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Research Article

A Phytocannabinoid Formula Containing Palmitoylethanolamide, *Acmella Oleracea* Extract, and Bovine Colostrum Filtrate Inhibited Cytokine and Chemokine Production in Human Peripheral Blood Mononuclear Cells Stimulated Ex Vivo

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A phytocannabinoid formula containing copaiba essential oil, palmitoylethanolamide, Sichuan pepper extract, *Acmella oleracea* extract, cruciferous vegetable extracts blend, and bovine colostrum filtrate was tested for its ex vivo effect on inhibiting stimulated cytokine release in human peripheral blood mononuclear cells. Effects of the phytocannabinoid formula on the peripheral blood mononuclear cells viability were measured by alamarBlue™ to confirm no obvious cytotoxicity. The phytocannabinoid formula was compared to reference compounds for its ability to inhibit both the phytohemagglutinin-stimulated release of the cytokines from T-cells and the lipopolysaccharide-stimulated release of the cytokines from macrophages/monocytes in human peripheral blood mononuclear cells. The T cell cytokine responses measured were those of interleukins (interleukin-1β, interleukin-2, interleukin-4, interleukin-5, interleukin-6, interleukin-8, interleukin-10, and interleukin-13) and interferon-gamma, interferon gamma-induced protein 10, macrophage inflammatory protein-1α, and tumor necrosis factor-α. The macrophage and monocyte cytokine responses measured were of interleukin-1β, interleukin-6, interleukin-8, macrophage inflammatory protein-1α, and tumor necrosis factor-α. For the phytohemagglutinin-stimulated T cell production of cytokines, the peripheral blood mononuclear cells treated with the Phytocannabinoid formula at 2 µg/mL and 20 µg/mL showed significant inhibition of interleukin-2, interleukin-6, interleukin-5, interleukin-8, interleukin-10, interleukin-13, and interferon gamma-induced protein 10 production. The phytocannabinoid formula also significantly reduced the macrophage/monocyte production of interleukin-1β and interleukin-6. Results from the present study showed that the phytocannabinoid formula inhibited cytokines and chemokines production in ex vivo stimulated human peripheral blood mononuclear cells, suggesting a potential immunomodulatory effect.

Keywords: Cannabinoids, Cytokines, Immune system, Immunoglobulins, Peripheral blood mononuclear cells, Phytocannabinoids

Abbreviations Used: Analysis of variance, ANOVA; Dimethylsulfoxide, DMSO; Endocannabinoid system, ECS; Interferon-gamma, IFNγ; Interleukin-10, IL-10; Interleukin-13, IL-13; Interleukin-1β, IL-1β; Interleukin-2, IL-2; Interleukin-4, IL-4; Interleukin-5, IL-5; Interleukin-6, IL-6; Interleukin-8, IL-8; Interferon gamma-induced protein 10, IP-10; Lipopolysaccharide, LPS; Macrophage inflammatory protein-1α, MIP-1α; 1-methyl-4-phenyle-1,2,3,6-tetrahydropyridine, MPTP; Peripheral blood mononuclear cells, PBMCs; Palmitoylethanolamide, PEA; Phytocannabinoid formula, PF; Phytohemagglutinin, PHA; Per cent of control, POC; Δ9-tetrahydrocannabinol, THC; Tumor necrosis factor-α, TNF-α

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INTRODUCTION

Cannabinoids are bioactive compounds found in the cannabis plant that interact with the human endocannabinoid system (Liu et al., 2022). The cannabinoids produced naturally within the human body are generally referred to as endocannabinoids, while the cannabinoids of external or plant origin are called phytocannabinoids (Gertsch et al., 2010). The human ECS is a complex signaling system comprised of ligands, enzymes, and receptors. Specific cannabinoid compounds interact with the major endocannabinoid receptors, CB_1 and CB_2, to affect pain modulation, nervous system function, and other therapeutic benefits (Marzo et al., 2004). Of great interest are CB_2 receptor agonists, which bind specifically to CB_2 receptors, thus avoiding the psychoactive effects attributed to CB_1 receptor binding (Pacher et al., 2006). The CB_2 receptors are known to be found in certain cells involved in immune system response, including T cells and macrophages (Turcotte et al., 2016). Various studies show that the binding of the cannabinoids to the CB_2 receptors promotes an immunosuppressive response (Eisenstein and Meissler, 2015).

A recent study showed that various phytocannabinoids, both individually and in combination, have a significant CB_2-binding effect in vitro as compared to certain CBD oils (Lee et al., 2021). A significant binding interaction, however, does not necessarily constitute a positive immunological impact. Various response markers can be measured to more directly determine if a formula will support the immune system, or a healthy immune response.

