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Literature Reviews

Optimization of Extraction of Cannabinoids and Flavonoids Substances from Influence *Cannabis sp.* and their Biological Activities before and after Decarboxylation

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Abstract: Inflorescence of eight different varieties of *Cannabis sp.* (3 hemp varieties and 5 medical cannabis varieties) were subjected to extraction with n-hexane or ethanol as representatives of non-polar and polar solvents. Crude extracts were either used directly or after decarboxylation of cannabis acids for measurement of selected biological activities-cytotoxicity against carcinogenic line B16 (musculus skin melanoma cells), cytotoxicity against Human Dermal Fibroblast (HDF) as a control non-carcinogenic line, antimicrobial activity against Candida albicans (DBM, 2186), Staphylococcus aureus (ATCC, 25923) and Salmonella enterica (CCM, 4420) and antioxidant activity using the ORAC (Oxygen Radical Absorbance Capacity) assay. 32 samples with a different profile of cannabinoids measured by HLPC were thus obtained and evaluated. The content of total flavonoids that could exert synergic action on bioactivity was measured by the spectrophotometric method. The hexane extracts showed higher antimicrobial activity, decarboxylation of the samples led to an increase in cytotoxicity against the human skin fibroblast line in most of the studied samples, and to decrease in the antioxidant effects of the decarboxylated extracts. The obtained findings suggest a therapeutic potential of the selected cannabis extracts in the treatment of skin cancer, skin wounds, or surgical wound healing.

Keywords: Cannabinoids, Flavonoids, *Cannabis Sativa*, Entourage Effect, Cytotoxicity, Antimicrobial Effect, Antioxidant Activity

Introduction

Today, cannabinoids from cannabis plants are becoming increasingly popular in the pharmaceutical, cosmetics, and food industries. From a taxonomic point of view, hemp or marijuana belongs to the family of Cannabaceae, which also includes Humulus lupulus-hops. Cannabis plants can be divided into three species: Cannabis sativa, Cannabis indica, and Cannabis ruderalis. Technical hemp is a bred variety of Cannabis sativa with low content of Tetrahydrocannabinol (THC) (McPartland and Guy, 2017). Cannabinoids are compounds that have many effects on the human body and include molecules that are structurally similar to Δ9-Tetrahydrocannabinol (Δ9-THC), so they can interact with cannabinoid receptors. Cannabinoids affect the cellular response through different cellular pathways initiated by two types of G-Protein Coupled Receptors (GPCRs): The cannabinoid receptor type 1 (CB1) and the cannabinoid receptor type 2 (CB2) (Fonseca *et al*., 2017). These G-protein coupled receptors are associated with the inhibition of adenylate cyclase, which catalases the synthesis of the cyclic nucleotide-adenosine monophosphate (Rempel *et al*., 2013). Cannabinoids could be divided into three groups: Endocannabinoids, which are produced by the human body, next phytocannabinoids produced by plants mainly by *Cannabis sp.*, and synthetic cannabinoids that are synthesized artificially. All three types of cannabinoids

have a similar structure, the main difference between them is in the different cyclization of precursors. For example, in plants, phytocannabinoid biosynthesis follows from fatty acids and isoprenoids synthesis. This means that cannabinoids are polyketides of mixed biosynthetic origin. The first precursor-hexanoyl-CoA formed during the biosynthesis of fatty acids, is converted by the enzyme polyketide ligase to olivetol acid. This acid is further geranylated, with geranyl pyrophosphate to give Cannabigerolic Acid (CBGA). This acid is then converted by specific acid synthetases to THCA (tetrahydrocannabinolic acid) or CBDA (cannabidiolic acid), Fig. 1 (Park *et al*., 2017; Tsala *et al*., 2015; Morales and Reggio, 2019; Protti *et al*., 2019).

Cannabinoids both function as agonists and antagonists of cannabinoid receptors. Δ9-THC is a partial agonist of CB1 and CB2 receptors. The affinity of THC to these receptors is lower than the other endocannabinoids but higher than other phytocannabinoids (Pertwee, 2008). But THC can also act as an antagonist, which was unhappy before, of CB receptors and inhibited the effect of endocannabinoids. The affinity for CB1 and CB2 by CBD is not so high. CBD is more often known as an antagonist to CB1 and CB2 than an agonist. As an antagonist, CBD can inhibit anandamide uptake and metabolism. CBD works mainly at micromolar concentrations, which are at least 1,000-fold higher than those observed while using THC. Also, it has been suggested that at low concentrations, CBD acts as an inverse agonist binding to the same receptors as other agonists but causing a different physiological effect (Kluger *et al*., 2015; Tomko *et al*., 2020; Pertwee, 2008). First evidence that CBD can behave like an inverse agonist to CB receptor was proved firstly in the study by Kovalchuk and Kovalchuk (2020). The ability of cannabidiol to behave as a CB2 receptor inverse agonist may contribute to its documented anti-inflammatory properties (Kovalchuk and Kovalchuk, 2020).

