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3,4-Dihydroxymandelic Acid, a Noradrenalin Metabolite with Powerful Antioxidative Potential

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The decarboxylated noradrenaline metabolite 3,4-dihydroxymandelic acid [DHMA, 2-(3,4-dihydroxyphenyl)-2-hydroxyacetic acid] occurs in different mammalian tissues, especially in the heart. To elucidate the physiological function of DHMA, the antioxidative and radical scavenging activity was determined by physicochemical and cell-based test systems. In the 2,2-diphenyl-1-picrylhydrazyl assay it shows a 4-fold higher radical scavenging activity compared to the standard antioxidants ascorbic acid, tocopherol, and butylated hydroxytoluene. DHMA is also a very potent superoxide radical scavenger and shows a 5-fold smaller IC_{50} value compared to standard ascorbic acid. Again, in most cases the antioxidative power of DHMA against bulk lipid oxidation determined by accelerated autoxidation of oils is much higher than for the standard antioxidants. In soybean oil and squalene a DHMA/α-tocopherol mixture (1:1 w/w) shows a synergistic effect. Last but not least, 0.001 and 0.0005% levels of DHMA protect human primary fibroblasts against H_2O_2-induced oxidative stress as determined by the 2′,7′-dichlorofluorescein assay.

KEYWORDS: Antioxidants; radical scavengers; 3,4-dihydroxymandelic acid [DHMA, 2-(3,4-dihydroxyphenyl)-2-hydroxyacetic acid]; DPPH assay; DCF assay; lipid peroxidation

INTRODUCTION

Antioxidants play a crucial role in the protection of organisms or tissues as well as of nonliving systems against oxidative stress (*1*). It is assumed that aging of organisms (*2*) and many severe lesions or diseases such as skin sagging, arteriosclerosis, cataract formation, and cancer are caused or accelerated by oxidative stress triggered by reactive oxygen species (ROS), which can be generated by natural and artificial processes such as radiation, chemicals, and smoking (*3*).

A complex enzymatic and molecular antioxidant defense system has evolved in organisms, which protects against ROS (*4*). However, a high level of ROS can tilt the balance toward a prooxidant state. The resulting oxidative stress is responsible for the damage of cellular molecules, especially DNA, proteins, and unsaturated lipids. This situation leads to degeneration of cellular and interstitial structures and therefore to apoptosis or necrosis of cells or tissue (*5*). The most important molecular antioxidants in mammals are α-tocopherol, L-ascorbic acid, ubiquinone, lipoic acid, and glutathione, which are able to scavenge most ROS directly (*6*).

In food, oxidative stress damages many constituents, mainly lipids, proteins, colorants, and flavor compounds. In most cases this process leads to negative nutritive or sensory properties of many food products. For this reason antioxidants are added primarily to inhibit the autoxidation of highly unsaturated lipids or flavor compounds, especially unsaturated terpenoids. In particular, the autoxidation of unsaturated fatty acids generates typical flavors and sometimes even off-flavors in many foodstuffs. The long-chain polyunsaturated fatty acids are degraded to different kinds of aldehydes such as *n*-alkanals (e.g., 1-hexanal), related α,β-unsaturated aldehydes including *E*-2-alkenals or 2,4-alkadienals [e.g., (2*E*)-hexenal, (2*E*)-nonenal, (*2E*,*4E*)-2,4-nonadienal], and 4-hydroxy-2-alkenals (e.g., 4-hydroxy-2-nonenal) (*7*). The unsaturated aldehydes cause the characteristic rancid and metallic flavor of oxidized fats and oils (*8*). In addition, the α,β-unsaturated fatty aldehydes and their oxygenated metabolites show biological activity as alkylation agents and are suspected to be genotoxic (*9*).

In our efforts to develop antioxidants for food applications, we found that 3,4-dihydroxymandelic acid [DHMA, 2-(3,4-dihydroxyphenyl)-2-hydroxyacetic acid] shows a very high antioxidative potential.

