ATTENUATION OF LIPID AND PROTEIN OXIDATION BY *CHALCONE DERIVATIVES* IN NEUROBLASTOMA CELLS AGAINST H_2O_2-INDUCED OXIDATIVE STRESS

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Summary

It is by now well accepted that oxidative stress plays a vital role in initiation and progression of the brain lipid and protein oxidation of Alzheimer patients. Hydrogen peroxide (H_2O_2) ,as a pro-oxidant, interacts with transition metals via fenton reaction and produces hydroxyl radicals that initiate lipid peroxidation through malondialdehyde (MDA) production and protein carbonyl oxidation (PCO). Therefore, to back up the system under oxidative stress conditions, it might be beneficial to use antioxidants. Chalcone derivatives (1, 3-diaryl-2-propen-1-ones) are plant flavonoides with free radical scavenging capabilities. The main difference among these derivatives is on their hydroxyl substitution patterns especially on their phenolic rings. The objective of this study was to examine the influence of thirteen different synthetic chalcone derivatives (compound 8-20) on the activity of some of the antioxidant enzymes including CAT, SOD and GPx as well as on the levels of MDA, PCO and GSH against H_2O_2-induced damage on SK-N-MC cells. Our results indicated that chalcone derivatives especially vanilline analogues increased the activities of SOD, CAT and GPx in drug-pretreated relative to H_2O_2-treated cells. In addition, they were capable of reducing the formation of MDA and PCO. The destructive effect of H_2O_2 on the GSH level of the cells was almost totally restored by each of the derivatives. These properties probably are due to free radical scavenging activity of each of the derivatives and thus, it is expected these compounds would attenuate the destructive effects of oxidants.

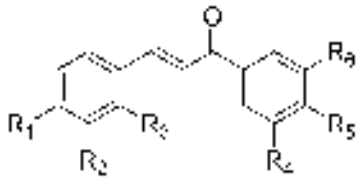
Keywords: Chalcone, Hydrogen peroxide, malondialdehyde, protein carbonyl oxidation, Reactive oxygen species, SK-N-MC cell.

Introduction

Reactive oxygen species (ROS) and free radicals have been associated with progression of many neurodegenerative disorders such as Alzheimer's disease (AD) [1]. The brain is considered the most vulnerable site of oxidative damage mainly due to the high content of polyunsaturated fatty acids in the brain [2]. Oxidative stress is generally defined as excess formation and/or incomplete removal of highly reactive molecules such as reactive oxygen species (ROS) [3]. ROS include free radicals such as superoxide ($O_2^{\bullet-}$), hydroxyl ($^{\bullet}OH$), peroxyl ($RO_2^{\bullet-}$) radicals as well as nonradical species such as hydrogen peroxide (H_2O_2) [4,5]. Hydrogen peroxide is a pro-oxidant agent which can lead to oxidative stress via fenton reaction and produces ROS especially hydroxyl radicals ($HO^{\bullet}$) [6,7]. Hydroxyl radicals ($HO^{\bullet}$) attack proteins and lipids, leading to the formation of protein carbonyl (PCO) via protein oxidation and 4-hydroxy-2-nonenal (HNE) and malondialdehyde (MDA) via lipid peroxidation [8, 9].

Antioxidant compounds can scavenge free radicals by retarding the process of lipid peroxidation and protein oxidation [10]. Biological antioxidants are natural compounds which can prevent the uncontrolled formation of free radicals and activated oxygen species, or inhibit their reaction with biological structures. These compounds include antioxidative enzymes, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR) and non-enzymatic antioxidants, such as glutathione (GSH), vitamin C and vitamin E [11].

Chalcones (1, 3-diaryl-2-propen-1-ones) constitute an important class of natural products related to the flavonoid family with interesting biological activities [12]. Our pervious in vitro study has shown that chalcone derivatives (compounds 8 to 20) can scavenge free radicals [13]. The free radical-scavenging activity of chalcone derivatives depends on the molecular structure and the substitution pattern of hydroxyl groups on phenolic rings [12,13]. Structurally, they are made of two aromatic rings which are joined by a three-carbon α, β-unsaturated carbonyl segment [14]. One of the rings is an acetophenone with different substitutions and the second ring is either a vanillin (4-hydroxy-3-mehoxybenzaldehyde (compounds 8-11), isovanillin (3-hydroxy-4-methoxybenzaldehyde (compounds 12-15), O-vanillin (2-hydroxy-3-methoxybenzaldehyde (compounds 16-19) and 3, 4-dimethoxy benzaldehyde (compound 20) (scheme 1 and Table 1) [15].



