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A preliminary study on radical scavenging abilities of two dihydroxy-coumarins by electron paramagnetic resonance (EPR) spectroscopy

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In current research were evaluated and compared the radical scavenging abilities of two coumarins differing in positions of ortho-dihydroxyl groups in their aromatic rings. Scavenging abilities towards superoxide ($\cdot O_2^-$) and hydroxyl (·OH) radical of 7,8-dihydroxy-4-methyl-2H-chromen-2-one (**a**$_1$) and 6,7-dihydroxy-4-methyl-2H-chromen-2-one (**b**$_1$) were studied. To realize the aim of the study proper Electron Paramagnetic Resonance (EPR) spin trapping spectroscopy was applied the only technique that allows scavenging, detecting and distinguishing of short live radicals such as $\cdot O_2^-$ and ·OH species. In studied Fenton system was demonstrated higher inhibiting activity against •OH for **a**$_1$ in comparison with **b**$_1$, while in hypoxanthine/xanthine oxidase system **b**$_1$ exhibited better inhibiting ability against $\bullet O_2^-$ generation.

Keywords: Dihydroxy-coumarins, EPR spin trapping, Superoxide anion radical, Hydroxyl radical

INTRODUCTION

Antioxidants are important species possessing ability to protect the living organisms from damages caused by free radical-induced oxidative stress [1]. Antioxidant was defined as any substance that directly scavenges reactive oxygen species (ROS) or indirectly acts to up-regulate antioxidant defense or inhibit ROS production" [2]. In a number of studies, have been demonstrated different antioxidants exhibiting selective scavenging activity towards various ROS [3]. Unfortunately, under various pathophysiological conditions, human antioxidative defense system, fails to eliminate the excess of ROS. Therefore, there is continuous demand for exogenous antioxidants in order to prevent oxidative stress, representing a disequilibrium redox state in favor of oxidation. However, high doses of isolated compounds may be toxic, owing to pro-oxidative effects at high concentrations or their potential to react with beneficial concentrations of ROS normally present at physiological conditions that are required for optimal cellular functioning. Coumarins are an important class of oxygen heterocycles, widespread in nature occurring in a lot of green plants as well as in fungi and bacteria [4, 5]. They greatly attract the attention of researchers because possess diverse pharmacological properties [6, 7]. For coumarins have been reported a remarkable range of biological activities that include inhibition of xanthine oxidase and direct scavenging of harmful ROS produced by enzymes other than xanthine oxidase [8, 9]. Moreover, EPR spectroscopy is the only analytical technique for direct and indirect detection of stable and unstable free radicals in chemical, physical and biological systems. Methods, based on EPR spectroscopy, are widely used because can detect paramagnetic species without interference from the sample properties, including the phase of the sample (solid, liquid or gas) [10]. As far as we are informed EPR spectroscopy studies on scavenging activity against the superoxide and hydroxyl radicals of 7,8 and 6,7-dihydroxy 4-methyl coumarins are quite scarce. All above facts prompted us to investigate the antioxidant activity of 7,8-dihydroxy-4-methylcoumarin (**a**$_1$) and 6,7-dihydroxy-4-methylcoumarin (**b**$_1$) to scavenge hydroxyl and superoxide radicals using EPR spin trapping technique and evaluate the effect of different positioning of o-dihydroxyl groups in their aromatic rings on radical scavenging ability.

EXPERIMENTAL

Materials

Coumarins: 7,8-dihydroxy-4-methyl-2H-chromen-2-one (**a**$_1$) and 6,7-dihydroxy-4-methyl-2H-chromen-2-one (**b**$_1$) (Fig. 1) were synthesized and characterized at the Department of Chemistry, University of Delhi, Delhi as described formerly [11-13].

Spin trap 5-*tert*-butoxycarbonyl-5-methyl-1-pyrroline *N*-oxide (BMPO), diethylene triamine pentaacetic acid (DTPA), hypoxanthine, xanthine oxidase, iron (II) sulfate heptahydrate, hydrogen

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peroxide and ethanol were purchased from Sigma-Aldrich Chemical Co, St. Louis, USA. All other chemicals used in this study were of analytical grade. Deionized and distilled water was used for all experiments.

