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Industrial hemp (*Cannabis sativa* L.) as a possible source of cannabidiol

Technické konopí (*Cannabis sativa* L.) jako možný zdroj kanabidiolu

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ABSTRACT

(-)-Cannabidiol (CBD) is a cannabinoid, which has unique pharmacological and biological activities.The aim of this study was to determine the CBD, cannabidiolic (CBDA), and canabigerolic acids (CBGA) content in parts of industrial hemp that are possible waste material in the production of hemp fiber, and could be used for CBD extraction. Two hemp varieties were sampled at the full-flowering stage, and at the ripening stage. The lyophilised material (leaves, roots, stems, and inflorescences/seeds) was extracted with ethylacetate and analyzed by HPLC. Hemp inflorescences and leaves are the most important source of CBD. CBDA and CBGA are the dominant compounds in these plant parts. They are the precursors of CBD. The CBD level is dependent on the variety and the environmental conditions. Dry cool weather with lower solar intensity increased the CBD level in 'Bialobrzeskie' - a monoecious variety potentially suitable as a material for CBD extraction with heat decarboxylation due to the high CBDA, CBGA, and CBD levels in the leaves and inflorescences.

Keywords: cannabinoid, variety, inflorescence, cannabidiolic acid, canabigerolic acid, leaf, HPLC

ABSTRAKT

(-) - Kanabidiol (CBD) je kanabinoid, který má jedinečné farmakologické a biologické účinky. Cílem této práce bylo zjistit obsah CBD, kyseliny kanabidiolové (CBDA) a kanabigerolové (CBGA) v jednotlivých částech technického konopí, které jsou možným odpadovým materiálem při výrobě konopných vláken a mohly by být použity k extrakci CBD. Vzorky ze dvou odrůd konopí byly odebrány ve fázi plného květu a ve fázi zrání. Lyofilizovaný materiál (listy, kořeny, stonky a květenství / semena) byl extrahován ethylacetátem a analyzován pomocí HPLC. Konopná květenství a listy jsou nejdůležitějším zdrojem CBD. CBDA a CBGA jsou dominantní sloučeniny v těchto rostlinných částech. Tyto látky jsou prekurzory CBD. Obsah CBD závisí na odrůdě a podmínkách prostředí. Suché chladné počasí s nižší intenzitou slunečního záření zvýšilo hladinu CBD v rostlinách. „Bialobrzeskie“ – jednodomá odrůda je potenciálně vhodná jako materiál pro extrakci CBD po tepelné dekarboxylaci z důvodu vysokého obsahu CBDA, CBGA i CBD v listech a květenstvích.

Klíčová slova: kanabinoid, odrůda, květenství, odrůda, kyselina kanabidiolová, kyselina kanabigerolová, list, HPLC

INTRODUCTION

(-)-Cannabidiol (CBD) is one of the main cannabinoid components from hemp (*Cannabis sativa* L.), which has no psychotropic effects, contrary to Δ9 - tetrahydrocannabinol (THC), as it does not activate the CB1 and CB2 cannabinoid receptors in the brain. CBD has sedative, antipsychotic, antiepileptic, analgesic effects, decreases the intraocular pressure, and also has antioxidant and neuroprotective properties (Zuardi, 2008, Grotenhermen and Muhler-Vahl, 2012). The anti-inflammatory effects of CBD in pneumococcal meningitis has been confirmed by Barichello et al. (2012). CBD also had potent anti-arthritic effects in murine collagen-induced arthritis (Malfait et al., 2000), has reversed cognitive impairment in a sepsis animal model (Cassol at al., 2010), has decreased inflammation in a murine model of lung injury (Ribeiro at al., 2012), has reduced brain damage in a hypoxic-ischemic model (Alvarez et al., 2008), has acted as an antiproliferative compound, inducing alossin viability in various tumor cell lines (Yang et al., 2014), and has suppressed murine intestinal inflammation (Burstein and Zurier, 2009). CBD could be used for diabetes treatment where it can inhibit and delay destructive insulitis and inflammation (Weiss et al., 2006), and it can prevent the decrease in autophagy induced by alcohol because it protects mouse liver from acute alcohol-induced steatosis through multiple mechanisms including attenuation of alcohol-mediated oxidative stress (Yang et al., 2014).