CB1 and CB2 receptors are very important for keeping homeostasis, important for the health of an organism. Cannabinoids that bind to endocannabinoid receptors can favorably affect neurodegenerative diseases as well as cardiovascular disease and cancer (Miller and Devi, 2011). Differences in the endocannabinoid system vary depending on the type of cancer, so they may differ. The level of anandamide is 2-3 times higher in adenomas and colorectal cancers than in normal mucosa (Michalak *et al*., 2016). In the study by Sarfaraz *et al*. (2008) it is stated that in the case of prostate cancer the increased densities of these receptors may correlate with a better prognosis too. CB1 receptors generally prevent cell death, CB2 receptors have anti-inflammatory effects, inhibit the production of inflammatory cytokines (IL-2, IFN-γ) and activate the function of anti-inflammatory cytokines (IL-4, IL-10) (Saroz *et al*., 2019). CB2 receptors also prevent cytotoxicity, especially by inhibiting T-cells. Inflammation plays an important role in the pathology of neurodegenerative diseases such as AD (Alzheimer's disease) or MS (Multiple sclerosis), so CB2 could be a possible control tool for inflammatory reactions and therapeutic interventions. The use of CB2 receptors for neuroprotection also positively induces psychotropic side effects of cannabinoids that are processed via CB1. CB2 receptors render microglia susceptible to modulation by cannabinoids. Cannabinoids inhibit the generation of cytotoxic molecules and suppress microglial activation while promoting their migration. The upregulation of CB2 on microglia is thus possibly a protective mechanism that limits inflammation and disease pathogenesis. This may not only be because CB2 blocks AB-induced microglial activation but also because CB2 restores the abilities of microglia to remove AB plaques. Thus, by reduction of AB, CB2 prevents AD-induced neurotoxicity (Walter and Stella, 2004).

Since many simple phenols show antimicrobial properties, it could be assumed that the resorcinol moiety of cannabinoids serves as the antibacterial pharmacophore, with the alkyl, terpenoid, and carboxylic appendices modulating its activity.

CBG (cannabigerol) and CBD (cannabidiol) are not psychotropic, because of their structure, they are often used in studies for their antimicrobial effect for use in pharmacy or cosmetics. Cannabinoid antibacterial chemotype is remarkably tolerant of structural modification of the terpenoid group and its positional relationship to the n-pentyl chain, suggesting that these residues serve mainly as modulators of lipid affinity and thus cellular bioavailability.

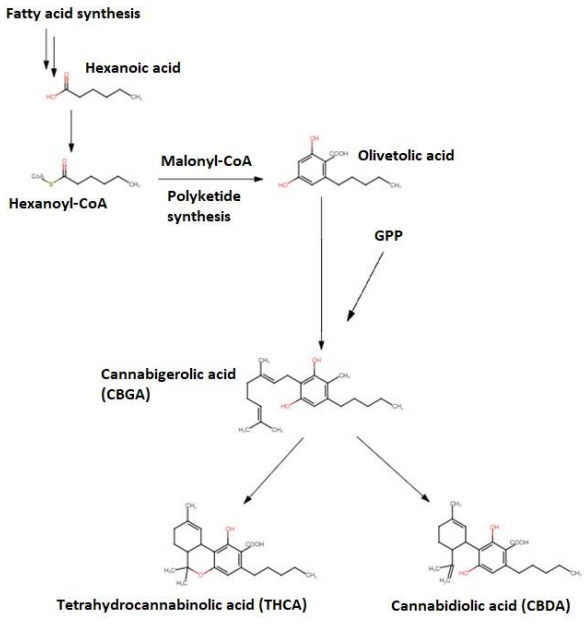

Fig. 1: Biosynthesis of CBDA and THCA (Tsala *et al*., 2015)

The high potency of cannabinoids suggests a specific interaction with a bacterial target, but whose identity is still in sight (Appendino *et al*., 2008). Given that technical hemp with a low THC content and especially species with a high content of non-psychotropic cannabinoids such as CBD or CBG are easily available, it is possible to use them as a source of substances having antibacterial effects, especially against pathogens and other harmful bacteria. Many microorganisms have already been tested for resistance to cannabinoids. Cannabinol showed potent activity against a variety of methicillin-resistant Staphylococcus Aureus (MRSA) strains (Appendino *et al*., 2008). Proved was also cannabinoids' antimicrobial effect against *Mycobacterium tuberculosis*, Gram-negative bacteria of the Coli-typhus group, *Pseudonymous aeruginosa* and *Proteus Vulgar*, acid-fast bacteria, yeastlike fungi, filamentous fungi, and a dermatophyte (Klahn, 2015; Lone and Lone, 2012; Zarina and Tan, 2013). Typical minimum inhibitory concentration and antibacterial activity of THC and CBD for staphylococci and streptococci in the medium are in the range of 1-5 $ug.ml^{-1}$. Cannabinoids also have strong antileishmanial activity and are effective in killing *Candida albicans*, a yeast that has an oral and genital area infection (Feldman *et al*., 2021). Aqueous extracts of cannabis leaves are also effective against bacteria such as *Cryptococcus reforms*, in a concentration less than 10 µg/mL and the same concentrations against *Vibrio cholera* (Lone and Lone, 2012).