Methods

In vitro EPR spectroscopy experiments

EPR measurements of studied coumarins were performed at room temperature (18-23°C) on an X-band EMX^{micro}, spectrometer Bruker, Germany, equipped with a standard resonator. Quartz capillaries were used as sample tubes. The capillary tubes were sealed and placed inside a standard EPR quartz tube (i.d. 3 mm) that was fixed in the EPR cavity. Due to insufficient coumarins quantity we had, it was not possible to determine their IC_{50} – the concentration inhibiting 50 percent of the generated superoxide ($\cdot O_2^-$**)** or hydroxyl (·OH) radicals. EPR experiments were carried out in triplicate. All results presented are based on averaging of three independent EPR measurements. Spectral processing was performed using Bruker WIN-EPR and SimFonia software.

Coumarins **a$_1$** and **b$_1$** were dissolved in ethanol at increasing concentrations as indicated in Tables 1 and 2.

EPR study on in vitro generated superoxide anion radicals

Superoxide anion scavenging activity was determined by the EPR method and hypoxanthine/xanthine oxidase system was used to generate $\cdot O_2^-$ [14]. Reaction was initiated by adding of xanthine oxidase (XO) and 5-*tert*-butoxycarbonyl 5-methyl-1-pyrroline *N* – oxide (BMPO) used as a specific spin trap for the superoxide anion radicals [15]. Final volume of reaction mixture was 200 µl and contained: 50 µl of 1mM of hypoxanthine dissolved in 50 mM phosphate buffer (pH 7.4, containing 50 µM of DTPA as a transition metal chelator), 20 µl of 100 mM BMPO in phosphate buffer, 50 µl of the studied coumarin concentration, 30 µl of phosphate buffer and 50 µl of xanthine oxidase 1 U/ml dissolved in phosphate buffer. Control sample contained 80 µl of phosphate buffer instead of 30 µl. EPR spectra were recorded at the 5th min after the reaction starts. The effect of **a$_1$** and **b$_1$** on *in vitro* generated superoxide anion radicals was evaluated according to the equation:

% O_2^- radicals scavenged by BMPO = [I/Io] × 100 %,

where: Io - double integrated plot of the EPR spectrum of BMPO/·OOH adduct registered in the control sample; I - double integrated plot of the EPR spectrum of BMPO/·OOH spin adduct registered after addition of the tested sample.

EPR settings were as follows: center field 3505 G, sweep width 100 G, microwave power 12.62 mW, modulation amplitude 10 G, receiver gain 2.52×10^4, time constant 40.96 ms, sweep time 40.96 s, 1 scan per sample.

EPR study on in vitro generated hydroxyl radicals (·OH)

To evaluate the effect on *in vitro* gen*erated ·OH both extracts were examined by the EPR method described by Wang *et al.* [16] with modifications. The reaction mixture contained 40 µl of 20 mM BMPO, 40 µl of 0.2 mM $FeSO_4$ freshly prepared, 80 µl of the studied coumarin concentration and 100 µl of 2mM H_2O_2. Control sample contained 80 µl of distilled water instead of coumarin tested. The EPR spectra were recorded 5 min after the start of reaction. The effects of both extracts on *in vitro* generated hydroxyl radicals were evaluated according to the equation:

% ·OH radicals scavenged by BMPO = [I / Io] × 100 %

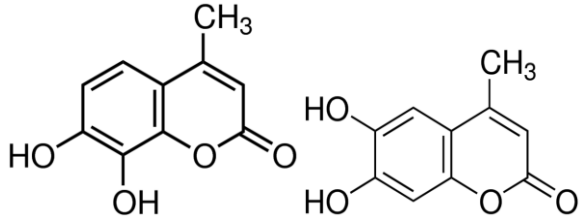
where: Io – double integrated plot of the EPR spectrum of BMPO/·OH spin adduct registered in the control sample; I - double integrated plot of the EPR spectrum of BMPO spin adduct registered after addition of the tested sample containing the corresponding coumarin concentration.

EPR settings were as follows: center field 3505 G, sweep width 100 G, microwave power 8.02 mW, gain 2.52×10^4, modulation amplitude 5 G, time constant 163.84 ms, sweep time 81.92 s, 5 scans per sample.

