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CBG, CBD, Δ9-THC, CBN, CBGA, CBDA and Δ9-THCA as antioxidant agents and their intervention abilities in antioxidant action

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ABSTRACT

Positive effect of some cannabinoids in the treatment and prophylaxis of a wide variety of oxidation-associated diseases and growing popularity of supplements containing cannabinoids, mainly cannabinoid oils (e.g. CBD oil, CBG oil), in the self-medication of humans cause a growing interest in the antioxidant properties of these compounds, especially those not showing psychotropic effects.

Herein, we report the antioxidant activity of cannabigerol (CBG), cannabidiol (CBD), Δ9-tetrahydrocannabinol (Δ9-THC), cannabinol (CBN), cannabigerolic acid (CBGA), cannabinolic acid (CBDA) and Δ9-tetrahydrocannabinolic acid (Δ9-THCA) estimated by spectrophotometric methods: ABTS, DPPH, ORAC, beta-carotene CUPRAC and FRAP.

The presented data prove that all the examined cannabinoids exhibit antioxidant activity manifested in their ability to scavenge free radicals, to prevent the oxidation process and to reduce metal ions. Although the intensity of these activities is not the same for the individual cannabinoids it is comparable for all of them with that of E vitamin.

As results from the research, the significance of the two types of electron sources presenting in examined cannabinoids, phenolic groups and double bonds transferring electrons, depends on the type of electron-accepting species - radicals/metal ions.

1. Introduction

The estimation of the influence of various compounds on the proper functioning of living organisms and searching for those able to effectively treat certain diseases are the most intensively developed and widely promoted areas in the pharmaceutical and nutrition sciences today. Special attention is paid in these studies to the bioactivity of plant and food components. The example of compounds that have recently been under the scrutiny of researchers are cannabinoids [1,2] the components of marijuana and hemp plants. Although Δ9-Tetrahydrocannabinol (Δ9-THC) is the most famous representative of this numerous compound group, recently much attention has been paid to another cannabinoid, namely cannabidiol (CBD), which - unlike Δ9-THC - does not show psychotropic effects.

It has been scientifically proven that CBD, a strong antioxidant, participates in preventing free radicals by interacting with the endocannabinoid system in the body [3]. As the endocannabinoid system is important for different health systems within the body, researchers got also interested in the bioactivity and antioxidant properties of other non-psychoactive cannabinoids, among them two neutral cannabinoids:

- cannabigerol (CBG) and
- cannabinol (CBN),

which are, in metabolic pathway of hemp plants, the precursor of CBD and the derivative of Δ9-THC, respectively. Other substances under frequent scrutiny are three cannabinoid acids:

- cannabigerolic acid (CBGA)
- cannabinolic acid (CBDA), and
- Δ9-tetrahydrocannabinolic acid (Δ9-THCA)

which are, in the hemp plant metabolic pathway, the precursors of CBG, CBD and THC, respectively.

The present paper discusses the antioxidant properties of CBG, CBN, CBDA, CBGA and Δ9-THCA which, beside CBD and Δ9-THC, are also

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supposed to be bioactive compounds useful in the therapeutic treatment of different diseases [4]. According to the literature [5], CBD and Δ9-THC exhibit strong antioxidant activity, stronger than vitamins C, A and E. To facilitate the comparison of the antioxidant activities of the examined cannabinoids with those of CBD and Δ9-THC, the latter were investigated as well, all of them by means of the same measurement methods in the same conditions.

2. Experimental

2.1. Materials

$CuCl_2$, $Fe_2(SO_4)_3{\cdot}7H_2O$, $FeCl_3{\cdot}6H_2O$, HCl, CH_3COONH_4, ethanol, methanol, chloroform CH_3COONa, CH_3COOH, $NaH_2PO_4{\cdot}H_2O$, $Na_2HPO_4{\cdot}7H_2O$ were supplied by the Polish Chemical Plant POCh (Gliwice, Poland). CBD, CBG, CBN, Δ9-THC, CBDA, CBDA and Δ9-THCA, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 2,2′-azobis(2-amidinopropane) dichloride) (AAPH), 2,2′-diphenylpicrylhydrazyl (DPPH), 2,4,6-tri(2-pyridyl)-*s*-triazine (TPTZ), di-potassium peroxdisulfate, fluorescein, linoleic acid, neocuproine - 2,9-dimethyl-1,10-phenanthroline, (Nc), Trolox, olivetol (OL), Tween 20,

β-carotene were purchased from Sigma Aldrich (Poznań, Poland). Deionized water was purified on a Milli-Q system from Millipore (Millipore, Bedford, MA, USA).

The chemical structures of examined antioxidants are presented in Fig. 1. The chemical structures of electron accepting species (radicals/metal ion complexes) applied in measuring systems for the antioxidant activity estimation of examined compounds are shown in Fig. 2.

2.2. Methods

2.2.1. β-carotene bleaching assay

The estimation of the β-carotene degradation in the presence of the examined cannabinoids or Trolox or OL was performed following the procedure described elsewhere [6]. The stock solution of β-carotene/linoleic acid emulsion in water consisting of linoleic acid (25 μL), Tween 20 (185 μL containing 200 mg of Tween) and chloroformic β-carotene solution (5 mL with β-carotene concentration equal to 0.5 mg/mL) was prepared. After evaporation of chloroform from the solution, the residue was dispersed in 100 mL of distilled water earlier saturated with oxygen for 30 min at flow rate equals 100 mL/min. The mixture was vigorously shaken and the obtained emulsion (2900 μL) was placed in an optical glass cuvette (1 × 1 × 3.5 cm) containing 100 μL methanolic solution of the examined substance. The cuvette (stoppered tightly and mixed) was put into a thermostated (45 °C) spectrophotometric system. The changes of β-carotene absorbance were monitored at 470 nm. The subsequent measurement readings were taken at constant intervals (10 min) until the orange color of the control sample disappeared (about 180 min). The control sample was prepared in the same way. The only difference was the absence of the examined substance, replaced by pure MeOH. The mixture composed of the emulsion without β-carotene (2900 μL) with MeOH (100 μL) was used to zero the spectrophotometer.

The antioxidant activity was expressed as inhibition percentage relative to the control using the following equation:

$AA = 100{\cdot}\frac{DR_C - DR_S}{DR_c}$ where:

AA – antioxidant activity.

DR_C – degradation rate of β–carotene in the control sample = {[ln (a/b)]/t}.

DR_S – degradation rate of β–carotene in the sample with antioxidant = {[ln (a/b)]/t}.

a = absorbance at time = 0.

b = absorbance at defined time (for example at 10, 20 …. to 180 min).

t = time.

2.2.2. DPPH method

The concentration of the DPPH radicals after their reaction with the examined component was estimated by the slightly modified Brand-Williams method [7]. The course of the measurements was as follows. $DPPH^{\bullet}$ methanolic solution of initial absorbance 0.7 ± 0.05 at 516 nm (2900 μL) was mixed with methanolic solution of the examined substance (100 μL) in a 4 mL test tube. The mixture, after vigorous shaking for 30 s, was transferred into an optical glass cuvette (1 × 1 × 3.5 cm), which was immediately placed in a spectrophotometer. The absorbance decrease at 516 nm was monitored continuously for 60 min. Pure methanol was used to zero the spectrophotometer.