In cannabis, CBD occurs naturally in the form of Cannabidiol Acid (CBDA), that under influence of heat decarboxylates to the more active form of Cannabidiol (CBD). Decarboxylation also occurs naturally during dry storage, so dried plant parts may have higher antioxidant properties than fresh ones. Antioxidant activity of cannabidiol itself has been already measured by many different methods, including the DPPH method (Huang *et al*., 2005), CUPRAC assay (Sethi *et al*., 2020), or ORAC assay (Viktorová *et al*., 2019). Data from these different methods proved that cannabinoids like CBD, Δ9-THC, CBG, CBN, CBGA, CBDA, and Δ9-THCA exhibit antioxidant activity (Dawidowicz *et al*., 2021). Although, the intensity of these activities for individual cannabinoids is not the same. Because it depends on the method applied in estimating the antioxidant properties of each cannabinoid (Sethi *et al*., 2020).

The effect known as the entourage effect is when a multitude of metabolites and related molecules modified the activity of the endogenous cannabinoids (Stout *et al*., 2012). This concept was extended to explain why whole botanical drugs could be often more effective than their isolated components alone (Pacher *et al*., 2020). Many compounds, such as cannabinoids or flavonoids, have been shown to have synergistic effects, for example with current chemotherapeutic agents. It must be said that such bioactive compounds combinations may show negative effects, so studying individual cases is necessary. Some studies have begun to evaluate the potential correlations of different compounds produced together within *Cannabis sp.* cultivars. Clusters of compounds are more than likely to have synergistic effects. These combinations have given plants unique profiles that can create better therapeutics (Anand *et al*., 2021).

Cannabinoids and other compounds contained in the cannabis plant such as flavonoids could have a positive impact on dermatological conditions and wound healing. The treatment of skin wounds and surgical wounds is a complex and difficult process and several natural substances can have a positive effect on the healing process, e.g., antioxidants and flavonoids have been reported to improve wound healing (Abdalla *et al*., 2021; Cheadle, 2006). Antimicrobial protection is also essential, especially in surgical wounds and preventing secondary infections that complicate, prolong, and increase the cost of treatment (Cheadle, 2006).

Materials and Methods

Experimental Materials

Inflorescence of Medical cannabis (Marijuana) plants-varieties: EUS4, EEA6, CCL, THE10, and E3D3 were previously preselected for potential therapeutic values to cover a range of high, medium, and low THC content and produced in an experimental indoor growth facility in 2020 by UCT Prague.

Inflorescence of Hemp plants-varieties: CAR, FED, and FUT were obtained from local Czech certified hemp farmers from the 2020 crop.

Preparation of extracts

Each dry sample of different varieties of *Cannabis sativa* inflorescence (EUS4, CCL, EEA6, CAR, FED, FUT, and THE10) was crushed by a grinder and weighed. Next, the extraction of each weighed sample was done by ethanol or hexane at a rate of 1:10 (v/v) for 18 h at a temperature of 30°C. Crude extracts were pressure filtered by the Büchner funnel, then divided in half. The solvents were evaporated from the extracts on a vacuum rotary evaporator at temperatures of 40°C, and 50 rpm. The second half of each dry extract was decarboxylated in a hot air oven at 140°C for 2 h. The solvent-free extracts, both decarboxylated and non-decarboxylated, were weighed and resuspended in DMSO at a rate of 1 mL DMSO per 100 mg dry extract.

The samples are labeled according to the code X-Y-deCO_2, where X is the designation of variety, Y is the solvent (EtOH or HEX) and deCO2 is attached if the extract has been decarboxylated.

Microorganism and Culturing

Cell compatibility and cytotoxicity tests against human cells were tested on the HDF control line (Human

Dermal Fibroblast) and the carcinogenic line B16 (Musculus skin melanoma cells).

The antimicrobial activity of the extracts was determined on *Candida albicans*, *Staphylococcus aureus* (ATCC, 25923), and *Salmonella enterica*.

Determination of Cannabinoids in Extracts by HPLC Methods

Resuspended DMSO extracts were analyzed by the HPLC method for determining cannabinoids. Preparation of samples: The amount of each extract was dissolved in 15 mL methanol, after 30 min shaking samples were filtered by 0,22 µm sterile filters. After that, the HPLC analysis was done. The content of selected cannabinoid in each extract are in Table III, the resolution is amount-mg/l in extracts, not in dissolved samples. LabSolutions LC software 5.96 by Shimadzu was used.

HPLC method parameters: The HPLC system used was a Shimadzu Nexera-I (Kyoto, Japan), chromatograph equipped with a solvent delivery unit (LC-2040C), an autosampler, a column oven, a degasser with a UV detector. Separation was conducted on a Shim-pack XR-ODS II (75 x 3 mm, Shimadzu Cooperation, Japan). The column temperature was set at 50°C. The mobile phase consisted of water containing 0,085% H_3PO_4 (A) and Methanol (B). The composition of the mobile phase was 0-5 min 60 % (B), 5-16 min 72% (B), 16-22 min 95% (B), 22-24 min 95% (B), 24-25 min 60% (B) and it was held for 5 min until the end of the analysis. The flow rate was 1,0 mL/min and the injection volume was 5,0 µL. The UV detector response was linear with concentrations of each measured cannabinoids in the range from 1,0-100,0 mg/L. See Fig. 2 - the standard (100 mg/L) of measured and identified cannabinoids separated by HPLC method which was used. In Table II can see retention times and other suitability parameters of these cannabinoids.

