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Supercritical carbon dioxide *Cannabis sativa* L. and *Humulus lupulus* extracts and their influence on human macrophages inflammatory state*

Ekstrakty *Cannabis sativa* L. oraz *Humulus lupulus* uzyskane metodą ekstrakcji nadkrytycznym dwutlenkiem węgla i ich wpływ na aktywność ludzkich makrofagów

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A Study Design
B Data Collection
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D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

Summary

Aim: Herbal extracts are promising immunomodulating compounds. Their standardization may improve clinical outcome in various conditions related to inflammatory state. The aim of this study was to assess the utility of *Cannabis sativa* L. and *Humulus lupulus* extracts obtained by supercritical carbon dioxide (scCO2) in the reduction of pro-inflammatory cytokines release after LPS stimulation in the in vitro model.

Material/Methods: After scCO2 extraction, the cytotoxic potential of the obtained compounds was determined. The highest non-cytotoxic concentrations were selected for further inflammatory testing. PMA-differentiated U937 cells were used as an LPS induced model of the inflammation to assess the extracts potential to decrease the level of the pro-inflammatory cytokines IL-1β, IL-6 and TNF-α.

Results: Either individually tested or in combination scCO2 extracts markedly reduced the level of released pro-inflammatory cytokines in comparison to LPS stimulated positive control.

Conclusions: Our results show that the usage of standardized *Cannabis sativa L.* and *Humulus lupulus* extracts might be beneficial in reducing the inflammatory state. Application of the mixed extracts not only reduces the need for a high concentration of pure compounds, but also broadens the possible therapeutic effect. Moreover, scCO2 extraction may serve as the efficient method of obtaining functional anti-inflammatory extracts from either hop cones or cannabis.

Keywords: **cannabidiol • *Cannabis sativa* L. • hemp • *Humulus lupulus* • hops • anti-inflammatory • supercritical carbon dioxide • extraction • herbal extracts**

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INTRODUCTION

Growing interest in natural therapies encourages the search for an effective process to obtain active ingredients from plants with a controlled pharmacokinetics. Proper extraction of compounds that are known to be pharmacologically active is crucial in the development of herbal drugs. The main difficulties related with the preparation of a natural active ingredient lie in eliminating the influence of plants breeding conditions and in maximizing the concentrations of the desired compounds. An underlying inflammatory state is the origin of pathophysiology for many diseases such as chronic low back pain and further spinal disc degeneration. According to recent studies, lower back pain incidence, which results from a local inflammatory state, linearly increases starting from the third decade of life and is one of the main reasons for seeking health care services by the elderly [25]. In recent years, public demand for medical cannabinoids favoured studies concerning the possible applications of *Cannabis sativa* L. extracts for various pathological conditions, which include inflammation control and its inhibition. Furthermore, the emerging role of the endocannabinoid system (ECS) in tissue homeostasis and its maintenance is highlighted in recent studies [7, 21, 37, 41]. ECS is comprised of so-called endocannabinoids (e.g. anandamide and 2-arachidonoylglycerol), their receptors (e.g. CB_1 and CB_2) as well as enzymes and synaptic transporters involved in a specific cellular response.

There are over 500 active compounds identified in a cannabis plant, along with phytocannabinoids, terpenes, flavonoids, alkanes, sugars, phenols, phenylpropanoids, steroids, fatty acids, and various nitrogenous compounds [26, 34]. Most widely-researched phytocannabinoids are Δ9-tetrahydrocannabinol (THC), cannabidiol (CBD), cannabigerol (CBG) and cannabinol (CBN), all of which are potential candidates to serve as functional analogues of endocannabinoids [4, 5, 11]. Elements of ECS are present in almost every cell in the body and are involved in controlling many processes such as pain sensation, memory, appetite regulation, bone formation and, most importantly, immune response [13]. Moreover, flaws in the activity of the ECS are believed to be related to the impairment of the immune system [12, 32]. Undesired psychoactive properties of THC and its general use remains controversial, which is why it is reduced or eliminated in the final herbal drug composition [22]. CBD, also present in *Cannabis sativa* L., is shown to not only decrease the psychoactive properties of THC, but also to possess a wide range of additional therapeutic actions. Up to date most research has focused on the CBD potential in epilepsy treatment. CBD based drug named Epidiolex (GW Pharmaceuticals, GB) is currently approved for the treatment of epilepsy syndromes in children. Its functionality in neurological disorders, hypoxia-ischemia, anxiety and psychotic disorders is also being tested. In addition, CBD was shown by *in vitro* and *in vivo* studies to have analgesic, anti-inflammatory and antitumor properties as showed [5, 10, 28, 29, 40].