Scheme 1: Chalcone derivatives: R1-R6 = H, OMe, F or -CH_2O-

The objective of this study was to examine the influence of chalcone derivatives (compound 8-20) on the activity of some of the antioxidant enzymes including CAT, SOD and GPx as well as on the levels of MDA, PCO and GSH against H_2O_2-induced damage on SK-N-MC cells. Our results indicated that chalcone derivatives especially vanillin analogues (compound 8-11) increased the activities of SOD, CAT and GPx relative to H_2O_2-treated cells. Based on the results

obtained in this study, chalcone derivatives would be capable of reducing the levels of MDA and PCO and consequently the GSH level within the cells under oxidative stress.

Table 1: Substituted 1, 3-Diphenyl-2-propen-1-ones derivatives (compounds 8-20) used in the present study. For the synthetic methods, please refer to Jung et al [15].

Compound	R_1	R_2	R_3	R_4	R_5	R_6	formula
8	OH	OMe	H	H	H	H	$C_{16}H_{14}O_4$
9	OH	OMe	H	H	-OCH_2O-		$C_{17}H_{14}O_5$
10	OH	OMe	H	F	F	F	$C_{16}H_{11}F_3O_3$
11	OH	OMe	H	F	OMe	H	$C_{17}H_{15}FO_4$
12	OMe	OH	H	H	H	H	$C_{16}H_{14}O_4$
13	OMe	OH	H	H	-OCH_2O-		$C_{17}H_{14}O_5$
14	OMe	OH	H	F	F	F	$C_{16}H_{11}F_3O_3$
15	OMe	OH	H	F	OMe	H	$C_{17}H_{15}FO_4$
16	H	OMe	OH	H	H	H	$C_{16}H_{14}O_3$
17	H	OMe	OH	H	-OCH_2O-		$C_{17}H_{14}O_5$
18	H	OMe	OH	F	F	F	$C_{16}H_{11}F_3O_3$
19	H	OMe	OH	F	OMe	H	$C_{17}H_{15}FO_4$
20	OMe	OMe	H	H	-OCH_2O-		$C_{18}H_{18}O_5$

Materials and methods

Materials

The cell culture medium (RPMI-1640), fetal bovine serum (FBS) and penicillin–streptomycin were purchased from Gibco BRL (Life Technology, Paisley, Scotland). Cell line was obtained from Pasteur Institute of Iran (Tehran, Iran). The culture plates were obtained from Nunc (Brand products, Denmark). Nicotinamide adenine dinucleotide reduced (NADH), nicotinamide adenine dinucleotide phosphate reduced (NADPH), phenazine methosulphate (PMS), nitroblue tetrazolium (NBT), H_2O_2 and 2-thiobarbituric acid (TBA) were obtained from Merck (Germany). Triton x-100 was purchased from Pharmacia LKB Biotechnology (Sweden). Glutathione (GSH), TCA (Trichloroacetic acid) and phenylmethylsulphonyl fluoride (PMSF) were from Sigma Chem. Co (Germany). Ethylenediaminetetraacetic acid (EDTA) was from Aldrich. Monochlorobimane (mBCl) was purchased from Molecular probe (Eugene, Oregon, USA).

The Chalcone derivatives (1, 3-diphenyl-2-propen-1-ones) have been synthesized as reported previously [15]. They were dissolved in a minimum amount of dimethyl sulfoxide (DMSO) and then diluted with the culture medium to get the desired concentration. The concentration of DMSO in the culture medium has been kept lower than 0.1% and the control cells have been treated with the vehicle containing the same amount of DMSO.