It is possible to find CBD in all hemp species and varieties (varying from nearly 0 to 95% of the total cannabinoids). CBD has an inverse relationship with THC. For that reason, industrial hemp has a low THC content but a high CBD content (Guy et al., 2004). The CBD and THC contents are controlled by independent genes. Inheritance of the cannabinoid content is sex-linked through the maternal line (Sytnik and Stelmah, 1998). So, it is possible to influence the CBD content in hemp by selection of the chemocultivar type (type I low CBD content, type II and III high CBD content) (Guy et al., 2004). Fiber hemp (*C. sativa*) landraces from Turkey, used for the fiber production, are an example of a rich source for pure CBD (95% proportion of total cannabinoids). However, the CBD and THC contents, and the ratio of both compounds, are also depend on environmental conditions (Fusar-Poli et al., 2009, Grotenhermen and Muhler-Vahl, 2012).

Hemp varieties with a low Δ9-THC content (and potentially high content of CBD or other phenolic compounds) could not only be used for fiber, seed, or energy production but could also be used for medicinal purposes (Guy et al., 2004).

Gas and liquid chromatography are the most commonly used methods for CBD determination in hemp. HPLC-DAD was chosen because the thermal conversion of acidic cannabinoids that occurs in gas chromatography systems has been demonstrated as providing incomplete and non-reproducible results, this because only neutral forms of cannabinoids are detected (De Backer et al., 2009).

The aim of this study was to determine the CBD, cannabidiolic (CBDA), and canabigerolic acids (CBGA), content in parts of industrial hemp that are possible waste material in the production of hemp fiber, and could be used for CBD extraction.

MATERIALS AND METHODS

Field experiment

Hemp (*Cannabis sativa* L.), varieties 'Finola' (dioecious) and 'Bialobrzeskie' (monoecious) were grown on plots (each plot 12 m^2) in České Budějovice (380 m a.s.l., cambisol with pH 5.6) in both 2012 (average annual temperature 9.3 °C, average annual sum of precipitation 799 mm) and in 2014 (average annual temperature 10.3 °C, average annual sum of precipitation 621 mm). Both varieties fulfilled the 0.2% limit for THC content established by EU regulations (Council Regulation (EC) No. 1420/98), and therefore the THC level was not investigated. The randomized complete block design with three repetitions was used in the field experiment.

The basic meteorological data during the growing period are given in Table 1. The content of nutrients in the soil were as follows: nitrogen 17 mg/kg DM, phosphorus 82 mg/kg DM, potassium 128 mg/kg DM, magnesium 108 mg/kg DM, and calcium 1102 mg/kg DM in 2012; with nitrogen 18 mg/kg DM, phosphorus 91 mg/kg DM, potassium 144 mg/kg DM, magnesium 125 mg/kg DM, and calcium 1 056 mg/kg DM in 2014.

In both years, seeds were sown on 22nd May by a machine for exact sowing in lines 25 cm wide with a growth density of 250 seeds per m^2. During the vegetation period one mechanical treatment of the stands was performed - weeding at the stage the 5th pair of leaves unfolded. Unfortunately, the 'Finola' variety stand was destroyed by heavy rain at an early growth stage in 2014.

First, half of one plot area (6 m^2) was used for sampling at the full-flowering stage of hemp, and the second half (6 m^2) was sampled at the ripening stage. The number of plants per square meter of each plot were determined before plant sampling. All plants from 1 square meter were sampled twice from every repetition. The plant samples were divided into their individual parts: leaves, roots, stems, and inflorescences/seeds. Next, the dry matter of the biomass was determined by oven drying for 2hr at 135 °C.

Sample preparation for HPLC analysis

Samples of 30 plants per plot for HPLC analysis were taken randomly five times (6 plants) diagonally in the plot at the flowering stage. The heights of the whole plants were also measured, and then they were divided into: the inflorescences (flowers with leaves without a petiole with small branchlets without parts of the main stem), stems, leaves (only green leaves with a petiole), and roots. Samples of the stems and inflorescences were taken separately from both female and male plants in the case of the dioecious variety 'Finola'. Leaves from the female and male plants were not sampled due to the fast withering of male plants after flowering. Individual plant parts were cut into approximately 1 cm lengths, mixed, and a representative sample of about 150g was immediately frozen and lyophilised (-50 °C).

Seeds (about 100 g from every plot) were sampled at the stage of full maturity after the harvest by a small-plot combine harvester. All seed materials were immediately frozen and lyophilised (-50 °C). The dried material was stored in a freezer (-18 °C). The dry matter of individual plant parts were determined for an estimation of potential CBD production by the hemp stand on one hectare at sampling time. The potential CBD production was calculated by multiplying the mean number of plants per hectare by the mean CBD level in the given dry plant part of one plant.

