Synthesis and In Vitro Antioxidant Evaluation of New bis α - Aminoalkylphosphinic Acids Derivatives

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Supplemental Materials

BIOLOGICAL ASSAY

Antioxidant Activity

Hydroxyl radical ($\bullet\text{OH}$) scavenging activity. 2-deoxy-D-ribose oxidative degradation through hydroxyl radicals²⁹ was used with the changes in order to verified the compounds' hydroxyl radical-scavenging ability. The reaction mixture, which included different compound dilutions from 50 to 500 $\mu\text{g/mL}$ in ethanol with sodium hydroxide (0,1 M), 150 μL of 3mM 2-deoxy-D-ribose, 60 μL of FeCl_3 (2mM), 30 μL of EDTA (1mM) 150 μL of H_2O_2 (1 mM) and 60 μL of ascorbic acid (5mM) was vortexed. Then, 2.5 ml KH_2PO_4 buffer (pH: 7.4) was added and the mixture was incubated at 37 °C for a duration of one hour. Thenceforth, thiobarbituric acid (1 mL of 2%) in trichloroacetic acid (5%) was added. Then the mixtures were vortexed and heated in a water bath at 100 °C for 30 min. The reaction was incubated by a 5 min. ice water bath. The absorbance was measured at 532 nm. The following formula was used to calculate the scavenging effect of hydroxyl radicals.

Hydroxyl radical scavenging activity (%) = $(A_0 - A_1/A_0) \times 100$, where A_0 the absorbance of control tube, which contains all reagents except the test compound, at 532 nm and A_1 is the absorbance in the presence of the samples or standards. *IC*50 (50% inhibitory concentration of compound) was calculated from the curve drawn by plotting the inhibition percentage against sample concentration. Antioxidant activity of synthesized compounds was compared with that of synthetic antioxidant BHT and ascorbic acid as the reference standard. (Table S 1)

Superoxide radical scavenging activity. The reported method with minor modifications was used with slight changes in order to create the superoxide radicals³⁵. Approximately 1 mL of nitrobluetetrazolium (NBT) solution (600 μM NBT in 100 mM phosphate buffer, pH 7.4), 1 mL

NADH solution (468 μM in 100 mM phosphate buffer, pH 7.4) and 0.1 mL of solution of compounds at different concentrations from 50 to 500 $\mu\text{g/mL}$ in ethanol with sodium hydroxide (0,1 M) were vortexed. 100 μL of phenazinemethosulphate (PMS) solution (60 μM PMS in 100 mM phosphate buffer, pH 7.4) was added to the mixture to induce the reaction. The reaction mixture was incubated at 25 $^{\circ}\text{C}$ for 5 minutes. Then the absorbance at 560 nm was measured against blank samples. Decreased absorbance of the reaction mixture meant that superoxide anion scavenging activity increased. All of the data were obtained by calculating the average of the triplicate analyses. As the reference standard antioxidant, ascorbic acid, α -tocopherol and BHT were used and IC_{50} values were calculated with the same way with above.

Diphenylpicrylhydrazyl (DPPH) radical scavenging activity. DPPH radical scavenging activity of the samples was assessed by reported method³⁶ with minor modifications. Scavenging of the stable free radical molecule of DPPH in presence of electron or hydrogen donor antioxidants was detected by spectrophotometric measuring due to change in typical purple color³⁷. Synthesized organic molecules were prepared in ethanol with sodium hydroxide (0,1 M) with various concentrations (50 to 500 $\mu\text{g/mL}$) and mixed with diluted DPPH inside methanol solution (0,004%) which was then incubated for 30 min and measuring of absorbance is concluded in 517 nm wavelength. BHT and ascorbic acid were used as reference standard antioxidants and IC_{50} values were calculated for each substance.

Metal chelating activity. The reported method with minor modifications was used to calculate the chelation of ferrous ions by the synthesized compounds and standards³⁸. The synthesized compounds (50-100-200- 500 $\mu\text{g/mL}$) in ethanol with sodium hydroxide (0,1 M) were added to a 2 mM solution of FeCl_2 (0.05 mL). The reaction was began by adding 5 mM of

ferrozine (0.2 mL) and the mixture was vibrated strongly, and then left to mature at room temperature for 10 minutes. The absorbance of the solution was measured at 562 nm using a spectrophotometer after the mixture reached stability. All of the test and analyses were performed in triplicate and then their average was calculated. Decreased absorbance of the reaction mixture meant that the metal chelating activity increased. The following formula gave the percentage of inhibition of ferrozine- Fe^{2+} complex formation: % Inhibition = $(A_0 - A_1/A_0) \times 100$, where A_0 is the absorbance of the control, and A_1 is the absorbance in the presence of the samples or standards. α -Tocopherol and BHT were chosen as reference standard antioxidant, then IC_{50} was calculated.

