduces patient noncompliance, and they also decrease the possibility of reaching toxic levels of drugs at the application sites [25].

There is currently limited information on the mechanism of antibacterial action of phytocannabinoids. However, some studies have shown that the lipophilic side chain and phenolic hydroxyl moieties of cannabigerol and cannabidiol-type cannabinoids are crucial for anti-staphylococcal activity [13]. These moieties disrupt the integrity and the function of the cytoplasmic membrane of Gram-positive bacteria [15]. In addition, a molecular docking, computational modelling study of cannabinoids against potential targets (iso-tyrosyl RNA synthetase, penicillin-binding protein, and DNA gyrase) suggested that the antibacterial activity may be related to the inhibition of penicillin-binding proteins, affecting the Gram-positive cell wall, but not iso-tyrosyl RNA synthetase and DNA gyrase [26]. A radiolabeled macromolecular synthesis assay found that cannabidiol inhibits protein, DNA, RNA, peptidoglycan, and phospholipid synthesis in *S. aureus* [27].

CONCLUSION

Cannabis extract showed good activity against *S. pseudintermedius*. The bactericidal activity with MIC values in the range of 3.13–12.50 µg/ml showed the MIC₅₀ and the MIC₉₀ values of 6.25 µg/ml and 12.50 µg/ml, respectively. A time-kill assay using extract concentrations 1–20 times the MIC showed that cannabis extract had a time- and not a dose-dependent antibacterial effect. Topical solutions containing 0.5, 1, 2, 5, and 10% w/w cannabis extracts were less effective against *S. pseudintermedius in vitro*. This study demonstrated that cannabis extract has the potential to be developed as an antibacterial agent. Further studies should be conducted to determine the stability, *in vivo* therapeutic efficacy,

and skin irritation/sensitization of topical formulations against *S. pseudintermedius* causing canine superficial pyoderma in animal studies.

Appendix A. Supplementary data

Supplementary data associated with this article can be found at <http://dx.doi.org/10.2306/scienceasia1513-1874.2022.053>.

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Appendix A. Supplementary data

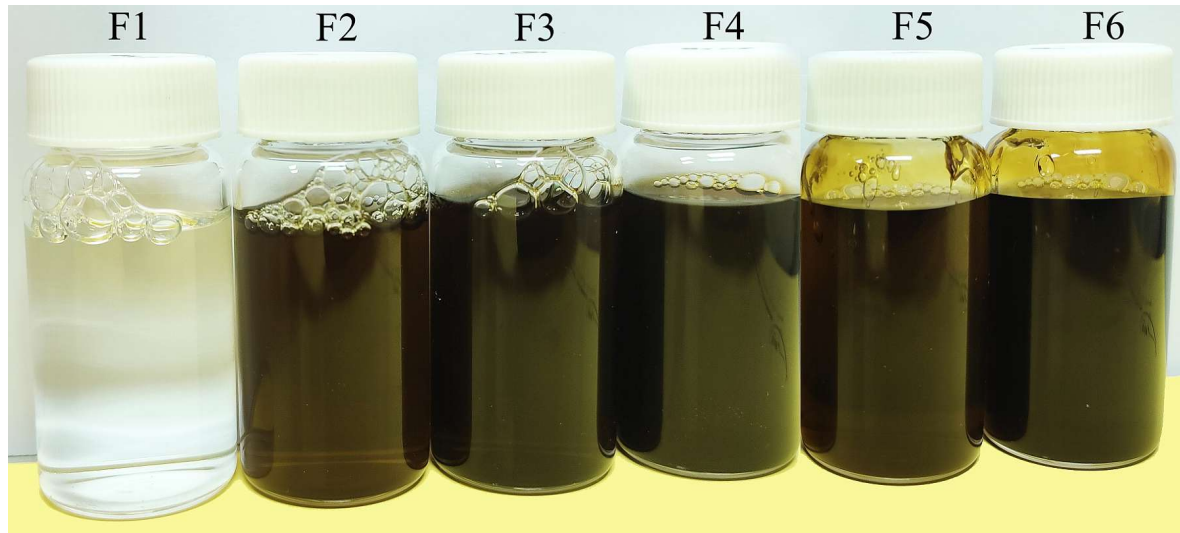


Fig. S1 The appearance of cannabis extract solution formulations: (F1) solution base; (F2) 0.5% w/w cannabis extract; (F3) 1% w/w cannabis extract; (F4) 2% w/w cannabis extract; (F5) 5% w/w cannabis extract; and (F6) 10% w/w cannabis extract.