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Chemical composition and antioxidant potential of *Cannabis sativa* L. roots

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ABSTRACT

Cannabis sativa L. has long been exploited for multiple purposes. Whereas all parts of the shoots are extensively used and well investigated, the roots have always received less attention. The phytochemical spectrum of the roots differs significantly from the rest of the plant, as no significant amounts of cannabinoids are found, whereas triterpenes as well as phytosterols are abundantly present. To shed light on the unique phytochemistry of hemp roots and the related industrial potential, three chemovars were investigated for the secondary metabolite composition and antioxidant activities by using *in vitro* and *in vivo* methods. Five triterpenes, ten phytosterols and five aliphatic compounds were identified by GC–MS analysis. Glutinol, ß-amyrone, stigmastanol, fucosterol, stigmasta-3,5-diene, stigmasta-3,5,22-triene, and oleamide were described for the first time in cannabis root extracts. The predominant triterpenoids friedelin (0.100−0.709 mg/g) and epifriedelinol (0.059−0.205 mg/g) were quantified in dependence of chemovar, harvest times, drying conditions, and extraction efficiency with ethanol, *n*-hexane, and supercritical CO_2.

1. Introduction

Cannabis sativa L. (hemp) is one of the oldest cultivated plants in history with multifarious applications, ranging from the textile, construction and paper industries to the nutritional, pharmaceutical and cosmetic sectors. While the stems provide cellulosic and woody fibres of very high quality, and the seeds are a rich source of fatty acids and proteins for the feed and food industries, the leaves and inflorescences are a gold-mine for phytochemicals. The rich spectrum of bioactive compounds can be exploited for several pharmaceutical applications (Ryz et al., 2017). The plant is known for its therapeutic usage as antiemetic, analgesic, and appetite stimulant or to treat epilepsy, glaucoma, and Tourette's syndrome (Amar, 2006). In total, a broad spectrum of more than 500 phytochemicals has been identified from the leaves, flowers, bark, seeds, and roots. This includes numerous cannabinoids, flavonoids, and terpenoids, as well as sterols (Jin et al., 2020), which are of industrial interest. The phytochemical spectrum, however, varies significantly with chemovar and plant part (Jin et al., 2020), and also with agronomic and environmental factors (Backer et al., 2019).

Traditionally, stems, inflorescence and seeds were the most used plant parts. In medicine, the major focus has always been on cannabidiol (CBD) and Δ^9-tetrahdydrocannabinol (THC) as bioactive compounds, which are mainly present in the flowers, as well as the leaves. Thus, the roots have been investigated less with respect to the reported pharmaceutical potential. Nonetheless, the roots have historically been used for the treatment of fever, inflammation, infections, as well as arthritis (Ryz et al., 2017). Recently, the presence of phytocannabinoids has been reported in hairy roots for the first time, although in almost negligible amounts compared with the rest of the plant (Andre et al., 2016; Gul et al., 2018).

Hemp roots are particularly known to contain considerable amounts of pentacyclic triterpenoids (Ryz et al., 2017). Naturally occurring triterpenoids are described as being of therapeutic value because of their anti-cancer, anti-inflammatory, antiulcerogenic or antiviral activities (Dzubak et al., 2006). The first characterized triterpenoids from ethanolic root extracts were friedelin and epifriedelinol, reported in 1971 (Slatkin et al., 1971). Recently β-amyrin was discovered to be accumulated in hemp roots as well (Jin et al., 2020). Of the identified

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triterpenoids, friedelin seems to be the most abundant (Jin et al., 2020; Slatkin et al., 1971), which was reported to exhibit anti-inflammatory, antipyretic and analgesic effects in mice and rats (Antonisamy et al., 2011).

Triterpenoids have been extracted from cannabis roots by conventional extraction with ethanol (EtOH), ethyl acetate (EtOAc), *n*-hexane, and petroleum ether (Elhendawy et al., 2018; Jin et al., 2020; Sethi et al., 1977; Slatkin et al., 1971). Supercritical fluid extraction (SFE) has not yet been described for triterpenoids from cannabis roots. However, the extraction with supercritical carbon dioxide in combination with EtOH has been reported for triterpenes from other plants (Felföldi-Gáva et al., 2012). Furthermore, SFE can be considered an environmentally friendly and highly efficient alternative, compared with volatile solvent extraction (Woźniak et al., 2016).

Antioxidant activity of naturally occurring triterpenoids has been determined in several studies. Cai et al. (2019) observed DPPH (2, 2-diphenyl-1-picrylhydrazyl), ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)), and superoxide anion free radical scavenging activity in extracts from medicinal fungus *S. sanghuang*. In particular, friedelin, isolated from *Azima tetracantha* Lam. leaves showed very promising scavenging effects on DPPH, hydroxyl, superoxide, nitric oxide, and suppressive effects on lipid peroxidation (Sunil et al., 2013). Additionally, phytosterols are known to be antioxidants and β-sitosterol, campesterol, as well as stigmasterol, have been reported to act as modest radical scavengers in solution (Yoshida and Niki, 2003). Currently there are no studies available on the antioxidative capacity of hemp root extracts, where triterpenoids and phytosterols have been identified.

