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Research Article

CB_2 Receptor Binding Affinity of Various Nutraceutical Ingredients and Their Combinations

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ABSTRACT

Introduction: Cannabidiol (CBD)-containing products have become very prevalent in the food, nutraceutical, and cosmetic industries. One reason for the popularity of these CBD products is their therapeutic effects on the endocannabinoid system due, in part, to their CB_2 receptor binding. Studies suggest benefits including immune modulatory effects, as well as emotional and physical support. Other plant-derived nutraceutical ingredients have been shown to have similar CB_2 receptor binding potential.

Objectives: The purpose of this study is to measure and compare the CB_2 receptor binding affinity of nutraceutical ingredients, both alone and in combination, to determine which may have greater potential for CB_2 receptor activity, and as a result, a greater potential for regulating the endocannabinoid system.

Methods: In this study, *in vitro* cellular and nuclear receptor functional assays were used to measure the CB_2 binding affinity of various nutraceutical ingredients both alone and in combination. The nutraceuticals and compounds tested were copaiba essential oil, palmitoylethanolamide (PEA), Sichuan pepper extract, *Acmella oleracea* extract, a blend of cruciferous vegetable extracts, bovine colostrum filtrate, three commercial CBD oil products, and a purified CBD reference standard.

Results: The results of the *in vitro* study showed agonist, antagonistic, or inverse agonistic CB_2 receptor binding. Comparisons for CB_2 receptor binding affinity of different combinations of nutraceutical ingredient were also determined. The nutraceutical with the highest CB_2 receptor binding affinity was *Acmella oleracea* extract, while the combination with the highest affinity was a blend of PEA, Sichuan pepper extract, *Acmella oleracea* extract, cruciferous vegetable extract blend, and bovine colostrum filtrates. Additional studies should be enacted with these same ingredients and blends to confirm similar CB_2 binding affinities *in vivo*.

Conclusion: Certain phytocannabinoid ingredient combinations were shown to have a higher CB_2 receptor affinity than several leading brand CBD oils. This suggests that therapeutic benefits may also be provided by these phytocannabinoid ingredients and formulas.

Keywords: Phytocannabinoid; CB_2 receptor; *Acmella oleracea*; Palmitoylethanolamide; Diindolylmethane; Colostrum ultra-filtrate

INTRODUCTION

The CB_2 receptor is mainly expressed in peripheral immune cells such as macrophages, dendritic cells, and B cells, suggesting that the endocannabinoid system has an immunomodulatory role [1]. Numerous studies also have reported that genetically modified mice lacking the CB_2 receptor have severe immunomodulatory deficiencies [2]. This gives credence to therapeutic strategies aimed at modulating CB_2 signaling for the treatment of various immune conditions.

Cannabidiol (CBD), one of the cannabinoids produced naturally in the marijuana plant, is a popular ingredient in many dietary supplement products on the market. None of the non-pharmaceutical products containing CBD, however, are considered allowed dietary supplements by the Food and

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Drug Administration (FDA) [3]. Several studies have shown the relationship between CBD, management of the endocannabinoid system and improved mood and decreased pain, due in part to the CB_2 receptor binding affinity of this cannabinoid [4-10]. Other compounds may be suitable substitutes for CBD and CBD-containing ingredients in nutraceutical products because they are not held to the same level of regulatory scrutiny by the FDA [11].

Several studies have investigated potential alternative ingredients to CBD with similar CB_2 receptor binding affinity that may also provide similar benefits; palmitoylethanolamide (PEA), a blend of cruciferous vegetable extracts, Sichuan pepper extract, *Acmella oleracea* extract, and copaiba oil are some of the more researched compounds that interact directly or indirectly with CB_2 receptors [12-23]. Some of the active components present in these ingredients are shown in Figure 1.

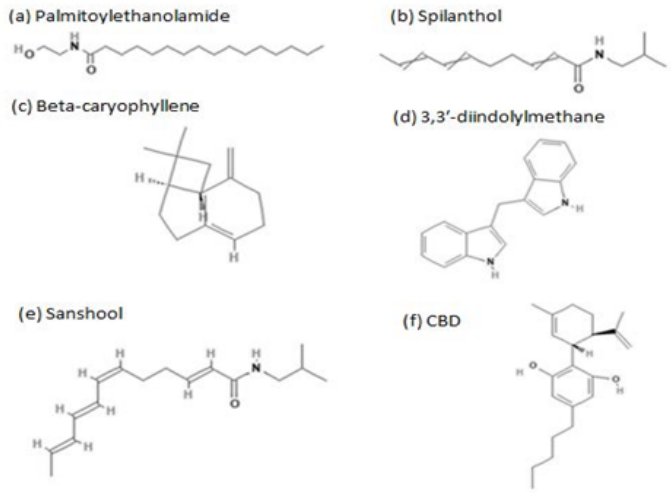

Figure 1: Chemical structures of several active compounds found in the ingredients selected for this study that purportedly interact directly or indirectly with CB_2 receptors.