Another plant widely used in folk medicine is *H. lupulus.* Extracts from this plant are known for their antioxidant, anti-inflammatory and sedative activities and are known to be one of the most potent phytoestrogens [17]. Active ingredients from the hop cones, such as α-acids (isohumulones) and β-acids (lupulones) along with xanthohumol are capable of reducing the production of proinflammatory prostaglandin E2 (PGE2) and cytokines, decrease oxidative stress, as well as inhibit acute local inflammation *in vivo* [3, 20, 23, 39]. Early studies suggested that moderate beer consumption might reduce the risk of coronary diseases [14, 36]. This theory has been recently called into question, mainly because of the alcohol malignancy. However, there is still a possibility that consumption of alcohol-free beer might be beneficial [2].

One of the main obstacles in the commercial use of herbal extracts in medicines is the reduced availability of these compounds in their pure forms [27]. Extraction of cannabinoids and hop bitter acids can be performed with use of organic solvents; however, they may leave toxic residues. Supercritical fluid extraction is a promising technique that results in pure herbal extracts. This method offers high efficiency and at the same time it reduces the risk of extraction of undesired toxic compounds. Supercritical fluid extraction is based on a well-known phenomenon, where a gas in a supercritical state acquires specific physical properties, such as high density and high resolution that transforms it into a very good solvent. At the same time, gas in supercritical state retains its high diffusivity, low viscosity and lack of surface tension. Depending on substances used, fluids have

different critical parameters and will also have different polarity. In this study, pure carbon dioxide in a supercritical state was used for extraction of hemp (*Cannabis sativa L.*) and hops (*Humulus lupulus*). Supercritical carbon dioxide ($scCO_2$) solubility changes depending on temperature and pressure used during the process. Extraction with $scCO_2$ is a very effective method of preparing the natural active ingredients for the following reasons:

- $scCO_2$ solubility is easy to control and may be adjusted for each individual component of an extract.
- It allows the extraction process to be run at low temperatures.
- It is completely non-toxic and solvent is completely removed from the extract.
- It allows for the fractionation of substances during their extraction.
- It proceeds without air, which reduces the risk of oxidation of susceptible substances
- It ensures high selectivity, which results from a different solubility of CO_2 extraction products.
- It is cost effective due to solvent recirculation.
- $ScCO_2$ transportation is inexpensive and its use does not generate harmful waste, which classifies it as "green chemistry" technology.

Because of all the potential benefits related to $scCO_2$ extraction as a method for highly purified natural active ingredient preparation from *C. sativa* L. and *H. lupulus*, in this research, we aimed to evaluate the ability of standardized *C. sativa* L. and *H. lupulus* $scCO_2$ extracts to inhibit inflammatory response.