This study presents the extraction of phytochemicals from hemp roots and the identification of heretofore undescribed secondary metabolites to ascertain the exploitation potential of this plant part, which is usually treated as waste. The predominant triterpenoids friedelin and epifriedelinol were directly quantified from the root extracts of three different hemp chemovars by GC–MS/FID analysis. Moreover, the extraction efficiency of the target triterpenoids by conventional extractions with EtOH and *n*-hexane as well as a supercritical CO_2 extraction is discussed herein for the first time. Furthermore, the influence of different harvest times and drying conditions on the triterpenoid concentration for one chemovar was monitored. In addition, *in vitro* (ABTS assay and ferric reducing antioxidant power assay: FRAP) and cellular antioxidant activity assays of the ethanolic cannabis root extracts were measured for the first time, due to the reported antioxidant activities of the accumulated secondary metabolites in hemp roots.

2. Material and methods

2.1. Plant material

The roots of three type III *Cannabis sativa* L. chemovars (example provided in Fig. S1), Futura 75 (France), Felina 32 (France), and Uso 31 (Netherlands), were cultivated in the fields (7°41′23.4″N 16°56′26.7″E) of BioBloom (Apetlon, Austria) in 2019. The crop was planted in rows with an average plant density of 35 plants per m^2. All three chemovars were grown organically in close proximity on a 60 ha plot. For Futura 75, three individual samples, which varied in harvest times and drying conditions were analysed.

The hemp roots of Futura 75 (sample A), were collected in July 2019, air dried and stored at room temperature. For comparison of chemovars, Futura 75 (sample B), Felina 32 (sample D), and Uso 31 (sample E), were harvested in August 2019 and received the same postharvest treatment as sample A. The third sample of Futura 75 (sample C) was harvested on an agricultural scale in October 2019 after the vegetative period and after the harvest of the aerial parts. Sample C was heavily washed with the help of a steam cleaner and dried for 30 h at 45°C in an agricultural drying facility and stored at room temperature until analysis.

For analysis, the complete hemp roots were washed with water and chopped to smaller sized parts. The pieces were shock frozen with liquid N_2 and milled by a Retsch ZM 100 with sieve (1 mm i.d.) at 14,000 rpm (F.Kurt Retsch GmbH & Co.KG, Haan, Germany). The pulverized material was lyophilised until constant weight and stored in a dark place for further experiments.

2.2. Conventional and supercritical CO_2 extraction

For the conventional extractions with EtOH and *n*-hexane, 0.50 g of freeze-dried hemp root powder were placed in 20 mL glass vials with Teflon screw caps. The roots were extracted for 3 h with a volume of 8 mL at room temperature under magnetic stirring (Elhendawy et al., 2018; Slatkin et al., 1971).

Supercritical carbon dioxide extractions were performed with a Jasco scCO$_2$ device (Jasco Corporation, Tokyo, Japan). Liquid CO_2 (>99.995 % purity; with ascension pipe; Messer GmbH, Vienna, Austria) was pressurized by two CO_2-pumps (PU-2086, Jasco Corporation, Tokyo, Japan) with cooled heads (CF40, JULABO GmbH, Seelbach, Germany). An HPLC pump (PU-2089, Jasco Corporation, Tokyo, Japan) supplied solvents. A heating coil and one HPLC-cartridge (L 127 mm, 10 mm i.d.) filled with hemp root powder were placed in an oven (CO-2060, Jasco Corporation, Tokyo, Japan). A back-pressure regulator (BP-2080, Jasco Corporation, Tokyo, Japan), a gas/liquid separator (HC-2086−01, Jasco Corporation, Tokyo, Japan), and a product collector (SCF-Vch-Bp, Jasco Corporation, Tokyo, Japan) were used to obtain the extracts. The samples were diluted to a defined volume with ethanol for the analysis. For the extraction, 0.5 g freeze dried hemp root powder was placed in the extraction reactor. The extraction was carried out for 2 h (1 h static / 1 h dynamic) at 20 MPa and 60°C. The flow was set to 3 mL/min with 10 vol% EtOH as a co-solvent. The method was developed and modified according to corresponding literature (Felföldi-Gáva et al., 2012).

2.3. Gas chromatographic analysis

Qualitative and quantitative analysis of the chemical constituents was carried out with an Agilent 7890A GC-System coupled to a mass detector and a flame ionization detector (FID). An Agilent HP-5MS GC-column (5%-phenyl-methylpolysiloxane, 30 m length, 250 µm i.d., 0.25 µm film thickness; Agilent Technologies, Santa Clara, CA, USA) was used for the separation. The initial flow was set to 1.3 mL/min and helium was used as a carrier gas. The samples were injected without split. The temperature program for the analysis was as follows: 1 min at 100°C as initial conditions, 10°C/min ramp up to 325°C, and 15 min hold at 325°C. The FID was operated at 350°C. Electronic ionization (E =70 eV) was used for the detection mass spectrometry. Source and single quad temperature were 230°C and 150°C. The total ion current (TIC) was measured between 35 to 750 *m/z* after a solvent delay of 6.5 min. The method was developed and modified according to recent literature for the separation of triterpenoids (Jemmali et al., 2016).

For the quantification with GC-FID, an analytical grade standard of friedelin was purchased from Sigma-Aldrich (St. Louis, MO, USA). A stock solution (1 mg/mL) in chloroform was prepared and diluted for calibration. The triterpene epifriedelinol was expressed as mg friedelin equivalent per g dried hemp root.