2.2.3. ABTS method

The ability of the examined compounds to scavenge ABTS cation radicals was estimated spectrophotometrically registering the concentration change of the radicals at 744 nm. The measurements were

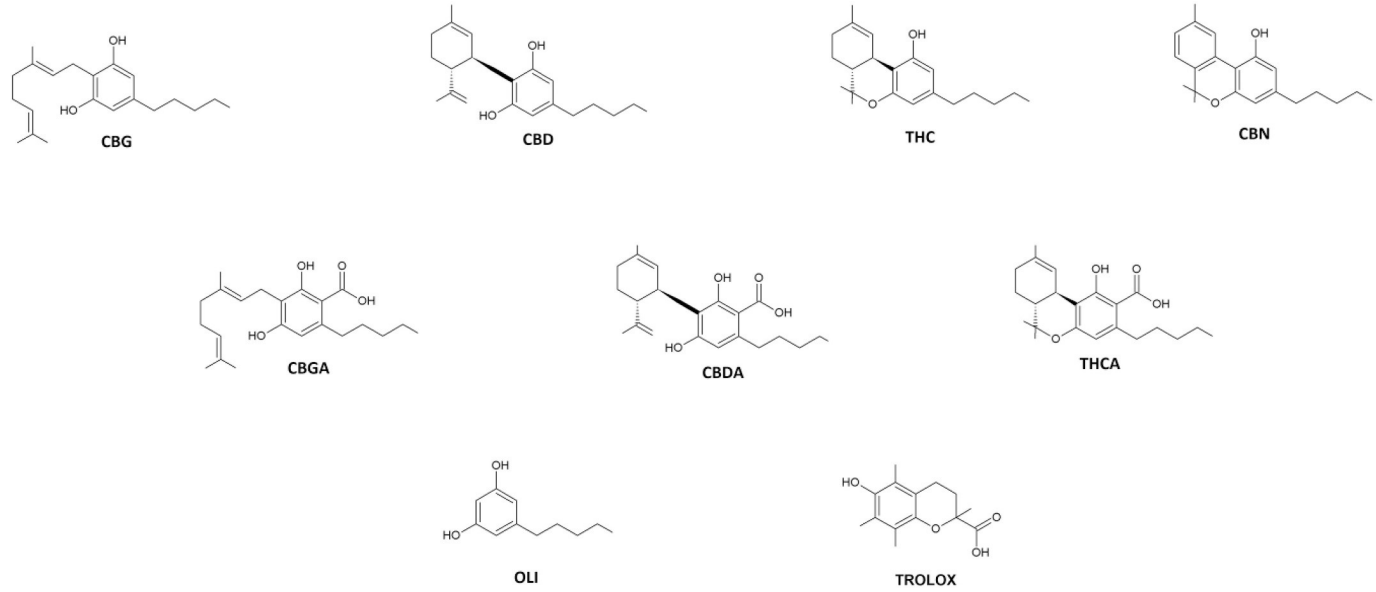

Fig. 1. Chemical structures of examined antioxidants.

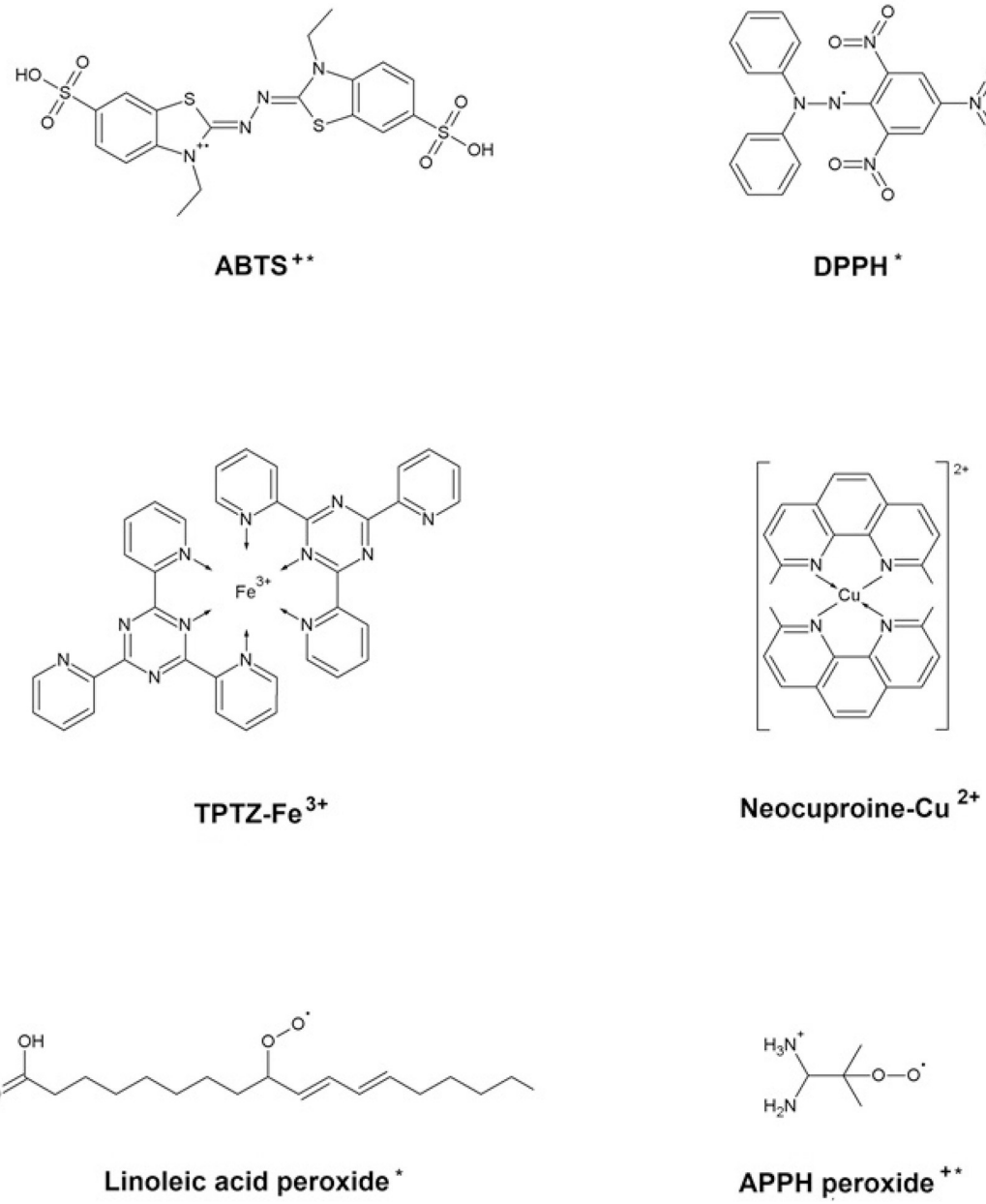

Fig. 2. Chemical structures of electron accepting species (radicals/metal ion complexes) present in measuring systems applied for the antioxidant activity estimation of examined cannabinoids.

performed according to the procedure described in the literature [8]. First, a mixture composed of 7 mM aqueous ABTS solution (5 mL) and 140 mM potassium persulfate ($K_2S_2O_8$) (88 μL) was prepared. After 16 h incubation in the dark, it was diluted with methanol to absorbance equal 0.7 ± 0.05 measured at 744 nm. The obtained solution (2900 μL) was subsequently mixed with methanolic solution of the examined compound (100 μL) in a 4 mL test tube and, after shaking, put an into optical glass cuvette (1 × 1 × 3.5 cm), which was immediately placed in a spectrophotometer. The absorbance decrease was monitored in a continuous manner for 60 min. Pure methanol was used to zero the spectrophotometer.

The inhibition percent of $DPPH^{\bullet}$ or $ABTS^{\bullet+}$ was calculated according to the following equation:

$$I(\%) = \left(1 - \frac{A_{60}}{A_0}\right) \cdot 100\%$$ where: A_0 and A_{60} are the values of $DPPH^{\bullet}$ or $ABTS^{\bullet+}$ absorbance at 0 and 60 min of the radical neutralization reaction, respectively.