MATERIAL AND METHODS

Cannabis extract

Raw materials intended for research were purchased in a specialized company that deals with the marketing of plant materials. The extraction process of hemp and hops was carried out with the use of laboratory installation with a 1 dm^3 extractor capacity (SITEC, Switzerland). Maximum operating parameters of a laboratory plant were set to pressure: 100 MPa, temperature: 200 °C. At first, dried hemp was milled with a planetary ball mill equipped with a 1 mm sieve (PM 100, Retsch, Germany). Later, milled raw material was decarboxylated to convert acidic forms to neutral ones, because the acidic forms of cannabinoids extract are harder than the neutral forms. $ScCO_2$ extraction of acidic forms promotes the CBD losses and increases the costs of extraction. Decarboxylation was carried out at 140 °C in a laboratory dryer (Memmert GmbH + Co. KG, Germany). Six marked samples were prepared from the same batch of raw material, which were sequentially decarboxylated for 1 to 6 h. Raw materials prepared with this method were subjected to extraction with a laboratory plant designated for extraction of vegetable raw materials. Extraction parameters were set at follows: pressure: 350 bar, temperature: 40 °C. The highest concentration of cannabinoids in an extract calculated to be CBD was obtained from decarboxylation, of a sample for 3 h and amounted to 21.642% (Table 1). It was observed that the extract contained mainly neutral forms, while the concentration of acidic forms was reduced to less than 0.5% by mass. It was also found that $scCO_2$extraction does not allow to increase the concentration of CBD above a certain level. Selected hemp extract names are mentioned in Tables 1 and 3.

In order to enrich the CBD content in the extract, distillation of a crude extract was carried out using aKDL5 laboratory plant manufactured by UIC, Germany. Tests were carried out using optimized parameters shown in Tab. 2. Test was used for the analysis of: CBDA-0.071%, CBD-6.741%, THC-0.141%.

Hop extract

Hop cones, Magnat variety, were harvested in the lubelski region (Poland) in autumn 2016. Fresh cones were dried and commercially pelletized. The obtained pellets were used for hop extract production process. Commercial type 90 hop pellets were produced by New Chemical Syntheses Institute (INS) in Pulawy, Poland. Content of α-acids in dry matter was 8.5 % wt. $ScCO_2$ hop extract containing 50.10 % of α-acids, and 10.91 % of β-acids was further used as a raw material for preparation of other extracts. The crude extracts were then subjected to

Table 1. Parameters of decarboxylation and extraction of hemp material. There were no statistical differences between CBD concentration among different extracts (1 Way ANOVA test with post hoc Dunn's comparison test)

Specification	Extract name	Decarboxylation temperature [°C]	Decarboxylation time [min]	Extraction temperature [°C]	Extraction pressure [bar]	Total CBD concentration [%]
Decarboxylation 140 °C and then extraction	KDE2	140	120	40	350	17.754
	KDE3	140	180	40	350	21.642
	KDE4	140	240	40	350	17.880
Simultaneous decarboxylation and extraction	gKDE2	X	X	115	200	13.12
	gKDE3	X	X	145	200	14.31

Table 2. Process parameters of vacuum distillation of hemp extract

Process parameters	Value
Feed system temperature [°C]	50
Residue system temperature [°C]	75
Evaporator temperature [°C]	170
Internal condenser temperature [°C]	55
Feeding speed [ml/h]	950
Pressure in the plant [mbar]	$1x10^{-3}$
Weight of the batch [g]	1350
Weight of the distillate [g]	555

distillation, which resulted in higher content of α-and β-acids. Relevant data under the names D1 and D2 are shown in Table 3. The differences in composition of α--and β-acids in distillates D1 and D2 resulted from use of different temperatures of evaporator.

Cell cultures

Human monocytic cell line U937 (European Collection of Authenticated Cell Cultures, UK) was maintained in RPMI 1640 cell medium supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Thermo Fisher Scientific, US), 100 U/ml penicillin and 100 mg/ml streptomycin (Sigma Aldrich, USA) at 37.0 °C with 5% CO_2 in humidified atmosphere.

