

# Comparative evaluation of antioxidant activity of *Cannabis sativa* L. using FRAP and CUPRAC assays

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*Cannabis* is one of the oldest plants on earth, which is known and used for medical purposes. There are many articles on hempseed oil research in the scientific databases, while the antioxidant activity of *Cannabis sativa* L. herb extracts has not been extensively studied yet, to our knowledge. In the present study, antioxidant properties of different *Cannabis sativa* L. varieties from different regions of Lithuania were examined. Spectrophotometric FRAP (ferric reducing antioxidant power) and CUPRAC (cupric reducing antioxidant capacity) methods were used for determination. It was found that *Cannabis sativa* L. herb extracts possess antioxidant activity. The strongest antioxidant activity was evaluated in the Futura variety and the lowest in Manoica. The obtained results showed that a statistically significant ( $p < 0.05$ ) higher reductive power was determined by analysing the raw material by the spectrophotometric FRAP method. According to the hemp growth location in Lithuania, the highest TEAC values were estimated in the samples from the North region.

**Keywords:** cannabis, antioxidant activity, FRAP, CUPRAC

## INTRODUCTION

There are various oxidative processes in the human body. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are constantly generated in vivo for physiological purposes and often over-produced in pathological conditions, resulting in oxidative stress [1, 2]. Ultraviolet rays, radiation, tobacco smoke and environmental toxins are sources of in vivo ROS production [3]. Oxida-

tive stress increases the production of free radicals or disrupts their neutralization, resulting in damage to biomolecules (proteins, lipids, DNA), cells and tissues, leading to many chronic diseases such as atherosclerosis, cancer, diabetics, rheumatoid arthritis, post-ischemic perfusion injury, myocardial infarction, cardiovascular diseases, chronic inflammation, stroke and septic shock, aging and other degenerative diseases in human [4, 5].

Substances with antioxidant properties – antioxidants – help to protect against harmful effects of oxidative stress. Antioxidants are classified as

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exogenous (natural or synthetic) or endogenous compounds, both responsible for removal of free radicals [4]. There are a wide range of natural antioxidants found in nature, they are found in many foods, including fruits and vegetables. Nowadays, attention is paid to plant raw materials that store compounds with antioxidative properties such as carotenoids, terpenes and polyphenols [6]. Many phenolic compounds have been reported to possess potent antioxidant activity and to have anticancer or anticarcinogenic/antimutagenic, antiatherosclerotic, antibacterial, antiviral and anti-inflammatory activities [7, 8]. Some phenolic compounds are even more powerful as antioxidants than vitamins C, E in vitro and significantly bioavailable as demonstrated by animal and human studies [1]. Terpenes, one of the most extensive and varied structural compounds occurring in nature, display a wide range of biological and pharmacological activities – due to their antioxidant behaviour they provide relevant protection under oxidative stress conditions in different diseases including liver, renal, neurodegenerative and cardiovascular diseases, cancer, diabetes as well as in ageing processes [9, 10].

*Cannabis* is one of the oldest plants on earth, which is known and used for medical purposes, fiber, feed production, fuel and cosmetics for more than 10,000 years. *Cannabis sativa* L. contains chemical compounds of various classes, e.g. mono- and sesquiterpenes, sugars, hydrocarbons, steroids, flavonoids, nitrogenous compounds and amino acids, among others [11]. Mono- and sesquiterpenes have been detected in flowers, roots and leaves of *Cannabis* [3]. Monoterpenes dominate generally the volatile terpene profile, sesquiterpenes occur also to a large extent in *Cannabis* extracts [3]. In *Cannabis*, about 20 flavonoids have been identified, mainly belonging to the flavone and flavonol subclasses [12]. Flavones and flavonoids in *Cannabis* have a wide range of biological effects, including terpenic and cannabinoid properties. *Cannabis* demonstrated positive health benefits, including alleviating constipation, lowering cholesterol, cardiovascular health benefits, immunomodulatory effects, and dermatological disease amelioration effects. Furthermore, *Cannabis* showed a strong antioxidant effect and the potential to improve the impaired learning and memory induced by chemical drugs in mice [13].

FRAP (ferric reducing antioxidant power) and CUPRAC (cupric reducing antioxidant capacity)

assays are commonly used for antioxidant properties that rely on the electron transfer potential of antioxidants present [14]. Evaluation of antioxidant activity is complicated by the prooxidative effect of antioxidants in the presence of unsequestered metal ions such as iron and copper. Since iron and copper are sequestered by proteins in vivo, there has been no conclusive evidence which shows that an antioxidant acts as a prooxidant in vivo by reducing metal ions, and it may be misleading to state that some antioxidants act as prooxidants under these conditions.