The lyophilised material was grounded (Retsch MM200 mill), and 0.25 g of the sample was extracted with 3 ml of ethylacetate for 30 min in a water bath at 40 °C. After centrifugation (1800 g for 10 min) the sediment was twice re-suspended in 1 mL of ethylacetate, all supernatants were pooled, the ethylacetate evaporated under a stream of nitrogen, and the residues were dissolved in 1 ml of methanol.

Table 1. Meteorological data during the growing period in 2012 and 2014

Year	Mean daily air temperature (°C)		Total precipitation (mm)	
	2012	2014	2012	2014
May	15.0	10.6	73.7	116.2
June	18.0	12.8	168.2	32.2
July	18.7	17.6	141.8	115.2
August	18.9	19.7	137.2	112.4
September	14.0	16.4	61.4	75.0

Heating of hemp extracts due to decarboxylation was performed in a universal laboratory oven (UF 260, Memmert GmbH) at 50 °C for 180 min, and then at 145 °C for 15 min.

HPLC analysis

Samples were analyzed by HPLC HP 1050 (Hewlett-Packard, U.S.A.) with a Phenomenex Luna C18 (2), 3 µm, 2 x 150 mm column (Phenomenex, U.S.A.) and DAD detector G1315B. The mobile phase A was: 5% acetonitrile + 0.1% o-phosphoric acid; mobile phase B was: 80% acetonitrile + 0.1% o-phosphoric acid.

The samples (5 µl) were measured in isocratic flow: 17% A + 83% B for 15 min, with a flowrate of 0.25 ml/min at 35 °C. The spectra were scanned in the range of 190 - 600 nm during the analysis. CBD, cannabidiolic acid (CBDA), and cannabigerolic acid (CBGA) were determined by confirmation of their retention times; and the DAD spectra with an authentic standard. The content of CBD, CBGA, and CBDA in the samples were calculated from the calibration curve at 220 nm. Limit of detection (LOD) was 0.013 µg/ml, limit of quantification (LOQ) was 0.044 µg/ml.

LC-MS analysis

LC-MS measurement was performed using an LCQ Accela Fleet (Thermo Fisher Scientific, U.S.A) atmospheric pressure chemical ionization (APCI) and photodiode array detector. The column and gradient were the same as in the HPLC, but in the LC-MS mobile phase, formic acid was used instead phosphoric acid. APCI capillary temperature was 275 °C, APCI vaporizer temperature was 400 °C, sheath gas flow was 58 L/min, auxiliary gas flow was 10 L/min. The negative polarity source voltage was 6 kV, source current 10 µA, and capillary voltage -10 V; for the positive polarity source, the voltage was 6 kV, source current 5 µA, and capillary voltage 43 V.

Chemicals

The standards of CBD, CBDA, CBGA were purchased from Sigma-Aldrich (Czech Republic); acetonitrile and methanol were purchased from Merck (Czech Republic), ethylacetate, formic and o-phosphoric acid from Fluka (Czech Republic).

Statistical analysis

The basic calculations and statistical tools (MS Office Excel) were used for confirmation of linearity, calibration, and other calculations. The influence of year, variety, and plant part was evaluated by analysis of variance with the post hoc Tukey HSD test (Statistica 12.0).

RESULTS

The influence of hemp plant parts, sex and variety on the CBD level

The cannabidiol level in the individual hemp plant parts were significantly different. The inflorescence parts had the highest CBD level (Table 2). According to Turner et al. (1980), hemp flowers (bracts) have the highest number of glands on their surface; therefore, the production of cannabinoids (and also CBD) was highest here. CBD levels in inflorescences is only influenced by the proportion of bracts and their age. In other plant parts, the CBD level decreased in the following order: leaves > seeds ≥ stems > roots. The CBD level in the leaves ranged from 16 to 125 mg/kg DM. Similarly, Turner et al. (1980) found the CBD level from 10 to 80 mg/kg DM in leaflets less than 5 cm long. Stems and roots had a low CBD level, or they were under the limit of detection (Table 2).