Cytokine production is a response that is measured to determine how a compound may affect immune system's action (Shaw et al., 2018). A controlled production of the cytokines within the human body allows for controlled inflammation, which is essential for a healthy response to infection (Pahwa et al., 2018). Inflammation allows the human body to focus on repairing areas that are damaged or under attack. However, overstimulated or prolonged inflammatory responses can cause additional damage to the body. During an inflammatory response, macrophages release the cytokines and chemokines to inform other cells in the body of the infection or foreign pathogens (Chen et al., 2017).

The cytokines function as a form of communication between cells and play a key role in accelerating the immune response (Zhang and An, 2007). Interleukins are some of the most common cytokines that help regulate the human immune system. Interleukins have a wide range of properties including proinflammation, anti-inflammation, signaling, and growth and function of other immune cells (Bernardi et al., 2018). Chemokines are a subgroup of the cytokines that have chemotactic activity or activity in response to chemical stimulus (Vilgelm and Richmond, 2019). Their specific roles include activation and movement of the immune cells, cell growth, involvement in the inflammation response, and apoptosis. The cannabinoids interacting with the CB_2 receptors have been shown to reduce cytokine and chemokine production by the immune cells (Miller and Stella, 2008). Because of these effects, it is believed that the cannabinoids have potential to modulate and assist with controlling damage during an immune response (Turcotte et al., 2016).

The present study was designed to determine the effects, ex vivo, that a PF, composed of the CB_2 receptor agonists, would have on T cell cytokine production in human PBMCs and macrophage/monocyte cytokine production in the PBMCs stimulated ex vivo.

MATERIALS AND METHODS

Two different assays were run in this study. The first protocol was for measuring the effects of the PF on T cell cytokine production in human PBMCs stimulated ex vivo. The second protocol was for the effects of the PF on macrophage/monocyte cytokine production in the human PBMCs stimulated ex vivo.

To ensure that results of the studies were not significantly impacted by cell death due to potential cytotoxicity of the test compounds, a supplementary cytotoxicity protocol was employed. The Cytotoxicity Assay Protocol employed was as follows. For treatment of the PBMCs, the assay protocol was followed as mentioned earlier, with the same parameters and treatments. A separate plate was seeded with cells (2×10^5 per well) to establish a baseline reading. AlamarBlue™ reagent (1:10) was added to this plate and incubated for 4 h 37°C, with 5% CO_2. This plate was then read on a fluorescence plate reader at 530 nM (530–570) excitation and 595 nM (580–610) emission. The reference control and staurosporine, a nonselective inhibitor or protein kinases that induces apoptosis, were resuspended in DMSO at 1 mM to make a working stock solution. The staurosporine control was used at 1 µM final concentration. The PF was added to the PBMCs in volumes mentioned earlier (T-cell Inflammation Inhibition Assay Protocol) to give final assay volumes of 200 µL. Controls were added to the PBMCs in volumes of 10 µL of diluted working stock, followed by the addition of 10 µL of appropriately diluted vehicle, to mimic compound addition, to give final assay volumes of 200 µL. The final DMSO vehicle concentration in the assay was 0.1%. The plates were incubated at 37°C, with 5% CO_2 for 48 h. At 44 h before the end of the incubation period, the plates were removed, alamarBlue™ reagent was added (1:10), and the plates were incubated until 48 h 37°C, with 5% CO_2. The plates were read on a fluorescence plate reader at 530 nM (530–570) excitation and 595 nM (580–610) emission. Relative fluorescence units were measured to allow calculation of cell viability as percentage of control values, using the following equation:

$$\frac{\textit{Mean relative fluorescence value of test sample}}{\textit{Mean relative fluorescence value of vehicle (0.1\% DMSO) control}}\times 100\%$$

T Cell Inflammation Inhibition Assay

The human PBMCs used in these studies were donated by a healthy male subject free of blood-borne viruses. Immuno-phenotypic characterization was done by flow cytometry, and simulation studies were used to qualify the donor. The PBMCs were isolated from fresh normal blood leukapheresis by standard Ficoll ingredient method, washed, and then cryopreserved. The cryopreserved human PBMCs were drip thawed and diluted to the appropriate density and seeded into three 96-well polystyrene plates (2×10^5 cells per well) with 150 µL per well of culture medium (RPMI 1640, 10% heat-inactivated FBS, 1% penicillin/streptomycin, 2 mM L glutamine). The cells were incubated at 37°C, with 5% CO_2 for 1 h prior to the addition of the PF. The ingredients contained in the PF were:

(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 ultrafiltered (to less than 10 kDa) whole colostrum and containing no less than 37% peptides