There are many articles on hempseed oil research in the scientific databases, while the antioxidant activity of *Cannabis sativa* L. herb extracts has not been extensively studied yet to our knowledge. Thus, our study puts importance on raising awareness about antioxidant properties of all *Cannabis sativa* L. raw material.

In the present study, spectrophotometric ferric reducing antioxidant power assay (FRAP assays) and cupric reducing antioxidant capacity assay (CUPRAC assay) were employed for the determination of antioxidant activity of different *Cannabis sativa* L. species – Finola, Felina, Futura, Manoica, Secuieni Jubileu, Virtus Rugo, KC-Dora – from various areas in Lithuania, as a promising source of antioxidants.

## EXPERIMENTAL

The object of this research is the upper part of different species (Futura, Felina, Finola, S. Jubilejum, Manoica, Virtus Rugo, KC-Dora) of *Cannabis sativa* L. Raw materials were collected in different regions of Lithuania (north, south, east) and dried at 25°C for chemical analysis.

Extraction solvent methanol (99%) was purchased from Sigma-Aldrich (Buchs, Switzerland). For FRAP reagent production, iron chloride hexahydrate ( $\text{FeCl}_3 \times 6\text{H}_2\text{O}$ ) and sodium acetate ( $\text{NaCH}_3\text{COO}$ ) were purchased from Sigma-Aldrich Chemie (Steinheim, Germany), glacial acetic acid (99.8%) from Standard (Poland), concentrated hydrochloric acid (Con HCl) from Fluka Chemie (Buch, Switzerland) and 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ) from Alfa Aesar (Germany). Acetonitrile was purchased from Sigma-Aldrich (Buchs, Switzerland), trifluoroacetic acid (99.8%) from Fluka Chemie (Buchs, Germany). For CUPRAC

reagent production, copper (II) chloride dihydrate ( $\text{CuCl}_2 \times 2\text{H}_2\text{O}$ ) was purchased from Alfa Aesar GmbH & Co. KG (Karlsruhe, Germany), neocuproine from Sigma-Aldrich Chemie (Germany) and ammonium acetate ( $\text{NH}_4\text{CH}_3\text{COO}$ ) from Sigma-Aldrich (Belgium). Standards for Trolox ( $\geq 98\%$ ) were from Fluka (Buch, Switzerland).

Prior to preparing of extract, *Cannabis sativa* herb was ground in an electric mill D-47906 Clatronic (Kempen, Germany). The powdered material (200 mg) was placed in a 10 ml volumetric flask and extracted with a 10 mL extraction solvent (methanol and trichloromethane (9:1)) in an ultrasonic bath BioSonic UC100 (Maui, USA) for 30 min. The extract was filtered through a 0.22  $\mu\text{m}$  microfilter into a dark glass vial. The vial was stored in a refrigerator until the day of extraction [15].

- Production of 300 mM acetate buffer (solution A): 3.1 g of  $\text{NaCH}_3\text{COO}$  is transferred to a 1000 mL volumetric flask and dissolved in 16 mL glacial acetic acid. The prepared solution is diluted with distilled water to the mark. The pH of the solution should be 3.6.

- Production of 10 mM TPTZ (2, 4, 6-tripyridyl-triazine) solution in HCl (solution B): 40 mM HCl solution (50 mL of distilled water mixed with 0.1695 mL of concentrated HCl) is added to 0.1562 g of TPTZ powder and dissolved.

- Production of 20 mM  $\text{FeCl}_3$  solution (solution C): 0.2703  $\text{FeCl}_3$  is dissolved in 50 mL of distilled water.

- Solutions A, B and C are mixed in a ratio of 10:1:1. The prepared solution is stored in a dark glass bottle. Before the analysis, the working FRAP solution is heated to 37°C.

- Production of  $\text{CuCl}_2$  solution (solution A): 0.17 g  $\text{CuCl}_2 \times 2\text{H}_2\text{O}$  is dissolved in water and diluted to 100 mL.

- Production of neocuproine solution (solution B): 0.1566 g neocuproine is dissolved in 70% methanol and diluted to 100 mL with water.

- Production of ammonium acetate buffer (pH 7) (solution C): 0.077 g  $\text{NH}_4\text{CH}_3\text{COO}$  is dissolved in water and diluted to 1000 mL.

- Solutions A, B and C are mixed in a ratio of 1:1:1. The reconstituted solution is stored at room temperature for 1 h, protected from light exposure.