In the case of the dioecious variety, the influence of sex on the CBD level in the plant parts was evident ($P<0.001$). The female inflorescences reached 2x higher levels of CBD than the male inflorescences of the 'Finola' variety (Table 2). These results agree with Hemphill et al. (1980) who found that the pistillate plant parts contained from 0 to 2.79 mg/100g DM of CBD, and staminate plant parts from 0 to 1.48 mg/100g DM CBD. According to Ranali (1999), sexual differentiation is caused by a greater density of glands on bracts subtending the female flowers. The difference in the CBD level between stems of male and female plants was not possible to evaluate due to the low level of CBD (under the detection limit).

Table 2. The mean canabidiol level in *C. sativa* in mg/kg DM (mean ± standard deviation) in 2012

	'Finola'	'Bialobrzeskie'	Var. diff.
Roots	0.85±0.02a	x	
Leaves female plant	16.54±1.20b	56.71±4.66b	***
Stems, female plant	x	4.27±0.39 a	
Stems, male plant	x		
Inflorescence, female plant	135.41±8.64d	262.92±10.57c	***
Inflorescences, male plant	61.82±17.34c		***
Seeds	14.80±0.32b	17.07±1.38a	N.S

x – The CBD level under LOD; a-d: differences among plant parts after Tukey HSD test; n.s. - nonsignificant; ***, P<0.001; *, P<0.05

The influence of the hemp variety on the CBD level in both leaves and inflorescences was significant (P<0.001). Hillig and Mahlberg (2004) determined the CBD level in inflorescences of different hemp chemotypes, with an average of 3.8 ± 1.7% CBD found in varieties of chemotype II. Although both tested varieties belonged to chemotype II, the average CBD level in the inflorescences of the 'Bialobrzeskie' variety agree with the results of Hilling and Mahlberg (2004); however, the 'Finola' variety had a CBD level below this range (Table 2). The 'Finola' variety has been especially bred for seed production, so this selection could be the cause of the low CBD level. It follows that the varieties used have an important influence on CBD production.

The environmental conditions influence on the the CBD level

Environmental conditions had an important influence on the CBD level. Differences between years were evident and statistically significant (P<0.001). Sikora et al. (2011) found that precipitation had a negative influence on the CBD level, and soil temperature at 5 cm had a positive effect on the CBD level as growing degree days. According to Pacifico et al. (2006), hemp plants grown in a dry place or during a dry season contained higher levels of cannabinoids, and CBD production decreased with increased temperature (32 °C). This agrees with results in this study; the growing season in 2014 was drier but cooler (Table 1), and the CBD level in the plants that year was higher compared with the 2012 growing season (Figure 1). Plants that exhibited water stress in 2014 were half of the height compared to plant height in 2012 (Table 3). Ranalli (1999) noticed a negative correlation between plant height at harvest and THC level. According to results of this study, the negative correlation between CBD level and plant height can also be applied to the CBD level.

Solar irradiation, especially UV-B intensity, has an important influence on the CBD level. According to Lydon and Teramuta (1987), 1320 kJ/m^2/day of UV and visible radiation caused chemical lability of CBD, with a gradual and consistent decline in the resin from floral tissues. In this experiment, the sum duration of sunshine for the growing period (V-IX) reached 938 h in 2012 and 625 h in 2014. Thus, lower sunshine duration could be connected with a higher CBD level in hemp plants.

The next important influence on the CBD level after weather conditions is the soil mineral balance of the stand, especially the nitrogen, phosphorus, potassium, and calcium levels in the soil (Bocsa et al., 1997, Rannali, 1999). However, in this study this influence was minimal due to very similar basic elemental contents in the soil at the experimental location in both years (see section Field experiment).

Table 3. Production of dry matter and potential CBD production of two varieties of hemp (mean ± standard deviation)

Variety	'Bialobrzeskie'		'Finola'	Var. diff.
Year	2012	2014	2012	
Dry matter of leaves (t/ha)	1.5 ± 0.36A	0.9 ± 0.29A	1.2 ± 0.37	n.s
Dry matter of inflorescences (t/ha)	1.5 ± 0.42A	0.9 ± 0.31A	2.4 ± 0.56	n.s.
Total production of biomass dry matter (t/ha)	12 ± 1.5B	5 ± 1.3A	6 ±1.2	*
Plant height (cm)	205 ± 23B	101 ± 18A	75 ±14	***
CBD production in leaves (kg/ha)	84 ± 2.0B	112 ± 9.2A	19 ±1.5	***
CBD production in inflorescences (kg/ha)	395 ± 1.1B	287 ± 11.5A	162 ±10.6 (female) 74 ± 20.7 (male)	***
Total CBD production (kg/ha)	479 ± 1.9B	399 ± 8.4A	256 ± 10.9	*

