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ORIGINAL PAPER

Antioxidant Activities of Some New Chromonyl-2,4-Thiazolidinediones and Chromonyl-2,4-Imidazolidinediones Having Chromone Cores

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Abstract The antioxidant properties of 11 new synthesized chromonyl-2,4-thiazolidinediones and chromonyl-2,4-imidazolidinediones (CBs) were investigated. The antioxidant activities and mechanisms of the CBs interaction with reactive oxygen species (ROS) were clarified using various in vitro antioxidant assay methods including superoxide anion radical ($O_2^{\bullet-}$), hydroxyl radical ($HO^{\bullet}$), 1,1-diphenyl-2-picryl-hydrazyl free radical ($DPPH^{\bullet}$) scavenging activity and the iron (II)-ferrozine complex formation. The potassium superoxide/18-crown-6 ether dissolved in dimethylsulfoxide (DMSO) was applied as a source of superoxide anion radical. Hydroxyl radicals were produced in the Fenton-like reaction Fe(II)+H_2O_2. Chemiluminescence, spectrophotometry, and electron paramagnetic resonance (EPR) spectroscopy using 5,5-dimethyl-1-pyrroline-1-oxide (DMPO) as spin trap were applied as the measurement techniques. The CBs examined that exhibited good free radical scavenging activity also showed strong total antioxidant power capacity. Possible mechanisms of antioxidation are proposed to explain the differences in the experimental results between the chromone derivatives with imidazolidine-2,4-dione ring and those with thiazolidine-2,4-dione ring. In conclusion, some of the new CBs are promising to be applied as inhibitors of free radicals.

Keywords Chromonyl-2,4-thiazolidinediones · Chromonyl-2,4-imidazolidinediones · Radical scavenging activity · Chemiluminescence · Electron paramagnetic resonance

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Introduction

Chromone (2,3-benz-γ-pyrone) forms the basis skeleton of many biologically important naturally occurring compounds, for example, the group of vitamins E.

An important group widely distributed in plants are derivatives of 2-phenylchromone, termed flavone. There is overwhelming evidence to indicate that hydroxy derivatives of flavone have been implicated in the protection of the human body from oxidative stress. The compounds may neutralize oxygen-derived reactive species (ROS), which include radical species such as superoxide anion radical ($O_2^{\bullet-}$), hydroxyl radical ($HO^{\bullet}$), peroxy radical ($RO_2^{\bullet}$), alkoxy radical ($RO^{\bullet}$), and nonradical species, such H_2O_2 or the oxygen molecule in the electronically excited state, called singlet oxygen ($^1O_2$). All these species have been implicated in the etiology of degenerative diseases including atherosclerosis and Alzheimer's diseases, diabetes, cancer, abnormal, aging, under oxidative stress conditions (prooxidant/antioxidant imbalance) [1–3]. This is due to the fact that ROS easily oxidize proteins, carbohydrates, lipids, and DNA and are considered as both

initiating and promoting factors of tumors. Antioxidants exhibit antioxidant activity by donation of hydrogen atoms or the single-electron transfer to a radical [4]. Due to the importance of polyphenolic antioxidants bearing chromone skeleton, it is worth noting that they are redox-active compounds and they can either act as antioxidants or as prooxidants [4].

Because of the importance of the chromone-based compounds, such as anti-inflammatory [5], antimicrobial [6], anticancer activities [7, 8], there identification in plant tissues and synthesis of derivatives play an important role in many scientific field.

In this study, a series of new chromone derivatives with a C-3 imidazolidine-2,4-dione or thiazolidine-2,4-dione substitutions connected with phenyl ring bearing various electrophilic substitutions were evaluated for their free radical and antioxidant activities. The chemiluminescent method (CL), DPPH free radical scavenging assay, total antioxidant activity test, and electron spin resonance (EPR) and spin-trapping method were applied.

Materials and Methods

Chemistry

Chromonyl-2,4-thiazolidinediones (CB1-CB6) and chromonyl-2,4-imidazolidinediones (CB7–CB11) (Fig. 1) were prepared via Knoevenagel reaction between the chromone-3-carboxaldehyde and appropriate substituted-2,4-thiazolidinediones or substituted benzyl-2,4-acetic acid glacial as described in our previous paper [9]. The structure of these new synthesized chromone derivatives was elucidated by elementary analysis, ^{1}H NMR and mass spectral data. All reagents for the compound preparation were