The PF blend contained, in decreasing order by percent of total formula weight, palmitoylethanolamide (35–40%), *A. oleracea* extract (20–25%), cruciferous extract (10–15%), copaiba essential oil (10–15%), bovine colostrum filtrate (10–15%), and Sichuan pepper extract (5–10%). The PF was solubilized in DMSO and further diluted to 20X with a cell culture medium. The PF was then added to the PBMCs in volumes of 10 µL (1X), in triplicate, and incubated for 1 h at 37°C, with 5% CO_2. Dexamethasone reference compound, 10 µL @ 100 nM final concentration, was added to the control wells according to the plate map and incubated for 1 h at 37°C, with 5% CO_2 (Smits et al., 1998). After 1 h of incubation, 40 µL of phytohemagglutinin (PHA) (10 µg/mL) was added to the PF wells or controls wells and incubated for 48 h at 37°C, with 5% CO_2 (Fan et al., 1998). The plates were centrifuged at 200X gravity for 10 min. Cell culture supernatants were collected and stored at −80°C and later read utilizing flow cytometry on a Luminex® device for the simultaneous measurement of multiple cytokines (Hildesheim et al., 2002; Prabhakar et al., 2002; Sullivan et al., 2000; Visser et al., 2001). All the cytokines were diluted to fall within the readable range of the analytical instruments and then multiplied by their respective dilution factor to obtain the result. Specific dilutions for the cytokines of the T cell assay were 1:2 dilution (IL-2, IL-4, IL-5, IL-10, IL-13, IL-1β, and IP-10), 1:8 dilution (IFNγ, IL-6, MIP-1α, and TNFα), and 1:20 dilution (IL-8).

Macrophage/Monocyte Inflammation Inhibition Assay

The macrophage/monocyte assay was very similar to that of the T cell assay. Initially, the cryopreserved human PBMCs were drip thawed and were then diluted to the appropriate density (2×10^5 per well) and seeded into 96-well polypropylene plates with 150 µL per well of culture medium (RPMI 1640, 10% heat inactivated FBS, 1% penicillin/streptomycin, 2 mM L-glutamine). The cells were incubated at 37°C, with 5% CO_2 for at least 1 h prior to the addition of test compounds or controls. The PF was suspended in DMSO at 20 mg/mL concentration. It was further diluted to 20X assay concentrations with the cell culture medium and added to the human PBMCs in volumes of 10 µL. The reference compound dexamethasone (100 nM) was added to the control wells. The cells were incubated for 1 h at 37°C, with 5% CO_2. The positive control, LPS (from *E. coli* O111:B4, Cat # L3024, Millipore Sigma) was resuspended in endotoxin-free water at 5 mg/mL to make the working stock solutions (Janský et al., 2003). After the 1-h incubation, 40 µL of the diluted working stock (LPS from *E. coli*, final assay concentration 50 pg/mL) was added to the PF wells and reference compound wells to give final assay volumes of 200 µL. The working stocks of the controls were added to the PBMCs in volumes of 40 µL, followed by addition of 10 µL of appropriately diluted vehicle, to mimic compound addition, to give final assay volumes of 200 µL. LPS (*E. coli*) was used at 50 pg/mL. The plates were incubated at 37°C, with 5% CO_2 for 24 h. The plates were then centrifuged at 200X g for 10 min. The cell culture supernatants were collected and stored at −80°C and later read utilizing flow cytometry on a Luminex® device for the simultaneous measurement of multiple cytokines (Chandok and Farber, 2004; Chen, 2005; Kellar et al., 2001; Tsang et al., 2006; Welker et al., 1996). Human IL-1β, IL-6, IL-8, MIP-1α, and TNFα were all diluted 1:8 so that their results would fall within the readable range of the fluorescence plate reader. Afterward, they were multiplied by their dilution factor to obtain the final measurements.

Data Analysis and Limits of Detection for Analyte

Luminex® assessments of the cytokine levels in the cell culture supernatants were performed per manufacturer's protocol using the Human Cytokine/Chemokine Magnetic bead panel from Millipore Sigma (catalogue No. HCYTOMAG-60K) with standards range of 3.2, 16, 80, 400, 2,000, and 10,000 pg/mL. Levels of induction of each cytokine were interpolated from a standard curve, using a 5-point nonlinear regression analysis, where y = (A + ((B–A)/(1 + (((B–E)/(E–A))*((x/C)^D))))). The raw data in the form of median fluorescence units were interpolated into test concentrations (picogram/milliliter). Outlier calculations were performed using the Grubbs' maximum normed residual test available in GraphPad Prism Version 8.0. All data are presented as the average of three replicates with standard deviation. All data are normalized and compared to the cytokine level in untreated PHA-stimulated T cells in the human PBMCs (standardized to 100%) or in untreated LPS-stimulated macrophage/monocyte cells in the human PBMCs (standardized to 100%). Statistical differences were calculated by the ANOVA test.