Cytotoxicity assay

U937 cells were seeded on 96-well plates with a seeding density of $1x10^4$ in triplicates. For differentiation, 10 ng/ml of phorbol-12-myristate-13-acetate (PMA, Sigma Aldrich, USA) was added to cell media, and the plates were incubated at 37 °C, 5% CO2, 95% relative humidity for 72 h. After 72 h, the cells were washed with sterile PBS, replaced with fresh complete cell medium and then incubated for 24 h prior to the addition of the selected *C. sativa* L. or *H. lupulus* extracts in 4 dilutions. The cells were then incubated again for 24 h. Metabolic activity was assessed with MTT assay, in which tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma Aldrich, USA) is reduced to insoluble formazan. Precipitated formazan was solubilized by isopropanol. Results obtained after reading the optical density at 570 nm on spectrophotometer (Synergy H1, Biotek, USA) were compared to control cells (100% viability) incubated with the complete culture medium. Results that were below 70% were considered cytotoxic.

Herbal extracts-mediated inhibition of lipopolysaccharide induced inflammation in macrophage in vivo model

U937 cells were seeded in 24-well plates for 72 h differentiation with 10 ng/ml of phorbol-12-myristate-13-acetate (PMA). The cells were washed three times with sterile PBS, then fresh culture medium was added, and the cells were incubated for 24 h. The next day, culture medium was replaced with selected herbal extracts (individually and in combination). After 3 h, 1 μg/ml of lipopolysaccharide was added and left for 24 hours of total incubation. Untreated cells served as a negative control, whereas cells treated with LPS were considered as a positive control. At desired time points, cell culture supernatant was collected, centrifuged and frozen for further interleukin 1β (IL-1 β), interleukin 6 (IL-6) and tumour necrosis factor α (TNF-α) analysis with commercial enzyme-linked immunosorbent assay kit (ELISA) performed according to the manufacturer's instructions (R&D Systems, USA).

Table 3. The content of the active ingredients in the crude hop and hemp extracts and vacuum distillates. There were no statistical differences between CBD concentration among different extracts (1 Way ANOVA test with post hoc Dunn's comparison test)

Plant	Name	Specification	α-acids concentration, % wt.	β- acids concentration, % wt.	Total CBD concentration, %
Hop	Magnat	CO_2 extract	50.10	10.91	X
	D1	Distillate	71.19	16.03	X
	D2	Distillate	58.80	14.69	X
Hemp	D1.30Y	CO_2 extract	X	X	25.48
	D2.30Y	CO_2 extract	X	X	25.78

Statistics

Data analysis was performed using Prism 5.0 (GraphPad Software, CA, USA). All results were expressed as mean ± standard error of mean (S.E.M.). For inflammatory test results, the significance of difference was estimated by the Kruskal-Wallis test followed by Dunn's post-hoc test. Values of $p < 0.05$ indicated a significant difference.

RESULTS

Extraction and distillation

Purification processes were carried out on extracts D1.30Y, D2.30Y that came from the same raw material but from two different batches. First, the extracts were subjected to a de-waxing process, followed by a decarboxylation process. After initial purification, the extracts were subjected to a vacuum distillation. In all cases, evaporator temperature was 170 °C. The THC content was determined to be 0.253% and 0.279%, and the results showed in Table 3 were obtained in individual runs. The CBD content in individual samples slightly differed.

An attempt was made to decarboxylate and extract in one run. Two tests were carried out using a hemp raw material from the same supply using a laboratory research plant. The experiments were carried out at a pressure of 200 bar, and CO_2 consumption of 48 kgCO_2/kg of dry mass. The results are shown in Table 1.

Experiments carried out on a laboratory scale showed that the decarboxylation process of acidic forms can be carried out simultaneously with the extraction process. Unfortunately, existing industrial plants do not allow for such a process to take place due to the inability to heat extractors to temperatures above 100 °C.

Cytotoxicity of C. sativa L. and H. lupulus extracts

The performed analysis showed that among *C. sativa* L. extracts the strongest cytotoxic effect was found for D2.30Y. The influence on viability was diminished below the concentration of 10 µg/ml, whereas incubation with other extracts in these concentrations did not have a negative effect on cells. Remarkably, extract D1.30Y increased the metabolic activity of cells in all tested concentrations (Figure 1A). D1.30Y lower cytotoxicity may have occurred due to differences in other than CBD, cannabinoids concentration, as well as their overall ratio. However, determination of all the active ingredients concentration was beyond the aims of the current study. For standardization purposes, CBD concentration was concerned suitable, as it was the most abundant and potent extract ingredient.

