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In vitro antioxidant activity of thiazolidinone derivatives of 1,3-thiazole and 1,3,4-thiadiazole

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ABSTRACT

The initial steps in preclinical drug developing research concern the synthesis of new compounds for specific therapeutic use which needs to be confirmed by *in vitro* and then *in vivo* testing.

Nine thiazolidinone derivatives (numerically labeled ***1–9***) classified as follows: 1,3-thiazole-based compounds (***1*** and ***2***); 1,3,4-thiadiazole based compounds (***3*** and ***4***); substituted 5-benzylideno-2-adamantylthiazol[3,2-b][1,2,4]triazol-6(5*H*)ones (***5–8***); and an ethylaminothiazole-based chalcone (**9**), were tested for antioxidant activity (AOA) by using three *in vitro* assays: *DPPH* (1,1-diphenyl-2-picrylhydrazyl scavenging capacity test); *FRAP* (ferric reducing antioxidant power test); and *TBARS* (thiobarbituric acid reactive substances test). Compounds ***1–4*** and ***9*** in particular are newly synthesized compounds. Also, traditional antioxidants Vitamins E and C and α-lipoic acid (α-LA) were tested.

The results of *DPPH* testing: Vitamin C 94.35%, Vitamin E 2.99% and α-LA 1.57%; compounds: ***4*** 33.98%; ***2*** 18.73%; ***1*** 15.62%; ***5*** 6.59%; ***3*** 4.99%; ***6–9*** demonstrated almost no AOA. The results of *TBARS* testing (% of LPO inhibition): Vitamin C 62.32%; Vitamin E 36.29%; α-LA 51.36%; compounds: ***1*** 62.11%; ***5*** 66.71%; ***9*** 60.93%; ***4***, ***6*** and ***7*** demonstrated ~50%; ***3*** and ***8*** displayed ~38%; ***2*** 23.51%. By *FRAP* method, Vitamins E and C showed equal AOA, ~100%, unlike α-LA (no AOA), and AOA of the tested compounds (expressed as a fraction of the AOA of Vitamin C) were: ***2*** and ***4***–75%; ***8***, ***3*** and ***1***–45%; ***5–7*** and ***9***–27%.

Different red-ox reaction principles between these assays dictate different AOA outcomes for a single compound. Vitamin C appeared to be the superior antioxidant out of the traditional antioxidants; and compound ***4*** was superior to other tested thiazolidinone derivatives. Vitamin C appeared to be the superior antioxidant out of the traditional antioxidants; and compound ***4*** was superior to other tested thiazolidinone derivatives. Phenyl-functionalized benzylidene, amino-carbonyl functional domains and chelating ligand properties of the thiazolidinone derivatives correlated with AOA.

1. Introduction

Antioxidant therapy of diseases associated with oxidative stress (OS) has been proven to be safe and effective. The intake of antioxidants has been shown to reduce the risk of cancer as well as neurological and cardiovascular pathologies among others [1]. The subtle regulation of free radicals is immensely important to the maintenance of the organism's homeostasis. Disturbances in an organism's red-ox balance (the natural consequence of free radical overproduction and/or their insufficient sequestration by antioxidant defense mechanisms) is associated with a number of diseases as well as with aging [2,3]. Under physiological conditions, the concentration of free radicals – including reactive oxygen, nitrogen, sulfur and carbon species (ROS, RNS, RSS and RCS) – is regulated by antioxidant defense systems. The inability of

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antioxidant defense systems to mediate the concentration of free radicals within the body results in oxidative, nitrosative, thilyl or carbonyl stress. Free radicals indiscriminately oxidize any substance within range, causing injury to endogenous as well as exogenous substances, including all classes of biomolecules (proteins, lipids, DNA), disrupting normal cell signaling mechanisms, devastating cellular energy supplies, depleting reducing equivalents (reduced nicotinamide adenine dinucleotide and glutathione) and causing cell death by eventually inducing apoptosis [3–6].

Compounds with 1,3-thiazole or thiazolidinone structures impose a broad spectrum of biological activities including the neutralization/sequestration of ROS and RNS [7–12]. The antioxidant activity (AOA) of thiazole derivatives has been acknowledged recently [13]. Kavitha et al. synthesized some novel 4-(4-chlorophenyl)-2-aryl substituted metheniminothiazoles as possible antioxidant agents [14]. Kachroo et al. reported the antibacterial and AOA of newly synthesized *N*-[(4E)-arylidene-5-oxo-2-phenyl-4,5-dihydro-1H-imidazol-1-yl]-2-(2-methyl-1,3-thiazol-4-yl) acetamide [15]. Gull et al. synthesized 2- amino-6-arylbenzothiazoles and investigated, *inter alia*, their RNS scavenging activities [16]. Sarkanj et al. synthesized 4-methyl-7-hydroxycoumarin derivatives (substituting at positions 7 - thiosemicarbazide and 4 - thiazolidinone) and evaluated their potential AOA [14,17]. Another interesting core is thiazolidinone with a wide spectrum of biological activities, including antioxidant [18–20].

In view of the considerable importance of thiazoles and thiazolidinones, which are the core structures in a variety of pharmaceuticals with a broad spectrum of biological activity, specifically referencing their ability to prevent ROS formation, the present work is intended to synthesize new heterocyclic compounds bearing the thiazole moiety [8]. The substitutions in the chosen derivatives were intended to stabilize the radicals formed from compounds via resonance (as shown in Figs. 4–8), or to donate labile hydrogens from the 1,3-thiazolidinone system. These hydrogens could be donated to the DPPH radical to form stable DPPH molecules. *In vivo* confirmation of the *in vitro* documented AOA for derivatives of thiazole, thiadiazole, or thiazolidinone is the next step in the preclinical phase of drug development research [9].

The goal of our study was to evaluate the AOA of newly synthesized compounds (***1–4*** and ***9***) as well as that of compounds ***5–8*** which were previously synthesized by three *in vitro* tests [21]. The compounds that were tested in this study were categorized as follows: ***group I***: 1,3-thiazole based thiazolidinones (compounds ***1*** and ***2***); ***group II:*** thiadiazole-based thiazolidinone (compounds ***3*** and ***4***); ***group III:*** thiazolidinone derivatives fused to a 1,2,4-triazol heterocyclic system (compounds ***5–8***); and ***group IV:*** an ethylaminothiazole-based chalcone (compound ***9***) [21,22] (Fig. 1).

We used three *in vitro* tests that make use of different principles of red-ox reactions: a) 1,1-diphenyl-2-picrylhydrazyl scavenging capacity test, *i.e. DPPH radical* scavenging capacity (or *DPPH*) assay [23]; b) *ferric reducing antioxidant power* (*FRAP*) assay [24]; and c) *thiobarbituric acid reactive substances (TBARS)* assay [25,26].

2. Material and methods

2.1. Chemicals

Melting points were determined with a MELTEMP II capillary apparatus (LAB Devices, Holliston, MA, USA) without correction. Elemental analyses were performed on a Perkin–Elmer 2400 CHN elemental analyzer. All compounds synthesized were documented to be within 0.4% of theoretical values. IR spectra were recorded as Nujol mulls on a Perkin Elmer Spectrum BX. Wave numbers collected from IR spectra are given in cm^{-1}. ^{1}H NMR. ^{13}C NMR spectra of the newly synthesized compounds in DMSO-d6 or $CDCl_3$ solution were recorded on a Bruker AC 300 instrument (Bruker, Karlsruhe, Germany) at 298 K. Chemical shifts are reported as δ (ppm) relative to TMS which was used as an internal standard. Coupling constants (***J***) are expressed in Hertz (Center of Instrumental Analysis of the University of Thessaloniki). The reactions were monitored by thin layer chromatography (TLC) plates on F_{254} silica-gel coated sheets (Merck, Darmstadt, Germany) and each of the purified compounds showed a single spot. Solvents, unless otherwise specified, were of analytical reagent grade or of the highest quality commercially available. Synthetic starting materials, reagents and solvents were purchased from Aldrich Chemie (Steinheimm, Germany).

1,1-Diphenyl 2-picryl hydrazyl (*DPPH*), 2,4,6-tripyridyl-*s*-triazine (*TPTZ*), Vitamin C (L-ascorbic acid), Vitamin E (α-tocopherol) and α-lipoic acid (α-LA) were obtained from Sigma Chemical Co. (St. Louis, USA); thiobarbituric acid (TBA), ferric chloride, ferrous chloride, trichloroacetic acid (TCA) and ethylenediaminetetraacetic acid (EDTA) were purchased from Merck (Darmstadt, Germany). All reagents were of analytical grade.

2.2. Chemistry

Compounds ***5–8*** were synthesized and described in our previous paper [21]. The mechanisms used to synthesize compounds ***1–4*** are as portrayed in the generic synthetic Scheme 1, while compound ***9*** was synthesized as illustrated in Scheme 2.

2.2.1. Synthesis of N-((5-adamantan-1-yl)-1,3,4-thiadiazol-2-yl)-2-chloroacetamide (Cb)

Anhydrous sodium carbonate (0.0302 mol, 3.2 g) was added with stirring into a solution of (2-amino-4-adamantane) - 1,3,4-thiadiazole (Ab) (0.0266 mol, 6.251 g) in anhydrous dimethylformamide (DMF) (64 mL). Afterwards, chloroacetyl chloride (B) (0.0798 mol, 9.02 g) in anhydrous DMF (34.9 mL) was added dropwise into the mixture. The reaction was stirred at room temperature for 3 h. The course of the reaction was controlled by TLC. After the reaction was complete, ice was added and the precipitate was filtered and washed thoroughly with water. The solid product (Cb) was recrystallized from ethanol (yield: 93.4%), m.p.179–180 °C. IR: 1562 (aromatic), 1712 (C = O), 3087 (NH). ^{1}H NMR: (δ ppm, DMSO-d6, 300 MHz): 1.26 (s, 6H, adamantane), 1.52–1.57 (d, 9H, adamantane), 3.91 (s, 2H, CH2), 7.46 (s, 1H, NHCO).