RESULTS

Positive controls elicited significant secretions of all the cytokines, while the negative controls did not elicit cytokine secretion. PHA is a plant lectin that is commonly used for human lymphocyte stimulation. The PHA-positive controls significantly induced secretion of all the cytokines/chemokines examined (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, IFNγ, IP-10, MIP-1α, and TNFα) while dexamethasone-inhibition controls decreased the cytokine and chemokine secretion confirming performance and validity of the assay. Dexamethasone is a potent drug used to treat various inflammatory conditions and is often used in similar studies as an inhibition control. The positive control for the macrophage and monocyte cytokine assay was lipopolysaccharides (LPS from *E. coli* O111:B4, Cat # L3024, Millipore Sigma). LPS is a sugar that is common in the cell walls of Gram-negative bacteria and has been shown to stimulate the release of the various cytokines in most cell types. This positive control also successfully stimulated the cytokine release of all measured cytokines (IL-1β, IL-6, IL-8, MIP-1α, and TNFα) from the human PBMCs. Dexamethasone served as the inhibition reference control for the macrophage and monocyte assay. It inhibited the cytokine release within the expected ranges.

Little to a total absence of toxicity of the PF on the T cell and the macrophage was observed in monocyte assay on the PBMCs. The T cell cytotoxicity of the PF showed 86% of the effect of the LPS percent of control at the concentration of 2 µg/mL, and 71% of the POC at the concentration of 20 µg/mL. In the macrophage

and monocyte assay, the PF exhibited 103.8% the effect of the LPS POC at the concentration of 2 µg/mL, and 112.2% the POC at the concentration of 20 µg/mL. The cytotoxicity results of the PF were close to that of the control at both concentrations in both the assays, indicating no obvious cytotoxicity of the PF in the PBMCs.

A POC at 75–100% was considered weak to no cytokine response suppression. A POC of 50–75% was considered moderate cytokine response suppression. A POC of less than 50% was considered strong cytokine response suppression. One-factor ANOVA was also run to determine which treatments were significantly different from the untreated PHA and LPS-stimulated controls for each cytokine response measured.

The PF had the following impact on the stimulated T cell production of the tested cytokines: PF (2 µg/mL) significantly and strongly inhibited the production of IL-2 (59% decrease), IL-6 (54% decrease), IL-10 (66% decrease), and IP-10 (76% decrease) as compared to the PHA-stimulated positive control. The PF (2 µg/mL) significantly inhibited the production of IL-5 (26% decrease), IL-8 (42% decrease), and IL-13 (31% decrease). The PF (2 µg/mL) had a nonsignificant weak to no inhibition on the production of IL-1β, IL-4, IFNγ, MIP-1α, and TNFα.

At the higher concentration of 20 µg/mL, the PF significantly and strongly inhibited IL-2 (58% decrease), IL-6 (53% decrease), IL-10 (66% decrease), and IP-10 (90% decrease). The PF (20 µg/mL) significantly inhibited cytokine production of IL-5 (29% decrease) and IL-13 (43% inhibition). The PF (20 µg/mL) had a nonsignificant weak to no inhibition on the production of IL-1β, IL-4, IL-8, IFNγ, MIP-1α, and TNFα. Comparative results for the cytokine production of the stimulated T cell assay are shown in Figures 1 and 2.