D1 had the safest cytotoxic profile out of all *H. lupulus* extracts of low concentrations. Above 50 µg/ml all tested substances had similar effects, resulting in a decrease in viability below 20% (Figure 1B). The highest non-cytotoxic concentrations were selected for further inflammatory testing.

The influence of C. sativa L. and H. lupulus extracts on LPS-induced inflammation

For further anti-inflammatory testing, two hemp and four hop extract concentrations with the highest non-cytotoxic response were selected. Increase in cell basal cytokine production by LPS exposure served as a positive control. Pre-incubation with *C. sativa* L. extracts resulted in the reduction of IL-1β, IL-6 and TNF-α concentration by almost 10x, which was comparable to the result obtained for a negative control (Figure 2). Extract D2.30Y with the highest concentration used had the most promising anti-inflammatory effect, whereas D1.30Y needed a 10x greater concentration to obtain a similar outcome. There was no clear connection between cannabidiol concentration in the extract and the overall anti-inflammatory effect. For D2.30Y relation was dose-dependent. Only selected concentrations of *H. lupulus* extracts were able to reduce inflammatory cytokines production and a dose-dependent effect was not observed. Among those able to reduce IL-1β and IL-6 production were 5 µg/ml

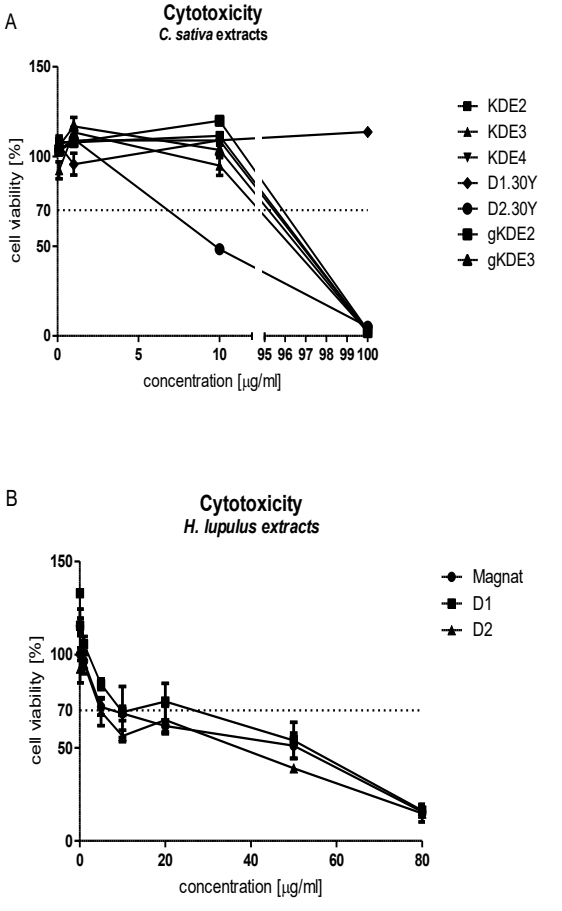

Fig. 1. Cytotoxicity of C. sativa L. (A) and H. lupulus (B) extracts in human U937 cell culture differentiated to macrophages. There were no statistically significant differences between IC50 values for each extract (data not shown). Overall results show that most C. sativa L. and H. lupulus extracts loss their cytotoxicity in concentrations below 10 and 5 µg/ml respectively