RESULTS AND DISCUSSION

It is well known that coumarins as natural products found in plants possess remarkable bioactivities including XO inhibition [17]. It should be noted that unlike most other XO inhibitors they act as radical scavengers against ROS produced by enzymes other than XO [18]. The superoxide anion radical ($\cdot O_2^-$), called the "primary" ROS is considered the most important physiologically generated radical [3,19,20]. *In vivo* overproduced $\cdot O_2^-$ directly interacts with other molecules, as well as through enzyme- or metal-catalyzed processes and causes generation of the "secondary" ROS such as H_2O_2, peroxyl (ROO^-),·OH. In general, the least ambiguous technique for detecting and characterizing free radicals at *in vitro* and *in vivo* conditions is EPR spectroscopy. However, direct detection of short lived radicals (e.g. superoxide

and hydroxyl radicals) is very difficult or impossible at room temperature. EPR spin trapping technique is the only one that allows scavenging, detecting and distinguishing of such species. Spin traps used in EPR spectroscopy are not radicals but can form stable radical adducts with short-lived radicals, generated *in vitro* or *in vivo*. In the present research BMPO was selected for *in vitro* evaluation of the radical scavenging abilities of the studied coumarins, because it can form stable and distinguishable spin adducts with superoxide and hydroxyl radicals [15]. In the system generating $\cdot O_2^-$ containing only BMPO (control sample) an EPR spectrum consisting of four spectral lines with equal intensity was recorded and identified as a BMPO/·OOH spin adduct (*conformer I*, Fig. 2A) [15]. The effect of $\mathbf{a_1}$ and $\mathbf{b_1}$ on the levels of generated $\cdot O_2^-$ in hypoxanthine/xanthine oxidase system is given in Table 1.



a1 **b1**

Figure 1. 7,8-dihydroxy-4-methyl-2H-chromen-2-one (**a1**) and 6,7-dihydroxy-4-methyl-2H-chromen-2-one (**b1**).

As can be seen, with an increase in $\mathbf{a_1}$ or $\mathbf{b_1}$ concentration, the percentage of $\cdot O_2^-$ radicals scavenged by BMPO decreases, which means that coumarins ability to scavenge $\cdot O_2^-$ also increases. Paya and co-workers [4], using a spectrophotometric technique, studied the reduction of ferricytochrome ***c*** to ferrocytochrome ***c*** depending on the amount of $\cdot O_2^-$ radicals generated in a hypoxanthine/xanthine oxidase system. They found $\mathbf{a_1}$ and $\mathbf{b_1}$ at a concentration of 100 μM and lower than 10 μM were not capable to scavenge $\cdot O_2^-$. Other research groups using systems containing hypoxanthine or xanthine and xanthine oxidase also demonstrated the abilities of *ortho*-dihydroxy coumarins to inhibit $\cdot O_2^-$ generation [8, 9]. By *in vitro* assays Hofmann and co-workers [8] investigated the inhibitory potencies against isolated XO of 18 coumarins differing in number and position of the hydroxyl groups at C6, C7, C8 and substituents at C4. They found that esculetin possessing a hydroxyl group at C6 and C7 exhibited the highest XO inhibitory potency comparing to the rest coumarins including those possessing OH groups at C7 and C8. Using computational ligand docking the same authors demonstrated snugly esculetin accommodation to the binding (active) site of XO due to formation of a hydrogen bond between the hydroxyl group at esculetin's C6 and the side chain of Glu802 whereas a second one involved the carbonyl oxygen and the guanidine group of Arg880 in enzyme [8]. Šeršeň and Lácová [9] reported very good scavenging against $\cdot O_2^-$ either for 7,8-dihydroxylated or 6,7- dihydroxylated coumarins but in contrary found almost twice higher activity for $\mathbf{a_1}$ comparing to esculetin. Current research shows that $\mathbf{a_1}$ and $\mathbf{b_1}$ can inhibit superoxide generation at a concentration of 10 μM and lower. Based on the other authors' and current results, we accept that two mechanisms can be involved through which both coumarins reduce superoxide generation: a) direct scavenging of $\cdot O_2^-$ and b) XO inhibition by binding the corresponding coumarin to enzyme active side. We believe that higher IC_{50} of the two coumarins (> 10μM) reported by the other authors [8, 9] are due to the use of spectrophotometric techniques whose sensitivity is considerably lower comparing to EPR spectroscopy used in current research. The fact that $\mathbf{b_1}$ exhibits higher inhibiting activity comparing to $\mathbf{a_1}$ at every studied concentration (see Table 1) we can explain by more effective XO inhibition due to C6 hydroxyl group presents in $\mathbf{b_1}$ structure like as in esculetin [8].