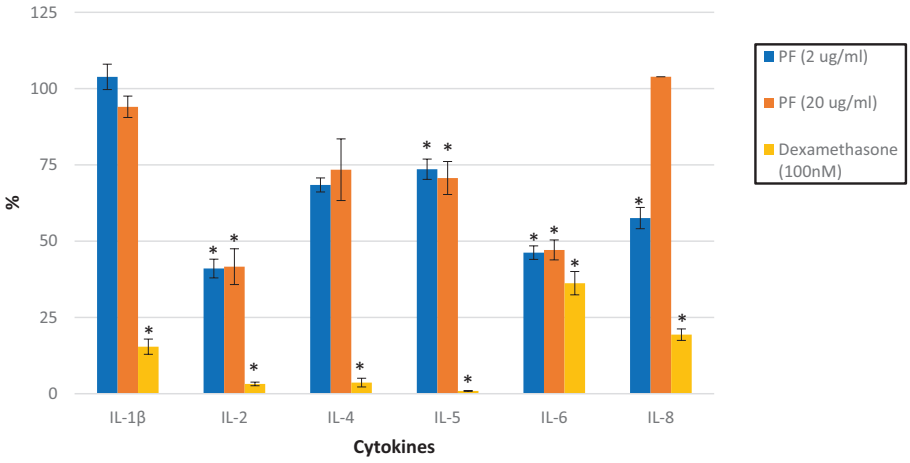


FIGURE 1 | Cytokine (IL-1β, IL-2, IL-4, IL-5, IL-6, and IL-8) levels produced by PHA-stimulated T cells in human PBMCs. Blue bars indicate treatment with low concentration (2 µg/mL) of PF, orange bars indicate treatment with high concentration (20 µg/mL) of PF, and yellow bars indicate treatment with dexamethasone (100 nM). All data are presented as the average of three replicates with standard error (black lines). All data are normalized and compared to cytokine level in untreated PHA-stimulated T cells in human PBMCs (standardized to 100%). Bars with an asterisk represent treated cytokine responses that are significantly different (P-value ≤ 0.05) from the untreated PHA-stimulated T cells.

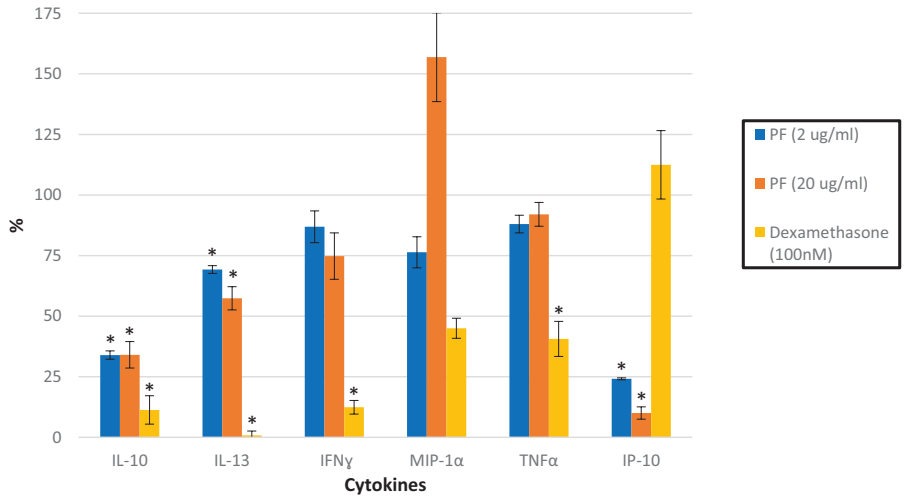


FIGURE 2 | Cytokine (IL-10, IL-13, IFNγ, MIP-1α, TNFα, and IP-10) levels produced by PHA-stimulated T cells in human PBMCs. Blue bars indicate treatment with low concentration (2 µg/mL) of PF, orange bars indicate treatment with high concentration (20 µg/mL) of PF, and yellow bars indicate treatment with dexamethasone (100 nM). All data are presented as the average of three replicates with standard error (black lines). All data are normalized and compared to cytokine level in untreated PHA-stimulated T cells in human PBMCs (standardized to 100%). Bars with an asterisk represent treated cytokine responses that are significantly different (P-value ≤ 0.05) from the untreated PHA-stimulated T cells.