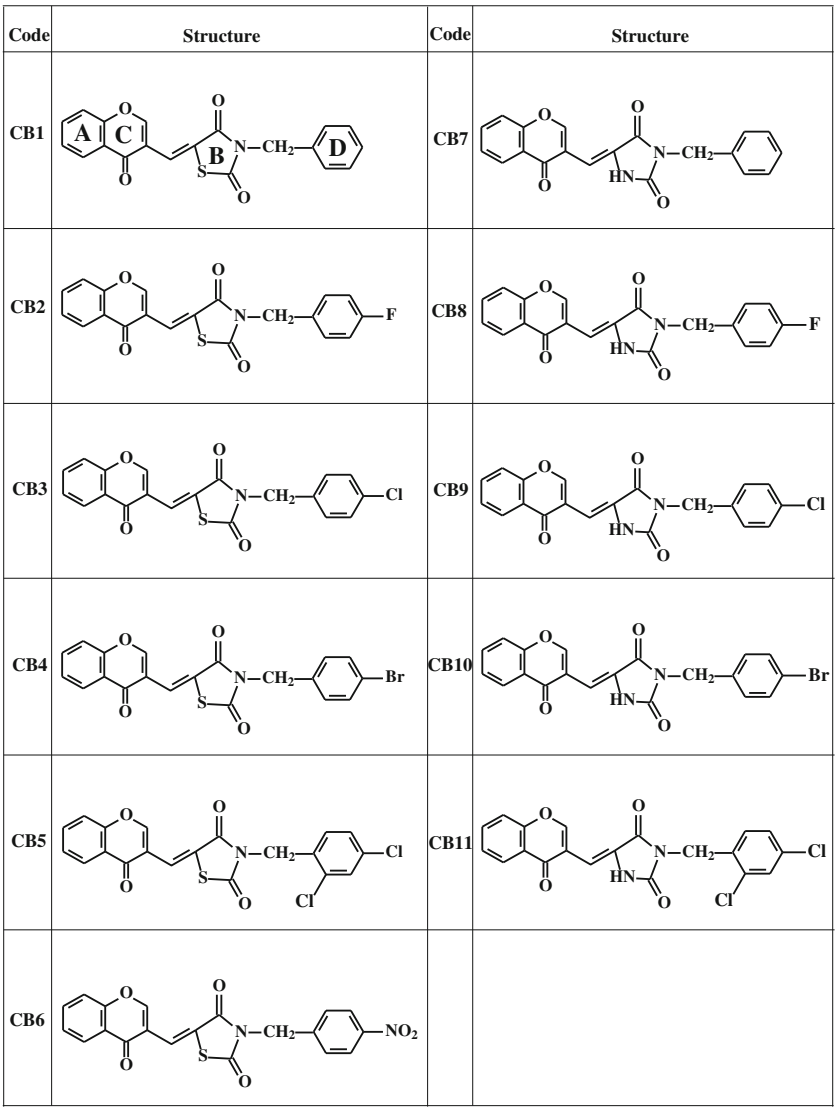

Fig. 1 Chemical structures of benzyl substituted-2,4-thiazolidinediones-2,4-imidazolidinediones having chromone core (CBs)

purchased from E. Merck (Darmstadt, Germany) and Aldrich (Milwaukee, MI, USA).

Antioxidant Activity Studies

5,5-Dimethyl-1-pyrroline-1-oxide (DMPO), (1,4,7,10,13,16)-hexaoxacyclooctadecane (18-crown-6), tiron (4,5-dihydroxy-1,3-benzene-disulfonic acid), $NH_4Fe(SO_4)_2 \cdot 12\ H_2O$ (molecular weight=482.2), $FeCl_3\ 6\ H_2O$, CH_3COOH, CH_3COONa, were from E. Merck. Dimethylsulfoxide (DMSO) and trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were purchased from Aldrich (Milwaukee MI, USA). The stable radical DPPH (2,2′-diphenyl-1-picrylhydrazyl) and potassium superoxide KO_2 were from Fluka (Buchs, Switzerland). Other reagents were from POCH (Gliwice, Poland). All reagents used in the study were of analytical grade and were used without further purification. They were prepared fresh daily before use and stored in darkness.

Superoxide Anion Radical Scavenging Activity

Chemiluminescence Method

Chemiluminescence measurements (CL) of the effect of CBs on superoxide anion radical were carried out using an EMI 9553Q photomultiplier with a S20 cathode sensitive in the range 200–800 nm interfaced with a personal computer (detailed data may be found in Ref. [10]). The procedure of Valentine et al. [11] was used as a source of superoxide anion radicals. Briefly, 60 mg 18-crown-6 was dissolved in 10 mL dry DMSO and then 7 mg KO_2 was added quickly. This solution was stirred for 1 h to give a pale yellow solution of 10 mmol/L superoxide anion radical. The effect of tested CBs was studied using both relative CL intensity (I) and high sum ($\sum$I) detected over 1 min $\left(\sum I = \int_0^1 I(t)dt\right)$. The reaction mixture contained 1 mmol/L superoxide anion radical in DMSO in the absence (blank) and presence of the CB compounds (2 mmol/L). Temperature 295 K. The radical scavenging activities was calculated by the following equations: $Q_1=[(I_0-I)/I_0]\times 100\%$ and $Q_2=[(\sum I_0-\sum I)/\sum I_0]\times 100\%$ where I_0 and I are the relative light intensity measured in the absence of a CB compound and I is that measured in the presence of the CB compound. $\sum I_0$ and $\sum I$ represent the CL sums in the absence of a CB compound and in its presence, respectively. The light sum from 1 mmol/L superoxide anion radical after an addition of 0.5 mL of DMSO was considered as a control. Tiron (1 mmol/L) was used as the standard control in the CL measurements.