Bovine colostrum filtrate has been demonstrated to have strong immune cell activity, though the mechanism is not fully understood [24-26]. Colostrum, the first milk produced by mammal mammary glands, may contain small amounts of certain endocannabinoids, which could be partially responsible for colostrum's immune related effects, *via* the CB_2 receptor [27,28]. For this reason, we include the proprietary bovine colostrum filtrate.

The purpose of this study is to measure and compare the CB_2 receptor binding affinity of the above mentioned nutraceutical ingredients, both alone and in combination, to determine which may have greater potential for CB_2 receptor activity, and as a result, a greater potential for regulating the endocannabinoid system.

MATERIALS AND METHODS

Compounds tested were as follows:

(a) Copaiba essential oil, standardized to contain no less than 50% beta-caryophyllene

(b) Palmitoylethanolamide (PEA), 99% purity by HPLC

(c) Sichuan pepper extract, supercritical fluid CO_2 extracted (not standardized)

(d) *Acmella oleracea* extract, standardized to 20% spilanthol

(e) A blend of cruciferous vegetable extracts, consisting of broccoli, cabbage and kale extracts and containing no less than 42% indole content, including 3, 3' diindoylmethane

(f) Bovine colostrum filtrate, from defatted and ultra-filtered (to less than 10 kDa) whole colostrum and containing no less than 37% peptides

(g) Three commercial CBD oil products, standardized to CBD with varying concentrations varying from 2 to 10 mg/mL. (CBD metabolite values listed are for the specific lot used in these experiments. Metabolites below 1 mg/mL or 1 mg/g are not listed; ND=Not detected.)

(a) Market brand CBD oil #1–(CBD, 11.4 mg/g; CBC, 1.9 mg/g; THC, 0.03%)

(b) Market brand CBD oil #2–(CBD, 835 mg/g; CBD-A, 1.1 mg/g; CBD-V, 3.9 mg/g; THC, ND)

(c) Market brand CBD oil #3–(CBD, 8.1 mg/mL; CBD-A, 0.5 mg/mL, CBG, 1 mg/mL; CBC, 0.7 mg/mL; THC, 0.04%)

(h) Purified CBD reference standard, 99.9% by HPLC

All compounds and their combinations were tested at 20.0 µg/ml for both agonist and antagonistic effects on CB_2 receptors from human recombinant Chinese Hamster Ovary (CHO) epithelial cells. A similar test was done for determining activity with CB_1 receptors. Phytocannabinoid Formula 5 was also tested on non-expressed cell lines to test for off target effects.

The method focused on evaluation of the agonist and antagonist activity of compounds at the human CB_2 receptor expressed in transfected CHO cells, determined by measuring their effects on cAMP modulation using the homogenous time-resolved fluorescence (HTRF) detection method for both agonist and antagonist effect assays.

The procedure for the agonist effect assay is as follows: the CHO cells were suspended in HBSS buffer (Invitrogen) complemented with 20 mM HEPES (Invitrogen Cat# 14025; pH 7.4), then distributed in microplates at a density of 7.5×10^3 cells/well in the presence of one of the following: HBSS (basal control), the reference agonist at 100 nM (stimulated control) or various concentrations (EC_{50} determination), or the test compounds.

Thereafter, the adenylyl cyclase activator NKH 477 (Tocris Cat# 1603) was added at a final concentration of 3 µM. We used NKH 477 to activate cyclases. This effect of NKH 477 is what we call the background signal of the assay (0% effect). It is measured in each experiment in several wells. Following 10 min incubation at 37°C, the cells were lysed and the fluorescence acceptor (D2-labeled cAMP) and fluorescence donor (anti-cAMP antibody labeled with europium cryptate) was added. After 60 min at room temperature, the fluorescence transfer was measured at λex=337 nm and λem=620 and 665 nm using a microplate reader (EnVision® , Perkin Elmer). The cAMP concentration was determined by dividing the signal measured at 665 nm by that measured at 620 nm (ratio). The results are expressed as a percent of the control response to 100 nM WIN 55212-2 (Sigma Cat# W102). The standard reference agonist is WIN 55212-2, which is tested in each experiment at several concentrations to generate a concentration-response curve from which its EC_{50} value is calculated [29].