Cell culture and experimental treatment

Human SK-N-MC neuroblastoma cells, obtained from Pasteur Institute (Tehran, Iran), were cultured at a density of 5×10^4/ml RPMI 1640 medium supplemented with FBS (10%, v/v), streptomycin (100 µg/ml) and penicillin (100 U/ml) and kept at 37 °C in a 5% CO_2 humidified

atmosphere. Drug treatments were usually done 24 h after seeding the cells. To induce the oxidative stress, H_2O_2 was freshly prepared from 8.5 mM stock solution prior to each experiment. SK-N-MC cells were incubated with 20 μM of chalcone derivatives (compound 8-20) for 3 h before exposure to 300 μM H_2O_2 [13].

Catalase activity assay

The CAT activity was measured by the method of Aebi [16] in which the rate of decomposition of H_2O_2 was determined spectrophotometrically at 240 nm. The enzyme activity was expressed as $\times 10^{-1}$ k/mg protein, where k represents the rate constant of the first order reaction of catalase. Protein concentration was determined by the method of Lowry et al [17].

Superoxide dismutase activity assay

The SOD activity was determined according to the method of Kakkar et al [18], based on the extent inhibition of amino blue tetrazolium formazan formation in the mixture of nicotinamide adenine dinucleotide, phenasine methosulphate and nitroblue tetrazolium (NADH-PMS-NBT). One unit of enzyme activity was defined as the amount of enzyme which caused 50% inhibition of NBT reduction/mg protein.

Glutathione peroxidase assay

GPx was assayed in a cuvette containing 0.890 mL of 50 mM potassium phosphate buffer (pH 7.0), 1mM EDTA, 1mM NaN_3, 0.2 mM NADPH, 1 U/mL GSH reductase and 1mM GSH. Cell lystate was added to make a total volume of 0.9 mL. The reaction was initiated by the addition of 100 μL of 2.5 mM H_2O_2, and the conversion of NADPH to $NADP^+$ was monitored with a spectrophotometer at 340 nm for 3 min. GPx activity was expressed as nmoles of NADPH oxidized to $NADP^+$/(min mg) protein, using a molar extinction coefficient of 6.22×10^6 (cm^{-1} M^{-1}) for NADPH [19].

Determination of protein carbonyl content

Oxidation of protein is accompanied by the formation of carbonyl protein which has been used widely as an index of protein oxidation. Treatment of the cells was achieved as described above, then washed twice with PBS, homogenized in 0.5 ml of lysis buffer (10% Triton X-100 in PBS) and centrifuged to remove debris. Then, 1 volume of 10% (w/v) streptomycin solution was added to 9 volume of supernatant and allowed to stand for 15 min. Centrifugation was carried out at 5000 rpm for 10 min, and the supernatant was taken for assay of protein-bound carbonyl groups [20]. The cell-free lystate (1 mg protein) was added in duplicate to phosphate buffer (10 mM, pH 6.8) to a final volume of 1 ml. One of the duplicate samples was treated with 0.2 ml of 2, 4-dinitorophenylhydrazine (DNPH, 10 mM in 2 N HCl). The other sample was treated with 0.2 ml 2 N HCl and it was used as the blank. The mixture was incubated at room temperature for 1 h, 0.25 ml 20% TCA was added followed by standing on ice for 10 min. The samples were centrifuged at 3000 rpm for 10 min, and the protein pellet was washed twice with 3 ml mixture of ethanol: ethyl acetate (1:1, v/v). The protein pellet was dissolved in 1 ml of 6 M guanidine hydrochloride. The absorbance around at 360 nm was used to quantitate protein carbonyl content, using a molar absorption coefficient of 22.0×10^3 cm^{-1} M^{-1} for aliphatic hydrazones. Data are expressed as amount of nmole carbonyl protein formed per mg total amount of protein.

