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THE SUPEROXIDE SCAVENGING ACTIVITY OF DIHYDROQUINOLINE TYPE DERIVATIVES (CH402 AND MTDQ-DA)

LÁSZLÓ PRÒNAI, ANNA BLÅZOVICS, MÓNIKA HORVÅTH, ISTVÁN LÅNG and JÁNOS FEHÉR

2nd Department of Medicine, Semmelweis University Medical School, 1088 Budapest, Szentkirályi u. 46, Hungary

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Electron paramagnetic resonance/spin trapping studies were applied, to verify the superoxide radical scavenging activity of two non-toxic, water soluble dihydroquinoline type antioxidants, CH402 (Na-2, 2-dimethyl-1,2-dihydroquinoline-4-yl methane sulphonate and MTDQ-DA (6,6-methylene bis 2,2-dimethyl-4-methane sulphonic acid: Na-1,2-dihydroquinoline). Results were compared with other indirect methods such as the amperometric, spectrophotometric and luminometric methods, respectively. Both dihydroquinoline type antioxidants scavenged superoxide in vitro specifically. MTDQ-DA scavenged superoxide an order of magnitude faster than CH-402. Neither CH402 nor MTDQ-DA affected the hypoxanthine/ xanthine oxidase superoxide generating system, nor did they inhibit xanthine oxidase directly.

KEY WORDS: Superoxide scavenger, dihydroquinoline type antioxidant, electron paramagnetic resonance/spin trapping, MTDQ-DA, CH402.

INTRODUCTION

Synthetic antioxidants are widely employed both as food-preservatives and anti-inflammatory, immune-modulating agents.[1,2] Previous, in vitro and animal studies have suggested an antioxidant effect of two dihydroquinoline type compounds, CH-402 (Na-2,2-dimethyl-1,2-dihydroquinoline-4-yl methane sulphonate) and MTDQ-DA (6,6-methylene bis 2,2-dimethyl-4-methane sulphonic acid: Na-1,2-dihydroquinoline), and have described an oxygen free radical scavenging effect as a major route of action of these compounds.[3-6]

The so called "antioxidant effect", however, is a complex reaction, including the inhibition of lipid peroxidation, interference with the free-radical-chain reaction, repairing damaged sites of biopolymers caused by free radicals, and/or direct scavenging of reactive oxygen radicals. Since the previous studies were based on indirect assays, the aims of the present study were; 1) to reconfirm these studies by using a selective and specific method for superoxide radicals, the electron paramagnetic resonance (EPR)/spin trapping method,[7] and 2) to compare these results with three other indirect methods such as the amperometric, spectrophotometric and luminometric methods, since EPR/spin trapping is not generally available in smaller laboratories.

Correspondence: László Prónai, M.D., Ph.D. 2nd Dept. Medicine, Semmelweis Univ. School of Medicine, 1088 Budapest, Szentkirályi u 46, HUNGARY, tel: (36 1) 210-0799, fax: (36 1) 210-0799

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MATERIALS AND METHODS

1. Chemicals

Xanthine oxidase (XO) was obtained from Boehringer-Mannheim GmbH (Mannheim, Germany); cytochrome c, xanthine, hypoxanthine (HT) and desferrioxamine (DFO) from Sigma Chem Co. (St. Louis, U.S.A.); 5,5-dimethyl-pyrroline-N-oxide (DMPO) from Shonan Analytic Center, (Tokyo, Japan); and recombinant human superoxide dismutase (SOD) from Nihon Kayaku (Tokyo, Japan). CH402 was obtained from Chinoin (Budapest, Hungary), MTDQ-DA was a gift from Dr. Vilmos Bâr (Chemist, Budapest). The chemical structures of MTDQ-DA and CH402 are shown in Figure 1. Fk-71 buffer pH 7.0 was obtained from RADELKIS, and all other chemicals used were REANAL (Budapest) products and were used without any further purification.