Hydrogen peroxide scavenging activity. The reported protocol with minor modifications was followed for the hydrogen peroxide scavenging activity³⁹. The solution of 43 mM H_2O_2 was prepared in 0.1 M phosphate buffer (pH 7.4). Various compound concentrations (50-100-200-500 $\mu\text{g/mL}$) in ethanol with sodium hydroxide (0.1 M) and 3.4 mL phosphate buffer were added to 0.6 mL H_2O_2 solution (43 mM), and then the absorbance of the reaction mixture was recorded at 230 nm. The sodium phosphate buffer without H_2O_2 was included in a blank solution. All of the test and analyses were performed in triplicate and then their average was calculated. Decreased absorbance of the reaction mixture meant that the Hydrogen peroxide scavenging activity increased. The following formula was used in order to calculate the percentage of H_2O_2 scavenging by compounds and standards = $(A_0 - A_1/A_0) \times 100$. As reference standard antioxidant, Ascorbic acid and BHT were used, then IC_{50} was calculated.

Table S 1. *IC*₅₀ values of standards and tested compounds from various in vitro antioxidant activity methods.

<i>Test compounds</i>	<i>IC</i> ₅₀ Values ± Standard Deviation				
	Hydroxyl Radical Scavenging Activity Method	Superoxide Radical Scavenging Activity Method	DPPH Radical Scavenging Activity Method	Metal Chelating Activity Method	Hydrogen Peroxide Scavenging Activity Method
4a	55,25 ± 15,06*	62,10 ± 17,97*	483,02 ± 14,28*	134,73 ± 5,95*	293,19 ± 3,06**
4b	60,56 ± 15,54*	385,06 ± 8,31*	61,20 ± 8,71*	353,68 ± 10,33*	333,41 ± 3,91*
4c	48,15 ± 15,31*	51,62 ± 9,14*	420,70 ± 9,99*	37,51 ± 3,01*	60,87 ± 4,54*
4d	54,85 ± 16,14*	61,86 ± 29,47*	476,45 ± 11,81*	62,61 ± 3,16*	251,51 ± 6,71**
4e	58,11 ± 14,79*	52,41 ± 18,16*	37,03 ± 8,07*	406,09 ± 15,41*	254,63 ± 18,25*
4f	48,40 ± 15,94*	55,91 ± 18,94*	424,22 ± 9,97*	48,46 ± 14,39**	60,07 ± 17,05*
4g	54,66 ± 16,87*	95,48 ± 12,67**	38,59 ± 11,33*	77,96 ± 9,92**	238,32 ± 8,51*
4h	52,45 ± 16,28*	66,44 ± 14,37*	43,73 ± 6,25*	66,85 ± 12,41*	201,23 ± 14,89*
BHT	38,27 ± 11,36*	39,70 ± 7,88*	75,35 ± 11,04*	45,24 ± 17,98*	201,15 ± 28,68*
Ascorbic acid	32,90 ± 10,63*	44,43 ± 10,65*	19,238 ± 1,25*	-	52,28 ± 9,64*
α-tocopherol	-	57,40 ± 14,11*	-	35,12 ± 2,94*	-

Linear Regression (*Cubic model **Linear Model)

FT-IR, ^1H , ^{13}C , ^{31}P NMR, and DTA Spectra for 4a-f (Figures S 1 – S 21) **Figure S 1.** ^1H -NMR spectrum of 4a

Figure S 2. ^{13}C -NMR 4a

Figure S 3. ^1H -NMR spectrum of 4b

Figure S 4. ^{13}C -NMR 4b

Figure S 5. ^1H -NMR spectrum of 4c

Figure S 6. ^{13}C -NMR 4c

Figure S 7. ^1H -NMR spectrum of 4d

Figure S 8. ^{13}C -NMR 4d

Figure S 9. ^1H -NMR spectrum of 4e

Figure S 10. ^{13}C -NMR 4e

Figure S 11. DTA Spectra of 4e

Figure S 12. ^1H -NMR spectrum of 4f

Figure S 13. ^{13}C -NMR 4f

Figure S 14. FT-IR spectrum of 4g

Figure S 15. ^1H -NMR 4g

Figure S 16. ^{13}C -NMR 4g

Figure S 17. ^{31}P -NMR 4g

Figure S 18. DTA Spectra of 4g

Figure S 19. ^1H -NMR 4h

Figure S 20. ^{13}C -NMR 4h

Figure S 21. ^{13}P -NMR 4h