Effect of extraction solvent on total polyphenols content and antioxidant activity of industrial hemp (*Cannabis sativa* L.)

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In this study, water and various concentrations of ethanol in water (30%, 50%, 70%, and 90%) were used as a solvent in the extraction of two different samples of hemp (*Cannabis sativa* L.). The extraction yield, total phenols content, total flavonoids content, antioxidant activity, and reductive capacity were determined in the obtained extracts. The extraction yield was from 8.16 to 19.56%, the content of total phenols was in the range from 5.85 to 17.05 mg GAE/g dw, and content of total flavonoids was in the range from 5.85 to 9.25 mg CE/g dw. Antioxidant activity was tested by DPPH assay and EC_{50} values were from 0.1331 to 0.7563 mg/mL, while EC_{50} values obtained by reducing power test ranged from 0.4450 to 1.1980 mg/mL. Ethanol/water mixture (50%) was determined to be the best solvent for the extraction of phenolic compounds from both hemp samples. Total phenols content in 50% ethanolic extracts were 17.05 mg/g dw and 9.25 mg/g dw for young and mature hemp, respectively.

Key words: *cannabis, phenols, antioxidant activity, flavonoids*

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1. INTRODUCTION

Hemp (*Cannabis sativa* L.) is an herbaceous annual dioecious plant recognizable for their characteristic spiky leaves from Cannabaceae family. Complete taxonomic classification within the genus *Cannabis* remains under considerable dispute. Some authorities claim that all plants from genus *Cannabis* belong to the species *C. sativa*, with subspecies, such as *C. sativa* subsp. *indica* and *C. sativa* subsp. *sativa* (Small and Cronquist, 1976; Quimby, 1974). Other authors claim that morphological differentiation and different cannabinoids content within plants of European origin, as compared with plants in India, indicate two species, *C. sativa* and *C. indica* (Schultes and Hofmann, 1992). A recent investigation on allozyme, enzyme that differs by one amino acid from other forms of the same enzyme, variation within 157 populations of *Cannabis* strongly suggests that the genus *Cannabis* consists of only two species, *C. sativa* and *C. indica* (Hillig, 2005). Generally, *Cannabis* species is divided in two type based on the difference in the content of the psychoactive molecule Δ^9-tetrahydrocannabinol (THC): drug type, best known as marijuana or hashish, and nondrug type, commonly referred as industrial hemp or hemp. The drug type contains THC in concentration between 1% and 20%, while nondrug type, according to the European Monitoring Centre for Drugs and Drug Addiction in the European Union countries, contains THC in a concentration less than 0.2% on dry matter (Pollastro et al., 2018). THC content in Serbia is allowed up to 0.3%. Hemp has been known since antiquity in almost all parts of the world and it has been mostly known as a source of fiber (Kalant, 2001). It has a long tradition in Asian medicine, particularly in India (Happyana et al., 2013). Many uses of hemp in Indian medicine are similar to those which is recognized today by many authors such as analgesia, appetite stimulation, antipyretic and antibacterial effects (Kalant, 2001).

Cannabis sativa L. is a complex plant with more than 480 compounds which can be divided into diverse phytochemical classes, and the most studied class is the cannabinoids (ElSohly, 2007). The predominant cannabinoids are psychoactive cannabinoids, such as THC and its natural occurring degradation product cannabinol (CBN), as well as non-psychoactive cannabinoids such as cannabidiol (CBD), cannabichromene (CBC) and cannabigerol (CBG). Other constituents are terpenoids, nitrogen-containing compounds, carbohydrates, flavonoids, non-cannabinoid phenols, simple alco-