For the antagonist effect assay, the CHO cells were suspended in HBSS buffer (Invitrogen) complemented with 20 mM HEPES (pH 7.4), then distributed in microplates at a density of 7.5 × 103 cells/well and pre incubated for 5 min at room temperature in the presence of one of the following: HBSS (stimulated control), the reference antagonist AM 630 (Tocris Cat# 1120) at 100 µM (basal control) or various concentrations (IC_{50} determination), or the test compounds. Thereafter, the reference agonist WIN 55212-2 and the adenylyl cyclase activator NKH 477 were added at respective final concentrations of 3 nM and 3 µM. For basal control measurements, WIN 55212-2 was omitted from the wells containing 30 µM AM 630. Following a 10 min incubation at 37°C, the cells were lysed and the fluorescence acceptor (D2-labeled cAMP) and fluorescence donor (anti-cAMP antibody labeled with europium cryptate) were added. After 60 min at room temperature, the fluorescence transfer was measured at λex=337 nm and λem=620 and 665 nm using a microplate reader (EnVision® , Perkin Elmer). The cAMP concentration was determined by dividing the signal measured at 665 nm by that measured at 620 nm (ratio). The results are expressed as a percent inhibition of the control response to 10 nM WIN 55212-2. The standard reference antagonist is AM 630, which is tested in each experiment at several concentrations to generate a concentration-response curve from which its IC_{50} value was calculated.

Subsequently, five different combinations of PEA, Sichuan pepper, *Acmella oleracea*, cruciferous vegetable extract blend, and bovine colostrum filtrate were tested for additive or synergistic effects on CB_2 receptor binding. All compounds and their combinations were tested at 20.0 µg/ml for both agonist and antagonistic effects on CB_2 receptors using the same methods above.

In each experiment and if applicable, the respective reference compound was tested concurrently with the test compounds, and the data were compared with historical values determined at Eurofins. The experiment was carried out in accordance with Eurofins validated Standard Operating Procedures.

The results are expressed as a percent of control agonist response or inverse agonist response:

$$\frac{(measured\ response - background\ response)}{(control\ response - background\ response)} * 100$$

And as a percent inhibition of control agonist response:

$$100 - \frac{(measured\ response - background\ response)}{(control\ response - background\ response)} * 100$$

Obtained in the presence of the test compounds.

The EC_{50} values (concentration producing a half-maximal response) and IC_{50} values (concentration causing a half-maximal inhibition of the control agonist response) were determined by non-linear regression analysis of the concentration-response curves generated with mean replicate values using Hill equation curve fitting:

$$Y = D + \left(\frac{A - D}{1 + \left(\frac{C}{C_{50}}\right)^{nH}}\right)$$

Where, Y=response, A=left asymptote of the curve, D=right asymptote of the curve, C=compound concentration, and C_{50}=EC_{50} or IC_{50}, and nH=slope factor.

This analysis was performed using software developed at Cerep (Hill software) and validated by comparison with data generated by the commercial software SigmaPlot® 4.0 for Windows® (© 1997 by SPSS Inc.).

For the antagonists, the apparent dissociation constants (KB) were calculated using the modified Cheng Prusoff equation:

$$K_B = \frac{IC_{50}}{1 + \left(\frac{A}{EC_{50A}}\right)}$$

Where, A=concentration of reference agonist in the assay, and EC_{50A}=EC_{50} value of the reference agonist.

Results showing stimulation or an inhibition higher than 50% are considered to have strong effect, whereas stimulation or an inhibition between 25% and 50% are indicative of weak to moderate effects. Results showing stimulation or an inhibition lower than 25% are not considered significant and mostly attributable to variability of the signal around the control level. These ranges and cutoffs were selected largely due to conclusions drawn from two distinct pharmacological papers [30,31].

RESULTS

For the agonist assay, the individual ingredients with a strong stimulation effect for CB_2 receptor binding were *Acmella oleracea* (78.5%), cruciferous vegetable blend (59.1%), and two of the market brand CBD oils (52.5%, 50.5%) (Figure 2). The only

compound with a weak to moderate effect was Sichuan pepper (25.9%). The copaiba essential oil showed a strong inverse agonist effect (-125.7%). This test was primarily enacted as a means of screening individual ingredients with potential for CB_2 receptor binding in a multi-ingredient formula. However, relatively small standard error values with only having duplicate measurements add confidence to the results of binding effects.