2.2.4. FRAP assay

FRAP assay was carried out using the method described in the literature [9]. The following components were used to prepare FRAP test solution: $FeCl_3 \cdot 6H_2O$ in distilled water with the final Fe(III) concentration equaling 20 mM, TPTZ in 40 mM HCl with the final TPTZ concentration equaling 20 mM, and 0.3 M CH_3COOH/CH_3COONa buffer (pH = 3.6). The three reagents were mixed together with volume ratio 10:1:1, respectively Estimating the antioxidant properties of the examined components, the aliquot of FRAP reagent (2900 μL) was mixed in a 4 mL test tube with the methanolic solution of a given antioxidant (100 μL) and, after shaking for 30 s, was left in the dark for 60 min at 37 °C. Then, the mixture was poured into an optical glass cuvette (1 × 1 × 3.5 cm) and immediately placed in a spectrophotometer to measure the absorbance at 593 nm. To zero the spectrophotometer, the mixture of FRAP reagent and MeOH without antioxidants was used.

2.2.5. CUPRAC assay

In CUPRAC method the ability of an antioxidant to the reduction of the cupric neocuproine complex (Cu(II)-Nc) to the cuprous form (Cu(I)-Nc) is measured [6]. CUPRAC test solution was prepared using $CuCl_2$ water solution with final Cu(II) concentration equaling 10 mM (740 μL), ethanolic solution of neocuproine with its final concentration equaling 7.5 mM (740 μL), 1.0 M CH_3COOH/CH_3COONH_4 buffer solution, pH = 7.0 (740 μL) and distilled water (680 μL). The test solution was mixed with the methanolic solution of the examined antioxidant (100 μL). The mixture was vigorously shaken for 30 s, left in the dark for 60 min and then poured into an optical glass cuvette (1 × 1 × 3.5 cm) to measure its absorbance at 450 nm. A mixture of CUPRAC reagent and MeOH without antioxidants was used to zero the spectrophotometer.

UV Probe-2500 Spectrophotometer (Shimadzu, Kyoto, Japan) was applied in all the described procedures.

2.2.6. ORAC assay

0.04 μM fluorescein solution in 0.075 M phosphate buffer, pH = 7.0 (2600 μL) was mixed with the methanolic solution of the examined antioxidant (100 μL) in a 4 mL test tube. The reaction mixture was allowed to equilibrate for 30 min at 37 °C. Antioxidant properties were determined examining fluorescence decay initiated by the addition of a radical generator. For this reason 300 μL of 200 mM AAPH solution in 0.075 M phosphate buffer, pH = 7,0 was added to the above mixture and after shaking poured to optical glass cuvettes (1 × 1 × 3.5 cm). Fluorescence measurements were performed at 37 °C using RF-6000 spectrofluorometer (Shimadzu, Kyoto, Japan). Excitation and emission wavelengths were 485 nm and 520 nm, respectively. The fluorescence was monitored in a continuous manner for 60 min. 0.04 μM fluorescein solution in 0.075 M phosphate buffer, pH = 7.0 (2600 μL) with addition of 0.075 M phosphate buffer, pH = 7.0 (100 μL) was used as a blank sample.

The antioxidant capacity, expressed as the AUC, was calculated as follows [10]:

$$AUC = 1 + f_1/f_0 + \ldots\ldots + f_{n+1}/f_0$$

where

AUC – area under the fluorescence decay curve

f_0 – the initial fluorescence reading at 0 min and.

f_n – the fluorescence reading at time n.

The solution concentration of the examined cannabinoids, Trolox and OL in all the experiments was the same and equalled 0.1 mM.

2.3. Statistical analysis

All results are presented as the mean value of five independent measurements (n = 5) ± SD. The antioxidant activities were compared using analysis of variance (ANOVA). Antioxidant activity differences in the studied groups were considered as significant for $p \leq 0.05$ and $F_{crit} < F_{exp}$.

3. Results and discussion

Although the antioxidant properties of the examined compounds can be considered in terms of their preventive, interventional, or restorative effects, the most frequently assessed their interventional activity i.e. the ability to free radical scavenging, to reduce metal ions and to retard and/or delay the target molecules oxidation processes. To estimate all these intervention activities a few methods are usually required. The spectrophotometric methods are used most willingly [11]. The antioxidant activity in the free radical scavenging process is most frequently assessed by ABTS and/or DPPH methods, the antioxidant ability to reduce metal ions is usually examined by CUPRAC and/or FRAP, whereas β-carotene bleaching and/or ORAC methods allow to assesses the ability of an antioxidant to protect the target molecule against its oxidation process.

As there is no universal scale of antioxidant activity, the antioxidant activity of the compound under scrutiny is usually compared with that for the compound adopted as a standard. The antioxidant properties of the examined cannabinoids were related to Trolox, (a water-soluble analog of vitamin E used in biological and biochemical applications to reduce oxidative stress or damage), which was treated as an antioxidant standard in the reported experiments.

The chemical structure of the examined cannabinoids (see Fig. 1) suggests that their antioxidant properties mainly results from the presence of phenolic groups easily oxidized to quinoid forms [12] and of unsaturated bonds found in non-olivetolic fragments of some cannabinoid molecules. In order to present the impact of the individual structural fragments in the examined cannabinoids on their antioxidant activity and to make the discussion clearer and more exhaustive, the antioxidant properties of the examined cannabinoids were related to the antioxidant properties of OL.

3.1. Antioxidant activity assessment with use of ABTS radical cations

The antioxidant activities of the neutral and acidic forms of the examined cannabinoids calculated in relation to the antioxidant activity of Trolox, all assessed by ABTS method, are shown in Fig. 3A and B, respectively. The presented results not only confirm the known high free radical scavenging ability of CBD and Δ9-THC but also prove that the other examined cannabinoids, except Δ9-THCA, are also very active in this respect. According to ABTS results, the scavenging ability of cation radical by each examined cannabinoid, except Δ9-THCA, is greater by more than 25% than that of Trolox. A small decrease of the radical scavenging ability by neutral cannabinoids CBG, CBD, THC and CBN in descending order ($F_{crit} = 3.24 < F_{exp} = 4.06$), and clear decrease by the acidic cannabinoids CBGA, CBDA and Δ9-THCA in descending order ($F_{crit} = 4.10 < F_{exp} = 161.06$) is understandable and results from the decreasing number of phenolic –OH groups in individual cannabinoids and from the interaction of OH groups with electrons of double bond occurring in non-olivetolic fragments of the examined compounds. In consequence, the transport ability of electrons from cannabinoid molecules to the ABTS cation radical is lowered and/or hindered in the mentioned order.

Analogous conclusions can be drawn relating the antioxidant power of the individual examined cannabinoids to that for OL (see Fig. 3C and D). The diagrams confirm the inhibiting influence of non-olivetolic fragment in cannabinoid molecules on their abilities to transport electron to the ABTS cation radical - the relations are lower than 1 and diminish in the sequence CBG, CBD, Δ9-THC CBN and CBGA, CBDA, Δ9-THCA. An extremely low antioxidant activity of Δ9-THCA results from the presence of only one OH group, additionally engaged in the formation of hydrogen bond with COOH groups.

The comparison of the data for CBG and CBGA and/or CBD and CBDA (see Fig. 3A and B and/or Fig. 3C and D) proves that the carboxyl group in acidic forms of cannabinoids does not have any essential impact on electron transport from cannabinoid molecules to the ABTS cation radical. It is more evident in Fig. 3E illustrating the relation between the antioxidant activities of the acidic and non-acidic forms of the examined cannabinoids.