hols, aldehydes, ketones, acids, esters, and lactones (ElSohly, 2007). In recent years, the antioxidant activity of many plants has been thoroughly investigated, because of their potential health benefits. It has been reported that increased intake of natural antioxidant, especially phenolics, decreased the risk of degenerative diseases, particularly cardiovascular diseases and cancer (Pérez-Jiménez et al., 2008). Plant extracts abundant in antioxidants can be used in the preparation of dietary supplements, pharmaceuticals and cosmetic products (Dai and Mumper, 2010). Type of solvent, extraction temperature, the particle size of plant material, and extraction time are the parameters which have the highest impact on classical solid-liquid extraction process (Ramić et al., 2015). The effect of solvent type on the extraction process can be considered as one of the most important factors (Naffati et al., 2017). The properly selected type of solvent enables the production of extracts rich in bioactive compounds that are cost-effective and safe to use. Methanol, ethanol, acetone, and their mixtures with water are reported by many authors for extraction bioactive compound from plant material, especially the phenolic compounds (Do et al., 2014; Sultana et al., 2009) Bushra et al., 2009). Water/ethanol mixtures are reported as the best combination of solvents for the extraction of phenolic compounds by several authors (Bahorun et al., 2004; Durling et al., 2007)) and they are also safe for use (Dent et al., 2013).

The aim of this study was to investigate the effect of different water/ethanol mixtures and pure water on the quality of *Cannabis* nondrug type extracts obtained by classical extraction. The extraction yield, total phenols content, total flavonoids content, antioxidant activity, and reductive capacity were determinate in the obtained extracts.

2. MATERIALS AND METHODS

2.1. Plant material

Two different samples of *Cannabis sativa* L. commercial variety Helena were used in this study. The first sample was aerial parts of young hemp, and the second sample was aerial parts of mature hemp. The plant material was obtained from Department for organic production and biodiversity of the Institute of Field and Vegetable Crops, Backi Petrovac, Serbia. Before the extraction, herbal material was grounded using a blender. The average particle size of material prepared in this way was determined by sieving and it was 0.4378 mm (CISA Cedaceria Industrial, Spain).

2.2. Chemicals

Two regents, Folin-Ciocalteu and 1,1-diphenyl-2-picryl-hydrazyl-hydrate (DPPH), were purchased from Sigma (Sigma-Aldrich GmbH, Steinheim, Germany). Both standard compounds, (±)-catechin and gallic acid, were purchased from Sigma (St. Louis, MO, USA). Potassium ferricyanide was purchased from Merck (Darmstadt, Germany). All other chemicals used in this study were of analytical reagent grade.

2.3. Extraction procedure

For classical extraction efficiency and the selection of the best extraction solvent, five different experiments were set. In each experiment, 10.0 g of investigated material was extracted with a different solvent (200 mL). Extraction was performed at room temperature for 24 h. Distilled water and following ethanol/water mixtures were used for the extraction: 30%, 50%, 70%, and 90% ethanol. After extraction, extracts were immediately filtered through the filter paper with pore size 4-12 µm (Schleicher & Schuell, Dassel, Germany) under vacuum. Obtained liquid extracts were collected into the glass flask and stored in the freezer until the analysis. The content of total phenols (TP), total flavonoids (TF), antioxidant activity, reductive capacity, and extraction yield (EY) in the obtained extracts were measured.

2.4. Extraction yield

The yield of obtained extract (EY) was determined by the gravimetric method, drying the sample to constant mass. All experiments were replicated three times and results are expressed as mean values. EY has been expressed in percent (%).

2.5. Total phenols content

The content of total phenols (TP) in obtained liquid extracts was determined by the Folin-Ciocalte spectrophotometric procedure (Kähkönen et al., 1999; Singleton and Rossi, 1965). Absorbance was measured on a spectrophotometer (6300 Spectrophotometer, Jenway, UK) at 750 nm. The content of total phenols has been expressed as mg of gallic acid equivalent per g dry weight hemp (mg GAE/g dw). All experiments were replicated three times and results are expressed as mean values.

2.6. Total flavonoids content

The content of total flavonoids (TF) was determined by the aluminum chloride spectrophotometric method described by Harborne (1984). The sample absorption was measured at a wavelength of 510 nm. Based on the calibration curve of a standard catechin solution, the content of total flavonoids was determined. The total flavonoid content was expressed as mg of catechin equivalent per g dry weight hemp (mg CE/g dw). All experiments were replicated three times and results were expressed as mean values.