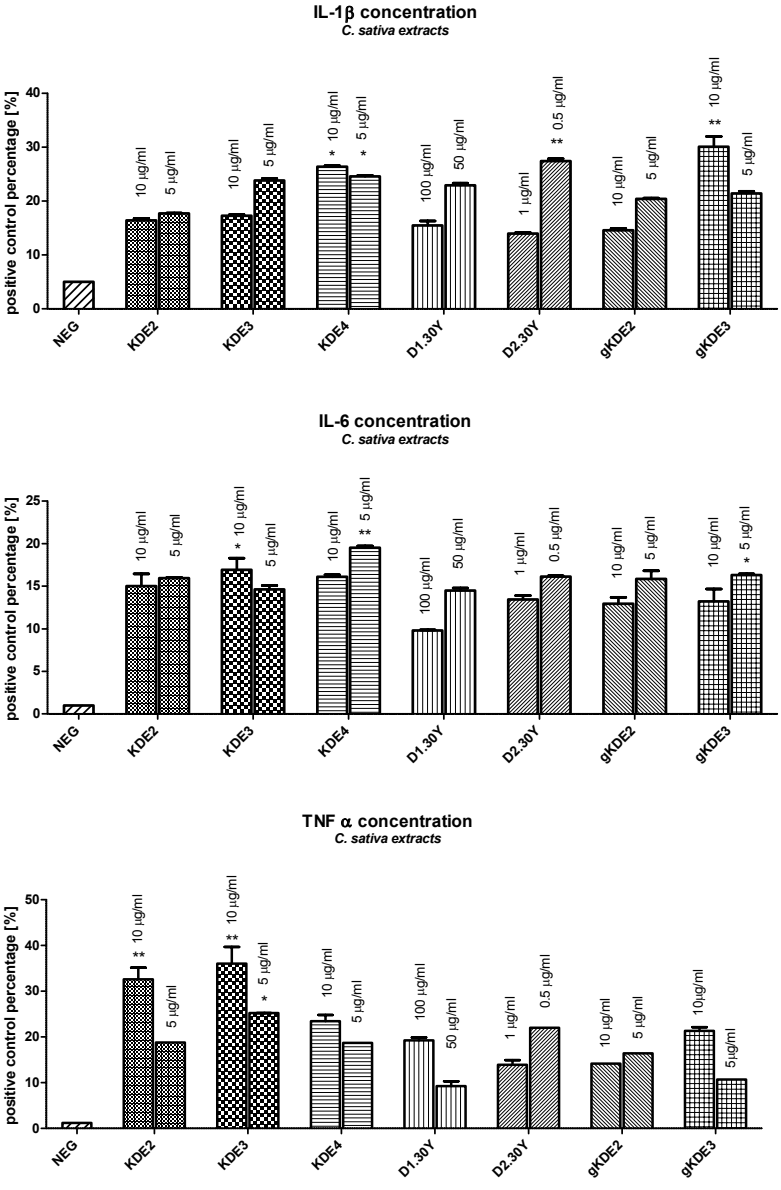

Fig. 2. Inhibition of the IL-1β (A), IL-6 (B) and TNF-α (C) production by C. sativa L. extracts in inflammatory model of human macrophages stimulated with LPS. Due to differences in the basal interleukin production after LPS stimulation in positive control between different experiments, results were shown as a positive control percentage. Concentrations marked with a star were significantly different from LPS control. Individual concentrations tested for each extract were marked above corresponding bars

of Magnat, 2.5 μg/ml of D1 and 2.5 μg/ml ofD2 extract. Slight reduction of TNF-α release was obtained for 5 μg/ml of Magnat, 1 μg/ml of D1 and 2.5 μg/ml of D2 extract. For remaining concentrations, a decrease of TNF-α levels could not be observed (Fig. 3).