EPS Spectroscopy and Spin Trapping

Superoxide anion radicals were generated using the procedure given by Valentine et al. as described above. Electron paramagnetic resonance spectroscopy (EPR) with a nitroso spin trap DMPO [12] was used for monitoring the ability of CBs to scavenge the superoxide anion radical. In this method superoxide anion radical reacts with DMPO to yield DMPO–OOH in aprotic solvents of which the half-time reach 91 s at pH5, and the stability decreases with increasing pH. In the presence of SOD or other antioxidants inhibition in the intensity of the EPR signal is easily observed. The EPR spectrum results from the reaction of an unpaired electron with the primary nitrogen atom and with the secondary β- and γ-protons [12]. In the presence of a superoxide anion radical inhibitor the EPR signal intensity decreases. The reaction mixture contained 0.1 mol/L DMPO, 1 mmol/L superoxide anion radical, 0.5 mmol/L a CB compound dissolved in ethanol. (80 % v/v DMSO/20 % v/v C_2H_5OH). The control reaction contains only DMPO and superoxide radical and ethanol (25 % v/v). The spectra were recorded using a quartz cuvette with an optical path length of 0.25 mm at room temperature. The spectra were analyzed after 1 min from the start of the reaction. The percentage of inhibition of superoxide anion radical was calculated by using the following equation: $Q=[(H_0-H)/H_0]\times 100\%$, where H_0 is the relative height of the second peak in the spectrum of the control and H is the intensity in the presence of a CB compound. The conditions of EPR measurement were as follows: microwave power, 20 mW; modulation amplitude, 0.5 mT; time constant 0.5 s, and receiver gain 4×10^4.

Hydroxyl Radical Scavenging Activity

The reactivity of CBs towards hydroxyl radical was evaluated by the use of a nitroso spin trap DMPO, which forms a stable free radical during reaction with unstable hydroxyl radical, and the EPR method [12].

Hydroxyl radical was generated by the Fenton reaction $Fe(II)+H_2O_2 \rightarrow HO^{\bullet}+HO^{-}+Fe(III)$ [13, 14]. For the generation of hydroxyl radical, 100 μL of 0.1 mol/L DMPO (aqueous solution), 100 μL of 2 mmol/L H_2O_2, 50 μL of 80 mmol/L sodium trifluoroacetate buffer (pH 6.15), and 100 μL DMSO were mixed. The reaction was started by adding 50 μL of 0.5 mmol/L ammonium ferrous sulfate. EPR spectra were recorded in quartz flat cell with an optical path length of 0.25 mm at room temperature. The spectra were recorded after 1 min from the beginning of the reaction and analyzed after 3 min. The reaction mixture consisted of 0.125 mmol/L DMPO, 0.5 mmol/L H_2O_2, 10 mmol/L sodium trifluoroacetate, pH 6.15 and 62.5 μmol/L $FeSO_4(NH_4)SO_4$ without (control) or with 2.5 mmol/L of CB dissolved in 0.1 mL of DMSO. EPR parameters were microwave power 20 mW, modulation amplitude 0.5 mT, time constant 0.1 s, receiver gain 4×10^4. The inhibition ratio (R) was defined as $R=[(H_0-H)/H_0]\times 100\%$, where H_0 is the relative height of the second peak in the spectrum of the spin adduct of the control reaction containing

all reagents except the examined compound and H is the relative height of the second peak in the spectrum in the presence of the tested compound. Trolox was used as a control standard of $HO^{\bullet}$ inhibition.

DPPH$^{\bullet}$ Method

The DPPH radical inhibiting capacity of the chromone derivatives was evaluated using the method of Nanjo et al. [15], except for the use of the solvent mixture, DMSO (25 % v/v C_2H_5OH) (75 % v/v) instead of ethanol. The DPPH free radical is very stable compound which exhibits a typical EPR spectrum. The spectrum decreases significantly on exposure to proton radical scavengers, i.e. compounds acting as hydrogen atoms or electron donors. Inhibition of DPPH radical was calculated using the equation $R=[(H_0-H)/H_0]\times 100\%$, where H is the relative height of the third peak in the EPR spectrum in the presence of the tested probe, H_0 is the relative hight of the third peak in the spectrum of the control. The percentage of antiradical activity R(%) was plotted against the CB concentration in order to obtain the concentration of the CB sample required to cause 50 % scavenging of DPPH$^{\bullet}$ (IC_{50}). The reaction contained 0.125 mmol/L of DPPH$^{\bullet}$, 2.5 mmol/L of CBs and 1.25 mmol/L of trolox. The control contains only DPPH$^{\bullet}$. EPR setting were: microwave power 20 mW, modulation amplitude 0.2 mT, time constant 0.2 s, receiver gain 3.2×10^4. Temperature 293 K.

All EPR spectra were recorded on a standard X-band spectrometer operating at 9.3 GHz with a 100 kHz modulation frequency of the steady magnetic field.

Total Antioxidant Activity

The ferric-ferrozine method called TAC (total antioxidant capacity) was used to measure the antioxidant properties of the examined CBs, according to the procedure given by Berker et al. [16]. The method is based on an electron transfer from an antioxidant to ferric ion of the iron (III)-ferrozine complex, which is reduced to Fe(II) showing an absorbance at 562 nm. The ferric-ferrozine complex containing 2 mmol/L Fe(III) and 10 mmol/L ferrozine was prepared in aqueous solution as follows: 0.024 g of $NH_4Fe(SO_4)_2\cdot 12H_2O$ was dissolved in 1 mL of 1 mol/L HCl and then mixed with a separate aqueous solution of 0.123 g ferrozine. The obtained mixture was diluted to 25 mL with distilled water. 0.1 mL of a CB compound (DMSO solution) was mixed with 0.4 mL of C_2H_5OH (96 %) and further mixed with 1.5 mL of ferric-ferrozine solution, 2 mL of pH 5.5 buffer (0.2 M/L CH_3COOH/CH_3COONa) and lastly 0.5 mL of water was added to obtain the final volume 4.5 mL. The mixture was let to stand at room temperature. After a 30 min standing period the absorbance was measured at 562 nm. The CB 3 compound concentration was 10 times lower than those of the remaining CB compounds 22 μg/L and 220 μg/L, respectively. Trolox (22 μg/L) was used as a control standard.