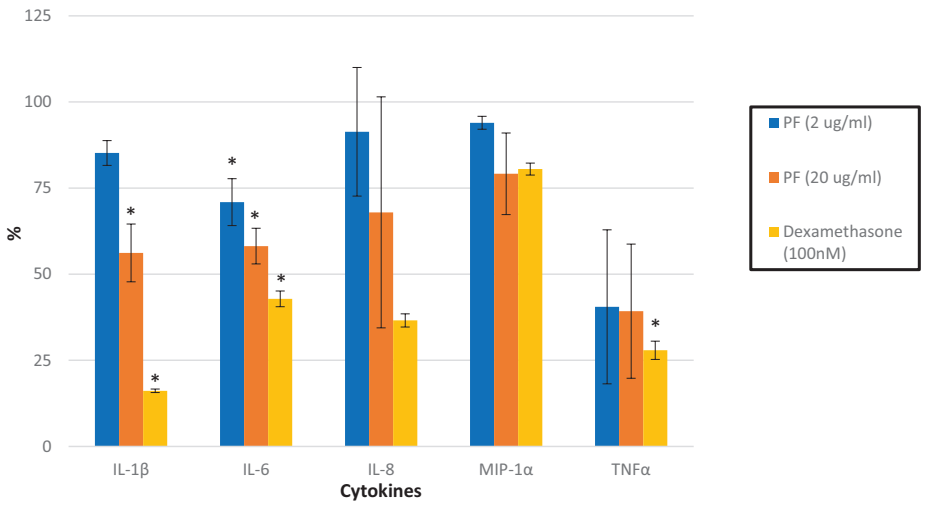


FIGURE 3 | Cytokine (IL-1β, IL-6, IL-8, MIP-1α, and TNFα) levels produced by LPS-stimulated macrophage/monocyte cells in human PBMCs. Blue bars indicate treatment with low concentration (2 μg/mL) of PF, orange bars indicate treatment with high concentration (20 μg/mL) of PF, and yellow bars indicate treatment with dexamethasone (100 nM). All data are presented as the average of three replicates with standard error (black lines). All data are normalized and compared to cytokine level in untreated LPS-stimulated macrophage/monocyte cells in human PBMCs (standardized to 100%). Bars with an asterisk represent treated cytokine responses that are significantly different (P-value ≤ 0.05) from the untreated LPS-stimulated T cells.

TABLE 1 | Summary of significant T cell and macrophage/monocyte cytokine production inhibition in human PBMCs treated with phytocannabinoid formula at two concentrations (2 μg/mL and 20 μg/mL) as compared to positive controls (PHA for T cell assay; LPS for macrophage/monocyte assay).

Cytokines	T cell assay		Macrophage/Monocyte assay	
	2 μg/mL	20 μg/mL	2 μg/mL	20 μg/mL
IL-1β	No inhibition	No inhibition	No inhibition	44%*
IL-2	59%*	58%*	Not measured	
IL-4	No inhibition	No inhibition	Not measured	
IL-5	26%*	29%*	Not measured	
IL-6	54%*	53%*	29%*	42%*
IL-8	42%*	No inhibition	No inhibition	No inhibition
IL-10	66%*	66%*	Not measured	
IL-13	31%*	43%*	Not measured	
IFNγ	No inhibition	No inhibition	Not measured	
IP-10	76%*	90%*	Not measured	
MIP-1α	No inhibition	No inhibition	No inhibition	No inhibition
TNFα	No inhibition	No inhibition	No inhibition	No inhibition

*P-value < 0.05.

The PF had the following impact on the stimulated macrophage/monocyte production of the tested cytokines:

PF (2 μg/mL) did significantly inhibit the production of IL-6 (29%). The PF (2 μg/mL) had a nonsignificant weak to no inhibition on the production of IL-1β, IL-8, MIP-1α, and TNFα. At the higher concentration, the PF (20 μg/mL) still did not significantly inhibit the production of any of the measured cytokines. The PF (20 μg/mL) did significantly inhibit the production of IL-1β (44% decrease) and IL-6 (42% decrease). The PF (20 μg/mL) had a nonsignificant weak to no inhibition on the production of IL-8, MIP-1α, and TNFα. Results for the cytokine production of the stimulated macrophage/monocyte assay are shown in Figure 3. A complete summary of results for both the assays is shown in Table 1.

DISCUSSION

Existing studies have shown that several of the ingredients included in the PF have the CB_2 receptor binding activity (Lee et al., 2021). The aforementioned study discusses in detail the CB_2-binding affinity of the individual ingredients contained in the PF, as well as different ratios of combinations of the ingredients. Some of the ingredients are standardized to the actives that are thought to promote CB_2 binding. However, other bioactive compounds are present in the blend, thus the potential cytokine suppressing effects must be attributed to the PF formula, and not necessarily to its individual constituents. Although their CB_2-binding activity has been studied, the downstream immune effect of the CB_2-receptor binding is unclear. The results of the present study shed light on the impact of the PF on various cytokines and chemokines produced by the stimulated T cells and macrophage/monocyte cells. The following discussion identifies previous investigations that focus on the impact of certain cannabinoids or the CB_2 agonists on the production of various cytokines by different stimulated cell types and compares those results to the present study: monocytes and macrophages are the primary immune producers of IL-1β. It is generally released during forms of cellular trauma such as cell injury, infection, or inflammation. IL-1β helps regulate immune response due to its anti-inflammatory characteristics as wells as its ability to stimulate the production of proinflammatory cytokines (Zhang and An, 2007; Kaneko et al., 2019). There is evidence from a past investigation that certain cannabinoids have the potential to inhibit the secretion of IL-1β by THP-1 cells (Klegeris et al., 2003). The present study did not show a significant decrease of T-cell stimulated production IL-1β (at both 20 and 2 μg/mL concentrations) production as compared to the positive control. The PF did not show suppression on the production of IL-1β in the macrophage and monocyte at concentrations of 2 μg/mL but did show moderate suppression at 20 μg/mL (44% decrease). The results of the suppression of monocyte-stimulated production of IL-1β partially concur

with that of the past investigation, as the THP-1 cells are monocytic cell lines.