According to the literature [13], the presence of a carboxyl group attached to the aromatic ring of the phenolic group leads to the charge delocalization and causes the reduction of the electron density, facilitating the formation of the phenolic radical. Thus, the obtained results (Fig. 3E) are in contradiction with the this statement. However, it should be remembered that the electron transfer from the antioxidant molecule to the ABTS cation radical is accompanied by proton detachment form phenolic —OH group (SET mechanism). In the case of acidic forms of cannabinoids, the active phenolic —OH group and the —COOH group are *ortho* position. In consequence, they are involved in the formation of intramolecular hydrogen bonding. This hinders the detachment of the

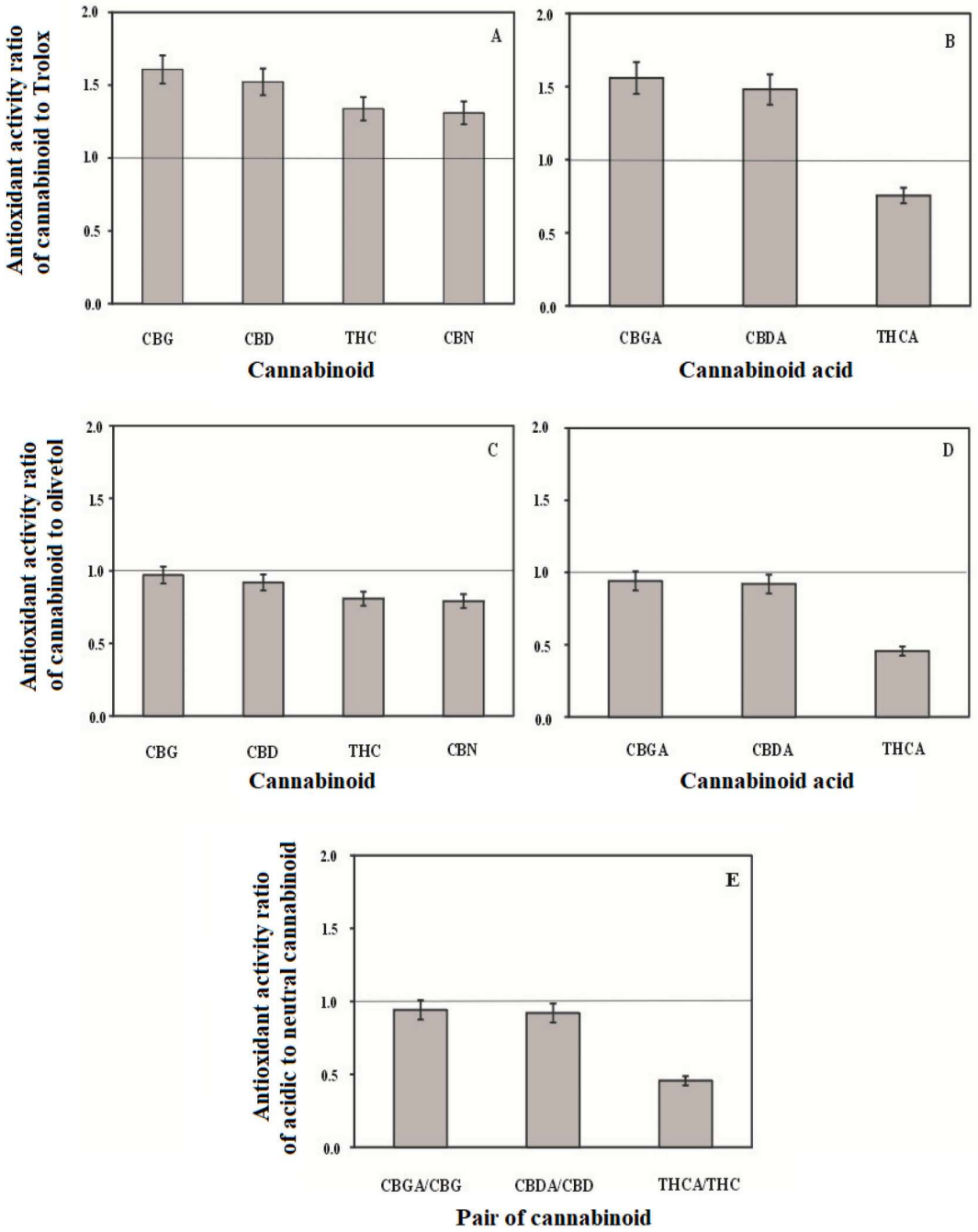

Fig. 3. Relative antioxidant activity of CBG, CBD, Δ9-THC, CBN, CBGA, CBDA and Δ9-THCA estimated in ABTS measurements.

proton from the phenolic group and reveals by the observed lack of the —COOH group influence on the antioxidant properties of acid cannabinoids in relation to neutral. An example conforming the lack of essential influence of the COOH group on the estimation of antioxidant activity of acidic cannabinoids by ABTS could be very low antioxidant properties of benzoic/salicylic acid. The proper experiments are not shown in this paper, however, these compounds used in the same concentration as the examined cannabinoids (0.1 mM) scavenge only 1% of the ABTS cation radical during 1 h reaction showing on their negligible antioxidant properties.

As appears from Fig. 3E, the relation for CBGA/CBG and CBDA/CBD pairs is about 1. In this context the low values for the Δ9-THCA/Δ9-THC pair looks strange; however, taking into account the observed difference in these relations (i.e. about 1 for CBGA/CBG and/or CBDA/CBD and about 0.5 for Δ9-THCA/Δ9-THC) and the structures of the examined cannabinoids, it can be concluded that only one OH group in each individual cannabinoid takes part in the process of electron transfer to the ABTS cation radical. The low value of the antioxidative relation for the Δ9-THCA/Δ9-THC pair results from the presence of only one antioxidatively active OH group, which in Δ9-THCA is engaged in the hydrogen bond with COOH.

3.2. Antioxidant activity assessment using DPPH radicals

only CBG and Δ9-THC exhibit stronger antioxidant activity than Trolox ($F_{crit} = 5.32 < F_{exp} = 21.50$ for CBG; $F_{crit} = 5.32 < F_{exp} = 61.47$ for Δ9-THC) (see the relations in Fig. S1A and B);-

in almost all the examined cannabinoids, except CBN and Δ9-THCA,

a more or less positive impact of the non-olivetolic fragment on their antioxidant properties is noticeable (see the relations in Fig. S1C and D);-

the presence of COOH group in the examined cannabinoids diminishes their antioxidant power in varying degrees (the ratio of antioxidant activity of acidic to neutral form of given cannabinoid is lower than 1 – see Fig. S1E).

Fig. S1 shows the antioxidant activities of neutral and acidic forms of the examined cannabinoids calculated in relation to the antioxidant activity of Trolox (Fig. S1A and B) and of OL (Fig. S1C and D), using the DPPH data. The antioxidative relation between the acidic and non-acidic forms of the examined cannabinoids are illustrated in Fig. S1E. The analysis of the results from Fig. S1 leads to the following conclusions:

- only CBG and Δ9-THC exhibit stronger antioxidant activity than Trolox ($F_{crit} = 5.32 < F_{exp} = 21.50$ for CBG; $F_{crit} = 5.32 < F_{exp} = 61.47$ for Δ9-THC) (see the relations in Fig. S1A and B);
- in almost all the examined cannabinoids, except CBN and Δ9-THCA, a more or less positive impact of the non-olivetolic fragment on their antioxidant properties is noticeable (see the relations in Fig. S1C and D);
- the presence of COOH group in the examined cannabinoids diminishes their antioxidant power in varying degrees (the ratio of antioxidant activity of acidic to neutral form of given cannabinoid is lower than 1 – see Fig. S1E).