2.2.2. Synthesis of N-((5-adamantan-1-yl)-1,3,4-thiadiazol-2-yl-imino)-thiazolidin-4-one (Eb)

While stirring, ammonium thiocyanate (D) (0.1 mol, 7.5 g) was added into the *N*-[(5-adamantan-1-yl) -1,3,4-thiadiazol-2-yl] chloroacetamide (Cb) (0.05 mol/16.7 g in 50 mL of 95% ethanol). The mixture was boiled in a water bath and refluxed under stirring for 1 h. The course of the reaction was controlled by TLC. The product (Eb) remained unchanged overnight and was then filtered, washed with water and recrystallized from ethanol (yield 61%), m.p. 258–259 °C. IR: 1602 (aromatic), 1740 (C = O), 3360 (NH). ^{1}H NMR: (δ ppm, DMSO-d6, 300 MHz): 1.75 (s, 6H, adamantane), 1.99–2.06 (d, 9H, adamantane), 4.07 (s, 2H, CH2 thiazolidinone), 12.20 (s, 1H, NHCO) [27].

Synthesis: Aryl aldehyde (6 mmol) was added into a well-stirred solution of 0.8 g of 4/5-substituted (thiazol-2-ylimino) thiazolidin-4-one (4 mmol) in acetic acid (35 mL) previously buffered with sodium acetate (8 mmol). The solution was refluxed for 4 h and then poured into ice-cold water. The precipitate was filtered and washed with water. The crude product was purified by recrystallization from dioxane [27].

2.2.2.1. Synthesis of (2E,5Z)-((5-adamatane-1-yl)-1,3,4-thiadiazol-2-yl)-imino)-5-(4-hydroxybenzylidene)-thiazolidin-4-one ***(3)***. Yield: 48.9%, m.p. 258–259 °C. Rf = 0.71 (toluene-EtOH 7:3). IR (cm^{-1}, Nujol): 1570 (aromatic), 1708 (C = O), 3089 (NH). ^{1}H NMR (δ ppm, DMSO-d6, 300 MHz): 1.77 (s, 6H, adamantane), 2.03–2.07 (d, 9H, adamantane), 6.95–6.98 (d, 2H, C3′-C5′), 7.51–7.53 (d, 2H,C2′- C6′), 7.67 (s, 1H, C = C), 10.22 (s, 1H, -OH), 12.690 (s, 1H, NHCO). Anal. Calc. for C22H22N4O2S2 (MW 438): C: 60.25%; H: 5.06%; N: 12.78%. Found: C: 60.13%; H: 5.11%; N: 12.75%.

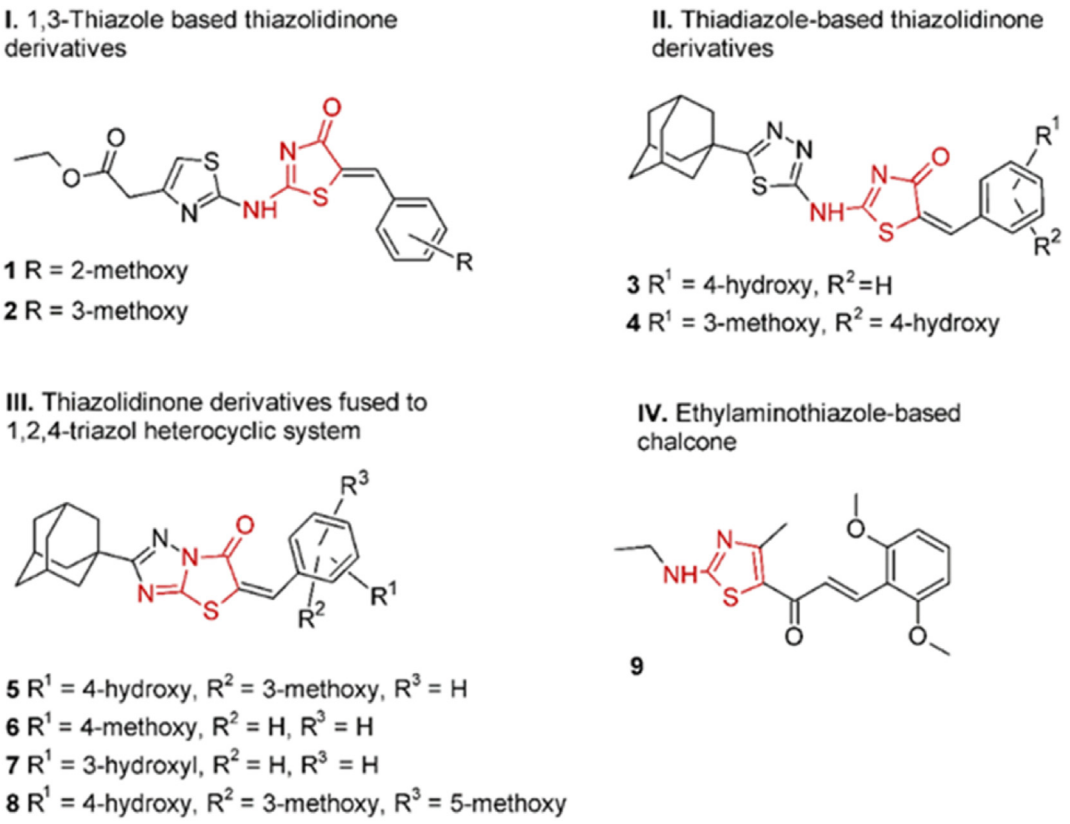


Fig. 1. Structures of nine tested thiazolidinones.

*2.2.2.2. Synthesis of (2E,5Z)-((5-adamatane-1-yl)-1,3,4-thiadiazol-2-yl)-imino)-5-(4-hydroxy-3-methoxybenzylidene)-thiazolidin-4-one (**4**).* Yield: 62.3%, m.p. 264–266 °C. IR (cm^{-1}, Nujol): 1600 (aromatic), 1711 (C=O), 3089 (NH). ^{1}H NMR (δ ppm, DMSO-d6, 300 MHz): 1.77 (s, 6H, adamantane), 2.03–2.08 (d, 9H, adamantane), 3.85 (s, 3H, -OCH3), 7.00–7.03 (d, 1H, C5′), 7.15–7.17 (d, 1H, C6′), 7.29 (s, 1H, C2′), 7.72 (s, 1H, C=O), 9.95 (s, 1H, -OH), 12.69 (s, 1H, NHCO). Anal. Calc. for C23H24N4O3S2 (MW 468): C: 58.95%; H: 5.16%; N: 11.96%. Found: C: 59.03%; H: 5.21%; N: 12.05%.

2.2.3. Synthesis of 1-(2-ethylamino)4-methylthiazol-5-yl)-ethanone (J)

An acetone solution of 3-chloracetylacetone (2.26 mL, 0.02 mol) (5 mL) was added dropwise into an acetone solution of 1-methyl-thiourea/ethylthiourea (1.8 g, 0.02 mol) (50 mL). The mixture was refluxed for 1.5 h and the solid product was filtered and recrystallized from ethanol [28,29].

*2.2.3.1. (E)-3-(2,6-dimethoxyphenyl)-1-(2-(ethylamino-4-methylthiazol-5-yl)-prop-2-en-1-one (**9**).* Yield: 47.9%, m.p. 157–159 °C, Rf: 0.58 (CHCl3:MeOH, 9.5:0.5). IR (cm^{-1}, Nujol): 3199 (NH), 3072 (C-H vinyl.), 1632 (C=O), 1596 (C=C), 1560 (C-H arom). ^{1}H NMR: (δ ppm, DMSO-d6, 300 MHz): 1.18 (t, 3H, CH3-C-N), 2.55 (s, 3H, thiazole-4′-CH3), 3.25–3.28 (q, 2H, C-CH2-N), 3.77 (s, 3H, Ar-5′-′CH3), 3.84 (s, 3H, Ar-2′-OCH3), 6.97–7.05 (m, 2H, Ar. 3′,4′), 7.27 (s, 1H, Ar. 6′), 7.37 (d, J = 15 Hz, 1H, CO-CH), 7.73 (d, J = 15 Hz, 1H, Ar-CH), 8.51 (s, 1H, NH). Anal. Calc. for C17H20N2O3S: C: 61.42%; H: 6.06%; N: 8.43%. Found: C: 61.45%; H: 6.06%; N: 8.46%.

2.3. Evaluation of compounds' antioxidant activity

Stock solutions of the tested thiazolidinones were prepared in ethanol (1 mg/mL). Measurements were repeated five times for chosen concentrations of the test compounds for all three assays as is consistent with the literature.

2.3.1. DPPH assay

The reducing properties of the examined compounds were evaluated by their ability to transform violet 1,1-diphenyl-2-picrylhydrazyl radical (*DPP*', i.e. *DPPH radical*) into its pale, yellow reduced form (*DPPH)*. The decrease in absorbance is related to the AOA of the compound in question (the loss of violet color was measured at 517 nm) [23].