The identification of the compounds was performed by comparing fragmentation patterns with an intern mass spectrometric library, National Institute of Standards and Technology (NIST) database (Lindstrom and Mallard), and corresponding literature data or the purchased pure standard substance. The structures of the identified compounds, mass spectra, and calibration curves are provided as Supplementary material (Figs. S2−25).

2.4. Antioxidant activity

For the determination of the antioxidant activity of the ethanolic hemp root extracts, three complementing antioxidant activity assays

were carried out.

The first method is based on the scavenging of the ABTS radical 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) and the radical cation was produced by reacting 3.5 mM ABTS with 1.2 mM $K_2S_2O_8$ in H_2O. Before use, the mixture was stored in darkness at room temperature for 12–16 h and then diluted with EtOH to a working solution with an absorbance of 0.700 at 734 nm. Vitamin C standards (1−50 µg/mL) were freshly prepared in EtOH for the calibration. After the addition of diluted ABTS working solution (1.7–1.9 mL) to diluted samples or standard (100−300 µL), the absorbance was recorded after 30 min (Kim et al., 2002; Re et al., 1999).

The test for ferric reducing antioxidant power, or FRAP, is based on the reduction of Fe^{3+} to Fe^{2+} by the antioxidant compound, which forms a coloured complex with an absorption maximum at 593 nm with 2,4,6-tripyridyl-*s*-triazine in acetate buffer at pH 3.6. The working solution was freshly prepared with 25 mL of 300 mM acetate buffer (pH 3.6), 2.5 mL of 10 mM 2,4,6-tripyridyl-*s*-triazine in 40 mM HCl, and 2.5 mL 20 mM $FeCl_3{\cdot}6H_2O$ in deionized water (Benzie and Strain, 1996). Between 30−100 µL hemp root extract was mixed with 2 mL FRAP working solution and the absorbance was recorded after 30 min.

In vitro antioxidant activity assays were carried out on a Lambda 25 UV/VIS spectrometer (Perkin Elmer, Waltham, MA, USA). The calibration was performed with vitamin C, which was purchased from Sigma Aldrich (St. Louis, MO, USA). The results are expressed in vitamin C equivalent mg per 100 g dried hemp roots.

Cellular antioxidant activity was determined with *Saccharomyces cerevisiae* ZIM 2155 as a model system following the procedures described in Slatnar et al. (2012), which estimates intracellular oxidation by fluorometric measurements using the ROS-sensitive dye 2′, 7′-dichlorofluorescin (H2DCF). The ethanolic hemp root extracts (50 µL) were incubated with 10 mL yeast suspension at their stationary phase in phosphate buffered saline (PBS, Merck, Vienna, Austria) at a density of 10^8 cells/mL at 28°C and 220 rpm for 2 h. Thereafter, 2 mL of yeast suspension was centrifuged at room temperature for 5 min at 14,000 x g. The resulting pellet was washed three times with 50 mM potassium phosphate buffer (pH 7.8) and was finally resuspended in 9 volumes 50 mM potassium phosphate buffer (pH 7.8). The suspension was kept for 10 min at 28°C and 220 rpm in the dark before addition of 10 µL H2DCF (1 mM stock solution in 96% ethanol). After incubation for further 30 min at 28°C and 220 rpm, the fluorescence of the yeast cell suspension was measured on a GloMax® Multi Microplate Reader (Promega, Madison, USA) using excitation and emission wavelengths of 490 and 520 nm, respectively. Values of fluorescence intensity were compared with a control, in which the sample was replaced with ethanol. Data are expressed as relative fluorescence intensity according to the control, where the values obtained with the control are defined as 1. Values lower than 1 indicate a higher antioxidant activity than the control, values above 1 indicate prooxidant behaviour (Slatnar et al., 2012).

3. Results and discussion

3.1. Identification of compounds in C. sativa extracts

Extracts from hemp roots were analysed for the presence of phytochemicals by GC-mass spectrometric analysis. In total, 20 secondary metabolites were identified and were numbered from **1-20** according to their retention times (Fig. 1) and the corresponding mass spectrometric data is presented in Table 1. This includes five triterpenes and ten phytosterols, of which two triterpenoids and four phytosterols were identified for the first time in hemp root extracts. In addition, five aliphatic compounds were identified, of which one was a novel compound in *C. sativa* extracts (Fig. 2), whereas the others were putative artefacts. All identified structures and the corresponding mass spectra are shown in the Supplementary Material.

Several triterpenes are known to be present in *C. sativa* roots, in particular friedelin (**20**), epifriedelinol (**19**) or the recently discovered β-amyrin (**15**) (Jin et al., 2020; Slatkin et al., 1971). Besides β-amyrin, another oleanane skeleton based triterpenoid, namely β-amyrone (**13**) was firstly discovered in this study in the root's extracts. Furthermore, the presence of the pentacyclic triterpenoid glutinol (**17**) can be reported. Glutinol and amyrone based pentacyclic triterpenoids are known to be accumulated in root barks of *Maytenus cuzcoina* (Reyes et al., 2017).