In addition to normetanephrine and vanillomandelic acid, DHMA is one important metabolite of noradrenaline as outlined in **Figure 1**. Usually, noradrenaline is O-methylated in a first metabolic step to normetanephrin and further oxidized to vanillomandelic acid. Without the O-methylation DHMA is generated directly by oxidative deamination; DHMA was found in plasma and excreted urine in concentrations of 0.3−0.6 pg

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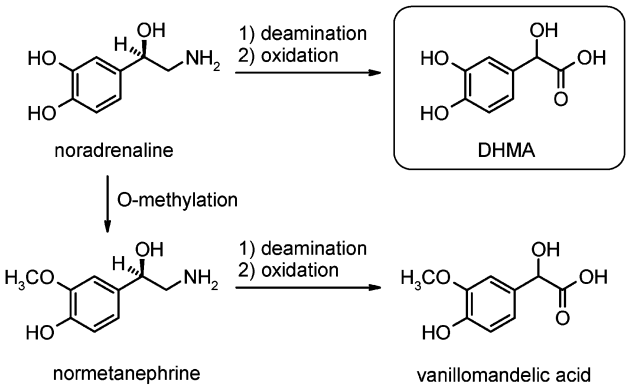

Figure 1. Formation of vanillomandelic acid and DHMA by oxidative metabolism of noradrenaline.

m/L (*10*). In addition, DHMA is a product of the tyrosinase-catalyzed oxidation of 3,4-dihydroxyphenylacetic acid and is further oxidized to 3,4-dihydroxybenzaldehyde (*11*). In rats, DHMA especially accumulates in aged individuals in the heart muscle (480 pmol/g; noradrenalin, 203 pmol/g); in the other tissues evaluated (aorta, kidney, and liver) the concentration is 4−20-fold smaller (*12*). In a further study DHMA was found in the skin excretions of cattles (*13*). Until now it is unknown whether DHMA shows important functionality in cells or is only metabolic "garbage".

MATERIALS AND METHODS

Squalene was obtained from E. Merck (Darmstadt, Germany); evening primrose oil (EPO) and soybean oil were obtained from Henry Lamotte (Bremen, Germany). Dulbecco's modified Eagle's medium (MEM), penicillin/streptomycin solution, Hank's balanced salt solution (HBSS), and *N*-(2-hyroxyethyl)piperazine *N*′-(2-ethanesulfonic acid) (HEPES) were obtained from Biochrom (Berlin, Germany). Fetal calf serum (FCS) was purchased from Life Technologies (Karlsruhe, Germany); 6-carboxy-2′,7′-dichlorohydrofluorescein diacetate, di-(acetomethyl ester) (C-H_2DCF-DA/AM) was from Molecular Probes (Eugene, OR). The cytotoxicity detection kit was obtained from Roche Diagnostics (Mannheim, Germany). DHMA, vanillomandelic acid, 3-(4,5-dimethylthiazol-2-yl)-2,5-dipenyltetrazolium bromide (MTT), and all other chemicals were obtained from Sigma-Aldrich (Deisenhofen, Germany) or Lancaster Synthesis (Mülheim, Germany). The oxidative stability of bulk lipids was measured using the Rancimat 679 (Deutsche Metrohm GmbH, Filderstadt, Germany). Chemiluminescence was determined using a Berthold LB96P (Perkin-Elmer GmbH, Überlingen, Germany). Photometric determinations were conducted using a lambda 12 UV−vis spectrophotometer (Perkin-Elmer, Überlingen, Germany). Cytotoxicity assays were performed using an iEMS multiwell plate reader (Labsystems, Frankfurt, Germany); the 2′,7′-dichlorofluorescein (DCF) assay was conducted using a Fluoroskan Ascent FL multiwell plate reader (Labsystems).

DPPH Assay. The relative ability of test compounds to abstract hydrogen radicals was evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. In disposable polystyrene cuvettes 2.5 mL of a methanolic DPPH solution (100 μmol/L) was mixed with 500 μL of the test solutions of different concentrations (methanol as control), and the decrease in the concentration of the purple radical was determined photometrically at 514 nm until the decrease was <2% per hour when measured against negative control (steady state). The concentration-dependent activity of the test compounds as free radical scavengers was calculated using the following equation:

$$\text{activity}_{\text{concn}}\,(\%) = 100 - (\text{remaining DPPH}) \qquad (1)$$

The remaining DPPH was calculated from absorptions by

$$\text{remaining DPPH}\,(\%) = \text{absorption}_{(\text{test at steady state})}/\text{absorption}_{(\text{control at } t_0)} \qquad (2)$$

The activity (percent) in a series of dilutions of one test compound was used to calculate the effective relative concentration EC_{50} (based on the starting concentration of DPPH, $EC = c_{\text{test}}/c_{(\text{DPPH at } t_0)}$), at which 50% of DPPH has been removed. The assay was performed in triplicate, and the absorptions were averaged before calculation. α-Tocopherol, L-ascorbic acid, and BHT were used as standards.