In brief, 100 µL of the stock solution was diluted to 2 mL with ethanol (0.1 mg/2 mL) and mixed with 0.5 mL of *DPPH radical* solution (0.5 mM), resulting in a final concentration of the tested compound of 0.04 mg/mL according to Das et al. [30]. The solutions were incubated for 30 min in a dark place at room temperature. The absorbance was measured at 517 nm. The mixture of ethanol and 0.5 mM *DPPH radical* solution (1:4, v/v) was used as the control. The capacity of the tested substance to reduce *DPPH radical* to *DPPH* corresponds to its AOA and was expressed as a percentage of *DPPH radical* inhibition [I (%)] according to the following equation:

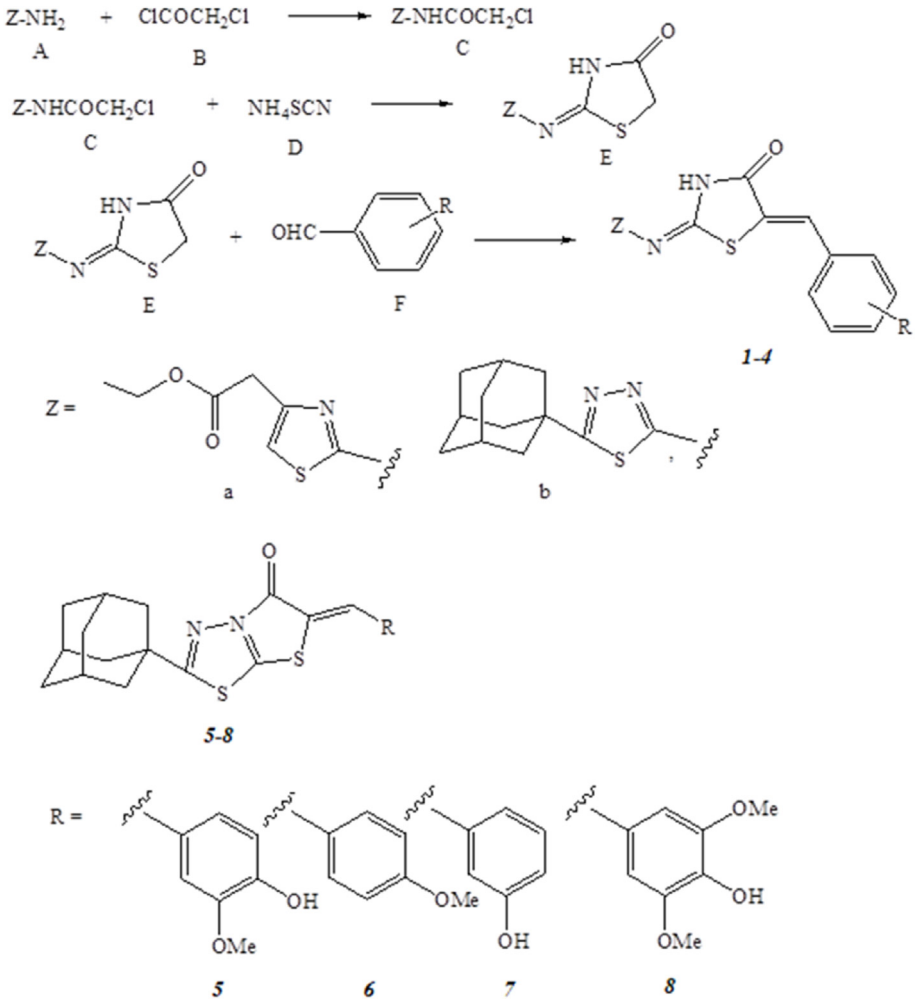
$$I(\%) = \frac{Ac - Aa}{Ac} \times 100$$

Legend: inhibition of DPPH radical - I; absorbance of the control (Ac) and tested samples (Aa).

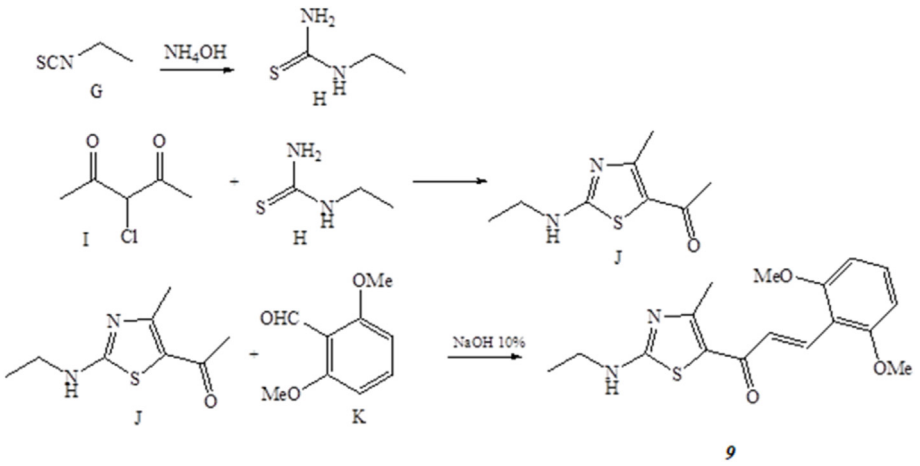
2.3.2. FRAP assay

The principle of *FRAP* assays is to assess a reactant's ability to reduce ferric tripyridyl triazine [*Fe(III)-TPTZ*] [Fe^{3+} complexed with 2,3,5-triphenyl-1,3,4-triaza-2-azoniacyclopenta-1,4-diene chloride] into blue colored *Fe(II)-TPTZ*, measurable at 593 nm, at pH 3.6 (to maintain iron solubility by the action of electron-donating antioxidants) [24,31–35].

FRAP reagent was freshly prepared by mixing acetate buffer (300 mM, pH 3.6), TPTZ solution (10 mM TPTZ in 40 mM HCl) and 20 mM $FeCl_3$ water solution (20 mM) in the ratio 10:1:1. 100 µL of the stock solution was mixed with *FRAP* reagent up to 3 mL (final



Scheme 1. A. Synthesis of the compounds ***1-4***.



Scheme 2. A. Synthesis of the compound ***9***.

concentration was 0.033 mg/mL) and incubated for 30 min at 37 °C. The absorbance was measured at 593 nm, using *FRAP* working solution as a blank.

2.3.3. TBARS assay (inhibition of lipid peroxidation)

The principle of the method is based on the formation of a pink-colored complex between carbonyls [decomposition products of oxidatively damaged poly unsaturated fatty acids (PUFA), including malondialdehyde (MDA) and other aldehydes] and TBA reagents (in ratio 1:2), measureable at 532 nm. The reaction takes place at a low pH and at an elevated temperature [25].

Different substrates (as a source of MDA) can be used to mimic cell membranes in this test, such as lecithin liposomes, free fatty acids, low density lipoproteins, etc. In this case, lecithin liposomes were used as a surrogate for a cell membrane's phospholipid bilayer. Lecithin water solution (in final concentration of 3%) was prepared from 10% lecithin

commercial product ("Lipotech 10") and kept it in an ultrasonic bath for 30 min to obtained liposomes [26]. The reaction mixtures (total volume of 4 mL) containing 3.9 mL of 0.075 M $FeSO_4$ (0.1043 g $FeSO_4 \times 7H_2O$ in 5 mL of water), 50 μL of liposome suspension, 10 μL of the ethanol stock solutions (0.1 mg of the tested compound), 20 μL of 0.1 M L-ascorbic acid (0.0882 g in 5 mL of water) and 20 μL of phosphate buffer (pH 7.4, I = 0.1) were incubated at 37 °C for 1 h. Then, 0.2 mL of 0.1 M EDTA and 1.5 mL of TBA reagent (0.375 g TBA, 15 g TCA and 2.1 mL $HClO_4$ in 100 mL of distilled water) were added into the reaction mixtures and heated at 100 °C for additional 15 min. The final concentration of the tested compounds equals 1.75 μg/mL. The control reaction mixture contained 10 μL of ethanol in place of a test compound. The blank mixture did not contain $FeSO_4$ and L-ascorbic acid. Further on, samples were cooled on ice, centrifuged at 3000 rpm for 10 min and the absorbance of supernatants was measured at 532 nm. The results were expressed as a percentage of lipid peroxidation (LPO) inhibition [I (%)] and calculated according to the following equation:

$$I(\%) = \frac{Ac - Aa}{Ac} \times 100$$

Legend: inhibition of LPO - I; absorbance of the control (Ac) and tested samples (Aa).

2.4. Statistics

Statistical software SPSS version 7.0 was used for the statistical analysis. Descriptive statistical analysis (x, STDEV, med, SEM) and Gaussian distribution of data were performed as well as a one-way ANOVA test to compare differences between AOA of the tested compounds between the three methods. Afterwards, a post-hoc Tukey test was used to analyze the associations between the tested compounds. The results were presented as an average ± STDEV. A Spearman correlation was tested for using Graph Pad Prism Version 5.0 in order to measure the associations between the results of the different methods according to individual compounds.

3. Results

3.1. Antioxidant activity of thiazolidinone derivatives obtained by DPPH assay

According to the equation for the percent of *DPPH* radical inhibition, Vitamin C reached an inhibition of 94.35%, Vitamin E 2.99% and α-LA 1.57%. Compounds ***4***, ***2*** and ***1*** resulted in 33.98%, 18.73% and 15.62% inhibition respectively; compounds ***5*** and ***3*** demonstrated 6.59% and 4.99% inhibition, while the rest of the derivatives showed almost no detectable AOA (Graph 1). Overall, the tested thiazolidinones showed lower AOA compared to Vitamin C ($p < 0.0001$) via the *DPPH assay*.