The principle of this method is based on the reduction of a ferric-tripyridyltriazine complex to its ferrous, coloured form in the presence of antioxi-

dants. For the analysis of *Cannabis sativa* L. extract 2 mL of the prepared FRAP reagent was mixed with 100  $\mu\text{L}$  of the extract. Absorption was measured by a spectrophotometer HALO DB-20 UV-vis Dynamica GmbH (Switzerland) at 593 nm for 30 min after preparation of the sample. Each sample was analysed 3 times. The antioxidant activities were expressed as TEAC values (Trolox mg/mL).

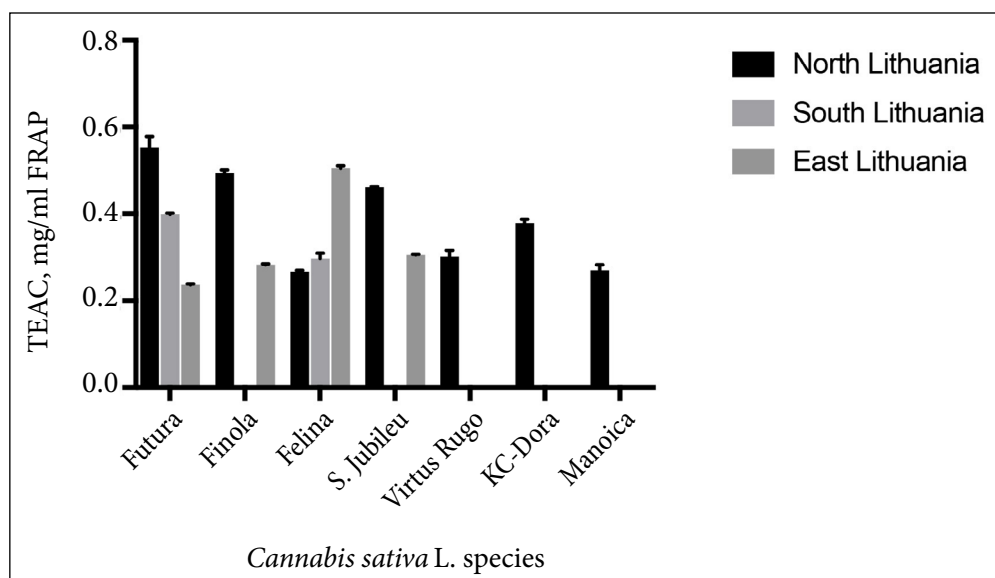
The principle of this method is based on conversion of phenolic hydroxyls to the corresponding quinones in the CUPRAC redox reaction, producing a chromogen of Cu(I)-neocuproine absorbing at 450 nm. For analysis 3 mL of the prepared CUPRAC reagent was mixed with 10  $\mu\text{L}$  of *Cannabis sativa* L. extract. Absorption was measured by a spectrophotometer HALO DB-20 UV-vis Dynamica GmbH (Switzerland) at 450 nm. Each sample was analysed 3 times. The antioxidant activities were expressed as TEAC values (Trolox mg/mL).

The data is expressed in averages  $\pm$  standard deviation (SD). The standard relative deviation (SRD) was estimated for survey data. The statistically significant differences between the distributions were determined using the 'Student t' criterion and the nonparametric Wilcoxon criteria for dependent sampling. A value of  $p < 0.05$  was taken as the level of significance.

## RESULTS AND DISCUSSION

The spectrophotometric FRAP and CUPRAC methods were used in further investigations of methanolic-tricholmetane extracts of different *Cannabis sativa* L. species herb with the goal of evaluating the input of potential reducers of medical plants raw material to the oxidant activity of raw material. The antioxidant activities were expressed as TEAC values (Trolox mg/mL). It has been established that all these species possess antioxidant activity.

Figures 1 and 2 show the collecting regions and antioxidant activities of different species of *Cannabis*. The antioxidant activity varied widely, ranging from 0.262 to 0.533 mg/mL using the FRAP method, and 0.143 to 0.467 mg/mL using the CUPRAC method. As shown in Fig. 1, the TEAC values of the samples from North Lithuania, other than Felina, were statistically higher. Meanwhile, the Felina species from East Lithuania possess stronger antioxidant activity than from the South of North region.