All tests were performed at least in triplicate and average values were taken. Experimental results are presented as means ± SD.

Results and Discussion

Eleven chromone derivatives (CBs), previously synthesized, were explored for their antioxidant activity, i.e. the ability to deactivating or stabilizing free radicals. Two methods were used to evaluate reactivity of CBs compounds with superoxide anion radical. Figure 2 (Parts a and b) summarizes results the scavenging activity of CB derivatives obtained from CL measurements. The CL detection for the evaluation of antioxidative activities against superoxide anion radical in hydrophobic medium had been used for first time in our laboratory and successfully applied to evaluate several antioxidants [17, 18]. The principle of this method is that there is a notable CL response upon addition of antioxidant to the superoxide anion radical/DMSO system. We have found previously, that a pale yellow solution of superoxide anion radical in DMSO emits a ultra-weak CL (blank, the inset of Fig. 2) and that the observed CL originates from singlet oxygen [10]. This electronically excited form of molecular oxygen may be generated in DMSO during oxidation of superoxide anion radical [19, 20]:

$$2\ O_2^{\bullet-} + 2(CH_3)_2SO \rightarrow {}^1O_2 + (CH_3)_2SO_2 + CH_3SO(CH_2)^- + OH^- \quad (1)$$

$$O_2^{\bullet-} \rightarrow {}^1O_2 + \text{electron} \quad (2)$$

The CBs tested, excepting compounds CB5 and CB6, showed a notable CL response against superoxide anion radical, increasing or decreasing of light emission. Typical CL responses are shown in Fig. 2(a), curves 2 and 3, respectively. Compounds B1–B4 and B7 demonstrated the direct significant superoxide anion radical scavenging, causing reduction of both the light intensity and the CL sum. In turn, compounds CB8–CB10 exhibited increase in the CL intensity and its sum, resulting from the transformation of the radical into another oxygen species. The reference compound tiron-that reacts with superoxide anion radical with high rate constant (~10^8 L mol^{-1} s^{-1} [2]), exhibited 49 % quenching at concentration of 1 mmol/L. This indicate for high antioxidant capacity at least several tested CBs.

The superoxide radical is a free radical with negative charge showing a multiplicity of chemical reactions [21, 22]. The species can promote proton transfer from much weaker acids than water, thus to act as an oxidant. The species is able