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2. Measurement of Superoxide Scavenging Activity by Electron Paramagnetic Resonance (EPR)/Spin Trapping Method

Superoxide scavenging activity in the hypoxanthine/XO system was measured as described elsewhere.[8] Kajihara *et al.* showed earlier that the EPR/spin trapping method can be applied for the detection of SOD-like activity in vitro.[9] First a standard curve was made by adding 0.05 ml of human recombinant SOD (2-20 U/ml) to the mixture containing 0.5 mM of HT, 0.5 mM DFO, and 34 or 68 mM of DMPO in phosphate buffer (pH 7.4, final volume 0.2 ml). The reaction was started by the addition of XO (final 0.05 U/ml) to the system. For the measurement of superoxide scavenging activity, a 0.05 ml of sample (diluted in phosphate buffer) was used instead of SOD. The reaction mixture was then stirred for 3 seconds and transferred into a flat quartz cell (JEOL Co. Ltd., Tokyo, Japan; 0.18 ml). All spectra were recorded at 40 sec after the addition of XO. Both DMPO-OOH and a smaller DMPO-OH signal were detected (Figure 2). The DMPO-OH signal did not decrease when catalase was added to the system indicating that DMPO-OH arises not from trapping hydroxyl radical, but from the decomposition of DMPO-OOH. Using a computer-controlled system, the height of DMPO-superoxide and DMPO-hydroxyl adducts could be measured continuously up to 5 min (Figure 3). The height of the DMPO-superoxide adduct at 40 sec. was compared with that of the internal standard, manganese peak. EPR spectra were recorded on a JES-FE 2XG (JEOL Co.

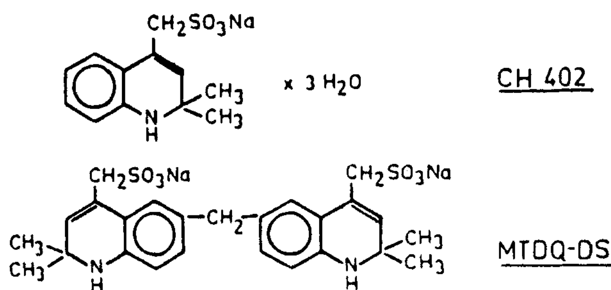

FIGURE 1 Chemical structure of CH402 and MTDQ-DA.

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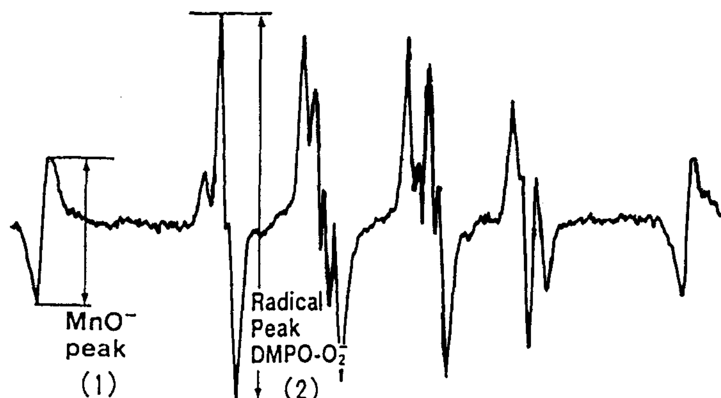

FIGURE 2 Representative electron paramagnetic resonance spectrum of DMPO-OOH and DMPO-OH spin adducts generated by the hypoxanthine/xanthine oxidase system. The first peak (1) is the internal standard manganese peak and the second peak (2) is the low magnetic field peak of DMPO-OOH spin adduct. The reaction mixture contained 0.5 mM hypoxanthine, 0.5 mM desferrioxamine, 0.05 U/ml xanthine oxidase and 34 mM DMPO, final volume 0.2 ml (in phosphate buffer; pH 7.4).

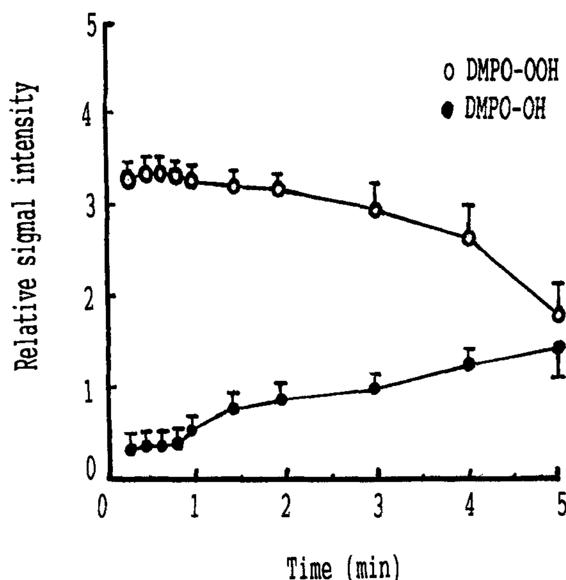

FIGURE 3 Time course of relative signal intensities of DMPO-OOH and DMPO-OH spin adducts in the hypoxanthine/xanthine oxidase system. Conditions for measurement were the same as shown in Figure 2.