Determination of lipid peroxidation

Thiobarbituric acid reactive substances (TBARS), as indicators of lipid peroxidation, were assayed as described in the literature [21]. Briefly, after exposure of SK-N-MC cells to chalcone derivatives (compound 8-20) for 3 h, cells were exposed to 300 μM H_2O_2 for 24 h. MDA levels were measured by the double heating method [22]. The method is based on spectrophotometeric measurement of the purple color generated by the reaction of thiobarbituric acid (TBA) with MDA. Briefly, the cells were mixed with 0.5 ml of tricholoroacetic acid (TCA, 10%, w/v) solution followed by boiling in a water bath for 15 min. After cooling to room temperature, the samples were centrifuged at 3000 rpm for 10 min and 0.5 ml of each sample supernatant was transferred to a test tube containing 0.25 ml of TBA solution (0.67%, w/v). Each tube was then placed in a boiling water bath for 15 min. After cooling to room temperature, the absorbance was measured at 532 nm with respect to the blank solution. The concentration of MDA was calculated based on the absorbance coefficient of the TBA-MDA complex ($\boldsymbol{\varepsilon} = 1.56 \times 10^5$ cm^{-1} M^{-1}) and it was expressed as nmol/mg protein [23].

Intracellular evaluation of reduced glutathione

Monochlorobimane (mBCl), which is a UV fluorogen bimane probe, reacts specifically with GSH through glutathione-S-transferase to form a fluorescent derivative [24]. In the present study, cells were pretreated with various concentrations of chalcone derivatives (20μM). Three hour later, H_2O_2 (300 μM) was added to the cells followed by incubation at 37° C for an additional 24 h. The mBCl diluted solution was added directly to the living cells (100 μM) for 15 min as previously described [25]. After incubation, cells were harvested and washed twice with PBS and their fluorescence was measured at an excitation wavelength of 360 nm and emission wavelength of 480 nm using a Varian spectrofluorometer, model Cary Eclipse.

Statistical analyses

Data are expressed as mean ±SD of three independent experiments and statistically analyzed using Student's t-test.Values of $p<0.05$ were considered significant.

Results

Effects of chalcone derivatives on endogenous antioxidant enzymes (CAT, SOD, GPx)

To study whether the effect of chalcone derivatives (compound 8-20) is related to the alteration of intracellular antioxidant status, the activities of CAT, SOD and GPx, as the most responsive antioxidant enzymes of the biological system, were determined at 20 μM of each drug (compound 8-20) among the H_2O_2-treated cells. For this goal, we pretreated SK-N-MC cells for 3 h with 20 μM of chalcone derivatives. Then, the treated cells were exposed to 300 μM H_2O_2 for 24 h. As shown in Fig. 1, 300 μM H_2O_2 reduced CAT, SOD and GPx activity by 54, 32 and 51%, respectively. However, pre-treatment of the cells with each of the derivatives (compounds 8-20) with a single dose (20 μM) increased the CAT activity by 19.6, 17.4, 22.6, 19.6, 2.4, 3.6, 8.8, 10.6, 24.3, 8.8, 24.3, 18.6, 1.6 % , SOD activity by 14.3, 11.3, 12.3, 10.6, 2.3, 4.2, 8.3, 1.2, 12.2, 10.9, 10.1, 1.3, 0.3% and GPx activity by 26.7, 25.3, 16.3, 15.6, 6.7, 24.2, 8.3, 2.7, 24.1, 29.3, 12.9, 21.3 and 1.3%, respectively relative to cells treated solely with H_2O_2.