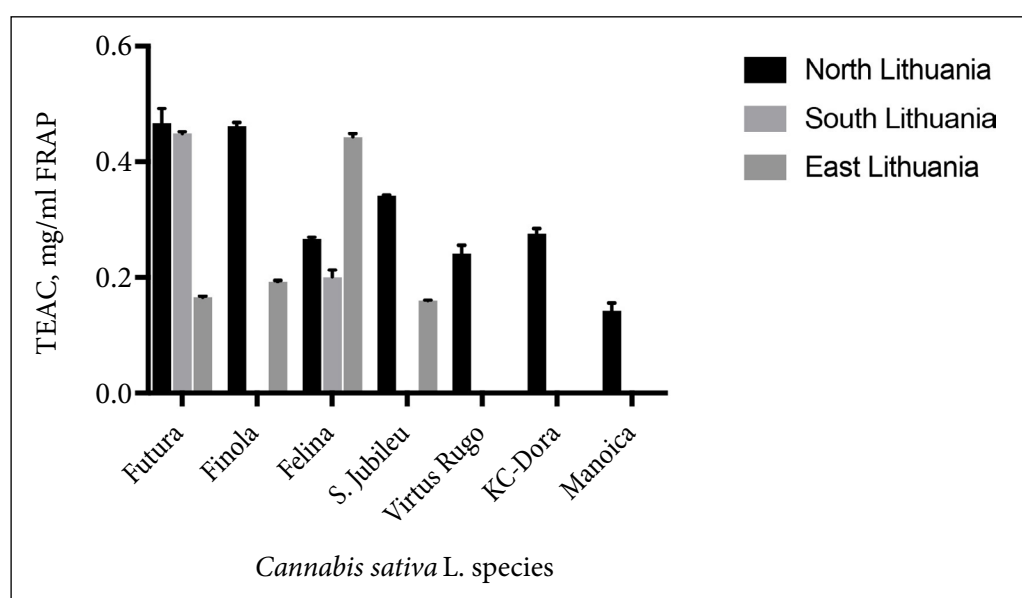


**Fig. 1.** The species collection region and antioxidant activity of *Cannabis sativa* L. using FRAP spectrophotometry

The hemp composition varies according to the hemp growth location. Anwar et al. previously reported that *Cannabis sativa* oilseeds concentration was highest (31.50%) in the seed samples collected from a wet mountainous region of Pakistan, whereas the seeds assayed from hemp plants grown in the zone with hot summers and cold winters were the lowest in oil content (26.90%) [16]. The average oil content of hempseed from Pakistan was slightly lower than that from Germany (30.00%) and from Turkey (31.79%) [16]. Furthermore, Chen et al. (2010) reported that

physicochemical properties of hempseed oil, oil contents, protein, fatty acid composition and tocopherols vary depending on cultivar and planting areas [17]. The results showed that the best planting areas – Southwest and Central China – because of a relatively high content of oil and protein appear to be the best varieties for oilseed and protein source based on kernel yield [17].

Hilling et al. analysed a small number of marijuana samples and determined that enhanced levels of particular terpenes may be useful for determining the country of origin [18].



**Fig. 2.** The species collection region and antioxidant activity of *Cannabis sativa* L. using CUPRAC spectrophotometry

Ahmad et al. have investigated that different noncannabinoid compounds like p-coumaric acid, m-coumaric acid, quercetin and cinnamic acid were in higher concentration in the whole *Cannabis sativa* plant sample [19]. Antioxidant properties of these phenolic compounds are reported [20, 21]. It is important that the antioxidant activity of the herb can be defined not only by phenolic components, but also by the essential oil or their interaction with other components. In the present study, the total antioxidant activity of *Cannabis sativa* L. was established.

The total antioxidant activities of different species of *Cannabis sativa* L. were expressed as TEAC values (Trolox mg/mL) and are presented in Fig. 3. The results showed statistically significant differences between the antiradical response of *Cannabis sativa* species and varied between 0.415 to 0.270 mg/mL using the FRAP method, and from 0.414 to 0.143 mg/mL using the CUPRAC. The highest antiradical response was obtained in Futura species ( $0.415 \pm 0.008$  mg/mL and  $0.414 \pm 0.013$  mg/mL) and the lowest in Manoica ( $0.270 \pm 0.013$  mg/mL and  $0.143 \pm 0.016$  mg/mL).

Significant differences of the antiradical response of different species of *Cannabis sativa* L. explained by FRAP and CUPRAC assays have different sensitivities for specific antioxidants [14]. Literature data shows that the FRAP method measures only the hydrophilic antioxidants, while the CUPRAC method is capable to assay both hydrophilic and lipophilic

antioxidants [16]. Meanwhile, according to our results, the antioxidative properties of different varieties of *Cannabis sativa* L., the statistically significant ( $p < 0.05$ ) amounts of all samples of varieties, with the exception of Futura, were determined using the spectrophotometric FRAP method. The reason for this difference in detection could be due to the reason that the FRAP reacts in an acidic pH while the CUPRAC can react only under a physiological pH of 7 [4]. Meanwhile, the TEAC value determined in the Futura variety using the FRAP method corresponded to the TEAC value set by the CUPRAC method –  $0.415 \pm 0.008$  mg/ml and  $0.414 \pm 0.013$  mg/ml, respectively.