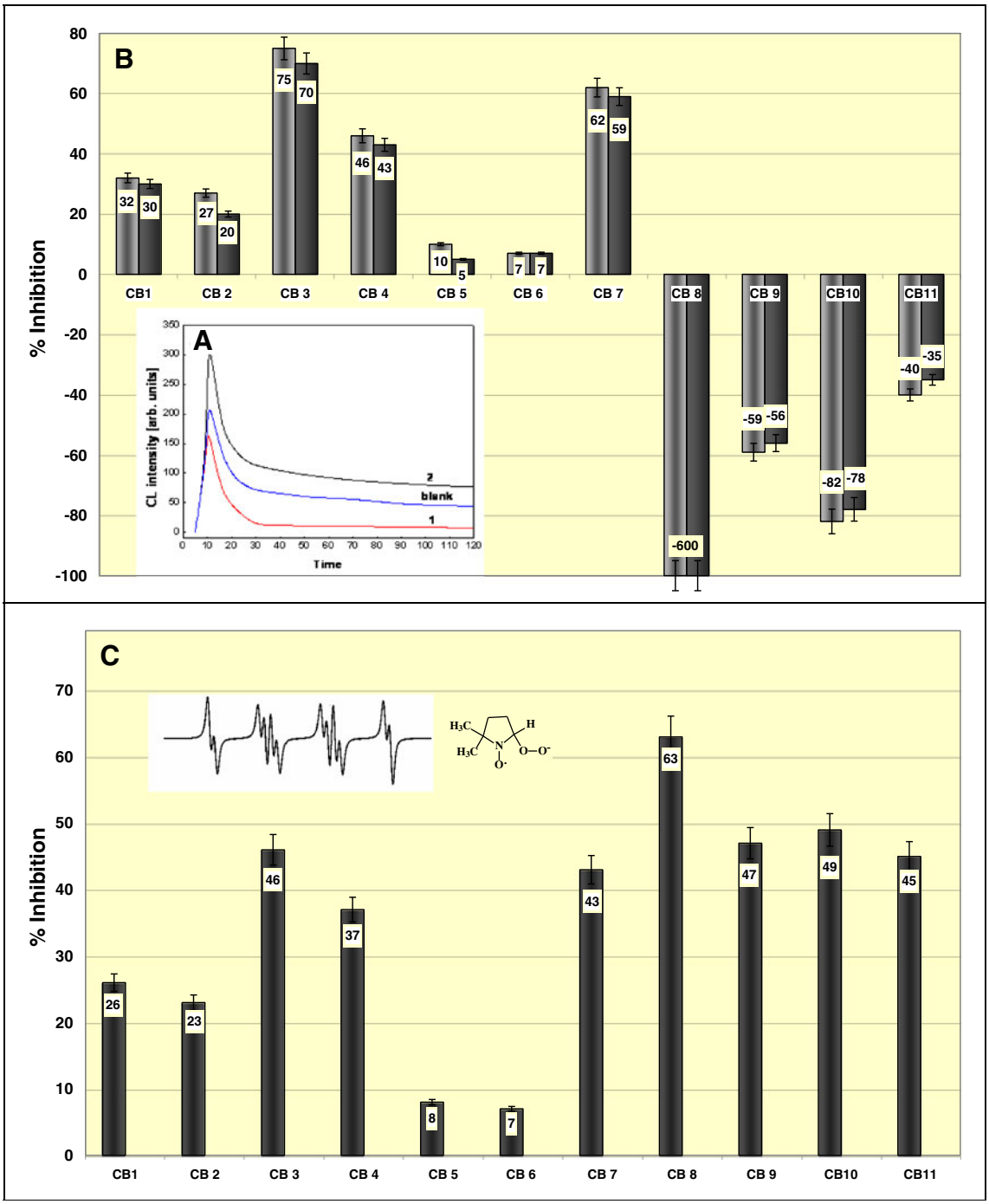

Fig. 2 Superoxide anion radical scavenging activity of the CBs compounds. Part (**a**) The kinetic curves of CL decay in the superoxide anion radical/DMSO system (blank); curve 1- the CL recorded under the same conditions as the blank but in the presence of compound CB3; curve 2- in the presence of compound CB9. Part (**b**) The inhibitory effect of the tested CBs compounds on the CL intensity and sum. *Clear bar* deals with the CL intensity, and *dark bar* the CL sum. Part (**c**) The inhibitory effect of CBs compounds exerted on the spin adduct of superoxide anion radical during the reaction with DMPO

to act as a one-electron reducing agent in aprotic solvents and shows also a high nucleophility towards the typical S_N2 substitution reaction. The nucleophilicity of superoxide anion radical and its reducing activity have been strongly supported [23]. It should be noted that deprotonation of organic compounds by superoxide anion radical is of a high efficiency when the compounds are in high concentration or are solvents. According to the main mechanisms through which compounds may express their antioxidant activity by a radical ($R^{\bullet}$) scavenging [4] and to the chemistry of superoxide anion

radical [2]:

$$CB + R^{\bullet} \rightarrow CB^{\bullet +} + R^{-} \quad \text{single electron transfer} \tag{3}$$

$$CB + R^{\bullet} \rightarrow CB^{\bullet} + RH, \quad \text{H–atom transfer} \tag{4}$$

the CBs containing thiazolidine-2,4-dione ring (CB1-CB6) can act directly as redactors for solution conditions, thereby quench the light emission:

$$CB + O_2^{\bullet -} \rightarrow CB^{\bullet +} + O_2^{-2} \tag{5}$$

Reaction 5 is followed by the stabilization of the O_2^{-2} species by DMSO which serves as a proton source [24]:

$$O_2^{-2} + 2H^{+} \rightarrow H_2O_2 \tag{6}$$

The second group of the chromone tested, those containing imidazolidine-2,4-dione ring (CB7–CB11), can donate a hydrogen atom from the N–H group to superoxide anion radical thus quench the CL directly (compound CB7)

$$CB(H) + O_2^{\bullet -} \rightarrow HO_2^{-} + CB^{\bullet} \tag{7}$$

and/or donate proton:

$$CB(H) + O_2^{\bullet -} \rightarrow CB^{-} + HO_2^{\bullet} \tag{8}$$

increasing the light emission (CB8–CB11) as follows [25]

$$2\ HO_2^{\bullet} \rightarrow {}^1O_2 + H_2O_2 \tag{9}$$

In this case the CBs behaviour may be compared to the SOD antioxidant activity.

Compound CB8 was the most effective of the group, reaching about 600 % light emission enhancing effect at the highest tested concentration. Compound CB10 reached about 80 % effect. From this group of CBs tested, compounds containing nitrobenzene or dichlorobenzene ring were weekly effective in this respect.

The observed differences in the light emission enhancing potency may be due to benzene ring carrying different substituents. The electrophilic substituents, such as halogens (Cl, F, Br) and NO_2 pull a pair electrons from the aromatic ring, and this effect is associated with the positive charge of the ring D. In this case, the dipole moments of the substituted benzene derivatives are different, and the substituent bearing carbon atom in the ring D is less positive, so less sensitive to nucleophilic attack. In addition, the substituent interacts not only with the benzene ring but also with one another through the benzene ring, and thus changes the stabilization of the products arising from CB derivatives and their antioxidant potential.

This finding seems to be in agreement with the results of Phosrithong et al. who reported that the chromone skeleton of the antioxidant molecule is not responsible for the scavenging capacity. The chromone nucleus plays an important role as a stabilizator of the antioxidant during hydrogen or/and electron transfer to a free radical [26].