DISCUSSION

Pro-inflammatory cytokines IL-1β, IL-6 and TNF-α are one of the main signalling elements involved in pain alleviation. They all act through NF-κB nuclear factor, which is essential in the maintenance of cellular homeostasis [19]. Activation of NF-κB pathway is linked to the pathogenesis of such diseases as atherosclerosis, rheumatoid arthritis, cystic fibrosis as well as carcinogenesis [38]. Van Cleemput and colleagues showed that incubation with isolated α- and β-acids from hops significantly reduced TNF-α and IL-6 production on the protein and transcriptional level in a dose-dependent manner. This result is inconsistent with our findings, where we showed that raw hop extracts have no dose-dependent effect on the reduction of TNF-α and IL-6 production. Hop extracts used in our study were composed of a mixture of both α- and β-acids, whereas in the study performed by Van Cleemput et al. pure substances were used. Other active components of hop extract may eliminate the dose-dependent effect. However, to obtain the desired

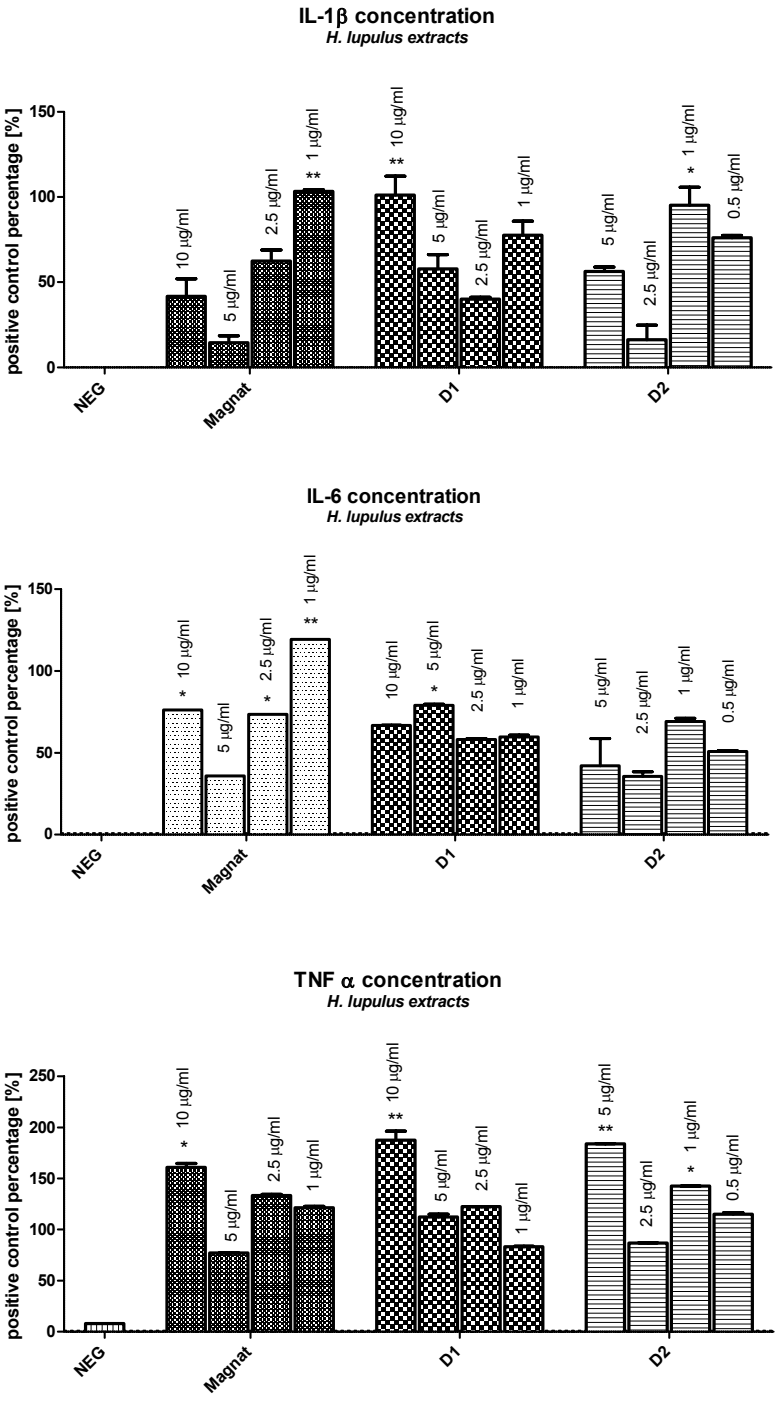

Fig. 3. Inhibition of the IL-1β (A), IL-6 (B) and TNF-α (C) production by H. lupulus extracts in inflammatory model of human macrophages stimulated with LPS. Due to differences in the basal interleukin production after LPS stimulation in positive control between different experiments, results were shown as a positive control percentage Concentrations marked with a star were significantly different from LPS control. Individual concentrations tested for each extract were marked above corresponding bars

therapeutic activity, isolated compounds must be used in higher concentrations due to additive biological interaction of the mixed extracts.

Humulones and lupulones from hop cones had an inhibitory effect in the model of the Epstein-Barr virus early antigen (EBV-EA) activation in Raji cells, served as an evidence of its anti-inflammatory and cancer-chemopreventive properties [1]. A standardized $scCO_2$ extract from hops was used to evaluate its ability to inhibit cyclooxygenase (COX), one of the enzymes involved in the inflammatory response. Results obtained in this study showed that hop extract is a selective inhibitor of a COX-2, but has no effect on the proteoglycan synthesis as well as joint swelling in mice [15].