A-B: differences among years after Tukey HSD test, n.s. - nonsignificant; ***, P<0.001; *, P<0.05

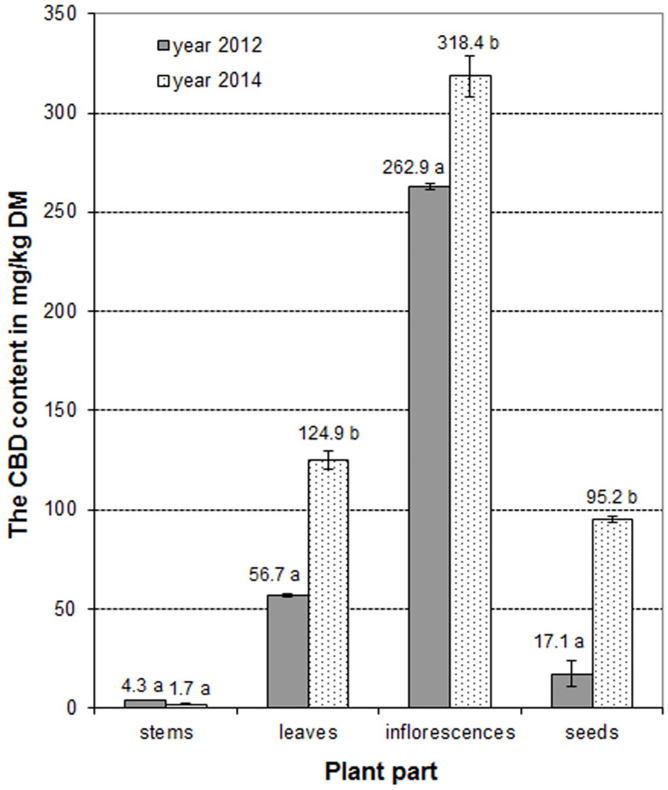

Figure 1. Interannual differences in the CBD level in different hemp parts of Bialobrzeskie variety (mean ± standard deviation). Different small letters (a - b) represent statistically significant differences (P <0.05) between the years in the plant part

Dominant cannabinoids and technics for increasing of the CBD level

For medicinal use, the dominant contents of effective compounds of interest in the plant materials are important. Therefore, a cannabinoid profile is necessary to take into account when varieties are selected for CBD extraction. In this study, CBD was not the dominant constituent of the cannabinoids. The dominant compound in HPLC separation (UV spectrum, retention time) were the cannabidiolic (CBDA) and canabigerolic acids (CBGA).

Identification of these compounds was confirmed using LC-MS (CBDA [M-H]- 357, CBGA [M-H]- 359, CBD [M+H]+ 315). In this study, CBDA was dominant in inflorescences and leaves. The stems and seeds contained low levels, with the roots only containing traces of CBDA as well as CBGA and CBD (Tables 4 and 5). The influence of environmental conditions on the content of CBDA and CBGA were only significant in the case of CBDA content in the leaves (4267.18 x 6419.77 mg/kg DM). Varietal differences were significant in inflorescences for both acids, as well as in the leaves and stems in the case of CBDA.

According to Wang et al. (2016), it is possible to use CBDA after decarboxylation under the influence of heat (110 °C for 50 min) as a source of CBD, because CBD in Cannabis sativa L. is predominantly biosynthesized from CBDA. This fact can be used in CBD extraction, and after heating of the extracts it is possible to get more CBD than the plant material originally contained. Heating of the extracts at 50 °C for 180 min. increased the CBD level only 7.8 times, on average. CBDA and CBGA levels were almost unchanged (Table 6). However, heating of the

extracts at 145 °C for 15 min. increased the CBD level 42 times, on average (Table 6).The highest content of CBD (13 459 mg/kg DM) was determined in the extract of the 'Bialobrzeskie' variety from inflorescences.