IL-2 is produced by specific activated T cells, as well as dendritic and thymic cells (Ross and Cantrell, 2018). The PF at a concentration of 2 µg/mL and 20 µg/mL significantly suppressed the stimulated T cell IL-2 cytokine production as compared to the PHA-positive control. IL-2 helps promote the production of the T cells during the immune response and suppresses the activity of certain inflammatory cells. Because IL-2 plays roles in both initiating and terminating the inflammatory immune response, the fact that the PF significantly reduced IL-2 cytokine production suggests that it may help in the modulation of IL-2's inflammatory effects (Hoyer et al., 2008). Additionally, a study utilizing 2-arachindonyl glycerol, a nonspecific endocannabinoid, showed IL-2 suppression is independent of the CB_1 and CB_2 receptor interaction, suggesting that certain inflammation-modulating effects from certain cannabinoids might be through mechanisms independent from the endocannabinoid system (Rockwell et al., 2006). Another study targeted T cell lines in rats using a CB_2-selective agonist known as ABK2. The results of the T cell line interaction with ABK2 significantly decreased the production of IL-2, suggesting anti-inflammatory potential (Tang et al., 2021a).

IL-4 is primarily produced by the activated T cells to help regulate allergic inflammation (Junttila, 2018) as well as to suppress proinflammatory environmental components (Chatterjee et al., 2014). The PF's impact on the stimulated T cell IL-4 cytokine production caused little to no cytokine suppression (27% inhibition at 20 µg/mL; 32% inhibition at 2 µg/mL) and was not statistically different from the PHA-positive control. A study on the rats involved in an ovalbumin (OVA)-induced asthma model treated with a selective CB_2 agonist showed that the agonist reduced several inflammatory markers, including IL-4 (Parlar and Arslan, 2020). Another study in the PBMCs and T cell lines from patients with relapsing-remitting multiple sclerosis and healthy patients showed that COR167, a selective CB_2 agonist, caused a slight reduction of both IL-4 and IL-5 production, suggesting its immunomodulatory potential (Annunziata et al., 2017).

IL-5 impacts the production and maturation of white blood cells, specifically eosinophils (Kouro and Takatsu, 2009). The PF significantly and moderately suppressed the stimulated T-cell production of IL-5 (29% inhibition at 20 µg/mL concentration; 26% inhibition at 2 µg/mL concentration) as compared to the untreated PHA-stimulated control. This is also in agreement with one prior study (Annunziata et al., 2017), where a selective CB_2 agonist had a similar reduction of both IL-4 and IL-5 secretion.

The key roles of IL-6 are in neural reactions to nerve injury, microglial and astrocytic activation, and neuronal neuropeptides' expression. It is generally recognized as a proinflammatory cytokine. When tested on the stimulated T cells in the human PBMCs, the PF significantly and strongly decreased IL-6 production (53% inhibition at 20 µg/mL; 54% inhibition at 2 µg/mL) in the present study. Similar statistically significant results were found for the stimulated macrophage/monocyte production of the cytokines with a 29% reduction of IL-6 production at a concentration 2 µg/mL and a 42% reduction at a concentration of 20 µg/mL, falling in the range classified as moderate cytokine suppression. A cannabinoid agonist, JWH-015, was shown in a different study to suppress the production of IL-6 in morphine stimulated microglial cells (Merighi et al., 2012). Another study showed similar IL-6 attenuating results with the CB_2 receptor agonist COR167 (Contartese et al., 2012).

IL-8 is an active cytokine involved in acute inflammation produced by various cells such as phagocytes and mesenchymal cells (Gonzalez-Aparicio and Alfaro, 2020; Fousek et al., 2021). The stimulated T cell production of IL-8 when treated with the PF showed no statistically significant inhibition in the present study. The stimulated macrophage/monocyte production of IL-8 was also not significantly suppressed. A previous study by Yang et al. (2015) tested THC on an osteosarcoma cell-line stimulated by LPS. The treatment with the THC cannabinoid resulted in the suppressed release of TNFα, IL-1β, IL-6, and IL-8 suggesting potential inflammation-mediating effects.

IL-10 cytokines take part in the processes that occur through the human innate immune system and can limit bacterial and viral damage. The action of IL-10 can suppress proinflammatory responses, which could lead to tissue damage (Mu et al., 2005; Wenjun et al., 2011). The results of the present study showed a strong and significant suppression of the stimulated T cell production of IL-10 of the cell lines treated with the PF: 66% inhibition at both 2 and 20 µg/mL concentrations. Correa et al. (2010) found that the endocannabinoid anandamide treatment in LPS stimulated microglial cells increased IL-10 production, suggesting its potential for treating neuroinflammatory disease. Another in vitro study treated peritoneal macrophages with cannabidiol and found a decrease in IL-10 production, where a CB_2 receptor antagonist in the same study prevented IL-10 decrease (Sacerdote et al., 2005). It appears that the result of IL-10 production is highly dependent on the type of cell line and cannabinoid used in the study.