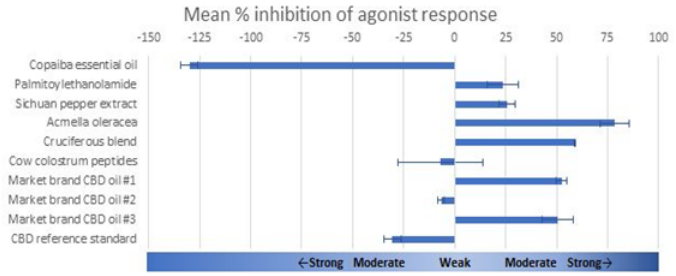

Figure 2: Histogram for mean CB_2 agonist effect (n=2) of individual ingredients and CBD oils. Cellular agonist effect of each tested sample was calculated as a % of the control response (100 nM WIN 55212-2 cannabinoid receptor agonist). Results showing stimulation or an inhibition higher than 50% are considered to have strong effect, whereas stimulation or an inhibition between 25% and 50% are indicative of weak to moderate effects. Less than 25% is considered to have no effect.

From the antagonist assay measured by percent inhibition of control agonist response, none of the individual ingredients or CBD oils showed significant, moderate or weak effects as antagonists for CB_2 receptor binding (Figure 3).

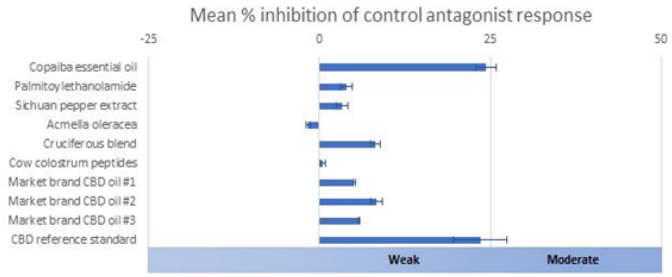

Figure 3: Histogram for mean CB_2 antagonist effect (n=2) of individual ingredients and CBD oils. Cellular antagonist effect was calculated as a % inhibition of the 3 nM WIN 55212-2 response in each well. Results showing a stimulation or an inhibition higher than 50% are considered to have strong effect, whereas a stimulation or an inhibition between 25% and 50% are indicative of weak to moderate effects. Less than 25% is considered to have no effect.

The specific percentage combinations of individual ingredients that were tested are found in Table 1. The ingredient formulas were developed based on results from the agonist data in Figure 2.

Table 1: Individual ingredient percentage ranges in different formulas that were tested for CB_2 agonist and antagonist effects. Ingredient ranges in red denote a decrease from the base formula, green denote an increase from the base formula. Specific blends are proprietary, but general CB_2 binding activity changes can be understood based on increase or decrease of specific compounds in the formulas.

	Copaiba essential oil	Palmitoyl-ethanolamide	Sichuan pepper extract	*Acmella oleracea* extract	Cruciferous extract blend	Bovine colostrum filtrate
Base Formula	20%-25%	35%-40%	10%-15%	10%-15%	5%-10%	10%-15%
Formula 2	0%-5%	45%-50%	15%-20%	15%-20%	5%-10%	15%-20%
Formula 3	0%-5%	50%-55%	15%-20%	15%-20%	5%-10%	0%-5%
Formula 4	20%-25%	30%-35%	10%-15%	20%-25%	10%-15%	10%-15%
Formula 5	10%-15%	35%-40%	5%-10%	20%-25%	10%-15%	10%-15%
Formula 6	45%-50%	0%-5%	0%-5%	50%-55%	0%-5%	0%-5%

Figure 4 shows the ingredient formulas with a significant stimulation effect as agonists for CB_2 receptor binding. The blend with the highest percent of control agonist response was Formula 2 (62.9%). This blend contained PEA, Sichuan pepper extract, *Acmella oleracea* extract, the blend of cruciferous extracts and bovine colostrum filtrate. The blend containing all six of the ingredients featured in Table 1 with the highest percent of control agonist response was Formula 5 (56.7%). There were no strong antagonistic effects of the formulas (Figure 5).

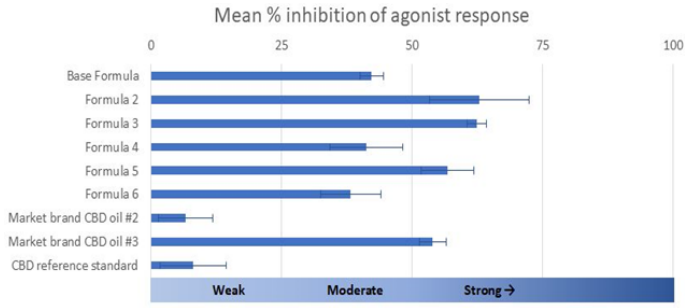

Figure 4: Histogram for mean CB_2 agonist effect (n=2) of ingredient formulas and CBD oils. Cellular agonist effect of each tested sample was calculated as a % of the control response (100 nM WIN 55212-2 cannabinoid receptor agonist). Results showing stimulation or an inhibition higher than 50% are considered to have strong effect, whereas stimulation or an inhibition between 25% and 50% are indicative of weak to moderate effects. Less than 25% is considered to have no effect.