For technical hemp of the variety Carmagnolla, the entire extraction and decarboxylation process was performed twice to determine the repeatability of the process.

Based on HPLC analysis, the extracts were diluted in DMSO to contain 10 g/L of total cannabinoids content, and the bioactivities were measured.

Determination of Flavonoids in Extracts

The content of total flavonoids was determined by spectrophotometric method, using the color reaction of flavonoids (which have 3´ and 4´ hydroxyl groups) with the chromogenic system: $NaNO_2$-$Al(NO_3)_3$-NaOH. Absorbance was measured at wavelength $\lambda = 420$ nm. The measurement was performed using a Bioscreen C. The individual measured samples were prepared as follows: First 20 µL of the measured extract/calibration solution was added, then 14 µL of $NaNO_2$ (5% V/V), 200 µL 30% ethanol, stirring was continued for 5 min, then 14 µL $AlCl_3$ (10% V/V), stirring was continued for 6 min, then 100 µL 1 mol/L NaOH and finally 152 µL 30% EtOH was added. Before measurement, the samples were incubated for 40 min at room temperature in the dark. All measurements were performed in three parallels. Blank samples were prepared in the same way, but distilled water was added instead of the extractor calibration solution (Zarina and Tan, 2013).

Bioactivity of Extracts

Selected biological activities of cannabis extracts were determined using a robotic station consisting of an automatic pipetting station Biomek FXP with 8-channel and 96-channel head (Beckman Coulter), a modular microplate reader SpectraMax i3 x MiniMax (Molecular Devices), a microplate centrifuge Sigma 6-16KRL, an automatic CO_2 incubator Cytomat 2 was used for testing. C-LIN (Thermo Fisher) and microplate washer 405 LSUVS (Biotek). The individual modules are connected by a SCARA robotic system (Beckman Coulter). This system allows the measurement of the in vitro activities of a large number of samples at once on microtiter plates.

Cytotoxicity

Cell compatibility and cytotoxicity tests against human cells were performed on the HDF control line (Human Dermal Fibroblast) and the carcinogenic line B16 (Musculus skin melanoma cells). The cell lines were grown in an EMEM culture medium containing 10 % Fetal Bovine Serum (FBS) and 1% of the antibiotic mixture (penicillin, 100 IU/mL, and streptomycin, 100 g/mL) at 37°C in a 5% CO_2 humidified incubator. The cells were counted by a Cellometer Auto T4 (Nexcelom Bioscience, Lawrence, MA) and were seeded (1 × 105 cells/mL) in the 96-well plate and incubated for 24 h. The cell culture medium was then discarded from each well and the tested extracts were added to assess the effect on the cytotoxicity. After 72 h of incubation, a standard resazurin assay (Tran *et al*., 2020) was performed to determine cell viability. The results were expressed as a percentage of viable cells in comparison to the control (taken as 100%).

Antimicrobial Activity

Antimicrobial activity was tested by a microdilution method, which consists in diluting the test substance with a bacterial culture. In this way, the value corresponds to the IC50 (concentration that inhibits 50 %) i.e., the concentration killing half of the bacterial cell population. By default, this method is evaluated after 24 h of cultivation as the difference in absorbance ($\lambda = 500$ nm) before and after cultivation. Due to the toxicity of the solvents used, it was possible to test as a maximum concentration of 100 × diluted stock concentration to avoid higher than 1% solvent content in the tested cultures. If at this concentration the samples showed weak

antimicrobial activity, which did not cause the death of half of the population, but only a small part, their activity is expressed by the abbreviation "RA" indicating the relative viability of the cells. Antimicrobial activity was tested against these strains: *Candida albicans*, *Staphylococcus aureus* (ATCC, 25923), and *Salmonella enterica* (CCM, 4420).

Antioxidant Activity

The antioxidant potential of cannabis extracts was determined using the classical biochemical method ORAC (Oxygen Radical Absorbance Capacity) based on the neutralization of free radicals with fluorimetric detection. The antioxidant activity was tested using the standard ORAC assay. The crude extracts were analyzed using binary dilution in the concentration range of 0.1-2500 mg/L in 3 replicates. The ability of samples to absorb the generated radicals was monitored by measuring the fluorescence (excitation/emission 485/535 nm), recorded at 5 min intervals for 2 h using a SpectraMax i3 x microplate reader (Molecular Devices, San Jose, CA, USA). The IC50 values were determined according to the concentration range using the AAT Bioquest IC50 calculator (Viktorová *et al*., 2019).