Ltd., Tokyo, Japan) with field set at 355 ± 5 mT, modulation frequency of 9.42 GHz, modulation of 0.125 mT, amplitude of 1000, response of 0.3 sec, sweep time of 2 min and microwave power at 8 mW. Results were conducted in triplicate and expressed as mean ± SD.

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3. The Principle of Superoxide Scavenging Activity (SSA)

The principle of superoxide scavenging activity measurement was originally described by Prònai *et al.*[10] Briefly, when a superoxide ($O_2^{-}\cdot$) scavenger (Scav) and DMPO are present in an $O_2^{-}\cdot$ generating system, Scav and DMPO scavenge $O_2^{-}\cdot$ competitively according to Eqs (1) and (2).

$$\text{DMPO} + O_2^{-}\cdot + H^{+} \xrightarrow{k_D} \text{DMPO-OOH} \quad (1)$$
$$\text{Scav} + O_2^{-}\cdot + H^{+} \xrightarrow{k_S} \text{Product} \quad (2)$$

In the case that the concentrations of DMPO and Scav are much higher than that of $O_2^{-}\cdot$, pseudo-first-order rate constants, k_D and k_S in Eqs (1) and (2), can be defined and the following equation can be derived:

$$I_0/I = 1 + (k_S/k_D)([\text{Scav}]/[\text{DMPO}]) \quad (3)$$

where I_0 and I are the signal intensity of DMPO-superoxide adduct without and with the scavenger, respectively, and [Scav] and [DMPO] are the initial concentration of scavenger and DMPO, respectively. Thus, the SSA of Scav can be assayed by utilizing a linear correlation between I_0/I and [Scav].

Since any compound measured may also affect the $O_2^{-}\cdot$ generating enzymatic system, it is important to separate this effect of the compound from its direct scavenging effect. For this purpose, it is necessary to use at least two different concentrations of DMPO (34 or 68 mM) in this system. The compound which does not affects the enzyme system, should give a similar slope of regression line between I_0/I and S/D. Therefore, the superoxide scavenging activity of compounds measured was estimated by the following modified equation:

$$I_0/I = (1 + rS)(1 + {}^{k}/ko^{S}\ /D), \quad (4)$$

where "r" is the inhibition coefficient of substrate against the $O_2^{-}\cdot$ generating system. The intensity ratio (I_0/I) is proportional to S/D and k/k^{O}. Scav can be estimated from the slope of a regression line between I_0/I and S/D. If the substrate, however, also inhibits the $O_2^{-}\cdot$ generation in proportion to the concentration of the substrate, the linearity between I_0/I and S/D is disrupted.

For simplicity, if the two regression lines at two different concentrations of DMPO are identical, the compound react with $O_2^{-}\cdot$ only, and does not affect the superoxide generating system.

4. Measurement of Oxygen Concentration (Amperometric Method)

Amperometric studies for the direct measurement of oxygen concentration, were carried out using an OP-Gl-7113S "Clark" type electrode set (RADELKIS electrochemical instruments, Budapest, Hungary). An OP-960 type adapter (RADELKIS, Hungary) provided the polarizing voltage required for operation of the electrode and converted the output signal for RADELKIS digital OP-271 pH meter. Principally, the generation of superoxide in the xanthine/XO system was followed by measuring the oxygen consumption in the system. Over the specified concentration range, there was a linear correlation between the measured signal intensity and the xanthine concentration in the system.

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5. *Spectrophotometric Method (SPECORD UV-VIS, Zeiss, Germany)*

The effect of CH402 and MTDQ-DA on the xanthine/XO reaction was studied by the method of McCord and Fridovich.[11] Reduction of cytochrome-c was monitored by following the increase in absorbance at 550 nm. The reaction mixture contained 50 μM cytochrome-c, 10^{-4} M xanthine and 5 μl xanthine oxidase of 1 IU/ml in 50 mM phosphate buffer (pH 7.5).