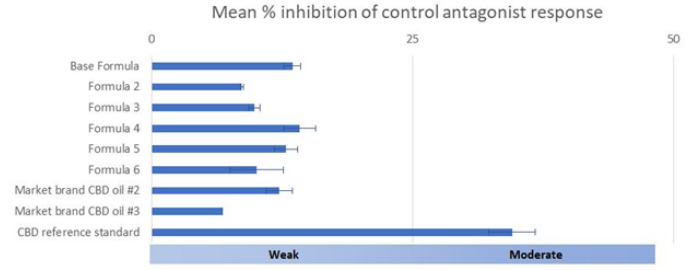

Figure 5: Histogram for mean CB_2 antagonist effect (n=2) of ingredient formulas and CBD oils. Cellular antagonist effect was calculated as a % inhibition of the 3 nM WIN 55212-2 response in each well. Results showing stimulation or an inhibition higher than 50% are considered to have strong effect, whereas stimulation or an inhibition between 25% and 50% are indicative of weak to moderate effects. Less than 25% is considered to have no effect.

Formula 5 had a reported EC_{50} of 13.2 ug/ml on CB_2 expressed CHO cell lines, while its EC_{50} on non-expressed cell line was >100 ug/ml. This data supported the hypothesis that receptor activity was driven primarily through CB_2 receptors. Test measuring the agonist response towards CB_1 receptors for Formula 5 did show some activity, like that of the CBD reference standard. This response is majorly attributed to the *Acmella oleracea* extract present in Formula 5 due to evidence from earlier studies on CB_1 receptor binding of individual phytocannabinoid ingredients.

DISCUSSION

Previous studies showed that several of the tested nutraceutical ingredients and compounds had an affinity for CB_2 receptor binding [11-22]. One study suggested a significant CB_2 binding effect for PEA [32]. However, additional studies conclude there is no direct binding between CB_2 receptors and PEA but that certain effects of PEA mediated by CB_2 receptors may be present [33,34]. The results of our study support the conclusion that direct PEA binding to CB_2 receptors is not significant *in vitro* at the tested concentration.

Acmella oleracea or electric daisy has been found to have CB_2 binding potential and be effective both orally and topically due to the specific alkylamide it contains called spilanthol, also known as affinin [35]. A study conducted by Gertsch et al. tested the potential of CB_2 receptor binding of various sources of N-alkylamides, including *Acmella oleracea* [21]. *A. oleracea* extracts did not have the highest binding affinity in their experiments, though it did show some affinity, as we found in our experiments. The *A. oleracea* used in our study was standardized to 20% spilanthol, the primary alkylamide present in this botanical. Difference in binding affinity between the two studies may be due to alkylamide concentration in the extracts.

Perhaps one of the more interesting points of discussion is the potent inverse agonist effect shown by the copaiba essential oil. The active cannabinoid constituent in copaiba is beta-caryophyllene [36]. The copaiba oil in our study contained ~50% beta-caryophyllene. In a mouse study by Bahi et al., beta-caryophyllene was identified as a CB_2 receptor agonist and shown to have positive behavioral effects on the mice in this model [37]. Another study supported beta-caryophyllene as a dietary cannabinoid showing that it was more potent than a high-affinity CB_2 receptor-selective agonist [15]. The effect shown by the copaiba oil in our study strongly suggests that copaiba oil is an inverse agonist which differs from data on beta-caryophyllene in other studies in that beta-caryophyllene is a direct agonist [37,38]. Inverse agonists bind to the CB_2 receptors but induce a response opposite of that of the agonists. Antagonists, on the other hand, differ from inverse agonists because antagonists simply bind to receptors, preventing the binding of agonists without eliciting an opposing response. Our findings support beta-caryophyllene as having an affinity for CB_2 receptors, but by causing a response which was different than an agonistic response.

The blend of cruciferous vegetable extracts had a strong CB_2 receptor agonist binding effect. The cruciferous extract blend contains a minimum of 42% 3,3'-diindoylmethane, indole-3-carbinol, and ascorbigen combined, with the major constituent being 3,3'-diindoylmethane. In a study by Yin et al., 3,3'-diindolylmethane was determined to be a CB_2 receptor partial agonist [23]. This result supports our data concerning the cruciferous vegetable blend. Differences in the strength of the agonist behavior could be due to the additional presence of the indole-3-carbinol and ascorbigen in our cruciferous extract, whereas the compound tested in the Yin et al. study was pure 3,3'-diindoylmethane.