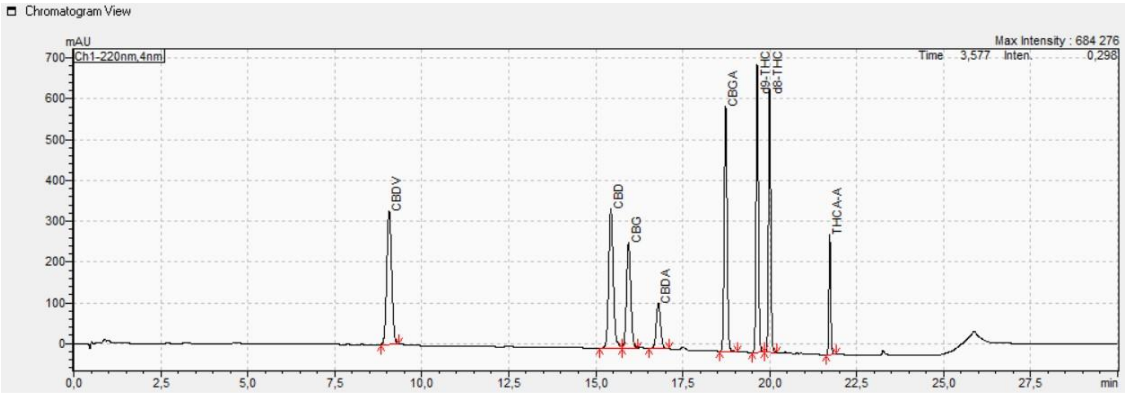

Fig. 2: Chromatogram of cannabinoids standard-100 mg/L was separated by a method which was used

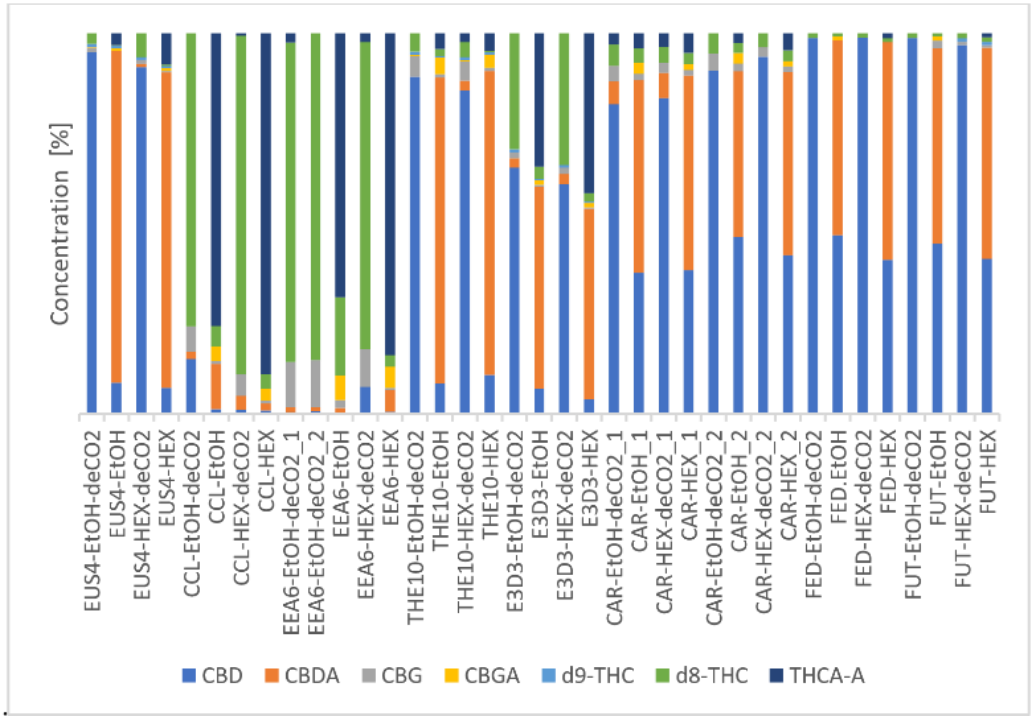

Fig. 3: Percentages of measured cannabinoids in the extracts (LabSolutions LC software 5.96 by Shimadzu)

Table I: Different varieties of *Cannabis sativa* inflorescence, were used

Cannabis	Varieties	Code
Marijuana	CBD USA Euforia	EUS4
	Critical	EEA6 CCL
	CBD Therapy	THE10
	E3D	E3D3
Hemp	Carmagnolla	CAR
	Fedora	FED
	Futura	FUT

Table II: Suitability parameter of cannabinoids standard – 100 mg/l separated by method which were used

	Ret. Time [min]	Peak Area	Peak Height	k'	Concentration [mg/l]
CBDV	9,062	3194761	326920	0,000	100
CBD	15,434	3133807	341725	0,703	100
CBG	15,944	2209551	258159	0,759	100
CBDA	16,802	970336	110716	0,854	100
CBGA	18,733	3432676	601074	1,067	100
Δ9-THC	19,641	3399874	702388	1,167	100
Δ8-THC	19,996	2901858	641880	1,207	100
THCA-A	21,726	1036991	293147	1,398	100
Total		20279853	3276008		

Table III: Content of total flavonoids and selected cannabinoids in extracts (before dilution in DMSO to 10 g/l of total cannabinoid content for following measurement of biological activities)