3.2. Antioxidant activity of thiazolidinone derivatives obtained by FRAP assay

According to the *FRAP* method, Vitamins E and C showed maximal and almost equal AOA (812.40 μM Fe^{2+} vs. 868.40 μM Fe^{2+}, respectively), though α-LA achieved no AOA (Graph 2). The AOA of the tested compounds was lower compared to Vitamins C and E ($p < 0.0001$) and higher when compared to α-LA ($p < 0.0001$). Expressed as a percentage of Vitamin C's AOA reached by this method, the AOA of the derivatives was classified in three categories as follows: compounds ***2*** and ***4*** (72–78%); compounds ***8***, ***3*** and ***1*** (43–46%); and compounds ***5***, ***6***, ***9*** and ***7*** (25–29%) (Graph 2).

3.3. Antioxidant activity of thiazolidinone derivatives obtained by TBARS assay

According to the equation, the % of LPO inhibition reached by the conventional antioxidants Vitamins C and E and α-LA was 62.32%, 36.29% and 51.36% respectively. Compound ***5*** demonstrated the highest observed AOA (66.71%), even higher than Vitamin C (62.32%). Compounds ***9*** (60.93%) and ***1*** (62.11%) showed almost identical AOA as Vitamin C. Slightly less AOA was recorded from compounds ***6***, ***4*** and ***7***, around 50%, ($p < 0.0001$), with α-LA and compounds ***8*** and ***3*** demonstrating around 38% inhibition ($p < 0.0001$), similar to that of Vitamin E (36%). Compound ***2*** demonstrated the lowest AOA (23.51%) ($p < 0.0001$) (Graph 3).

The data regarding the AOA of the synthesized compounds and the conventional antioxidants collected via the three *in vitro* methods applied are summarized in Table 1.

The AOA of the tested compounds, expressed as the fraction of the AOA of conventional antioxidants, were quantitatively categorized into five categories (0–21%, 22–43%, 44–65%, 66–87%, 88–109%) within the assays. Overall, out of the traditional antioxidants, Vitamin C had the greatest antioxidant effect across all three tests. Vitamin E showed no AOA in *DPPH* testing, moderate effects in *TBARS* and maximal effects in *FRAP*. By contrast, α-LA displayed any meaningful antioxidant effects in the *TBARS* assay. Results regarding the AOA of the tested compounds are mixed and will be covered under Discussions.

Spearman correlation analysis showed that *FRAP* and *TBARS* assays were perfectly correlated (Slope = 1, $r^2 = 1$) for each individually tested compound and reference standards except for the *DPPH* assay.

4. Discussion

The interpretation and the evaluation of AOA for the tested compounds (***1–9***) was done with respect to Vitamins C and E and α-LA, their chemical structures and metal chelating potency and the principles and thermodynamics of the underlying test reactions [36,37].

Vitamin C's AOA was evaluated through a two-step reduction and Vitamin E's through a one-step reduction [38,39]. Stable semi-dehydroascorbic acid radical ($Asc^{2-}/Asc^{\bullet-}$) (Scheme 3) spontaneously reduces compounds with a standard reducing potential ($\Delta E^{\circ\prime}$) higher than +282 mV and +174 mV, which corresponds to ascorbic monoanion/radical and dianion/radical ($AscH^{\bullet}$, $H^+/AscH^-$ and $Asc^{\bullet-}/Asc^{2-}$) (pH = 7) respectively [40].

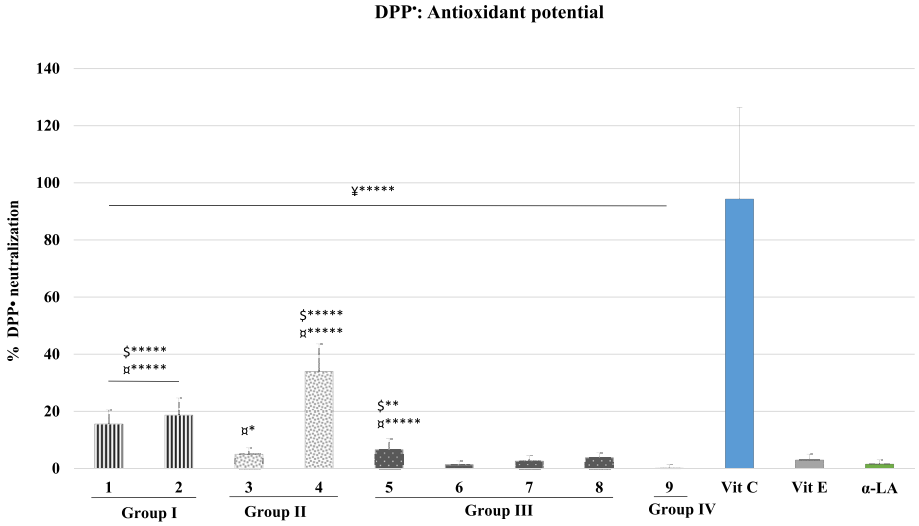
Vitamin E (TOH) can reduce compounds with $\Delta E^{\circ\prime} > +500$ mV, which corresponds to the couple tocopheroxyl ($TO^{\bullet}$), H^+/TOH, by a one-step reduction mechanism (Scheme 4). The phylyl side chain of Vitamin E makes it liposoluble [40,41].

The reduced form of α-LA, dihydrolipoic acid (DHLA), is a potent reducing agent (α-LA/DHLA, $\Delta E^{\circ\prime} = -320$ mV) with amphiphilic properties (Scheme 5). Additionally, DHLA is capable of forming a complex with Fe^{3+} [36].

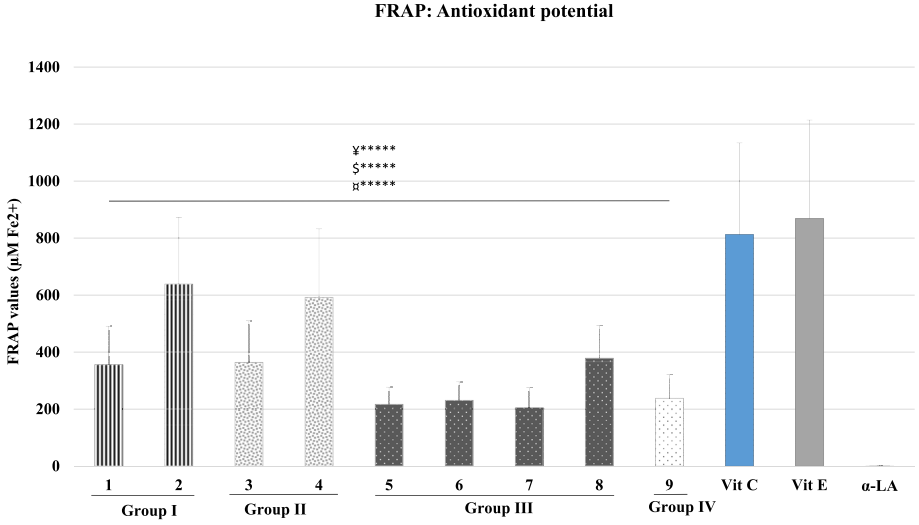
4.1. DPPH assay

Transfer of $H^{\bullet}$ from the tested compound to the $N^{\bullet}$ of *DPPH radical* reagent follows SET and/or HAT types of antioxidant mechanisms (Scheme 6) [23].

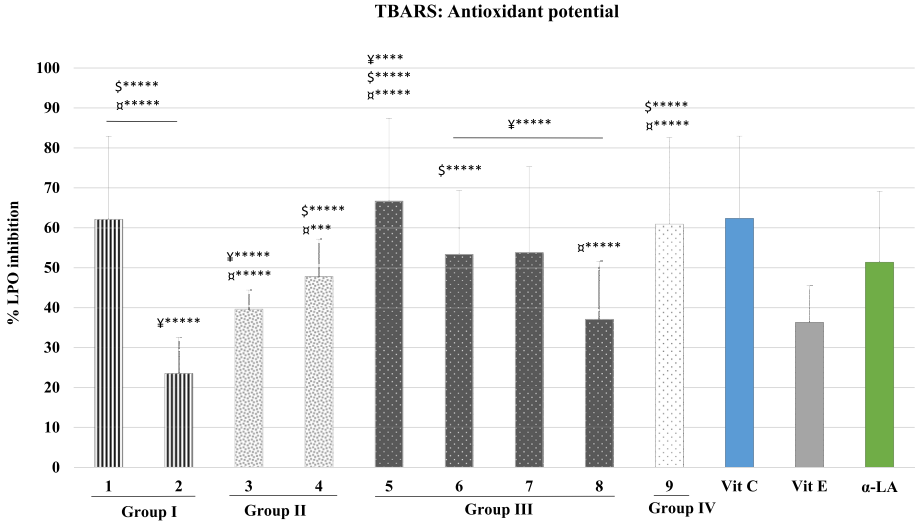
The extremely high AOA of hydrophilic Vitamin C (confirmed by all three *in vitro* assays, Graphs 1-3) can be attributed to the two OH groups on adjacent carbon atoms of the gamma-lactone ring, responsible for the two-step transfer of H^+ and e^-. Vitamin E did not achieve AOA by *DPPH* assay, as the reduction of $DPP^{\bullet}$ by Vitamin E is thermodynamically unfeasible ($TO^{\bullet}$, H^+/TOH: $\Delta E^{\circ\prime} = +500$ mV; $DPP^{\bullet}/DPPH$: $\Delta E^{\circ\prime} \sim +250$ mV) [42]. Dihydrolipoic acid is a strong reducing agent (DHLA/α-LA $\Delta E^{\circ\prime} = -320$ mV), thus the lack of AOA can be explained by the presence of its oxidized form, α-LA, in the reaction mixture, and α-LA is not capable of donating $H^{\bullet}$ to $DPP^{\bullet}$ [36]. Also, the overall



Graph 1. Antioxidative activity of 1,3-thiazolone and 1,3-thiazole derivatives and reference standards assessed by *DPPH* assay. Number of measurements: n = 5. Final concentrations of the tested compounds equal 0.04 mg/mL. Values are expressed as mean ± STDEV. Values are expressed as a % of DPP• inhibition (i.e. its sequestration by tested compounds). Legend: ¥-compared with vitamin C, $-compared with vitamin E, ¤- compared with α-lipoic acid. Levels of significances were graduated as: * ($p < 0.05$); ** ($p < 0.02$); *** ($p < 0.004$); **** ($p < 0.003$); ***** ($p < 0.0001$).