6. *Luminometric Method*

Light emission was measured by a chemiluminescence method as described by Blázovics *et al.*[5] Measurements were conducted in a CLD-1 Medicor luminometer with MMT microprocessor (MEDILAB, Budapest, Hungary). Measurements were carried out in separate phases of a double-part cuvette. The xanthine/XO reaction mixture in the upper part of the cuvette contained 0.05 ml of 0.01 M xanthine in 0.02 M K-phosphate buffer (pH 7.5) and 2 μl of 1 U/ml xanthine oxidase. The reagent solution in the lower part of the cuvette (volume 1 ml) consisted of 0.7 mM luminol, 3.8 μM hemin, and 11.8 mM Na_2CO_3 adjusted to pH 10–11 and bubbled with N_2 gas. To avoid any non-specific changes, MTDQ-DA or CH402 (0.1 ml) were added just 30 sec prior to the initiation of the reaction with XO.

Statistical analysis was done by a two-tailed t-test with a preset probability level of $p < 0.05$. Mean values represent the data obtained from five experiments.

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RESULTS AND DISCUSSION

Two dihydroquinoline type antioxidants, MTDQ-DA and CH402 have been shown to have membrane protecting action in experimental hyperlipidaemia[6] and in the CCl_4 and galactosamine induced acute liver lesions[3]. Previous studies, including a pulse radiolysis study,[5] demonstrated the antioxidant properties of CH402 and MTDQ-DA and suggested a superoxide scavenging effect as one of the major actions of these drugs. The present work was performed to examine the direct superoxide scavenging action of CH402 and MTDQ-DA in systems where superoxide was generated enzymatically. Comparisons with the three indirect methods, the amperometric, spectrophotometric and luminometric methods were also performed, since these methods are simple and less expensive, and EPR/spin trapping is not generally available in smaller laboratories.

Because of the known complexity of DMPO-OOH kinetics,[12] the stability of DMPO-OOH radical was studied in the EPR experiments. Using the previously established computer-controlled system for the parallel measurement of DMPO-OOH and DMPO-OH,[8,10] the stability of DMPO-OOH and its decomposition to DMPO-OH were followed for up to 5 min (Figure 3). DMPO-OOH was relatively stable for 3 min, and DMPO-OH increased slowly in the system. Peak intensities were not affected by adding catalase to the system, indicating that DMPO-OH arises from the decomposition of DMPO-OOH (data not shown).

Both CH-402 and MTDQ-DA had a direct superoxide scavenging activity in cell free systems (Figure 4 A–B). Since the two regression lines observed at two different concentrations of the spin trap, DMPO were identical, both compounds can be considered superoxide scavengers which do not affect the hypoxanthine/XO, superoxide generating system. This fact was also supported by the observation that the

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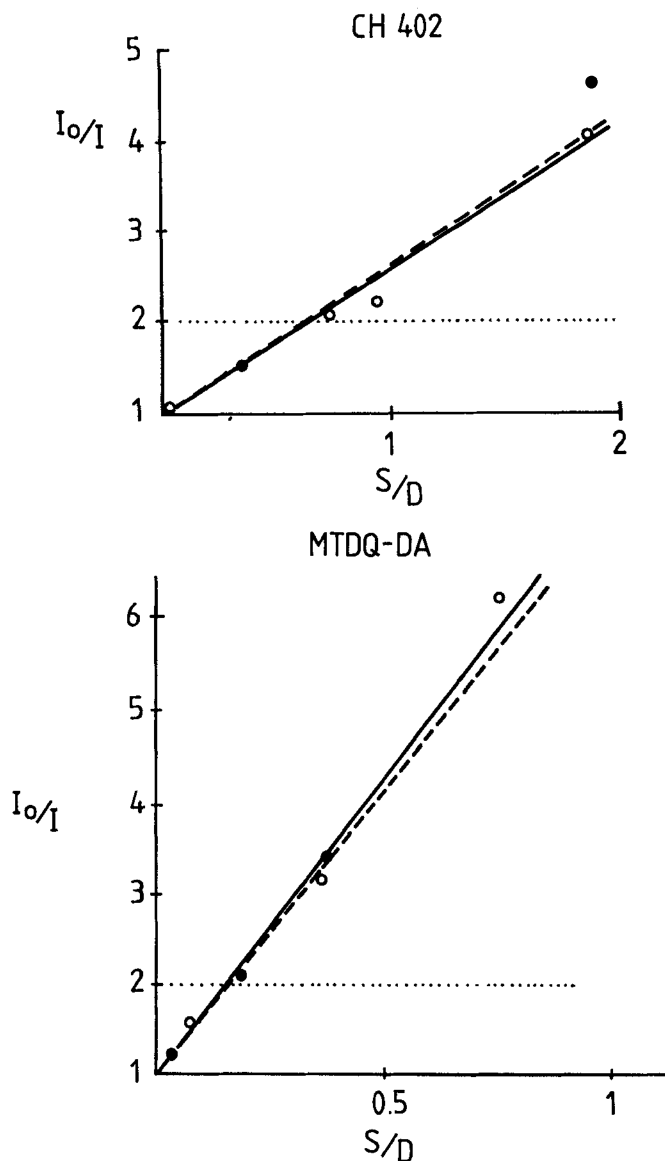