An *in vivo* models of carrageenan-induced acute inflammation showed that isolated CBD does not have as strong an effect as other cannabinoids also present in the raw plant extracts [8]. Additionally, the application method is pivotal in obtaining the effective dose due to the first pass effect that reduces CBD bioavailability to less than 20% [16]. In the study, models of complete Freund's adjuvant and chronic constriction injury of the sciatic nerve in rats, with a measured outcome of thermal and mechanical hyperalgesia, where an oral CBD administration of 20 mg/kg dosage, were used and the physiological thresholds of hind paw withdrawal was restored. Additionally, it significantly supressed lipid peroxide overproduction and the concentration of the end-products of NO oxidation. CBD was unable to decrease TNF-α concentration in a dorsal root ganglia and to modify NF-κB upregulation [9]. Similar results were shown in the study done by Kozela, where CBD was able to reduce the IL-17, IL-6 secretion and increase the anti-inflammatory IL-10 production, whereas it did not affect TNF-α and IFN-γ levels [18]. Similarly to the earlier described isolated hops active compounds, CBD alone might not be as potent in a mixed plant extract. This might explain the observed differences of TNF-α reduction between our study and studies performed with CBD in its pure form.

However, further studies performed on murine models of the LPS-induced inflammation, acute lung injury and multiple sclerosis showed that a dosage exceeding 1 mg/kg was able to reduce the production of inflammatory cytokines TNF-α, IL-6 and IL-1β [6, 24, 31]. These results strongly support findings obtained with our *in vitro* model of inflammation.

Cannabinoids act as an antagonists of various pain receptors such as a transient receptor potential cation channel subfamily V member 1 (TRPV1) and a transient receptor potential channels of ankyrintype-1 (TRPA1), directly resulting in pain decrease [4, 30, 33, 40]. Moreover, cannabidiol has the potential to act as a functional antagonist to the GPR55 that regulates bone cell functions and modulates the inflammation, since it is present on monocytes and microglia cells [35]. Combined antagonism of pain receptors and inflammatory response suppression by CBD prevent the appearance of osteoarthritis symptoms in a rat model [28]. Altogether, those results stay in line with ours and confirm the broad therapeutic potential of cannabis plant extracts.

Our results show that the use of *Cannabis sativa* L. and *Humulus lupulus* extracts might be beneficial in reducing inflammatory state. Application of extract blends not only reduces the need for a high concentration of pure compounds, but also broadens the possible therapeutic effect. Moreover, $scCO_2$ extraction may serve as the efficient method of obtaining functional anti-inflammatory extracts from either hop cones or cannabis.

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