In addition to the group of triterpenoids, ten phytosterols were identified in the extracts. Slatkin et al. (1975) described the steroids, campesterol (**8**), stigmasterol (**9**), and β-sitosterol (**10**), as well as the steroid ketones 4-campestene-3-one (**14**), stigmasta-4,22-dien-3-one (**16**), and stigmast-4-ene-3-one (**18**) in cannabis roots. Furthermore,

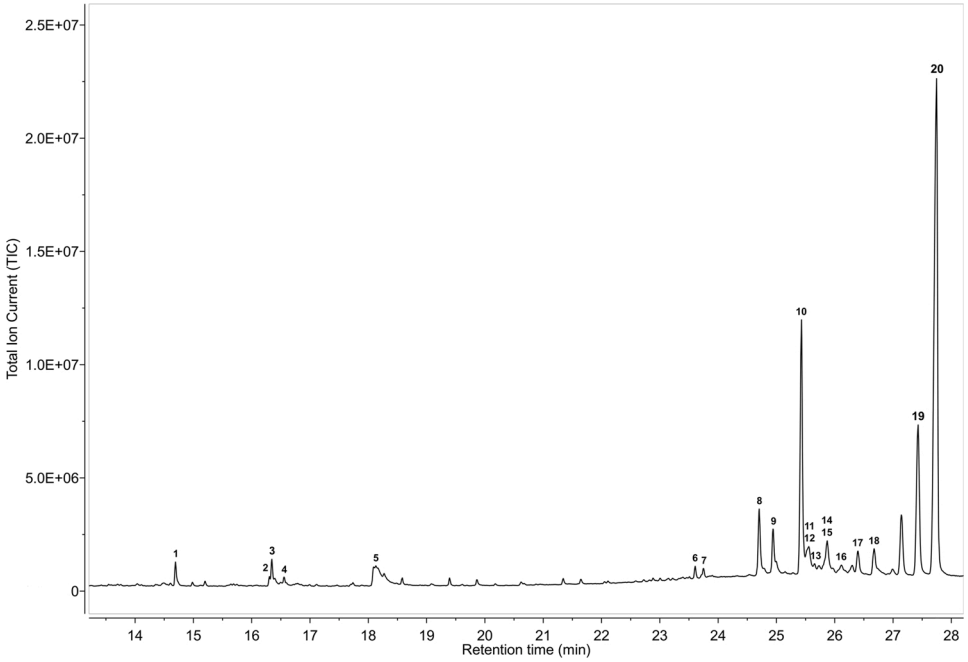

Fig. 1. Total ion current (TIC) chromatogram of the ethanolic *C. sativa* root extract of sample E.

Table 1
Identification of the compounds found in *C. sativa* L. roots by GC–MS.

Nr.	Name; M_W	*m/z* (rel. Intensity, %)	Ref.
1	Ethyl palmitate; 284	41 (25), 43 (37), 55 (26), 57 (20), 73 (18), 88[a] (100), 101 (56), 157 (17), 239 (8), 284[b] (9)	4
2	Ethyl linoleate; 308	41 (55), 45 (28), 54 (37), 55 (73), 67[a] (100), 81 (86), 95 (58), 109 (30), 135 (14), 263 (11), 308[b] (7)	4
3	Ethyl elaidate; 310	41 (68), 43 (61), 55[a] (100), 69 (69), 83 (53), 88 (52), 97 (48), 101 (37), 111 (22), 123 (17), 180 (11), 222 (14), 264 (19), 265 (17), 310[b] (4)	4
4	Ethyl stearate; 312	43 (69), 55 (41), 73 (21), 88[a] (100), 101 (56), 157 (15), 267 (5), 312[b] (9)	4
5	Oleamide; 281	41 (44), 43 (41), 55 (52), 59[a] (100), 72 (66), 281[b] (5)	4
6	Stigmasta-3,5,22-triene; 394	43[a] (100), 55 (76), 81 (74), 91 (44), 105 (42), 135 (88), 143 (73), 394[b] (91)	6
7	Stigmasta-3,5-diene; 396	41 (55), 43[a] (100), 55 (74), 57 (82), 69 (68), 81 (86), 91 (57), 105 (65), 147 (86), 213 (23), 275 (16), 381 (29), 396[b] (99)	3,6
8	Campesterol; 400	43[a] (100), 55 (67), 57 (46), 95 (57), 105 (60), 107 (63), 119 (42), 145 (56), 213 (51), 289 (52), 315 (51), 367 (35), 382 (45), 400[b] (92)	4,6
9	Stigmasterol; 412	43 (42), 55[a] (100), 69 (63), 81 (70), 83 (72), 105 (43), 145 (39), 159 (42), 255 (50), 271 (40), 300 (27), 412[b] (55)	4,6
10	β-Sitosterol; 414	43[a] (100), 55 (70), 57 (58), 69 (45), 81 (59), 95 (56), 107 (60), 119 (40), 145 (50), 303 (36), 329 (37), 396 (37), 414[b] (63)	4,6
11	Stigmastanol; 416	43[a] (100), 55 (82), 57 (69), 69 (64), 81 (74), 95 (72), 121 (45), 135 (35), 147 (38), 165 (41), 215 (88), 233 (64), 401 (31), 416[b] (55)	4,6
12	Fucosterol; 412	41 (42), 55 (97), 69 (65), 81 (59), 95 (54), 229 (35), 299 (27), 314[a] (100), 412[b] (7)	4
13	β-Amyrone; 424	41 (17), 55 (27), 69 (24), 81 (20), 95 (23), 109 (20), 119 (16), 135 (14), 189 (16), 203 (58), 218[a] (100), 409 (5), 424[b] (8)	7,8
14	4-Campestene-3-one; 398	43 (54), 55 (44), 69 (28), 81 (30), 95 (39), 107 (30), 124[a] (100), 135 (27), 147 (28), 229 (45), 275 (19), 398[b] (33)	2
15	β-Amyrin; 414	43 (31), 55 (35), 69 (34), 81 (29), 95 (33), 109 (24), 119 (19), 135 (18), 189 (17), 203 (48), 218[a] (100), 411 (3), 426[b] (4)	4,8
16	Stigmasta-4,22-dien-3-one; 410	41 (44), 43 (60), 55[a] (100), 69 (63), 81 (63), 95 (61), 107 (37), 123 (32), 147 (35), 269 (51), 298 (28), 367 (24), 396 (28), 410[b] (34)	4
17	Glutinol; 426	41 (31), 55 (56), 69 (59), 81 (49), 95 (65), 109 (47), 119 (40), 134 (48), 150 (23), 173 (23), 205 (32), 259[a] (100), 274 (94), 426[b] (4)	1
18	Stigmast-4-ene-3-one; 412	43 (43), 55 (32), 69 (20), 81 (21) 95 (27), 107 (22), 124[a] (100), 229 (41), 289 (17), 370 (12), 412[b] (31)	4
19	Epifriedelinol; 428	41 (36), 43 (30), 55 (67), 69 (89), 81 (76), 95[a] (100), 109 (79), 125 (59), 165 (68), 177 (30), 231 (25), 275 (23), 413 (15), 428[b] (7)	5
20	Friedelin; 426	41 (40), 55 (73), 69[a] (100), 81 (77), 95 (93), 109 (78), 125 (59), 135 (28), 163 (35), 179 (28), 205 (34), 273 (30), 302 (15), 411 (8), 426[b] (16)	9