The second assay applied to monitor the CBs reactivity towards superoxide radical was based on the ability of the compounds to inhibit the DMPO radical adduct formation. The spin trapping involves the covalent reaction of superoxide radical with DMPO. A typical EPR spectrum of the DMPO–OOH is shown inside of Fig. 2(c). The spectrum formed by reaction of superoxide radical with the trap was completely distinct from the DMPO–OH spectrum. Similarly to other [12], we observed hyperfine couplings a_N=14.2 G, a_H=11.5 G and a_H^{γ}=1.2 G. All tested CBs compounds inhibited the intensity of the signal ranging from about 7 % to 63 %. The order of inhibiting power for CBs evaluated with this method is in good accordance with that using the CL technique. We also observed that the production of DMPO–OOH was prevented by tiron (1 mmol/L, 55 % quenching). These finding show that the both method are sensitive and convenient to examine the superoxide anion scavenging activity of CBs compounds.

The EPR method in conjunction with the spin trapping technique was also used to determine the CBs scavenging potency towards hydroxyl radicals of the species having an extremely short half-life. The radicals were produced using the Fenton reaction in the presence of a spin trap DMPO. It has been calculated that the rate constant for reaction of hydroxyl radical with DMPO is very high (about $3.4\times10^9 Lmol^{-1}\ s^{-1}$) [27]. DMPO forms a stable long-lived free radical DMPO–OH by reacting with a short-lived hydroxyl radical seen after at least 91 s from the reagents mixing. Similarly to Finkelstein and co-workers [27], we have observed EPR spectrum of the DMPO–OH spin adduct having a high intensity and a characteristic 1:2:2:1 quartet (nitrogen, a_N, and β-proton, a_H, hyperfine coupling constants $a_N=a_H$=14.9 G). When the similar reaction was carried out in the presence of CBs, the EPR signals had lower intensity. The production of the DMPO–OH adduct was also strongly inhibited by 12.5 % ethanol (by 70 %). Hydroxyl radicals react with ethanol to form α-hydroxymethyl radicals, $C^{\bullet}H(CH_3)OH$, which can react further with DMPO forming a new DMPO–$CHCH_3OH$ spin adduct of different splitting constants than those observed for hydroxyl radical (data not shown). This latter observation was confirmed by the addition to the Fenton reaction of catalase (300 μg/mL) which decomposes hydrogen peroxide to H_2O. In the presence of catalase no EPR signal was observed at any time of incubation, indicating that the hydroxyl radical production was dependent on the presence of hydrogen peroxide. We found that the method was sensitive and convenient to examine the hydroxyl radical scavenging activity of CBs. The examined chromones showed 16.5–41.7 % inhibition of the EPR spectrum amplitude with chromones CB3 and CB8 which were the most active inhibitors (Fig. 3).

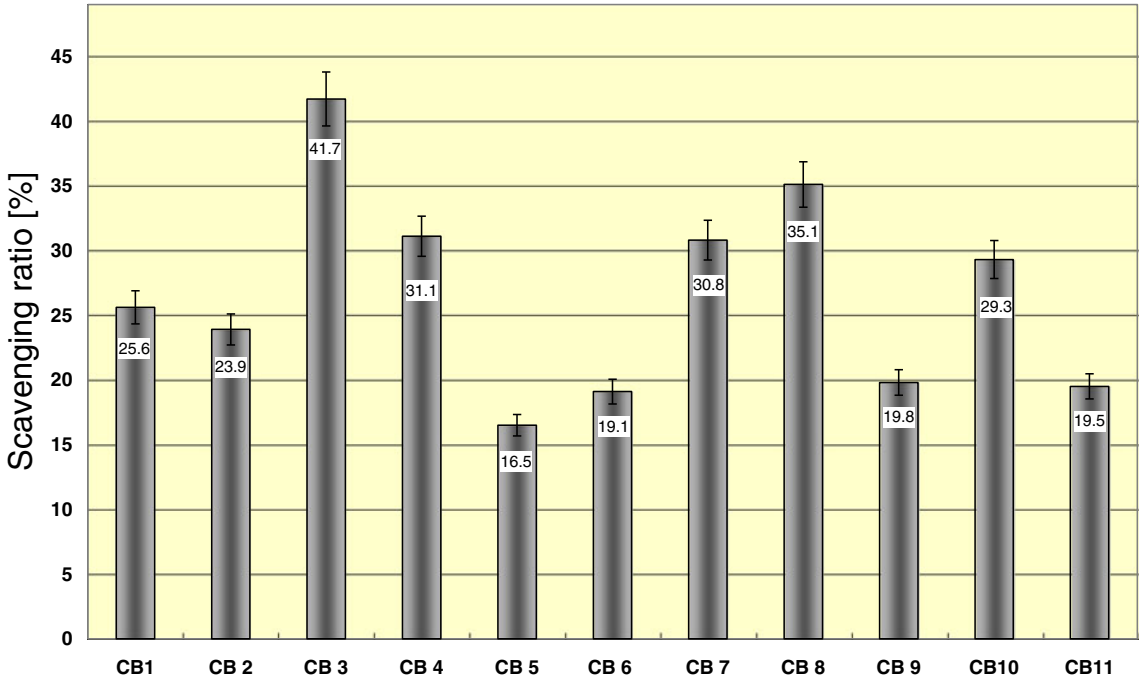

Fig. 3 The inhibitory effect of CBs compounds exerted on the DMPO–OH spin adduct production

Compounds CB4, C7 and CB10 exhibited about 30 % inhibition. The hydroxyl radical exhibits the high reactivity and low selectivity with a large variety of organic compounds (rate constants usually exceed 10^9 L mol^{-1} s^{-1} [1]). Three basic modes of this radical reaction are distinguished: (1) addition to a double bond, (2) hydrogen abstraction and (3) an electron transfer. The latter reaction is especially rapid with halogen anions. As shown in Fig. 3 compounds CB5, CB6, CB9 and CB11 have the lowest antioxidant capacity towards hydroxyl radical. It is not clear whether the D ring with two chlorine groups or with the nitroso group confounded the antioxidant effect by a lower stabilizing the resulting free radical form, however, such possibility exists through conjugation from the D ring to the B ring.