IL-13 receptors are found on a variety of different immune cells: B cells, basophils, mast cells, and others, but have not been found on the human T cells. IL-13 seems to be involved in allergic inflammation regulation and defined as being anti-inflammatory (Bieber, 2021). The PF significantly inhibited the stimulated T cell cytokine production of IL-13 (43% inhibition at 20 µg/mL concentration; 31% inhibition at 2 µg/mL concentration). In another study, the LPS stimulated human gingival fibroblasts and oral mucosa epithelial cells were treated with β-Caryophyllene resulting in an increased expression of IL-13 as compared to the LPS control (Picciolo et al., 2020). This result is consistent with expected outcome, considering the shared anti-inflammatory role of IL-13 and CB_2 receptor activation (Ma et al., 2015), yet does not concur with the effect that the PF elicited from our PBMC line.

The chemokine IP-10 is often activated by the activity of IFNγ and generally classified as proinflammatory. It binds to specific immune receptors, which promotes the presence of various leukocytes during an immune response (Gotsch et al., 2007). The application of the PF to the stimulated T cell production of cytokines resulted in a strong and statistically significant suppression of IP-10 (90% inhibition at 20 µg/mL; 76% inhibition at µg/mL concentration). In another model, the usage of a selective CB_2 receptor agonist, JWH-133, prevented MPTP-induced degeneration of dopamine neurons in the substantia nigra in a mouse model of Parkinson's disease.

One of the ways in which it did this was through the suppression of several proinflammatory cytokines, including IP-10 (Chung et al., 2016). These results, albeit with a different cannabinoid and cell culture, coincided with the strong suppression of IP-10 cytokine production found in the present study.

IFNγ is primarily proinflammatory. A study analyzing the effect of β-caryophyllene as an oral treatment on mice with dextran sulfate sodium induced colitis showed a significant reduction of IFNγ, IL-1β, and TNF-α (Bento et al., 2011). These results differed from those of the PF at both high and low concentrations since there was little to no significant reduction of the stimulated T cell production of IFNγ.

MIP-1α, a member of the C-C chemokine subfamily, is known to promote inflammation as one of its major functions (Oglodek, 2018). In the present study we analyzed the effect PF had on both the stimulated T cell production of MIP-1α, and the stimulated macrophage/monocyte production of MIP-1α. Interestingly enough, in the stimulated T cell assay, neither the lower concentration (2 μg/mL) of treatment with the PF resulted in a significant suppression of MIP-1α production nor the higher concentration (20 μg/mL). In the stimulated macrophage/monocyte production of MIP-1α, treatment with the PF also failed to significantly decrease the cytokine production at both a high and low treatment concentration. In a study by Tang et al. (2021b) the CB_2 selective agonist ABK5-1 was shown to significantly decrease levels of MIP-1α in the LPS stimulated microglia cell line BV-2. Another study showed similar results in suppressing MIP-1α, with JWH-133 being the cannabinoid treatment agonist (Bátkai et al., 2007). The present study, in conjunction with past studies, showed different results, suggesting that the ability of cannabinoid agonists and phytocannabinoids to mediate the production of MIP-1α during inflammatory responses is likely dependent on the cannabinoid treatment as well as the treated cell line.

The PF failed to significantly reduce the stimulated T-cell production of TNFα at both the higher and lower concentrations. It also did not significantly reduce the stimulated macrophage/monocyte production of TNFα at concentrations of 2 μg/mL (59%) and 20 μg/mL (61%), though this portion of the study likely merits repetition due to high variation of the suppression of TNFα production. The cannabinoids from the cannabis plant have been shown in a previous study to inhibit the monocytic THP-1 cell stimulated production of TNFα (Klegeris et al., 2003). Another study with a specific CB_2 receptor agonist showed similar effects in decreasing serum TNFα levels (Parlar et al., 2018).

CONCLUSION

The PF demonstrated a strong inhibitory effect on levels of the cytokines and chemokines, including IL-2, IL-6, IL-10, and IP-10 that are released by the stimulated T cells in the human PBMCs. The stimulated macrophage/monocyte cells treated with the PF resulted in significant and moderate decrease of IL-1β and IL-6. The demonstrated immunomodulatory activities in the human PBMCs suggest that the PF might be a potential natural product for immune support and that some of its immune benefits may stem from or correlate with its CB_2-binding potential.

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CONFLICT OF INTEREST DECLARATION

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

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