SO Scavenging Assay. The test compounds were diluted starting from a dimethyl sulfoxide stock solution (10 mmol/L) using phosphate-buffered saline (PBS). Each well of a 96-well white polystyrene plate was subsequently charged with 50 μL of luminol (200 μmol/L in PBS), 50 μL of horseradish peroxidase (HRPO; 1 unit/mL in PBS), and 50 μL of the test solutions of different concentrations (e.g., 40 μmol/L). The mixtures were incubated at 37 °C for 5 min. Finally 50 μL aliquots of H_2O_2 (40 μmol/L) were added and the chemiluminescence was recorded immediately for 10 s. The activity was calculated as inhibitor concentration IC_{50}, at which 50% of the generated superoxide was depleted. L-Ascorbic acid was used as standard.

Rancimat Assay. Prior to the assays the lipids were purified as follows: Soybean oil, EPO, and squalene were dissolved in *n*-heptane. The solutions were passed through a glass column charged with activated alumina type N by pressurized nitrogen. The eluent was collected under nitrogen. The solvents of the purified solutions were distilled off under reduced pressure. In the case of squalene, a solution of α-tocopherol in *n*-heptane was added to give a final concentration of 1 ppm α-tocopherol prior to evaporation.

The test compounds were dissolved in methanol or acetone (15 mg/mL for soybean oil and EPO and 1.5 mg/mL for squalene), and 100 mL of these solutions was added to a pre-prepared 3 g lipid sample in the reaction vessels of the Rancimat apparatus. Pure solvent was added to control samples. A constant dry air stream (20 L/h) was blown through the sample during heating to 100 °C for soybean oil and EPO and to 80 °C for squalene. The volatile oxidation products were collected in the measuring vessels containing water, and the conductivity of the aqueous solution was measured continuously and recorded. The induction periods (IP) were calculated automatically by the Rancimat. All tests were run in duplicate and averaged before calculation. The following equation was used to calculate the AOI:

$$\text{AOI} = \text{IP}_{\text{test}}/\text{IP}_{\text{control}} \qquad (3)$$

α-Tocopherol and butylated hydroxytoluene (BHT) were used as standards.

Cytotoxicity Assay. Prior to the DCF assay, the cytotoxicity of DHMA was proven. Therefore, human dermal fibroblasts were seeded in 96-multiwell plates at a density of 5×10^3 cells per well. Cells were cultured under normal cell culture conditions (37 °C, 5% CO_2) using Dulbecco's MEM including 10% FCS and 100 units/100 μg/mL penicillin/streptomycin. After reaching confluency, cells were supplemented with different concentrations of DHMA (0.01, 0.001, and 0.0001%). After supplementation periods of 24, 48, and 72 h, the LDH concentration in the supernatant and cellular MTT conversion were measured.

DCF Assay. Human dermal fibroblasts were seeded in a 48-multiwell plate at a density of 1×10^4 cells per well and cultured under normal cell culture conditions (37 °C, 5% CO_2) until confluency. Afterward, cells were supplemented with different concentrations of DHMA (0.001, 0.0005, and 0.0001%) and incubated for 48 h. DHMA was added to the cell culture by preparing a stock solution using 5% DMSO in medium and further dilution.

After two washings with HBSS containing 20 mmol/L HEPES, the cells were stained with 2.5 μmol/L C-H_2DCF-DA/AM for 45 min and again washed carefully with HBSS/HEPES. The cells were covered with 200 μL of HBSS/HEPES, and background fluorescence was measured over a period of 15 min. For the measurement of the antioxidative capacity, oxidative stress was induced extracellularily by the addition of 100 μmol/L H_2O_2. The subsequent intracellular radical

Table 1. Radical Scavenging Action Determined Using DPPH and SO Scavenging Assay and Antioxidative Activity against Accelerated Autoxidation of Bulk Lipids of DHMA Compared to Vanillomandelic Acid and Standards Ascorbic Acid, α-Tocopherol, and BHT