The mutual relations between antioxidant activities of individual cannabinoids established with the DPPH method are less unambiguous

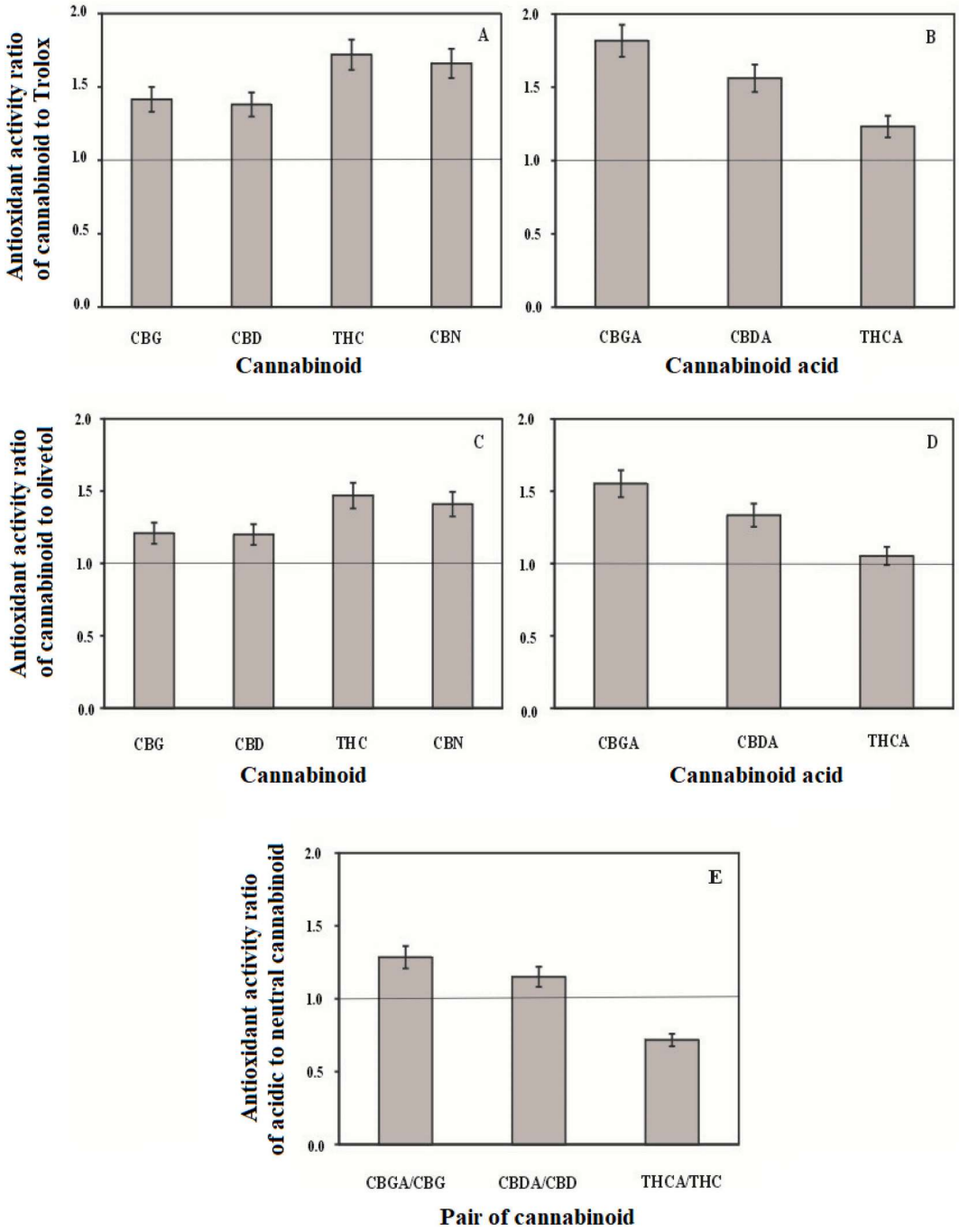

Fig. 4. Relative antioxidant activity of CBG, CBD, Δ9-THC, CBN, CBGA, CBDA and Δ9-THCA estimated in CUPRAC measurements.

those found with the ABTS method, which makes their scientific interpretation much more difficult for a few reasons. In the case of phenolic antioxidants dissolved in methanol, SPLET mechanism dominates in the process of electron transfer from the antioxidant to the radical. Electron transfer requires appropriate mutual orientation of the antioxidant molecule and the radical. Moreover, access to the active center of the DPPH radical is more difficult than in the case of the ABTS cation radical [14,15]. Due to the limited access to the active center of the DPPH radical, achieving its proper orientation with respect to the molecules of each examined cannabinoid, whose structures are complex and different, is hindered to varying degrees. The above explanation of the mutual relations between the antioxidant activity of individual cannabinoids, established with the DPPH method, is plausible if one takes into account the noticeably lower ability of the examined compounds to scavenge DPPH radicals than ABTS cation radicals.

DPPH radicals can also be neutralized by compounds with double bonds in the adduct formation mechanism [16,17]. As results from the diagrams in Fig. S1C and D, a less or more important role in the antioxidant activity of the examined cannabinoids, estimated by the DPPH method, is played by their non-olivetolic fragment. It indicates that double bonds also participate in the neutralization of DPPH radicals. If so, an appropriate mutual orientation of the antioxidant molecule and the radical is also required.

Thus, a simple interpretation of the mutual relations of the antioxidant activity of individual cannabinoids is made difficult due to the coexistance of two different, more or less dependent sources, namely OH groups and double bonds, transmitting electrons to the DPPH radical in two different ways, the dominance of which may depend on the antioxidant structure.

3.3. Antioxidant activity assessment by the FRAP and CUPRAC methods

The antioxidant activity of the examined cannabinoids in relation to that of Trolox, OL, and of the acidic forms of some cannabinoids in relation to their neutral forms, estimated by the FRAP and the CUPRAC methods, is presented in Figs. S2 and 4A, respectively. Considering the data corresponding to the neutral forms of cannabinoids, it should be observed that both methods confirm the strong, albeit slightly diverse, antioxidant power of these compounds (stronger than that of Trolox – see Figs. S2A and 4A). A more precise analysis of the diagrams from Figs. S2A and 4A indicate on:

- almost identical values of the ratios of antioxidant activity of individual cannabinoids with the activity of Trolox estimated by FRAP and CUPRAC ($F_{crit} = 5.32 > F_{exp} = 0.07$ for CBG; $F_{crit} = 5.32 > F_{exp} = 4.28$ for CBD; $F_{crit} = 5.32 > F_{exp} = 0.07$ for Δ9-THC; $F_{crit} = 5.32 > F_{exp} = 0.77$ for CBN), and
- the same relations between the values of the antioxidant ratios for individual cannabinoids: lower but almost identical values for CBG and CBD ($F_{crit} = 4.10 > F_{exp} = 1.27$ in CUPRAC measurements; $F_{crit} = 4.10 > F_{exp} = 0.06$ in FRAP measurements), and higher but also almost the same ones for Δ9-THC and CBN ($F_{crit} = 4.10 > F_{exp} = 1.58$ in CUPRAC measurements; $F_{crit} = 4.10 > F_{exp} = 0.17$ in FRAP measurements).