FIGURE 4 (A, B) Superoxide scavenging activity (SSA) of CH402 and MTDQ-DA. I_0/I represents the ratio between the intensity of DMPO-OOH spin adduct in the absence (I_0) of the scavenger and that in the presence (I) of the agent. S/D is the molar ratio between the scavenger (S) and DMPO (D). Solid lines with open circles and dashed lines with dots represent the SSA determined at 34 mM or 68 mM of DMPO, respectively. Values represent the mean of three measurements. Correlation was higher than 0.985 in each cases.

decay of DMPO-OOH and the increase of DMPO-OH was not influenced by either MTDQ-DA or CH402 (data not shown).

The SOD-like activity of compounds was also calculated, and is shown in Figure 5. MTDQ is a stronger scavenger than CH-402 in this system.

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The I_{50} inhibition concentrations of both compounds were calculated (Table 1). MTDQ-DA and CH402 were five times and twice as effective in scavenging superoxide as the spin trap, DMPO.

Based on the reported pseudo-first order kinetic rate constant of superoxide with DMPO: k_{DMPO} = 10–30 $M^{-1}s^{-1}$,[12,13] and assuming that the reactions are running as postulated in eqn. 3 (—), the calculated rate constant for the reaction between superoxide and MTDQ-DA is: $k_{MTDQ\text{-}DA} = 0.7 - 2.1 \times 10^2\ M^{-1}s^{-1}$, and that for the reaction of superoxide with CH402 is: $k_{CH402} = 1.514.5 \times 10\ M^{-1}\ s^{-1}$. The calculated values in this system, were about five to six times smaller than that reported in our previous work.[5] One explanation for the different values observed, could be the differences in pH values in the two studies. The EPR experiments were conducted at pH 7.4, which is lower that those used in pulse radiolysis (pH 8.2). The major difference, however, was that while in the pulse radiolysis studies superoxide was produced directly in a non-enzymatic system, in the EPR experiments superoxide was produced enzymatically. Furthermore, it is important to notice that in our hypoxanthine/XO system not only superoxide but also hydrogen peroxide were produced and that might also influence the calculations. In addition, the rate constants

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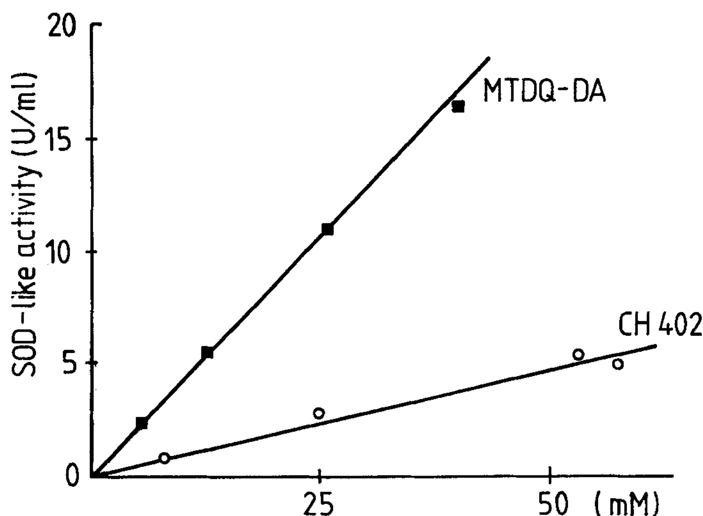

FIGURE 5 Comparison between the SOD-like activity of CH402 and MTDQ-DA. Values are expressed as U/ml. The order of SSA intensities are; MTDQ-DA > CH402.