Graph 2. Antioxidative activity of 1,3-thiazolone and 1,3-thiazole derivatives and reference standards assessed by FRAP assay. Number of measurements: n = 5. Final concentrations of the tested compounds equal 0.033 mg/mL. Values are expressed as mean ± STDEV. Values are expressed as a FRAP units, i.e. μM Fe^{2+}. Legend: ¥-compared with vitamin C, $-compared with vitamin E, ¤- compared with α-lipoic acid. Level of significance was ***** ($p < 0.0001$).



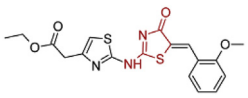
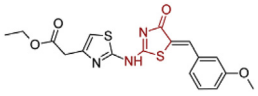
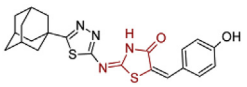
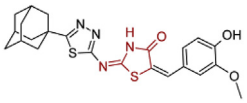
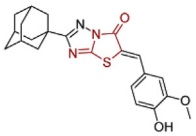
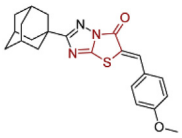
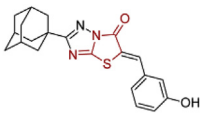
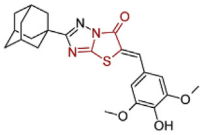
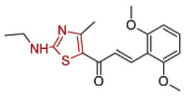
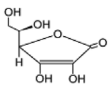
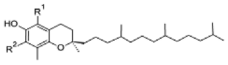
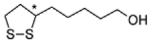
Graph 3. Antioxidative activity of 1,3-thiazolone and 1,3-thiazole derivatives and reference standards assessed by TBARS method. Number of measurements: n = 5. Final concentrations of the tested compounds equal 1.75 μg/mL Values are expressed as mean ± STDEV. Values are expressed as a % of LPO. Legend: ¥-compared with vitamin C, $-compared with vitamin E, ¤- compared with α-lipoic acid. Levels of significances were graduated as: * ($p < 0.05$); ** ($p < 0.02$); *** ($p < 0.004$); **** ($p < 0.003$); ***** ($p < 0.0001$).

reaction would require the generation of an intermediate between *DPP*• and α-LA which just so happens to be stereochemically unfeasible (Scheme 7).

Group I (derivatives ***1*** and ***2***) accomplished moderate AOA (Graph 1) likely attributable to the donation of an H• from the amino group (NH) and *N*-radical intermediate stabilization which enables the reaction (Fig. 2).

The only difference between compounds ***1*** and ***2*** relates to the

Table 1
In vitro determined AOA of title compounds, vitamins C and E and α-lipoic acid. Number of measurements: n = 5. Concentration of ethanol stock solution of the tested compound was 1 mg/mL (Final concentrations in AOA, *in vitro* tests were: DPPH: 0.04 mg/mL; FRAP: 0.033 mg/mL; and TBARS; 1.75 μg/mL). Values are expressed as mean ± STDEV.

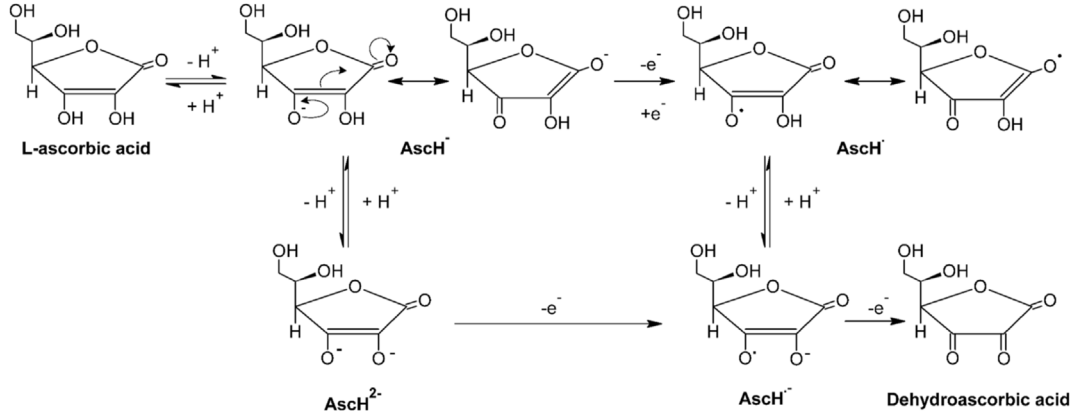
Compounds		Name	Structure	Antioxidative potential		
				DPPH	FRAP	TBARS
1,3-Thiazole based thiazolidinone derivatives (**Group I**)	***1***	Ethyl 2-{[(5Z)-5-(2-methoxybenzylidene)-4-oxo-4,5-dihydro-1,3-thiazol-2-yl]amino}-1,3-thiazol-4-yl-acetate		15.62 ± 4.91	365.40 ± 134.54	62.11 ± 20.79
	2	Ethyl 2-{[(5Z)-5-(3-methoxybenzylidene)-4-oxo-4,5-dihydro-1,3-thiazol-2-yl]amino}-1,3-thiazol-4-yl-acetate		18.73 ± 5.98	639.40 ± 233.33	23.51 ± 9.04
Thiadiazole-based thiazolidinone derivatives (**Group II**)	***3***	5-(4-Hydroxybenzylidene)-2-[(5- adamantyl-1,3,4-thiadiazol-2-yl)imino]-1,3-thiazolidin-4-one		4.99 ± 2.24	364.40 ± 145.89	39.64 ± 4.76
	4	5-(4-Hydroxy-3-methoxybenzylidene)-2-[(5-adamantyl-1,3,4-thiadiazol-2-yl)imino]-1,3-thiazolidin-4-one		33.98 ± 9.65	592.40 ± 240.12	47.80 ± 9.35
Thiazolidinone derivatives fused to 1,2,4-triazol heterocyclic system (**Group III**)	***5***	5-(4-Hydroxy-3-methoxybenzylidene)-2-adamantyl-1,3-thiazolo [3,2-b]1,2,4-triazol-6(5H)-one		6.59 ± 3.84	217.40 ± 59.90	66.71 ± 20.69
	6	5-(4-Methoxybenzylidene)-2-adamantyl-1,3-thiazolo [3,2-b]1,2,4-triazol-6(5H)-one		1.36 ± 1.29	231.40 ± 64.46	53.33 ± 16.01
	7	5-(3-Hydroxybenzylidene)-2 adamantyl-1,3-thiazolo [3,2-b]1,2,4-triazol-6(5H)-one		2.62 ± 1.88	205.40 ± 70.01	53.8 ± 21.51
	8	5-(4-Hydroxy-3,5-dimetoxybenzylidene)-2 adamantyl-1,3-thiazolo [3,2-b]1,2,4-triazol-6(5H)-one		3.80 ± 1.69	379.40 ± 114.48	37.10 ± 14.51
Ethylaminothiazole-based chalcone (**Group IV**)	***9***	3-(2,6-Dimethoxyphenyl)-1-[2-(ethylamino)-4-methyl-1,3-thiazol-5-yl]prop-2-en-1-one		0.15 ± 1.20	238.40 ± 82.90	60.93 ± 21.67
Reference standards		Vitamin C		94.35 ± 32.05	812.40 ± 321.85	62.32 ± 20.63
		Vitamin E		2.99 ± 2.04	868.40 ± 345.87	36.29 ± 9.26
		α-Lipoic acid		1.57 ± 1.48	0.16 ± 1.42	51.36 ± 17.78

position of a methoxy group (OMe) (*orto* OMe in compound ***1*** and *meta* OMe in compound ***2***), which could be the reason for the higher observed AOA of compound ***2***. Namely, stabilization of the N$^{\bullet}$ by delocalization of electrons towards carbonyl group (C=O) (electron withdrawing group, or EWG) is more pronounced in the case of the *meta* OMe derivative (compound ***2***), as electron delocalization occurs only inside the benzylidene ring and does not extend towards C=O (Fig. 3).

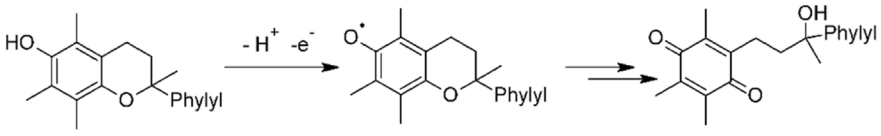
From ***group II***, compound ***4*** accomplished the highest AOA out of all tested compounds, though the observed AOA was moderate in comparison to Vitamin C (Graph 1). By combining the properties of the phenol (PhOH) and NH functional groups, ***group II*** compounds can employ concomitant phenoxy/amino radical reaction mechanisms, contributing to its higher AOA, comparing to the other derivatives (Figs. 4 and 5).

Phenol groups are stronger electron-donating groups (EDG) than NH, thus an electron delocalization toward C=O orchestrated by *para* OH probably exceeds the N$^{\bullet}$ intermediate stabilization in compound ***3***. The presence of *meta* OMe in compound ***4*** which enhances the stabilization of the *O*-radical intermediate is the only differentiating factor between the two compounds and the most likely cause of its six-fold higher AOA [43].