2.7. Antioxidant activity

The antioxidant activity of the obtained liquid extracts was analyzed using the DPPH assay, previously described by Espín et al. (2000). A certain volume of diluted sample/liquid extract was mixed with 95% methanol and 90 µM DPPH solution in order to obtain different final concentrations of the test sample. The absorption of the test sample was then measured at a wavelength of 515 nm, after 60 minutes of incubation at the room temperature.

The antioxidant activity obtained by the DPPH method was firstly expressed as Radical Scavenging Capacity (RSC) and further as the EC_{50} value. The EC_{50} value represents the concentration of the test sample which inhibits 50% DPPH radicals present and which is required to obtain 50% RSC. All experiments were replicated three times and results were expressed as mean values. Value of EC_{50} was expressed as mg dry weight hemp per mL test mixture (mg/mL).

2.8. Reductive capacity

The reductive capacity of obtained liquid extracts was determined by reducing the power test described by Oyaizu (1986). This method is based on the transformation of ions, Fe^{2+} into Fe^{3+}. This transformation can be provoked by the polyphenol antioxidants. According to the experimental procedure, 1 mL of extract (different dilutions) was mixed with 1 mL of 0.2 M phosphate buffer and 1 mL 1% potassium ferricyanide in glass tubes. The prepared reaction mixture was incubated for 20 minutes at a temperature of 50 °C.

After incubation, 1 mL of 10% trichloroacetic acid solution was added to the reaction mixture. Tubes were centrifuged at 3000 o/min for 10 minutes after 2 mL of supernatant was mixed with 2 mL of bidistilled water and 0.4 mL of 0.1% ferric chloride solution. The absorbance was measured at a wavelength of 700 nm. The reductive capacity of extracts is expressed as EC_{50}, the concentration of the test solution that provides 50% Fe3+ ion reduction. All experiments were replicated three

Table 1. Experimentally obtained values for all investigated parameters in obtained extracts of aerial parts of young and mature hemp

Solvent	EY[a] [%]	TP [mg GAE/g dw]	TF [mg CE/g dw]	EC_{50} (DPPH) [mg/mL]	EC_{50} (RC) [mg/mL]
Young hemp					
90% Ethanol	8.28	6.43	3.90	0.4353	0.8840
70% Ethanol	12.36	10.84	4.96	0.1659	0.5790
50% Ethanol	15.68	17.05	11.20	0.1331	0.5327
30% Ethanol	18.36	10.63	5.01	0.1321	0.4450
Water	19.56	8.44	2.36	0.3561	0.7005
Mature hemp					
90% Ethanol	8.16	5.85	3.18	0.7563	0.8561
70% Ethanol	9.96	7.84	2.70	0.2585	1.0517
50% Ethanol	14.52	9.25	5.21	0.2055	0.7301
30% Ethanol	15.60	7.68	3.38	0.2305	0.8752
Water	16.88	6.21	1.83	0.7276	1.1980

[a]EY - extraction yield, TP – total phenols content, TF – total flavonoids content, EC_{50} (DPPH) – effective concentration obtained by DPPH assay and EC_{50} (RC) – effective concentration obtained by Reducing power assay.

times and results reported as mean values. Value of EC_{50} was expressed as mg dry weight hemp per mL test mixture (mg/mL).

3. RESULTS AND DISCUSSION

3.1. Effects of solvent type on extraction yield

The criteria for extraction efficiency are the EY and the content of the target compounds. Extraction efficiency depends on the chemical nature of target compounds, extraction method used, sample particle size, the polarity of the used solvent, pH, extraction temperature, extraction time, and composition of the sample (Stalikas, 2007). Under the same extraction parameters (time and temperature), it is possible to study the effect of the solvent type on the extraction efficiency.

The extraction yields are given in Table 1. For aerial parts of young hemp EY was from 8.28 to 19.56%, and for aerial parts of mature hemp, it was from 8.16 to 16.88%. EY of extracts obtained by different solvents decreased in the following order: water, 30% ethanol, 50% ethanol, 70% ethanol, 90% ethanol. This means that the increase in the polarity of the solvent increases the yield of the extraction. The distilled water was found to be most efficiency regarding EY for both *C. sativa* samples. This can be explained by the fact that in addition to phenolic compounds, other compounds such as proteins and carbohydrates, which have higher solubility in water than in ethanol, can lead to higher yields (Do et al., 2014).