Free radical scavenging activity of the CBs compounds have been confirmed in DPPH assay. This method is based on the reduction nitrogen centered radicals such as DPPH$^{\bullet}$ in alcoholic solution in the presence of antioxidants. The stable DPPH radicals react with antioxidants via two different ways: (1) a direct abstraction of antioxidant H - atom due to the formation of the non-radical form, DPPH–H and (2) an electron transfer from antioxidant to the DPPH radical. The mechanism of DPPH reduction depends on polarity of solvents and/or the redox potentials of the compounds in the reaction [28]. In polar solvents such as ethanol an electron transfer mechanism is predominant, whereas in apolar solvents the H - atom transfer becomes more important [28]. Therefore, DPPH$^{\bullet}$ assay is one of the commonly used method for the evaluation of the radical scavenging ability of antioxidants. Figure 4 illustrates a decrease in the concentration of DPPH$^{\bullet}$ followed by the EPR signal decrease, due to the scavenging activity of CBs and the referent compound. Three types of the kinetic curves behaviour are observed. Only trolox, vitamin C (data not shown) and CB3 reacted rapidly with DPPH$^{\bullet}$ reaching a steady state during t<2 min. For compounds CB8, CB4 and CB7, the steady state was reached after approximately 3, 4 and 5 min, respectively. Compounds CB10, CB1, CB2 and CB9 reacted more slowly with the DPPH radical, the steady state was reached after about 6–8 min. The most slow kinetics were observed for compounds B11, B5 and B6. These kinetic curves were of the hyperbolic shape taking from 10 to 20 min to reach a steady state. The antiradical capacity of CBs

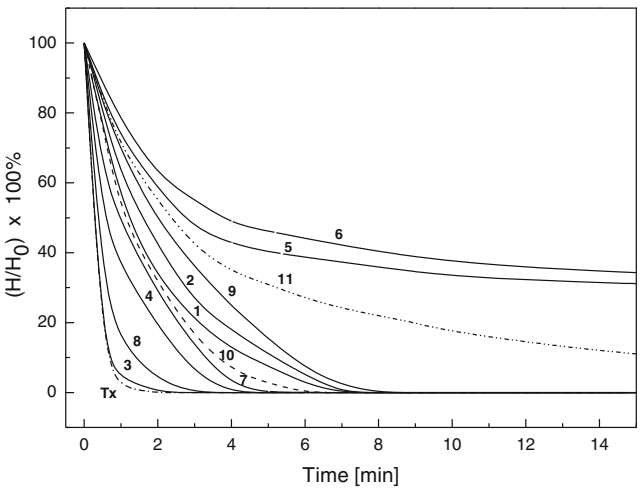

Fig. 4 DPPH$^{\bullet}$ scavenging activity of the chromone derivatives and trolox

was evaluated as the amount of a CB compound necessary to decrease the initial DPPH radical concentration by 50 % (Table 1) after 2.5 min of reaction time. In the case of those compounds-showing rapid kinetic behaviour (trolox, ascorbic acid, CB3) the difference in IC_{50} were at the limit of error. For compounds showing slower kinetics an IC_{50} determined at 2.5 min will be erroneous because the reaction was still continued.

Ascorbic acid and trolox were used as reference compounds for radical inhibition. From Table 1 we can see that all chromones exhibited less activity than trolox and their IC_{50} were also higher than that of vitamin C except for chromone CB3. A higher IC_{50} value means a lower $DPPH^{\bullet}$ scavenging ability. Thus, chromone CB3 was more potent radical scavenger than that known antioxidant vitamin C. IC_{50} values for the chromone and the reference compounds decreased in the order of CB10 > CB7 > CB4 > CB8 > vitamin C > CB3 > trolox. The remaining CBs compounds needed more time to react with $DPPH^{\bullet}$, and their IC_{50} were higher than 1 mmol/L. As can be seen in Table 1, IC_{50} values for CBs are extremely diversed, ranging from 194 μmol/L for CB3 to 2,841 μmol/L for CB6. In the case of trolox the IC_{50} value we measured is in accordance with some authors [26, 28]. As shown in Table 1 most of the CBs tested react slowly with $DPPH^{\bullet}$ and mechanisms their reaction are complex. It has been found that time needed to reach a steady state depends on the reactivity of an antioxidant and its concentration [28]. For this reason some authors account the TIC_{50} parameter, defined as "the time at equilibrium reached with a concentration of antioxidant equal to IC_{50}" [28].