	DPPH	SO	Rancimat AOI		
compound	EC_{50} (mol/mol)	IC_{50} (nmol/L)	100 °C, soybean oil, 0.05%	80 °C, squalene, 0.005%	100 °C, EPO, 0.05%
DHMA	0.062 ± 0.005	130	9.5 ± 0.2	34 ± 2	6.7 ± 0.5
vanillomandelic acid	0.315 ± 0.007	550	1.2 ± 0.1	1.9 ± 0.1	
α-tocopherol	0.25 ± 0.02	>10000	5.1 ± 0.1	39 ± 4	3.4
ascorbic acid	0.27 ± 0.02	700	1.2 ± 0.1	0.7 ± 0.1	
BHT	0.24 ± 0.01	>10000	4.6 ± 0.7	19 ± 1	

production was detected by fluorescence (FL) measurement of oxidized DCF. Antioxidative capacity was calculated by

$$\text{antioxidative capacity (\%)} = (FL_{stress} - FL_{background})_{blank}/(FL_{stress} - FL_{background})_{test} \quad (4)$$

RESULTS AND DISCUSSION

To determine the antioxidant and radical scavenging action, we have used several established or modified assays, which cover different aspects of radical species and mechanistic details. For radical scavenging measurement we chose DPPH (*14*) and superoxide anion (SO) quenching assays, and for antioxidative power in lipid systems the accelerated autoxidation of bulk oils was measured using the Rancimat. Last but not least an in vitro cell culture model using human primary fibroblasts was used to determine the antioxidative potential of DHMA in living systems.

For evaluation of physiological antioxidants it is very important to measure their SO scavenging activity. SO plays a pivotal role in oxidative stress under physiological conditions, because it is generated by leakage of the respiratory chain located in mitochondria (*15*). In healthy organisms SO is deactivated by the superoxide dismutase/catalase enzyme system, but if the balance is moved to oxidative stress, it is important to scavenge this ROS, because SO is converted to the highly aggressive hydroxyl radical. Hydroxyl is a very oxidizing species; nearly all of its reactions have negative Gibbs energy (*16*). Therefore, hydroxyl is trapped by many other organic cell "bulk" compounds, for example, lipids, carbohydrates, DNA, and proteins. For the SO scavenging assay the superoxide radical was generated in a hydrogen peroxide/horseradish peroxidase system (*17*). The SO was detected by recording chemiluminescence amplified by luminol. The SO assay is applicable only for water soluble antioxidants.

As summarized in **Table 1** DHMA is a powerful antioxidant and/or radical scavenger in all performed test systems. In DPPH assay it shows a superior EC_{50} compared to vanillomandelic acid and the standard antioxidants ascorbic acid, tocopherol, and BHT. DHMA reaches the steady state after 1 h, α-tocopherol after 20 min, ascorbic acid after 10 min, BHT after 7 h, and vanillomandelic acid after 23 h. Because the kinetic behavior of the DPPH radical is very complex, detailed knowledge of the meaning of the steady state is not available; for only some reference compounds was the mechanism of antioxidative activity deduced from DPPH data (*18*). As shown in **Figure 2** a plot of the remaining DPPH concentration against the logarithm of the effective DHMA concentration results in a clear dose−response relationship and reaches a plateau at EC = 0.13 mol/mol. According to Brand-Williams (*14*) the antiradical power (ARP) was calculated as the reciprocal of EC_{50}; DHMA has an ARP of 16 compared to 4 for ascorbic acid, BHT, and tocopherol and 3.2 for vanillomandelic acid. DHMA is also a very potent SO scavenger and shows a 5-fold smaller IC_{50} value compared to that of standard ascorbic acid.

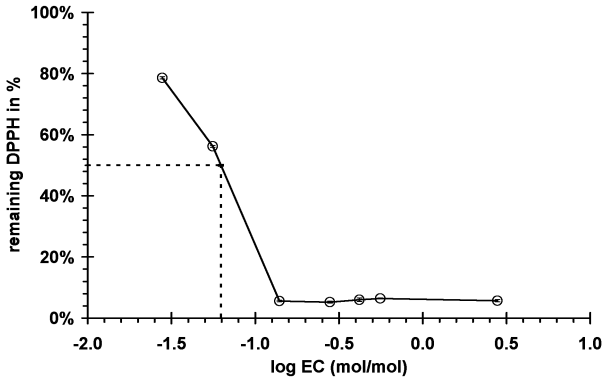

Figure 2. Dose−response plot for DHMA in the DPPH assay. Remaining DPPH was calculated after reaching steady state (1 h). DPPH concentration was determined photometrically at 514 nm.

