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Analysis of Anti-inflammatory and Antioxidant Properties of Commercial Cannabinoids extracted from *Cannabis sativa*

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Abstract

The recent commercialization and legalization of cannabis products nationwide calls for a significant amount of research into the potential risks and rewards of cannabis usage. The chemicals found in the *Cannabis sativa* plant, called cannabinoids, present possible new sources of treatments for a variety of conditions. The most common cannabinoids include tetrahydrocannabinol (THC), a psychoactive compound, and cannabidiol (CBD), a non-psychoactive compound found mainly in hemp. To explore the medicinal properties of these common cannabinoids, three commercial samples of hemp oil were analyzed for CBD and THC content by Gas Chromatography-Mass Spectroscopy (GC-MS), for anti-inflammatory capabilities by a Human Inflammation Antibody Array-Membrane assay, and for antioxidant power by a Ferric Reducing Antioxidant Power (FRAP) assay. While there was no detectable amount of THC, the CBD concentration of the three samples ranged from 0.0278 mg/mL to 17.87 mg/mL, and each sample contained one or more unknown contaminants. The *in vitro* bioassays indicated all the hemp oil samples had varying degrees of anti-inflammatory and antioxidant properties, and all exceeded the capabilities of a CBD standard alone. These results suggest cannabinoids could serve as potential sources for anti-inflammatory and antioxidant drugs, however other compounds other than CBD also contribute to the anti-inflammatory and antioxidant properties of hemp oil. Future studies will focus on the effects of other compounds within hemp oil, varying methods of consumption that may impact the effect of cannabinoids, and the long-term effects of cannabinoid usage.

Keywords: Cannabinoids, Anti-inflammatory, Antioxidant

1. Introduction

After years of being outlawed, the infamous cannabis plant has become the newest interest in the pharmaceutical and medical industries. The cannabis market has increased by an estimated 700% in recent years and market intelligence reports sales could exceed 2.1 billion dollars in 2020[1]. This economic boom is a result of many states passing laws to broaden the use of marijuana including 11 states and the District of Columbia which have legalized the recreational use of marijuana and numerous other states which have legalized medicinal marijuana. Recent commercialization and legalization of cannabis products nationwide calls for a significant amount of research into the potential risks and rewards of cannabis usage.

The utilization of the 400 natural compounds found in the *Cannabis sativa* plant in medicine can be traced back as early as 5,000 years ago[2]. Research into a class of 60 of these compounds known as cannabinoids suggests these chemicals function through an endogenous cannabinoid system consisting of specific receptors CB1 and CB2 as well as endocannabinoids such as arachidonoyl ethanolamide (or anandamide) and 2-arachidonoyl glycerol (2-AG)[3,4]. The most abundant cannabinoids in *Cannabis sativa* are tetrahydrocannabinol (THC) known for its psychoactive properties and cannabidiol (CBD) which has been speculated to hold multiple medicinal properties[4]. Studies examining these two cannabinoids have shown THC functions through activation of the CB1 and CB2 receptors, whereas CBD does not. Additionally, structural differences between THC and CBD cause contrasting functions including psychoactivity,

which constitute CBD as a better source for various disorders compared to THC (Figure 1). The endogenous cannabinoid system has been indicated to play a role in the central nervous system affecting memory, cognition, and pain perception[6] and thus is of particular interest in many sectors of medical research.

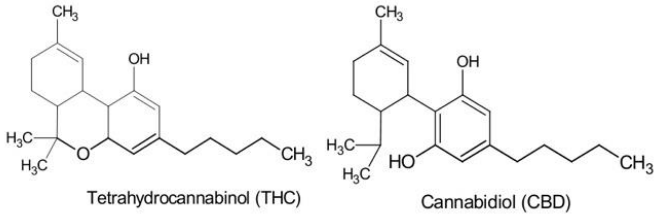

Figure 1. Structures of the two most abundant cannabinoids present in *C. sativa*, THC and CBD. Image courtesy of CBD Oil Review (2015).

Modern research into the medicinal potential of cannabinoids first began in the 1970s when Raphael Mechoulam investigated cannabinoids' effect on the body and conditions like epilepsy[2]. This study soon resulted in THC-based products such as Marinol® and Cesamet® in the 1980s, which were created to increase appetite and reduce nausea and vomiting in chemotherapy patients[4]. Further research has indicated a cannabinoid application in the treatment of various other conditions including insomnia and anxiety. These studies implored the development of a cannabis-based drug, Sativex®, which contains both THC and CBD, to induce sleep in patients with chronic pain conditions such as multiple sclerosis[5]. Lastly, almost four decades after Mechoulam's initial research, in June 2018, the U.S. Food and Drug Administration approved its first CBD-based drug, Epidiolex®, to treat rare seizure disorders. The clinical trials leading to its approval showed that Epidiolex® with other medications reduced the number of seizures in over 40% of patients with Lennox-Gastaut syndrome or Dravet syndrome by half [6,7]. Although clinical trials are producing positive results in support for CBD-based medicine, CBD treatments are not effective for everyone. With numerous possible mechanisms, more research is needed to understand the CBD pathways and how to utilize cannabinoids in medicine.