TABLE 1
Superoxide scavenging activity of compounds determined by EPR/spin trapping method

Compounds added	50% inhibition rate (I_{50})		Direct effect on the HT/XO system
	DMPO		
	34.0 mM	68.0 mM	
MTDQ	6.35 mM	12.70 mM	no effect
CH402	23.0 mM	46.0 mM	no effect

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calculated from electron spin resonance studies should be cautiously evaluated, since it is known that the electron spin resonance, spin trapping system with the spin trap DMPO has a low sensitivity for superoxide, DMPO traps only a minor portion of superoxide generated and DMPO-OOH decomposes by superoxide itself.[12]

Chemiluminescence is a property of excited states of luminol formed in a medium containing free radicals. The data in Table 2 indicate a reduced production of free radicals originating from the xanthine-xanthine oxidase enzyme system, when CH402 or MTDQ-DA were present at increasing concentrations.

CH402 and MTDQ-DA reduced the superoxide production in the xanthine-xanthine oxidase reaction, which was detected spectrophotometrically with a cytochrome-c assay (Figure 6). The calculated rate constants were $k_{CH402} = 2 \times 10^3$ $M^{-1}s^{-1}$ for the reaction of superoxide with CH402 and $k_{MTDQ-DA} = 7 \times 10^3$ $M^{-1}s^{-1}$ for the reaction of superoxide with MTDQ-DA, respectively.

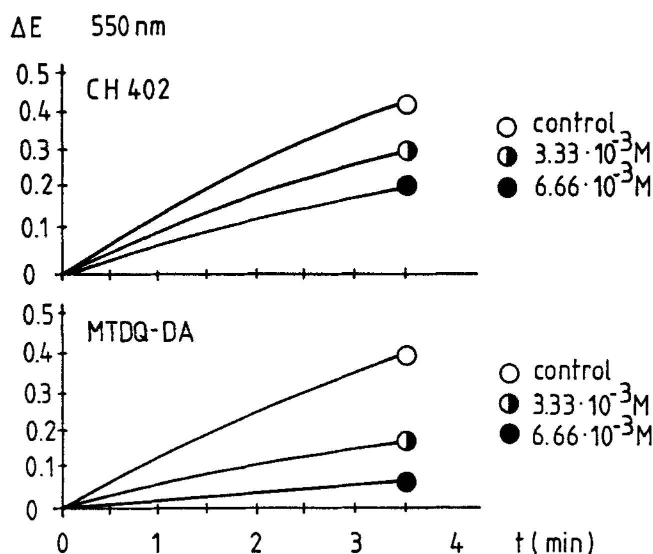

FIGURE 6 Inhibitory effect of antioxidants on the reduction of cytochrome-c by superoxide in xanthine/xanthine oxidase system. Temperature: 20°C (○) control; (◑) 3.33×10^{-3} M CH402 or MTDQ-DA; (●) 6.66×10^{-3} M CH402 or MTDQ-DA

TABLE 2
Scavenger effect on superoxide anion by CH402 and MTDQ-DA in xanthine/xanthine oxidase system

Concentrations (M)	Chemiluminescence intensity (mVs) Antioxidants CH402	MTDQ-DA
0	182.918	182.918
8×10^{-6}	233.390	101.459
8×10^{-5}	97.210	36.390
8×10^{-4}	20.824	5.124
10^{-3}	0.000	0.000

Measurements were performed in triplicate. SD was less than 5%. Temperature was 20°C. Reaction time was 60 sec.