It is worth remembering that the SET mechanism dominates in the reduction process of Fe+3 and/or Cu+2 in FRAP and CUPRAC. In this process, electron from the phenolic group takes place. Although only one OH group occurs in Δ9-THC and CBN, it is bonded to the aromatic ring which, as in Trolox, is connected with the aliphatic etheric grouping. It shifts the electron cloud in the aromatic ring towards the aliphatic structure. Consequently, the bond between oxygen and hydrogen in the phenolic group is weakened, which facilitates the transfer of an electron from the antioxidant to the iron or copper complex. Hence, higher antioxidant properties of Δ9-THC and CBN than of CBG and CBD in the SET mechanism are not surprising. Moreover, as the structures of Δ9-THC and CBN are more rigid than those of CBG and CBD, it cannot be ruled out that the stiffer structures more easily assume a more favourable spatial arrangement with the iron or copper complexes in the SET process.

Analogous conclusions and explanation can be offered analyzing the antioxidant activities of the examined non acidic forms of cannabinoids in relation to OL – see Figs. S2C and 4C. They confirm the significant impact of the non-olivetolic part of the cannabinoid molecules, especially the one with ether oxygen, on the antioxidant power of all the tested neutral cannabinoids.

Slightly different antioxidant activities are revealed by the FRAP and CUPRAC methods in the examined cannabinoid acids (see Figs. S2B and D and 4B and D). Both methods reveal a decrease of the antioxidant power of the compounds in the sequence CBGA, CBDA and Δ9-THCA. Such sequence results from the loss of the number of phenolic —OH groups in the molecules of individual cannabinoid acids and from the formation of a hydrogen bond between the phenolic group and the carboxyl group. More striking, however, is the fact that only CUPRAC reveals a greater reduction ability of these acids than that of Trolox and/or OL. Yet it seems obvious if one takes into account pK_a of individual cannabinoid acids (see Table 1) and pH of the redox reaction environment: pH = 3.6 in FRAP and pH = 7.0 in CUPRAC. As results from [18], the increase of the dissociation degree of the carboxyl group in the vicinity of the phenolic —OH group weakens the strength of the bonding between oxygen and hydrogen in —OH group and facilitates electron detachment. While the carboxyl group of cannabinoid acids in the CUPRAC environment (pH = 7.0) is almost totally dissociated, its dissociation degree in the FRAP environment (pH = 3.6) is only partial - about 50%. In consequence, the oxygen-hydrogen bond in the phenolic —OH group at pH = 3.6 is stronger than at pH = 7.0. Hence, the electron transfer from cannabinoid acids to the Fe+3 complex is more difficult than the electron transfer from cannabinoid acids to Cu+2 complex. This is why the FRAP method reveals weaker antioxidant power of the examined cannabinoid acids than the CUPRAC method. Neutral cannabinoids exhibit strong antioxidant activity in the neutral and the basic environments, so their almost identical antioxidant power estimated by FRAP and CUPRAC is not surprising.

3.4. Antioxidant activity assessment by the beta-carotene bleaching method

One of the most popular estimating method of the antioxidant activity of food ingredients is the β-carotene bleaching assay. Peroxyl radicals occurring in the measuring system of the β-carotene bleaching assay are neutralized by antioxidant molecules, transferring electrons from their double bonds according to the adduct formation mechanism [19] and/or from phenolic groups according to the PCET mechanism [20], if only such reacting centres (double bonds and/or phenolic groups) are present in the molecule. Due to the presence of both types of centres in most of the examined cannabinoids, the simultaneous

Table 1
pK_a of examined cannabinoids.

Lp	Compounds	pKa
1	CBG	9.16
2	CBD	9.13
3	THC	10.60
4	CBN	9.40
5	CBDA	3.41
6	CBGA	3.42
7	THCA	3.89
8	Olivetol	9.59
9	Eugenol	10.19
10	Benzoic acid	4.19
11	Resorcylic acid	3.11
12	Trolox	3.77
13	Limonene	–

occurrence of both mentioned mechanisms in the reaction between peroxyl radicals and some cannabinoids is probable. Hence, the observed decrease of the antioxidant power of neutral cannabinoids in the order CBG, CBD, Δ9-THC and CBN ($F_{crit} = 3.24 < F_{exp} = 166.71$) (see Fig. 5A) is understandable and results from the decrease of the number of phenolic –OH groups and/or of double bonds in the molecules of individual cannabinoids. It seems strange, however, that all the neutral cannabinoids in the β-carotene assay show markedly weaker antioxidant activities than Trolox ($F_{crit} = 3.24 < F_{exp} = 1212.60$ for CBG; $F_{crit} = 3.24 < F_{exp} = 1024.19$ for CBD; $F_{crit} = 3.24 < F_{exp} = 1018.27$ for Δ9-THC; $F_{crit} = 3.24 < F_{exp} = 1362.47$ for CBN), whose antioxidant power results from the presence of only one phenolic group. One might think that the double bond present in the non-olivetolic fragment of the CBG, CBD and Δ9-THC molecule is not involved in the neutralization process of peroxyl radicals. This is contradicted, however, by the higher antioxidant activity of CBG than of OL (see Fig. 5C). A highly probable participation of the double bond from non-olivetolic part of cannabinoids in the neutralization process of peroxyl radicals may also be indicated by a considerably high antioxidant activity of Δ9-THCA in relation to that of CBGA and/or of CBDA (see Fig. 5B). Due to a strong hydrogen bond between the phenolic —OH group and carboxyl group, the antioxidant activity of CBGA and CBDA is lower than that for their corresponding neutral equivalents, CBG and CBD (compare corresponding bars in Fig. 5A and B). The same cannot be said for Δ9-THCA. Its antioxidant activity, despite a strong hydrogen bond between the phenolic —OH group and carboxyl group, is almost the same as of Δ9-THC (the antioxidant activity ratio for the Δ9-THCA/Δ9-THC pair equals 1 - see Fig. 5E). This points to the insignificant importance of the only —OH