3.2. Effects of solvent type on total phenols and total flavonoids content

TP and TF contents for both samples of hemp are given in Table 1. TP content of aerial parts of young hemp and aerial parts of mature hemp was from 6.43 to 17.05 mg GAE/g dw and from 5.85 to 9.25 mg GAE/g dw, respectively. TF content of aerial parts of young hemp and aerial parts of mature hemp was from 2.36 to 11.20 mg CE/g dw and from 1.83 to 5.21 mg CE/g dw, respectively. The highest content of TP and TF was found in the extracts obtained with 50% ethanol. 50% ethanol concentration was reported by several authors as a solvent with higher efficient extraction of phenolic compounds from other ethanol/water mixtures (Ćujić et al., 2016; Galvan d'Alessandro et al., 2012; Gavaric et al., 2018). TP content was decreased in the following order: 50% ethanol, 70% ethanol, 30% ethanol, water, and 90% ethanol. TF content was decreased in the following order: 50% ethanol, 30% ethanol, 70% ethanol, 90% ethanol and water for aerial parts of young hemp, and 50% ethanol, 90% ethanol, 30% ethanol, 70% ethanol and water for aerial parts of mature hemp.

There was a significant difference in TP and TF content between hemp samples used in the studies. TP and TF content was about 2 times higher in aerial parts of young hemp. This can be explained with higher content of cannabinoids in the mature plant. Cannabinoids are synthesized by the glands that cover the surface of aerial parts of the plant, dominantly bracts, and they are formed by condensation of terpene and phenol precursors (Mahlberg and Kim, 2004).

3.3. Effects of solvent type on antioxidant activity and reductive capacity

The results of DPPH assay and reductive capacity of the obtained extracts are given in Table 1. Antioxidant activity was expressed through EC_{50} value and it was in the range from 0.1331 to 0.4353 mg/mL for aerial parts of young hemp and from 0.2055 to 0.7563 mg/mL for aerial parts of mature hemp. The value of EC_{50} is inversely related to its antioxidant activity and it presents the concentration of the test sample which neutralizes 50% of DPPH radicals. The lowest value of EC_{50} means the highest antioxidant activity. It has been found that extract obtained with 50% ethanol has the highest antioxidant activity for both hemp samples. The extracts obtained with 70% and 30% ethanol from both industrial hemp samples have a high antioxidant activity, and their EC_{50} value was similar to the EC_{50} value of extracts obtained with 50% ethanol. Extracts obtained with 90% ethanol showed the lowest antioxidant activity and it was about 3.5 times lower than the activity of the extracts obtained with 50% ethanol.