			Cannabinoids [mg/l]							
No.	Extract	∑Flavonoids [mg/l]	CBD	CBDA	CBG	CBGA	Δ9-THC	Δ8-THC	THCA	∑Cannabinoids
1	EUS4-EtOH-deCO2	2113	56412	0	646	111	520	1636	0	59324
2	EUS4-EtOH	1424	3752	39632	0	398	234	89	1429	45534
3	EUS4-HEX-deCO2	1433	65489	582	731	0	555	4521	0	71877
4	EUS4-HEX	1442	4014	48691	182	466	315	228	4825	58721
5	CCL-EtOH-deCO2	1642	6197	801	2873	0	0	33077	0	42949
6	CCL-EtOH	1202	650	6006	377	1879	0	2766	38937	50615
7	CCL-HEX-deCO2	1482	482	1555	2432	0	0	38479	272	43219
8	CCL-HEX	1398	395	843	316	1363	0	1695	39266	43877
9	EEA6-EtOH-deCO2	1678	247	456	4831	0	0	33544	0	50625
10	EEA6-EtOH	1629	0	687	862	2731	0	8876	29786	42943
11	EEA6-HEX-deCO2	1433	4252	0	5922	0	0	48467	1396	60037
12	EEA6-HEX	1420	354	3431	334	3360	0	1807	50977	60262
13	THE10-EtOH-deCO2	1993	46997	0	2887	127	469	2573	0	53053
14	THE10-EtOH	1664	3727	37476	412	1991	217	850	1938	46611
15	THE10-HEX-deCO2	2664	54352	1685	3175	203	543	2495	1528	63980
16	THE10-HEX	1869	4875	37870	376	1689	215	200	2211	47435
17	E3D3-EtOH-deCO2	2207	33017	1248	781	0	434	15531	0	51011
18	E3D3-EtOH	1833	3245	26130	249	598	182	1529	17249	49183
19	E3D3-HEX-deCO2	1611	36273	1585	1010	0	425	20819	0	60111
20	E3D3-HEX	1718	1998	25596	198	589	186	1112	21523	51201
21	CAR-EtOH-deCO2	1976	16586	0	819	0	0	992	0	21755
22	CAR-EtOH	1802	7029	6580	313	418	0	399	382	18492
23	CAR-HEX-deCO2	953	10490	0	274	0	0	411	0	18983
24	CAR-HEX	1384	12502	14473	435	406	0	866	1339	17457
25	FED-EtOH-deCO2	838	21718	0	0	0	0	283	0	22233
26	FED-EtOH	1429	9850	10745	0	225	0	169	0	20989
27	FED-HEX-deCO2	1718	26008	0	0	0	0	307	0	26559
28	FED-HEX	1411	9770	13776	0	0	0	248	324	24117
29	FUT-EtOH-deCO2	1584	22576	0	0	0	0	290	0	22865
30	FUT-EtOH	2193	10279	11831	448	262	0	189	0	23009
31	FUT-HEX-deCO2	1656	34009	0	281	0	320	478	0	35291
32	FUT-HEX	1287	13789	18795	241	0	280	401	365	33871

Results and Discussion

Determination of Cannabinoids and Flavonoids in Extracts Biological Activities

Five varieties of medical cannabis and three varieties of EU-certified hemp varieties with a wide range of major cannabinoids profile were selected for extraction with solvents of different polarity, namely ethanol and n-hexane. The extracts were then decarboxylated and resuspended in DMSO, from each cannabis variety were created samples with different compositions-hexane decarboxylase, hexane non-decarboxylated, ethanol decarboxylated, and ethanol non-decarboxylated.

The measured data show that hemp contains significantly fewer cannabinoids than medical cannabis/marijuana varieties. The varieties of hemp contain mainly the CBDA, which decarboxylates to the CBD, as well as the varieties of medical cannabis EUS4 and THE10. In contrast, the CCL and EEA6 varieties have a high content of THC derivatives, but at the same time contain the largest amounts of CBG and CBGA. The E3D3 variety has a balanced ratio between THC and CBD derivatives. There are differences in the total concentration of cannabinoids of 20% between individual samples, which may be caused mainly by inaccuracy in weight and due to low weights of extracts, but the percentage of individual CBD, CBG, and THC derivatives are almost the same. In general, hemp contains lower amounts of cannabinoids than varieties of medical cannabis. CBD and CBDA contain mainly varieties of hemp (CAR, FED, FUT) and also varieties of medical cannabis varieties EUS4 and THE10. In contrast, CCL and EEA6 varieties have a high content of THC derivatives, but also contain the highest amounts of CBG and CBGA.

The content of total flavonoids ranged from 0,8 to 3 mg.100 mg^{-1} DW of inflorescences. The content of flavonoids in decarboxylated extracts was not different from the flavonoids in non-decarboxylated extracts. The total flavonoid content was approximately ten to forty times lower than the cannabinoid content for the individual extract. As far as the total content of extracted substances is concerned, it cannot be said directly that the extracted content of cannabinoids correlates with the resulting amount of flavonoids content. The largest contains flavonoids were in extracts of medical cannabis varieties: THE 10 and E3D3. The largest contains flavonoids was in extracts of this hemp variety Carmagnolla. Overall, the hexane extract: THE10-HEX-deCO2_10g/L, number 15, had the highest content of flavonoids (2664 mg/L), in the case of the cannabinoid content, this extract contains the second-highest amount of total cannabinoids (63980 mg/L), all quantified and identified content od cannabinoids in extracts are in Table III, as well as total content of flavonoids. Percentages of measured cannabinoids in the extracts are illustrated in Fig. 3.