For the determination of the antioxidative potential against lipid autoxidation we have used the accelerated autoxidation of bulk oils by air with or without test compound (*17*, *19*). Oxidation of the unsaturated lipids proceeds only very slowly for the induction period (IP) and then suddenly increases in the propagation period. The oxidation was carried out in the Rancimat apparatus with stripped soybean oil, EPO, or squalene. Squalene was chosen as a highly oxidizable lipid found in human sebum and olive oil. Squalene shows an IP of 0.8−1 h at 80 °C, and soybean oil and EPO have IP values of 2−3 h at 100 °C. IP determinations by the Rancimat method correlate very well with other established measurements of oxidative stability of bulk oils, for example, chemiluminescence, peroxide value by iodometric titration, and shift of double bond (*20*); Gordon and Mursi showed that the IP values obtained at 100 °C correlate highly with oil stability as measured by peroxide value during storage at 20 °C (*21*).

Again, the antioxidative power of DHMA against lipid oxidation determined by the Rancimat method is much higher than for vanillomandelic acid and the standards, with the exception of the experiment in squalene, which resulted in nearly identical AOI values compared to α-tocopherol. In **Figure 3**, the results of the synergistic action of a 1:1 mixture of tocopherol and DHMA are shown. Tocopherol and DHMA were used as single components and as a 1:1 mixture in the same concentration. The theoretical activity was calculated by $AOI_{calcd} = 1 + [(AOI_{toco} - 1) + (AOI_{DHMA} - 1)]$. In fact, in the experiment the 1:1 mixture has a much higher AOI in soybean oil and squalene compared to the calculated AOI and shows a real synergistic effect.

As shown earlier for other polyphenolic antioxidants (*14*, *17*, *22*), the catechols show higher antioxidant activity than their monophenolic counterparts in most cases. Comparison of DHMA to vanillomandelic acid shows that the *o*-dihydroxy

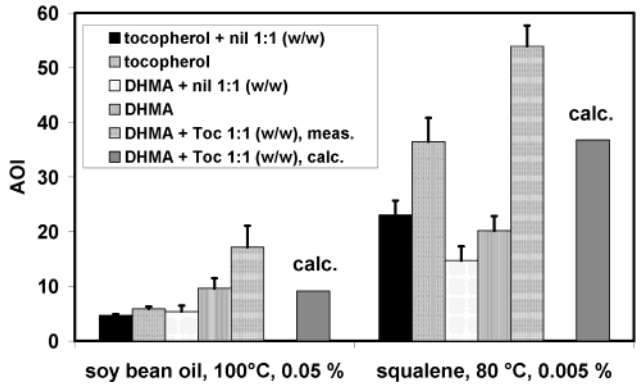

Figure 3. Synergistic effect of a DHMA/α-tocopherol mixture in stripped soybean oil and stripped squalene (stabilized with 1 ppm of tocopherol) determined by accelerated autoxidation in the Rancimat. Individual AOI values were calculated by $AOI = IP_{test}/IP_{blank}$; theoretical activity was calculated by $AOI_{calcd} = 1 + [(AOI_{toco} - 1) + (AOI_{DHMA} - 1)]$.

moiety seems to be the most important cause of the high efficacy. As suggested in **Figure 4** DHMA can be oxidized by reactive radicals in some subsequent steps; the intermediates are stabilized by resonance and can scavenge further radicals. In a first step a phenolic hydrogen is abstracted from DHMA by a reactive radical. The resulting semiquinone radical is stabilized by intramolecular hydrogen bonding (*23*), and this effect is obviously absent in the monophenolic antioxidants. In contrast to non-catechols a second phenolic hydrogen can be abstracted and an *o*-quinone is generated, which can tautomerize to a quinonemethide. Furthermore, one-electron oxidations or hydrogen abstractions from enolic hydroxyls can occur as well as decarboxylation and Michael reactions at the α,β-unsaturated ketone moieties.