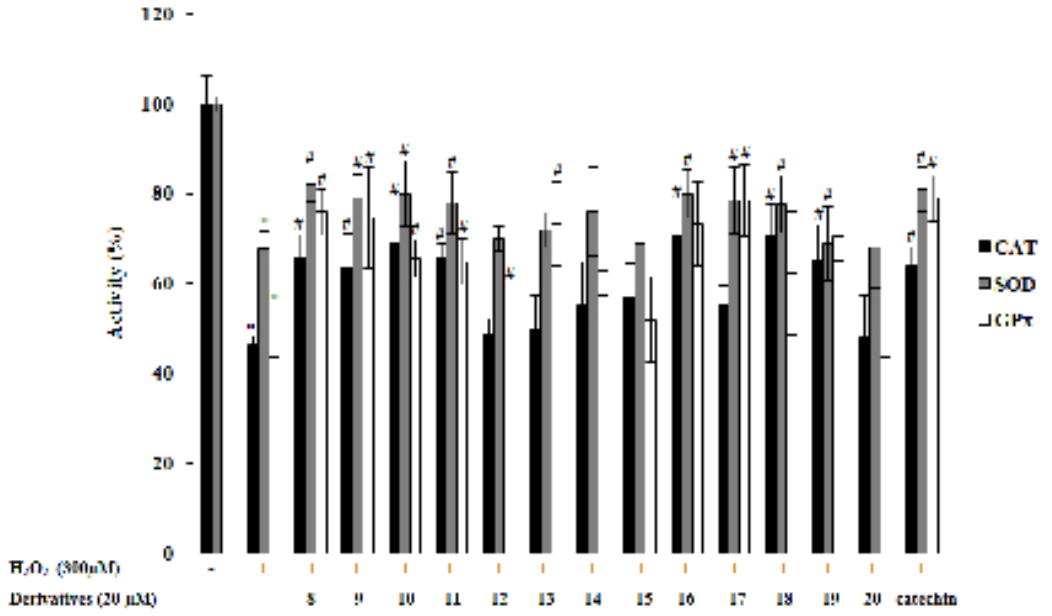


Fig 1. Effect of chalcone derivatives (compound 8-20) on the intracellular activities of CAT, SOD and GPx. SK-N-MC cells pretreated with 1,3 diphenyl-2-propen-1-one derivatives (20 μM) for 3 h before addition of 300 μM H_2O_2 for 24 h. Catechin was used as the positive control. Each value represents the mean ± SD (n=3). *Significantly different from untreated control cells ($p < 0.05$). #Significantly different from H_2O_2-treated cells ($p < 0.05$).

Inhibitory effects against protein oxidation

The oxidative protein damage, provoked by free radicals, has been demonstrated to play a significant role in aging and several pathological events [26]. Radical mediated damages to proteins might be initiated by electron leakage, metal-ion dependent reactions, and autoxidation of lipids and sugars [27]. Major molecular mechanisms, leading to structural changes in proteins are free-radical mediated protein oxidation characterized by carbonyl formation (PCO). Indeed, measurement of PCO has been used as a sensitive assay for oxidative damages of proteins [20]. Protein oxidation was used as another method to measure hydroxyl radical scavenging activity of chalcone derivatives assay by incubating with H_2O_2. The oxidation was determined in terms of PCO formation. As shown in table 2, in SK-N-MC cells treated with H_2O_2 for 24 h, the level of PCO was 9.47 nmol/mg protein, which was about 5.1 fold higher than that of untreated cells (1.86 nmol/mg protein). Chalcone derivatives (compound 8-20) exhibited inhibitory effects on PCO formation by 55, 51, 61, 47, 31, 39, 17, 13, 42, 37, 56, 11 and 6% at the concentration of 20 μM relative to cells treated only with 300 μM H_2O_2.

Inhibition of lipid peroxidation

Lipid peroxidation is an oxidative alteration of polyunsaturated fatty acids in the cell membranes that generates a number of degradation products. MDA, one of the products of lipid peroxidation, has been studied widely as an index of lipid peroxidation and as a marker of oxidative stress [28]. The addition of 300 μM H_2O_2 to SK-N-MC cells for 24 h significantly

increased the extent of MDA formation, compared to the control sample (0.68 nmol/mg protein versus 0.14 nmol/ mg protein). However, as shown in Table 2, adding each of the derivatives at a concentration of 20 μM to cells significantly reduced MDA formation. The MDA levels in cells pretreated with chalcone derivatives (compound 8-10) were 0.34, 0.31, 0.26, 0.37, 0.61, 0.45, 0.61, 0.69, 0.55, 0.33, 0.32, 0.62, and 0.67 nmol/mg protein, respectively. In other words, chalcone derivatives (compound 8-20) were capable of inhibiting MDA formation by 50, 54, 61, 46, 10, 34, 10, 0, 19, 51, 52, 9 and 1% relative to H_2O_2-treated cells.

Table 2. Inhibitory effects of chalcone derivatives (compound 8-20) on the H_2O_2-induced protein oxidation (PCO) and lipid peroxidation (MDA) after 24 h in H_2O_2-treated SK-N-MC cells. Catechin was used as the positive control. Each value represents the mean ± SD (n=3) *Significantly different from untreated control cells ($p < 0.05$). #Significantly different from H_2O_2-treated cells ($p < 0.05$).