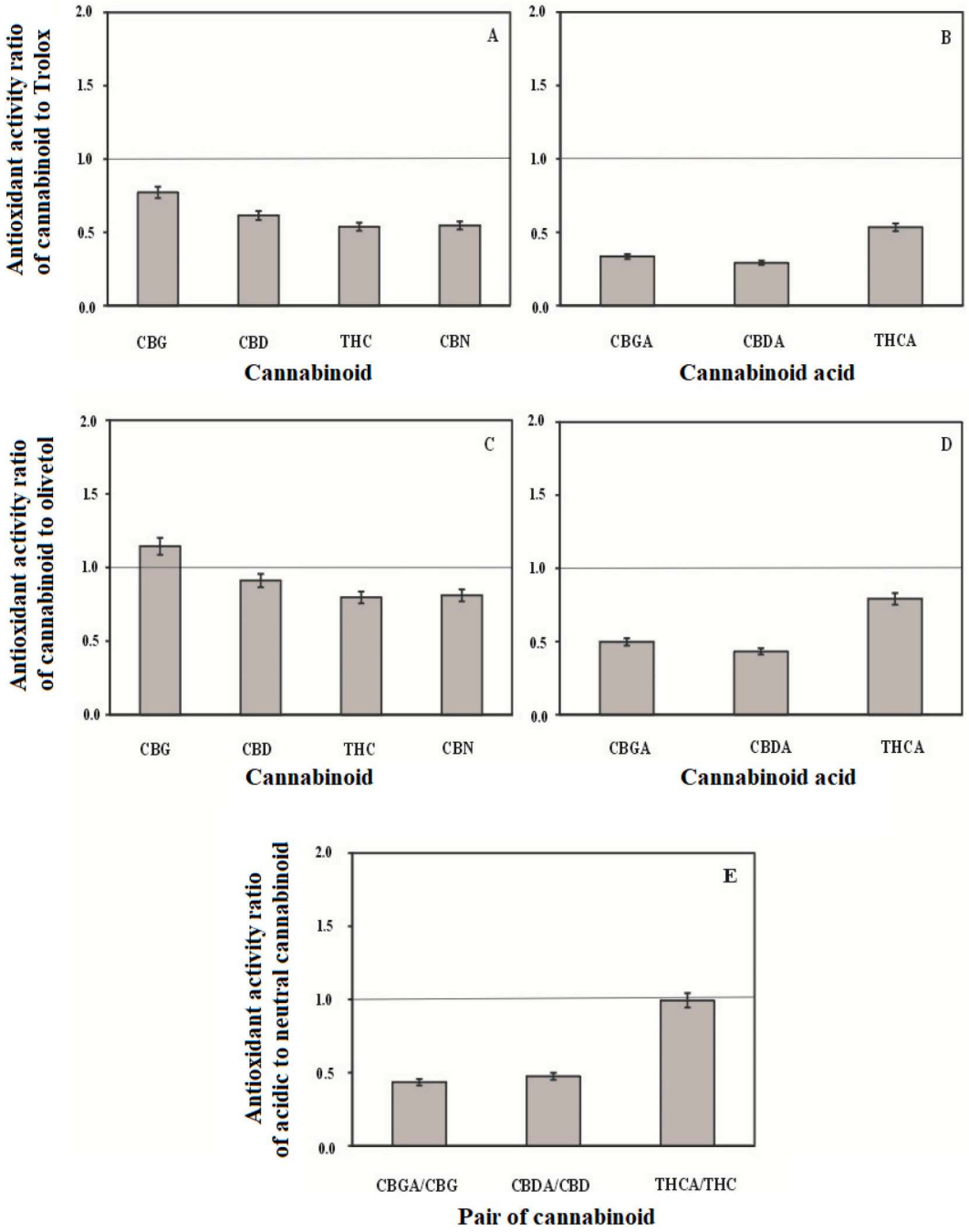

Fig. 5. Relative antioxidant activity of CBG, CBD, Δ9-THC, CBN, CBGA, CBDA and Δ9-THCA estimated in beta-carotene bleaching assay measurements.

group presenting in the Δ9-THC and Δ9-THCA molecules in the neutralization process of peroxyl radicals and indicates that the antioxidant activity of these compounds mainly results from the presence of the double bond in the aliphatic ring belonging to their non-olivetolic fragment. At this point, another process comes to mind, that of limonene oxidation to the oxirane form with the use of H_2O_2 [21]. According to the cited paper [21] the oxidation of the double bond in the cyclic structure is privileged due to the higher order of carbon atoms. In this context, the weaker antioxidant properties of CBDA than of CBD seem to be puzzling as their molecules also contain an unsaturated aliphatic ring. However, CBDA and CBD are less stiff than the Δ9-THC and Δ9-THCA ones, and the unsaturated aliphatic ring contained in them may rotate. The rotation of the ring in these molecules may hinder an attack of the peroxyl radical on the double bond, which is manifested by their lowered antioxidant activity.

The antioxidant activities of cannabinoids estimated by β-carotene assay are worth to consider taking also into account the methodology of gaining results. The peroxyl radicals in the measuring system of the β-carotene method are scavenged in two competitive reactions - carotene oxidation and oxidation of the examined antioxidant. The first process involves only the adduct formation mechanism, whereas the second reaction, in all the examined cannabinoids except CBN and Δ9-THCA, can involve adduct formation as well as the PCET mechanism, or both mechanisms simultaneously. For CBN, only the PCET mechanism seems to be most probable, whereas for Δ9-THCA – the only adduct formation mechanism. It cannot be ruled out that the competitiveness of the two reactions (i.e. carotene oxidation and oxidation of the investigated antioxidant) is different:

- if they follow the same mechanism,
- if they follow different mechanisms, or
- one of them involves a mixed mechanism with a different degree of dominance –depending on the type of cannabinoid.

3.5. Antioxidant activity assessment by the ORAC method

Fig. S3 shows the antioxidant activity of the neutral and acidic forms of the examined cannabinoids calculated in relation to the antioxidant activity of Trolox (Fig. S3A and B) and of OL (Fig. S3C and D), based on the data obtained by the ORAC method. The antioxidative relation between the acidic and non-acidic forms of the examined cannabinoids are presented in Fig. S3E. As results from the diagram in Fig. S3A, the antioxidant activity of all the neutral examined cannabinoids revealed by ORAC is greater than that of Trolox. Apart from CBN, mutual relations between antioxidant activities of other neutral cannabinoids are the same as those established by the ABTS and beta-carotene bleaching methods – i.e. the decrease of the activity in the order CBG, CBD and Δ9-THC ($F_{crit} = 4.10 < F_{exp} = 12.71$). In the ORAC measurement system, the peroxyl radicals originating from AAPH disintegration are scavenged by two competing system components: the tested antioxidant and fluorescein. The activity of the tested antioxidant is determined on the basis of the disappearance of fluorescein by monitoring its concentration by fluorescence ($E_{Ex} = 485$ nm, $E_{Em} = 520$ nm). Although the precise reaction mechanism of the radical with fluorescein is not known, it is assumed that the radical is scavenged by fluorescein, which oxidizes its OH group according to the HAT mechanism and double bounds according to the adduct formation mechanism. The same mechanisms can be involved in the radical scavenge process by the examined cannabinoids. Hence, the observed decrease of the antioxidant activity in the order CBG, CBD and Δ9-THC is obvious and results from the decrease of the number of OH groups and double bond in individual molecules. In this context, the extremely high antioxidant power of CBN looks strange. Yet it should be remembered that fluorescein is easily able to form π-complexes with components containing aromatic rings. The formation of π-complexes between fluorescein and the tested antioxidant in the ORAC measuring system leads to experimental results indicating a higher radical scavenging activity of the examined antioxidant than it really is [10]. It is highly probable in the case of CBN, as its molecule is better predestined to form π-complexes with fluorescein than other examined neutral cannabinoids. The likelihood of the CBN-fluorescein complex formation is partly confirmed by the data from Fig. S3C showing that the non-olivetolic part in the examined neutral cannabinoids has a positive influence on their antioxidant activity only in the case of CBN.

Analyzing the results from Fig. S3, attention was paid to the extremely high antioxidant activity of the acidic forms of the examined cannabinoids (see Fig. S3B). Their high antioxidant activity is not surprising, given that the peroxyl radicals formed from the decay of AAPH have basic character. The presented diagrams prove that acid-base interactions between antioxidant molecules and radicals stimulate the scavenging process of the latter. The diagrams in Fig. S3E prove that the importance of acid-base interactions in the radical scavenging process for individual acids is similar ($F_{crit} = 4.10 > F_{exp} = 1.20$).