The objective of this exploratory study is to investigate possible medicinal properties of cannabinoids, specifically CBD. Because hemp seeds are CBD-rich, three samples of commercial hemp oil were purchased and analyzed by GC-MS for CBD and THC content and by *in vitro* bioassays for anti-inflammatory and antioxidant properties. These introductory experiments have the capability to expand the medicinal capacity of CBD and cannabinoids as well as give insight into the regulation of cannabis products.

2. Materials and Methods

2.1. Determination of CBD and THC Concentrations

In this preliminary study, the concentrations of CBD and THC in commercial hemp oil samples purchased from Amazon were determined using GC-MS. The samples included: (1) *Cannabis sativa* "cold-pressed" Hemp Oil, (2) Rocky Mountain Hemp Oil Company Supplement, and (3) House of Hemp Premium Organic Hemp Oil. Standards of CBD and THC (1.0 mg/mL) were each diluted to 16.4, 32.3, 47.6, 62.5, 76.9, and 90.9 μg/mL in 99.9% HPLC-grade hexane. Three dilutions of each hemp oil sample were prepared by adding 10, 50 and 250 μL of sample into 1.5 mL of hexane. Each standard and sample dilution was then processed through a Shimadzu GCMS-QP2010 under the following experimental conditions: a pressure of 14.2 psi, a total flow of 82.9 mL/min, a column flow of 1.31 mL/min, a linear velocity of 42.5 cm/sec, a 5-minute hold at 100°C and a 10-minute hold at 320°C. Under these conditions, the THC and CBD standards were run to determine relative elution times, 13.0 minutes and 12.6 minutes, respectively. From the chromatogram data, the area under the peak in mega absorbance units (mau) was collected and used to plot concentration calibration curves for each THC and CBD standard. The concentration of each cannabinoid in each sample was then calculated from the linear regression and converted to mg/mL.

2.2. *In Vitro* Anti-inflammatory Bioassay

Using the Human Inflammation Antibody Array-Membrane assay kit from Abcam® and the provided protocol, each sample of hemp oil was tested for the detection of 20 human inflammation factors and compared to a CBD standard. The provided membranes were integrated with positive (biotin-conjugated IgG) and negative (blank) controls (Figure 3). Four membranes were placed in an 8-well plate and incubated at room temperature with 2 mL of 1X Blocking Buffer for 30 minutes. The buffer was extracted, and 1.000 mL of undiluted hemp oil sample was added to separate membranes while a 1:2 dilution of CBD standard in methanol was added to the final membrane and all were incubated at 6°C for 2 days. Once the samples were removed from the wells, each membrane was washed with a large volume wash of 20 mL of 1X Washing Buffer I at room temperature for 30 minutes on a rocker. The membranes were washed again with 2 mL of 1X Washing Buffer I for 5 minutes 3 times and with 2 mL of 1X Washing Buffer II for 5 minutes twice. One milliliter of the 1X Biotin-Conjugated Anti-Cytokines were added to each membrane and incubated overnight at room temperature on the rocking bed. The Anti-Cytokines were removed from each well and the washing procedure was repeated without the large volume wash, and 2 mL of 1X HRP-Conjugated Streptavidin was pipetted into each well and incubated overnight at room temperature on the rocking bed. Once the Streptavidin was extracted, the membranes were washed following the same procedure and blotted with Kim Wipes to remove excess buffer. Detection Buffers C and D were combined in a 1:1 ratio, and 500 μL of the mixture was added to each membrane and incubated for 2 minutes before being imaged on a ChemiGelDoc. Each image was adjusted by contrast to better visualize the dots and was analyzed by relative dot intensity.

2.3 *In Vitro* Antioxidant Bioassay

Using the Ferric Reducing Antioxidant Power (FRAP) assay kit from Abcam® and the provided protocol, each sample of hemp oil was tested for antioxidant abilities and compared to a CBD standard. In this assay, the antioxidants in the samples reduce Fe^{3+} to Fe^{2+} resulting in a colored ferrous complex at low pH, which can be measured by absorbance at 595 nm.