Sichuan pepper extract, from the plant genus *Zanthoxylum*, have been studied for interactions with both CB_1 and CB_2 receptors [39]. Sichuan peppers contain components called sanshools which have been isolated as promising compounds that interact with CB_2 receptors [40]. Results from our study found no significant agonist effect of Sichuan pepper extract on CB_2 receptor binding.

Concentration and purity of the sanshools in the extracts of these peppers may vary, which could have a significant impact on their CB_2 receptor binding result.

Bovine colostrum filtrate, to our knowledge, has not been previously tested as a CB_2 receptor agonist. It was included in this study due to its known effects on the immune system, and we hypothesized that it may act through the CB_2 receptor. There was no significant agonist effect in our experiments. The lack of activity for this extract could be related to the highly complex nature of the mixture, which is comprised of immuno-modulatory proteins and peptides, carbohydrates, oligosaccharides, fats, vitamins and minerals [41]. Moreover, the ultrafiltration process of the raw colostrum, where the lipid-rich portion that presumably contains CB_2-active components is removed from the starting material, most likely reduces the presence of endocannabinoids.

Results for each ingredient were used to formulate combinations that might produce additive or synergistic effects on the CB_2 receptor. A base formula was established and then modified to evaluate the impact of decreasing and increasing individual ingredients. For instance, the relative amount of copaiba essential oil was generally decreased across most of the formulas owing to its strong inverse agonist effect. Conversely, the relative amount of *Acmella oleracea* extract was generally increased across the formulas due to its strong agonist activity. The other ingredients were adjusted up or down to optimize activity. As expected, the formulas with less copaiba essential oil, more *Acmella oleracea* extract, and more of the other ingredients yielded a stronger agonist effect compared to the base formula. No synergistic effects were observed in any of the formula combinations.

Results for marketed CBD oil products were consistent across the two sets of experiments, giving confidence in the reproducibility of the CB_2 assays. Compared to one another, however they demonstrated a large disparity in CB_2 agonist activity. Two of the marketed products had significant agonist effect percent response, while one brand had almost no measurable effect. Though CBD oils are marketed similarly, the concentration of CBD and the presence of CBD-like compounds may vary widely and is not always consistent with what is claimed on the label [42]. Our results were consistent with the degree of CBD standardization; that is, the two products that were standardized to a higher concentration of CBD had greater responses, where the lower concentration did not [43]. Pure CBD has been shown to display inverse agonism towards the human CB_2 receptor, which is consistent with the purified CBD reference material in these experiments [44].

CONCLUSION

This study is the first to measure the CB_2 receptor binding affinity of various individual ingredients, CBD oils, and ingredient mixes at the same time using a similar metric *in vitro*. Because of the format of the study, active comparisons of ingredient CB_2 binding affinity was possible, as well as binding effects of combinations of several of these ingredients.

CB_2 receptor binding was highest in *Acmella oleracea* extract. Other ingredients that showed significant CB_2 binding affinity were a blend of cruciferous vegetable extracts and two commercial CBD oil products. Copaiba essential oil showed a strong inverse agonist effect, which requires further investigation. The combination of PEA, Sichuan pepper extract, *Acmella oleracea* extract, cruciferous vegetable extract blend, and bovine colostrum filtrate had the highest agonistic effect, though the affinity was still lower than that of the *Acmella oleracea* alone.

Several of the phytocannabinoid ingredient combinations were shown to be as effective, if not more, than several leading brand CBD oils. This suggests that therapeutic benefits, such as positive physical and emotional support, may also be provided by these phytocannabinoid ingredients and formulas. Additional studies in vivo are necessary to substantiate any effects that these compounds may have on the human endocannabinoid system.

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AUTHOR DISCLOSURE STATEMENT

The authors are employees of 4Life Research USA, LLC.

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REFERENCES

1. Turcotte C, Blanchet M, Laviolette M, Flamand N. The CB_2 receptor and its role as a regulator of inflammation. Cell Mol Life Sci. 2016;73(23):4449-4470.
2. Ziring D, Wei B, Velazquez P, Schrage M, Buckley NE, Braun J. Formation of B and T cell subsets require the cannabinoid receptor CB_2. Immunogen. 2006;58(9):714-725.
3. Mead A. The legal status of cannabis (marijuana) and cannabidiol (CBD) under U.S. law. Epilepsy Behav. 2017;70B:288-291.
4. Pertwee RG. Pharmacology of cannabinoid CB_1 and CB_2 receptors. Pharm Ther. 1997;74(2):129-180.
5. Guindon J, Hohmann AG. Cannabinoid CB_2 receptors: A therapeutic target for the treatment of inflammatory and neuropathic pain. British J Pharmacol. 2008;153:319-334.
6. White CM. A review of human studies assessing cannabidiol's (CBD) therapeutic actions and potential. J Clin Pharmacol. 2019;59:923-934.
7. McPartland JM, Duncan M, Di Marzo V, Pertwee RG. Are cannabidiol and Δ9-tetrahydrocannabivarin negative modulators of the endocannabinoid system? A systematic review. Br J Pharmacol. 2015;172:737-753.