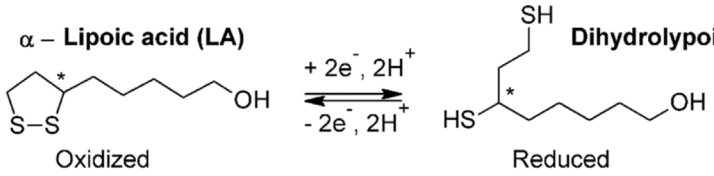
Group III compounds showed poor AOA as compared to Vitamin C by *DPPH* assay. The only possible source for H$^{\bullet}$ donation refers to the PhOH of the benzylidene ring as there is no NH available in ***group III*** derivatives. The benzylidene moiety of compound ***5*** closely resembles that of compound ***4*** (*para* OH and *meta* OMe) and is probably the reason for the fact that a higher AOA was recorded for compound ***5*** compared to the other ***group III*** derivatives (Fig. 6) [43].



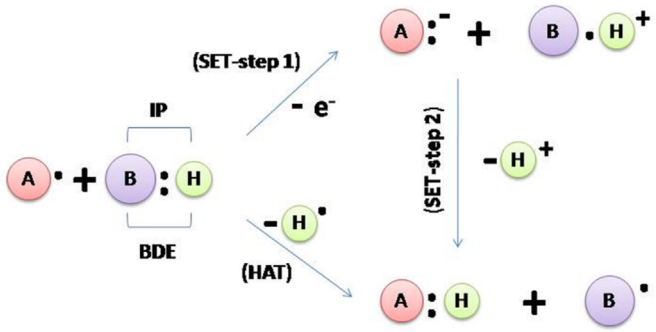
Scheme 3. Two steps reduction mechanism of vitamin C.



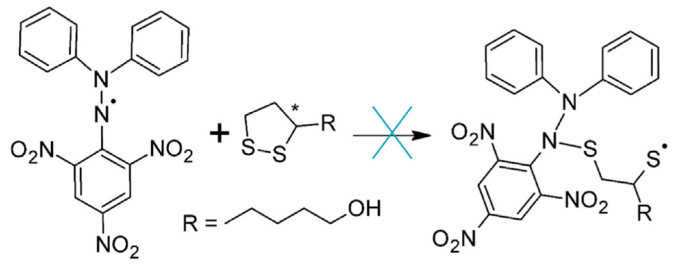
Scheme 4. One step reduction mechanism of vitamin E.



Scheme 5. Two steps reduction mechanism of α-lipoic acid.



Scheme 6. SET and HAT AO mechanisms.



Scheme 7. Stereochemically unfeasible reaction between *DPP*• and α-LA.

The negligible AOA of other ***group III*** derivatives can be explained by the lack of any H• (***6***); sterically hindered resonance between *para* OH in the benzylidene moiety and the C=O group (*para* OH is surrounded by two OMe at positions 3 and 5) (***8***); and the *meta* OH fixed electron delocalization within the benzylidene ring and not toward the EWG C=O (***7***) (Fig. 7).

The donation of H• from NH did not result in any AOA of compound ***9***, probably because its *N* • intermediate is less stabilized due to the presence of the ethyl group instead of an aromatic ring (Fig. 8).

To conclude, the compounds labeled ***1–9*** accomplished weak AOA by *DPPH* assay as compared to Vitamin C. It is possible that concomitant resonant stabilization of the N-radical and the boosted stabilization of the *O*-radical intermediate of the *para* OH and *meta* OMe groups in the benzylidene ring contributed to the enhanced AOA of compound ***4***. The somewhat lower AOA of compounds ***1*** and ***2*** are likely attributable to the presence of the NH group and OMe substituent in the *orto* and *meta* position of the benzylidene ring respectively. The negligible AOA of compound ***5***, though higher than that of other ***group III*** derivatives, is probably due to the presence of the benzylidene moiety (as in compound ***4***). Compound ***9*** did not accomplish AOA, perhaps due to poor *N* • intermediate radical stabilization.

4.2. FRAP assay

The antioxidant mechanism of *FRAP* test concerns only e^- transfer. The reaction between Vitamins C and/or E and Fe^{3+} occurs spontaneously at low pH since their reducing potentials are lower than for Fe^{3+}/Fe^{2+} ($\Delta E^{\circ\prime} = +770$ mV) [39,41,44]. α-Lipoic acid did not react with Fe^{3+} in spite of the strong reducing potential of α-LA/α-DHLA ($\Delta E^{\circ\prime} = -320$), probably because the potential of DHLA to form a complex with Fe^{3+} dominated [39].

Compounds ***2*** and ***4*** exhibited the highest AOA by the *FRAP* method (around 75% of the Vitamins C and/or E) compared to other thiazolidinone derivatives (Graph 2). Both compounds exhibit chelating ligand activities that could potentially replace bidentate heterocyclic ligands of *FRAP* reagent, whereas a SET mechanism would occur intramolecularly. The formation and the stability of –S→Fe←S– and –S→Fe←N– types of coordinate bonds, associated with intramolecular cyclisation forming 5-membered rings in compounds from ***groups I*** and ***II***, are responsible for their higher AOA, while the weaker –O→Fe←S– coordinate bond and the formation of a 4-member ring contributed to

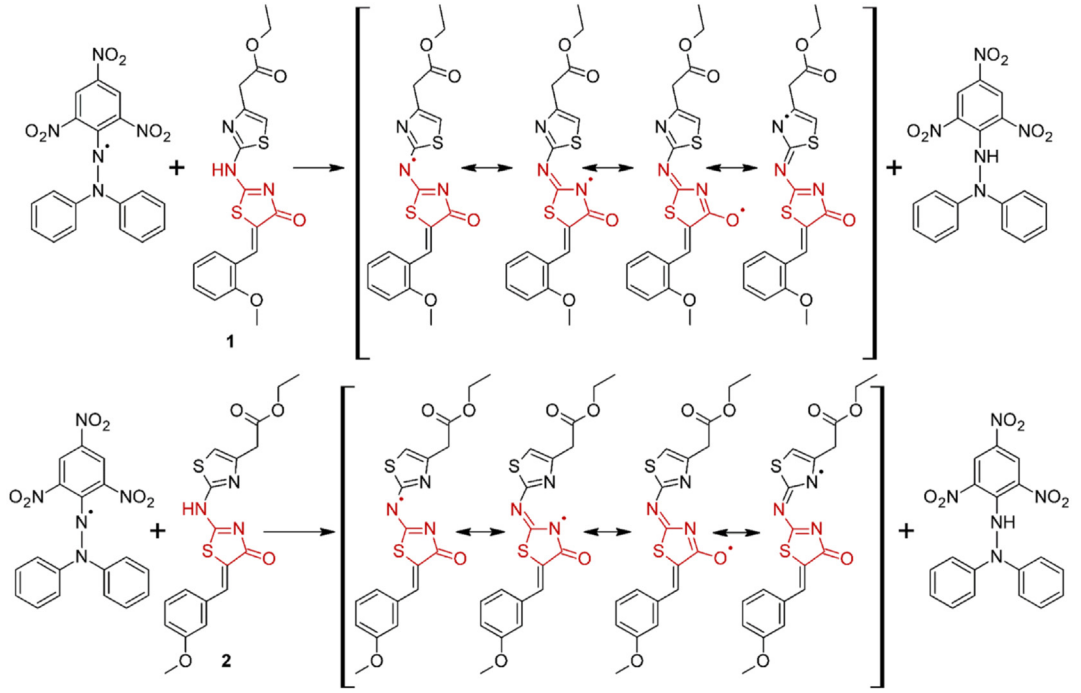


Fig. 2. a,b. Resonant stabilization of *N*-radical in *group I* derivative, *1* (a) and *2* (b).

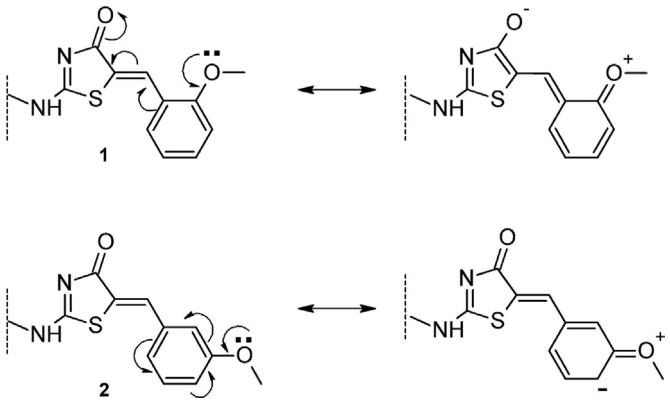


Fig. 3. Resonant stabilization of ***1*** and ***2*** *O*-radicals.

the lower level of AOA of compound ***9***.

Within ***group III***, chelating ligand activities are diminished in condensed heterocyclic rings. Compound ***8*** (in which a *para* OH is surrounded by two *meta* OMe in a benzylidene moiety) demonstrated the highest level of AOA in this group, suggesting a type of reaction involving ligand exchange occurred.

However, the presence of *meta* OMe in the benzylidene rings of compounds ***2*** and ***4*** (which accomplished the highest AOA of all compounds by this method) indicated the importance of NH and *meta* OMe in benzylidene ring above that of the enhanced O-radical intermediate (which was suggested to be the crucial factor in achieving high levels of AOA in the *DPPH* assay).