A standard curve was prepared using the supplied 2 mM Ferrous Standard, which was diluted to concentrations of 0, 4, 8, 12, 16, and 20 nmol/well in assay buffer. Each sample of commercial hemp oil was diluted by adding 5, 25 and 50 μL of sample to 190 μL of Reaction Mix (Assay Buffer, FRAP Probe, $FeCl_3$ Solution). The CBD standard (90.9 μg/mL) was used in concentrated form and then diluted 1:10 and 1:100 in concentrated hexane. The supplied positive control was diluted 2:5 in assay buffer. The background reaction control was prepared using 342 μL of the assay buffer and 38 μL of FRAP probe. In a 96-well plate, each standard, sample, and control were plated in duplicates and incubated at 37°C for one hour. The absorbance was measured at 595 nm on a plate reader and averaged for each duplicate. The measured absorbance for each sample and positive control was converted to ferrous equivalents (Equation 1).

$$Ferrous\ Equivalents\ (mM) = Standard\ Curve\ Ferrous\ x\ \left(\frac{Dilution\ Factor}{Volume\ added}\right) \quad (1)$$

3. Results

3.1 GC-MS Analysis

Three commercial samples of hemp oil were analyzed for CBD and THC content by GC-MS. A standard curve of common cannabinoids such as CBD and THC was constructed from the measured area in mau. While none of the hemp oil samples contained a detectable amount of THC, there was a wide range of CBD content. The cheapest sample, *C. sativa* "cold-pressed" hemp oil, had the lowest concentration of CBD, 0.0278 mg/mL, and the most expensive sample, House of Hemp hemp oil, contained the most CBD with a concentration of 17.87 mg/mL (Table 1). This data follows what any knowledgeable consumer knows: the higher quality products cost more money. The samples investigated and the respective companies did not list or advertise the amount of CBD in the products and therefore, the experimental values could not be compared to the commercial listing. The GC-MS data also showed additional peaks beside CBD including one consistent absorbance of an unknown compound around 11.5 minutes on

each hemp oil sample (Figure 2). Speculatively, this peak could represent the presence of cannabinol (CBN), a mildly psychoactive cannabinoid that may form as a breakdown product of CBD under storage conditions[8]. The other unknown peaks are most likely contaminants from the extraction process including common pesticides, solvents, and trace amounts of THC. These contaminants would become more concentrated in the final CBD oil and could cause serious health effects[9]. Regulation of the quality and contents of cannabis products is lacking and could be extremely harmful to consumers if proper and safe extraction processes as well as contaminant detection and identification are not developed.