8. Di-Marzo V, Piscitelli F. The endocannabinoid system and its modulation by phytocannabinoids. Neurotherap. 2015;12:692-698.

9. Corroon J, Felice JF. The endocannabinoid system and its modulation by cannabidiol (CBD). Altern Ther Health Med. 2019;25(S2):6-14.

10. Ashton CH, Moore PB. Endocannabinoid system dysfunction in mood and related disorders. Acta Psychiatr Scand. 2011;124:250-261.

11. Corroon J, Kight R. Regulatory status of cannabidiol in the United States: A perspective. Cannabis Cannabinoid Res. 2018;3(1):190-194.

12. Hill MN, Miller GE, Carrier EJ, Gorzalka BB, Hillard CJ. Circulating endocannabinoids and N-acyl ethanolamines are differentially regulated in major depression and following exposure to social stress. Psychoneuroendo. 2009;34:1257-1262.

13. Locci A, Pinna G. Stimulation of peroxisome proliferator-activated receptor- by N-palmitoylethanolamine engages allopregnanolone biosynthesis to modulate emotional behavior. Biol Psych. 2009;85:1036-1045.

14. Sugiura T, Kondo S, Kishimoto S, Nakane S, Kodaka T, Suhara Y, et al. Evidence that 2-arachidonoylglycerol but not N-palmitoylethanolamine or anandamide is the physiological ligand for the cannabinoid CB_2 receptor. Comparison of the agonistic activities of various cannabinoid receptor ligands in HL-60 cells. J Biol Chem. 2005;275:605-612.

15. Gertsch J, Leonti M, Raduner S, Racz I, Chen JZ, Xie XQ. Beta-caryophyllene is a dietary cannabinoid. Proc Natl Acad Sci USA. 2008;105:9099-9104.

16. Dossou KS, Devkota KP, Morton C. Identification of CB_1/CB_2 ligands from *Zanthoxylum* bungeanum. J Nat Prod. 2013;76:2060-2064.

17. Brellenthin AG, Crombie KM, Hillard CJ, Koltyn KF. Endocannabinoid and mood responses to exercise in adults with varying activity levels. Med Sci Sports Exerc. 2017;49:1688-1696.

18. Stone NL, Millar SA, Herrod PJJ, Barrett DA, Ortori CA, Mellon VA, et al. An analysis of endocannabinoid concentrations and mood following singing and exercise in healthy volunteers. Front Behav Neurosci. 2018;12:269.

19. Bahi A, Al Mansouri S, Al Memari E, Ameri MA, Nurulain SM, Ojha S. -Caryophyllene, a CB_2 receptor agonist produces multiple behavioral changes relevant to anxiety and depression in mice. Physiol Behav. 2014;135:119-124.

20. Zhang M, Wang J, Zhu L, Li T, Jiang W, Zhou J, et al. *Zanthoxylum* bungeanum Maxim. (Rutaceae): A systematic review of its traditional uses, botany, phytochemistry, pharmacology, pharmacokinetics, and toxicology. Int J Mol Sci. 2017;18.

21. Gertsch J, Raduner S, Feyen F. Exploration of natural alkylamides and synthetic analogs as source for new ligands for the cannabinoid type-2 receptor. Planta Med. 2006;72:970-971.

22. Urasaki Y, Beaumont C, Workman M, Talbot JN, Hill DK, Le TT. Fast-acting and receptor-mediated regulation of neuronal signalling pathways by copaiba essential oil. Int J Mol Sci. 2020;21:2259.

23. Yin H, Chu A, Li W, Wang B, Shelton F, Otero F. Lipid G protein-coupled receptor ligand identification using beta-arrestin Path Hunter assay. J Biol Chem. 2009;284(18):12328-12338.

24. Vetvicka V, Vetvickova J. Effects of transfer factor supplementation on immune reactions in mice. J Nutr Health Sci. 2019;6(3):301.

25. Vetvicka V, Fernandez-Botran R. Non-specific immune-stimulatory effects of transfer factor. Int Clin Pathol J. 2020;8(1):1-6.