The present study shows the ability of *Cannabis sativa* L. herb extracts to perform antioxidant activity. It is important for raising awareness about antioxidant properties of *Cannabis sativa* L. raw material.

The determination of the antioxidant activity of different varieties of *Cannabis sativa* L. from different regions of Lithuania using spectrophotometric iron (FRAP) and copper (CUPRAC) methods was evaluated. The obtained results showed that a statistically significant ( $p < 0.05$ ) higher reductive power was determined by analysing the raw material by the spectrophotometric FRAP method. The highest value was estimated in the Futura and the lowest in the Manoica variety by both assays.

The antioxidant activity of different varieties of *Cannabis sativa* L. varies according to the hemp

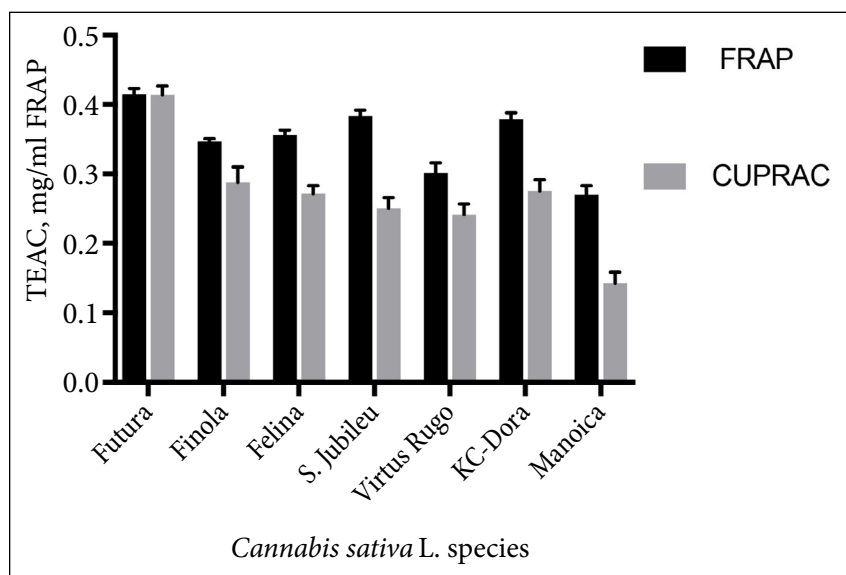


Fig. 3. Antioxidant activity of different *Cannabis sativa* L. species

growth location in Lithuania. The highest values were estimated in the Futura, Finola and S. Jubileu from the North region, while the Felina variety showed a higher antioxidant activity from the East region.

Received 26 March 2020

Accepted 16 April 2020

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## PLUOŠTINIŲ KANAPIŲ (*CANNABIS SATIVA* L.) VEISLIŲ ANTIOKSIDACINIO AKTYVUMO NUSTATYMAS IR PALYGINIMAS SPEKTROFOTOMETRINIŲ FRAP IR CUPRAC METODAIS

### Santrauka

Kanapės – vienas seniausių augalų žemėje, žinomas ir naudojamas medicininiais tikslams. Mokslinėje literatūroje plačiai aprašoma kanapių aliejaus nauda, tačiau tyrimų apie jų antioksidacinį aktyvumą nėra daug. Tyrimo tikslas – įvertinti Lietuvoje auginamų skirtingų *Cannabis sativa* L. veislių antioksidacinį aktyvumą spektrofotometriu metodu, naudojant du skirtingus reagentus – FRAP (geležies redukcijos antioksidacinė galia) ir CUPRAC (vario jonų redukcijos antioksidacinė geba) – ir palyginti gautus rezultatus. Nustatyta, kad *Cannabis sativa* L. pasižymi antioksidaciniu aktyvumu. Lyginant skirtingų *Cannabis sativa* L. veislių žaliavų antioksidacinį aktyvumą paaiškėjo, kad didžiausia redukcine geba pasižymi 'Futura', o mažiausia – 'Manoica'. Statistiškai reikšmingai ( $p < 0,05$ ) didesni antioksidacinėmis savybėmis pasižyminčių junginių kiekiai nustatyti tyrimui naudojant FRAP reagentą. Įvairiuose Lietuvos regionuose atlikus pluoštinių kanapių skirtingų veislių antioksidacinio aktyvumo tyrimą paaiškėjo, kad didžiausiu TEAC kiekiu pasižymi Šiaurės Lietuvoje auginama žaliava.