The DCF assay is a highly sensitive fluorescence-based assay that allows the determination of antioxidative capacities in living cells (*24*). The cell-based approach not only takes the bioavailability of DHMA into account but also considers cell-specific regulatory mechanisms that may be triggered by stimuli such as oxidative stress. Human dermal fibroblasts were presupplemented with DHMA. Prior to the experiment excessive DHMA was removed from the supernatant. Antioxidative capacity was determined by staining the cells with the nonfluorescent dye C-H_2DCF and subsequent induction of oxidative stress using hydrogen peroxide. The ROS oxidize the dye, which leads to a characteristic fluorescence emission. Cells with a high antioxidative capacity can prevent the dye from being oxidized, which results in a lower fluoresence emission than that of cells with a low antioxidative capacity.

As indicated in **Figure 5** the cytotoxicity assays performed prior to the DCF assay revealed no cytotoxic effect for 0.001 and 0.0001% DHMA. Neither LDH release nor MTT conversion rate differed from those of control cells. In contrast, an acute cytotoxic effect was found for the highest dose. After a supplementation period of 24 h, 0.01% DHMA caused a 2.45-fold increase in extracellular LDH concentration relative to that of the control cells. During the next 48 h extracellular LDH decreased to 120% of the control value, which may be due to instability of LDH in media at 37 °C. Concomitant to the LDH release, MTT conversion rate differed in cells supplemented with 0.01% DHMA compared to control cells with an increase after 24 h of supplementation and a decrease after 48 and 72 h. As one referee suggested, the cytotoxicity found for 0.01% at 24 h could be due to cell culture adaptation. Thus, DHMA below 0.001% is considered not to be cytotoxic, so the DCF assay

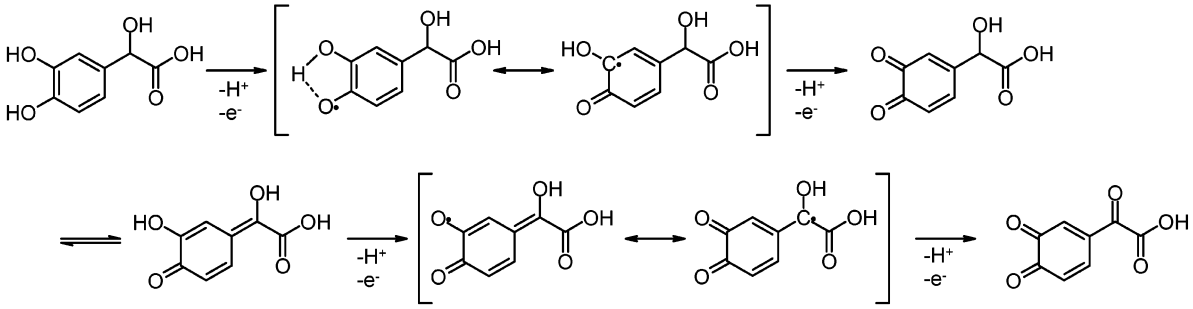

Figure 4. Proposal for possible oxidation reactions of DHMA; the radicals are stabilized by resonance. Further reactions, for example, decarboxylation or Michael addition at the α,β-unsaturated ketone moieties, can occure.