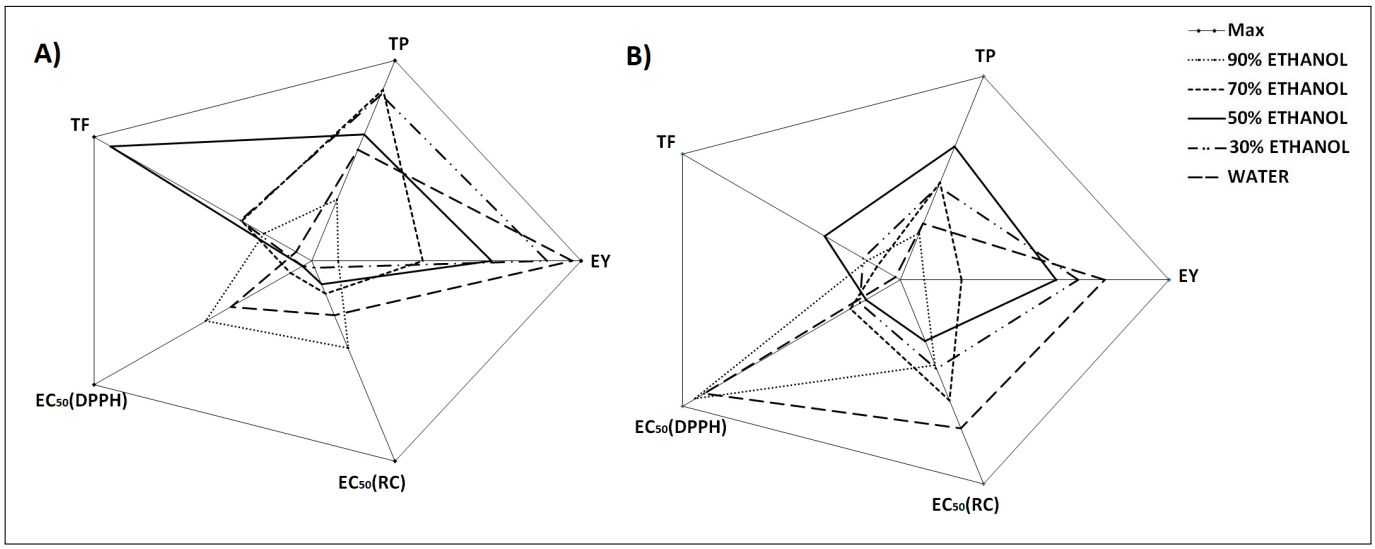

Fig. 1. Radar chart for all investigated parameters in obtained extracts of aerial parts of A) young hemp and B) mature hemp; EY - extraction yield, TP – total phenols content, TF – total flavonoids content, EC_{50} (DPPH) – effective concentration obtained by DPPH assay and EC_{50} (RC) – effective concentration obtained by Reducing power assay

The reductive capacity of the extract may serve as a reflection of its antioxidant activity. Reductive capacity was expressed by EC_{50} value and it was in the range from 0.4450 to 0.8840 mg/mL for aerial parts of young hemp, and from 0.7301 to 1.1980 mg/mL for aerial parts of mature hemp. EC_{50} present concentration of the test solution that provides 50% Fe^{3+} ion reduction and the lowest EC_{50} value means the highest reductive capacity. Extract from aerial parts of young hemp with the lowest EC_{50} value was obtained with 30% ethanol, while an extract from aerial parts of mature hemp with the lowest EC_{50} value was obtained with 50% ethanol. Extracts obtained with 90% ethanol and water for aerial parts of young hemp and aerial parts of mature hemp, respectively, exhibit the lowest reductive capacity. The content of TP and antioxidant activity were well correlated ($R^2 > 0.7$) for both hemp samples. Phenols and flavonoids are carriers of antioxidant activity. The antioxidant activity of phenols is in correlation with their redox properties. They are hydrogen donors and singlet oxygen quenchers (Djeridane et al., 2006). Many studies reported that TP content is directly proportional to their antioxidant activity (Djeridane et al., 2006; Katalinic et al., 2006; Paixao et al., 2007; Liu et al., 2009; Šeruga et al., 2011).

Mkpenie et al. (2012) were investigated the influence of methanol, acetone and their 50% aqueous solutions on polyphenols extraction from hemp leaves at room temperature for different times (2, 8, and 18 h). Phenols content was from 0.09 to 0.556 mg GAE/g dw, and antioxidant activity determined by reducing power assay was from 0.202 to 0.866 mg/mL. It can be concluded that pure distilled water and different ethanol/water mixtures are a better solvent for extraction phenols from industrial hemp. By considering all investigated parameters, 50% ethanol was the most suitable solvent for both hemp samples since using 50% ethanol provides an extract of the highest quality in terms of polyphenol content and antioxidant activity (Fig.1)

CONCLUSION

The aim of this study was to determine the impact of different solvents on EY, TP content, TF content, antioxidant activity, and reductive capacity of *C. sativa* extracts obtained from young and mature herbal material. In general, EY increased with increasing water content in ethanol/water mixture and it is the highest when the pure distilled water was used, while the best solvent for extraction of phenolic compounds was 50% ethanol for both plant samples. Extracts obtained with 30% and 50% ethanol for young and mature hemp, respectively, showed the highest antioxidant activity and reductive capacity. The extracts from young industrial hemp have a higher TP and TF content than extracts from a mature plant. Antioxidant activity and reductive capacity were higher in young industrial hemp extracts.

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