References: Choudhary et al., 2005[1]; Georges et al., 2006[2]; Kasim et al., 2009[3]; Lindstrom and Mallard[4]; Manoharan et al., 2005[5]; Marques et al., 2008[6]; Sandison et al., 2003[7]; Yam-Puc et al., 2019[8], Reference Substance[9].

[a] Base Peak.

[b] Molecular Ion.

two yet undescribed phytosterols in the roots, stigmastanol (**11**) and fucosterol (**12**), were extracted in this study. Both sterols were identified in other roots, stigmastanol has been reported in *D. cinnabari* and fucosterol was found in *Hordeum vulgare* L. (Masaoud et al., 1995; Sheden et al., 2016). An additional new group of steroids can be reported herein. Thus, two steroid hydrocarbons, namely stigmasta-3,5-diene (**6**) and stigmasta-3,5,22-triene (**7**) were extracted and identified by GC—MS analysis. Compounds **6** and **7** have been isolated in other roots before, respectively in *Cordia rothii* and *Moringa oleifera* roots (Faizi et al., 2014; Khan et al., 2016).

Further investigation of the mass spectrometric data led to the identification of five aliphatic lipophilic compounds. As a result, oleamide (**5**) could be determined in the roots of *C. sativa* for the first time. The derived oleic acid amid has been reported in ethanolic root extracts of *Arctium lappa* L. (Yang et al., 2016). In addition, the fatty acid ethyl esters ethyl palmitate (**1**), ethyl linoleate (**2**), ethyl elaidate (**3**), and ethyl stearate (**4**) were identified. It can be assumed that compounds **1** to **4** are derived from their respective fatty acids and possibly resulted during the extraction with EtOH. Fatty acids have been isolated from hemp roots before (Elhendawy et al., 2018).

3.2. Friedelin and epifriedelinol in hemp roots

The triterpenoids friedelin and epifriedelinol are the prevalently present compounds and have been associated with a broad spectrum of health-related effects (Russo and Marcu, 2017). Although largely neglected so far, this makes hemp roots an interesting additional product for exploitation by the hemp industry. Utilization of further plant parts would increase the income of growers and the virtual absence of phytocannabinoids would overcome legal hurdles. Therefore, the amounts of friedelin and epifriedelinol in three chemovars were determined and compared within the studied varieties. The alcoholic extraction of sample A–E yielded 0.100−0.709 mg/g DW of friedelin, with the lowest yield in sample C and the highest yield in sample E. In addition, the highest yield of epifriedelinol was 0.205 mg/g DW in sample B and the lowest amount of 0.059 mg/g DW was observed in sample C (Table 2). Slatkin et al. (1971) reported 150 mg of column chromatographically purified friedelin and 100 mg epifriedelinol from ethanolic extracts of 4.7 kg air dried hemp roots. In another study the author V. Sethi isolated 20 mg and 49 mg of crystallized friedelin and epifriedelinol, respectively, from dried hemp roots, extracted with petrolether (Sethi et al., 1977). Hence, both studies reported significantly lower yields of the investigated triterpenoids, due to the purification with column chromatography and subsequent recrystallization. A recent study described higher contents of friedelin and epifriedelinol, ranging from 0.083−0.135 mg/mg% and 0.033−0.092 mg/mg%, respectively, in ethyl acetate extracts from hemp roots of three different chemovars, which resulted in slightly higher amounts of both triterpenoids, compared with this study. In this case, the roots were air dried for 24 h and medicinal varieties were analysed, which predominantly accumulate THC with a CBD:THC ratio of 1:2 (Jin et al., 2020). In contrast, herein, the roots were freeze dried until a consistent weight was achieved before subsequent analysis, and industrial varieties were used, which have a low THC limit.