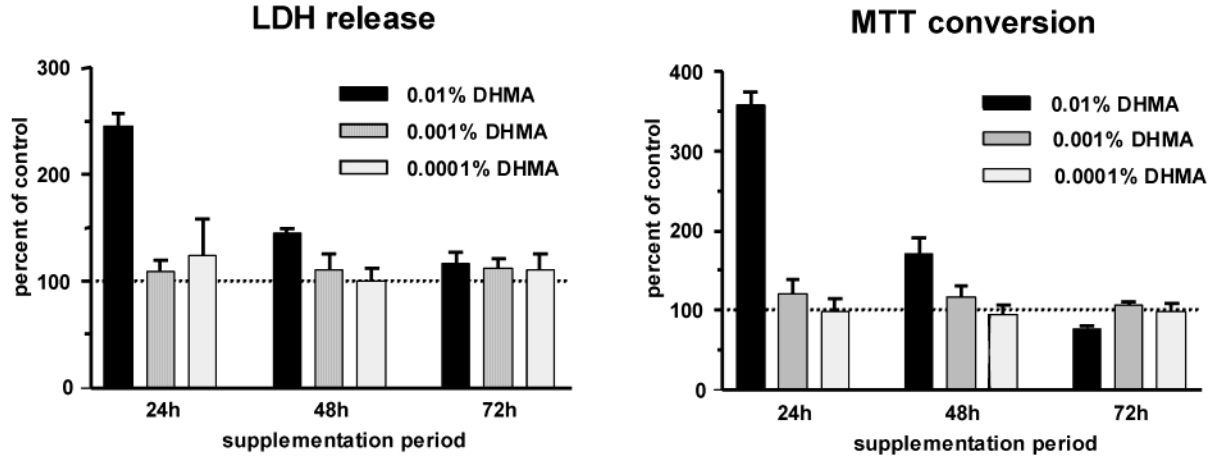

Figure 5. Results of cytotoxicity determination on human dermal fibroblasts incubated at 37 °C and 5% CO_2. After supplementation periods, LDH concentration in the supernatant and cellular MTT conversion were measured.

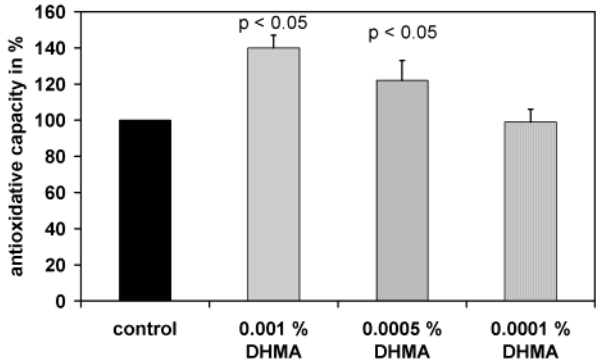

Figure 6. Antioxidative capacity in human dermal fibroblasts of DHMA against control. Cells were incubated with DHMS at 37 °C and 5% CO_2 for 48 h, washed, stained with C-H_2DCF-DA/AM for 45 min, and again washed carefully. After measuremtn of background fluorescence, H_2O_2 was added and fluorescence was measured. Antioxidative capacity was calculated by $(FL_{stress} - FL_{background})_{blank}/(FL_{stress} - FL_{background})_{test}$; statistical significance was calculated by paired *t* test.

was performed with concentrations between 0.001 and 0.0001% DHMA.

In the cell-based DCF assay, DHMA showed a significant and dose-dependent antioxidative potency (**Figure 6**). Supplementation with 0.001% DHMA improved the antioxidative capacity of human fibroblasts by 40%, and 0.0005% DHMA gave rise to a 20% increase in the antioxidative capacity. The increase in the antioxidative capacity by use of 0.001% DHMA is comparable to that observed by the use of 25.0 μmol/L α-tocopherol (33% increase; own observations, data not shown). However, addition of 0.0001% DHMA to the cell culture medium showed no antioxidative effect. These results not only confirm the antioxidative effects found in the cell-free assays mentioned above but also clearly demonstrate the bioavailability of DHMA.

In conclusion, DHMA is a potent radical scavenger and a powerful antioxidant for lipids. Tocopherol and DHMA combinations seem to be very efficient synergistic antioxidants for bulk lipids. DHMA in a concentration below 0.001% is not cytotoxic, supports intracellular antioxidative status, and therefore may be important for the defense system of cells against oxidative stress. Because DHMA is readily available (*25*), it may be used as an antioxidant, but for food and cosmetic applications further research is needed to evaluate its toxicological and pharmacokinetic properties.

ABBREVIATIONS USED

ARP, antiradical power; AOI, antioxidative index; BHT, butylated hydroxytoluene; C-H_2DCF-DA/AM, 6-carboxy-2′,7′-dichlorohydrofluorescein diacetate, di(acetomethyl ester); DCF, 2′,7′-dichlorofluorescein; DHMA, 3,4-dihydroxymandelic acid; DPPH, 2,2-diphenyl-1-picrylhydrazyl; EC, effective concentration; EPO, evening primrose oil; FCS, fetal calf serum; FL, fluorescence; HEPES, *N*-(2-hydroxyethyl)piperazine *N*′-(2-ethanesulfonic acid); HBSS, Hank's balanced salt solution; HRPO, horseradish peroxidase; IC, inhibitor concentration; IP, induction period; LDH, lactate dehydrogenase; MEM, modified Eagle's medium; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; ROS, reactive oxygen species; SO, superoxide radical anion.

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