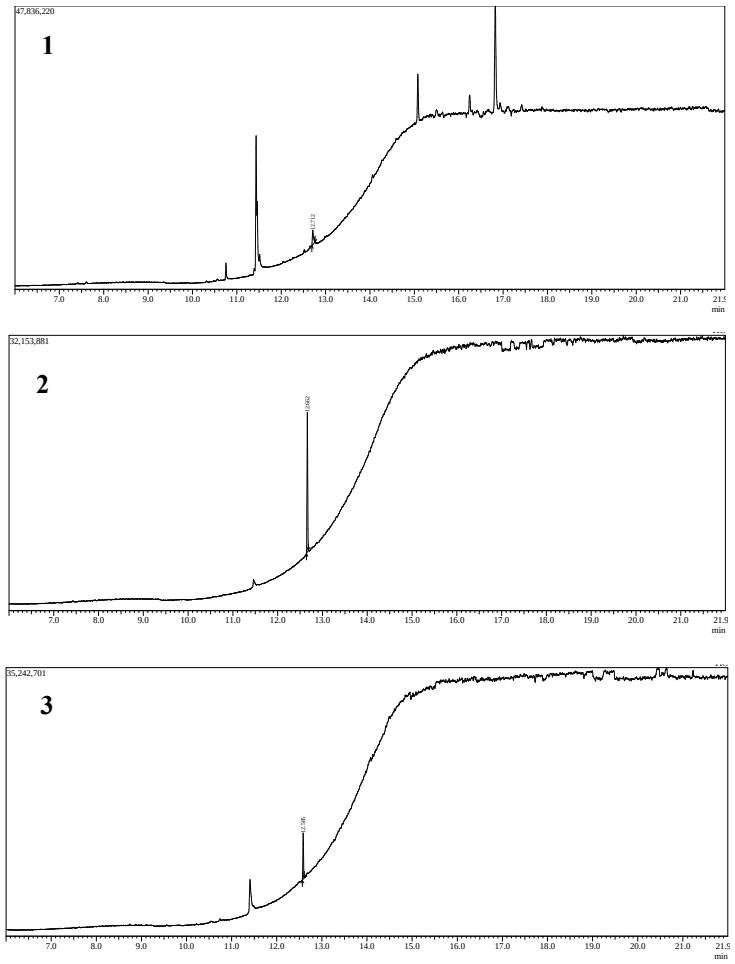

Figure 2. GC-MS data for each of the hemp oil samples. (1) C. sativa "cold-pressed" hemp oil, (2) Rocky Mountain hemp oil, (3) House of Hemp hemp oil. The area under the peak at 12.5 minutes representing the amount of CBD for each sample was adjusted for each dilution and then used to determine the CBD concentration.

Table 1. CBD concentrations for commercial hemp oil samples. GC-MS was used to separate and measure the quantity of the contents of each sample. The concentration of CBD in each sample was calculated from the linear regression of the CBD standard and accounted for splits during GC-MS analysis.

Sample	Area Under Peak (mau)	CBD Concentration (mg/mL)	Price
C. sativa "cold-pressed" Hemp Oil	4.877	0.0278	$13.99
Rocky Mt. Hemp Oil	6.336	7.22	$66.49
House of Hemp Oil	17.423	17.87	$149.99

3.2 Anti-inflammatory Properties

To qualitatively assess the anti-inflammatory capability of CBD from hemp oil, a Human Inflammation Antibody Array-Membrane assay was performed. Each area on the membrane tested for a different inflammation cytokine, which aid in cell-to-cell signaling in the immune system. All of the commercial hemp oil samples tested positive for activation of the antigen-specific antibodies to varying degrees (Figure 4). Typically, greater intensity of color indicates a higher concentration. The most abundant antibodies detected across the three hemp oil samples included those for cytokines IL-11, IL-12p40, IL-12p70, Eotaxin-2, and GM-CSF. The interleukin (IL) family of cytokines consists of proteins that can have pro- or anti-inflammatory properties and are crucial in the regulation of proliferation, maturation, migration, and adhesion of immune cells[10]. Although the CBD standard had less intense spots, it tested positive for a majority of the antibodies including significantly more for IL-11, Eotaxin-2, and IL-3. The discrepancy between the CBD and the hemp oil results may be accounted for by other compounds within the hemp oil shown by the GC-MS chromatograms. Because other compounds likely effect the anti-inflammatory potential of the hemp oil samples, quantification of the protein levels of the cytokines tested wouldn't yield significant results relevant to the anti-inflammatory properties of CBD. For these reasons, the extent of CBD's and hemp oil's anti-inflammatory properties should be further investigated individually.

POS	POS	NEG	NEG	Eotaxin	Eotaxin-2	G-CSF	GM-CSF
POS	POS	NEG	NEG	Eotaxin	Eotaxin-2	G-CSF	GM-CSF
IFN-γ	IL-1α	IL-1β	IL-2	IL-3	IL-4	IL-6	IL-7
IFN-γ	IL-1α	IL-1β	IL-2	IL-3	IL-4	IL-6	IL-7
IL-8	IL-10	IL-11	IL-12p40	IL-12p70	IL-13	I-309	TIMP-2
IL-8	IL-10	IL-11	IL-12p40	IL-12p70	IL-13	I-309	TIMP-2
						NEG	POS
						NEG	POS

Figure 3. Human inflammation factors array map for 20 different inflammation factors, where POS represents the positive control and NEG represents the negative control.

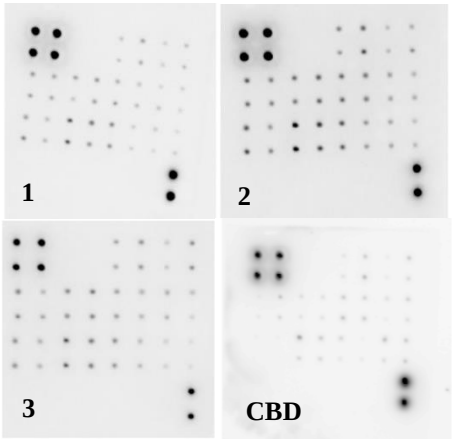

Figure 4. Anti-inflammatory antibody arrays for three samples of commercial hemp oil samples and a CBD standard. (1) *C. sativa* "cold-pressed" hemp oil, (2) Rocky Mountain hemp oil, (3) House of Hemp hemp oil. The CBD standard was diluted in concentrated hexane; all other samples were used in concentrated form. For orientation of inflammation factors reference Figure 3. All images were captured using a ChemiGelDoc.