The three analysed chemovars, Futura 75, Felina 32 and Uso 31, are commonly used industrial varieties, from the approved list of industrial hemp in the EU according to article 17 of the guideline 2002/53/EC. Futura 75 and Felina 32 are French varieties, largely used by industrial hemp growers in Central Europe for production of grain and fibers. Uso 31 is of Dutch origin with a particularly low THC content and especially suitable for grain production with a shorter vegetation period (European Commission, 2002). For comparison, these chemovars were harvested at the same time and extracted with EtOH, which led to no significant difference in the yields of epifriedelinol. However, from Felina 75 roots significantly more friedelin (0.709 mg/g DW) was extracted compared with Futura 75 as well as Uso 31 ($p < 0.05$, Table 2). Thus, Felina 32 accumulated the highest total levels of triterpenoids and demonstrates potential for yield optimization of bioactive compounds in hemp roots, by careful selection of varieties.

Flavonoids and cannabinoids could not be detected, as reported before. However, both triterpenoids have been described in the stem bark, indicating that the analysed triterpenoids accumulate in the outer tissue layer of the roots and stem (Jin et al., 2020). Depending on the

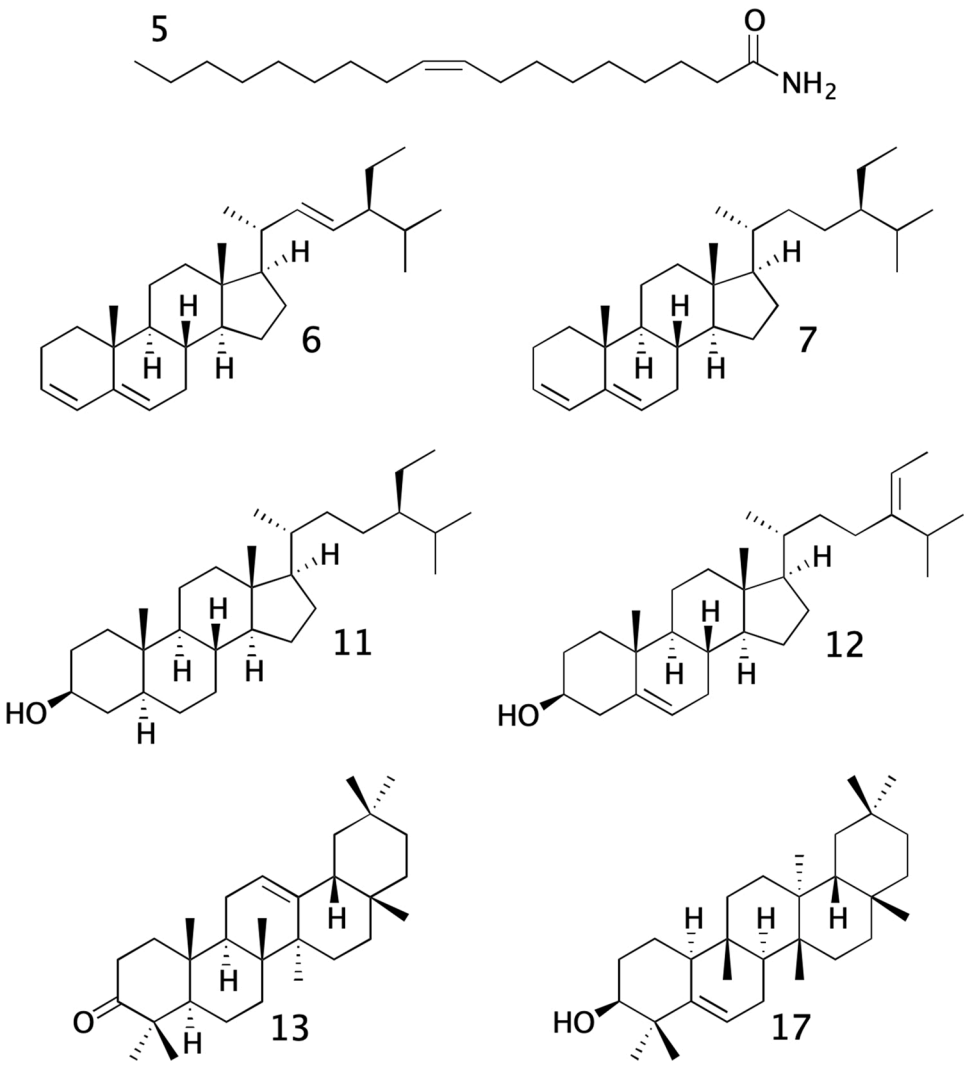

Fig. 2. Novel compounds in cannabis roots. (**5**) Oleamide, (**6**) stigmasta-3,5,22-triene, (**7**) stigmasta-3,5-diene, (**11**) stigmastanol, (**12**) fucosterol, (**13**) β-amyrone, (**17**) glutinol.

Table 2
Yields of friedelin and epifriedelinol in the extracts of *C. sativa* (n = 3, mean ± SD).