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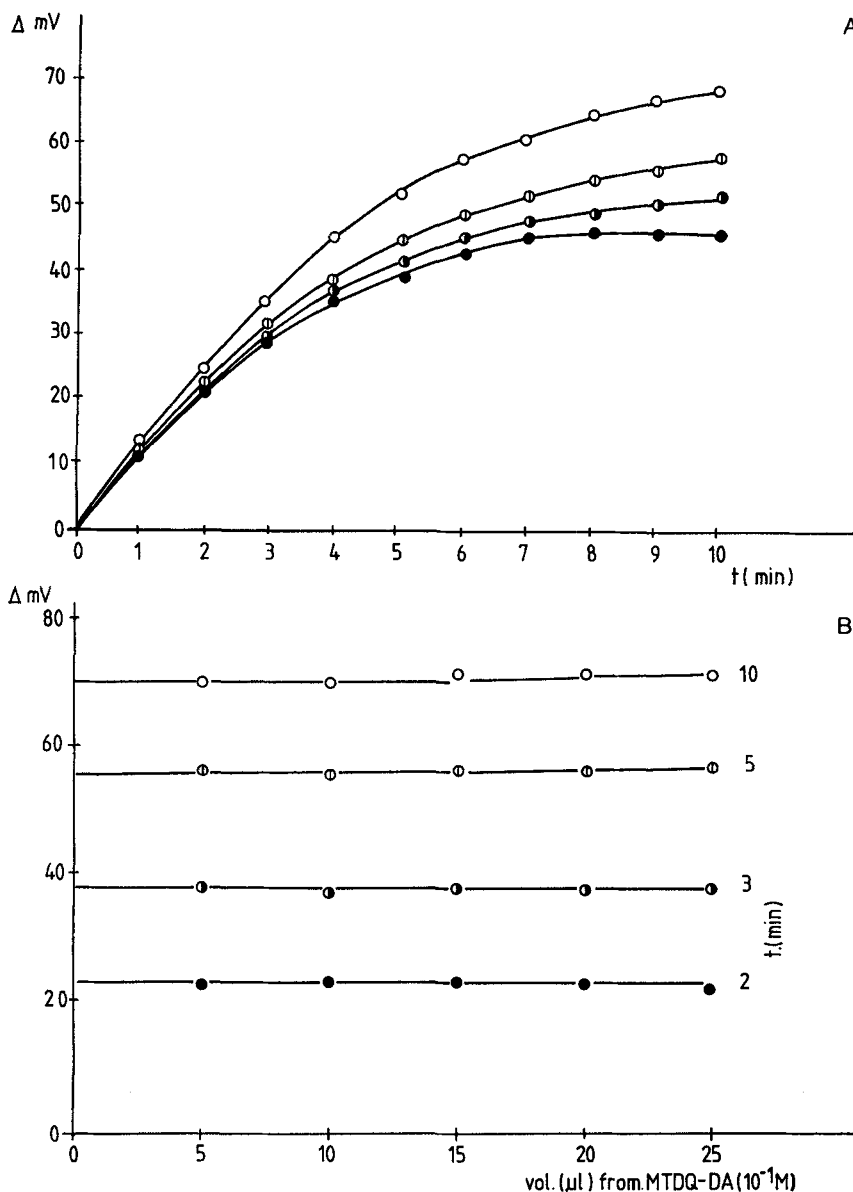

FIGURE 7 A: Amperometric measurement of the oxygen concentration in xanthine/xanthine oxidase system (○) 50 μl of xanthine (10^{-2} M); (◐) 40 μl of xanthine (10^{-2} M); (◑) 30 μl of xanthine (10^{-2} M); (●) 20 μl of xanthine (10^{-2} M). B: Effect of MTDQ-DA or CH-402 on xanthine oxidase Xanthine concentration: 7.14×10^{-5} M, total volume: 7000 μl Fk-71 buffer (pH 7.0), 30 μl 0.1 U/ml xanthine oxidase, temperature 25°C.

To study the direct effects of CH402 and MTDQ-DA on the xanthine oxidase enzyme system, we also employed an amperometric study. Figure 7A shows the calibration curves of xanthine concentrations, transformed into mV with time. Figure 7B indicates that these derivatives do not inhibit the xanthine–uric acid

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enzymatic conversion even at high concentration, 3.5×10^{-4} M. In this experiment, we could not detect any inhibition mechanism in the measured concentration range of the derivatives, even if we changed the xanthine concentration or the reaction time, respectively.

All the results shown above are consistent with the direct $O_2^{-\cdot}$ scavenging activity of water soluble dihydroquinoline type antioxidants, MTDQ-DA and CH402. Based on EPR studies, these compounds may act as direct scavengers of superoxide dominantly in the aqueous phase of biological samples such as the human plasma and extracellular fluids.

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