		Content (nmol/mg protein)	
Compounds	**H_2O_2**	**MDA**	**PCO**
-	-	0.14 ± 0.01	1.86 ± 0.86
-	+	0.68 ± 0.06 *	9.47 ± 0.45 *
8	+	0.34 ± 0.05 #	4.20 ± 0.27 #
9	+	0.31 ± 0.04 #	4.60 ± 0.36 #
10	+	0.26 ± 0.04 #	3.66 ± 0.04 #
11	+	0.37 ± 0.06	4.97 ± 0.67 #
12	+	0.61 ± 0.05	6.50 ± 0.81
13	+	0.45 ± 0.07 #	5.75 ± 0.97 #
14	+	0.61 ± 0.03	7.90 ± 0.54
15	+	0.69 ± 0.09	8.20 ± 0.45
16	+	0.55 ± 0.08	5.43 ± 0.66 #
17	+	0.33 ± 0.01 #	5.96 ± 0.11 #
18	+	0.32 ± 0.02 #	4.11 ± 0.76 #
19	+	0.62 ± 0.07	8.41 ± 0.80
20	+	0.67 ± 0.06	8.90 ± 0.55
Catechin	+	0.38 + 0.08#	4.21 + 0.65#

Chalcone derivatives prevents H_2O_2-induced GSH depletion

GSH is one of the most important members of the endogenous non-enzymatic antioxidants and plays important roles in the maintenance of cellular redox homeostasis [29]. Fluorescence techniques using the mBCl probe provide a sensitive tool to measure the intracellular GSH

content and have been shown to yield similar results as the standard spectrophotometric assays. Major advantages of this approach include its ability to measure GSH in intact living cells and its specificity for GSH via the conjugation reaction catalyzed by glutathione-S-transferase [24]. The intracellular GSH level was decreased by 47.5% in 24 h after treatment with H_2O_2 (300 µM) (Fig. 2). The intracellular GSH concentration of SK-N-MC cells, following pretreatments with 20 µM of chalcone derivatives (compound 8-20), increased by 20.9, 22.6, 28.9, 19.6, 5.4, 23.7, 3.8, 5.1, 17.3, 6.3 and 25.6% relative to H_2O_2-treated control cells, respectively (Fig. 2).

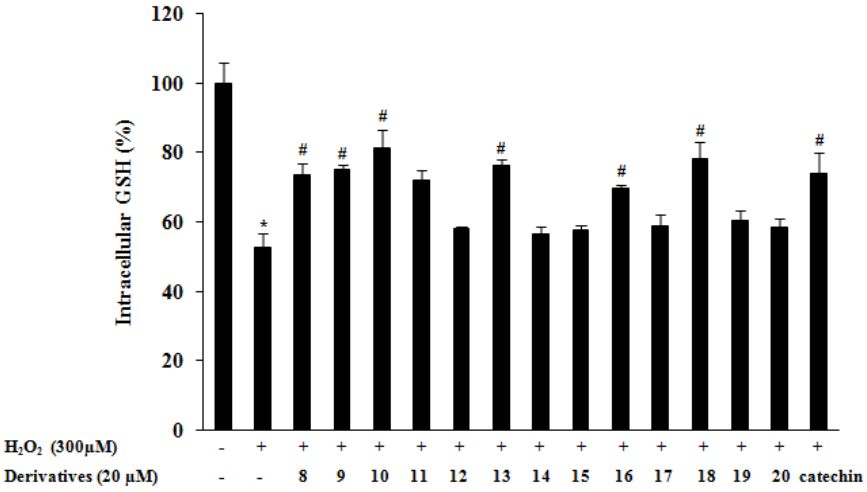


Fig 2. Intracellular evaluation of reduced glutathione (GSH) with the fluorescence probe, monochlorobimane (mBCL) in SK-N-MC cells. SK-N-MC cells were pretreated with 20 µM of each of the derivatives (compounds 8-20) for 3 h, and then they were exposed to 300 µM H_20_2 for 24 h. Cells were incubated with mBCL (100 µM) and then fluorescence intensity was measured at Ex 360 nm and Em 480 nm. The GSH content of the untreated cells was expressed as 100%. Catechin was used as the positive control. Each value represents the mean ± SD (n=3) *Significantly different from untreated control cells ($p < 0.05$). $^{\#}$Significantly different from H_2O_2-treated cells ($p < 0.05$).