3.3 Antioxidant Properties

Using a Ferric Reducing Antioxidant Power (FRAP) assay, the antioxidant power of each hemp oil sample was determined by reducing Fe^{3+} to Fe^{2+} resulting in a colored ferrous complex at low pH, which was measured by absorbance at 595 nm. From the standard curve of the ferrous standards, the ferrous equivalent or FRAP was calculated for each commercial hemp oil sample. While all the samples including a CBD standard produced positive results for antioxidant abilities, the House of Hemp sample had the greatest FRAP (Figure 5). The House of Hemp sample showed twice as much antioxidant power as the other samples and the positive control. This anomaly could be a result of a higher concentration or other compounds within the sample that contributed to the antioxidant activity. The latter is more likely since the CBD standard had a lower FRAP; thus, this discrepancy could be accounted for by additional compounds aiding in reducing the ferrous ions. While this *in vitro* assay suggests CBD and hemp oil have antioxidant properties, other studies report cannabinoids including CBD may have pro-oxidant qualities as well. In fact, one article states that CBD and other cannabinoids are known to oxidize purines and pyrimidines and to induce micronuclei, which cause chromosomal damage and may indicate potential carcinogenic properties[1].

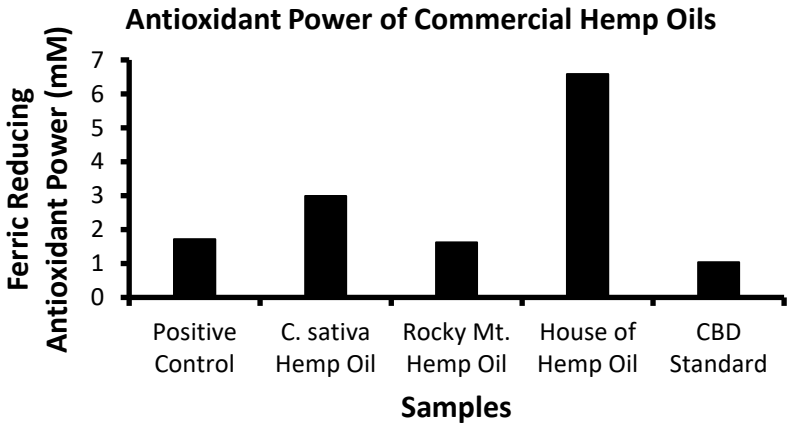

Figure 5. Antioxidant power of commercial hemp oil samples. FRAP was calculated for each sample using the first dilution absorbance and the linear regression of the ferrous standards. Graph was produced by Excel for Mac 2018.

4. Conclusion

As cannabis products have become more popular and further legalized in the US, the intriguing capabilities of compounds within these plants including cannabinoids are still merely anecdotal. Consumers should be aware of the lack of scientific data when purchasing commercial products containing cannabinoids. Recent research supports CBD as a promising compound for pharmacotherapy due to its direct and indirect antioxidant effects on oxidative stress, which regulates redox imbalances and inflammation in diseases such as cancer, inflammatory diseases, and neurodegenerative diseases. Possible antioxidant and therefore anti-inflammatory mechanisms of CBD include reducing reactive oxygen species, regulating the level and activity of oxidants and antioxidants, and reducing oxidative conditions by preventing the formation of superoxide radicals[11]. This exploratory study attempted to test the effect of CBD's anti-inflammatory and antioxidant properties using three commercial samples of hemp oil in order to evaluate the quality of the remedies widely available to consumers. Conclusively, there is a lack of regulation of cannabis-based products that leaves consumers vulnerable. Current legislation does not require toxicological or long-term effects testing of natural substances, and therefore the medicinal properties of cannabinoids may be trivial compared to the long-term consequences. Additionally, the *in vitro* bioassays indicated variable anti-inflammatory and antioxidant properties of cannabinoids, however the hemp oil mixture showed greater medicinal properties than CBD alone. Therefore, while cannabinoids could serve as potential sources for new drugs for many conditions, the other compounds within hemp oil and their effect on medicinal properties of cannabinoids should be investigated. Little is known about the mechanisms by which CBD interacts with the body, but new safety studies suggest CBD targets molecules outside of the endocannabinoid system including those in metabolic and redox pathways. Thus, consumers should consult medical professionals on potential adverse drug events and drug-drug interactions[12]. Further studies are needed to evaluate the safety, the rewards, and the risks of regular usage of cannabis-based products. Furthermore, the different strains of cannabis, extraction process, and method of consumption (i.e. inhalation versus digestion) may also impact the effects of these erratic compounds and should be investigated.

5. References

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