The theoretical CBD production

A variety suitable for CBD production should have a good yield of leaves and inflorescences (in dry matter per unit area). The 'Finola' variety reached a lower height (Table 3) and total yield of biomass, but its advantage

Table 4. The mean CBDA level in *C. sativa* in mg/kg DM (mean ± standard deviation) in 2012

Variety	'Finola'	'Bialobrzeskie'	Var. diff.
Roots	7.9±0.20a	1.0±0.1a	n.s
Leaves female plant	1792.1±27.26c	4267.18±64.80c	*
Stems, female plant	125.1±2.50b	367.07±12.24b	*
Stems, male plant	30.4±0.04a		***
Inflorescence, female plant	8707.7±47.62e	11519.54±1544.17d	*
Inflorescence, male plant	4561.6±1.18d		***
Seeds	49.40±16.07ab	190.41±8.59b	n.s

x – The CBDA level under LOD; a-d: differences among plant parts after Tukey HSD test; n.s. - nonsignificant; ***, P<0.001; *, P<0.05

Table 5. The mean CBGA level in *C. sativa* in mg/kg DM (mean ± standard deviation) in 2012

Variety	'Finola'	'Bialobrzeskie'	Var. diff.
Roots	0.998±0,06a	x	
Leaves female plant	14.35±0.10a	17.00±1.20a	n.s
Stems, female plant	9.49±0.50a	5.55±0.18a	n.s
Stems, male plant	2.35±0.16a		n.s
Inflorescence, female plant	1345.43±1.26c	905.40±104.73b	***
Inflorescence, male plant	468.31±9.75b		***
Seeds	0.72±0.25a	5.25±0.23a	n.s

x – The CBDA level under LOD; a-d: differences among plant parts after Tukey HSD test; n.s. - nonsignificant; ***, P<0.001; *, P<0.05

Table 6. The change of the CBD, CBDA, and CBGA content after heating of extracts from hemp materials in 2012 (in % of the initial content)

	CBDA		CBGA		CBD	
Temperature (°C) /time of heating (min)	50/180	145/15	50/180	145/15	50/180	145/15
'Bialobrzeskie' leaves	108.9	3.4	56.6	x	1054.5	4428.6
'Bialobrzeskie' inflorescences	119.1	1.0	114.6	x	846.3	3788.2
'Finola' leaves female plant	89.2	0	x	x	223.3	2439.9
'Finola' female inflorescences	116.0	8.7	118.3	10.1	996.0	6173.5

x- The content after heating under the limit of detection

Table 7. Seed and hull production and potential CBD production in seeds of two hemp varieties (mean ± standard deviation) in 2012

	'Finola'	'Bialobrzeskie'	Var. diff.
Mean weight of seeds per a plant (g)	56.5 ± 1.5	55.0 ± 1.5	n.s.
Seed yield (t/ha)	0.32 ± 0.06	0.49 ± 0.08	n.s
Hull production (t/ha)	0.11 ± 0.02	0.17 ± 0.03	n.s
CBD production (g/ha)	1.61 ± 0.04	9.52 ± 0.64	*

n.s. - nonsignificant; *, P<0.05

is the high proportion of inflorescence dry matter and its fast development; female plants started to flower 37 days after sowing. However, the lower CBD level in the plant parts also caused lower CBD production from green biomass per unit area (Table 3). The 'Bialobrzeskie' variety had a high total biomass yield (12 t/ha) and high CBD production in both leaves and inflorescences, but the proportion of inflorescence dry matter is low (30% proportion of leaves and inflorescences out of total biomass for 'Bialobrzeskie'; 60% for 'Finola'). The mean CBD production in the leaves and inflorescence dry matter harvested at the flowering stage was 440 kg/ha. According to Mueller (2014), it is possible to extract up to 90% of the contained CBD from industrial hemp. From this point of view, the 'Bialobrzeskie' variety could be suitable for CBD extraction not only for its higher CBD content but also for its higher CBGA and CBDA levels. So, this variety is justifiedly considered as a variety of combined efficiency. Hemp plants harvested at the flowering stage could have the inflorescences with leaves (top of the plants) pulled off from the stems for CBD extraction, and the stems used for fiber production.

The CBD level in the seeds is determined by resin production in any given year that sticks on the seed surface. So, a possible source of CBD could also be the hulls, the waste after hulling of the hemp seeds. The seed coat consists of about 35±7% of the total weight of the seed. Based on this data, the mean yield of hulls in this study reached 0.14 t/ha (Table 7). Thus, the CBD, CBDA, and CBGA production in the seeds is considerably lower (5.6 g/ha on average), compared with production in the leaves and inflorescences.

CONCLUSIONS

It is possible to conclude that for CBD extraction, it is possible to utilize the raw materials of suitable varieties bred for fiber production. Industrial hemp harvested at the flowering stage could produce material for CBD extraction as well as for fiber production from extraction. A significant increase in CBD content is achieved by heating extracts from the hemp materials.

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