Table 1 Percent scavenged $\cdot O_2^-$ radicals by BMPO in presence of studied coumarins.

Concentration (μmol/ml)	**a1*** (% scavenged O_2^- by BMPO)	**b1*** (% scavenged O_2^- by BMPO)
2.5	62.71	27.99
5	54.85	13.68
10	45.05	7.3

***Averaged value of three independent measurements with ±SD ≤ 5 %

Despite the reaction with •OH is not as specific as that with DPPH and $\cdot O_2^-$, it is used in many studies to assess the antioxidant activity of natural extracts, fractions and substances [21-25]. In the present research after addition of BMPO to the system generating •OH the typical EPR spectrum of a spin adduct between hydroxyl radical and the spin trap BMPO was registered (Fig. 2**B**) and identified as BMPO/•OH *conformer II* [15, 26].

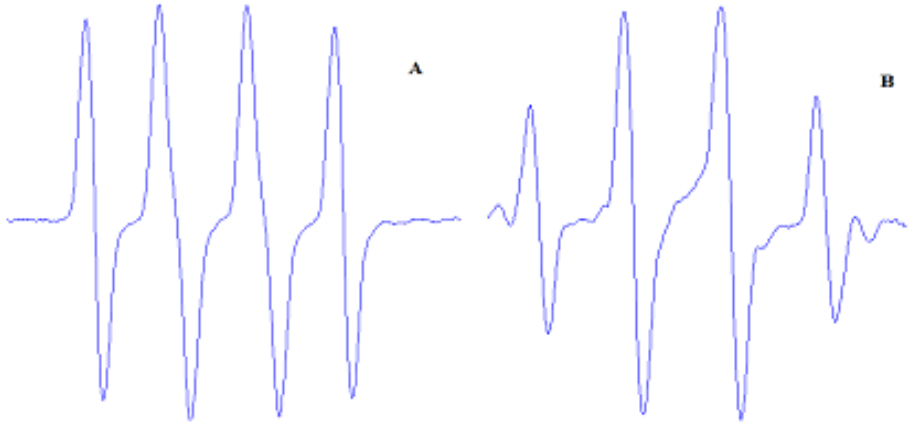


Figure 2. **A)** EPR spectrum of BMPO/•OOH spin adduct (*conformer I*) and **B)** EPR spectrum of BMPO/•OH spin adduct (*conformer II*)

As is seen in Table 2, when $\mathbf{a_1}$ and $\mathbf{b_1}$ concentrations added to the control sample increased, the amount of hydroxyl radicals scavenged by BMPO decreased, demonstrating the antioxidant behavior of the studied coumarins.

Table 2. Percent scavenged·OH radicals by BMPO in presence of studied coumarins.

Concentration (μmol/ml)	$\mathbf{a_1}$* % scavenged ·OH by BMPO	$\mathbf{b_1}$* % scavenged ·OH by BMPO
2.5	41.68	85.24
5	34.58	45.97
10	32.25	19.67