Sample (Chemovar)	Harvest	Drying	Extraction	$Yield_{Friedelin}$ /(mg/g DW)	$Yield_{Epifriedelinol}$[a] /(mg/g DW)
A (Futura 75)	07/2019	Air	EtOH	0.373 ± 0.012c	0.144 ± 0.009g
B (Futura 75)	08/2019	Air	EtOH	0.434 ± 0.038bc	0.205 ± 0.016e
C (Futura 75)	10/2019	30 h at 45°C	EtOH	0.100 ± 0.005d	0.059 ± 0.004h
D (Uso 31)	08/2019	Air	EtOH	0.422 ± 0.037bc	0.203 ± 0.016e
E (Felina 32)	08/2019	Air	EtOH *n*-hexane $scCO_2$	0.709 ± 0.036a 0.698 ± 0.078a 0.548 ± 0.073b	0.188 ± 0.007ef 0.179 ± 0.024efg 0.148 ± 0.016fg

Mean values with different letters (a, b, c, *etc.*) within the same column are statistically different ($p < 0.05$).
No cannabinoids and flavonoids were found in the ethanolic extracts with HPLC analysis (Data not shown).
Abbreviations: EtOH: ethanol; $scCO_2$: supercritical carbon dioxide.
[a] Calculated in friedelin equivalents.

growth conditions, root harvest techniques and sample preparation, the results differ in the amount of fine root structures, which mainly contribute to the root surface, correlating with the amount of bark per weight. Currently, a possible positive correlation between the concentrations of cannabinoids and terpenes, particularly mono and sesquiterpenes, is a matter of debate (Andre et al., 2016). The comparable rates of triterpenoids of the industrial varieties herein and the medicinal varieties used by Jin et al. (2020) do not point to a correlation of the concentrations of triterpenoids and cannabinoids.

For chemovar Futura 75 the effects of harvest time as well as the

influence of subsequent drying were analysed. No significant reduction of friedelin was observed between sample A and B, however for sample A, significantly lower amounts of epifriedelinol were obtained ($p < 0.05$), which was harvested one month earlier than sample B. In addition, sample C was dried at 45°C for 30 h directly after the harvest, which correlates with the significant reduction from 0.434 to 0.100 mg/g DW friedelin and from 0.205 to 0.059 mg/g DW epifriedelinol ($p < 0.05$, Table 2). Therefore, it can be assumed that drying at higher temperature and storage over several weeks have an impact on the targeted triterpenoids. Additionally, the reduced overall concentration of both triterpenoids could be attributed to the seasonal difference in the harvesting time points. With the harvest in October, as in sample C, the hemp plants are already in the process of senescence with starting degradation processes and rearrangement of the metabolome.

For comparison of extraction efficiency, sample E was extracted with ethanol, *n*-hexane and supercritical CO_2 combined with ethanol as a modifier (Fig. 3). No significant difference between the conventional extractions for the target triterpenoids was observed. Significantly less friedelin (0.548 mg/g DW, $p < 0.05$) and slightly less epifriedelinol (0.148 mg/g DW) were yielded by SFE. However the lower yields of extracted epifriedelinol were insignificant. It can be assumed that the supercritical CO_2 extraction conditions at 60°C, 20 MPa and 10 vol% of EtOH are sufficient for epifriedelinol, but not for friedelin, due to the structural difference and can be further optimized for a dual extraction. Supercritical fluid extraction of triterpenes is reported to be favoured at high pressure, high temperature and with EtOH as a modifier from *Alnus glutinosa* (L.) Gaertn. (Felföldi-Gáva et al., 2012). Furthermore, conventional extractions herein provided higher extraction yields with smaller amounts of solvent compared with SFE. Therefore, EtOH seems the best choice for extracting friedelin and epifriedelinol due to its extraction efficiency, environmental benignity and experimental simplicity.

3.3. Antioxidant activity of ethanolic hemp root extracts

In this study, the antioxidant activity of ethanolic hemp root extracts was determined by *in vitro* ABTS and FRAP assays, as well as cellular antioxidant activity assay using *S. cerevisiae* as model system for studying aspects of oxidative stress in eukaryotic cells (Slatnar et al., 2012).

Hence, FRAP resulted in 20.6–97.1 VCE mg/100 g DW and ABTS ranged from 22.9 to 89.1 VCE mg/100 g DW. Sample A had the significantly highest antioxidant activity for both assays ($p < 0.05$). For ABTS the lowest activity was observed for sample D and for FRAP in sample E (Table 3), however sample E showed the highest content of friedelin,

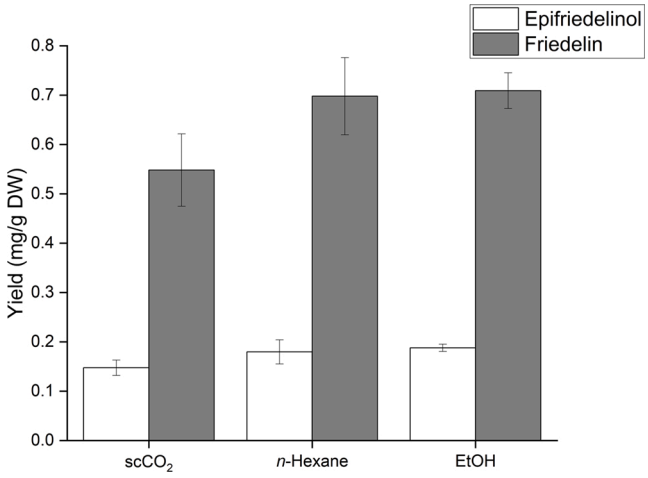

Fig. 3. Yield of friedelin and epifriedelinol in mg/g DW by extracting sample E with EtOH, *n*-hexane and $scCO_2$ (n = 3, mean ± SD).