Discussion

Reactive oxygen species (ROS), with high chemical reactivity, cause destructive and irreversible damage to cellular components [30]. Antioxidants are compounds, which at low concentration could prevent biomolecules (proteins, nucleic acids, polyunsaturated lipids, sugars) from undergoing oxidative damage through free radical mediated reactions [31]. In AD brain, the levels of the antioxidants are lower than normal and the extent of protein oxidation and lipid peroxidation are oftenly augmented [32]. Therefore, antioxidant-based therapeutical approaches might assist and attenuate oxidative damage among the AD patients.

Chalcones (1, 3-diphenyl-2-propene-1-one) belong to the largest class of plant secondary metabolites. They have in which two aromatic rings which are linked by a three carbon α, β-unsaturated carbonyl groups [12]. In general, the radical-scavenging of chalcone derivatives depends on the molecular structure and the hydroxyl group substitution pattern. The hydroxyl

group enhances the antioxidant activity of chalcone derivatives mainly due to its easy conversion to phenoxy radicals through the hydrogen atom transfer mechanism [12, 13]. Generally, chalcone compounds with para hydroxyl substitution tarnsact better than those with ortho-hydroxyl substitution and these act better than meta-substituted ones [13]. In the present study, the antioxidant activity of chalcone derivatives (compound 8-10) were investigated by assessing their activities in terms of CAT, SOD and GPx and measuring the extent of lipid peroxidation (MDA), protein oxidation (PCO) and evaluating the GSH content.

The biological effects of ROS are controlled in vivo by a wide spectrum of enzymatic and non-enzymatic defense mechanisms such as SOD which catalyzes dismutation of superoxide anions into H_2O_2 and CAT and GPx which detoxify H_2O_2 and convert lipid hydroperoxides to nontoxic alcohols [33]. H_2O_2 can lead to oxidative stress possibly through different factors including increase in ROS level and decrease in activity or expression of anti-oxidant enzymes. Our data indicated that H_2O_2 decreased the activity of these enzymes. However, pre-treatment of cells with chalcone derivatives especially vanillin analogues (compound 8-11) enhanced the activitiy of SOD, CAT and GPx with respect to H_2O_2-treated cells.

ROS causes oxidative damage to proteins and lipids, if the efficiency of the antioxidant enzymes is altered. Oxidation of proteins is accompanied by the formation of protein carbonyl which has been used widely as an index of protein oxidation [34]. Lipid peroxidation generates a number of degradation products, such as MDA which is one of the causes of cell membrane destruction and cell damage [30]. The present study demonstrated that exposure of cells to 300 μM H_2O_2 could enhance protein oxidation levels in terms of PCO formation and chalcone derivatives were capable of reducing protein oxidation, probably due to their antioxidant activities. Chalcone derivatives were also capable of quenching the extent of lipid peroxidation caused by H_2O_2. The most important function of chalcone derivatives are their ability to scavenge hydroxyl radicals. In addition, chalcone derivatives can scavenge lipid peroxy radicals generated through lipid peroxidation so they can decreased the formation of lipid peroxidation products.

In biological systems, GSH plays a key role in the maintenance of intracellular oxidative balance of cells. The reduced form of GSH provides a large portion of the intracellular reducing power available to the cell and therefore determines to a large extent the cell's ability to eliminate potentially harmful ROS and metabolic by-products [29]. Fluorescence studies using mBCL indicated that H_2O_2 causes depletion of GSH content among SK-N-MC cells. Regarding these data, chalcone derivatives can certainly increase GSH level due to their free-radical scavenging capabilities.

In conclusion, our data indicated that GSH depletion along with significant reduction in the activities of CAT, SOD and GPx occur in cells exposed to H_2O_2. On the other hand, pretreatment of cells with chalcone derivatives significantly increased the GSH level with enhanced CAT, SOD and GPx activity. Furthermore, H_2O_2 significantly decreased MDA and PCO formation in the cells. Therefore, chalcone derivatives can interfere with the oxidation process by reacting with free radicals, chelating free catalytic metals and also by acting as oxygen scavengers.

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