Medicinal cannabis extracts, especially extracts of the EEA6 variety, were more cytotoxic for the control line than for the carcinogenic line. On the contrary, extracts with significantly higher cytotoxicity against the tumor line include mainly non-decarboxylated extracts of hemp and non-decarboxylated extracts of medical cannabis of varieties THE 10 and E3D3. In general, decarboxylated extracts can be seen to be more cytotoxic to the control line.

The ratio between the cytotoxicity of the extracts against HDF and B16 expresses the safety of the application of the extract to the skin and its therapeutic index. whose cytotoxicity to the control line was higher than to the tumor line (HDF/B16 ratio is less than 1).

Table IV: The results of Cytotoxicity, Antimicrobial activity, and Antioxidant activity in diluted extracts to a content of 10 g/L of total cannabinoids

		Cytotoxicity B16		HDF			Antimicrobial activity Viability [%]			Antioxidant activity ORAC assay	
No.	Extract	IC50 (%)	SEM	IC50 (%)	SEM	HDF/B16	*C. albicans*	*S. aureus*	*S. enterica*	IC50 (%)	SD
1	EUS4-EtOH-deCO2_10g/l	0,08	0,01	0,07	0,01	0,95	>80%	74,26	>80%	0,20	0,02
2	EUS4-EtOH_10g/l	0,02	0,00	0,05	0,00	2,56	>80%	56,21	78,50	0,08	0,01
3	EUS4-HEX-deCO2_10g/l	0,04	0,00	0,05	0,00	1,21	>80%	69,41	>80%	0,11	0,00
4	EUS4-HEX_10g/l	0,04	0,00	0,05	0,01	1,19	>80%	55,27	>80%	0,08	0,01
5	CCL-EtOH-deCO2_10g/l	0,07	0,00	0,06	0,00	0,77	>80%	>80%	>80%	0,09	0,01
6	CCL-EtOH_10g/l	0,05	0,01	0,05	0,00	1,04	>80%	62,96	>80%	0,14	0,00
7	CCL-HEX-deCO2_10g/l	0,09	0,00	0,07	0,00	0,74	>80%	59,90	>80%	0,23	0,03
8	CCL-HEX_10g/l	0,04	0,00	0,05	0,01	1,31	>80%	56,58	>80%	0,09	0,01
9	EEA6-EtOH-deCO2_1_10g/l	0,09	0,00	0,06	0,00	0,66	>80%	>80%	>80%	0,12	0,02
10	EEA6-EtOH_10g/l	0,09	0,00	0,08	0,01	0,89	>80%	74,96	>80%	0,08	0,01
11	EEA6-HEX-deCO2_10g/l	0,07	0,01	0,05	0,00	0,80	>80%	72,19	>80%	0,16	0,00
12	EEA6-HEX_10g/l	0,04	0,00	0,07	0,01	1,92	>80%	62,52	>80%	0,09	0,00
13	THE10-EtOH-deCO2_10g/l	0,06	0,00	0,08	0,01	1,33	>80%	75,87	>80%	0,15	0,02
14	THE10-EtOH_10g/l	0,02	0,00	0,05	0,00	1,95	>80%	63,17	>80%	0,08	0,01
15	THE10-HEX-deCO2_10g/l	0,04	0,01	0,06	0,00	1,29	>80%	68,55	>80%	0,20	0,01
16	THE10-HEX_10g/l	0,02	0,00	0,05	0,00	2,46	>80%	69,28	>80%	0,06	0,01
17	E3D3-EtOH-deCO2_10g/l	0,04	0,01	0,06	0,00	1,43	>80%	>80%	>80%	0,14	0,00
18	E3D3-EtOH_10g/l	0,03	0,00	0,10	0,00	2,92	>80%	68,41	>80%	0,08	0,00
19	E3D3-HEX-deCO2_10g/l	0,08	0,00	0,06	0,00	0,72	>80%	56,31	>80%	0,20	0,02
20	E3D3-HEX_10g/l	0,03	0,00	0,06	0,00	2,17	>80%	59,63	>80%	0,06	0,01
21	CAR-EtOH-deCO2_1_10g/l	0,05	0,00	0,05	0,00	1,09	>80%	>80%	>80%	0,10	0,02
22	CAR-EtOH_1_10g/l	0,03	0,00	0,06	0,00	1,90	>80%	>80%	>80%	0,06	0,00
23	CAR-HEX-deCO2_1_10g/l	0,04	0,01	0,05	0,00	1,52	>80%	76,16	>80%	0,20	0,01
24	CAR-HEX_1_10g/l	0,04	0,00	0,09	0,00	2,46	>80%	69,98	>80%	0,05	0,01
25	FED-EtOH-deCO2_10g/l	0,05	0,00	0,05	0,00	1,08	>80%	74,06	>80%	0,08	0,01
26	FED-EtOH_10g/l	0,04	0,00	0,08	0,01	1,99	>80%	67,77	>80%	0,08	0,01
27	FED-HEX-deCO2_10g/l	0,05	0,00	0,05	0,00	1,09	>80%	58,30	>80%	0,38	0,01
28	FED-HEX_10g/l	0,04	0,00	0,08	0,00	2,25	>80%	62,07	>80%	0,10	0,02
29	FUT-EtOH-deCO2_10g/l	0,03	0,00	0,05	0,00	1,90	79,7	>80%	>80%	0,13	0,01
30	FUT-EtOH_10g/l	0,03	0,00	0,07	0,01	2,26	>80%	74,27	>80%	0,07	0,01
31	FUT-HEX-deCO2_10g/l	0,05	0,00	0,05	0,00	1,12	>80%	58,38	>80%	0,27	0,03
32	FUT-HEX_10g/l	0,03	0,00	0,05	0,00	1,41	>80%	55,63	>80%	0,17	0,01