Table 3
Results of *in vivo* and *in vitro* antioxidant activity tests of the ethanolic root extracts. (n = 3, mean ± SD).

Sample (Chemovar)	FRAP / (VCE mg/100 g DW)	ABTS / (VCE mg/100 g DW)	Intracellular oxidation[a]
A (Futura 75)	97.1 ± 3.8a	89.1 ± 2.5e	0.809 ± 0.013j
B (Futura 75)	25.4 ± 2.2cd	59.5 ± 1.0f	1.048 ± 0.048i
C (Futura 75)	33.5 ± 2.7b	29.7 ± 0.8g	1.024 ± 0.051i
D (Uso 31)	20.6 ± 16d	27.0 ± 1.1g	0.967 ± 0.095i
E (Felina 32)	31.0 ± 1.0bc	23.0 ± 0.3h	0.979 ± 0.014i

Mean values with different letters (a, b, c, *etc.*) within the same column are statistically different ($p < 0.05$).
Abbreviations: FRAP: ferric reducing antioxidant power assay; ABTS: 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid).
[a] Data are expressed as relative fluorescence intensity according to the control, where the values obtained with the control are defined as 1.000.

which is reported to have radical scavenging effects (Sunil et al., 2013).

In addition, the values of the intracellular oxidation ranged between 0.809–1.024 for the investigated ethanolic extracts. In agreement with the *in vitro* assays, sample A exhibited the highest cellular antioxidant activity. Compared with the control, whose oxidation was nominally defined as 1.000, a 20% decrease in intracellular oxidation was observed. Treatment of cells with other samples (B–E) did not show any changes compared with the control. The latter could be a result from a limited uptake of the relevant compounds by the yeast cells or a low aqueous solubility (Slatnar et al., 2012). In general, antioxidant and other biological activities of compounds depend on the rate of their incorporation into cells, which is related to the balance between their lipophilicity and hydrophilicity (Fiuza et al., 2004). Furthermore, when bioactive compounds enter a cell, their antioxidative activity is influenced by such factors as their reduction potential and the antioxidant defence activity endogenous to the cell itself (Lü et al., 2010). Thus, while *in vitro* assays can be used as a first step in screening compounds for possible antioxidative activity (Pilar de Torre et al., 2019), subsequent testing in a cellular environment provides insight into the actual effects in a natural physiological setting. The simple, yet eukaryotic nature of S. cerevisiae presents a good model for these preliminary tests before moving on to more complex organisms (Pilar de Torre et al., 2019). The stationary phase yeast cells used in the assay are a particularly useful model system for the study of damage that occurs during oxidative stress and aging (Zakrajšek et al., 2011), because they resemble cells of multicellular organisms in important aspects: (i) most energy comes from mitochondrial respiration, (ii) cells are in the G_0 phase, (iii) damage accumulates over time (Longo et al., 1996) and has the same defense mechanisms as higher eukaryotes (Moradas-Ferreira et al., 1996; Gralla and Kosman, 1992).

No linear relationship between the content of the target triterpenoids and the antioxidant activity of the FRAP Assay ($r^2 = 0.04$), ABTS assay ($r^2 = 0.001$) and the intracellular oxidation ($r^2 = 0.006$) was observed. Therefore, it can be assumed that other secondary metabolites, *e.g.* phytosterols are responsible for the antioxidant activity in the extracts, which have been reported to have a moderate antioxidant potential (Yoshida and Niki, 2003).

4. Conclusions

Besides leaves and inflorescences, hemp root is an interesting target for phytochemical exploitation, which provides added value to the growers. In total 20 secondary metabolites were identified, which includes the firstly described β-amyrone, glutinol, fucosterol, stigmastanol, stigmasta-3,5-diene, stigmasta-3,5,22-triene, and oleamide in hemp roots.

In addition, a comparison of various extraction methods led to the assumption that conventional and supercritical CO_2 extraction methods can yield the same amounts of epifriedelinol as well as comparable

amounts of friedelin. Furthermore, it has been observed that air drying preserves both analysed triterpenoids in the roots.

Ethanolic extracts of all investigated chemovars exhibited a moderate antioxidant activity in *in vitro* FRAP and ABTS assays, whereas the cellular antioxidant activities were more promising, but seem to strongly depend on chemovar and external factors such as harvest time. A correlation between the antioxidant potential and the targeted triterpenoids was not observed.

E-Supplementary data

E-supplementary data of this work can be found in the online version of the paper and comprises structures and mass spectra of the identified compounds as well as calibration curves.

Credit author statement

Christoph Kornpointner: conceived the research, designed and performed the experiments, analysed the data, wrote the orignial draft, edited and reviewed the manuscript. **Heidi Halbwirth:** conceived and supervised the research, designed the experiments, analyzed the data, edited and reviewed the manuscript. **Aitor Sainz Martinez:** designed and performed the experiments, edited and reviewed the manuscript. **Silvija Marinovic:** designed and performed the experiments, analysed the data. **Christian Haselmair-Gosch:** designed the experiments, analysed the data. **Christian Löfke:** provided the plant material, edited and reviewed the manuscript. **Polona Jamnik:** analysed the data, edited and reviewed the manuscript. **Katharina Schröder:** supervised the research, designed the experiments. All authors read and approved the final manuscript, analyzed the data

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.indcrop.2021.113422.

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