26. Vieira-Brock P, Anderson A, Vaughan B. Method development for the analysis of PBMC-mediated killing of K562 cells by bovine colostrum and various fractions. J Immunol. 2019;202(1).

27. Mechoulam R, Berry EM, Avraham Y, Marzo VD, Fride E. Endocannabinoids, feeding and suckling--from our perspective. Int J Obes (Lond). 2006;30:S24-S28.

28. Malek N, Popiolek-Barczyk K, Mika J, Przewlocka B, Starowicz K. Anandamide, acting *via* CB_2 Receptors, alleviates LPS-induced neuroinflammation in rat primary microglial cultures. Neural Plast. 2015;2015:130639.

29. Felder CC, Joyce KE, Brilet EM, Mansouri J, Mackie K, Blond O, et al. Comparison of the pharmacology and signal transduction of the human cannabinoid CB_1 and CB_2 receptor. Mol Pharmacol. 1995;48:443.

30. Bowes J, Brown AJ, Hamon J, Jarolimek W, Sridhar A, Waldron G, et al. Reducing safety-related drug attrition: the use of *in vitro* pharmacological profiling. Nat Rev Drug Discov. 2012;11(12):909-922.

31. Jenkinson S, Schmidt F, Rosenbrier-Ribeiro L, Delaunois A, Valentin JP. A practical guide to secondary pharmacology in drug discovery. J Pharmacol Toxicol Method. 2020;106869.

32. Lambert DM, Di-Marzo V. The palmitoylethanolamide and oleamide enigmas: are these two fatty acid amides cannabimimetic? Curr Med Chem. 1999;6(8):757-773.

33. Farquhar-Smith WP, Jaggar SI, Rice AS. Attenuation of nerve growth factor-induced visceral hyperalgesia *via* cannabinoid

CB(1) and CB(2)-like receptors. Pain. 2002;97(1-2):11-21.

34. Saliba SW, Jauch H, Gargouri B, Keil A, Hurrle T, Volz N. Anti-neuroinflammatory effects of GPR55 antagonists in LPS-activated primary microglial cells. J Neuroinflam. 2018;15(1):322.

35. Boonen J, Baert B, Roche N, Burvenich C, Spiegeleer BD. Transdermal behaviour of the N-alkylamide spilanthol (affinin) from Spilanthes acmella (Compositae) extracts. J Ethnopharmacol. 2010;127(1):77-84.

36. Soares DC, Portella NA, Ramos MF, Siani AC, Saraiva EM. Trans-β-caryophyllene: an effective antileishmanial compound found in commercial copaiba oil (Copaifera spp.). Evid Based Compl Alternat Med. 2013;2013:761323.

37. Yang M, Lv Y, Tian X. Neuroprotective Effect of β-Caryophyllene on Cerebral Ischemia-Reperfusion Injury *via* Regulation of Necroptotic Neuronal Death and Inflammation: In Vivo and *in Vitro*. Front Neurosci. 2017;11:583.

38. Klauke AL, Racz I, Pradier B, Markert A, Zimmer AM, Gertsch J, et al. The cannabinoid CB_2 receptor-selective phytocannabinoid beta-caryophyllene exerts analgesic effects in mouse models of inflammatory and neuropathic pain. Eur Neuropsychopharmacol. 2014;24(4):608-620.

39. Dossou KSS, Devkota KP, Morton C. Identification of CB_1/CB_2 ligands from *Zanthoxylum* bungeanum. J Nat Prod. 2013;76(11):2060-2064.

40. Sharma C, Sadek B, Goyal SN, Sinha S, Kamal MA, Ojha S. Small molecules from nature targeting G-protein coupled cannabinoid receptors: potential leads for drug discovery and development. Evid Based Compl Alternat Med. 2015;2015:238482.

41. Godhia ML, Patel N. Colostrum - its composition, benefits as a nutraceutical- a review. Curr Res Nutr Food Sci. 2013;1(1):37-47.

42. Wakshlag JJ, Cital S, Eaton SJ, Prussin R, Hudalla C. Cannabinoid, terpene, and heavy metal analysis of 29 over-the-counter commercial veterinary hemp supplements. Vet Med (Auckl). 2020;11:45-55.

43. UNODC. Degree of CBD standardization for the marketed products was determined by review of certificate of analyses for the specific lots tested in our experiments. UN. 2009.

44. Thomas A, Baillie GL, Phillips AM, Razdan RK, Ross RA, Pertwee RG. Cannabidiol displays unexpectedly high potency as an antagonist of CB_1 and CB_2 receptor agonists *in vitro*. Br J Pharmacol. 2007;150(5):613-623.