4.3. TBARS assay

The inhibition of LPO (i.e. prevention of MDA production) in the *TBARS* assay evaluates the flow of reducing equivalents between $Asc^{2-}/Asc^{\bullet-}$, molecular oxygen (O_2), Fe^{3+}/Fe^{2+}, and PUFA radical intermediates on the one hand and the tested compounds on the other [39,40,45]. The product of Asc^{2-} autoxidation with molecular oxygen (O_2) is the superoxide anion radical ($O_2^{\bullet-}$) according to the Marcus theory of electron transfer (Reaction 1). Autoxidation of the $AscH^{-}$ is much slower, with $k \sim 10^6\ M^{-1}s^{-}$ at a pH of 7.4 [37]. The production of hydrogen peroxide (H_2O_2) (Reaction 3) precedes the production of the most potent of the hydroxyl radicals, $HO^{\bullet}$, which forms either by homolytic cleavage or through a Fenton reaction (Reaction 4). The protonated form of $O^{\bullet-}$, the perhydroxyl radical ($HO_2^{\bullet}$, pKa = 4.7) or $HO^{\bullet}$ can initiate LPO by the abstraction of bis-allylic H^{+} from PUFA (Reactions 2, 5-10) [40]. The thermodynamic reactions described below could possibly promote LPO (initiation, propagation and termination) and precede MDA formation [39,40].

The propagation of LPO is comprised of the formation of PUFA radical ($PUFA^{\bullet}$), PUFA peroxyl ($PUFA\text{-}OO^{\bullet}$), PUFA alkoxyl ($PUFA\text{-}O^{\bullet}$) and PUFA hydroperoxides (PUFA-OOH), which undergo β-scission reactions, or intramolecular cyclisation, followed by decomposition into carbonyls (including MDA) in the termination phase of LPO [26].

The LPO inhibition accomplished by Vitamin C, α-LA and Vitamin E (62.32%, 51.36% and 36.29%, respectively), decreases in accordance with their reducing potentials and the chelating properties of DHLA (Graph 3). The strong LPO inhibition achieved by Vitamin C is attributable to its two-step reduction capability and low standard reducing potentials. Vitamin E terminates LPO through its reaction with $PUFA\text{-}OO^{\bullet}$ (reaction 12) that prevails thermodynamically and kinetically over reactions of LPO propagation (reaction 9, with a low rate constant) [40,41,44].

The strong reducing potential of α-LA, in combination with DHLA's chelating affinity with Fe^{3+}, contributes to LPO inhibition.

The tested compounds can inhibit LPO at initialization (assuming the reactions with Fe^{2+}, $O_2^{\bullet-}$ and $HO^{\bullet}$), propagation and termination (reactions with lipid peroxy radicals) [25]. The LPO inhibitory effect was observed, from highest to lowest, to be: compounds ***1***, ***5*** and ***9*** (between 61 and 67%); compounds ***4***, ***6***, ***7*** (~50%), equal to that of α-LA; compound ***8*** (~38%), equal to that of Vitamin E; and compound ***2*** (23.51%). The meta position of the OMe group within compound ***2*** decreases its lipophilicity as compared to the ortho position of the OMe group in compound ***1***, possibly hindering its AOA in the TBARS assay [46].

Compounds with PhOH/NH showed better AOA, particularly in cases when *N*-/*O*-radicals are stabilized by electron delocalization [44].

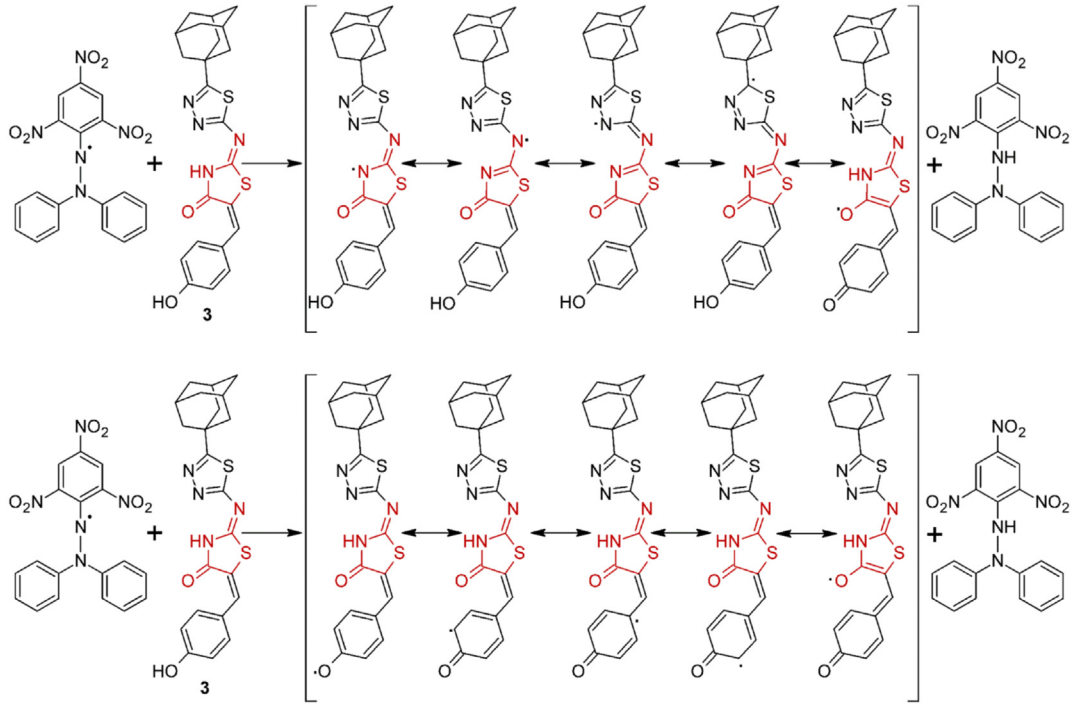


Fig. 4. a,b. Resonant stabilization of *N*- and *O*- radical intermediate of ***3***.

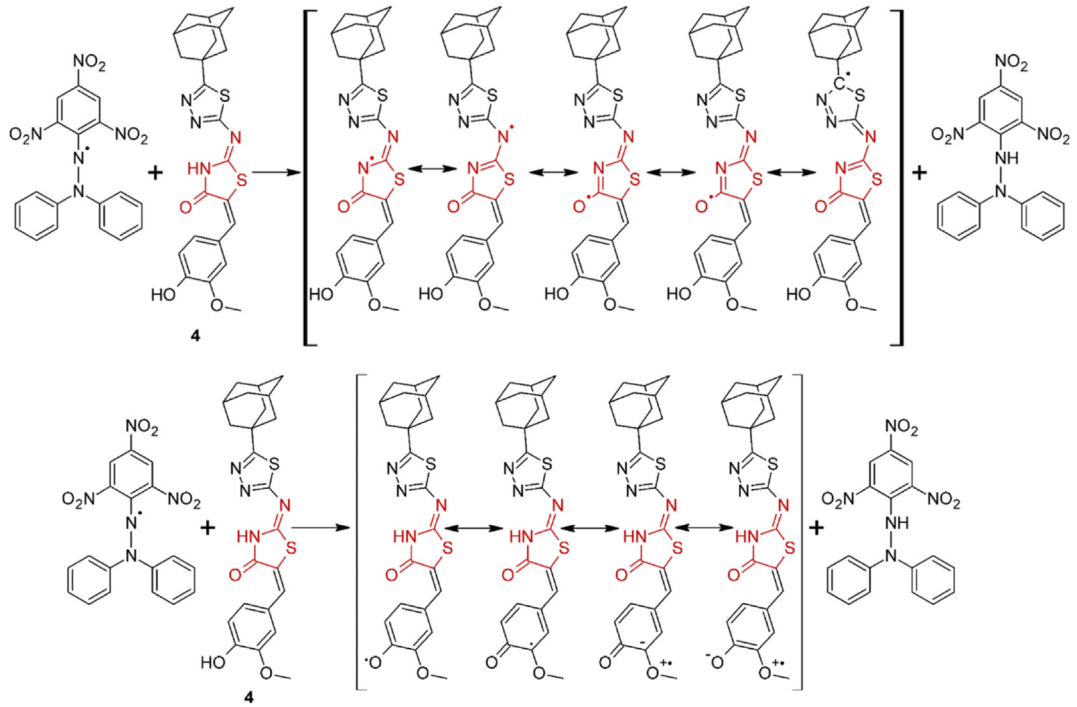


Fig. 5. a,b. Resonant stabilization of *N*- and *O*- radical intermediate of ***4***.

It is possible that the chelating ability of the tested compounds contributed to the AOA profile generated. Accordingly, compounds ***1*** and ***9*** probably combine both N-radical stabilization and chelation of Fe^{2+} within their mechanisms, while the stabilization of the phenoxy radical and its reaction with $O_2^{\bullet-}$ dictated strong LPO inhibitory effect of compound ***5***.