Hemp extracts showed no antimicrobial activity against *C. Albicans* and *S. enterica*. Almost all cannabis extracts showed antimicrobial activity against *Staphylococcus aureus* to some extent at the highest possible concentration, but cell death never exceeded 50%. The highest activity against *S. aureus* was measured in non-decarboxylated extracts of the EUS4 variety, hexane extracts of the CCL and E3D3 varieties, and hexane extracts of the Futura and Fedora hemp varieties.

The results of cytotoxicity measurements against bacterial strains of *Candida albicans*, *Staphylococcus aureus*, and *Salmonella enterica* are shown in Table IV. Due to the toxicity of the solvents used, it was possible to test as a maximum concentration the 100 × diluted stock concentration to avoid higher than 1% solvent content in the tested cultures. If at this concentration the samples showed weak antimicrobial activity, which, however, did not cause the death of half of the population, but only a small part, their activity is expressed by relative cell viability in%. If the viability of the cells was higher than 80%, it is assumed that the extract does not show antimicrobial activity against the given strain.

Almost all medical cannabis extracts showed antimicrobial activity against *Staphylococcus aureus* to some extent at the highest possible concentration, but cell death never exceeded 50 %. The highest activity against *S. aureus* was measured in non-decarboxylated extracts of the EUS4 variety, in hexane extracts of the CCL and E3D3 varieties, and in hexane extracts of the Futura and Fedora hemp varieties. Activity against *S. aureus* was not demonstrated in ethanolic decarboxylated extracts of CCL, EEA6, E3D3, and Carmagnola and Futura and the ethanolic non-decarboxylated extract of Carmagnola.

All hemp and medical cannabis extracts showed high antioxidant activity even when a small amount was added to the free radical solution. Non-decarboxylated extracts usually showed higher antioxidant activity than decarboxylated extracts. Hexane decarboxylated extracts usually showed the least antioxidant activity. Thus, it is likely that during decarboxylation, some of the substances in hemp extracts that are responsible for the antioxidant effect are degraded. The results of measuring the antioxidant activity of cannabis extracts using the ORAC method are shown in Table IV.

Conclusion

Overall, medical cannabis varieties had a higher content of cannabinoids, compared to hemp, the content was about half as high. Of the hemp, the Futura variety extract had the highest content of cannabinoids (35291 mg/L), which also had the high content of flavonoids (2193 mg/L).

The required cytotoxicity was better for non-decarboxylated extracts in the case of hemp. The best cytotoxicity ratio of HDF/B16 cell lines around 2,5 was found in extracts of THE10, E3D3, Fedora, and Futura varieties.

Studied medical cannabis and hemp extracts showed no antimicrobial activity against *C. Albicans* and *S. enterica*. They act mainly on G^+ bacteria (*S. aureus*). Between hemp and medical varieties, rather surprisingly extracts of hemp varieties mostly had a higher antimicrobial effect. *S. aureus* had lower viability due to non-decarboxylated extracts. Thanks to the hexane extracts of the hemp variety Futura, the viability of *S. aureus* was reduced to 50%, this variety was the best of the hemp varieties. Of the medical varieties, the viability of *S. aureus* was reduced most by the variety CCL. Generally, hexane non-polar extracts showed somewhat higher antimicrobial activity against *S. aureus*.

The antioxidant activity, measured by the ORAC assay, was higher in all extracts which were not decarboxylated. In the decarboxylated extracts, the total antioxidant capacity was reduced up to twice.

Obtained results suggest that extracts of several *Cannabis sp*. varieties show antioxidant activity as well as antimicrobial activity against *S. aureus,* while at the same time having low cytotoxicity against human dermal fibroblast skin cells and high cytotoxicity against B16 tumor cell line used as a model for human skin cancers. This suggests that these extracts have potential as topical dermatological agents for melanoma therapy or in the treatment of skin or surgical wounds, often infected with *S. aureus.* of extraction solvent and decarboxylation of the crude extract alter the chemical composition and biological activities of the extracts. As all tested extracts were diluted to a concentration of 10 g/L of a total of 7 major cannabinoids, it is obvious that individual differences in cannabinoids profile, probably along with varying content of flavonoids (and other plant metabolites, non-analyzed in this study) are responsible in changes of biological activities. Taking into account the complicated legislative status of medicinal cannabis with higher d9-THC contents, the dermatological use of extracts of technical cannabis, especially non-decarboxylated extracts of the Futura variety, seems interesting. The lower cytotoxicity and higher antioxidant effects of non-decarboxylated extracts found as a typical trend in most of the samples studied also indicate that decarboxylation commonly used in the recreational use of cannabis may not be suitable for other therapeutic applications.

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Author's Contributions

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Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved the manuscript and no ethical issues involved.

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