We further investigated antioxidant property of CB compounds applying assay of total antioxidant capacity using ferrozine as reagent. In the ferric-ferrozine antioxidant assay, the tested CBs were able to reduce the Fe(III) ion, in the presence of ferrozine, to the Fe(II)-ferrozine complex showing a very high absorbance at 562 nm. A ferric-ferrozine method of antioxidant activity measurement, proposed by Berker and coworkers [16], provides high sensitivity because the ferri-ferrozine reagent is able of oxidizing compounds having also a weak reducing potential. A ferric ion reducing abilities of the tested compounds are shown in Fig. 5. The reducing powers of CBs and the standard compound—trolox were as follow: trolox > CB3 > CB8 > CB4 > CB7 > CB10 > CB1 > CB2 > CB9 > CB1 > CB6 = CB5. Taken together, our data show that the hierarchy of antioxidant power for CBs measured with this method is in good accordance with those using the CL, EPR and spin-trapping techniques.

Several researchers had already reported the importance of structure—activity association for the activity of flavones [5, 6, 8, 16, 28]. The CBs compounds tested have not the catechol moiety in their structures. The absence of hydroxyl groups differentiates the CBs antioxidant power from flavonoids known as the good H - atom donors and often strong antioxidants. However, the presence of the 2,3 – double bond in conjugation with the 4-oxo function of a carbonyl group in the C-ring in the CBs structure enables them to an electron transfer reaction as the main mechanism, e.g. in the case of the thiazolidine-2,4-dione substitution. They can act as electron donors and convert free radicals to more stable products and further terminate chain reactions.

Table 1 DPPH radical scavenging activity of eleven chromone derivatives and trolox

Compound	IC_{50}[a] (μmol/L)	Compound	IC_{50} (μmol/L)
CB1	1,143±21.7	CB7	859±13.6
CB2	1,310±25.6	CB8	364±3.3
CB3	194±3.5	CB9	1,843±192
CB4	610±9.2	CB10	969±15.4
CB5	2,397±39.8	CB11	2,036±35.8
CB6	2,841±40.2	Trolox	17.5±0.5
		Vitamin C	346±28

DPPH 2,2-diphenyl-1-picrylhydrazyl, *IC_{50}* mean inhibition concentration

[a] Each value is mean ± SD (n=3)

Conclusion

The scavenging abilities for reactive oxygen species of 11 chromone derivatives were examined for the first time in the present study. The CB3, CB8, CB4 and CB7 compounds were found to be the effective antioxidants in several in vitro methods, including: CL, EPR spectroscopy and EPR spin trapping, and spectrophotometry. From the results obtained it is evident that the majority of CB compounds (beside of CB5, CB6, CB11) are effective as superoxide anion radical, hydroxyl radical, $DPPH^{\bullet}$ scavengers and posses the high reducing power. Chromones CB3 and B8 were found to be

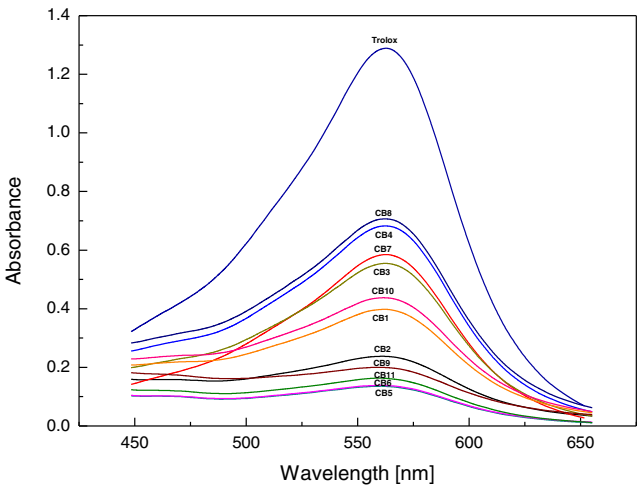

Fig. 5 Comparison of total antioxidant activity of the chromone derivatives and the standard antioxidant compound-trolox

the most active antioxidants. One question arises, whether these CB derivatives are better antioxidants than the standard antioxidants, such as ascorbic acid or trolox. We would suggest that compound B3 is better antioxidant than ascorbic acid but worse than trolox, in term of its antioxidant capacity. The antiradical reactivity of the examined compounds may occur by the hydrogen atom transfer and an electron transfer. Using superoxide anion radical, generated in hydrophobic medium in the CL assay, it was also possible found that some of CBs compounds can also act as donors of the protons. For a better understanding of the mechanisms involving in the assays used and to answer, why some of CBs compounds have a higher antioxidant activity, it would be interesting to separate the reaction intermediates and products by chromatography and to characterize them.

Free radical scavenging is one of the well recognized mechanisms by which antioxidants inhibit lipids and other biomolecules oxidation, i.e. oxidative stress. Regardless of that for the majority of the compounds tested the mechanism their reactions with $DPPH^{\bullet}$ is more complex, i.e. they react slowly with this radical, the results obtained confirm our previous finding [9] of the potential therapeutic value of CBs as substances of antidiabetic activity. Reactive oxygen species are believed to play a major role in chronic inflammation, which, in turn, can mediate diabetes. Because of low level of antioxidant enzymes, β-cells are particularly sensitive to hydroxyl radical and superoxide anion radical [29], therefore, the ability of the oxidative stress to cause the β-cells dysfunction and decrease insulin secretion is a highly probable. Thus, the present results suggest that some of the CBs tested are promising for treating diabetes that should be further explored.

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