The *FRAP* and TBARS methods turned out to be the most nuanced tests for AOA measurement, evidenced by the gradation in results recorded. A perfect correlation (Slope = 1, $r^2 = 1$) between *FRAP* and *TBARS* methods was observed which can be attributed to one-electron transfer mechanisms as well as to coordinate binding of Fe by the tested compounds (Fe is present in the reaction mixtures, in both methods). Different red-ox reaction principles of the applied assays dictate different AOA outcomes for a single compound (Table 2) [47]. Out of the tested compounds, ***4*** achieved the maximum AOA by *DPPH*, closely followed by compounds ***2*** and ***1***, with all other compounds showing negligible AOA. Compounds ***2*** and ***4*** reached the highest AOA by *FRAP* assay, with 2 achieving a greater AOA than 4. Results gathered from testing the other compounds produced either moderate or low AOA, with α-LA demonstrating no AOA. Almost all the compounds

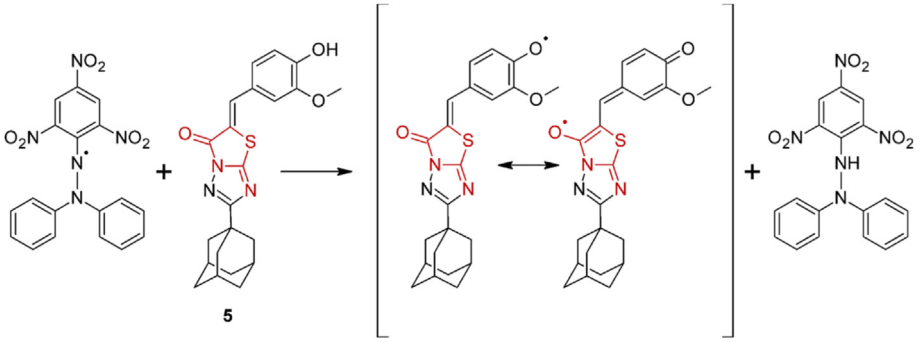


Fig. 6. Resonant stabilization of ***5*** *O*- radical.

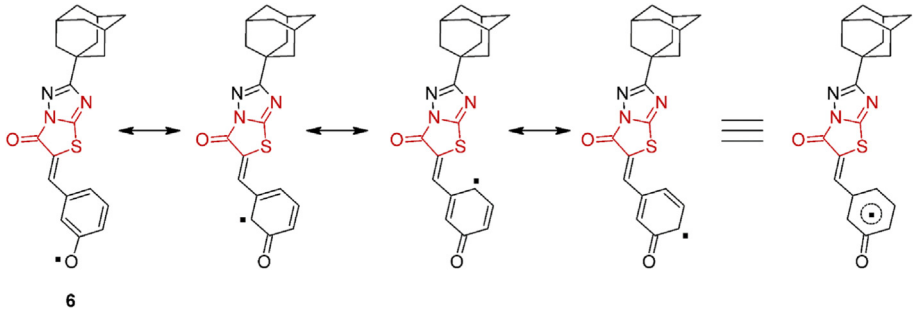


Fig. 7. Profile of electron delocalization directed by *meta* PhOL in ***7*** derivatives.

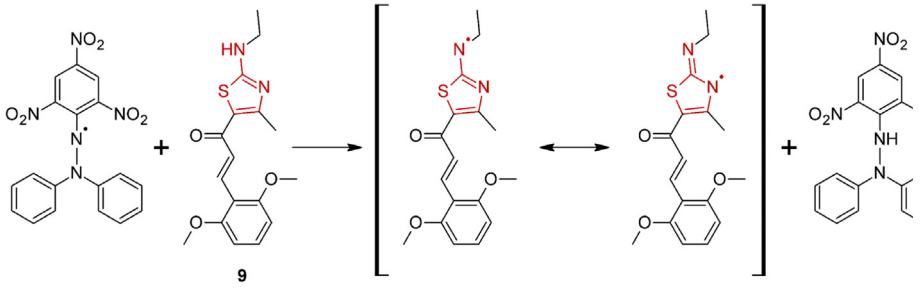


Fig. 8. Proposed resonance stabilization for ***9*** that requires considerable deal of energy.

demonstrated relatively high values of AOA according to the TBARS method except for compound 2.

We have confirmed through our results that compounds with PhOH/NH show better AOA according to *FRAP* and *TBARS*, especially in cases where N-/O-radicals were stabilized by electron delocalization. Accordingly, compounds ***1*** and ***9*** probably undergo both N-radical stabilization and chelation of Fe^{2+} which increases their AOA, while stabilization of the phenoxy radical and its reaction with $O_2^{\bullet-}$ determined the strong LPO inhibitory effect of compound ***5***. In regard to conventional antioxidants, Vitamin C was the superior of the three traditional antioxidants (ascertained by all three tests), unlike Vitamin E and α-LA which gained maximum of AOA only by *FRAP* and *TBARS* assays, respectively.

5. Conclusion

In this study we evaluated the synthesis and AOA of thiazolidinone derivatives of 1,3-thiazole and 1,3,4-thiadiazole, by three *in vitro* tests: *DPPH, FRAP* and *TBARS*. The *FRAP* and *TBARS* methods appeared to be the most sophisticated assays for AOA measurement. Different red-ox reaction principles of the applied assays predicted different AOA outcomes for the individual compounds (demonstrated by the traditional and tested compounds). The presence of an –OH group in the benzylidene ring, the position of the OMe in the benzylidene ring, amino-carbonyl functional domains [–NH–C=N–C(O)–] and the chelating ligand properties of the tested compounds determined their primary AOA mechanisms. Of the compounds being tested, compound ***4*** accomplished the highest overall AOA with results from other compounds being mixed. Accordingly, *in vitro* testing of the thiazolidinone derivative, compound ***4***, can be accepted as a model for future *in vivo* experiments within the preclinical phase of drug development research.

Reactions 1-12

$O_2^{\bullet-}$ was generated in the reaction of autoxidation between Asc^{2-} and O_2, based on Marcus theory of electron transfer (Reaction 1). Autoxidation of $AscH^-$ is much slower, $k \sim 10^6\ M^{-1}s^-$, at pH 7.4 [30]. The production of H_2O_2 (Reaction 3) precedes the production of the most potent $HO^{\bullet}$ by homolytic cleavage or Fenton reaction (Reaction 4). The initiation of LPO starts by the abstraction of bis-allylic H^+ from PUFA, protonated form of $O^{\bullet-}$, perhydroxyl radical ($HO_2^{\bullet}$, pKa = 4.7) (Reaction 2) or $HO^{\bullet}$ (Reactions 5-10) [33]. Reaction 12 relates to AOA of vitamin E [40].

$$Asc_2^- + O_2 \rightarrow Asc^{\bullet-} + O_2^{\bullet-}\ k = 10^2\ M^{-1}s^{-1} \quad \text{(Reaction 1)}$$

$$O2^{\bullet-} + H^+ \rightarrow HO_2^{\bullet} \quad \text{(Reaction 2)}$$

$$O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 \quad \text{(Reaction 3)}$$

Table 2
Antioxidant activity of the tested compounds across the *in vitro* assays. Percentage range of measured outcomes for each test: ○0–21%, ◔22–43%, ◑44–65%, ◕66–87%, ●88–109%). In vitro tests: DPPH (% DPPH radical neutralization, used concentration 0.04 mg/mL); FRAP: (μM Fe^{2+}, used concentration 0.033 mg/mL); and TBARS (% LPO inhibition, used concentration 1.75 μg/mL). Number of repetitions for one concentration of the tested compounds: n = 5.

The tested compounds	DPPH (%)	FRAP (%)	TBARS (%)
1	○ 17	◔ 42	● 100
2	○ 20	◕ 74	◔ 38
3	○ 5	◔ 42	◑ 64
4	◔ 36	◕ 68	◕ 77
5	○ 7	◔ 25	● 107
6	○ 1	◔ 27	◕ 86
7	○ 3	◔ 24	● 86
8	○ 4	◑ 44	◑ 60
9	○ 0	◔ 27	● 98
Vit C	● 100	● 94	● 100
Vit E	○ 3	● 100	◑ 58
α-LA	○ 2	○ 0	◕ 82

$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^{\bullet} + HO^{-}$ $\Delta E^{\circ\prime} = 320–110 = 210$ mV (*) (Reaction 4)

$HO_2^{\bullet} + PUFA\text{-}H \rightarrow PUFA^{\bullet} + H_2O_2$ $\Delta E^{\circ\prime} = 1060–600 = 460$ mV (*) (Reaction 5)

$HO_2^{\bullet} + PUFA\text{-}OOH \rightarrow PUFA\text{-}OO^{\bullet} + H_2O_2$ $\Delta E^{\circ\prime} = 1060–1000 = 60$ mV (**) (Reaction 6)

$HO^{\bullet} + PUFA\text{-}H \rightarrow PUFA^{\bullet} + H_2O$ $\Delta E^{\circ\prime} = 2310–600 = 1710$ mV (Reaction 7)

$PUFA^{\bullet} + O_2 \rightarrow PUFA\text{-}OO^{\bullet}$ $k = 3 \times 10^8\ M^{-1}s^{-1}$ (Reaction 8)

$PUFA\text{-}OO^{\bullet} + PUFA\text{-}H \rightarrow PUFA\text{-}OOH + PUFA^{\bullet}$ $\Delta E^{\circ\prime} = 1000–600 = 400$ mV; $k = 10\ M^{-1}s^{-1}$ (Reaction 9)

$Fe^{2+} + PUFA\text{-}OOH \rightarrow Fe^{3+} + PUFA\text{-}O^{\bullet} + HO^{-}$ $\Delta E^{\circ\prime} = 1000–110 = 890$ mV (**) (Reaction 10)

$PUFA\text{-}O^{\bullet} + PUFA\text{-}H \rightarrow PUFA\text{-}OH + PUFA^{\bullet}$ $\Delta E^{\circ\prime} = 1600–600 = 1000$ mV (Reaction 11)

$PUFA\text{-}OO^{\bullet} + TO\text{-}H \rightarrow PUFA\text{-}OOH + TO^{\bullet}$ $\Delta E^{\circ\prime\prime} = 1000–1500 = 500$ mV; $k = 8 \times 10^4\ M^{-1}s^{-1}$ (Reaction 12)

Legend: * Fenton reaction, **preferred site for $HO_2^{\bullet}$ attack, *** Fenton-like reaction.

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