4. Conclusions

The growing popularity of supplements containing cannabinoids, mainly cannabinoid oils (e.g. CBD oil and CBG oil), in the self-medication of humans and the increased interest in these compounds in different preclinical and clinical trials stimulates researcher to investigating of the bioactive properties of individual cannabinoids, including their antioxidant activities. The presented data prove that all the examined cannabinoids - CBG, CBD, Δ9-THC, CBN, CBGA CBDA and Δ9-THCA - exhibit antioxidant activity manifesting itself in their ability to scavenge free radicals, to protect oxidation process and to reduce metal ions. Although, the intensity of these activities for individual cannabinoids is not the same, it is generally comparable to that of E vitamin. It should be noticed, however, that the magnitude of the deviation from this approximate and simplified observation depends on the method applied in estimating the antioxidant properties of cannabinoids. Careful consideration of the obtained results leads to the following conclusions:

1. Two types of electron sources (i.e. antioxidant centers) transferring electrons to the reduced radical/metal ion can be distinguished in CBG, CBD, Δ9-THC, CBN, CBGA CBDA, and Δ9-THCA: phenolic groups and double bonds. Their significance depends on the type of electron-accepting species.
2. The antioxidant activity of the examined neutral cannabinoids (CBG, CBD, Δ9-THC and CBN) is higher than that of Trolox when radicals/metal ions are reduced by electron transfer from phenolic groups following the SET mechanism. It is evident from the results obtained by ABTS, CUPRAC and FRAP and slightly less so by DPPH.
3. The antioxidant activity of neutral cannabinoids is lower than that of Trolox when the adduct formation mechanism dominates in the radical scavenging process (see the results from beta-carotene assays).
4. Although the hydrogen bond between the phenolic —OH group and the carboxyl group in cannabinoid acids may account for their lower antioxidant activity, it is not necessarily the case. At the presence of basic radicals (see ORAC) or in an environment with pH greater than the pK_a of cannabinoid acids (see CUPRAC), their antioxidant activity is equal to or even greater than that of their neutral counterparts.
5. The analysis of the obtained results shows that in the case of cannabinoids with two hydroxyl groups, only one of them exhibits antioxidant activity when the SET and/or HAT mechanism dominates in the electron transfer process.
6. The importance of the cannabinoids' double bond in the radical scavenging process– is revealed in the adduct formation mechanism (see beta-carotene, DPPH and ORAC results).

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Declaration of Competing Interest

There are no conflicts of interest to declare.

References

[1] H. Pen, F. Shahidi, Cannabis and Cannabis edibles: a review, J. Agric. Food Chem. 69 (2021) 1751–1774, https://doi.org/10.1021/acs.jafc.0c07472.

[2] A. Tubaro, A. Giangaspero, S. Sosa, R. Negri, G. Grassi, S. Casano, R. Della Loggia, G. Appendino, Comparative topical anti-inflammatory activity of cannabinoids and cannabivarins, Fitoterapia 81 (2010) 816–819, https://doi.org/10.1016/j.fitote.2010.04.009.

[3] S. Atalay, I. Jarocka-Karpowicz, E. Skrzydlewska, Antioxidative and anti-inflammatory properties of cannabidiol, Antioxidants 9 (1) (2020) 1–20, https://doi.org/10.3390/antiox9010021.

[4] L. De Petrocellis, A. Ligresti, A.S. Moriello, M. Allarà, T. Bisogno, S. Petrosino, V. Di Marzo, Effects of cannabinoids and cannabinoid-enriched Cannabis extracts on TRP channels and endocannabinoid metabolic enzymes, Br. J. Pharmacol. 163 (7) (2011) 1479–1494, https://doi.org/10.1111/j.1476-5381.2010.01166.x.

[5] A.C.M. Hacke, D. Lima, F. De Costa, K. Deshmukh, N. Li, A.M. Chow, J.A. Marques, J.P. Pererira, K. Kerman, Probing the antioxidant activity of Δ9-tetrahydrocannabinol and cannabidiol in *Cannabis sativa* extracts, Analyst 144 (16) (2019) 4952–4961, https://doi.org/10.1039/c9an00890j.

[6] M. Olszowy, A.L. Dawidowicz, Essential oils as antioxidants: their evaluation by DPPH, ABTS, FRAP, CUPRAC, and β-carotene bleaching methods, Monatsh. Chem. 147 (12) (2016) 2083–2091, https://doi.org/10.1007/s00706-016-1837-0.

[7] A.L. Dawidowicz, D. Wianowska, M. Olszowy, On practical problems in estimation of antioxidant activity of compounds by DPPH method (problems in estimation of antioxidant activity), Food Chem. 131 (3) (2012) 1037–1043, https://doi.org/10.1016/j.foodchem.2011.09.067.

[8] N. Nenadis, L.F. Wang, M. Tsimidou, H.Y. Zhang, Estimation of scavenging activity of phenolic compounds using the ABTS + assay, J. Agric. Food Chem. 52 (15) (2004) 4669–4674, https://doi.org/10.1021/jf0400056.

[9] K.B. Pandey, S.I. Rizvi, Ferric reducing and radical scavenging activities of selected important polyphenols present in foods, Int. J. Food Prop. 15 (3) (2012) 702–708, https://doi.org/10.1080/10942912.2010.498547.

[10] D. Huang, O.U. Boxin, R.L. Prior, The chemistry behind antioxidant capacity assays, J. Agric. Food Chem. 53 (6) (2005) 1841–1856, https://doi.org/10.1021/jf030723c.

[11] M. Olszowy, A.L. Dawidowicz, Is it possible to use the DPPH and ABTS methods for reliable estimation of antioxidant power of colored compounds? Chem. Zvesti. 72 (2) (2018) 393–400, https://doi.org/10.1007/s11696-017-0288-3.

[12] D. Caprioglio, D. Mattoteia, F. Pollastro, R. Negri, A. Lopatriello, G. Chianese, A. Minassi, J.A. Collado, E. Munoz, O. Taglialatela-Scafati, G. Appendino, The oxidation of phytocannabinoids to cannabinoquinoids, J. Nat. Prod. 83 (2020) 1711–1715, https://doi.org/10.1021/acs.jnatprod.9b01284.

[13] J. McMurry, Organic Chemistry, 7th ed., Brooks/ColeThomson, Belmont, 2007.

[14] M. Antolovich, P.D. Prenzler, E. Patsalides, S. McDonald, K. Robards, Methods for testing antioxidant activity, Analyst 127 (1) (2002) 183–198, https://doi.org/10.1039/b009171p.

[15] A.L. Dawidowicz, M. Olszowy, The importance of solvent type in estimating antioxidant properties of phenolic compounds by ABTS assay, Eur. Food Res. Technol. 236 (6) (2013) 1099–1105, https://doi.org/10.1007/s00217-013-1982-1.

[16] A.M. Osman, Multiple pathways of the reaction of 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) with (+)-catechin: evidence for the formation of a covalent adduct between DPPH and the oxidized form of the polyphenol, Biochem. Biophys. Res. Commun. 412 (3) (2011) 473–478, https://doi.org/10.1016/j.bbrc.2011.07.123.

[17] K.A. Wojtunik, L.M. Ciesla, M. Waksmundzka-Hajnos, Model studies on the antioxidant activity of common terpenoid constituents of essential oils by means of the 2,2-Diphenyl-1-picrylhydrazyl method, J. Agric. Food Chem. 62 (37) (2014) 9088–9094, https://doi.org/10.1021/jf502857s.

[18] V. Bernal, L. Giraldo, J.C. Moreno-Piraján, Thermodynamic study of the interactions of salicylic acid and granular activated carbon in solution at different pHs, Adsorpt. Sci. Technol. 36 (3–4) (2018) 833–850, https://doi.org/10.1177/0263617417730463.

[19] A.L. Dawidowicz, M. Olszowy, Depletion/protection of β-carotene in estimating antioxidant activity by β-carotene bleaching assay, J. Food Sci. Technol. 52 (11) (2015) 7321–7328, https://doi.org/10.1007/s13197-015-1820-5.

[20] A.L. Dawidowicz, M. Olszowy, Influence of some experimental variables and matrix components in the determination of antioxidant properties by β-carotene bleaching assay: experiments with BHT used as standard antioxidant, Eur. Food Res. Technol. 231 (6) (2010) 835–840, https://doi.org/10.1007/s00217-010-1333-4.

[21] M.F.M.G. Resul, A.M. López Fernández, A. Rehmana, A.P. Harvey, Development of a selective, solvent-free epoxidation of limonene using hydrogen peroxide and a tungsten-based catalyst, React. Chem. Eng. 3 (5) (2018) 747–756, https://doi.org/10.1039/C8RE00094H.