*Averaged value of three independent measurements with ±SD ≤ 5 %

Although IC_{50} value was not determined, obviously, it is lower than 2.5 μM for $\mathbf{a_1}$ and is between 2.5 μM and 5 μM for $\mathbf{b_1}$ (see Table 2). Moreover, $\mathbf{a_1}$ manifested itself as a more active compound in the studied Fenton system. This result is supported by findings of other research teams using EPR spin trapping technique and a Fenton system with the same composition as ours [9] or spectrophotometric technique and a Fenton system with different constituents [4]. In several studies well expressed metal chelating abilities towards ferric [4] or ferrous ions [28] were found for coumarins possessing ortho-dihydroxy phenol structures. Using the same Fenton system and spin trapping EPR spectroscopy, Šeršeň and Lácová [9] determined 9.18 μM IC_{50} for $\mathbf{a_1}$ and 57.04 μM for esculetin (6,7-dihydroxycoumarin) values higher than ours. It should be noted that this research group used DMPO spin trap for ·OH scavenging instead of BMPO. As was mentioned above, spin trap BMPO is the most suitable one for the specific *in vivo* or *in vitro* detection of superoxide and hydroxyl radicals by EPR spin trapping spectroscopy. The well-established fact that the DMPO spin trap does not easily distinguish superoxide and hydroxyl radical because of spontaneous decay of the DMPO-superoxide adduct into DMPO-hydroxyl adduct [15, 29] made us believe that the results obtained using BMPO are more reliable than those of Šeršeň and co-workers [9]. Paya et al. [4], by spectrophotometric technique using two systems based on Fenton chemistry, evaluated interaction of coumarins with •OH generated in slow rate ($FeCl_3$-EDTA and H_2O_2) and rapid rate ($FeCl_3$-ascorbate and H_2O_2) system. In the first system they found accelerated hydroxyl radical formation (pro-oxidant activity) for all 6,7- and 7,8-dihydroxy coumarins including $\mathbf{a_1}$ and $\mathbf{b_1}$. Contrary, in the second (rapid rate) system containing $FeCl_3$-ascorbate and H_2O_2, all 6.7- and 7,8-*ortho*-dihydroxyl coumarins exhibit striking inhibition of site-specific deoxyribose degradation induced by iron ions similar to desferrioxamine - a typical iron chelator [4]. These authors explain the ·OH inhibiting activity of ortho-dihydroxy coumarins with their ability to chelate ferric (Fe^{3+}) ions. On the other hand, many studies in relation with metal chelating ability of different natural products and compounds demonstrated that their ferrous (Fe^{2+}) ions chelating ability was due to the presence of ortho-dihydroxy phenol structures. [29,30]. In relation to the mechanism that was involved in $\mathbf{a_1}$ and $\mathbf{b_1}$ abilities to reduce the amount of ·OH radicals generated we accepted that both coumarins were able to scavenge either directly·OH (confirmed by EPR spectroscopy) or through chelation of ferrous ions present in the system by their ortho-dihydroxy phenol structure. We also assumed that the higher inhibiting potency against •OH generation demonstrated by $\mathbf{a_1}$ was due to its higher chelating abilities towards Fe^{2+} ions. The significantly low concentrations at which both coumarins exhibited inhibitory effect against hydroxyl radical generation can be explained on

one hand with the high sensitivity of the EPR spectroscopic technique and on the other with the use of BMPO as a spin trapping agent.

CONCLUSION

For the first time, by EPR spectroscopy combined with an appropriate spin trap, it was demonstrated that at concentrations lower than 10 μM, the two coumarins **a₁** and **b₁** showed *in vitro* a well expressed inhibitory effect on superoxide and hydroxyl radical generation. Results obtained show that **a₁** exhibits a higher scavenging activity against $^{\bullet}OH$ than **b₁** in the Fenton system whereas in the hypoxanthine/xanthine oxidase system **b₁** exhibits a higher ability to inhibit formation of $^{\bullet}O_2^-$. Given the various biological effects reported for coumarins, we have planned further detailed EPR studies with **a₁** and **b₁** to determine their IC_{50} values, to investigate the exact mechanisms of interaction with various ROS and also to evaluate their abilities for reducing oxidative damages at *in vivo* conditions.

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ПРЕДВАРИТЕЛНО ПРОУЧВАНЕ НА РАДИКАЛ-УЛАВЯЩИТЕ СПОСОБНОСТИ НА ДВА ДИХИДРОКСИ-КУМАРИНА ЧРЕЗ EPR СПЕКТРОСКОПИЯ

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(Резюме)

В настоящото изследване са оценени и сравнени възможностите за радикал-улавящата способност на два кумарина, които се различават в позициите на орто-дихидроксилните групи в техните ароматни пръстени. Изследвана е способността на 7,8-дихидрокси-4-метил-2H-хромен-2-он (**а$_1$**) и 6,7-дихидрокси-4-метил-2H-хромен-2-он (**б$_1$**) да улавят супероксидния (·O_2^-**)** и хидроксилния (·OH) радикал. За да се осъществи целта на това изследване е използвана подходяща спин-улавяща EPR спектроскопия техника, която е единствената техника, позволяваща улавяне, откриване и разграничаване на краткоживущи радикали, като например: ·O_2^- и ·OH видове. Получените резултати показват, че **а$_1$** проявява по-висока радикал-улавяща активност срещу ·OH в сравнение с **б$_1$** във Fenton системата, докато в хипоксантино / ксантин оксидазна система **б$_1$** проявява по-висока способност да инхибира образуването на·O_2^-.